The Effect of Mechanics on Migration, Morphology and Matrix Production by Primary Human Corneal Fibroblasts: Long-Term Dynamic Observation

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Abstract
The Effect of Mechanics on Migration, Morphology and Matrix Production by Primary Human Corneal Fibroblasts: Long-Term Dynamic Observation

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Mechanical load is believed to play a critical role in tissue formation, growth, maintenance, and disease in vertebrate animals. Although many studies have investigated the effect of mechanical stimuli on biological processes, the dynamics of these processes have not been closely explored. Live long-term observation of the collective behavior of Primary Human Corneal Fibroblasts (PHCFs) exposed to mechanical load could provide important insight into the mechanisms used by fibroblasts while they migrate, produce multilayered constructs and manage collagen deposition during the synthesis of organized ExtraCellular Matrix (ECM). Previous studies have shown that PHCFs produce locally organized and aligned ECM similar to developing corneas when cultured in the presence of stabilized ascorbic acid. In this study, DIC imaging, live cell fluorescent labeling, traction force microscopy and our previously designed mechanobioreactor were employed to locally and globally capture PHCF’s behavior under cell culture conditions for up to two weeks. We have directly tracked and observed the morphology and migration of PHCFs under physiological conditions in the presence of uniaxial mechanical load directly on the microscope. The behavior of human corneal fibroblasts is observed from initial seeding through matrix production. The orientation of cells, the magnitude and direction of their velocity as well as forces exerted by PHCFs were tracked on substrates every 6 minutes. The analyzed data demonstrates that corneal fibroblasts are induced to align on loaded substrates at a relatively fixed angle to the applied force while corneal fibroblasts on unloaded substrates exhibit only local alignment. We conclude that applied external mechanical loads significantly affect the patterns of cell migration and culture morphology and that load abrogates local guidance signals. Tissue engineers should be able to take advantage of such mechanical guidance to produce highly organized tissues such as the corneal stromal matrix.

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Chapter 1

1.1 Introduction

Mechanical stimulus plays a critical role in many different biological and biochemical processes in cells such as migration, gene expression and stem cell fate. Deep understanding of the role of mechanical signals on the behavior of human fibroblasts would be beneficial in studying formation, growth, maintenance and healing of animal tissue. Although previous studies have shed lights on the effect of mechanical stress on endothelial or fibroblast cells, the mechanism of converting mechanical signals to biochemical responses by cells, mechanotransduction, resulting in specific cellular responses has remained elusive. This could be due to the fact that most studies on cell mechanotransduction examine the state of cells at fixed times rather than tracking cells during tissue formation and development dynamically. There is therefore a strong need in establishing proper protocols for real-time observation of structural reorganization of cells and alterations of protein expression under highly controlled mechanically stimulated conditions, as well as defining standard and relevant mathematical techniques for quantification of the dynamic processes. Our goal is to establish a technique to observe and analyze the dynamics of Primary Human Corneal Fibroblast (PHCF) behavior with or without mechanical load while simulating physiological conditions of animal tissues. Here, we designed a bioreactor that can operate in several uniaxial modes along with providing direct accessibility for live observation on an inverted or confocal microscope. The device is capable of applying uniaxial mechanical load on samples in static or dynamic load and strain control. This bioreactor will be used to achieve the following three Specific Aims (SAs):
Aim 1: To establish a general method to culture Primary Human Corneal Fibroblasts (PHCF) inside a uniaxial mechanobioreactor at aseptic conditions for long-term observation

The mechanobioreactor will first be tested mechanically in load and strain control modes to check the accuracy of the bioreactor. Then the bioreactor will be disassembled to be accessible in a laminar hood for sterilization by ethanol and UV. Finally, a cell culture method will be used to provide a proper environment for PHCFs during the experiments (long-term observation).

Aim 2: To investigate the effect of uniaxial mechanical load on the migration, motion and alignment of PHCF during tissue formation under physiological conditions

Here, we will analyze the behavior of cells, such as their patterns of migration and elongation, during tissue formation with and without mechanical stretching of their substrate. The migration, motion and alignment of corneal fibroblasts will be measured by Particle Imaging Velocimetry (PIV) and Fast Fourier Transform (FFT) and 2D spatial cross correlation methods.

Aim 3: To measure the physiological forces generated by PHCF on calibrated polyacrylamide gels during fibroblast migration and tissue formation.
Traction force microscopy will be used to assess the physical interactions between cells and their substrate as they approach organized tissue formation. Long-term continuous imaging will be beneficial in studying the evolution of these interactions over time, from the time the cells are seeded on the substrate until they adhere and form a confluent monolayer.

1.2 Background

Corneal diseases are the second leading cause of blindness worldwide, following cataract in importance. More than 10 million people world-wide are blind due to corneal scarring and the only available therapy for this problem is cornea transplantation. Corneal replacement is one of the most successful treatments for corneal blindness due to injury and disease, which improved the quality of patient’s life [1]. In the United States, 33,000 corneal transplants are performed each year [2]. Although corneal graft transplantation could help to restore vision, there are many issues and concerns. In addition to requiring substantial follow up care, the severe shortage of corneal donors raises serious issues. Bioengineered corneal substitutes could be a promising alternative to voluntary donation. Despite improvements in the field of tissue engineering, there remain many issues regarding fabrication of functional tissue replacements and major developments of the current approaches are needed for successful clinical trials. Here, we briefly introduce cornea and its structure, the importance of mechanics in cornea tissue formation and growth as well as the current methods for testing cell mechanobiology. We introduce a novel system that can be used to address some of the fundamental questions regarding cell biomechanics.
**Cornea**

Cornea is the transparent front part of the eye that covers the iris, pupil and anterior chamber and is named window of the eye (Figure 1). This tissue protects the eye from outside insults and allows transmission of light into the eye. The cornea is also responsible for providing two-thirds of the eye’s optical power. Human cornea is approximately 500 mm thick, 12 mm in diameter and 7.8 mm curvature. The cornea comprises several layers including the epithelium, Bowman’s layer, stroma, Descemet’s membrane, and the endothelium. The epithelial layer acts as a barrier to protect the cornea and prevents bacteria and viruses from entering corneal stroma and the epithelium. Bowman’s layer is composed of strong lamina and collagen fibers to protect stroma. In adult humans this layer is approximately 8-12 µm thick. The stroma, which constitutes more than 90% of the corneal thickness, is principally responsible for corneal function. It is composed of about 250-400 flattened lamellae [3]. Each lamella is 1.5-2.5 µm in thickness and is produced by mesenchymal cells during development [3].

Figure 1: a schematic of the eye and showing the location of cornea.
**Composition of corneal layers**

The cornea comprises five major layers: (i) an outermost epithelium containing multiple layers of cells, (ii) Bowman’s layer; an acellular layer of mostly collagen type I fibrils which are irregularly arranged, (iii) a stroma containing regularly arranged collagen fibrils and keratocytes, which are responsible for tissue repair, (iv) Descemet's membrane; an acellular layer of mainly collagen type IV fibrils and (v) an innermost monolayer of specialized endothelial cells. Mainly due to its particular structure, the cornea can provide a barrier to the outside environment and serves as the optical pathway of the eye.

**Corneal Stroma**

The stroma is the main component in the cornea (500µm thick, ~90% of corneal thickness) and comprises of around 250-400 flattened plates (lamella). Each lamella contains many highly-organized collagen fibrils. The stroma is responsible for three main functions: 1) physical protection; 2) optical refraction; and 3) optical transmission. These functions are due to the specific structural properties of the stroma. The organization, direction and concentration of collagen fibrils can significantly affect these functions.

Natural synthesis of the stroma is critically important in corneal tissue engineering. Since the functionality of stroma is dependent on its highly organized collagen fibrils, the assembly of collagen fibrils into aligned layers is one of the main focuses of cornea tissue engineering [3]. Because of this important property of the stroma and the fact that the corneal extracellular matrix is largely collagenous, some studies have suggested using
cell-free cross-linked collagen gels molded into implantable cornea-shaped scaffolds [4-7]. Implants synthesized with this approach are shown to be stable for 24 months after transplantation into patients, which suggests that biosynthesized tissue replacements could offer a safe alternative to implantation of donor tissue, although further optimization of the current approaches is necessary [4]. Our goal is to examine the role of mechanical strain as a controlling factor for matrix production by corneal fibroblasts, which have shown excellent potential, in our hands, to recapitulate stromal development \textit{in vitro} [8-11].

Figure 2: a schematic image of the stroma to show how these plates form the stroma. Each plate is composed of aligned collagen fibrils and also there is a uniform angle between the fibrils of two adjacent (neighboring) layers (A). A TEM image of bovine corneal stroma that show the real structure of these lamella and how they form the stroma (B). The magnified TEM image illustrates three collagen lamellae aligned at different directions. This structural arrangement has been generally observed in adjacent collagen lamella in the cornea (C).
**The structure of stroma in human cornea**

Human corneal stroma comprises hydrated type I/V collagen fibrils [12-14], glycosaminoglycans [15], keratan sulfate and dermatan sulfate [16], proteoglycan (PG) core proteins [17] and other proteins including collagen type VI and fibronectin. There are at least 10 of the 28 known collagen types and two types of proteoglycan detected in human cornea [17]. The stroma consists of 250-400 collagen lamellae combined from anterior to posterior direction [18]. The corneal stroma is a complex arrangement of collagen lamellar bundles stacked in an alternating lattice pattern. Each lamella is arranged in parallel and contains fibrils with a uniform diameter, while its adjacent lamellae (above and below) are aligned in different directions [18-20]. Figure 2 is a TEM image of bovine cornea in cross section. The image illustrates three different collagen fibril layers (collagen lamellae), which are aligned at different orientations. One of the main functions of stroma is its optical transparency. It has been suggested that the transparency of stroma relies predominantly on the organized spacing and uniform diameter of collagen fibrils in the stroma [21-24]. Another property of stroma, its significant strength in tension tangential to the surface, is a result of the “plywood-like” arrangement of the lamellae [25, 26]. The tensile strength of cornea (stroma) is dependent on the direction of measurement [27, 28] and interestingly correlates with the general collagen fibril direction (detected using x-ray scattering) [29]. In the human cornea, the collagen fibril orientation and the highest strength direction coincide with the maximum loading direction (rectus muscle lines and at the annular ring near the sulcus). Figure 3 demonstrates the link between corneal structure and mechanical strength. This
observation agrees with our earlier hypothesis that mechanical force may stabilize collagen fibrils against enzymatic degradation [30-38].

Figure 3: represent the link between adult corneal structure and mechanical strength. Figure A demonstrates a schematic of fibril orientation in adult cornea based on X-ray observation by Meek et al, 2004. Figure B shows different tensile strength of adult cornea in different directions. The tensile strength or modulus at circumferential direction is much higher than the other directions (horizontal and vertical directions). There is a strong correlation between the principal directions of the fibrils in the cornea (Fig A) and the tensile mechanical properties of the adult stroma (Fig B). Not surprisingly, both the directions of the fibrils and direction of applied loads are the same. The AP and LM strength lines up with the line of action of the rectus muscles while the circumferential band of collagen resists the force generated by the curvature changes at the sulcus. (Fig A modified from [29] and Fig B Image from [3])
**Development and maintenance of the corneal tissue**

Two major properties of cornea are its tissue strength and transparency, which are due to a unique structure of cornea tissue. Both tissue structures, namely the highly organized collagen fibrils, and specific protein expression by corneal cells are responsible for the tissue transparency. The optimal arrangement of these collagen fibrils allows the cornea to pass the light through with minimal optical scattering [3].

Corneal tissue formation is a complex process involving the development of multilayer with highly controlled structures: mainly the epithelium, endothelium and the stroma. Many different cytokines and growth factors are involved in the development of the cells that make up these different layers; the keratocytes, endothelial cells and nerve cells [39].

Cell-cell communications are also likely involved and are necessary in corneal development and maintenance [40-41]. For instance, stromal-epithelial interactions are suggested to be necessary for the maintenance of Bowman’s layer [42]. Endothelial cells are also suggested to communicate with keratocytes [43].

In addition to biochemical processes, mechanical force is suggested to be critical for proper corneal development and growth [44]. We believe that the intraocular pressure applies mechanical forces to the cells and tissue during corneal development and is at least partly responsible for the specific organization of corneal tissue.

**The role of mechanics in the production of corneas**

Mechanical force could regulate neural crest cells (derived from Mesenchymal cells) local and global organization from initial prospective stromal population through matrix production. Early investigations on the effect of mechanics on vascular endothelial cell
orientation under shear [45, 46] suggested that dynamic mechanical force applied to a substrate influences fibroblast orientation [47]. More recent studies of ligament fibroblasts on 3D collagen gels under mechanical load reported that the cells align in the direction of applied load and the secreted collagen fibrils by the cells are also aligned with the direction of load [48]. A unique kinetic footprint of different cell types has been suggested through short-term, in situ, dynamic imaging of cells (ligament fibroblasts and bone cells) on a stretchable substrate [49]. In 2011, our group demonstrated that mechanical force influences the orientation of corneal fibroblasts “en mass” on a uniaxially stretched collagenous membrane [50]. Two processes could be responsible for this behavior. One of these is through alterations in the local modulus of the substrate following the application of mechanical load, which results in an effective anisotropy in the mechanical properties promoting durotaxis [51, 52]. Durotaxis was first described by Lo [52] using 3T3 fibroblasts on polyacrylamide substrates with directional mechanical properties. Recent theoretical investigations into durotaxis suggest that cells sense changes in the substrate rigidity through focal adhesions and cytoskeletal components (e.g. actin stress fibers (ASFs)) [51] and migrate in the direction of higher stiffness presumably because a stiffer surface stabilizes their ASFs [53]. The other means by which the propagated force to the cells could alter cell orientation is through changing the orientation of substrate fibrils resulting in an effective anisotropy in the fibril direction, which promotes contact guidance, the motion of cells along aligned surfaces [54, 55]. Understanding the role of mechanical force on fibroblast migration and orientation can result in valuable information resulting in more control in matrix synthesis for tissue engineering and provide insight into pathologies such as fibrosis and metastasis [56, 59].
The methods used to test cell mechanobiology

Mechanical signals from the cell microenvironment are long believed to play a key role in various cellular processes including cell migration, differentiation and tissue formation. However, the mechanisms by which cells translate mechanical signals into cellular responses, a process known as mechanotransduction, are not well understood. Therefore, various methods have been established to investigate the effect of mechanical force in regulation of cellular response and function as well as the mechanism of its action. While some studies focused on quantifying the traction forces between cells and their substrate and the role of substrate stiffness on these forces, others are interested in investigating the role of mechanical stimuli on cellular processes by imposing external mechanical stretching on the substrate.

In addition to its benefits in understanding the role of mechanical stimuli in regulation of cell behavior in vivo, mechanical stretching of cell substrate is helpful in engineering tissues in vitro. Various studies have shown that stretching results in numerous cellular responses such as cell reorientation, cytoskeletal remodeling, and regulation of gene expression and synthesis of extracellular matrix proteins [60 - 62]. These studies most commonly use distinct custom loading devices that can apply uniaxial and biaxial stretching to various substrates [63-65]. Other techniques such as stretching a membrane over a fixed loading post using vacuum pressure are also carried out by commercially available devices [66]. Although these devices could be helpful for tissue engineering-type applications, they might not be proper for investigating the role of mechanical stimuli on cellular processes, as they cannot simultaneously provide imaging accessibility and the cell culture environment necessary for cell growth over time scales that are
relevant to studying processes like extracellular matrix synthesis and organization. One main approach that quantifies the forces between cells and their substrate uses deformable substrates with carefully designed microfabricated elastomeric substrates [67]. These techniques include gelatin, polyethylene glycol (PEG) PAA gel-based traction force microscopy (TFM), microfabricated horizontal cantilevers and elastomeric micropost arrays. Traction force microscopy techniques which utilize materials with tunable mechanical properties are mainly used to investigate the role of two-dimensional substrate stiffness on cell behavior [68-71]. They rely on cellular deformation of specifically calibrated gels used as substrates to quantify the forces that cells exert on their substrate. The tracking of the position of the beads embedded near the surface of the functionalized gels gives a two-dimensional map of the surface stress [68]. Micropost arrays are also used to measure traction forces using a substrate consisting of arrays of elastomeric posts of known stiffness [70, 72]. When cells adhere and migrate on such substrates they deflect these underlying posts. The amount of deflection of the posts is easily translated to the traction applied by the cells. As mentioned above, although these techniques are promising to quantify cell tractions, incapability to provide in situ optical accessibility and proper long-term cell culture conditions simultaneously limits their efficacy in studying cellular processes that are in time scales exceeding a couple of hours.

