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Understanding Cell Viability And Mechanics Of Actin Filament Response Of Nih/3T3 Fibroblasts Under Biaxial Stretch

Hamed Ghazizadeh
North Carolina Agricultural and Technical State University

Understanding Cell Viability and Mechanics of Actin Filament Response of NIH/3T3 Fibroblasts under Biaxial Stretch

Hamed Ghazizadeh

North Carolina A&T State University

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Hamed Ghazizadeh

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Approved by:

Dr. Shyam Aravamudhan Major Professor	Dr. Dennis R. LaJeunesse Committee Member
Dr. Albert M. Hung Committee Member	Dr. Sanjiv Sarin Dean, The Graduate School
Dr. Ajit Kelkar Department Chair	

Biographical Sketch

Hamed Ghazizadeh was born on September 22_{nd}, 1990 in Esfarayen, Khorasan, Iran. Hamed received his bachelor's degree in Mechanical Engineering from Khayyam Institute of Higher Education, Mashhad, Iran in 2012. He joined the master's program in Nanoengineering at North Carolina Agricultural and Technical State University in the Spring of 2013. He conducted a research on understanding cell viability and mechanics of actin filament response of NIH/3T3 fibroblasts under biaxial stretch since summer of 2013.

Dedication

This thesis is dedicated to my loving parents Ali Ghazizadeh and Soori Mohammadian for their love, patient and support through the entire process.

Acknowledgments

I would like to thank my advisor, Dr. Shyam Aravamudhan, for his educational guidance during my studies at North Carolina Agricultural and Technical State University. I deeply appreciate his dedication and commitment to my educational and professional training. Thank you also to my committee Dr. M. Hung and Dr. R. LaJeunesse whose time and understanding are invaluable.

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Abstract

Cells are constantly subjected to mechanical stress during various physical activities. Understanding the role of the resultant mechanical stresses on cellular mechanotransduction is critical for considerate various cellular activities in the body such as control of cell growth, migration, differentiation, apoptosis and wound repair. The long-term goal is to understand whether it is possible to control cell functions through mechanical forces. Specifically, in this work, we report on the cellular and mechanistic response of NIH/3T3 fibroblastic cells (cultured on silicone membrane), when subjected to cyclic biaxial stretch generated in a custom-built stretching system, as described in Karumbaiah et al (Karumbaiah et al., 2012). The silicone membrane was first plasma-treated to increase its hydrophilicity, followed by coating a layer of Collagen type-I to increase cell adhesion to the membrane. Cell viability and morphological changes at the cell surface were studied in response to cyclic biaxial forces to determine the effect of time and amplitude on cell responses. In particular, the cell responses have been studied at 5% up to 10% strain with keeping frequency constant as low as 0.05 cycles/sec along with variable stretching time (6 and 24 hours) to model a situation closer to *in vivo*. Our results indicate that stretching cells under applied conditions have no considerable negative effect on cells viability while significantly increase the proliferation of cells. Also, there is a migration happened for cells from inner parts of the membrane to the corners which might be a result of the combination of shear stress (resulted from liquid movements during stretch) and the localized bending stress at the range of bending forces (when the membrane bends over glass indenters). Additionally, there is no evidence of alignment of the actin filament of cells under biaxial force whereas the spreading factor which is an indication of actin filament's response to the cell's mechanical environment was increased for stretched samples compared to the control.

CHAPTER 1

Introduction

1.1 Objective and Motivation

Cells, especially the fat and muscle cells are constantly subjected to mechanical stress during various physical activities or during exercise. Understanding the role of physical activity and the resultant mechanical stresses specifically on DNA methylation within muscle cells or on the regulatory and signaling Ca2+ molecule and generally on cellular mechanotransduction are critical for various cellular activities in the body such as control of cell growth, migration, differentiation, apoptosis and wound repair. There are well-known examples of cell lines being under mechanical stress *in vivo* such as lung cells during respiratory procedure, cells on blood vessel walls during circulatory procedure and re-modelling of living bone in response to changes in their mechanical environment. A number of recent studies have been investigated the exposure of various types of cell lines to different types of mechanical forces. However, to isolate the effect of different parameters, the current research has made assumptions such as applying regular waveform cyclic mechanical forces, which are not the case *in vivo*. The long-term goal is to understand whether it is possible to control cell behavior and its functions such as proliferation, differentiation and migration through applying mechanical forces (static and dynamic forces, cycle-by-cycle variability in amplitude of forces, etc.). However, the design of a well-isolated environment *in vitro* with the minimum weaknesses and the maximum control over the effective variables is a vital part of current studies.

3T3/NIH fibroblast cells are responsible for synthesizing the ECM of tissue and consequently are exposed to mechanical stimuli regularly *in vivo*. As a result, modelling an *in* vitro stretching system to better understanding the behavior of fibroblastic cell lines in response

to different types of mechanical forces can be helpful in wound healing and tissue engineering researches.

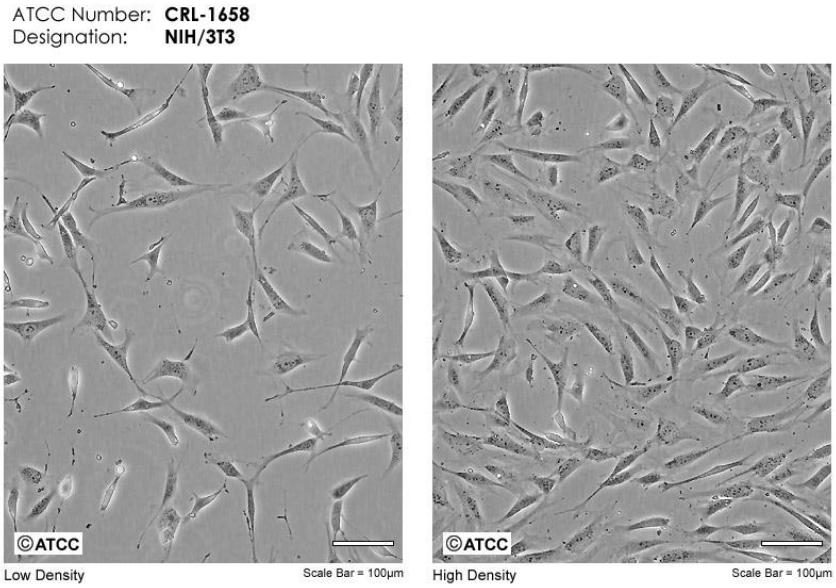


Figure 1. Morphology of adhered 3T3 fibroblast cell lines (low and high density)

1.2 Approach

3T3/NIH fibroblast cells were exposed to cyclic sinusoidal wave-form mechanical forces to further understand the effect of mechanical stimuli on cells behavior (viability, proliferation, migration, actin filament's alignment, etc.) *in vitro*. A custom built biaxial cell stretching setup (as previously explained by Karumbaiah et al.) was employed for this study in which the cells were cultured on a flexible silicon membrane and then the membrane was stretched over fixed glass indenters dynamically. Considering the fact that 3T3 fibroblast cells have to adhere to the culture dish surface to be able to survive, grow and split, the major challenge was to maintain the cells adhesion and confluency on flexible bottomed PDMS dishes even at the time of stretching. To overcome this challenge, the membrane was coated with collagen-I for at least 12 hours to

increase the adhesion of fibroblasts to silicon membrane. However, the hydrophilicity of silicon membrane has to increase for collagen fibers to stick to the surface. This was done using a plasma etch machine to plasma threatening the silicon membrane for 30 seconds which is followed by immediate coating of the dish with collagen-I. The cells were then cultured on to the PDMS dishes 24 to 36 hours before the experiment starts. Cells were then exposed to 5%, 7.5% and 10% strain for 6 and 24 hours exposure time while keeping the frequency of stretch as low as 0.05 cycles/ sec to understand the effects of strain percentage and exposure time on cells behavior. Two types of imaging (live and fixed) were done using an inverted confocal microscope to study the changes in viability, proliferation, cells migration, spreading factor, actin filaments alignment and nucleus size of stretched cells in comparison with the control.

1.3 Thesis Organization

The thesis consists of four major chapters: literature review, methodology, results, and discussion and future works. The literature review chapter discusses major motivations for the mechanotransduction studies during last decades and also reviews and discusses the currently available cell stretching systems in their specific categories. In chapter 3, methodology, the custom built setup and the methods of maintaining confluency of cells are discussed along with the assays that were used to do image analysis. This is followed by the results chapter which includes the analysis of images were taken and finally, the last chapter that discusses the conclusion of represented results and future recommendations.

CHAPTER 2

Literature Review

2.1 Importance of Mechanobiology

Mechanotransduction of cells is the study of any kind of cell's response to their mechanical environment within the human body. Different types of cells in our body are sensing various forms of mechanical stimulation such as hydrodynamic shear flow (Chang et al., 2008; Chia-Ching Wu, 2007; Dalous et al., 2008), substratum strain (Clark, Burkholder, & Frangos, 2001) and cyclic forces due to either regular procedures like respiratory and circulatory within body or external and internal factors that cause tissue injuries (Imsirovic J, 2013). Bone cells response is critical to tissue engineering (Adachi, Sato, & Tomita, 2003), lung cells become stretched during breathing process all the time (Tsuda et al., 1999) and also cells that stick on blood vessel walls become stretched while blood is pumping through them (Berger et al., 1981), especially when the blood pressure is changing continuously. Some researchers have studied cultured fibroblast cells in gels (R. A. Brown, Prajapati, McGrouther, Yannas, & Eastwood, 1998; Nekouzadeh, Pryse, Elson, & Genin, 2008) to estimate their mechanical environments by comparing tensile properties of gels with or without cells (Matsumoto & Nagayama, 2012). However, the microscopic heterogeneous structure of arterial vessel walls, as an example, doesn't match with the homogeneous structure of gels. The best way to investigate these types of cell's responses is to stretch substrata on which cells are adhered (Iwadate & Yumura, 2009). Studying the response of these cells to their mechanical environment is critical to bioengineers and scientists due to various applications in tissue engineering and curing disease. For example, lots of studies revealed that living bone modifies and remodels its structure to be able to prevail against environmental changes in mechanical forces (Adachi, Murai, Hoshiai, & Tomita, 2001;

Kurata et al., 2001). However, it also was proved that there is a threshold value for some cells to sense their mechanical stimuli (Adachi et al., 2003). Mechanical stimuli cause vital changes in morphology, proliferation and differentiation of cells (Hu, Humphrey, & Yeh, 2009; Lee, Maul, Vorp, Rubin, & Marra, 2007; Maul, Chew, Nieponice, & Vorp, 2011) which is critical in tissue engineering. It was also showed that cell migration happens under mechanical stress while the principle of the action is not still understood completely and needs more investigations. For instance, cells migrate randomly in no mechanical stress environment whereas in shear flow stress condition, they migrate in the direction of the flow (Li, 2002).

2.2 Cellular Response to Different Types of Mechanical Stimuli

Most of researchers were focused on regular, simple, cyclic mechanical forces in isolated environments while the cells in body experience irregular stimulation with different amplitudes and frequencies, cycle-by-cycle which in turn results in complicated stretching patterns under these conditions (Arold, Bartolak-Suki, & Suki, 2009; Imsirovic J, 2013). For example, Imsirovic and Suki have used a custom built stretching device to investigate mRNA changes in fibroblasts and the results was indicating that when variable strains were being applied, more changes in mRNA production occur comparing to that of consistent cyclic stretching.

Another complex mechanical environment is the existence of a hole in the tissue which can be modeled through the introduction of a hole to a circular membrane. It was proved that the existence of a small center hole in a membrane could result in a stress gradient in response to applied stretching force. Also, this stress gradient would rise by increasing the size of the center hole (David, 2004). There are some studies (Raeber, 2007) showed that migration happens in both static and dynamic stretching states while cells' migration as a respond to 20 percent static state was more than that of 5 percent dynamic stretch (Jenna Leigh Balestrini, 2006).

2.3 Existing Stretching Systems and Current Techniques

Stretching devices are divided into two main groups: two-dimensional and three-dimensional systems. Both of them have their own advantages and limitations that need to be considered for specific purposes. Most of the three-dimensional stretching devices are not able to handle a wide variety of amplitudes or frequencies while they are capable of studying 3-D constructs like tissues (Imsirovic J, 2013).

Although two-dimensional stretching systems, lack in providing data on stress and strain applied to 3-D biological constructs such as tissues, most of them can be used for studying cell's responses under wide range of amplitudes and frequencies of mechanical forces with a constant sinusoidal pattern (Imsirovic J, 2013). There are different novel designs for applying 2-D stretching forces on cells with their own advantages and disadvantages. Therefore, it is critical to understand the design, test procedure and its limitations before choosing one over another for specific case study. Due to the critical environment (37^{o}C, 5% CO2) that cells need to grow in, most of these instruments are designed to fit inside incubators during the test procedure. Two-D devices are categorized based on directional uniformity and homogeneity of strain field stretching systems into two groups of Uniaxial and Biaxial testers (Sotoudeh, Jalali, Usami, Shyy, & Chien, 1998).

2.3.1 Uniaxial stretching devices. In uniaxial method, cells are usually cultured on rectangular membranes which is stretched only in one direction (mostly in longitudinal, XY Plane) (Moretti, Prina-Mello, Reid, Barron, & Prendergast, 2004). Tensile force is applied to one end while the other end is fixed securely using clamps (Clark et al., 2001; Moretti et al., 2004). Although this type of stretching depicts a closer condition to what is happening to cells in vivo, the strain field is usually nonhomogeneous due to existence of compression force in

perpendicular plane (XZ) to stretching direction which in turn results in a combination of stretching and compressing (not a pure stretch) (Moretti et al., 2004; Sotoudeh et al., 1998). Uniaxial stretching systems could be divided in to two main groups: stretch membrane with cells cultured on it and stretch cells directly using micropipettes and micromanipulators. The former group was used mostly to examine and measure cells mechanical properties such as stiffness while researches who were studying cells responses to mechanical forces have used first approach. The mostly used uniaxial stretching designs are:

2.3.1.1 Stretching cells cultured on smooth flexible membrane using apparatuses. A commercial available design for uniaxial stretching of an elastic membrane was used by C. Neidlinger-wilke et al. (Neidlingerwilke, Wilke, & Claes, 1994) and consisted of a rectangular elastic silicone membrane which was clamped using two brackets at each ends. One of the brackets (fixed bracket) was fixed while the other one (moving bracket) was connected to a motor through a steel rod. The steel rod was converting the rotary movement of the motor to a forward-backward movement of the moving bracket which in turn resulted in a cyclic stretching of the membrane. This design had two main advantages including its capability to stretch six samples at the same time which gave users the opportunity of having control samples and also offering precise control over the frequency and amplitude of stretching (Neidlingerwilke et al., 1994). However, the stretching design introduced later by Clark et al. (Clark et al., 2001) was capable of handling 8 samples while only 4 of them could be stretched and the other 4 had been used as control samples for further comparisons.