**Mechanobioreactor**

Many studies have shown that mechanical load can alter cell differentiation, migration orientation and organization. However, the dynamics of cell organization and cell-cell interactions under mechanical load have not been studied over long cell culture periods. A well-controlled bioreactor can help to observe the dynamics of cell behavior. The
current bioreactors are able to provide either direct, high magnification observation of
cells on a microscope or a mechanical tester inside the incubator to measure mechanical
properties of scaffolds under culture conditions. A bioreactor that can perform both of
these functions simultaneously has not been designed before. There are four main
requirements for the design a bioreactor with this capability: 1) long-term sterility, 2) good control of the physiological environment, 3) ability to apply mechanical stimulation and 4) a low volume chamber. Previous studies have shown many improvements in the
design of cell culture bioreactors [23-26]. Several successful experimental bioreactors
clearly demonstrate how the tissue engineering community could benefit from these
carefully designed culture systems. However, live direct observation along with
mechanical force application could provide an even better understanding of the role of mechanical stimuli and constraints on the final structure of matrix formed by cells. In this thesis, a mechanobioreactor that was designed and built in Extracellular Matrix Engineering Research Laboratory (EMERL), Northeastern University, Boston, MA by Paten and Zareian et al. [35] to satisfy three important requirements: 1) highly-controlled mechanical load/strain application 2) direct optical accessibility 3) minimal dead space, will be used. The bioreactor was designed to load specimens from 0 to 10N with 0.01N resolution. The strain resolution design should provide ~ 0.5% gage length (15mm). Specimens can be tested from 3 to 16mm gage length with 0.01-2mm thickness and 0.1-12 mm width. The bioreactor should provide a stable body temperature ~ 37C ± 0.05 for long-term experiments while providing operational perfusion ports and minimizing the chamber volume dead space. Finally, the device can be mounted with minimal
modification to a standard Inverted microscope stage and provide the ability to perform high magnification transmission optical microscopy (up to 60X objective).

In the following chapters, the mechanobioreactor will be tested at load and strain control modes with simultaneous high magnification in situ observation. The bioreactor is mounted on an inverted microscope (Nikon TE2000) to study the behavior of live cells over extended culture period. The bioreactor will be tested for growing human corneal fibroblasts for up to two weeks on different scaffolds in next chapter. A few sterilization steps will be performed to ensure proper physiological conditions for cell growth in long periods of time. The data captured from imaging will record the process of cell growth from the initial seeding to matrix production on different scaffolds. During our two-week observations, we will observe various cellular behaviors including 1) cell sheet motions on the scaffold, 2) degradation of collagen substrate by cells, 3) apoptosis behavior from corneal cells and 4) effects of uniaxial mechanical on the morphology and matrix production of human corneal fibroblasts. Further, using the new mechanobioreactor system along with traction force microscopy technique, we are able to track cell-cell and cell-substrate tractions and measure physical forces in extended culture periods (up to one week).
Reference:


[40] Corneal Cells: Chatty in Development, Homeostasis, Wound Healing, and Disease, Steven E, Wilson, Marcel Netto and Renato Ambro’sio.


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Chapter 2

Assessment of the performance of a custom mechanobioreactor in combination with a PHCF culture system

2.1 Introduction

The role of mechanics in tissue formation and structure has been recognized for many years [1-5]. Yet we are only just beginning to understand the processes by which mechanics can alter biological events. In vertebrate animals, strong evidence suggests that mechanical load plays an important role in the growth, formation and remodeling of musculoskeletal system components [6-10]. Also, significant research has focused on mechanobiology of the extra-cellular matrix [10-20]. However, there is no consensus agreement to suggest how a connective tissue grows during development. Studies have shown that mechanical load can significantly influence cell differentiation, migration and organization [10-22]. There is, however, little evidence depicting how the mechanical load alters the dynamic behavior in cell culture. This is mainly due to the fact that most research has focused on cell status at fixed time points when the effect of mechanical stimulation is likely already completed. Intermittent investigations will miss important dynamic behavioral changes that could provide information about how cells deposit different structural proteins such as collagen and how they build their extra-cellular matrix in the presence of mechanical load. To circumvent this limitation, a well-controlled cell culture bioreactor combination can help to discover fundamental questions about the dynamics of cell behavior.
Current bioreactors commonly used in tissue engineering are able to provide either direct high magnification observation of cells for at most a few days or mechanical stimulation without optical accessibility. A bioreactor that can perform both of these functions simultaneously would allow investigation of the effect of mechanical stimuli on the dynamics of cell behavior over the long-term [35]. Generally, there are four main requirements for the design of such a bioreactor; 1) stable sealing, 2) proper cell culture environmental controls, 3) ability to apply controlled mechanical stimulation 4) small volume chamber. Previous studies have shown many improvements in the design of cell culture bioreactors [23-27]. For instance, stem cell expansion in a perfusion culture system resulted in significant increase in cell output by constantly controlling flow rate inside the bioreactor (Wang et al., Multilineal hematopoiesis in a three-dimensional murine long-term bone marrow culture 1995). These perfusion culture systems employing bioreactors offer better transport of nutrients and waste. In cartilage tissue engineering, the effect of modifying the hydrodynamic environment on bovine calf chondrocytes has been investigated in spinner flasks. After seeding these cells on fibrous polyglycolic acid substrates and culturing them in static or turbulent mixing regimes, it was found that the hydrodynamic properties of cell environment can be important in extracellular matrix production. Briefly, the constructs exposed to turbulent mixing, regardless of the intensity of mixing, contained higher amounts of collagen, and while releasing more glycosaminoglycans (GAG) contained lower fractions of GAG relative to the ones in the static culture condition [28]. In spinal cord research, a micro stepper motor has been used to grow axon fibers between two membranes. Growing central nervous system axons was achieved only with the application of continuous mechanical
tension. [29-30]. Using dynamic compression bioreactors, it is shown that the compressing articular cartilage dynamically modulates protein biosynthesis. Uniaxial compression of cartilage at different frequencies was used to identify the physical and biological roles of dynamic compression on protein synthesis by cells, which can be beneficial in studying the effect of mechanical compression on cartilage remodeling. Briefly, it was found that at high frequencies, protein incorporation in the cartilage could be stimulated with strains as small as 1%, while protein synthesis was observed at lower frequencies only at higher amplitudes of strain [31]. A biaxial bioreactor has been designed to stretch and compress substrates simultaneously [32]. As opposed to non-stimulated constructs, mechanically stimulated constructs showed an increase in cell growth and matrix deposition (proteoglycan and collagen). This biaxial bioreactor has been used to fabricate implants for tissues in compression and tension modes [32]. In tendon tissue engineering research, mesenchymal cells were cultured on sponge constructs in the presence of cyclic tensile loads. Cyclic mechanical testers can be used to improve the mechanical properties of tendon and repair the tendon over time [39, 40]. The results have shown that loaded constructs (every 5 min for 8 h/day to a peak strain of 4% for 2 weeks) ended up 2.5 times stiffer than unloaded constructs [33]. These successful experimental bioreactors clearly demonstrate how much the community of tissue engineering could benefit from carefully designed mechanobiological culture systems. Over the past decade, studies using mechanical bioreactors have shown that mechanical load 1) significantly improves cell growth and proliferation; 2) enhances/modifies protein deposition by cells 3) enhances or modifies molecular incorporation into constructs and 4) improves the organization of cells as well as their
substrate. To better understand the dynamic mechanisms which underlie these effects, assessment of cell behavior in *real-time* is critical. For example, formation of cell-cell junctions has been shown to play a potentially important role in the production of highly-organized collagen by corneal cells [34]. However, the lack of long-term live imaging limits researchers understanding of how these cells communicate and how and when cell-cell connections are produced. Real-time studies could be beneficial in leading to a better understanding of such cellular processes. Additionally, live direct observation along with controlled mechanical load application could provide a better understanding of the role of mechanical stimuli and constraints on the final structure of matrix formed by cells.

In this chapter, a custom mechanobioreactor, that addresses the limitations of commonly used bioreactors in investigating the dynamics of cell function, is evaluated. The mechanobioreactor has been designed and tested to satisfy these requirements: 1) to control tensile mechanical load with high accuracy 2) to provide high magnification imaging capability, 3) to maintain the temperature of the chamber for long periods of time, and 4) to provide a sterile/aseptic environment for cells during the experimental time course. The device will be mounted on an inverted microscope to observe live constructs for periods as long several weeks.

**The aim of this study:**

To establish a general method to sustain Primary Human Corneal Fibroblast (PHCF) cultures in our uniaxial mechanobioreactor under aseptic conditions and in the presence of controlled tensile mechanical load application. The following two sub-aims represent specific goals in this study.
**Sub aim 1:** Determine the accuracy and repeatability of mechanobioreactor’s performance in load and strain control modes.

**Sub aim 2:** To successfully transfer our PHCF culture system into the mechanobioreactor. Three different substrates (bare glass coverslips, loose collagen gels on a glass coverslip and dense disorganized collagen constructs) will be seeded with PHCFs inside the mechanobioreactor and then observed under the microscope.

**Mechanobioreactor Description:**

Our mechanobioreator was previously designed and built by an undergraduate capstone team and refined by Jeffrey Paten. The device itself and the results of this chapter are published in the paper Paten and Zareian *et al* [35] which was published in Tissue Engineering Part C in 2011. Top and bottom views of the mechanobioreactor as mounted on an inverted microscope (TE2000 Nikon) are shown in Figure 4 We examine the mechanobioreactor to determine if it meets our general design criteria to address four important requirements: 1) optimal environmental control 2) mechanical load/strain application 3) direct optical accessibility 4) a low volume chamber.

**The design specification of the mechanobioreactor**

Figure 5 is a solid works drawing which depicts all the components of the mechanobioreactor. The device has an adjustable-height chamber that is formed by the mating of three individual components. Each part provides a specific function. These components include: 1) the chamber lid, 2) the chamber bottom and 3) the bioreactor housing. The chamber lid provides an optical window to observe the specimens and also
houses the temperature control components. The chamber bottom is a vertically adjustable viewport that lets the objectives (4X, 10X, 20X, 40X and 60X) approach the specimens and also permits changes in the volume of the chamber. The bioreactor housing provides mechanical support for the adjustable viewport, temperature control block, grips and load-sensing components. Also, the bioreactor housing is mounted on the microscope stage, which holds the linear motor and actuation transmission system (figure 5). To prevent contamination in the chamber, all components that interface with DMEM, specimens and cells are constructed from 316L-stainless steel, glass, polycarbonate and silicon rubber (to prevent any contamination or corrosion inside the chamber). All components can be cleaned and washed by 70% ethanol. The mechanism of gripping the dense disorganized collagen specimens has been shown in Figures 5 and 6. The tissue grips hold the specimens (Figure 6) in the center of coverslip. One of the grips is connected to load cell (Honeywell Sensotec Model 31 Low) to measure the load while the other one is connected to a linear motor (KT-NA08A50; Zaber) which is designed to stretch the samples via a custom labview program. There is a guide rail (Figure 5), which is connected to a linear motor and the grip. This guide rails helps the linear motor to transfer the backward and forward movement to the grip. Thermal input is provided by cartridge heaters (C1E14 - L36; Watlow) embedded in two copper inserts. To preheat fluid with a varying range of flow rates before entering the chamber, incoming fluid is transferred through the steel blocks. The temperature of the chamber is controlled by two thermocouples which are positioned on either side of the sample in the steel wall. A custom labview program was provided to record all parameters, and to control the linear
motor position. The program provides the ability to run the device in either load or strain control mode while it records the load, strain and temperature.
Figure 5: Solid works exploded-view drawing of the mechanobioreactor showing all the components. The device is set into the Perfect Focus stage of the microscope. The tissue grips are above the chamber bottom to hold the specimens over the coverslip. The specimen is connected by two tissue grips to the linear actuator (through the transmission linkage or guide rails) and to the load cell. The temperature components (heater block and cartridge heaters) are housed in the chamber lid. The whole system was held together with a stage adaptor, which is mounted on the microscope stage. [35]
Figure 6: Solid works drawing of the chamber bottom showing how the specimen was stretched inside the mechanobioreactor. Both tissue grips are made from 316L-stainless steel and hold the specimens in the center of coverslip. The grips work using a spring-loaded retaining bar that presses the sample into the grip face with a force. [35]
2.2 Materials and methods:

Sub aim 1: Determine the accuracy and repeatability of the mechanobioreactor’s performance in load and strain control modes.

2.2.1 Experimental protocol

1) Actuation accuracy and repeatability test: To test the accuracy and repeatability of the actuator (motor) we used our ability to continuously capture DIC (Differential Interference Contrast) images from specimens while operating the mechanobioreactor under an inverted microscope. A micropipette was fixed to the actuator (the grip connected to motor) and the mechanobioreactor was commanded to move a 20, 100, 400 or 800 micron step while the micropipette was tracked at 20X magnification. The position of micropipette was detected for forward and backward motions (N=3 for each set). The distances of the micropipette movement (backward or forward) were optically captured to produce DIC images that were compared to the measurement data reported by the mechanobioreactor (actuator reported data).

2) Strain control tests on a rubber band: We performed a series of mechanical experiments using calibrated rubber bands (6 mm width, 0.11 mm thickness) to verify the accuracy of the mechanobioreactor’s measurements. A custom made cutting die was used to generate accurate and repeatable test strips from the original rubber band sheet. Tensile specimens were cut and separated at three different gage lengths 1) short length - 3mm, 2) medium length - 5mm, 3) long length - 7mm). We analyzed the mechanical behavior of our specimens both with the mechanobioreactor and with the ELF 3100 (ElectroForce
Systems Group (Bose Corporation) – Minnesota USA) [36]. ELF 3100 Elector force (Bose) uniaxial mechanical tester was chosen to test the relative accuracy and repeatability of mechanobioreactor at load and strain control mode measurements against a commercial system. ELF 3100 Electro-force (Bose) machine is a commercial mechanical apparatus with the accuracy of 0.5% full-scale range (0.05V in a 0-10V) for load cell and displacement transducers. All rubber bands in this protocol were loaded in strain-control mode (fixed strain) while both strain and load were recorded. All specimens were positioned in the device and were stretched to detect an initial 0.01 N load from load cell. The resulting strain was taken to be the reference or zero strain. Mechanical strains of 1%, 2% and 4% were applied to three different gage lengths (3mm, 5mm and 7mm) at 0.01mm/s strain rate. At the end of experiments, the data obtained from the ELF 3100 machine were compared to those from the mechanobioreactor.

3) Preparation of dense disorganized collagen substrates (DDCS): The ability of the mechanobioreactor to accurately control the load and strain on a dense collagen specimen was tested. For these series of experiments, we employed DDCS constructs, which were produced in-house using our own protocol. Type I bovine collagen (3 mg/ml) (Advanced Biomatrix) was polymerized inside a 10000 MWCO dialysis cassette (Spectrum Laboratories). The collagen solution was mixed with polystyrene microbeads (10um beads, Polysciences) prior to polymerization to provide a direct optical measure of the applied strain. After polymerization, cassettes containing the bead/collagen mixture were immersed in 40% PEG (20kDa) overnight at 37°C. A custom made cutting die was used to generate accurate and repeatable test strips from DDCS (~ 15mm Length X 6mm
Width X 40um Thickness). DDCS strips, produced in this manner, were placed and fixed between two grips inside the assembled mechanobioreactor.

4) Strain control tests (DDCS): A DDCS specimen, prepared aforementioned (N=5), was mounted into the grips and preloaded to 0.01 N (as a reference or zero strain). The initial gage length was taken as the grip-to-grip distance, following a short equilibration period (120 seconds). To demonstrate the mechanobioreactor’s ability to control strain, the system was commanded to strain the tissue to 5.0% and the actual strain (optically tracked) was compared to the desired value and the reported value from the linear motor encoder. To achieve direct optical strain tracking, polystyrene microbeads were intentionally added to the DDCS during fabrication, allowing us to track the local positions of beads. Thus, we could, with high accuracy determine the actual strains for comparison to the desired and reported values. In addition to reporting the both static and time dependent strains, the presence of the load cell in the system allowed us to follow the load relaxation data simultaneously.

5) Load control tests (DDCS): A similar DDCS specimen (N=5) was clamped by the grips and stretched to 0.01N load (as a reference or zero strain). Then specimens were loaded to 0.1N constant load while the microscope tracked embedded 10µm beads in the constructs to report optical strain during the loading operation. The strain reported by labview program was recorded as the creep response of the tissue (tracked optically and via the motor encoder).
6) **Temperature control tests**: To assess the mechanobioreactor’s thermal control system, we examined its ability to achieve and maintain a fixed temperature during perfusion of the chamber (conditions: no specimen, chamber filled with fluid). The mechanobioreactor was assembled and mounted on the microscope. A thermocouple (T type, Omega Company) was attached at the center of the chamber to record temperature. To perform the test, room temperature 1X phosphate buffered saline (PBS) was perfused through the reaction chamber at 4 different flow rates (0, 4, 40 and 400 \( \mu \text{l/min} \)). Three runs at each flow rate were performed and the temperature was monitored with an additional thermocouple. We set the control temperature to a value that adjusted the temperature to 37°C in the center of the chamber. The dynamic temperature data captured for all flow rates was recorded and compared at the end of the experiments.

Sub aim 2: To successfully transfer our PHCF culture system into the mechanobioreactor.

**2.2.2 Experimental protocol**

1) **Preparation of the bioreactor to culture PHCF’s**: The mechanobioreactor is easily disassembled and assembled inside a laminar hood. However, for this experiment, the mechanobioreactor was disassembled outside the hood, where the chamber, load cell, frame and lid were washed with 70% ethanol and moved into the laminar flow hood. The O-rings, valves, grips and rods were kept in 70% ethanol and exposed to UV light overnight. The valve connectors and tubes were also washed in 70% ethanol and exposed to UV light overnight. The following day, the components were re-assembled and then 10ml of preheated DMEM (37°C) was passed through the assembled chamber and tubes.
to remove the ethanol from the bioreactor. All electrical wires (the actuator and load cell) were sealed with sterilized, autoclaved paper to protect them from the ethanol and DMEM during the sterilization process. Finally, the sterilized, assembled bioreactor was mounted on the microscope and connected to the supporting controllers for the heater, load cell, thermocouples, and actuator.

2) Preparation and expansion of PHCF’s: PHCF cells were extracted from a 12 year-old donor cornea using our standard cell extraction protocol [37, 38]. Human corneas were cut from sclera by sterilized razor blades and washed three times by 1X PBS in a sterilized dish. Then the epithelium and endothelium layers were gently removed from corneas by sterilized razor blades. The corneas (stroma) were cut into a few small pieces (2mm X 2mm square) and washed three times by 1X PBS then placed onto 6 well cell dishes. Sterilized DMEM enriched with 10% FBS and 1% Antibiotic (Antibiotic/antimycotic; mixed 10mg/ml Streptomycin and 25µl/ml Amphotericin B, Cellgro, Fisher Scientific) was added to corneal pieces and then the dishes were kept in an incubator for two weeks. Every day, 50% DMEM was gently extracted from each well and 50% fresh enriched DMEM was added. When the primary human corneal fibroblasts (PHCFs) grew and reached confluence in the dishes, they were trypsinized to be removed. They were cultured in different sterilized dishes and expanded in culture for 2 weeks. After 3 passages, PHCFs were moved into sterilized culture dishes until the day of experiment. A fresh culture flask was washed two times with 1X PBS then was incubated with preheated Trypsin/ETDA for 20 minutes. The suspended cells were then removed into a 15ml falcon tube. The cells were centrifuged (at 2000 rpm for 5 minutes) and
counted using a standard cell passage protocol. The final density of the solution was 1000 cells/µl for every experiment. These isolated fibroblast cells were used for all experiments in this chapter.

3) Culturing on glass substrate (coverslips): The mechanobioreactor was assembled inside the laminar hood and the center of the glass substrate was seeded with PHCFs (1000 cells/µl). The chamber was full of fluid at this point and then the mechanobioreactor was sealed and mounted on the microscope. All control wires were connected to their respective components. In this experiment we only used the temperature control system. DIC Images were captured from the center of glass substrate (at just one location) every 6 minutes at 20X magnification for two weeks. Preheated DMEM enriched with CO₂, 10% FBS and 1% Antibiotic/antimycotic (mixed 10mg/ml Streptomycin and 25µl/ml Amphotericin B, Cellgro, Fisher Scientific) was used to feed the cells at an 8 µl/min flow rate. The medium flow rate was adequate to exchange the media while keeping the shear stress well below 0.1 dyn/cm². Here, we calculate the shear stress within the chamber (Figure 7).

**Stress and strain**

\[
\text{Area} = (W) \times (T) = (6\, \text{mm}) \times (40\, \mu\text{m}) = 2.4 \times 10^{-7} \, \text{m}^2
\]

\[
\text{Stress} = \frac{F}{A} = \frac{0.012 \, \text{N}}{2.4 \times 10^{-7} \, \text{m}^2} = 50 \, \text{kPa}
\]

stress when the sample was 0.01N preloaded

\[
\text{Stress} = \frac{F}{A} = \frac{0.12 \, \text{N}}{2.4 \times 10^{-7} \, \text{m}^2} = 500 \, \text{kPa}
\]

stress at 5% strain
**Shear stress**

Viscosity water at 37 °C $\mu_w = 0.692 \times 10^{-3}$ Pa.s

Flow rate = $Q = 8$ µl/minutes

Height of chamber = 3 mm

Area = $(15\text{mm}) \times (3\text{mm}) = 45\text{ mm}^2$

(Velocity of DMEM in chamber) $V = \frac{Q}{A} = (8\text{ µl/minutes})/(45\text{ mm}^2) = 3 \times 10^{-6}\text{ m/s} = 3\text{µm/s}$

Water density at 37 °C = 993.37 Kg/m$^3$ $\rho = 3 \times 10^{-6}\text{ m/s}$ $h = 3 \times 10^{-3}\text{ m}$

Viscosity water at 37 °C $\mu_w = 0.692 \times 10^{-3}\text{ Pa.s}$

Reynolds Number $Re = \frac{\rho V h}{\mu_w} = \frac{(993.37)(3 \times 10^{-6})(3 \times 10^{-3})}{(0.692 \times 10^{-3})} = 0.0129$

0.0129 is **laminar** $< 1400$

$d$ (the length of the chamber and is independent in the calculation)

From fluid mechanics, we use the simplified **Navier-Stokes equation** with pressure gradient

$$d^2v/dy^2 = (1/\mu_w)(dp/dx)$$

Then: $V(y) = (1/2\mu_w)(dp/dx)(y^2 - hy)$

$dp/dx = 12V \cdot \mu_w / h^2$

$dp/dx = 12(3 \times 10^{-6})(0.692 \times 10^{-3})/(3 \times 10^{-3})^2 = 2.77 \times 10^{-3}\text{ Pa/m}$

Then: $V(y) = (0.722 \times 10^3)(2.77 \times 10^{-3})(y^2 - hy)$

$V(y) = 2(y^2 - hy)$ $h$ from 0 to 3 mm

Shear stress

$\tau = \mu_w . (du/dy) = \mu_w . (2)(2y-h)$

$\tau = (1.384 \times 10^{-3}).(2y-h)$ $h$ from 0 to 3 mm

Max shear stress 4.152 mPa = 0.0004152 Pa = 0.004152 dym/cm$^2$
4) **Culturing on collagen type I gels:** The coverslip of the mechanobioreactor was coated with 3mg/ml collagen type I solution (Advance Biomatrix Inc.) and maintained at 37°C in the incubator for 6 hours. This time span was sufficient for collagen to polymerize to become a white gel coating the coverslip. The thickness of the collagen gel was measured by the microscope and was approximately 100 µm. PHCFs were seeded (1000 cell/µl) on the top of the gel with a pipette. As before, the bioreactor was sealed and filled with enriched DMEM. The bioreactor was again mounted on the microscope for imaging and set up for thermal control. DIC Images of the cells on the collagen-coated coverslips were
captured from the center of collagen gel sample (one location) every 6 minutes using a 20X objective. The cells were fed with enriched DMEM as described above. Culture media was perfused at flow rate of 8µl/min.

5) Culturing on dense disorganized collagen substrates: A DDCS strip was generated as described in 2.2.1 number 3 and fixed between two sterilized grips inside the assembled mechanobioreactor. The PHCFs were counted and diluted in DMEM to a concentration of 1000 cells/µl. One milliliter of the PHCF solution was seeded on the topside of the DDCS (between two grips) inside the chamber using an Eppendorf manual pipetter. After the DDCS was seeded with PHCFs (drop by drop) for a half hour, the bioreactor was sealed and gently filled with enriched DMEM. The bioreactor was then mounted on the microscope and was perfused with enriched DMEM (10% FBS and 1% Antibiotic). The flow rate was kept at 8µl/min for consistency with the other experiments. DIC images were captured from the PHCF seeded DDCS specimens every 6 minutes and Z-scan images were captured from the constructs at the end of the experimental period. Live dynamic DIC microscopy was used to capture the dynamics of cell migration and proliferation. To avoid Z stage drifting the Nikon Perfect Focus System (PFS) was utilized. PFS is capable of holding Z plane at constant height during the experiment (the focus holds the image plane within a 50nm distance from the desired image plane). Without the PFS system, the fidelity that was achieved in our movies would not have been possible. NIS-elements- Nikon (version 3.2) was used to capture DIC images (by 20X objectives) from one location every 6 minutes.
6) PHCF Seeded DDCS with load: The DDCS substrates were generated as described in section 2.2.1 number 3 and then mounted on the tensile grips and seeded with 1000 PHCF cells/ul. The bioreactor was filled with DMEM supplemented with 10% FBS, 1% antibiotic and dissolved CO₂. The bioreactor was mounted on an inverted microscope and used in strain-control mode at 37°C to provide a suitable culture environment for the PHCFs. The bioreactor was prepared for culture, loaded with a DDCS and seeded with PHCFs as described in 2.2.2.-5. The DDCS was stretched to a preload 0.01 N (~50 kPa stress). This amount of stress was applied to specimens until the cells reached confluence across the entire specimen surface (about one week). The collagen fibrils of DDCS sample were not organized by this amount of stress when 0.01N load was applied. Then the specimen was strained in strain control mode (6% fixed strain with 100 um/s strain rate) while load data were recorded. As described in 2.2.1 number 2, the culture was continuously fed by DMEM supplemented with 10% FBS, 1% antibiotic and dissolved CO₂ at a fixed flow rate of 8ul/min and maintained at 37°C for up to two weeks. To support the production of hydroxylated collagen 0.05mg/ml stabilized ascorbic acid was added to DMEM from day 3 to 14. DIC images were taken every 6 minutes from one location at 20X, while the load and strain were tracked and recorded by the labview program.
2.3 Results:

2.3.1 Bioreactor Performance

1) Actuation accuracy and repeatability test results: Figures 8 A and B show the initial and final positions of a micropipette fixed into the system following a 400 µm commanded backward motion. The maximum error detected was 4.2 µm (about 1%) during the 400 µm backward motion (N=3). Figure 8C demonstrates the accuracy and repeatability of the micropipette movement for forward and backward position commands at 20, 100, 400, and 800 µm. The bar graph (Figure 8C) compares the optically sampled position error and standard deviation for each commanded step. The device operated well within our design specifications with minimal backlash (maximum of ~ 5 µm). The forward positional error was untraceable optically for the three larger commanded moves (< 1 µm). At the smaller distance (20µm), the forward positional error detected was ~ 1 µm (~ 5% error) [35].

2) Strain control comparison to Bose ELF 3100: Figure 8D shows our relative accuracy comparison with the commercial Bose ELF-3100. A total of 36 experiments were performed at different strains (1%, 2%, and 4%) and with specimen strips of different lengths (3, 5, and 7 mm). The plot demonstrates that the mechanobioreactor and the commercial system have a good agreement in measuring force during the dynamic and equilibrium strain. The total average force value difference between the systems was less than 8%. Also, the results from the dynamic and equilibrium strain have shown that the repeatability of the bioreactor is in good agreement. It can be readily seen that our custom mechanobioreactor performs well relative to the commercial system.
3) Strain and load control tests with Dense Disorganized Collagen Substrate (DDCS): Figure 9 demonstrates the results of strain and load control tests from the DDCS specimen experimental series. The image shows the DDCS in perfusion fluid in the bioreactor before and after the application of a commanded strain of 5%. Figures 9 A and B represent the continuous strain and load control test results with the DDCS constructs. The optically tracked strain was measured and compared to reported strain. For 5% commanded strain in strain control mode (Figure 9 C), the optical strain of 4.8% ± 0.1 (~ 3% error) was detected. The load data shows the classic nonlinear increase in force during the application of the 5% strain. Figure 9 D shows that our target load of 0.1N at load control mode is reached quickly and held stably. The evolved equilibrium strain response of the sample (measured optically) reaches a consistent value of 4.95% ± 0.1%.
Figure 8: A) Photograph of the initial position of the micropipette and B) position of the micropipette following a commanded backward motion of 400µm. C) Graph showing the average positional error for a series of forward and backward steps of varying distances. Error bars are standard deviations in the positional error. D) Graph comparing the (our bioreactor vs. Bose ELF-3100) results of a series of strain-control experiments (1%, 2%, and 4% strain) run on rubber bands cut to three different lengths. Force deviations and strain repeatability measurements are shown. [35]
Figure 9: A) DIC images showing the initial and final (B) positions of two beads embedded in the collagen strip following an applied displacement or force C) Strain-control test aggregate results showing ramp to 5% strain with corresponding load response. D) Load-control aggregate results showing ramp to 0.1N load with corresponding strain results. [35]
4) **Temperature control results**: Figure 10 reports the transient and steady state temperature control performance of the mechanobioreactor at four different nutrient flow rates. The plots represent three experimental runs for each flow rate. **No flow**, Center: 36.6°C ± 0.13°C, Control points: 37.7°C±0.11°C. (B) **4 mL/min**, Center: 36.6°C ± 0.09°C, Control points: 37.7°C ± 0.07°C. (C) **40 mL/min**, Center: 36.5°C ± 0.08°C, Control points: 37.6°C ± 0.08°C (D) **400 mL/min** Center: 36.5°C ± 0.06°C, Control points: 37.4°C ± 0.04°C.

At all flow rates, the temperature inside the chamber reached 37 °C in less than 5 minutes. An internal temperature variation of ± 0.5 °C with a standard deviation of ± 0.04 °C in the center of the chamber was detected at all flow rates. The results from different flow rates show that flowing fluid through the device appears to stabilize the temperature, since the standard deviation dropped at high flow rates.
Figure 10: The recorded temperature at four different flow rates from the bioreactor. The center of the chamber (dashed line) and two control thermocouples (solid lines) on either side of the chamber were monitored while temperature was reaching 37°C. The target temperature at the center of chamber was 37°C. All plots represent three experimental runs for each flow rate. (A) *No flow*, Center: 36.6°C ± 0.13°C, Control points: 37.7°C ± 0.11°C. (B) 4 mL/min, Center: 36.6°C ± 0.09°C, Control points: 37.7°C ± 0.07°C. (C) 40 mL/min, Center: 36.5°C ± 0.08°C, Control points: 37.6°C ± 0.08°C (D) 400 mL/min Center: 36.5°C ± 0.06°C, Control points: 37.4°C ± 0.04°C. [35]
2.3.2 Transfer of PHCF Cell Culture System into Bioreactor

1) PHCFs on Glass substrate (coverslips):

Figure 11 represents a sequence of images (taken daily) of the PHCFs’ behavior over a period of one week. The mechanobioreactor allowed continuous observation of the motion of PHCF’s from the initial seeding to tissue formation. The PHCFs began adhering on the coverslip surface at day zero and then fibroblasts elongated to the glass at day 2. Next, the PHCF layer became confluent and exhibited random motion at day 3. The PHCF sheet motion slowed down and the existence of multiple cell layers was detected at day 4. At day 7, at least two different cell layers moving in different directions were observed (black and white arrows in figure 11). The motion of PHCFs (~60µm/day) was measured using particles/debris on the PHCF sheet surface (highlighted by small black circles in Figure 11). Also, some individual fibroblasts from the second cell layer (the white arrow in Figure 11H) were detected with a higher velocity of movement. The second layer formed at a relatively fixed angle to the first cell layer. This experiment showed that we could culture PHCFs inside the mechanobioreactor at least for a week and monitor the PHCF’s migration behavior. Note on the imaging: As the experimental series progresses in this thesis, one will note vast improvements in our optical tracking of the cells. This first series of experiments had fairly degraded DIC optics.
Figure 11: DIC image sequence of PHCFs on the glass substrate. At day zero (A), the rounded cells started to adhere on the glass surface. The glass substrate was coated to confluence by PHCFs at day 1(B). A sheet of PHCFs was formed at day 3(D). The motion of this layer was monitored by debris (black circle). The debris appeared to keep pace with cell motion (E and F). The cell sheet was moving vertically while a horizontal motion of individual PHCFs was detected at day 6 and 7(G and H). The black arrows represent the sheet motion (upward in image) while the white arrow shows the horizontal motion of individual cells. Scale bars are 100µm. [35]
2) PHCFs on Collagen type I gels:

We were able to observe fibroblast behavior for long periods at high magnification on the collagen gel substrate. In this series of experiments, the fibroblasts did not grow to confluence but they adhered and elongated on the collagen-coated glass. Figure 12 shows PHCF behavior on the collagen-coated coverslip at 0, 1 and 2 weeks. The results from this experiment showed that there are two different morphological sets of cells in the culture system. The indirect immunofluorescent staining study was not performed on this sample to show the differentiation of PHCF’s to the other cell type. We just observe two different morphological sets of cells in this experiment. One set of cells shows what appears to be typical behavior of human corneal fibroblasts (small black arrows in Figure 12) that were elongated on the collagen substrate. The other type of cells were rounded and appeared to be degrading the local collagen gel. The degradation of collagen gel (small holes on the entire collagen gel) is highlighted with black arrowheads in Figure 12. It is not clear if the cells in the second state (rounded shape) are differentiated from PHCFs over time or if they originally existed in the cornea at the day of extraction.
Figure 12: The DIC images of PHCFs at day 0, 7 and 14 on a collagen gel-coated coverslip. Two types of PHCFs were detected in the culture. The rounded fibroblasts (small black circle) and elongated fibroblasts (small black arrows) are visible in all images. (A) A mix of rounded and elongated PHCFs at zero-week time point. (B) The elongated PHCFs appear to dominate the population although both types are detected at 1 week. (C) The rounded PHCF’s are more prevalent in this region and appear to be eroding the collagen coating (arrowheads) at 2 weeks (C and D). The dynamic movie suggests that the rounded PHCF’s are degrading the collagen gel at multiple locations (arrowheads). [35]
3) PHCFs on DDCS held between grips:

Figure 13 is a series of images which showing the initial seeding of PHCFs on a dense collagen substrate at day 0 (Figure 13A), followed by the formation of a single organized layer of PHCF’s at day 2 (the first layer, Figure 13B, the white arrow) and then an organized cell sheet motion at day 5 (the second layer, figure 13C and D, the small white arrows). The second layer was formed at angle to the first layer and is shown sliding over the first layer at day 8.