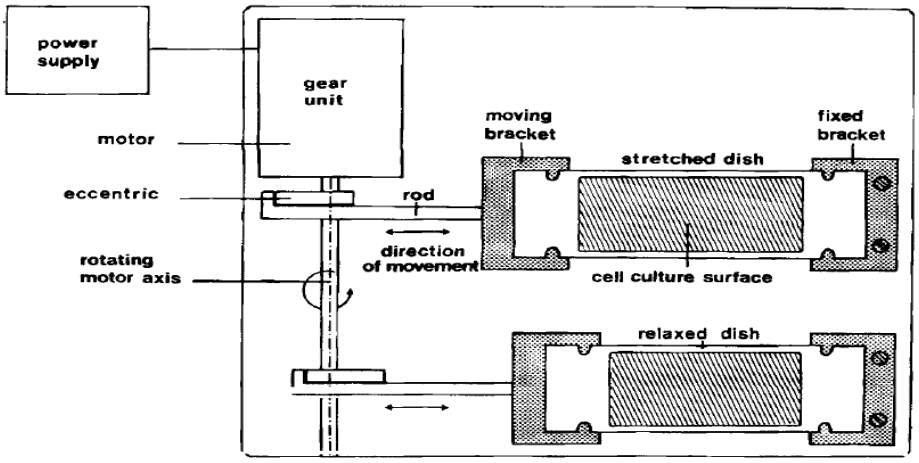


Figure 2. A schematic of the uniaxial stretching design used by C. Neidlinger-wilke et al. (Neidlingerwilke et al., 1994)

2.3.1.2 Stretching cells cultured on micro-grooved flexible membrane using apparatuses

apparatuses. Previous researchers demonstrated that cells tend to align perpendicularly to uniaxial stretching direction in order to reduce the force being sensed by them (Park et al., 2004). This type of responses would be a challenge in cell stretching studies. To address the issue, a novel method was introduced in which cells were cultured in a micro-grooved flexible bottomed dish. This approach allowed researchers to study cells behavior when the applied force is in both parallel and perpendicular direction of the micro grooves (Wang, Yang, & Li, 2005; Wang, Yang, Li, & Shen, 2004). In 2005, Loesberg et al. had cultured cells on micro-grooved flexible membrane and stretched them using the same tensile tester design that was used by C. Neidlinger-wilke et al. in 1994 (Loesberg, Walboomers, van Loon, & Jansen, 2005).

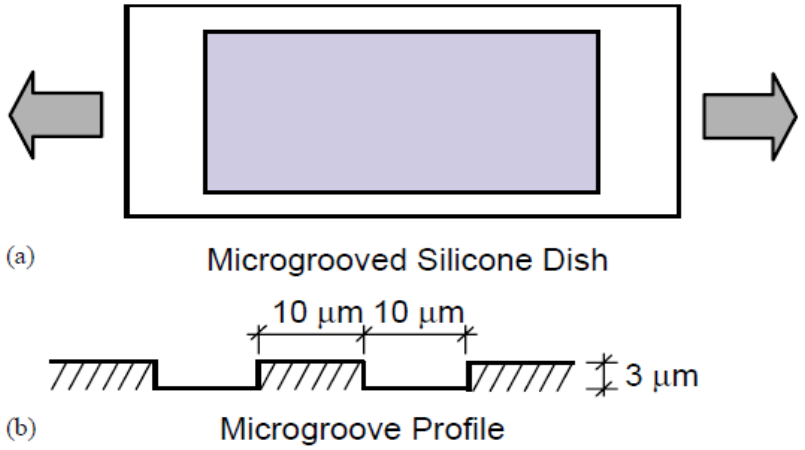


Figure 3. A schematic of the uniaxial stretching design used by Wang et al. (Wang et al., 2004)

2.3.1.3 Stretching cells cultured on smooth flexible membrane using vacuum suction. In 2010 D. Wang had introduced a tensile tester which was using vacuum suction to stretch cells cultured on PDMS membrane. The principle was to assemble membrane smoothly within the hole of a glass petri dish and using a thick layer of PDMS at the bottom of membrane to hold it. Vacuum tubes were placed in vacuum chamber at the bottom of thick PDMS while a thin layer of glass was covering the whole structure from the bottom to give the chance of live imaging. Elastic membrane would be stretched and spread on top of thin glass layer in response to applied vacuum suction from vacuum chamber. This instrument, for the first time, allowed researchers to study live cell response to mechanical stimuli on inverted high-resolution microscope (at the bottom of thin glass base) during the procedure of stretching. It also had the advantage of being used in either uniaxial or biaxial modes depending on chamber shape (Dong Wang, 2010).

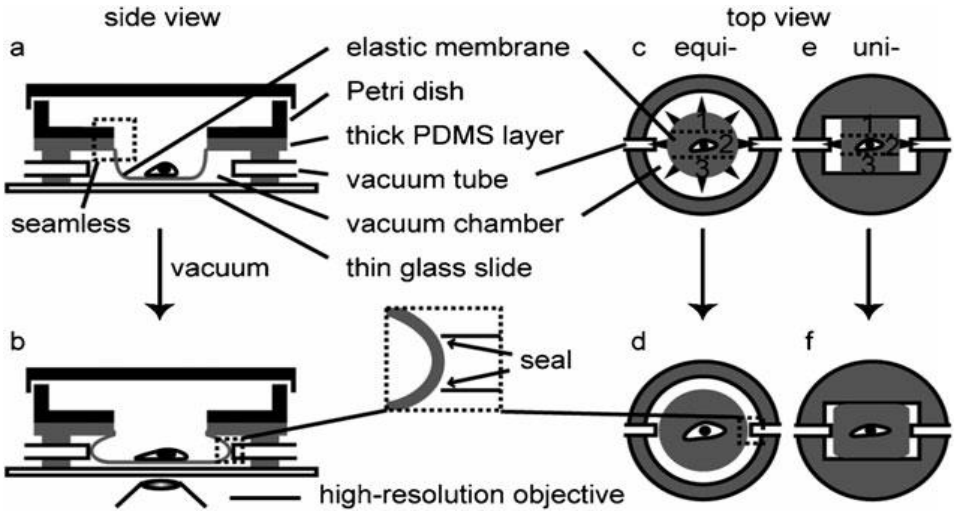


Figure 4. A schematic of the uniaxial stretching design used by Dong Wang (Dong Wang, 2010)

2.3.1.4 Stretching single cell using micropipettes and studying tensile properties.

Nagayama and Matsumoto designed and used a laboratory-made tensile tester in 2008 (Nagayama & Matsumoto, 2008) and 2010 (Nagayama & Matsumoto, 2010), respectively, which was capable of stretching single cells to study mechanical properties of them. The tester consisted of two micropipettes with diameters of 5-8μm that were designed to hold a single cell. One of them was controllable through an electro-micromanipulator by moving horizontally while the other one was stationary and used for measuring the amount of tensile force a single cell can sense. However, there was a big challenge in the process of cell detachment from the initial petri-dish and sticking it to the micropipettes. Using micropipettes to examine and determine the mechanical properties (viscoelasticity, fracture point, strength, etc) of cells is one of the most reliable approaches for data gathering.

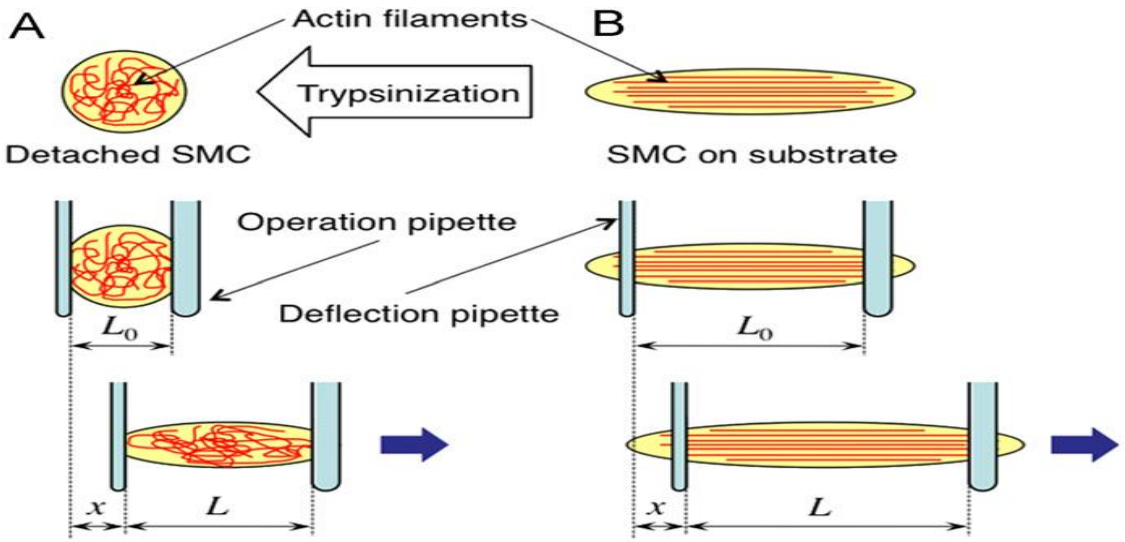


Figure 5. A schematic of the uniaxial stretching design used by Matsumoto & Nagayama (Matsumoto & Nagayama, 2012)

2.3.2 Biaxial stretching devices. Biaxial stretch, on the other hand, results in a homogeneous strain field in which circular membranes are used for culturing cells instead of rectangular ones (used in uniaxial ones). The whole procedure relies on applying a force at the center of the elastic membrane using different methods such as dynamic injection, vacuum suction, or fluid underneath the membrane to stretch it biaxially. However, the uniformity of strain field depends on the instrumentation and the type of applied load to stretch the cells. The main drawbacks of this method are the shear stress during stretching due to liquid movements on top of membrane and the difficulties involve in monitoring cells during the procedure due to the vertical movements at the center of membrane (Sotoudeh et al., 1998). M. S. Thompson et al. has studied this issue by using a FX-4000 cell stretcher and using computer simulation to address fluid-substrate interaction for the first time. He showed that shear stress is inhomogeneous which increases on the radius of circular membrane, linearly. It's been proved that shear stress

magnitude is linearly dependent on frequency which in turn resulted in a quadratic dependency of shear stress rate to frequency (Thompson et al., 2011). The mostly used biaxial stretching designs are:

2.3.2.1 Stretching cells cultured on circular smooth flexible membrane using apparatuses. The instrument that was introduced by Sotoudeh et al. in 1997 consisted of 3 plates carrying their components: the stationary plate, fixed plate, and the bottom plate. Sotoudeh has showed that the design had the advantages of uniformity as well as homogeneous equibiaxial strain field over previously reported designs. The mobile plate (Top plate) had four circular holes around the center which let the membranes to be fixed on top of these holes, being clamped to the surface of the plate using a silicon O-ring. Also, the plate was connected to the dc gear motor through a cam fixed at the center of it. The fixed plate (Middle plate) had also four holes around the center carrying indenter rings which were screwed in the holes and fixed tightly using aluminum nuts. This plate was connected to the top plate through stainless steel posts using self-aligning bearings to keep the plates level during the procedure and also connected to the bottom plate by connecting bars at the corners of the plate. The bottom plate was carrying the DC gear motor, Aluminum blocks and their precision ball-bearings, flexible coupling, and cam. Ball bearings transferred the movement to the cam via flexible coupling and also their shaft. Following this procedure, the cam also transferred the rotary movement of the DC gear motor to up and down movement of the mobile plate which in turn resulted in a sinusoidal motion of the top plate while the indenter rings were stretching the membranes (Sotoudeh et al., 1998). Karumbaiah (Karumbaiah et al., 2012) had also used CSD (Cell Stretch Device) in 2012 with almost the same principles as that of Sotoudeh et al. with some small modifications in the design.

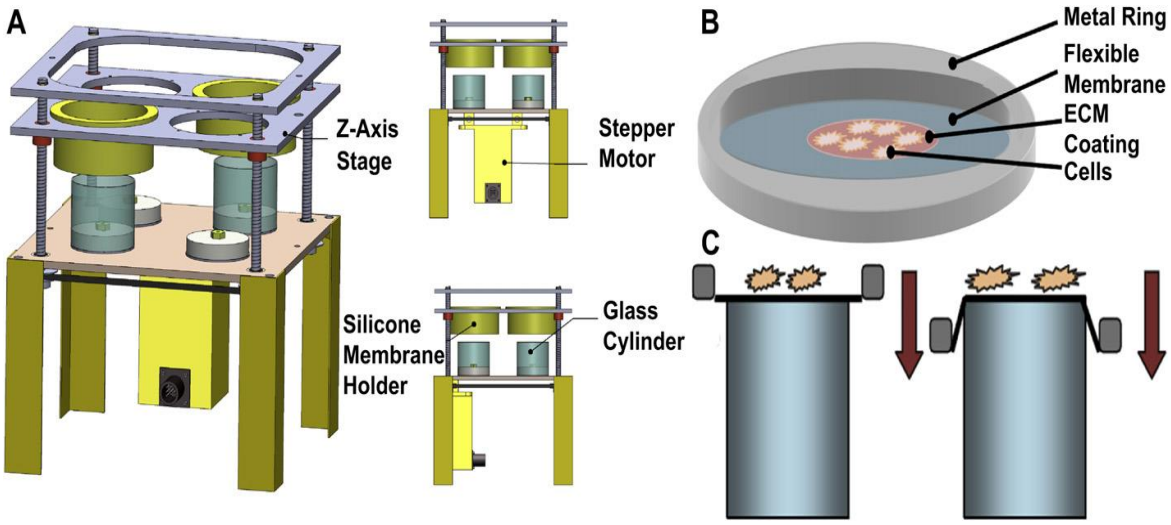


Figure 6. A schematic of the biaxial stretching design used in 2012 (Karumbaiah et al., 2012)

2.3.2.2 Stretching cells cultured on circular smooth membrane with a small center hole using apparatuses. Stretching cells cultured on flexible membrane with a small hole at the center is also of interest due to depicting a complex mechanical environment which is more similar to what is happening in vivo (G.David, 2004). In this case, cell migration is expected due to stretching gradient. S. Yazdani (Yazdani-Beioky, 2010) had used a stretching device with almost the same principle as that of Sotoudeh et al. (Sotoudeh et al., 1998) to stretch stained cells cultured on silicone membrane with a hole at the center for studying cell migration with respect to period of exposure to mechanical stimuli. Cells were imaged during procedure by employing a microscope at the bottom of the whole instrumentation.