In a second experiment (Figure 14), an initial formation of a single aligned sheet was not detected. Instead, the substrate became densely populated with a multilayered, disorganized colony of PHCFs. However, organization was arising in this structure, possibly through local, intra and interlayer cell contraction. However, at day 6, the tension/contraction in the colony became high enough to peel the culture off of collagen substrate. Notably, some individual PHCF’s were uncovered over the next three days (Figure 14) by the retreating cell colony. As the colony retreated, some cells attempted to catch the cell sheet while others began to die. We didn’t performed indirect immunofluorescence staining study for this sample. Therefore, we don’t know whether corneal fibroblasts were differentiated to myofibroblasts in the cell sheet (the multilayer cells) when cells migrated in complex cell sheet sliding motions.
Figure 13: A) The DIC images from initial seeding of PHCF on dense disorganized collagen gel at day zero. Rounded PHCFs were observed on the entire sample. B) First PHCF layer was formed vertically (elongated PHCF’s in the direction of white arrow) at day 2, C) A second layer was detected at day 5 forming a fixed angle with the first layer. D) An organized sheet motion of second layer of PHCF’s sliding over the first layer of PHCF’s at day 8. The rate of proliferation of PHCF’s and cell division was clearly observed over the entire sample (small white arrows) at day 8. [35]
Figure 14: The DIC images showing contraction of a multilayer of PHCFs on the dense organized collagen substrate at days 6, 7, 8 and 9. The direction of PHCF sheet motion is shown with white arrows in the images. A) The contracting PHCF sheet was formed at day 6, B) the PHCF layer was peeled off the collagen substrate and some individual PHCF’s were uncovered at day 7. C) The contracting PHCF sheet continued moving to right side of the image at day 8. More individual PHCFs were uncovered and showed necrosis/apoptosis behavior. D) At day 9, more uncovered PHCFs were observed on the collagen substrate and the motion of the contracting PHCFs sheet was slower compared to day 6, 7 and 8. The apoptotic/necrotic behavior of isolated PHCF’s was exposed by this contracting PHCF sheet motion. Scale bars are 100µm. [35]
5) Dense disorganized collagen substrates subjected to uniaxial strain results:

In the final experimental series, we seeded PHCFs on a preloaded DDCS specimen and observed the PHCFs behavior for up to two weeks. The DDCS sample was loaded to 0.01N load (less than 1% strain) at the beginning of the experiment (day zero) and we didn’t observe that the dense disorganized collagen substrate (DDCS) was organized by this amount stress/strain (0.01N load). The DDCS specimen was stretched to 6% strain when PHCFs reached confluence. The alignment of PHCFs (~40° respect to load direction) on the strained dense disorganized collagen was completed over a week and PHCFs generated a single cell layer on the entire collagen matrix (Figure 15A). After reaching cell confluence (at day 7), 6% uniaxial strain was applied to the DDCS sample. A cluster of embedded micro beads was identified to optically track the application of the 6% strain. The black and white circle in Figure 15B represent the location of the cluster of micro beads before and after strain is applied. Tracking the motion of the cluster of beads indicates that the mechanical strain results in an x-displacement of 5.6%. This amount of difference in commanded strain relative to optical strain (~ 0.4%) is consistent with our observations of strained DDCS during load and strain control tests (5% error, from previous results). Figure 15 C and D are high magnification images showing the optical strain from two targeted micro beads. We observed that the orientation of cells remained approximately at 45° to load direction (figure 15B), did not change dramatically and that the cells migrated approximately at the same orientation. In the second week, a second layer of PHCFs started to grow on the underside of the collagen substrate making a 130° angle to the load direction. The images from day 14 demonstrate
the existence of the two aligned layers of PHCFs (at 40° on top side and 130° on bottom side of DDCS) which were, at all locations, approximately perpendicular to each other.

Figure 15: The DIC images from PHCF on dense disorganized substrates before and after applying 6% strain. A) The image from the sample after 4 days of stretching at 0.01N. A cluster of microbeads has been identified to track following 6% strain application. (B) Tracking the motion of the cluster of beads indicates that the mechanical strain applied to the collagen substrate in an x-displacement with a concomitant vertical drift of the construct. The alignment of the PHCFs was observed at ~ 40° to the horizontal axis (big white arrow). The white and black circles represent the position of the bead cluster before and after 6% strain. The optical measurement of bead separation reported 5.6% strain instead of the commanded 6% strain. (C and D) The high magnification image of the collagen substrate shows the targeted beads before and after 6% strain respectively. The optical strain was measured based on the distance of these beads before and after 6% strain. Scale bars for low (A and B) and high (C and D) magnification are 100µm and 50µm. [35]
2.4 Discussion

Bioreactor operation confirmed in the absence of cells

Data from first aim of this study indicated that the mechanobioreactor was well-designed to provide the initial requirements for cell culture on an invented microscope. Our uniaxial mechanobioreactor is capable of providing 1) stable temperature control for long periods; 2) high-magnification continuous observation in one plane in conjunction with the PFS stabilized stage and 3) high accuracy strain and load measurements during substrate stretching. The maximum positional error of 6% was detected in backward movement of the grips at short distances (20 µm) while the amount of error was less than 1% for long distances. The results from the position accuracy test show that the actuator and transmission system operate consistency. Also, the mechanobioreactor measurements compare well to a commercially available system (Bose ELF 3100 Electroforce). The results verify that the bioreactor is also capable of accurately stretching the DDCS samples in both load and strain control modes. Moreover, we developed a method to optically measure strain by embedding 10µm beads into the DDCS. Temperature control of the environment inside the bioreactor was tested with four different flow rates. The time to reach the prescribed temperature value from cold start-up to 37°C was measured to be less than 5 minutes. The temperature controller could provide a stable temperature over long periods (with less variation than ± 0.5C) at all four different flow rates. We found that all flow rates (0, 4, 40 and 400 µl/min) stabilized the temperature of the chamber and provided a uniform temperature distribution inside the chamber.
Effective transfer of the PHCF culture into the mechanobioreactor system

**Glass substrate:** The transfer of our cell culture system of PHCFs was effective. The device provided a suitable environment to maintain PHCFs in culture for at least two weeks. During this time the bioreactor was mounted on our Nikon TE2000 permitting the close examination of cell migration. We effectively tested the viability of the PHCFs on three different substrates of long periods 7 – 14 days. The data show that human corneal fibroblasts were maintained at proper culture conditions and proliferated within the bioreactor. In addition to demonstrating the effectiveness of the mechanobioreactor, during these initial experiments we observed unexpected behavior of human corneal fibroblasts. The images from the glass substrate clearly revealed the morphological behavior of PHCF’s from the initial seeding (rounded fibroblasts) to multilayer formation. The results suggest that there is both full cell sheet motion and multiple fibroblast layers which display different orientations, directions, and speeds.

**Collagen coated glass:** The results from collagen coated glass substrate suggested the existence of two different morphological sets of human fibroblasts in the culture system. One set of cells demonstrated the typical behavior of fibroblasts that entailed an elongated cell profile and extensive filopodial exploration. PHCF’s in the second state however, were rounded and appeared to be degrading the local collagen gel. It is unclear whether the rounded cells are transformed PHCFs or if they came from cornea tissue when they were extracted.
Unloaded DDCS: In unloaded DDCS substrates inside the bioreactor, we were able to directly observe the initial populating of the collagen substrate by a single aligned layer of PHCFs (initial population). This observation was followed by stratification of the fibroblasts into layers which exhibited organized cell sheet motion. We observed the formation of an organized fibroblast layer on an unloaded disorganized substrate over a week. In a separate experiment under the same conditions, the unloaded substrate became densely populated with multilayered PHCFs. There was no initial formation of a single aligned sheet followed by stratification. Instead, over the first week the PHCFs formed a dense structure, multiple cells thick, with little organization. The cell mass then began to create visible organization via internal tensioning. At one location, the PHCF tension became so great that the entire cell sheet was pulled from the substrate. We observed that some fibroblasts attempted to catch the retreating cell sheet; whereas other exposed cells began to die quickly individual PHCFs were uncovered and began to die due to necrosis/apoptosis. Apoptosis is a naturally process of programmed cell death that a cell is directed to the death and is based on a genetic process inside the cell. At day 9, the population of exposed cells had suffered a high mortality rate.

Loaded DDCS: In the final experiment, we observed PHCFs on a lightly pre-loaded DDCS and after 4 days, they reached cell confluence. We then applied an extra uniaxial strain of ~ 6% to DDCS and observed fibroblasts for one more week. The results show PHCFs first populating the gel substrate and forming a generally aligned single layer of cells. Upon application of mechanical force, the cells appeared to reorient at uniform angle to the strain axis (about ~ 40°). Also, a second cell layer was formed on the bottom
side of the DDCS and made a ~ 130° angle (perpendicular to the top layer) to the load direction. This observation will be discussed in more detail in the next chapter of this dissertation.

**Limitations of the Mechanobioreactor**

However, there were some limitations, which could affect our ability to use the mechanobioreactor. The first limitation was the need to enrich cell media with CO₂. The bioreactor has a closed system design and we had to precondition the media with CO₂ overnight in an incubator. This preconditioned media was made and used for the bioreactor every day. In some cases, the preconditioned media released bubbles in the bioreactor, which caused some imaging difficulties. To circumvent this problem, a modification in the design of chamber lid can help to remove the bubbles from specimens during the experiment. One could also condition the media at slightly above 37° C to prevent outgassing. An important second problem was data storage capacity and software stability. Our two-week experiments entailed taking a large number of high magnification images. Storing this amount of data in one file produced instability in the microscope’s software (NIS Element, version 3.1; Dell inspiron performance. For example, we took a tiff image (6 Mbytes per image) every 6 minutes from one location on the sample for a period of two weeks. This means taking ~ 3000 images totaling 10 Gigabytes. To solve the file storage problem, Image J software was used to resize all images and reduce the file size.
2.5 Conclusions

The mechanobioreactor has demonstrated excellent capacity to perform our experiments. The device can load specimens in uniaxial tension from 0 to 10N with 0.01N resolution. The strain resolution is up to ~ 0.5% of gage length (15mm) while specimens of various dimensions can be tested (3 to 16mm gage length; 0.01-2mm thickness and 0.1-12 mm width). Ultimately, the mechanobioreactor provides a stable temperature control ~ 37C ± 0.05 for long-periods while providing operational perfusion access and minimization of the chamber volume dead space. Finally, the device mounts with minimal modification to a standard inverted microscope stage and provides the ability to perform high magnification transmission optical microscopy (using up to 60X objective). It is a powerful tool for which to use in the study of mechanobiology.
Reference


Chapter 3

Live, Long-Term Observation of Migration of Primary Human Corneal Fibroblasts on 3D Collagen Matrix Subjected to Uniaxial Mechanical Load

3.1 Introduction

To understand how cells build a tissue under mechanical load is one of the most important questions in tissue engineering [64]. Strong evidence supports the hypothesis that, in connective tissue, mechanical load is essential for tissue formation, growth and maintenance [1-7]. Indeed, some studies have demonstrated that cell behavior, such as cell migration and organization is dependent on these mechanical forces [8-13]. Understanding the role of mechanical force on cell migration and orientation can be highly beneficial for tissue engineers trying to control matrix synthesis and for developmental biologists attempting to understand connective tissue growth and can provide insight into pathologies such as fibrosis and metastasis [8, 14]. In “avian ocular globe” research, mechanics has been suggested to have a role in the growth and formation of tissue and its effect has been studied [14-15]. These studies have shown that the loss of intraocular pressure results in a decrease in corneal cell growth rate which leads to a condition called “small eye” [16]. Studies on corneal fibroblast mechanobiology have shown that fibroblasts exert physical forces (~ 0.1 µN) on collagen scaffolds [17] and they are responsive to mechanical stimulation applied to them by their surrounding matrix [18-20]. It is shown that corneal fibroblasts in 3D matrices tend to
release the imposed tensional or compressional stresses. In one study, the local ECM stress was manipulated by using embedded micro needles in the ECM (collagen gel) and the response of human or rabbit corneal fibroblasts in these 3D matrices was observed. It was concluded that corneal fibroblasts respond to tensional and compressional stresses and attempt to maintain a constantly tensional position in ECM. In addition, mechanochemical studies have demonstrated that mechanical load can stabilize and protect collagen fibrils against enzymatic degradation [21-25]. The results have shown that forces in the range of piconewtons (only at monomer level) can significantly reduce the enzymatic degradation of corneal tissue and can protect collagen fibrils during the degradation process [25].

Studying the effect of mechanical signaling on ECM stability could suggest a natural method to develop a practical treatment for damaged tissue. However, the role of mechanical load on the formation, growth and treatment of tissue is not completely clear. Live dynamic observation of the effect of mechanical load on cells over extended periods can answer critical questions about the mechanism of tissue formation, growth, maintenance, pathological progression and the role of mechanical signaling in these processes.

3.1.1 Known effects of mechanical stimulation on general cell behavior in culture.

Endothelial cells

Previous studies have shown that endothelial cell alignment is altered by the application of mechanical load or shear flow [26]. They have shown that the endothelial cells (from vessels and aorta) elongate under mechanical load or shear stress their longer
axis and their orientation is perpendicular to the load direction [27]. Others have shown that under mechanical load F-actin is rearranged to the direction of load [28-29]. They have shown that by applying uniaxial cyclic strain, the stress fibers of endothelial cells get reoriented rapidly in less than an hour and align at a specific angle with respect to the load direction and proportional to the load magnitude [30].

**Fibroblasts**

Several studies have reported significant correlation between changes in cell orientation and the magnitude and direction of mechanical load on several different native substrates [31-37]. Application of tensional strain on 3-D collagen gels has resulted in alignment of fibroblasts as well as their deposited collagen fibrils to the loading direction [65]. In 2011, short-term, live, dynamic imaging of ligament and bone fibroblasts on a stretchable scaffolds suggested that each cell type may exhibit a unique kinetic footprint [32].

### 3.1.2 Mechanical Stimulation of a Developmentally Mimetic Corneal Culture System

In previous studies, primary human corneal fibroblasts (PHCFs) were shown to produce organized collagenous constructs (similar to native stromal tissue when cultured in stabilized ascorbic acid [38]. Long-term observation of this developmentally mimetic culture system subjected to externally applied mechanical load can help us understand the role of mechanical stimuli on the deposition and organization of cell-secreted collagen fibrils during formation of corneal stromal tissue. Direct, sustained dynamic observation may elucidate whether mechanical load plays a significant role in matrix organization and/or cell behavior [3]. Mechanical stimuli (strain or load) applied to cell substrates can play two important roles: 1) they may alter the local modulus of scaffold, which can lead
to durotaxis [39, 40] and 2) alter the orientation of fibrils in the scaffold, which can lead to “contact guidance” resulting in cellular alignment with the fibril’s orientation [41, 42].

**Durotaxis:** Durotaxis was first introduced by Lo [40] using 3T3 fibroblasts on polyacrylamide substrates with directional mechanical properties. Recently, it has been shown that cells sense changes in the substrate rigidity through focal adhesions and cytoskeletal components such as actin stress fibers [39]. Cells migrate toward stiffer substrate, where the cells can anchor their actin stress fibers [43].

**Contact guidance:** It has been shown that fibroblasts are elongated and aligned with collagen fibrils and then they adhere to collagen fibrils through focal adhesion [68]. Based on this observation, silicon microgrooves at micro scale have been designed to mimic collagen fibrils. The goal of these patterned substrates is to study how fibroblasts respond to contact guidance by the grooves while under mechanical load along specific load direction. These microgrooves help fibroblasts to form a monolayer of cells aligned with the microgrooves. The growth of a second layer of highly organized cells was also observed on the top of the first layer, which were oriented at a different direction. It was concluded that these microgrooves help fibroblasts to organize their ECM in multiple cell layers [35]. Another study investigated the effect of these microgrooves while applying uniaxial mechanical load simultaneously [69]. They have shown that microgrooves help the fibroblasts alignment and that the applied mechanical load does not change the cell orientation after their organization on the microgrooves even under high amplitude strains. It was thus concluded that the orientation of fibroblasts depends on the patterned substrate surface which act as contact guidance, mimicking collagen fibers in vivo [69].
3.2 Materials and Methods:

**Aim 1:** To culture nucleus-labeled PHCFs on strained Dense Disorganized Collagen Substrate (DDCS) constructs coupled with long-term observation. Live Dynamic Differential Interference Contrast (DIC) microscopy and live dynamic Immunofluorescent microscopy are powerful methods for the observation of cells under mechanical load.