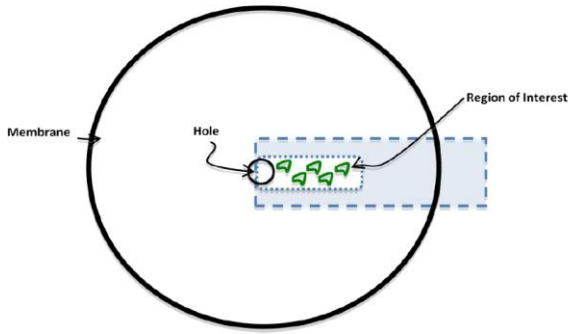


Figure 7. A schematic of the biaxial stretching design used by Yazdani (Yazdani-Beioky, 2010)

2.3.2.3 Stretching cells cultured on cruciform smooth flexible membrane using apparatuses. J. D. Humphrey et al. and Hu et al. had used a custom built tensile tester in 2008 and 2009 respectively, with cruciform membrane (instead of popular circular ones) which was the same as Norton et al. (1995) in design and principles (Hu et al., 2009; J. D. Humphrey, 2008). The instrument fit inside incubator and was made of stainless steel and polycarbonate. The specimen was in cruciform shape and could be made by attaching four suture brackets at four boundaries of square membrane using porous polyethylene. Considering the fact that these brackets were distributing force into the specimen and to have a biaxial stretch over membrane, size of the brackets had to be the same. These suture brackets were connected to load cells which themselves were attached to load plates via push rods surrounded by compression springs. Desired stretching was applied to specimen employing a set of four cams, gearboxes system and the amplitude of stretching was controlled using a DC motor. The rotary movements of cams were converted to a back and forth movement of push rod at the midpoint of each cam which in turn resulted in biaxial stretch of specimen. Also the specimen was set on a glass slide which permitted for intra-vital imaging by using a microscope stage of NLOM system (Hu et al., 2009; J. D. Humphrey, 2008; Waldman SD, 2005).

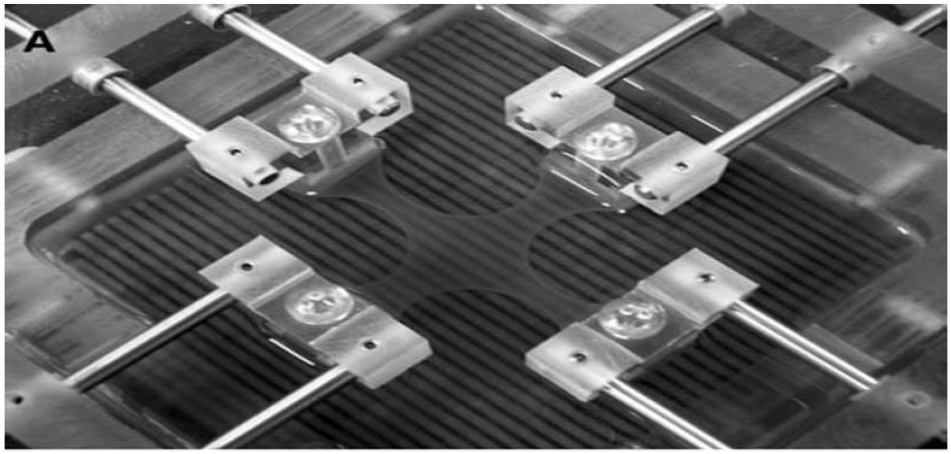


Figure 8. A schematic of the biaxial stretching design used by Jin-Jia Hu et al. (Hu et al., 2009)

2.3.2.4 Stretching cell cultured on circular smooth membrane using vacuum suction.

While using template displacement had been used as a promising way to stretch cells cultured on circular flexible membranes, the tendency of using vacuum suction as a novel way for stretching cells increased in the last decade. There are two basic vacuum stretching approaches. The circular membrane can be fixed on a hole using clamps and when vacuum suction applies at the bottom of membrane, it's been stretched downward. Repeating the procedure, results in a cyclic stretching of cells (T. D. Brown, 2000). In the other method, flexible circular membrane is set on a frictionless surface and vacuum suction applies at parts close to edges of the membrane which results in a homogeneous biaxial stretching at center of circular membrane (T. D. Brown, 2000). The most popular stretching devices based on vacuum suction are Flex products. FX-4000 Tension System is a computer-regulated bioreactor designed and was used to apply mechanical forces to flexible bottomed cell culture dishes by using vacuum pressure. Generally, cultured cells on 6-well flexible bottom plates (Bio-flex plates) could be exposed to either uniaxial or biaxial uniform strain through computer-controlled vacuum forces (Theresa R. Cassino, 2012).

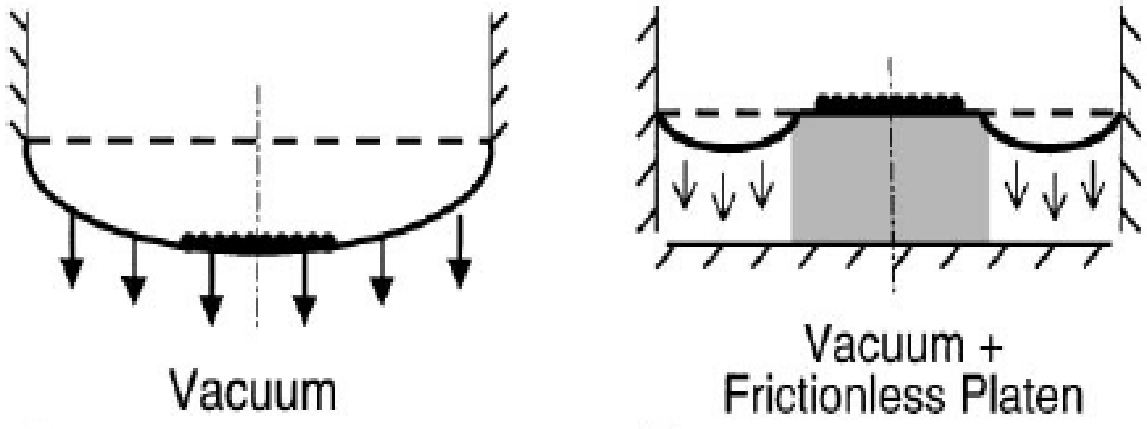


Figure 9. A schematic of the mostly used biaxial stretching systems (T. D. Brown, 2000)

Table 1

Typical advantages and disadvantages of two general types of stretching systems

Stretching systems	Advantages	Drawbacks
Uniaxial	1- Capability of cell stiffness study using micropipettes method. 2- Easy to monitor cells during the stretching process. 3- Capable to study the relation of cells alignment/force direction	1- Existence of compression stress. 2- Inhomogeneity of strain field.
Biaxial	1- Uniformity of strain over the membrane. 2- Homogeneous strain field.	1- Existence of shear stress due to liquid movements during stretch. 2- Difficulties in monitoring cells during the stretching process. 3- Existence of bending stress when the membrane bends over glass indenters.

CHAPTER 3

Methodology

To investigate cellular responses to various types of mechanical environments, cells were seeded on collagen coated silicon membrane. The membrane was then exposed to two types of equi-biaxial forces: cyclic sinusoidal force and cycle-by-cycle variable sinusoidal force. Fluorescent microscope was employed for observation of viability, proliferation and actin filament of cells after short-time (6 hours) and long-time (24 and 48 hours) stretching of membrane.

3.1 Cell Culture

3T3/NIH fibroblasts cell line were used mainly because they are primarily responsible for synthesizing the ECM of tissue, and consequently are being exposed to different types of mechanical forces in *vivo*. Considering the fact that fibroblast cells have to stick to the surface of culture dish to grow, the maintenance of confluent cell culturing on flexible silicon membrane became a vital part of the experiment. Pre-established cell culturing methods through sterile techniques were employed as previously used. Briefly, NIH/3T3 fibroblast cells were cultured in cell culture media consisting of Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum and 1% antibiotic. Cells were passaged onto PDMS dishes, specifically designed for this experiment, after being grown to 80-90% confluence in 25cm^2 flasks in humidified environment (37^oC) of incubators with 5% CO_2. Seeding density for cells on the PDMS dishes was kept at around 2000 cells/cm^2.

3.1.1 Preparation of PDMS dishes. The designed circular dishes consist of silicon membrane (SMI silicone sheeting, .010" NRV G/G 40D 12" X 12") as the flexible bottom with a thick enough PDMS wall around it for carrying medium purposes. The PDMS mixture was made

through mixing PDMS base and curing agent in the ratio of 10:1 which was followed by pouring it properly around the silicon membrane and curing it in the oven at 75^{o}C for 30 minutes. The dishes were then autoclaved. To prepare the flexible-bottomed dishes for culturing 3T3 cells, the silicon membrane had to be hydrophilic. A plasma-etch machine was employed to increase the hydrophilicity of silicon membrane through proper plasma treatment process (200 RF power and 30 seconds duration), followed by immediate coating of a layer of Collagen type-I (Cultrex® Rat Collagen I) film to increase cell adhesion to the silicone membrane. The collagen coated membranes were incubated in the same condition as that of live cells for about 12 hours (or over-night) and then the collagen film was removed and the samples were washed 3 times with D-PBS 1X just before passaging cells to the designed dishes.

3.1.2 Passaging protocol. 3T3/NIH fibroblast cells were removed from 25cm^2 flasks using 3ml of trypsin (gibco by life technologies, 1374942) and neutralizing in 6ml D-PBS 1X after 2 minutes of incubation. D-PBS 1X which is usually used for washing purposes during cell passaging procedure was prepared using D-PBS 10X (SIGMA, RNBB9331). This is, then followed by 5 minutes centrifuging at 1100 rpm and re-suspension of cells in 1ml growth medium. To control the density of passaging, the cells were counted using a hemocytometer as shown in *Figure 10*. The seeding density was kept at about 2000 cells/cm^2 which is about 88,500 cells across the entire membrane surface (44.18cm^2). Cells were then allowed to proliferate for at least 24 hours and no more than 36 hours to form cell-matrix connections and cell-cell networks before exposure to the mechanical force.

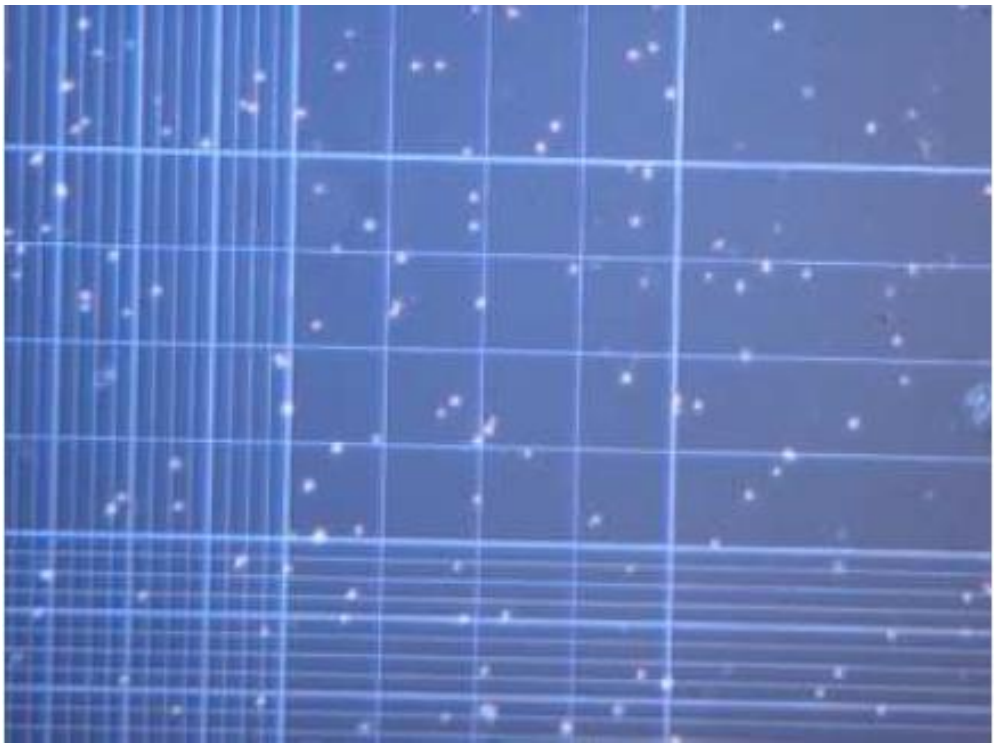


Figure 10. Cell counting procedure using hemocytometer

3.2 Biaxial Stretching System

The custom built cell stretching device is similar in the design and principles to previously reported systems with a few modifications. The whole instrument is capable of operating and fitting inside an incubator (37^{o}C and 5% CO_2) to provide the proper conditions for cell growth. The whole system was sterilized with alcohol and also exposed to UV light in a laminar flow hood for at least 3 hours before each experiment. All screws and nuts were used, are biocompatible stainless steel and were all sterilized along with glass indenters through autoclaving process.

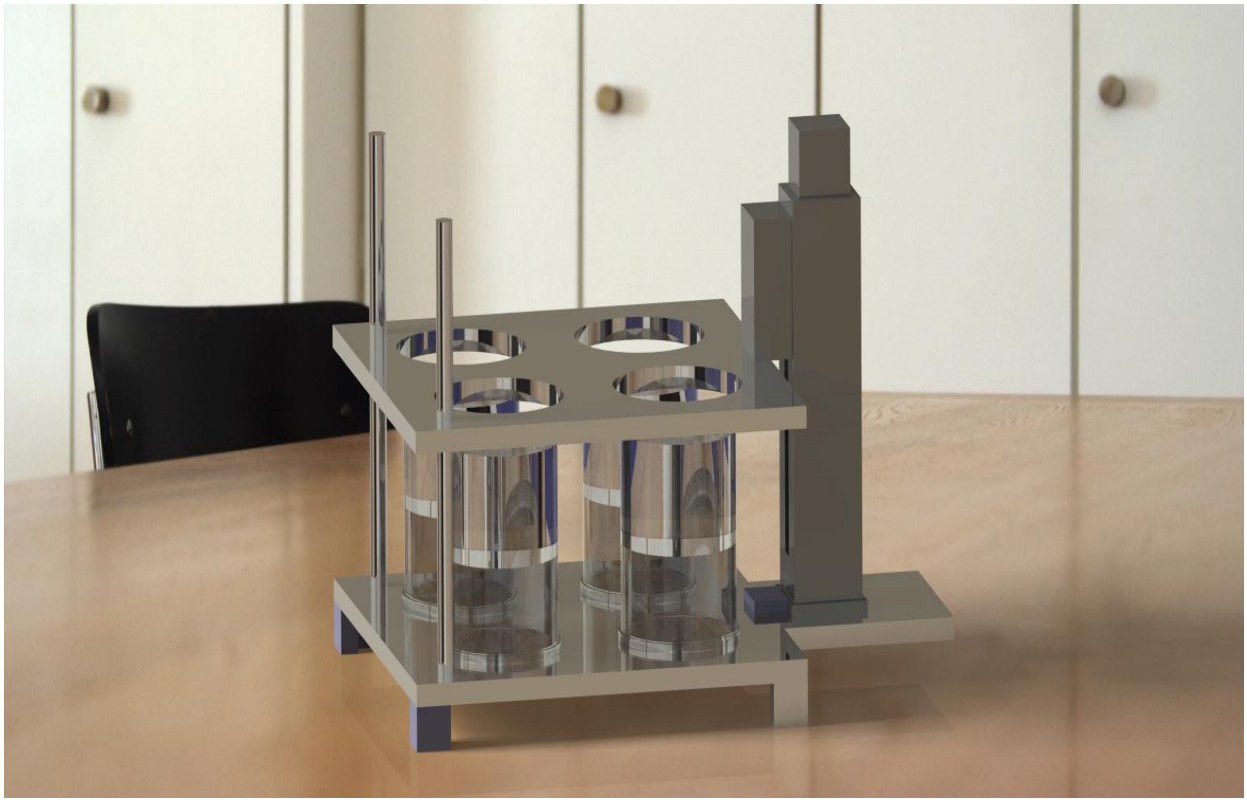


Figure 11. Rendered schematic of the stretching device designed in Catia software

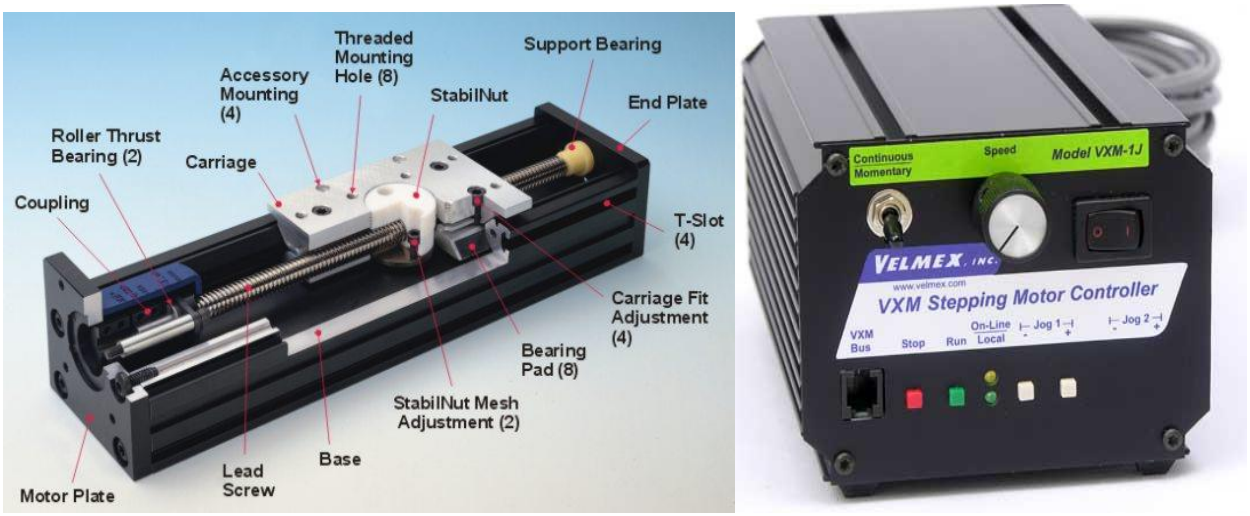


Figure 12. Velmex stepper motor tower and the controlling system

3.2.1 The design of stretching device. The stretching system is composed of two Aluminum plates carrying their own components and the stepper motor tower (Velmex) as shown in *Figure 12*. The stationary (bottom) plate supports two Aluminum rods and the stepper motor tower by providing a base for them while maintaining the position of four hollow glass cylinders at the bottom of holes of the top plate. The mobile (top) plate has 4 holes around the center on which PDMS dishes were fixed. The motion of the plate was controlled through the stepper motor and the tower's apparatus up and down movements while two straight aluminum rods support the motion. These up and down movements of mobile plate result in stretching of silicon membranes (one was control sample, no stretch, and the other three were stretched) over glass cylinders and consequently stretching of the cells cultured on it.

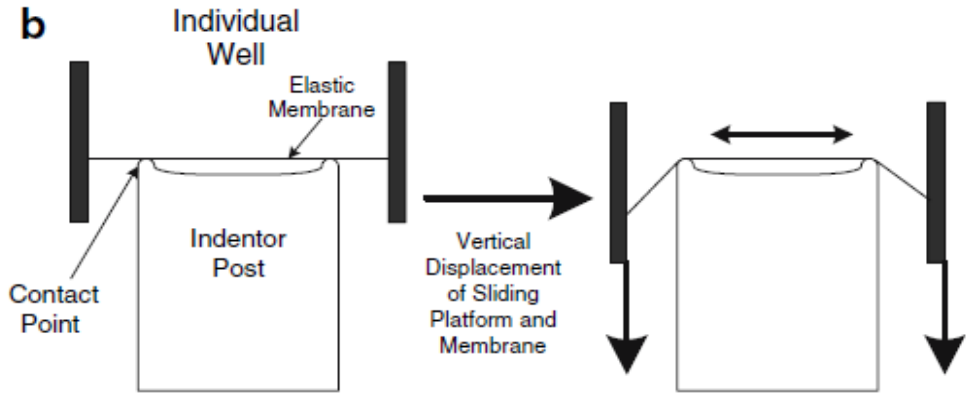
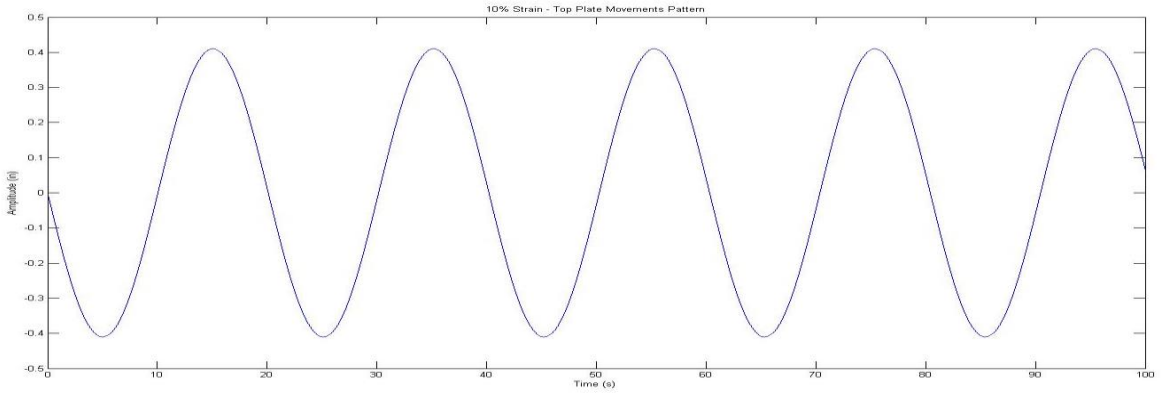


Figure 13. Schematic side view of vertical movement of membrane at contact and stretched points

3.2.2 Stretching patterns and controlling system. The stepper motor tower was responsible for controlling the movement of the mobile plate and consequently the amplitude of stretch. Cyclic biaxial force with a constant amplitude cycle-by-cycle was programmed via Cosmos software for studying cells responses to their mechanical environment (the patterns of

the two types of forces are shown in *Figure 14*). The applied stretch was as low as 5%, 7.5% and 10% with a frequency of 0.05cycles/second (to minimize the effect of shear stress that results from liquid movement on top of the membrane). Cells were exposed to mechanical stimuli for both short period of time (6 hours) and long period of time (24 hours) (One of the codes is attached as an example in *Appendix A*). The motion of the stepper motor was measured and it was found that every 50,000 steps equal to 1 inch in vertical movement of the top plate.

(a)



(b)

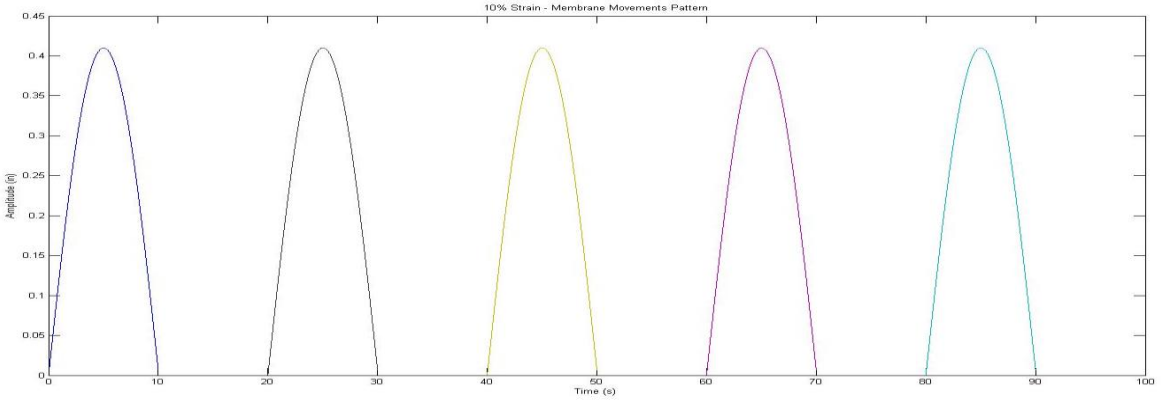


Figure 14. The movement patterns of (a) the mobile plate (down and up) and (b) the membrane (stretch and rest)

2.1.1.1 Measurements of strain field. A previously published method was used for the calculations of the degree of applied strain and displacement. Briefly, the interactions between glass intenders and membrane were predicted using Lagrangian strain method as:

$$\mathbf{E} = \frac{\mathbf{1}}{\mathbf{2}}\left[\left(\mathbf{1} + \frac{\boldsymbol{h-c}}{\boldsymbol{r}}\right)^{\mathbf{2}} - \mathbf{1}\right] \qquad \text{Where} \qquad \mathbf{h} = \sqrt{c^2 + d^2}$$

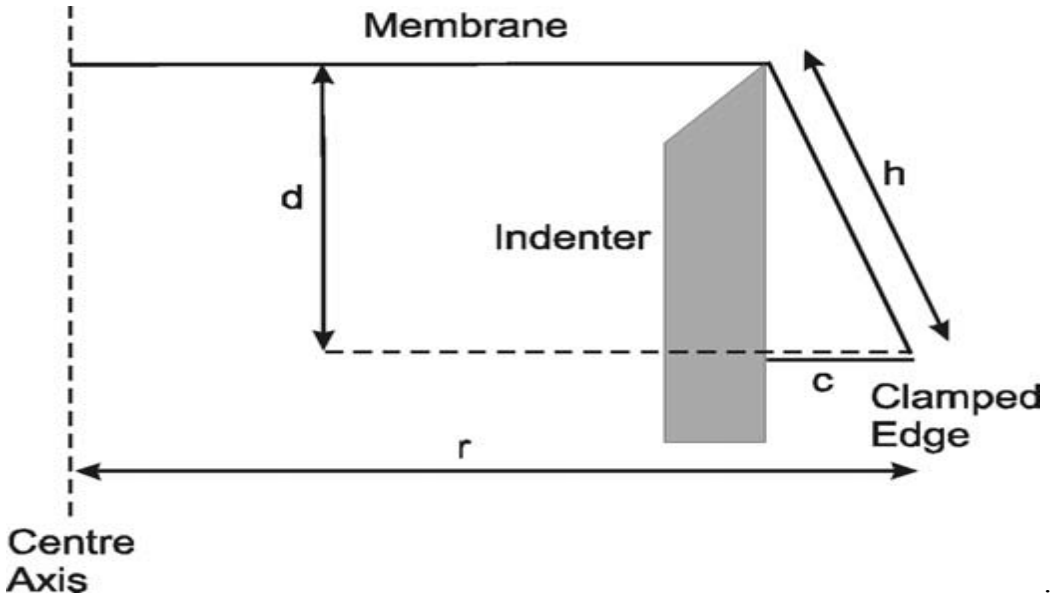


Figure 15. Schematic diagram of stretched membrane and geometrical analysis of strain

3.3 Microscopic Imaging

Cells were observed using an inverted confocal microscope. Two types of imaging were done in this experiment: live cell and fixed cell. A Live/Dead cell assay kit (molecular probes by life technologies, R37601) was used to image cells at 10X and 40X magnifications for analysis of viability and spreading factor. The staining process with live cells in green and dead cells in red color was done following the protocol of the kit. Since the membrane was too big for the

confocal stage, a part of the membrane that fit on the microscope's thin slides was cut along the diameter of the circular sample. Also, a Molecular Probes® Image-IT® Fix-Perm kit from Life Technologies™ was used to fix cells following the protocol of the kit and image them in 20X magnification to study the directionality changes in actin filament (green) and to analyze the size of the cells' nucleus (blue).

To be able to have a better analysis of cells behavior under applied mechanical forces, a rectangular part of the circular membrane was cut on the diameter and all the analysis were done for 4 independent spots as shown in *Figure 16*.

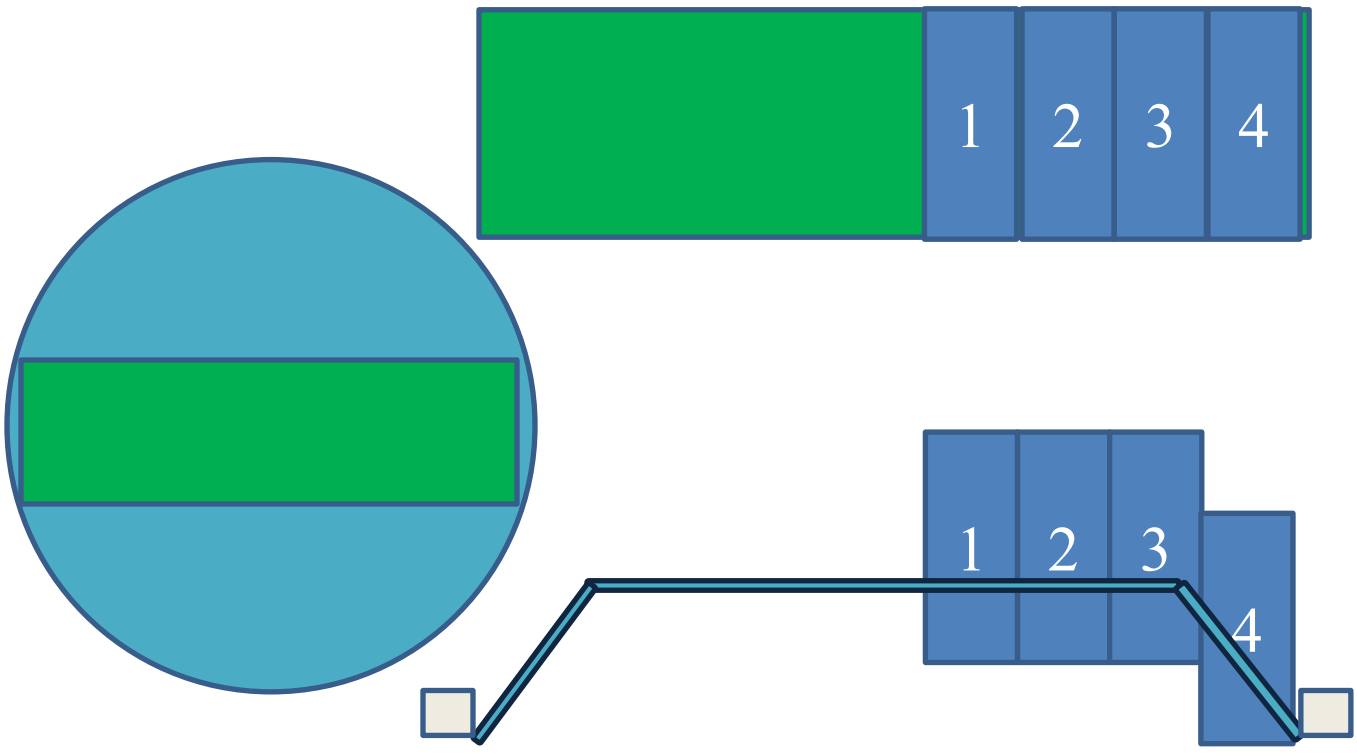


Figure 16. Schematic diagram of the circular membrane in blue, the rectangular part in green and also depicting four important spots on the rectangular part from both top view and side view

Table 2

Strain percentages and the exposure times of applied mechanical force

Periods / **Force**	Regular cyclic sinusoidal forces		
6 hours	5% strain; 0.5571 in (amplitude)	7.5% strain; 0.6994 in (amplitude)	10% strain; 0.8268 in (amplitude)
24 hours	5% strain; 0.5571 in (amplitude)	7.5% strain; 0.6994 in (amplitude)	10% strain; 0.8268 in (amplitude)

CHAPTER 4

Results

Cells were successfully stretched and survived from exposure to 5%, 7.5% and 10% for periods of 6 and 24 hours. Two types of imaging (live cell and fixed cell) were done after each experiment to determine the effect of mechanical stimuli on viability, actin filament alignment, spreading factor and migration of cells. Also, data showed with the mean ± SD from at least 50 cells for each sample, all data represented by averaging three independent samples.