**Experimental protocol:**

1) **Preparation and expansion of PHCFs (Primary Human Corneal Fibroblasts):**

PHCFs were extracted from a 12 year-old donor cornea using our standard cell extraction protocol as described in chapter 2 [49, 50]. The human corneas were cut from sclera by sterilized razor blades and washed three times by 1X PBS in a sterilized dish. Then the epithelium and endothelium layers were gently removed from corneas by sterilized razor blades. The corneas (stroma) were cut to a few small pieces (2mmX2mm square) and washed three times by 1X PBS to place and culture corneal pieces on 6 well cell dishes. Sterilized DMEM enriched by 10% FBS and 1% antibiotic was added to corneal pieces and then the dishes were kept in an incubator for two weeks. Every day, 50% DMEM was gently extracted from each well and 50% fresh enriched DMEM was added. When the primary human corneal fibroblasts grew to confluence in the dishes, they were trypsinized and subcultured in different sterilized dishes and expanded in culture for 2 weeks. After 3 passages, PHCFs were moved into sterilized culture dishes and kept in the incubator until the day of experiment. A culture flask was washed two times with 1X PBS then preheated Trypsin/ETDA for 20 minutes then suspended cells were removed.
into a 15ml falcon tube. The cells were centrifuged (2000 rpm for 5 minutes) and counted using a standard cell passage protocol. The final density of the solution was 1000 cells/µl for every experiment.

2) Preparation of bioreactor to culture PHCF:
Our custom bioreactor [51] was readily disassembled into its component parts for cleaning. The bioreactor was disassembled outside the laminar hood and the major components: chamber, load cell, frame and lid were washed for 10 minutes in 70% ethanol and moved into the hood. Smaller internal components such as the O-rings, valves, grips and rods were kept in 70% ethanol under UV light overnight. External small components, i.e. valve connectors and tubes were washed in 70% ethanol and remained under UV light overnight as well. The next day, all components were assembled and then 10 ml of preheated DMEM (37°C) was passed through the assembled chamber and tubing to remove the ethanol. All electrical wires (the actuator and load cell) were sealed with sterilized, autoclaved paper to protect them from the ethanol and DMEM during sterilization of the bioreactor in the hood. Finally, the sterilized, assembled bioreactor was removed from the hood, mounted on the microscope and connected to electrical support for the heater, load cell, thermocouples, and actuator.

3) Preparation of DDCS (Dense Disorganized Collagen Substrate):
Type I bovine collagen (3 mg/ml) (Advanced Biomatrix) was polymerized inside a 30000 MWCO dialysis cassette (Spectrum Laboratories). The collagen solution (8 ml collagen, 1 ml PBS 1X, 1 ml NaOH 0.1M) was mixed with polystyrene microbeads (10um beads,
Polysciences) before polymerization to provide a direct optical measure of the applied strain. After polymerization, cassettes containing the bead/collagen mixture were immersed in 40% PEG (20kDa) overnight at 37°C. A custom made cutting die was used to generate accurate and repeatable test strips from DDCS (~15mm Length X 6mm Width X 40µm Thickness). At the end, the DDCS strip (our specimen) was placed and fixed between two sterilized grips inside the assembled bioreactor while the assembled bioreactor was in the laminar hood.

4) Seeding of cells into the bioreactor system:

The bioreactor, as described in chapter 2, has been designed and built with two inlets and one outlet separately. The first inlet was connected to a syringe pump to feed corneal fibroblasts with a constant flow rate (the enriched DMEM) and the second inlet was used to seed the samples with suspended corneal fibroblasts at the beginning of the experiment. A sterilized syringe was filled with DMEM (mixed with the final density of PHCF’s (1000 cells/µl) and it was connected to the second inlet. Before the sterilized bioreactor was moved from the hood to the microscope, the loaded syringe (1000 cells/µl) was connected to the second inlet. When the bioreactor was mounted on the microscope and all wires were connected, the PHCFs inside the loaded syringe were gently injected to the chamber. This method was used to seed the specimens inside the bioreactor uniformly. After corneal fibroblasts were injected to the bioreactor, the second inlet was blocked by a sterilized value until the end of the experiment. The first inlet (to feed corneal fibroblasts) and the outlet (to collect the media) were unlocked to feed fibroblasts with 8µl/mins flow rate.
5) Note on co-culture in the bioreactor:

This series of investigations was performed in a bioreactor that not only houses the glass substrate, it also houses a set of grips which are designed to apply a uniaxial force to a second extensible substrate (a dense disorganized collagen substrate – DDCS) on which the PHCFs grow as well. In this study, the PHCFs which populated the glass are considered a secondary surface control series of experiments. The behavior of the PHCFs on the glass will be compared to PHCFs which populate a DDCS construct to which mechanical tension is applied. Thus, the observations reported here should be interpreted in the context of the whole experiment, which was in effect, a simultaneous co-culture experiment where the same cell source was grown on two different substrates.

6) Perfusion methodology:

The bioreactor is designed to operate independently of the incubator and directly on the microscope stage for extended period. Because of the closed system, changing the media as we normally do in our PHCF cell culture system is not possible (total media exchange once every two days). Instead, we used a constant perfusion method in which the media is preconditioned with CO₂. The 20ml DMEM (mixed with 10%FBS and 1% antibiotic) was added to a T75 cell culture flask every day and was kept in an incubator for at least 24 hours. The fresh enriched DMEM was loaded into a sterilized syringe every day and was pumped to the bioreactor by a syringe pump with 8ul/mins (flow rate) from day 0 to 3. Ascorbic acid (Vitamin C) at 0.05mg/ml (50 mg powder in 1000ml DMEM) was added to the enriched DMEM from day 3 to 14. The extracted DMEM from the outlet is collected with a sterilized, HEPA filter sealed bottle during the experiment. While it was
difficult to settle on a perfusion rate, we chose one that would exchange the media fairly rapidly (at least 7 chamber volumes per day). However, we were also concerned about the effect of shear-stress on the sample and the autocrine factors being swept away with the solution. In the end, the perfusion rate chosen was 8μl/min which resulted in a shear stress of 0.004 dym/cm² on the cell layer (this calculation has been shown in chapter 2). Thus we are confident that adequate media exchange was provided without unduly influencing the cells via shear-stress mechanotransduction or by removing critical autocrine factors [56, 57].

7) Experimental Series Conducted

1- PHCF Seeded DDCS with load:

The DDCS substrates (N=4) were mounted on tensile grips and seeded with 1000 cells/μl as previously described. The bioreactor was filled with DMEM supplemented with 10% FBS, 1% antibiotic and dissolved CO₂ and mounted on an inverted microscope. The temperature of the system was set to 37°C to provide a suitable culture environment for the PHCFs. To ensure that the substrate was within a single focal plane, all DDCS specimens were pre-stretched to 0.01 N load (50kPa stress). This amount of stress was applied to specimens until cell confluence was observed on the entire specimen (approximately one week). The bioreactor was shifted to strain control mode and a 6% fixed strain was applied at a 100 um/s strain rate) while the load data were recorded. The culture was continuously fed with DMEM supplemented with 10% FBS, 1% antibiotic and dissolved CO₂ at a fixed flow rate of 8ul/min and maintained at 37°C for the two week period, however at day 3, 0.05mg/ml stabilized ascorbic acid was added to the
media. DIC (DIC has been filtered out by a filter block from 385 to 420 excitation wavelength) images were taken every 6 minutes using the 20X objective, while live fluorescent images were taken every one hour. The lower rate of fluorescent imaging was used to protect cells from photo-bleaching, photo-toxicity and cell death [55].

2- PHCF Seeded DDCS without load:

While the coverslip control was useful, it was not a complete control and was not adequately designed to isolate the effect of strain on the substrate. To better control the applied mechanical strain, another set of DDCS substrates (N=3) were placed between the grips and seeded with 1000 cells/µl as described in “Seeding of cells into the bioreactor system” section. The bioreactor was perfused in the same manner as before and the experiments were run for one week. Because this experiment was a true “no load” control, we were unable to image the surface of the DDCS in case of sagging. To improve imaging, the DDCS samples were stretched just enough to bring them into the focal plane of the microscope (0.01 N load) once per day to allow a single image to be taken, before it was immediately unloaded. 0.05mg/ml stabilized ascorbic acid was added to the enriched DMEM beginning on day 3.

3- Unseeded DDCS with load (control test):

It is always the possibility that our DDCS substrates are intrinsically affected in the bioreactor. To avoid any changes in the DDCS over time, the DDCS substrates (N=3) were fixed between tensile grips and the bioreactor was perfused with DMEM supplemented with 10% FBS, 1% antibiotic and dissolved CO₂. The bioreactor was
mounted on the microscope and strained to 0.01 N load. All control specimens were fed with the supplemented DMEM for three days (flow rate 8ul/min) and then 0.05mg/ml stabilized ascorbic acid was added to the DMEM. At day 7, specimens were stretched to 6% fixed strain and load data were recorded the rest of experiments. This type experiment was designed to measure the natural stress relaxation behavior of the DDCS specimens over the period of the experiments (two weeks) and to present any slippage of specimens from the grips due to unforeseeable instability.

8) Live Cell Staining and Imaging:

In one experiment in series 1, PHCF nuclei were labeled with Hoechst 33342 (trihydrochloride, trihydrate - 10 mg/mL solution in water) according to the manufacturer’s protocol. The blue fluorescent Hoechst 33343 has multiple applications in cell visualization and the fluorescent signal from this dye is very sensitive to DNA conformation and chromatin state in cells. The dye binds to all nucleic acids, but AT-rich dsDNA fluorescent ~ 2-fold greater than GC-rich chromosome regions. With this live cell chromophore, we were able to stain cell nuclei directly on the microscope anytime. The dye solution was injected in the cell media in the bioreactor for 30 minutes without being image and then the whole chamber was washed with DMEM three times to remove any traces of the dye. After staining the PHCFs, the whole reactor chamber is filled with preheated DMEM supplemented with 10% FBS, 1% antibiotic, and 0.05mg/ml ascorbic acid and dissolved CO₂. Fortuitously, the PHCF cell staining can be refreshed every three days to augment the fluorescent signal from the stained nuclei over the two week. Images were taken of the cell culture at three locations every 60 minutes (exposure time 400
microseconds) using fluorescence microscopy. The imaging system employed a high-intensity white light source coupled to the optical system through a filter cube which permitted an excitation wavelength 343 nm and emission wavelength 483 nm) refer to invitrogen handbook [52]. The resulting image series were stitched into a movie using orientation and motion detection algorithms (see PIV and FFT methods below). To provide correlation with the behavior of the cell cultures which were not live-stained with the nuclear dye, DIC images were taken as well (and in the same locations). However, to minimize phototoxicity, the DIC imaging was performed using a light source in which the excitation frequency of the Hoechst 33342 stain was blocked.

9) PHCF Seeded coverslip:
The sterilized bioreactor was mounted on the microscope and the coverslips (N=4) were seeded with 1000 cell/µl. The culture was fed by DMEM supplemented with 10% FBS, 1% antibiotic and dissolved CO₂ at a fixed flow rate of 8ul/min and maintained at 37°C over two weeks while 0.05mg/ml stabilized ascorbic acid was added to DMEM from day 3 to 14. DIC images were taken every 6 minutes using the 20X objective for DIC imaging continuously, while live fluorescent images were taken every one hour as described in “Live Cell Staining and Imaging” section.

10) Indirect-immunofluorescence staining:
Two of the constructs were collected and fixed in 4% paraformaldehyde after 2 weeks. Immunofluorescence was performed as previously described [53, 54]. The constructs are fixed and incubated at 4°C overnight with the primary antibody—anti-type III collagen
(1:40: Southern Biotech; Birmingham, AL), anti-SMA (1:50: Dako North America; Carpinteria, CA), Type I collagen (1:50: Abcam; Cambridge, MA), and Fibronectin (1:800: Sigma; St. Louis, MO) —diluted in 1%BSA + 0.1%Triton-X. The constructs are then washed and incubated overnight at 4°C with the corresponding secondary antibody—donkey anti-goat IgG (1:200, type III Collagen), donkey anti-mouse IgG (1:200, SMA), donkey anti-rabbit IgG (1:200, Type I Collagen) and donkey anti-mouse IgM (1:200, Fibronectin) —diluted in 1%BSA + 0.1%Triton-X. Phalloidin-rhodamine (Invitrogen; Carlsbad, CA), which binds to the cell F-actin, is also used. Constructs are counterstained with TOPRO-3 iodide (1:1000, Invitrogen), a marker of cell nuclei. Negative controls, where the primary antibody is omitted, are run with all experiments. Constructs are washed, mounted with Vectashield Mounting Media (Vector Laboratories; Burlingame, CA), observed and photographed using a confocal TCS-SP2 Leica microscope (Leica Microsystems; Bannockburn, IL).

11) Transmission Electron Microscopy (TEM) - Post Processing:

All specimens were removed from bioreactor after 60 minutes and processed for TEM [67]. Briefly, the specimens were immersed in 1/2 strength Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1M Cacodylate buffer, pH 7.4) for 1 hour, post-fixed in 2% osmium tetroxide and serially dehydrated in graded ethanol. Following dehydration, specimens were embedded in Epon-Araldite and thin-sectioned perpendicular to the applied load in the center of the tensile specimen. Specimens were stained with Uranyl Acetate and Lead Citrate in methanol and viewed on the JEOL 1000 Transmission Electron Microscope (JOEL, Tokyo, Japan).
Primary Human Cornea Fibroblasts were provided and stained based on our protocols (see above). Then the labeled fibroblasts were seeded on different substrates (1-Coverslips, 2-strained DDCS and 3-unstrained DDCS). Figure 16 is a schematic our setup showing the two imaging locations, one on the DDCS and the other on the coverslip. Figure 17 is a merged DIC/fluorescent image from nuclei-labeled human corneal fibroblast on the coverslip sample. The PHCF’s are locally aligned on the coverslip. The coverslip has not coated by collagen and there is no load on fibroblast, but they are locally aligned.
Image processing - FFT (Fast Fourier Transform), PIV (Particle Imaging Velocimetry) and Spatial Autocorrelation Function (SAF):

Introduction of three methods:

FFT is a useful image-processing tool used to transform an image to series of sinusoidal components in the frequency domain. In the FFT image, each pixel represents a particular frequency. This method was helpful to analyze the orientation of different sections of images. With this method, we were able to continuously quantify human corneal fibroblasts orientation. Figure 18 represents a few simple images, which were imported into our MATLAB code and analyzed using FFT methods.
Particle Imaging Velocimetry (PIV) is an optical technique to track velocity vectors and related properties in fluid mechanics. Two velocity vectors (2D model, x and y axis) are measured at a specific cross-section by a camera. To achieve these measurements, micron-sized fluorescent particles were seeded into the flow to track and map the fluid flow. We used the PIV method to assess the velocity of nuclei-labeled PHCFs motion over the two week period.

Spatial Autocorrelation Function (SAF) is a technique in image processing to study a variable in order to be correlated with itself. This method gauges how a pair of variables are similar or dissimilar each other. When both variables are autocorrelated, the variables will be predictable at each location and nearby locations. Otherwise these variables are completely independent. With this property of spatial autocorrelation, we apply it to our images to highlight the cell alignment and cell organization on the different substrates when PHCF’s assemble to form layers on substrates.

3.2.2 Experimental protocol

1) Continuous Assessment of Cell Orientation, FFT (Fast Fourier Transform):

All DIC images (single or panoramic images) were imported to custom MATLAB programming software and were transferred to binary (black and white) images. All analyzed images were plotted as histograms and polar graphs. The Fast Fourier Transform (FFT) is a method to describe the anisotropy of fibers in binary image by using image processing analysis. In the other words, the aim of applied FFT method in this study is to graphically and dynamically quantify the orientation of cells. The graphical FFT method used spectral techniques with the aid of two-dimensional Fourier
transform in the form of vectors. We extended the FFT method to Z scan images collected from specimens to quantify the alignment of PHCF’s at each Z scan images. Images were converted to grayscale images; a custom MATLAB programming was developed and performed on images by the FFT methods to highlight the orientation of human corneal fibroblasts. The converted grayscale images were imported in the MATLAB program and then the output was a series of orientation arrangement of PHCF’s in the form of a histogram and polar images. The distribution of angles was graphically showed in polar plots and the orientation of fibers was compared at fixed intensity.