4.1 Live Cell Imaging

Live/dead cell assay kit was used to do live imaging of the cells immediately after stretch (the protocol is attached to the kit). 10X and 40X magnifications were used for live/dead cell imaging to do viability and spreading factor analysis, respectively. Live cells are green while dead cells are red in all images.

4.1.1 Viability. Viability was measured as the ratio of live cells to the total number of cells for three stretched and control samples. Our results indicate no significant difference in the viability of stretched samples compared to the control and all the differences were in the range of 3% for all strain percentages and exposure times. As a result, there was no evidence of the negative effect of biaxial stretch under applied conditions (applied strain% in range of 5% to 10% and frequency of 0.05 cycles/sec) for 3T3 /NIH fibroblasts.

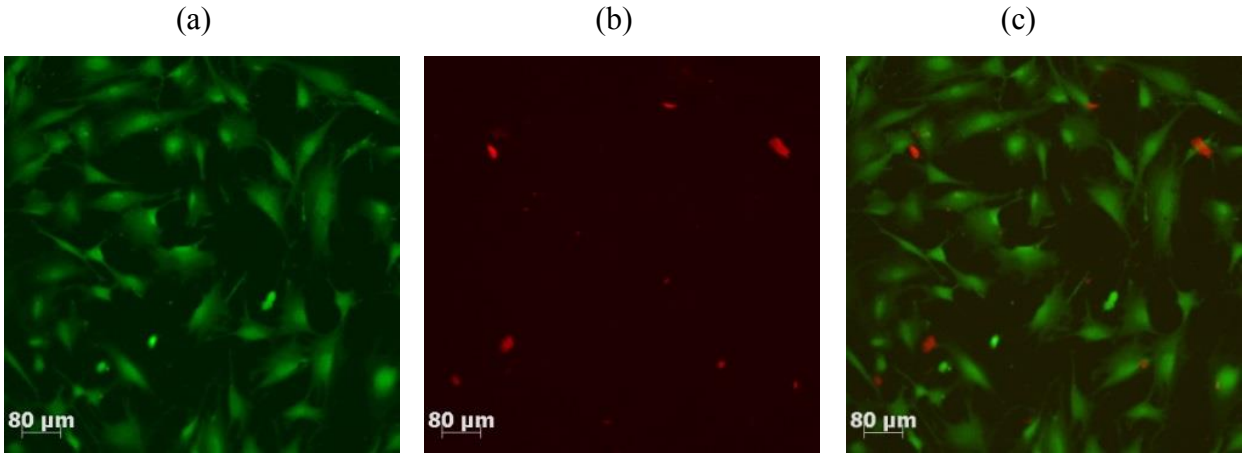
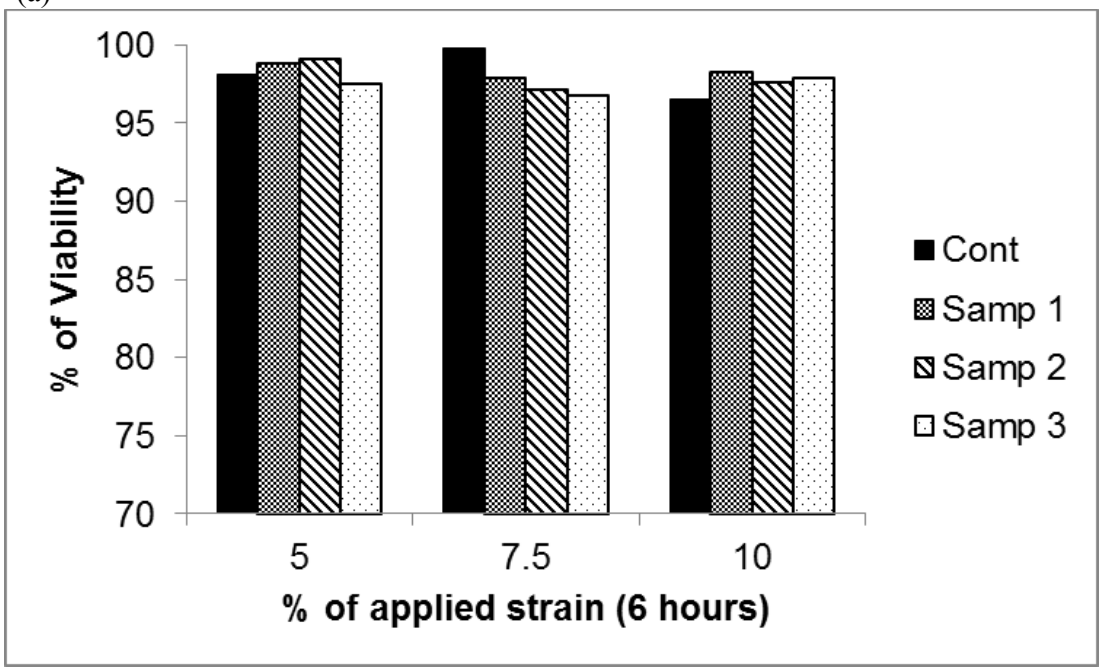


Figure 17. (a) green chamber, (b) red chamber and (c) the merge images were used for viability analysis (10X magnification).

(a)



(b)

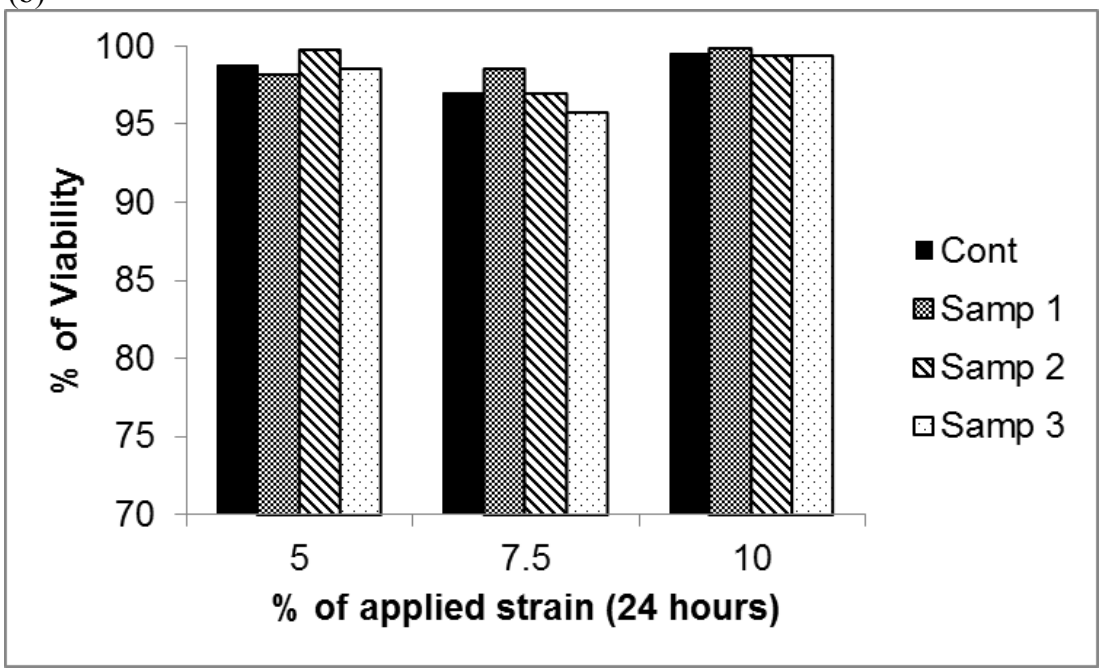


Figure 18. Viability % of control sample (Cont) and the other 3 stretched samples (Samp 1, 2 and 3) were shown for (a) 6 hours, and (b) 24 hours of exposure to 5%, 7.5% and 10% strain.

4.1.2 Proliferation. The total number of cells was counted for the control and stretched samples to understand the effects of stretch on the proliferation rate of fibroblasts. The percentage change of the total number of cells (total # of cells for stretch sample/total # of cells for the control) was depicted with respect to exposure time for stretched samples exposed to 5%, 7.5% and 10% strain. The graph shown, illustrated a slight rise in the total number of cells for the stretched samples as the strain% and exposure time increased. However, our results indicate a great increment in the total # of cells for 7.5% and 10% strain with respect to time. Considering the fact that viability didn't change for stretched samples, this increase in the total number of cells could be an indication of a faster proliferation rate of fibroblasts under biaxial stretch. We also have analyzed images spot by spot (as we have explained in methodology) to further understand the effect of stretch on fibroblast cells.

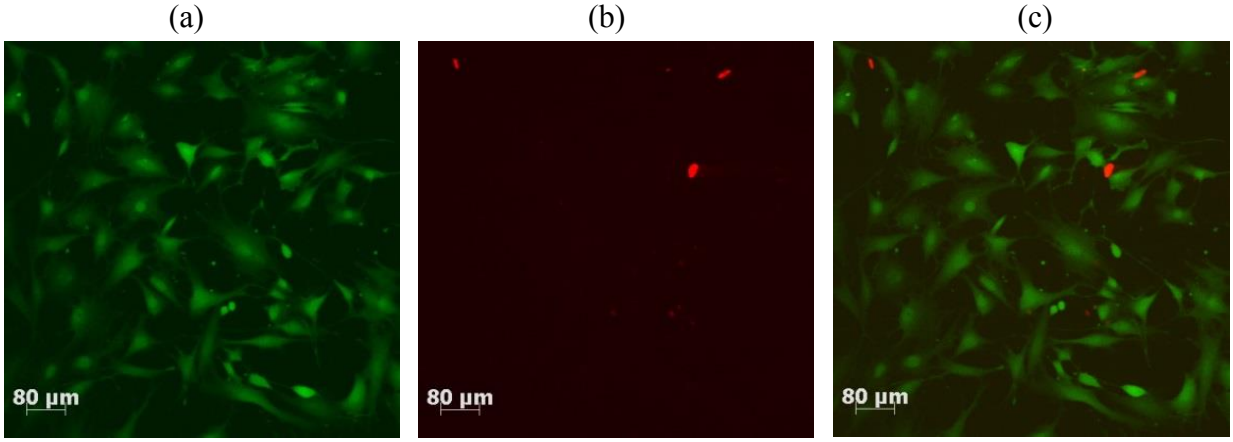


Figure 19. (a) green chamber, (b) red chamber and (c) the merge images were used for viability analysis (10X magnification).

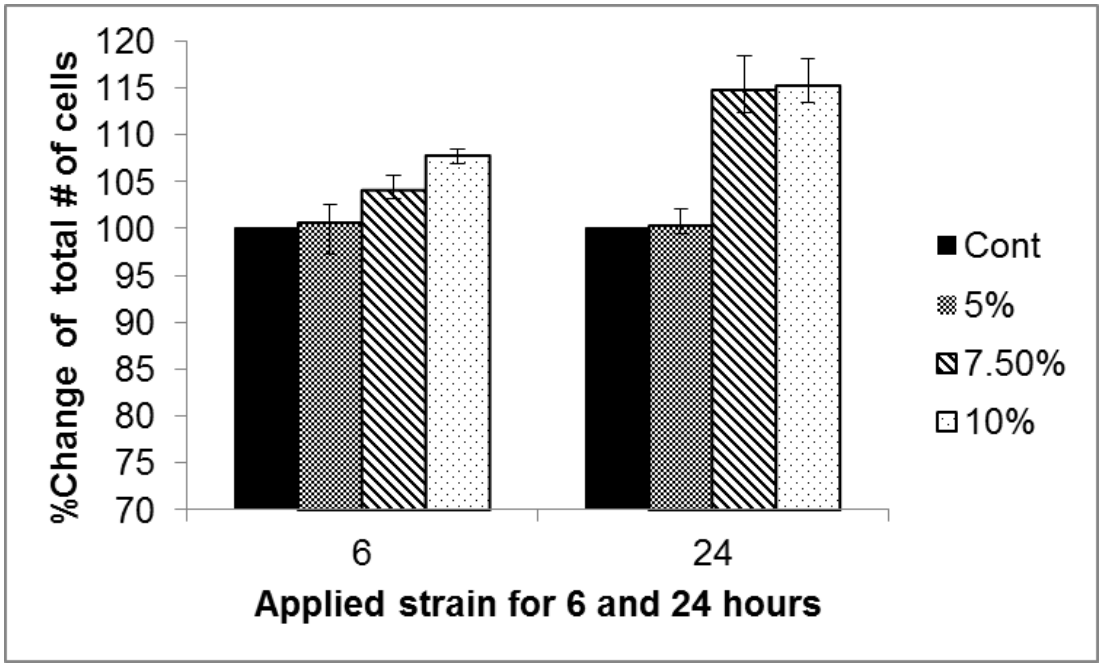


Figure 20. Percentage changes of total # of cells for control (Cont) and 5%, 7.5% and 10% stretch samples.

4.1.2.1 Number of cells/spot with respect to strain %. The represented graphs show the percentage change of the total number of cells (total # of cells for stretch sample/total # of cells

for the control) per spot (as explained in methodology) with respect to different strain percentages and for 6 hours and 24 hours exposure time, respectively. Our results show a huge decrease in the number of cells at spots 2 and 3 and a significant increment at spots 1 and 4 for stretched samples and these differences between the control and stretched samples have increased for higher strain percentages. Since our results showed a rise in the proliferation rate of cells and no considerable changes in viability, these regional changes in the number of cells on the diameter of the circular membrane could be an indication of migration happened for cells from spots 2 and 3 to spots 1 and 4. This migration might have happened as a result of shear stress resulted from liquid movements during stretch and also localized excessive bending stress at the range of the higher stress bending region (spot 3).