2) Continuous Velocimetry, PIV (Particle Imaging Velocimetry):
PIV (particle imaging velocimetry) is a combined computational and optical method which generates a velocity field in a fluid flow by following observable markers. The PIV method was adapted to track stained PHCFs in the bioreactor continuously over the whole fluorescent images. Fluorescent images obtained from the experimental series were transformed to binary (black and white, Figure 19) by Imagej software and then processed with a custom PIV algorithm (MATLAB) to extract the cell nuclei velocity field. Figure 19A shows a single nucleus stained PHCF on the coverslip sample and Figure 19B represents the binary transferred image from the original fluorescent image to black/white image by Imagej software. All fluorescent images were first transferred to binary images by Imagej software and then a mask (20 X 20 µm²) was fitted on the bright area as a unit in the whole image. This unit was assumed as an individual unit and tracked in the image. Two images (the first one at t = 0 hour and second one at t =1 hour) were
compared in the PIV algorithm and the individual bright unit was detected before and after movement. Based on time (hours) and dimension (µm), the displacement and velocity have been detected and the velocity (length and direction, green vector in figure 19C) plotted as a velocity of nucleus in the image. Figure 19C represents the final calculated velocity of a single stained nucleus in the fluorescent image. This algorithm has been performed for the whole images over the two weeks experiment [66]. The resulting vectors were projected and plotted in two dimensions and the data provided a continuous velocity vector field for cell nuclei motion over the two week period.
Figure 18: Three different images containing one line (B, one orientation), a few lines (A) and several lines with different orientations (C) to test and verify our custom MATLAB code based on FFT method. The orientation and alignment of images were illustrated as FFT diagrams (frequency domain) and histograms plots. There are some limitations for FFT method. The FFT method can not detect the length and width of fibers in the images. This method just highlights the range of fiber orientation. The polar plots magnify this range. If the plot is a thick and short (Figure 12C), we have a large range in fiber orientation. While the plot is narrow and elongated, we have a small range in fiber orientation (Figure 12 A).
Figure 19: Preprocessing images of the fluorescently-labeled human corneal fibroblasts. An image of a single corneal fibroblasts nucleus on the coverslip (A1) is transformed to a binary black/white image (A2) and tracked over time for cell velocity. The PIV program picks up a small area of 20X20 µm² (A3) to detect the change in white pixels location. The green arrow is the average direction of motion of pixels in the selected region. B1 shows the nuclei of corneal fibroblasts in the whole field of view on coverslip sample and B2 shows the transformed binary image. Scale bars are 15µm.
3) Assessment of Cell Alignment, SAF (Spatial Autocorrelation Function):

All panoramic DIC images were imported into custom MATLAB code and then the cell alignment are quantified by estimating the local orientation of cells in the pictures using MATLAB. After normalizing the image, the gradients of the image in both x and y direction are calculated. This is done by generating a Gaussian filter and its gradient in x and y directions and then filtering the image with this gradient. Afterwards the principal axis of variation in these gradients is found locally to estimate the local orientation of the intensity variations (in this case PHCF’s). The principal direction of cells in every point is analytically identified. Therefore, there is a principal direction for every point (pixel) of the image. Depending on the characteristics of the image we can sub-sample these principal directions to a desired number. The final data shown is the histogram of these directions (small blue lines in a white background). Figure 20 A and B represents the DIC image and the histogram of cell direction (small blue lines) based on this method. Then the histogram was imported to another custom MATLAB programming to calculate the spatial autocorrelation. The histogram was cross correlated with itself at X and Y direction. The result was plotted as normalized autocorrelation versus the distance to show the cell alignment from different substrates (Figure 20C).
Figure 20: A) DIC images from loaded DDCS sample at day 7 B) the histogram of cell alignment by edge detection method C) the result of spatial normalized autocorrelation from the histogram
3.3 Results

Images (Figure 21 A-H) from cells which settled on the coverslip were extracted and are depicted in a static sequence. PHCFs possessed a rounded shape at the initial of seeding (first day figure 21A), followed by rapid adhesion and elongation on the glass by day 3 (figure 21B and C). The fibroblast cells reached confluence at day 4 (figure 21D) and appeared to be locally aligned at day 6 (figure 21F). The images demonstrate dynamic re-organization and the general motion of a cell layer at day 9 (figure 21G), while by days 11 to 14 (figure 21H), a new layer of cells started to grow on the first layer. The culture system was exceedingly dynamic as cells were continually dividing, dying and migrating during two weeks. Figures 21 I-P show the cells settling on the collagen matrix film. Figure 21I shows rounded cells that were elongated at different orientations after 2 days (Figure 21J). By day 4, the PHCFs were confluent and locally aligned (figure 21K). The alignment of cells (135° to load direction) on pre-strained DDCS was completed and PHCF fibroblasts generated a single layer cell on the entire collagen matrix by day 6 (figure 21L). We didn’t detect that DDCS was organized or aligned by this amount of load (0.01N) in the first week. After reaching cell confluence, 6% strain was applied to the samples at day 7. We observed that the alignment of cells remained approximately at 135° to load direction and did not change in any distinct manner and that the cells migrated approximately at the same orientation. From day 7 to 14 a second layer of cells started to grow at the back of the collagen substrate at a 45° angle to the load direction. The images from day 14 demonstrate the alignment of PHCFs (at 135° on topside of DDCS) at nearly all locations on the substrate (locally and globally). The DIC images and dynamic fluorescent images captured from stained fibroblast cells on the coverslip.
and DDCS specimens were analyzed by FFT and PIV algorithms to evaluate cell orientation, as well as magnitude and direction of cell velocity.

Figure 21: Merged DIC/DAPI images of live, dsDNA-labeled HPCFs in the uniaxial loading mechanobioreactor. A-H Cells on coverslip from initial seeding to confluence (day 3) through matrix production (days 11-14) I-P Cells on DDCS substrate from seeding (day 0) to confluence (day 4) to load application (day 6 – white arrow shows the direction of load) through matrix production (days 11-14). Cells on the coverslip align locally while fibroblasts on loaded DDCS align at a fixed angle (135 degree) with respect to the direction of applied strain at nearly all locations. The white arrows represent the load direction. Scale bars represent 50μm.
Figure 22 demonstrates the analyzed data at two different locations in the experiment (coverslip and DDCS) with both methods. Figure 22 A and B show the distribution of ds-DNA labeled fibroblast motion by PIV on coverslip at day 0 and 14, while Figures 22 D and E show all motion on pre-loaded and loaded DDCS samples at day 0 and day 14 respectively. In each case the green and orange small arrows on the images display the direction of cell motion quantified by PIV method. This orientation changes day by day and the image at day 14 illustrates that the orientation of cell motion reaches an approximately constant angle on the DDCS. Figure 22C shows the mean velocity angle of cells on DDCS (orange dots) and coverslip (green dots) throughout the experiment. The orientation of cells (not the direction of their motion) analyzed by FFT method is also shown on the same plot from coverslip (blue dots) and DDCS (red dots) over two weeks. Using FFT, we measure the angle of cells at each point while PIV was used to calculate the orientation and velocity of cell motion at the same time. Both methods (FFT and PIV) verify that the cell motion and cell angle approximately have the same value at each point on DDCS sample while they do not match as perfectly on the coverslip from day 3 to 7 and then they converge. Also, cell motion (orange dots) and orientation (blue dots) on DDCS are more organized and close to the observed 135° angle compared to green dots (coverslip). Figure 22F and I demonstrate the FFT analyzed data from three different experiments on the coverslip and DDCS. These data suggest that the angle of PHCFs converges to 135° in all three different tested samples on the DDCS while this angle is different for each location on the coverslip. Figure 22H shows the mean velocity magnitude of the PHCFs analyzed by PIV method. These mean velocities illustrate that PHCFs migrate faster during the first 4 days and then plateau approximately 4 days after
cell seeding. The histograms of PHCF orientation quantified by FFT on the coverslip (G and H) and DDCS substrate (J and K) at day 0 (G and J) and 14 (H and K) are also shown in Figure 22. These data are consistent with the qualitative observations revealed by the video. To evaluate the alignment of PHCFs on the entire of DDCS and glass, panoramic DIC images were captured to verify the global cell alignment. Panoramic images from the entire DDCS (Figure 23A) and the coverslip (Figure 23B) demonstrate that the alignment of fibroblast cells on the DDCS is approximately uniform all over the substrate at a 135° angle with load direction while on the coverslip only local alignment of the cells was observed.
Figure 22: Live ds-DNA labeled PHCFs with overlaid particle velocimetry vector field of PHCFs on coverslip at day 0 (A) and 14 (D), vector field of the cells on a strained DDCS substrate at day 0 (B) and 14 (E). (C) Orientation of PHCFs on the same sample as determined by PIV and FFT methods versus time. FFT analysis of PHCFs on the coverslip at day 0 (G) and day 14 (J) and on strained DDCS at day 0 (H) and day 14 (K). Median angle of the cells on three different DDCS samples using FFT method and their average in red (F). Median angle of the cells on coverslip for three independent experiments (I); showing the variation of cell orientation on the coverslip as opposed to strained DDCS substrate. (L) Mean velocity of the cells on coverslip and loaded substrate showing a steady decline when the cells reach confluency state and continuous at constant velocity $\sim 6\mu$m/h in the second week when the matrix is being produced. Calculated mean velocity of PHCFs on coverslip (M) and loaded DDCS (N) specimens by PIV method from DIC images (N=3) and fluorescent images (N=1) respectively. The data from both methods demonstrate that the average mean velocity from DIC images is the similar to mean velocity from fluorescent images on both substrates.
Figure 23: FFT analysis of the global fibroblast alignment on the coverslip (A1) and the loaded DDCS construct (B1) at the end of two weeks of PHCFs culture. The panoramic montages (A1, B1) contain 144 images taken at 20x magnification. Clear global orientation is indicated by the integrated FFT plot for the fibroblasts on the strained DDCS (A2 and B2). Polar histogram plot corresponding to FFT results (A3 and B3) on coverslip and DDCS respectively. Alignment on the coverslip persists only locally while the cell alignment on DDCS is globally fixed at 135 with the direction of load. White arrow: load direction; black arrow: fibroblast orientation.
The Z-scan of samples demonstrate the existence of an aligned layer of PHCFs (oriented at 135° to the load direction) on the front side of the sample and another aligned layer (oriented at 45° to load direction) on the other side of the sample (Figure 24 A-E). Figure 24 is the Z-scan of DDCS sample from top to bottom layer with FFT analysis. The cell orientation from top (A) to bottom (F) side of DDCS has been analyzed by FFT method (our custom MATLAB code) and then the distribution of cell orientation throughout the scaffold.
image is shown by a polar histogram extracted from FFT results at each Z-plane. This behavior was consistent throughout our experimental series.

Figures 25, 27 and 29 present 2D FFT results of three different substrates (coverslip, loaded DDCS and unloaded DDCS) at day zero. Three separate sets of experiments were analyzed for each substrate. At day zero, 2D FFT diagrams and histogram plots from the three specimens (Figure 25A3-C3, 27A3-C3 and 29A3-C3) demonstrate that cell orientation is uniformly distributed from zero to 360. Figure 26 shows the results of three different experiments on coverslip at day 7. The cell orientation from three different experiments on coverslip samples show peaks in distribution at 90°, 135° or 20°. The different dominant orientations from coverslip samples confirm that corneal fibroblasts were locally aligned at each location but not globally and that the angle of cell alignment is not fixed on the coverslip. This observation was also confirmed by looking at panoramic images previously (Figure 23B1-3). Here we show that cells are locally aligned and the alignment is not the same across different sets of experiments. Figure 28 shows cell orientation data of three different loaded DDCS samples at day 7. Cells on all samples are aligned and oriented at approximately 135 degrees with respect to the load direction. This fixed alignment from loaded specimens shows that cells were aligned both locally and globally over the first and second week of the experiments and that the angle of cell orientation is not random. The panoramic image from the loaded DDCS sample (Figure 28A1-3) contains a larger area of loaded DDCS sample and supports our observations from three different DDCS samples. The unloaded DDCS samples, our control tests, are shown in Figure 30. The cells on unloaded DDCS samples are locally aligned but not globally. Three different experiments from three unloaded DDCS samples
prove that cell alignment is significantly different in each experiment and has peaks in orientation distribution at either $80^\circ$, $40^\circ$ or $90^\circ$ based on 2D FFT analysis.

Figure 25: Three different experiments were performed on coverslip samples. At day 0 (A1, B1 and C1), images were analyzed with 2D FFT method (A2, B2 and C2) to measure the cell orientation. The distributions of orientation direction are shown as polar histograms in A3, B3 and C3. The FFT diagrams and histogram plots demonstrate that cell orientation is almost uniformly distributed at zero to $360^\circ$. 
Figure 26: Three different experiments were performed on coverslip samples. The images of cells at day 7 (A1, B1 and C1) were analyzed with 2D FFT method (A2, B2 and C2) to measure the cell orientation at day 7. The distributions of orientation direction are shown as polar histograms in A3, B3 and C3. The FFT diagrams and histogram plots demonstrate that cell alignment has different peaks at each experiment at either 90°, 135° or 20°. The white arrows show the local cell orientation at each experiment.
Figure 27: Three different experiments were performed on loaded DDCS samples. The images of cells at day 0 (A1, B1 and C1) were analyzed with 2D FFT method (A2, B2 and C2) to measure the cell orientation at day zero. The distributions of orientation direction are shown as polar histograms in A3, B3 and C3. The FFT diagrams and histogram plots demonstrate that cells are oriented almost uniformly at every angle from zero to 360°.
Figure 28: Three different experiments were performed on loaded DDCS samples. The images of cells at day 7 (A1, B1 and C1) were analyzed with 2D FFT method (A2, B2 and C2) to measure the cell orientation at day 7. The distributions of orientation direction are shown as polar histograms in A3, B3 and C3. The FFT diagrams and histogram plots demonstrate that cells are aligned and oriented at 135° at each experiment. The white arrows show the local cell orientation.
Figure 29: Three different experiments were performed on unloaded DDCS samples. The images of cells at day 0 (A1, B1 and C1) were analyzed with 2D FFT method (A2, B2 and C2) to measure the cell orientation. The distributions of orientation direction are shown as polar histograms in A3, B3 and C3. The FFT diagrams and histogram plots demonstrate that cell orientation is distributed almost uniformly at every angle from zero to 360° in each case.
Figure 30: Three different experiments were performed on unloaded DDCS samples. The images of cells at day 7 (A1, B1 and C1) were analyzed with 2D FFT method (A2, B2 and C2) to measure the cell orientation. The distributions of orientation direction are shown as polar histograms in A3, B3 and C3. The FFT diagrams and histogram plots demonstrate that dominant cell orientation is different in each case and the distribution of cell orientation has peaks at $80^\circ$, $40^\circ$ or $90^\circ$ at different experiments. The white arrows show the local cell orientation.
Figure 31 shows a sequence of panoramic images (12X12 stitched small images) from coverslip and DDCS samples at day 3, 7 and 14. As we mentioned before, the cell alignment on the coverslip is only local and cells at each local location are aligned at different angles ranging from 0° to 180° while cells on DDCS substrates are aligned both locally and globally at 135°. We measured the spatial cross correlation of these panoramic images as function of a distance to quantify cell alignment as function distance on all samples. Figure 32 shows the normalized cross correlation from coverslip sample (in red) unloaded DDCS (in orange) and loaded DDCS sample (in blue) (Figure 32 D1 and D3) at days 3 and 7 respectively. The data (N=1) indicates that the cross correlation from loaded DDCS sample is higher than the coverslip sample as a function of time. The cross correlation of the coverslip and unloaded samples did not change for the experimental period while this parameter is higher at day 7 on the DDCS samples when the top and bottom cell layers grew. The diagrams of the cell orientation are shown for coverslip, unloaded and loaded DDCS samples respectively (Figure 32 A2, B2 and C2) at day 7. These diagrams represent the map of cell alignment for each sample.
Figure 31: The panoramic images from Coverslip (A1, B1, C1) and loaded DDCS samples (A2, B2, C2) at day 3, 7 and 14 respectively. The images show the local cell alignment on the Coverslip sample at day 3, 7 and 14 and both local and global alignment of cells on the loaded DDCS sample at day 7. DDCS sample was under 0.01N load (less than 1% strain) from the beginning of the experiment and this amount of load/strain was enough to impose cell alignment at a fixed angle (~135°, the black arrow on B2). After 7 days the DDCS sample was stretched an additional 6%. The orientation of cells didn’t change in the second week while PHCFs grew on the bottom side DDCS sample at 45° angle (the white arrow on C2). Scale bar is 100 µm.
Figure 32: The normalized 2D spatial cross correlation of images from the Coverslip, no load DDCS and loaded DDCS. The images (A1, B1 and C1) and (A2, B2 and C2) are DIC images and cell orientation map from Coverslip, unloaded and loaded DDCS at day 7. The plots (D1 and D2) represent the quantified cross correlation results (blue is loaded DDCS sample, orange is unloaded DDCS and red is Coverslip sample) as a function of distance (microns) at day 3 and 7 respectively. The data suggests that the cross correlation from the loaded DDCS sample is higher than Coverslip and unloaded samples at day 7. Scale bar is 100 µm.
The density of the live dsDNA-labeled PHCFs and the cell mean velocity as a function of time is plotted in Figure 33. The plot represents that the cell density and the cell mean velocity increases over the first week (200 cells /mm$^2$ and 20µm/hour) until they reach steady state values of ~ 400 cells /mm$^2$ and 6µm/hour when corneal fibroblasts reach confluence. Note that, the cell density on the DDCS is larger than on the coverslip. This could be due to the difference between the area of DDCS and coverslip. While the area that can be covered by cells on DDCS is ~ 48 mm$^2$ (a rectangular strip ~ 6 mm width X 8 mm length), this area for coverslip sample is 1256 mm$^2$ (~ round shape with 20 mm radius). Therefore, coverslip sample’s surface is 26 times larger than DDCS, which suggests that PHCFs have less space on the DDCS to cover proliferate over two weeks and they start to grow on the bottom side DDCS (double surface ~ 2X48 mm$^2$ = 96 mm$^2$ is still 13 times less than coverslip sample) in the second week. Interestingly, at the end of the second week, the two layers of PHCFs on the top and bottom side of DDCS are perpendicular to each other.
Figure 33: A) The density of live nucleus-labeled PHCFs per mm² on the Coverslip (red) and DDCS substrate (blue). B) The mean velocity of nucleus-labeled human corneal fibroblasts on the Coverslip and loaded DDCS sample. The data indicates that the mean velocity and cell density reach constant values of ~ 6µm/hour and 400cells/mm², when cells reach confluence. These plots have been taken from one experiment (N=1). C) The cell orientation on the top and back of DDCS at day 0, 7 and 14.
After two weeks, specimens (the coverslips and DDCS), were fixed in situ using 4% paraformaldehyde and then all specimens were transferred to Professor Zieske’s lab at Schepens Eye Research Institute, Harvard Medical School, Boston, MA. Specimens (all of them) were treated with primary and secondary antibodies to label Fibronectin, cytoskeletal Actin (F-Actin), smooth muscle Actin (SMA), Collagen type I and III. The images from labeling of the Collagen type I, III, Fibronectin, SMA and F-Actin on coverslip and DDCS substrates demonstrate that cells were healthy enough to produce extracellular matrix (ECM) in the bioreactor. Figures 34 and 35 show the difference in the local organization of selected components associated with the fibroblast culture at the end of a two week experiment. From the figure it is clear that the substrate/mechanical load combination produces a marked effect on the organization of the system at many levels relative to the coverslip control experiments. Figures 34 and 35 A–E represents immunostaining for Fibronectin, SMA, Col I, Col III and F-Actin of DDCS sample at the end of the experiment. The images demonstrate that the Col I, Fibronectin and F-Actin were produced in abundance by the PHCFs while SMA and Col III were produced at low levels during the tissue formation process. Figures 35A, C and E represent the alignment of Fibronectin, Col I and F-actin fibrils on the DDCS and show that the orientation of fibrils is similar to cell orientation. Figure 35C demonstrates the alignment of collagen type I deposited on the DDCS with the long axis of corneal fibroblasts. From our initial work with this culture system, we observed that collagen tends to align with the long axis of the fibroblastic cells, thus we expect the synthesized collagen to follow the cells, which are influenced strongly by the direction of load. While it has been shown from images of labeled collagen I on the DDCS in Figure 35 A2, we use a specific antibody (as
described in “Indirect-immunofluorescence staining” section) staining to highlight the produced human collagen type I from PHCF on the DDCS (this substrate is made from bovine collagen type I). The images indicate that collagen type I (green fluorescent lines in images) are aligned with the cell orientation 45° with respect to load direction) after two weeks under strain. Also, Figures 35B and D demonstrate minimal production of Col III and SMA by cells on strained DDCS specimens. In figure 36B an image of the DDCS specimen cross section reveals the existence of two PHCF layers (F-Actin in red and nuclei in blue) on the top and the bottom of DDCS sample (DDCS collagen is the black space ~ 40µm thickness). The image suggests that PHCFs did not penetrate into the DDCS specimens and instead migrated on the top and bottom of DDCS sample. Figure 36 shows two immunostained cross-sections (collagen type I in green, F-actin in red and nuclei in blue) of the coverslip construct and the DDCS samples (Figures 27 A and B) at the end of a two week experiment. The high-magnification images show the existence of two separate cell layers on DDCS sample (top and bottom sides) and a cell multi-layer on the coverslip sample. These images demonstrate that PHCFs were at comfortable culture conditions and both proliferate and generate Extra-Cellular matrix (ECM) inside the bioreactor.
Figure 34: Confocal imaging of immunohistochemical labels for standard corneal ECM components and fibrotic ECM components on coverslip samples. At the end of the experimental period (two weeks) five immunohistochemical antibody-based reporters were imaged using confocal microscopy (Fn, SMA, COL I, Col III and F-actin). The data is arranged in columns where the nuclear stain (DAPI-blue) is shown in column 1 (Coverslip), the antibody marker is in column 2. The columns 1 and 2 are merged in column 3 for Coverslip samples. A1-3) Fn (green); B1-3) SMA (red); C1-3) Collagen type I (green); D1-3) Collagen type III (green); E1-3) F-Actin (red). Bar is 50µm.
Figure 35: Confocal imaging of immunohistochemical labels for standard corneal ECM components and fibrotic ECM components on DDCS sample. At the end of the experimental period (two weeks) five immunohistochemical antibody-based reporters were imaged using confocal microscopy (Fn, SMA, COL I, Col III and F-actin). The data is arranged in columns where the nuclear stain (DAPI-blue) is shown in column 1 (Coverslip), the antibody marker is in column 2. The columns 1 and 2 are merged in column 3 for DDCS samples. A1-3) Fn (green); B1-3) SMA (red); C1-3) Collagen type I (green); D1-3) Collagen type III (green); E1-3) F-Actin (red). Bar is 50µm.
Figure 36: Confocal imaging (green collagen type I, red F-Actin and blue nucleus) of cross sections of coverslip constructs (A) and the DDCS samples (B) after two weeks of culture. On the coverslip sample collagen type I fibrils were deposited by corneal fibroblasts (A) Corneal fibroblasts and the deposited collagen fibrils are on the same layer (white arrows). In DDCS sample (B) two layers of cells were formed on the top and bottom of DDCS after two weeks of culture inside the mechanobioreactor.
Fluorescent and TEM images from unseeded DDCS have been shown in figure 37. In general, random organization of the collagen fibrils can be seen at the surface and inside the DDCS. Figure 37 B1 and B2 are high magnification TEM images, which demonstrate the banding of the collagen fibrils inside and at the surface of the DDCS. Densely packed collagen fibrils can be observed at the center and the surface of collagen films, which are perpendicular (dots) and parallel (lines) to the plane. Figure 37 also shows TEM images of DDCS which were seeded with PHCFs. One can see layered fibroblasts on top of collagen films (figure 37 C1-3 and D1-3).
Figure 37: Fluorescent images from FITC DDCS after exposure to 40% PEG at high (A1) and low (A2) magnification; (A3) FITC labeled fibrils before exposure to 40% PEG, (B1-B3) TEM cross-section from unseeded collagen films at different magnifications. TEM images of seeded collagen matrix cross-section from top (C1-3) and bottom (D1-3).
General Load versus time response of seeded and unseeded specimens

Mechanical response of samples to the strain control test (6% strain): All specimens were stretched to 0.01N load at day 0 and then strained to approximately 6% at day 7 (when cell confluence was reached). The initial stress obtained from all seeded and unseeded
specimens was 50KPa (less than 1% strain) based on the dimensions of the samples (8mm length X 6mm width and 40µm thickness). We observed that this amount of strain/stress significantly changes the cell orientation. The human corneal fibroblasts were under this amount of strain/stress during the first week, which resulted in their alignment at a fixed angle. All specimens then were strained 6% after one week and were observed for one more week. The DIC and fluorescent images show that the cell orientation didn’t change after applying this additional strain and that the cells maintained their previous orientation. The data recorded show stress relaxation with a long time constant (7 days) for both the loaded and unloaded samples (the second week is shown, Figure 38). The data from seeded cell samples indicates that the stiffness of DDCS had dropped in comparison to the unseeded samples.
3.4 Discussion

In this chapter, we set out to extend our initial work by culturing nucleus stained PHCFs (N=1) under physiological conditions in the presence of a controlled uniaxial mechanical load. The behavior and growth of human corneal fibroblasts are tracked by imaging cells at 6-minute intervals (DIC microscopy) and one hour intervals (fluorescent microscopy) for two weeks on 3D high-density collagen type I constructs. The present study not only observes PHCF cell but also evaluates and quantifies the migration and morphology of stained PHCF fibroblast such as their orientation, alignment, velocity and cell-ECM interactions. We successfully labeled the nuclei of PHCF cells to track cell alignment and cell-cell interactions on strained 3D dense disorganized collagen films and observed how human corneal fibroblasts are aligned on the entire collagen film and how ECM is organized with respect to the direction of mechanical load. Cell migratory behavior was quantified using particle imaging velocimetry (PIV) on the live, labeled cells. Cell orientation was quantified using 2D - Fast Fourier Transform (FFT). PHCF cultures were followed continuously from initial seeding through matrix production. The design of the bioreactor enabled us to refresh the labeling of corneal cells every two days without interrupting loading or imaging. We had a photobleaching problem during the fluorescent microscopy from PHCF’s over the experiments and the design of the mechanobioreactor (extra inlets) helped us to refresh the labeling and measure the cell velocity and alignment by the PIV method. The dsDNA-labeled corneal fibroblasts were successfully seeded onto preloaded DDCS samples (a ~40 micron thick type I collagen film, held in the tissue grips with 50KPa stress) and grown to confluence over a week and then were stretched at 6% strain after approximately one week (when fibroblasts reached
confluence). Two different cultures were grown simultaneously in the bioreactor; one on the DDCS construct and one on the coverslip in order to provide a uniform cell culture (human corneal fibroblasts from same donor, passage and environment) for both specimens. The data showed that cell alignment on the coverslip (i.e. no orientational cues) was only local while this alignment was both local and global on DDCS samples after one week. The 6% strain applied in the second week of fibroblast culture on DDCS did not significantly change the cell alignment and orientation. This was unexpected. The panoramic images from the entire samples (DDCS and coverslip) allowed us to detect both the local and global cell orientation in real time and in response to the applied uniaxial load. Here, a new approach was introduced and performed using our mechanobioreactor to track corneal fibroblasts and quantify the cell organization, alignment and velocity from initial seeding to matrix production. Two different image processing methods (PIV and FFT) were performed on the images to measure and quantify the orientation, motion and velocity of PHCFs. The results demonstrate that a small uniaxial strain (~ 50kPa) was adequate to alter the mechanism of cell alignment. Using FFT we quantified the local and global cell alignment on the samples. FFT was also applied to panoramic images from the DDCS and coverslip covering a larger area of the samples. Based on FFT results, the corneal fibroblasts on the top of DDCS specimens were aligned to ~ 130°-135° degree during first week and the new layer of cells that was grown during the second week of culture at the back of the sample was aligned to ~ 40°-45°, perpendicular to the top layer. The coverslip culture was essentially randomly oriented but locally aligned (due to spindle shaped cell packing). The result suggests that the mechanical load influenced the cell alignment and resulted in global alignment of
cells on DDCS samples. The DDCS sample was under 0.01N load (~ 50KPa) at the beginning of the experiment, which suggests that corneal fibroblasts are very sensitive to the small tension and sense this amount of forces during their migration (at piconewton range) on DDCS samples. This sensitivity of corneal fibroblasts supports previous studies, which claimed small forces (as small as piconewtons) can alter the formation of protein loops [58-60]. Our previous studies on collagen/enzyme mechanochemistry have shown this sensitivity, indicating that a few piconewtons per monomers of loading can alter the degradation rate of collagen monomers [21-25].

The local migration of corneal fibroblasts has been detected by the PIV method and cell velocity was simultaneously measured as function of time on both samples from initial seeding to matrix production. The data shows that fibroblasts on the coverslip are slower to settle on the local direction of the cell migration (along the cell alignment axis) while it takes the fibroblasts less time to settle at their fixed angle to the applied load on the DDCS. For both cultures, the velocity of cell migration slows over the first hundred hours, reaches steady state and then abruptly declines (possibly coincident with matrix production at about 10-11 days). We suspect that both confluence and synthesis of organized tissue slow the cells as they are likely forming cell-cell junctions [61]. Our results demonstrate the advantage of the relatively unique capabilities we have built to quantitatively examine the effect of mechanics on cell culture behavior in real-time and over long time periods. Qualitative observation of the cell culture system revealed startling culture dynamics which included cell proliferation and death, increasing levels of cellular organization, multilayer formation, organizational breakdown and remodeling (healing), velocity changes and matrix production. The analyzed data shows that single
fibroblast cells move relatively fast and have a random orientation at the first couple of hours after seeding. Cell average speed declined from an initial high of more than 0.5 µm/min to a relatively steady state following confluence (approximately 0.2 µm/min) of the culture and finished lower (about 0.1 µm/min) at two weeks when matrix production was beginning. We observed that the external stress (0.01 N load ~ 50KPa ~ native human intraocular pressure) significantly changes the patterns of migration and morphology of PHCFs during tissue formation. The data from loaded substrates demonstrate that the corneal fibroblasts are locally and globally aligned on the entire substrate at a relatively fixed angle to the applied force while corneal fibroblasts without the load/strain (unloaded substrates and coverslip samples) express a local alignment and orientation. We observed that the mechanical load changes the migration and morphology of PHCFs when compared to cells on the glass coverslip and unloaded collagen substrates. While local alignment of cells was observed on the coverslip and unloaded substrates, the orientation of PHCFs on the loaded constructs was uniform throughout the sample and constant in different experiments (N=5). From the initial papers on this corneal fibroblast culture system, we observed that collagen tends to align with the long axis of the fibroblastic cells [62-63], thus we expected the synthesized collagen to follow the cell orientation which means corneal fibroblast follow the mechanics. The previous studies have already confirmed deposition of col I and col V by this culture system on a type I collagen gel [63] and here, we have shown the deposition of collagen type I on strained DDCS sample. The images suggest that the collagen type I fibrils are aligned with the cell orientation (45° with respect to load direction) after two weeks of culture under tiny strain. It has been shown that myofibroblasts differentiate from fibroblasts and
can be identified by SMA markers [44, 45]. Myofibroblasts have been shown to expand a muscle contractile which leads to the formation of stress fiber and expression of SMA in the process of wound healing [46]. The lack of SMA and Col III from specimens suggests that PHCFs did not differentiate to myofibroblasts and that our matrix is unlikely to be a wound-healing or scarring process [47, 48]. In our system, we used 10% FBS and 0.05mg/ml stabilized ascorbic acid without any growth factors to maintain the physiological fibroblast over culture system. The data suggests that the mechanical load did not alter the production of Col III and SMA while it did change the cell orientation (~45°) which led to organization of the produced Col I and fibronectin fibrils. A few unloaded DDCS samples were also tested in the bioreactor over a week. The data from these tests (by FFT analysis) showed that the cell alignment was only local thus implicating the small applied pre-load as adequate to induce the organizational field. The results from unloaded samples show that corneal fibroblasts grown on unloaded DDCS at the physiological conditions did not differentiate to myofibroblast either (no evidence of wound healing process was observed). Also, the data from unloaded and loaded DDCS samples confirm that corneal fibroblasts were proliferated and grown under standard cell culture conditions, and that global cell alignment was reached by applying a very tiny strain.
Reference:


[66] [http://www.mathworks.us/matlabcentral/fileexchange/27659](http://www.mathworks.us/matlabcentral/fileexchange/27659)


Chapter 4

Continuous Measurement of Physical Forces During Human Primary Corneal Fibroblast Migration

4.1 Introduction:

It has been shown that cell orientation and migration are force dependent in vertebrates [1-4], however, the exact role of mechanical signals on different cellular processes is still under study. For instance, little is known about the mechanobiology of matrix production by cells. Corneal fibroblasts, for example, are observed to produce extracellular matrix \textit{in vitro} and while doing so they self-organize by executing a coordinated migration [5, 6]. Similar coordinated migration is observed in epithelial and endothelial cells. Additionally, these cells also apply forces to their substrate as well as to their neighbors simultaneously [7-9]. The corneal fibroblasts are shown to exert physical forces on the collagen substrates [10] and they are responsive to mechanical stimulation applied to their surrounding matrix [11-12]. If such forces are generated by fibroblasts during matrix production, then they may influence the deposition and retention of collagenous matrix [13-17]. However, it is very difficult to follow fibroblast behavior while measuring forces on time scales long enough. Although the forces extracted from single corneal fibroblasts to their collagen substrates has been measured previously, [18] studies have not been able to measure these physical forces for long periods of time from cell seeding to matrix production. Here, we present an \textit{in vitro} mechanobiological assay capable of directly
observing the behavior of PHCFs over time scales relevant to tissue formation (one week) while simultaneously measuring intrinsic (cell-substrate) physical forces. To investigate the dynamics of changes in the forces applied by cells, we combined our mechanobioreactor [19] with Fourier-transform traction microscopy [7, 8]. The traction microscopy, as originally described [20-23] and then fundamentally modified [7, 8], was performed using our mechanobioreactor [19]. We seeded Primary Human Corneal Fibroblasts (PHCF) at the center of a soft collagen-coated polyacrylamide gel with 1240Pa Young’s modulus and imaged the cells and their substrate for a week. The bioreactor was able to provide incubator conditions for fibroblasts over a week while fibroblasts proliferated and migrated on the gel to form a monolayer cell sheet. The traction forces and orientation of corneal fibroblasts were mapped while they migrated on a soft gel over a week.