(a) (b) (c)

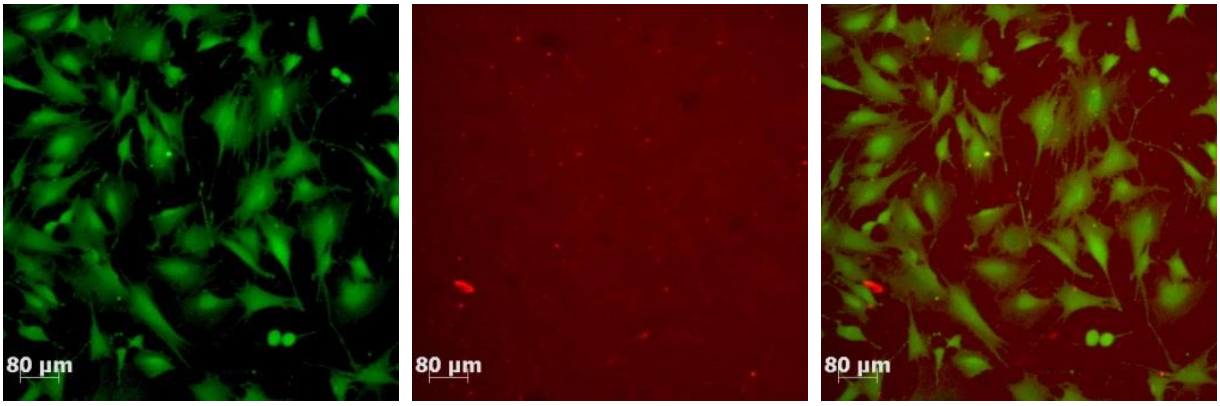
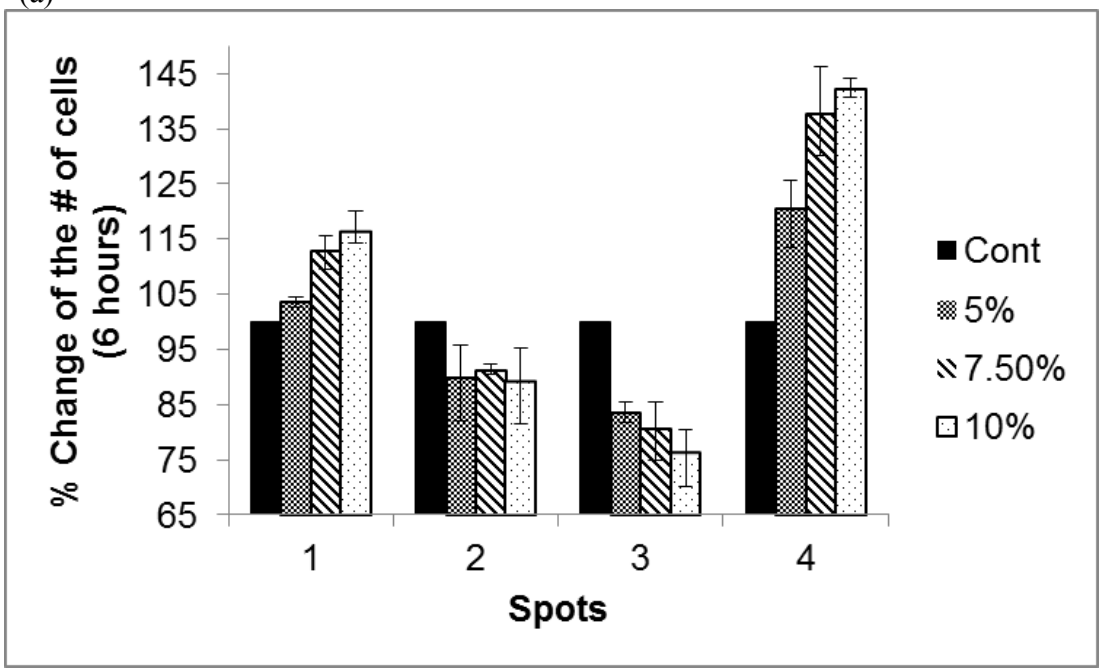


Figure 21. (a) green chamber, (b) red chamber and (c) the merge images were used for viability analysis (10X magnification).

(a)



(b)

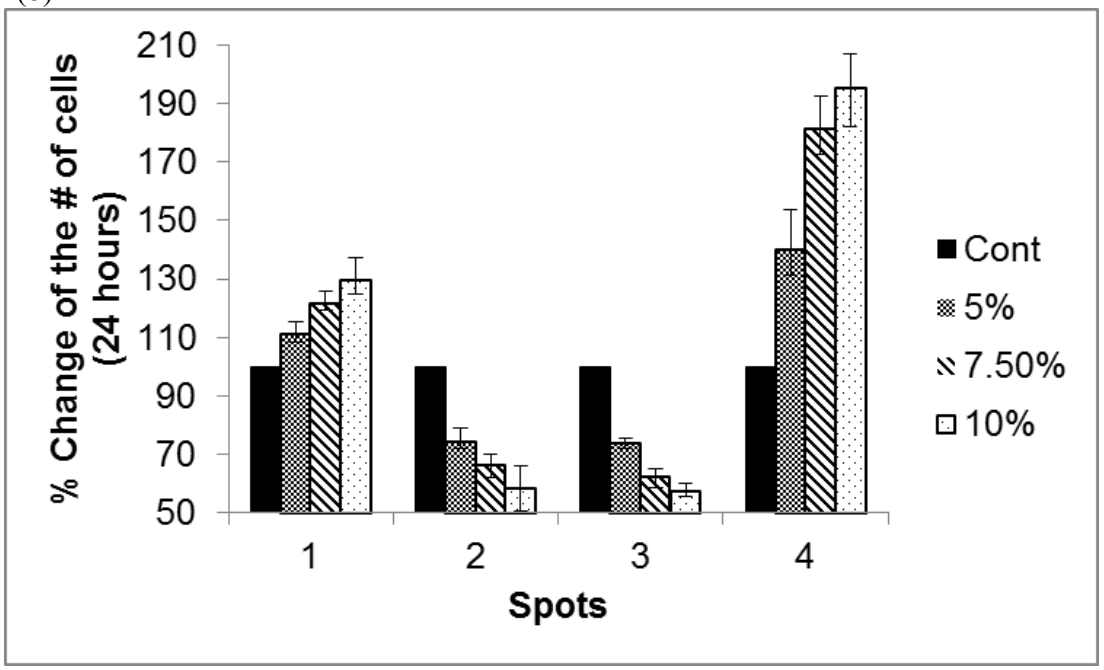
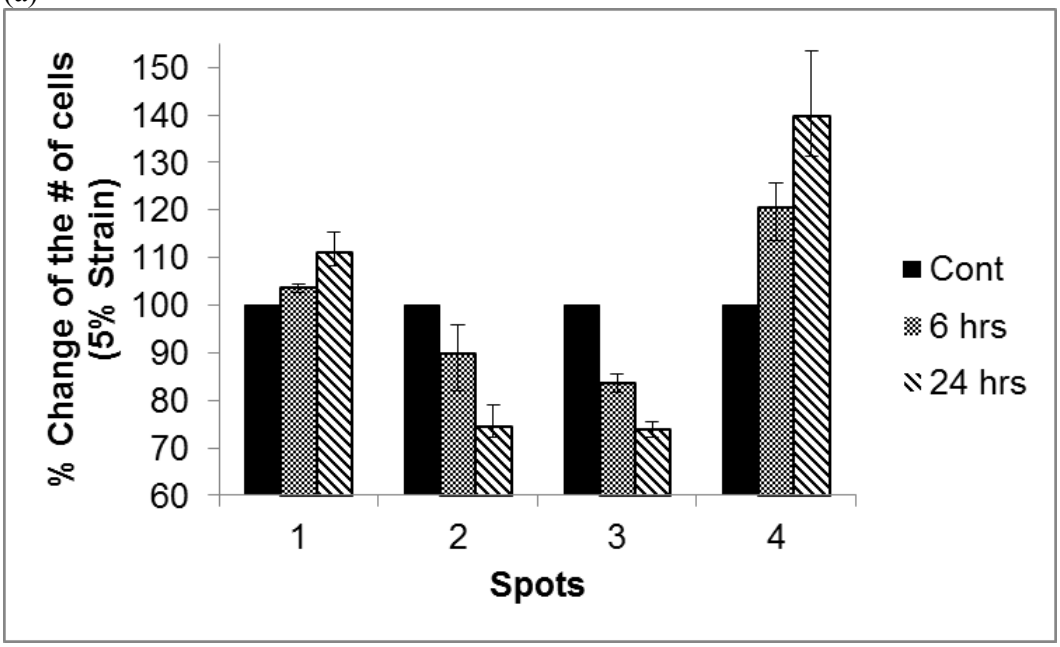


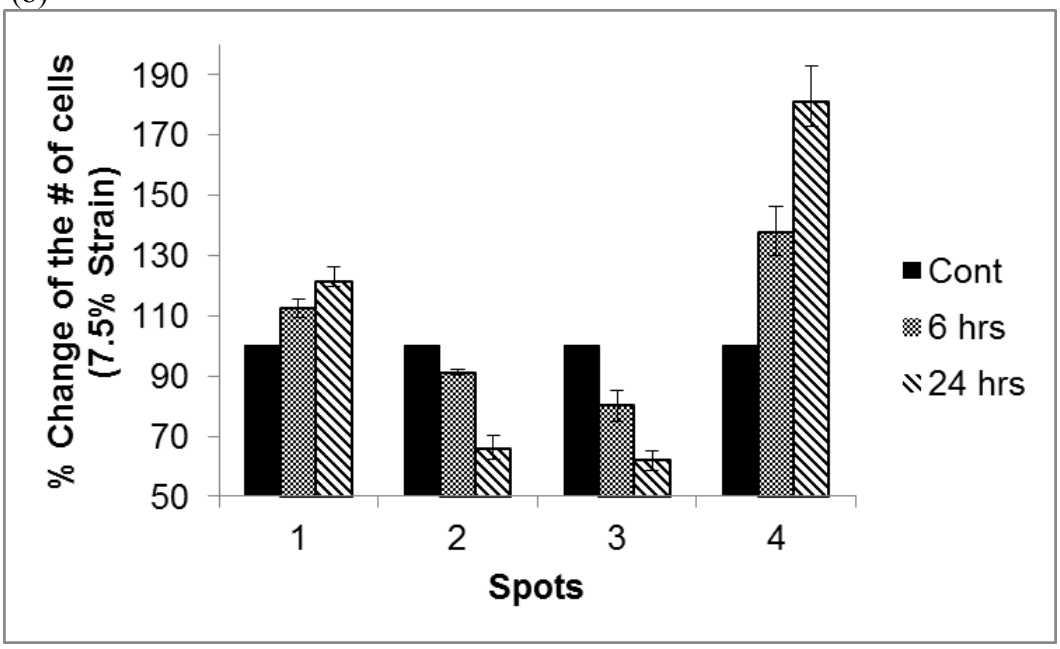
Figure 22. Percentage changes of the total # of cells per spot for stretched samples in comparison with the control sample (Cont) is shown for (a) 6 hours, and (b) 24 hours of exposure to 5%, 7.5% and 10% strain.

4.1.2.2 Number of cells/spot with respect to exposure time. The following graphs are showing the % changes in the total number of cells per spot and with respect to exposure time for 3 different applied strains, to better understand the effect of biaxial stretch on the total number of cells and the relation between exposure time and migration of cells. A rise in the number of cells was observed at spots 1 and 4 as the exposure time increased while the results for spots 2 and 3 is a downward trend. This could be an indication of increase in the migration of cells from spots 2 and 3 with respect to exposure time of biaxial stretch.

(a)



(b)



(c)

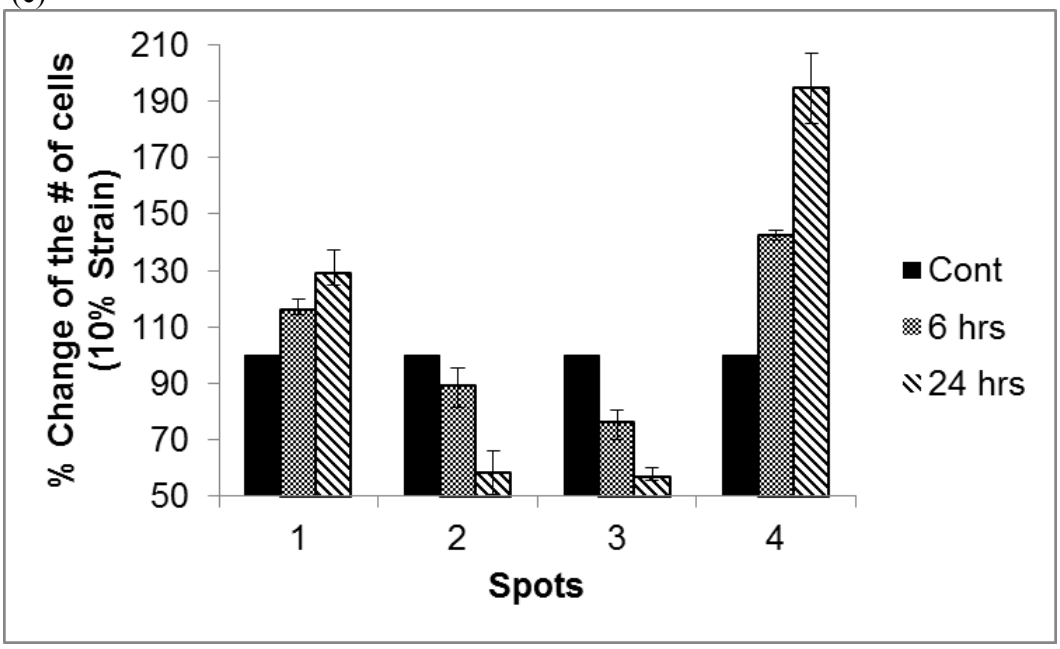
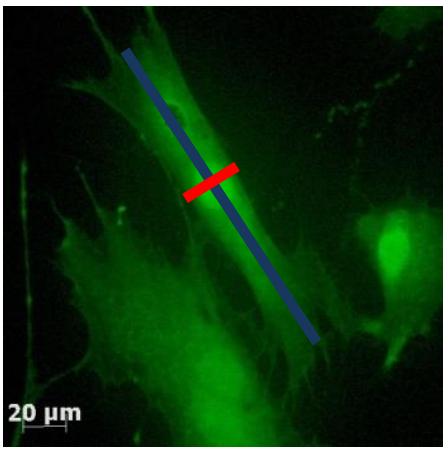


Figure 23. Percentage changes of the total # of cells per spot for stretched samples in comparison with the control sample (Cont) is shown for (a) 5%, (b) 7.5%, and (c) 10% strain for 6 hours and 24 hours exposure time.

4.1.3 Spreading factor. Spreading factor is the ratio of longitudinal to transverse length of the cells which is an indication of cell shape and cell adhesion to the surface of the dish.

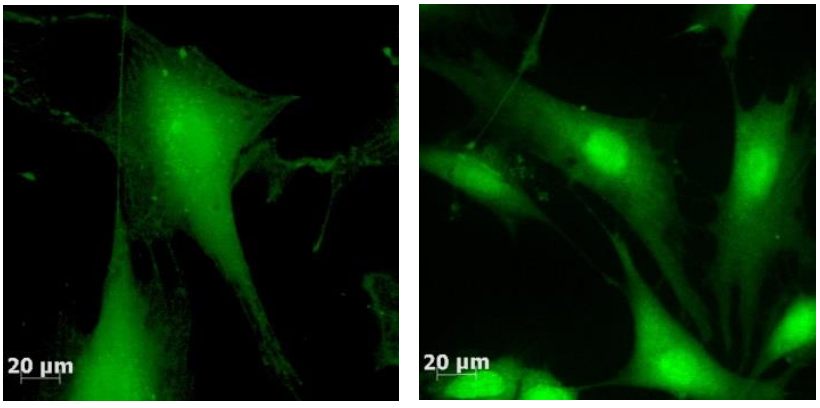


$$S.F = \frac{Length\ of\ the\ blue\ line}{Length\ of\ the\ red\ line}$$

Figure 24. Measurement of the spreading factor of the cells (40X magnification)

4.1.3.1 Percentage changes in spreading factor/spot with respect to strain %. As the graphs are showing, spreading factor has significantly increased under all of the applied conditions while this increase is slightly more at spots 1 and 4 compared to the other two spots (2 and 3). Also, as the strain percentage increased this difference between the spots has widened. These results showed that actin filament of exposed cells at spots 1 and 4 have responded more to the mechanical stimuli compared to the other spots, 2 and 3 which in turn might be a sign of migration of cells happened at spots 2 and 3.

(a) (b)



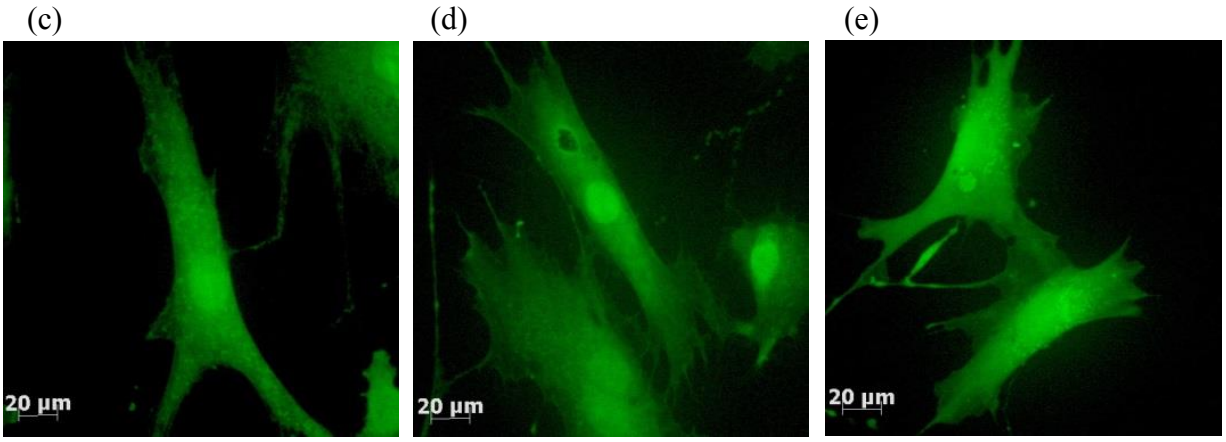
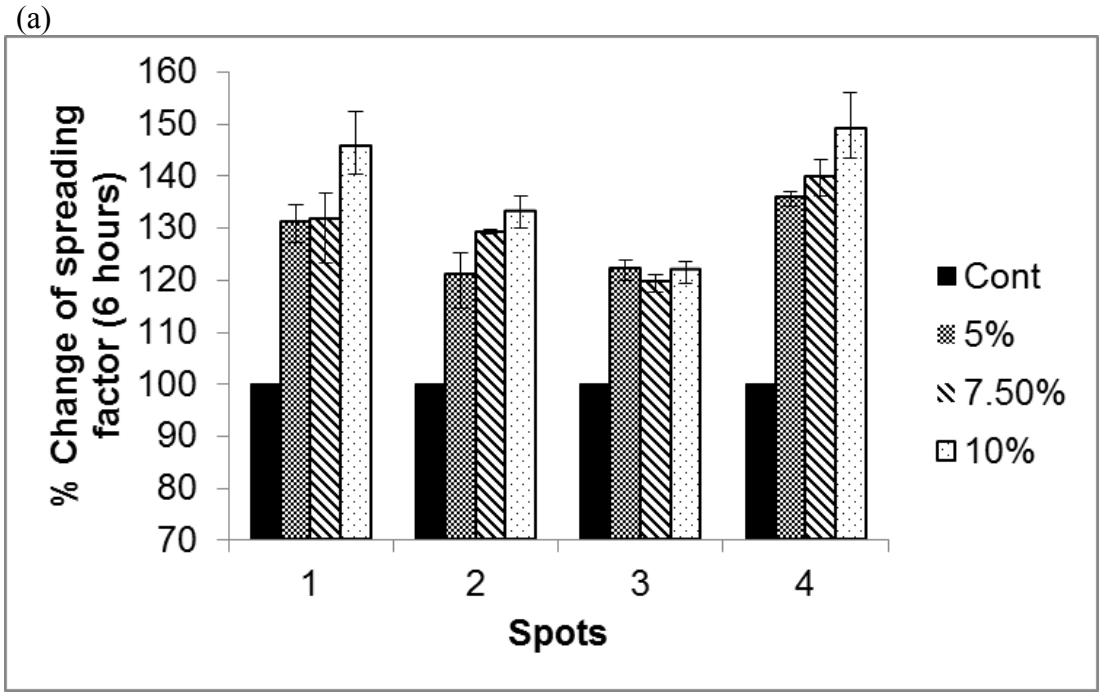


Figure 25. Images of stretched samples for the measurements of the spreading factor of the cells (40X magnification)



(b)

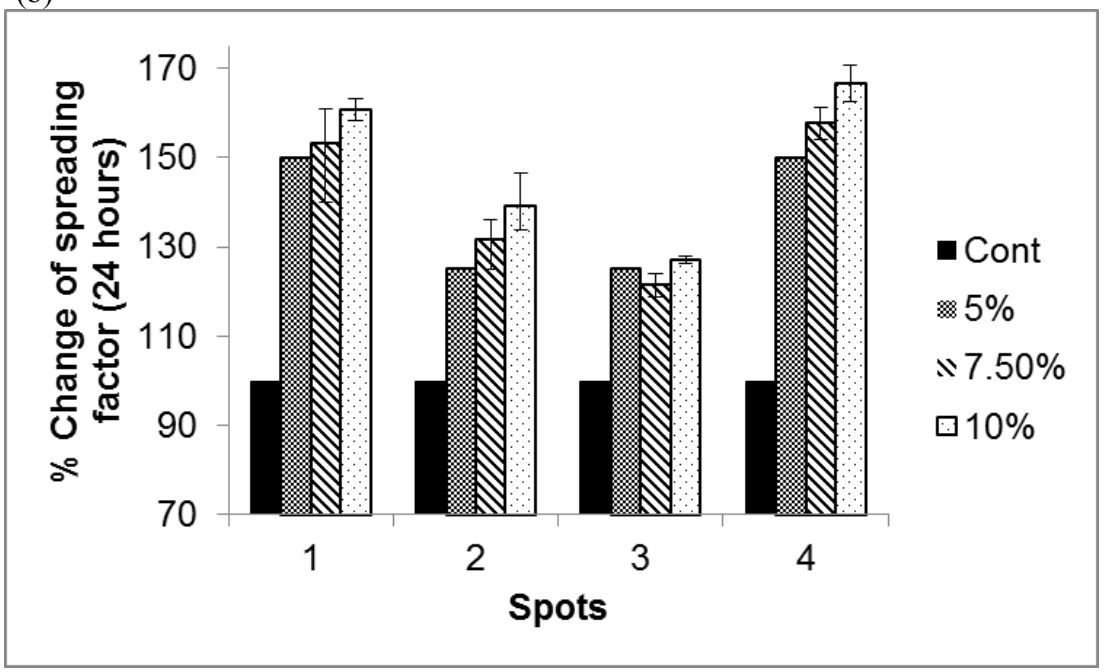
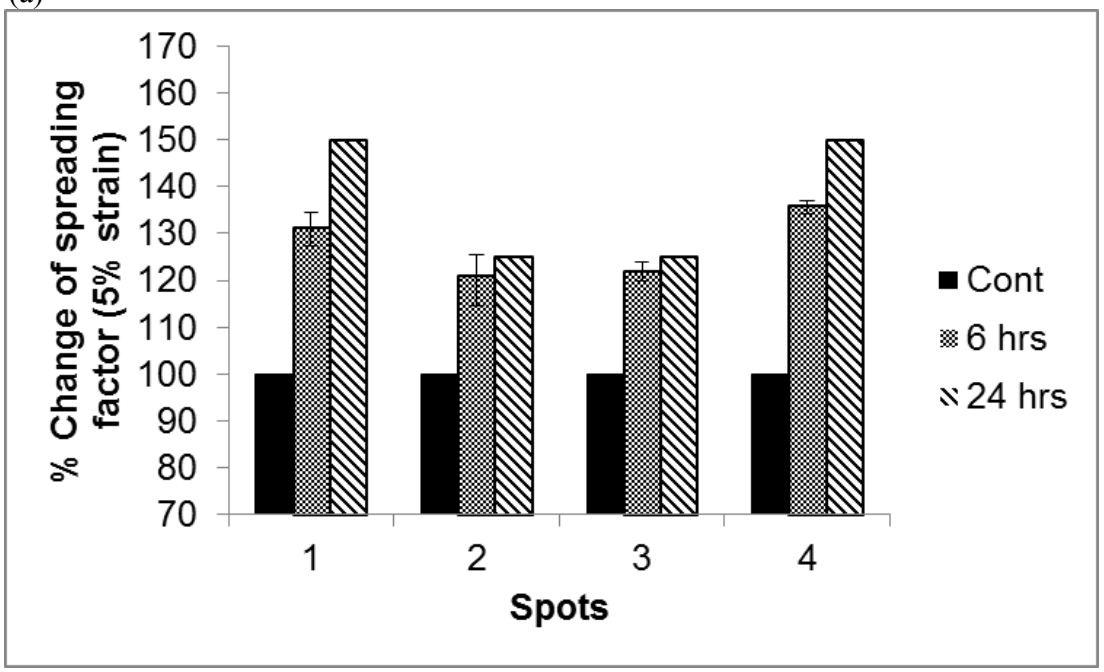


Figure 26. Percentage changes of spreading factor (S.F.) per spot for stretched samples in comparison with the control sample (Cont) is shown for (a) 6 hours, and (b) 24 hours of exposure to 5%, 7.5% and 10% strain.

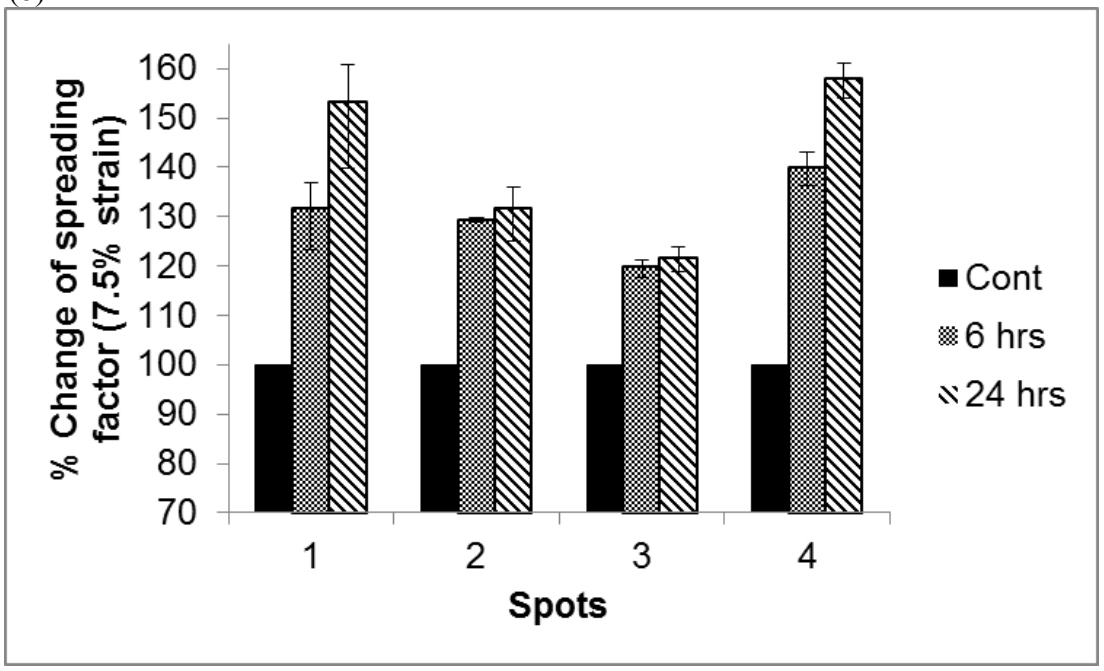
4.1.3.2 Percentage changes in spreading factor/spot with respect to exposure time.

Spreading factor (S.F.) has increased with respect to the exposure time while the amount of this increase is much more significant at spots 1 and 4 compared to the other two spots which show more response in the actin filament of cells at these spots.

(a)


% Change of spreading factor (5% strain)
170
160
150
140
130
120
110
100
90
80
70
1
2
3
4
Spots
Cont
6 hrs
24 hrs

(b)


% Change of spreading factor (7.5% strain)
160
150
140
130
120
110
100
90
80
70
1
2
3
4
Spots
Cont
6 hrs
24 hrs

(c)

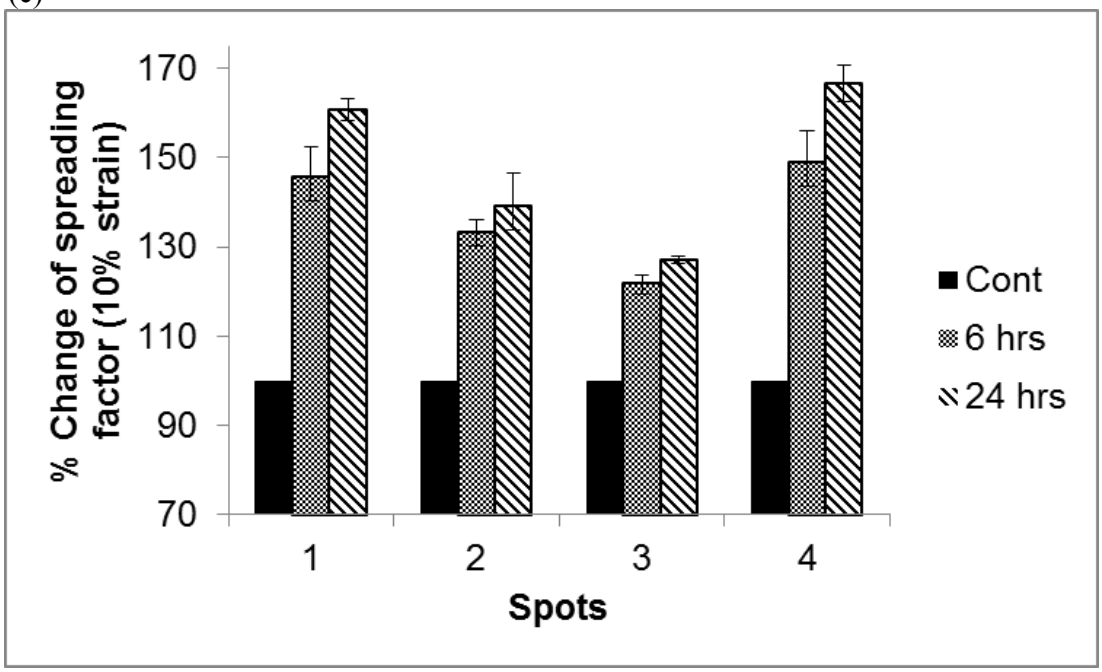


Figure 27. Percentage changes of the spreading factor (S.F.) per spot for stretched samples in comparison with the control sample (Cont) is shown for (a) 5%, (b) 7.5%, and (c) 10% strain for 6 hours and 24 hours exposure time.

4.2 Fixed Cell Imaging

A Molecular Probes® Image-IT® Fix-Perm kit from Life Technologies™ was used to do the fixed cell imaging immediately after stretch. 20X magnification used for nucleus size and actin filament alignments analysis. Actin filament is green while nucleus is blue in all images. Our results indicate no considerable difference in the nucleus size of the stretched samples compared to the control which is the proof for the fact that the actin filament has the responsibility to react to the mechanical environment of the cells and nucleus are minimally affected by mechanical stimuli. Also, there was no evidence of actin filaments alignment in response to mechanical stimuli for the system which makes sense with the biaxial nature of applied forces.

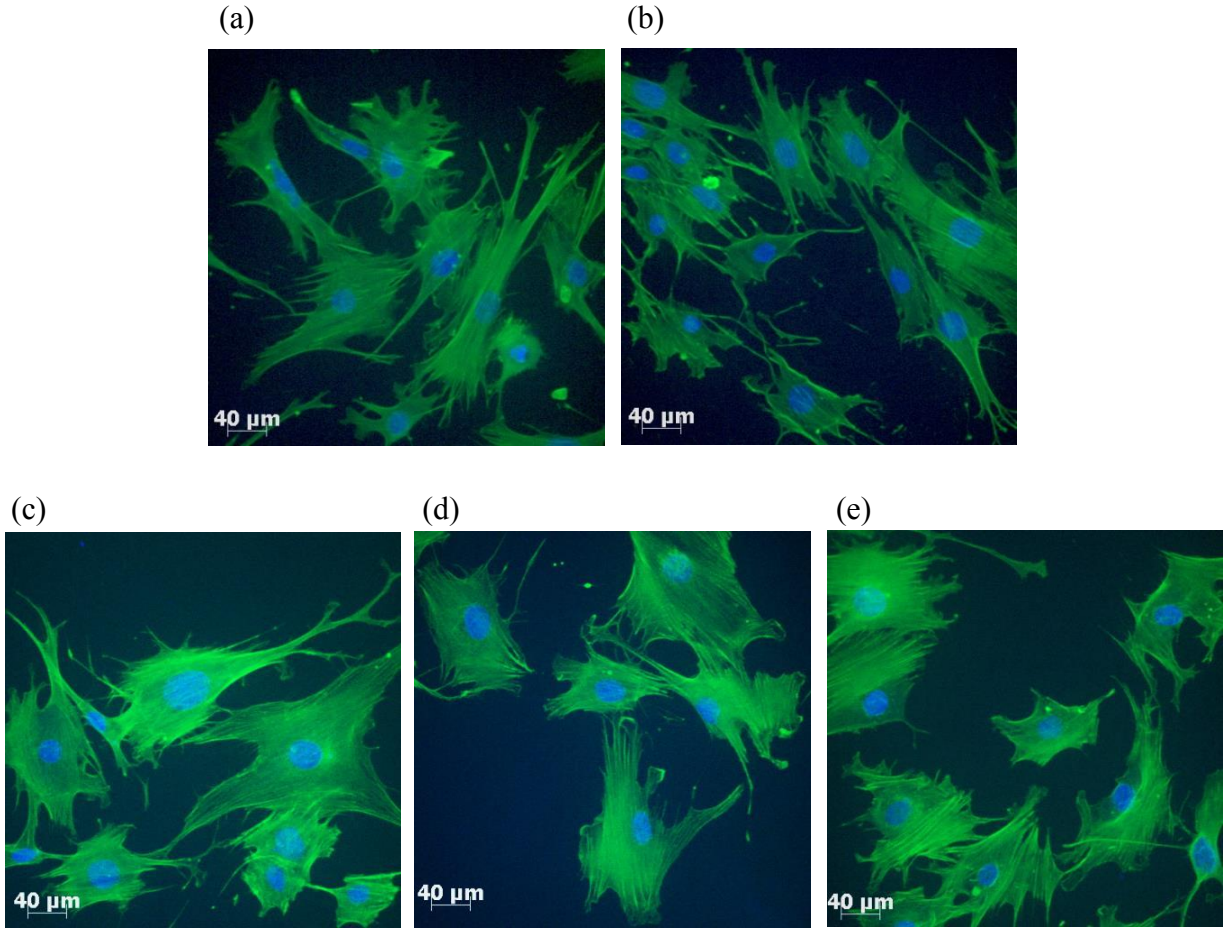


Figure 28. Images of stretched samples for the measurements of the nucleus size and actin filament alignments of the cells (20X magnification)

CHAPTER 5

Discussion and Future Research

Mechanobiology would play a vital role in wound healing and tissue engineering in general if the relations between specific cells types responses and the type of applied forces and conditions are known. Understanding these cell responses is the first step to take control over factors such as viability, proliferation and differentiation of cell eventually.

Cells were successfully cultured and grew on collagen coated silicon membrane and were exposed to 5% to 10% strain for 6 and 24 hours exposure time. While the strain field is uniform and homogeneous all over the circular membrane, the existence of shear stress due to liquid movements during stretching process along with the localized bending stress at the range of bending parts of the membrane over glass indenter (which is definitely more than the strain percentage at the center) result in a complex strain field. However, the effects of shear flow were minimized by choosing frequency value as low as 0.05cycles/sec and also to depict a condition closer to in *vivo*. The imaging process after exposure indicate the adherent of cells to the membrane and no significant changes in viability which makes it possible for stretching over 24 hours (under applied conditions). Live cell imaging was done using an inverted confocal microscope to further investigate cell responses. Also, a slight increase in the total number of cells was observed which indicates a faster proliferation rate for stretching cells in comparison with the control sample. This rise in the total number of cells is related to the strain percentage and exposure time directly which makes it possible to control fibroblast's proliferation rate choosing specific values for these factors.

Analysis of confocal images revealed a downward trend for the number of cells at the range of localized bending stress (spot 3) and an upward trend for the cells at the corner (spot 4) and the center (spot 1) of the membrane. This migration of cells is directly related to the applied strain percentages and the exposure time. While the applied strain field is uniform and homogeneous, the existence of shear stress resulted from liquid movements during stretching process and also the existence of localized bending stress which is definitely more than the applied strain might have resulted in the migration of cells have happened from spots 2 and 3 to the corner (spot 4) and center (spot 1) parts. The shear stress is directly related to stretch amplitude and frequency and for higher values of these controlling factors have the ability to take cells from spots 2 and 3 to the corner and the center via the back and forth movement of the liquid (We have minimize the effect of frequency by choosing frequencies as low as 0.05 cycles/sec). The combination of this shear stress and the localized bending stress might have resulted in a significant migration rate happened from spot 3 to the corner (spot 4) and also a noticeable migration rate from spot 2 to the center (spot 1).

Another important aspect is spreading factor (explain how to be measured in part 4-3-1) to better understand the effects of biaxial forces on actin filaments which are the responsible parts of the cells to react to the mechanical environment changes. The analysis of the confocal images showed a rise in the spreading factor of the cells at all the spots with respect to strain percentages and exposure time which is an indication of actin filament response to mechanical stress. However, the cells at the center (spot 1) and at the corner (spot 4) have shown more significant increases compared to the other two spots (2 and 3) which in turn means the cells at the corner and the center have responded to mechanical stimuli more than the others. This might

be an indication of the migration happened for the cells from spots 2 and 3 to the center and the corner of the membrane, respectively.

Finally, to make sure that actin filaments are responsible for changes in cell's mechanical environment, the changes in the size of nucleus were measured to understand the effect of mechanical stimuli on nucleus of the cells. There was no considerable difference in the size of nucleus of exposed cells in comparison with the control sample which is an indication of the responsibility of actin filament to sense and react to the cell's mechanical environment. Also, there was no evidence of alignment of the cells and actin filaments in response to biaxial stretch which was expected considering the nature of a biaxial force.

To conclude, the custom built stretching system is able to stretch cells cultured on collagen coated flexible silicon membranes while cells maintained their adhesion and confluency after exposure to strain percentages up to 10% and for 24 hours period of time.

This research provides the basis for further studies in the concept of cellular response to mechanical stimuli (biaxial stretch in this specific work). Despite the results of this work, we can say probably migration of cells and morphology changes would be more pronounced for higher migration, staining of cells while marking the membrane with a marker could be a good idea for future work. Also, to be able to model the shear stress during biaxial stretch would be a valuable future work to better model the stress and strain that the cells are sensing. Additionally, capability of controlling the differentiation of cells using the current setup would be a bright future work especially for the concept of stem cells which are able to be differentiated to most of other types of cell lines.

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Appendix

Cosmos software and the code sample:

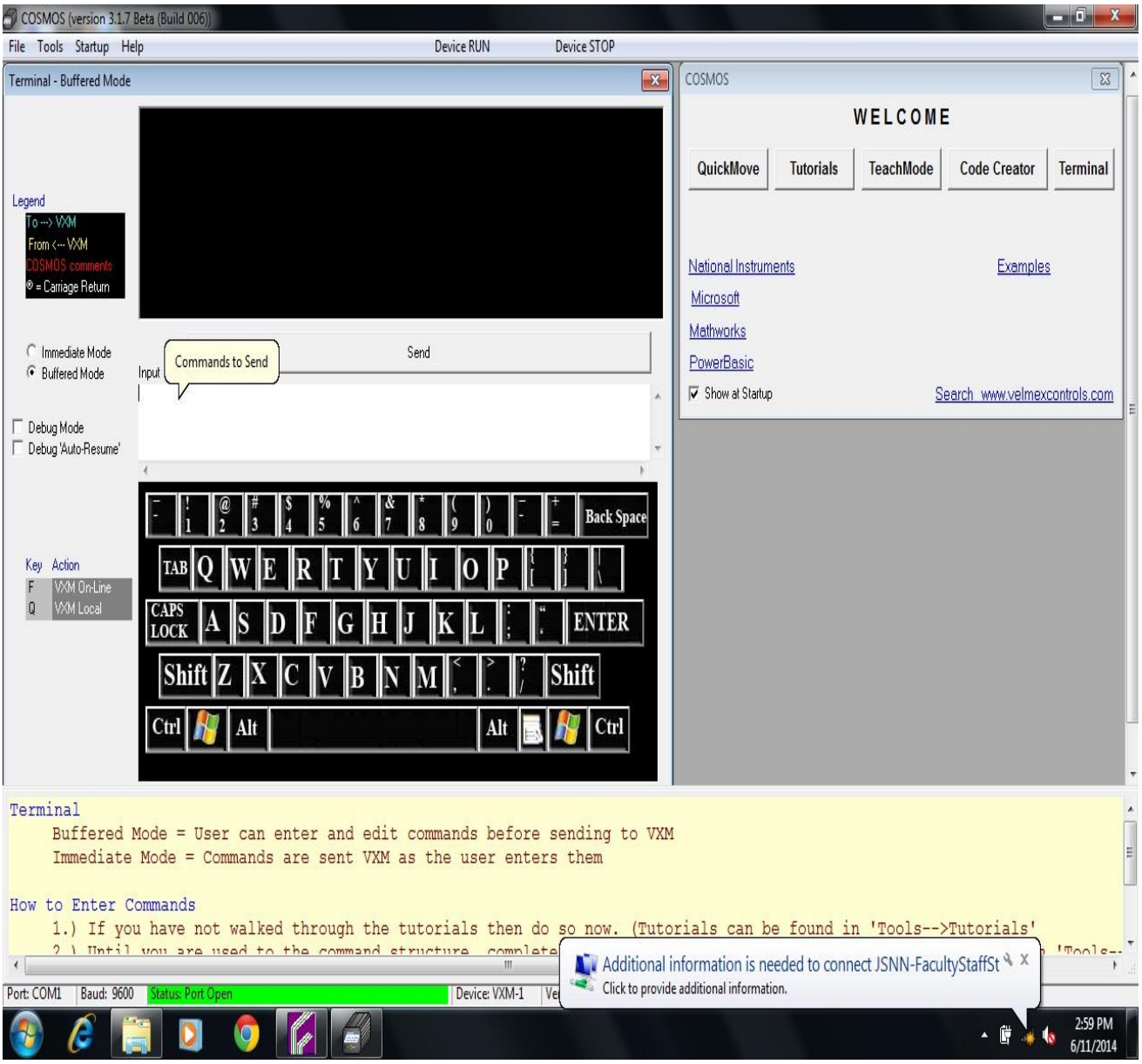

Figure 29. The terminal window of the software which commands to and control the stepper motor tower

C, (Clear all commands from currently selected program).

F, (Enable On-line mode with echo "off")

ImMx, (Set steps to incremental Index motor CW (positive), m = motor # (1 for current setup), x = 1 to 16,777,215 (20,670 for 10% strain)).

ImM-x, (Set steps to incremental Index motor CCW (negative), m = motor # (1 for current setup), x = 1 to 16,777,215 (20,670 for 10% strain)).

SmMx, (Set speed of motor, m = motor # (1 for current setup), x = 0 to 8000 steps/ sec. 0 being 1 step/ 2 sec. (5000 steps/ sec for 10% strain)).

Lx, (Loop from beginning or loop-to-marker x-1 times (x=2 to 65,535), when the loop reaches its last count the non-loop command directly preceding will be ignored).

R, (Run currently selected program).