4.2 Materials and methods:

1) To continuously measure and calculate natural forces exerted by PHCFs on calibrated gels in long-term.

It is not understood how human corneal fibroblasts produce and organize extracellular matrix during tissue formation and growth in vitro. Previous studies have shown how epithelial and endothelial cells apply physical forces to the substrate and neighbors over short periods of time. In this study, we developed a method to observe and analyze forces applied by PHCFs over time scales relevant for tissue formation observations. Then we investigated how these forces influence ECM production and organization. Fourier
transforms traction microscopy and monolayer stress microscopy [7, 8] was used to assess the forces applied by PHCFs.

2) To continuously observe calibrated polyacrylamide gel deformation during tissue formation by PHCFs.

We used calibrated polyacrylamide gels with known elastic modules to track gel deformation over extended period of time. This gel deformation helped us quantify the level of forces applied by PHCFs on their substrate for a week.

4.2.1 Experimental protocol

For this experimental series, we combined cell culture method in our sterilized mechanobioreactor and 100µm thick, collagen-coated, calibrated polyacrylamide gels [8] to observe the physical forces applied by cultures of PHCFs on their substrate. All calibrated gels were glued to the coverslip inside the bioreactor and were washed three times with 1X PBS and DMEM. The bioreactor was then filled with preheated DMEM enriched with CO2, 10% FBS and 1% antibiotics; the cells were fed with the media at 8µl/min for a week. Before seeding PHCFs on gels, fluorescent Z-stacks images were captured from micro beads to keep track of the initial location of micro beads. Suspensions of 1000 cells/µl in DMEM were gently injected into the bioreactor mounted on the microscope. Phase contrast and fluorescent images (4X) were collected every 5 minutes from a single location. At the end of one week, the bioreactor was washed three times with 1X PBS and preheated Trypsin was added to the system for 30 minutes to
detach corneal fibroblasts from the gels. Fluorescent Z-scan images were captured again from gels in order to find and save the final location of micro beads after a week. Our sterilized mechanobioreactor and polyacrylamide gels were used to track the forces applied by corneal fibroblasts on their substrate for a week. NIH-Image J and ELS-Elements software were used to transfer images from the microscope for post-processing.

To measure physical forces on polyacrylamide gels deformed by PHCFs during the formation of ECM and cell monolayer in vitro.

This method illustrates how PHCFs self organize for a week in vitro. The physical forces (cell-substrate) were measured using image processing methods.

4.2.2 Experimental protocol

All fluorescent microscopy images from polyacrylamide gels (red and green micro beads) were collected and transferred from NIS-Elements to NIH-image J to adjust the intensity of image sequences. Fourier traction microscopy was applied to the images to find gel deformation based on correlation-based particle image velocity. Briefly, gel displacement (x, y and z vectors) and stress vectors were calculated and transformed to Fourier space. Then using Hook’s law and calculating strain (the first derivative from displacement) were generated three fundamental equilibrium equations. These equations together with the mechanical properties of gels enabled us to calculate cell-substrate forces using MATLAB programming (Traction microscopy [8]). The intercellular forces were calculated based on the overall balance of cell-substrate traction across the mono-layer
(Newton’s laws). The plane internal stress tensors (plane stress) and shear stress exerted by fibroblasts on the gels were computed using the measured local traction forces. Then based on the assumption that cell mono-layer is a continuum and using boundary conditions, principal stresses were defined at each local orientation of these stresses. (Monolayer stress microscopy [8]). Fourier transforms traction microscopy and monolayer stress microscopy were be used to measure the physical forces from PHCFs while they grew and formed a tissue mono-layer on the calibrated gels.

In this specific aim, we observed the behavior of PHCFs on calibrated gels while physical forces were measured dynamically. This study illustrated the traction forces (cell-substrate) and the migration patterns of PHCFs during tissue formation. Fourier transform traction microscopy and monolayer stress microscopy enabled us to measure and quantify the traction forces locally while PHCFs organized. This study is highly beneficial in tissue engineering as it provides new information on fundamental biological process of cell morphogenesis, growth and tissue formation. The main concern was stage drifting at X, Y and Z directions. To calibrate stage drifting at a fixed plan (X and Y directions), a few 2µm fluorescent beads were fixed (FITC, green) at the back of the sample (Figure 39). These micro beads demonstrated the amount of X and Y drifting at each frame and this drifted amount was measured and subtracted from the motion of red micro beads using MATLAB. Moreover, perfect focus system (PFS) was hired to observe red micro beads at a fixed Z plane over a week. The other concern in this study was that Nikon TE2000 E was focused on red micro beads instead of PHCFs during the experiment, which might result in out of focus images captured from PHCFs. However, our data
showed that the resolution of phase images captured with 4X objective was high enough for analysis. Finally, NIH-image J software was used to adjust the mean intensity of fluorescent images (TITC and FITC) to avoid any inconstancy between the intensities of different frames.

Figure 39: A) A schematic representation of our setup of the mechanobioreactor. Two different micro beads are used to measure physical forces over the two-week experiment. The green micro beads (2µm) are imbedded at bottom side of the calibrated polyacrylamide gel to fix the X and Y stage drifting issue and the red micro beads are imbedded at the top of the gel to calculate the deformation of the gel caused by interactions with cells.
4.3. Results

For this experimental series, we used the mechanobioreactor to observe a 100µm thick, collagen-coated, calibrated polyacrylamide gel (E=1250 Pa - gel) and investigate HPCF mechanobiology. Forces applied to the substrate are interrogated continuously via embedded 0.5µm fluorescent beads. Phase contrast and fluorescent images (4X) were collected every 5 minutes from a single location for one week. Using Fourier transform traction microscopy the gel deformation was transformed into a continuous traction vector field applied by the cells to the substrate (cell-substrate forces). Over the time

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Figure 40: Phase contrast images and local orientation of maximum stress (red lines) at day 0, 4 and 7 (A1, B1, C1). Map of traction magnitude at day 0, 4 and 7 (A2, B2, C2). Traction along horizontal direction at day 0, 4 and 7 (a3, b3, c3) Traction along vertical direction at day 0, 4 and 7 (A4, B4, C4).
course of this experiment, the corneal fibroblasts actively elongated on the gel and then migrated over the gel surface and began to form a confluent monolayer. We observed that cells gradually built up tractions. When they self-organized, magnitude of this traction was on the order of 100Pa and increased as the time progressed. Figure 40 represents the map of traction magnitude at day 0, 4 and 7 along X and Y directions. Live, continuous images from cells show that cells possessed a rounded shape (Figure 40A1) at the beginning of seeding followed by rapid adhesion and elongation on the gel by day 3 (Figure 40B1). The cells appeared to be locally aligned at day 7 (Figure 40C1). The images demonstrate dynamic organization and alignment of a layer of cells (one week real-time movie). The traction forces between cells and their substrate are represented in Figures 40A2, 40B2, 40C2 at day 0, 3 and 7 of culture. Traction magnitude (stress) increased when cells reached a mono-layer on the gel at day 7 (Figure 40C2). The traction applied by the cells initially increased with time and stabilized after almost one week and the physical traction in the gel increased as the time progressed (Figure 40C2, 40C3 and 40C4). Figures 40C2 to 40C4 indicate apparent saturation of forces applied by cells on the substrate for up to one week (horizontal and vertical directions). The maps of traction magnitude are shown in Figures 40C2 to 40C4 to quantify the local physical forces from cells. They are also reminiscent of dynamic heterogeneity in jammed matter. Figure 41A and B show that Root Mean Square (RMS) of traction and strain energy of the gel increased over a week while corneal fibroblasts form a mono-layer. Traction magnitude (stress) increased when cells reached a mono-layer on the gel at day 7. The root mean square (RMS) of traction applied by the cells initially increased with time and stabilized after almost one week and the strain energy in the gel increased as the time
progressed (Figure 41E to F). Both plots quantify apparent saturation of forces applied by cells on the substrate up to one week. The distribution of tractions has exponential tails (Figure 41E and 13F). The appearance of exponential tails, as opposed to Gaussian, implied that extreme values are not uncommon; they are also reminiscent of dynamic heterogeneity in jammed matter. Although the source of such distribution is currently unknown, its observation in cell monolayer suggests that the source may not be the ability of cells to transmit forces over large distance through cell-cell junctions. Over the period of 7 days, the cell tractions remained exponentially distributed but surprisingly, the orientation of tractions spontaneously evolved into domains (Figure 41 C and D). Similar spontaneously evolved domains are also observed for orientation of cells (Figure 41 C and D). Moreover, the orientation of cells and the direction of tractions are strongly correlated (figure 42).

Figure 42 represents the cell and traction orientations over a week on the coated polyacrylamide (PA) gel. Over the time course of a week, the corneal fibroblasts formed a confluent monolayer (Figures 42 A1-C1 show cells at day 1, 4 and 7 respectively). While forming the monolayer, the cells actively migrate, and elongate, and simultaneously apply appreciable tractions on the substrate. The tractions have extreme spatial heterogeneity and the magnitudes of tractions can be as high as ~150 Pa. Figures 42 A2, 2B2 and 2C2 are the maps of cell orientation from PHCFs on the gel at days 1, 4 and 7 respectively. Surprisingly, the direction of traction and the orientation of cells are correlated. That is to say, tractions are aligned with the cell axis.
Figure 41: Apparent saturation of forces applied by cells on substrate (days 1-7). Plot of RMS of traction (A), and strain energy in the substrate (B) Distribution of traction direction (C) and orientation of cells (D) from day 2 (blue) to day 6 (red). Distribution of x-component of tractions (E), and y-component of traction (F) from day 1 (blue) to day 6 (red).
Figure 42: Phase contrast images (A1, B1 and C1) from fibroblasts on the polyacrylamide gel, maps of cell orientation (A2, B2 and C2) and maps of traction orientation (A3, B3 and C3) at day 1, 4 and 7 respectively.
Figure 43 (D1 and D2) demonstrates a spatial cross correlation from cell orientation and traction direction on polyacrylamide gels. The data suggest that PHCF’s are only locally organized when they were compared to our data from Coverslip, unloaded and loaded DDCS samples. The critical distance of cell alignment on polyacrylamide gel is less than 250 µm at day 7 (the highest value over the one week period). Our data from loaded DDCS reported the critical distance of cell alignment to be more than 1000µm after one week. The cross correlation as function a distance on traction orientation images over a week is plotted in Figure 43D2 and suggest that the traction orientation alters over 25µm to 200µm distances during a week. At day 7, the traction direction is the more organized and the critical distance of spatial correlation reaches 200µm. interestingly, critical distances of cross correlation from both cell orientation and traction orientation reach ~ 200µm at day 7, which suggests that the cell alignment and traction orientation change in space at the same scale. These measurements by the autocorrelation as function a distance support our observation in monolayer stress microscopy (figure 41 C and D) where we claimed that the orientation of cells and the direction of tractions are strongly correlated based on monolayer traction microscopy.
Figure 43: The images (A1, B1 and C1) and (A2, B2 and C2) are DIC images and maps of cell orientation from corneal fibroblasts on the polyacrylamide gel at day 1, 4 and 7 respectively. The auto-correlation of cell orientation (D1) and traction orientation (D2) on the polyacrylamide gels as a function of distance (µm) over a week. The data demonstrate that the cell alignment and traction orientation alignment were improved in time. Red (day 1), green (day 4) and blue (day 7).
4.4. Discussion:

We performed traction microscopy to measure physical forces exerted by cells to their substrate over long culture times. Here we presented an *in vitro* mechanobiological assay that enables direct observation of the cell behavior over time scales relevant to tissue formation (one week) while simultaneously measuring intrinsic (cell-substrate) physical forces. This method allowed us to study how PHCFs self organize by exerting physical forces (cell-cell and cell-substrate). The combination of our mechanobioreactor and traction microscopy technique permits direct, real-time measurements of physical forces applied by cells during migration and growth. The traction microscopy was originally described in [7, 8] and was used by our mechanobioreactor culture system. The system will ultimately enable dynamic, continuous and quantitative observation of the mechanobiology of corneal cells. Such a system will lead to a more profound understanding of the role of force in cell migration and retention *in vitro*. The traction microscopy data suggest that while fibroblasts undergo local alignment to form a cell monolayer, the strain energy in the substrate and RMS of tractions increase until they reach steady state after seven days. During this period, the tractions are not only distributed exponentially but also align spontaneously with neighboring traction vector forming self-organized domains. The results represent that the maximum forces generated by PHCF reached 150 Pa at some locations, suggesting that the fibroblasts do not form strong cell-cell contacts like epithelial or endothelial cells [7, 8]. However, traction maps of fibroblast share a key property observed for epithelial and endothelial cell monolayer – the exponential tails in traction distribution. The exponential tail implies that extreme values are not as uncommon as one would estimate if the distribution were
Gaussian instead. Although the source of such distribution is currently unknown, its observation in fibroblast monolayer suggests that the source may not be the ability of cells to transmit forces over large distance through cell-cell junctions. Over the period of 7 days, the tractions remain exponentially distributed, but surprisingly, the orientation of the tractions spontaneously evolves into domains. Similar spontaneously evolved domains are also observed for orientation of cells. Moreover, the orientation of cells and the direction of tractions are strongly correlated.
Reference:


Chapter 5

Conclusions

Cornea, as a connective tissue in the body, is the transparent front part of the eye that protects the fragile intraocular contents and covers the iris, pupil and anterior chamber. The cornea generally becomes damaged through diseases, infections or injuries which can result in irreversible scarring. These scars can interfere with vision by blocking or distorting light as it enters the eye. Injured or diseased cornea is the second leading cause of vision loss and more than 10 million people worldwide are blind due to corneal scarring. Corneal transplantation is the only currently available therapy for stromal scarring and is a surgical technique where damaged or injured corneal tissue is replaced by donated corneal tissue in its entirety (penetrating keratoplasty) or in part (lamellar keratoplasty). As of 2000, it was reported that approximately 33,000 corneal transplants per year performed in the United States. However, the number of corneas available for cornea transplantation is limited and the stimulation of host immune responses results in a fairly significant rate of tissue rejection. In addition, donor corneas with LASIK corrective surgeries do not qualify for cornea transplantation surgery. Thus, because of the lack of cornea donors and donor variability, the cornea tissue engineering community has been focused on the synthesis of corneal tissue in vitro. To succeed in this aim, we must discover methods to either directly organize collagen fibrils or find ways to guide the production of organized ECM by corneal stromal fibroblasts. The corneal stroma comprises 90 percent of the thickness of the cornea and is a very challenging tissue to reconstruct. Although a significant amount of recent research has been focused on the organizing of ECMs, there has been little success in direct synthesis of native-like highly-
organized ECMs such as the corneal stroma. This is likely because little is known about the mechanisms which govern the organization and morphology of collagen (the most important component in cornea) during its synthesis in vivo. In addition, there has been little emphasis in the corneal tissue engineering community on the use of mechanical force as a controllable parameter. Consequently, no studies have managed to reproduce the mechanical strength or organization of the natural corneal stromal matrix in vitro. The goal of this PhD dissertation was two-fold: 1) To provide a method which permits direct, long-term visual access to a cell-system (PHCFs) known to generate organized ECM and 2) to test the hypothesis that mechanical load plays a significant role in the production, growth, organization and maintenance of ECMs synthesized by the PHCF culture. Thus the dissertation was both a basic science investigation of corneal culture system behavior and a hypothesis-driven examination of mechanobiological signaling during matrix production and substrate remodeling.

In chapter 2, we introduced a low-volume and optically accessible uniaxial-loading mechanobioreactor (previously described in Paten et al) designed for culturing and continuously imaging cells subjected to mechanical load for long periods of time. The mechanobioreactor performance was thoroughly tested. Our data show that the temperature is stable and controllable for long-term operation. The bioreactor was tested independently in strain and load control modes. We found that its mechanical performance was at least as good as competitive commercial unit even while mounted on the microscope. A cell-culture protocol was successfully developed to maintain primary human corneal fibroblasts in the bioreactor for long-term observation. Finally, corneal fibroblasts were successfully grown on custom dense disorganized collagen type I films.
and on glass for up to 14 days. The results have shown that the bioreactor can maintain viable cell culture system long enough to observe self-organization and matrix formation. During the corneal fibroblast experiments on the different substrates, we made numerous observations. We found unexpected corneal fibroblast behavior which included differentiation into catabolic-like “clastic” cells, sliding sheet motion, cell sheet contraction and spontaneous cell death. We learned that the mechanobioreactor had the capacity to give us an unprecedented, long-term and high magnification window into the life of cells and cell culture systems. At the end of chapter 2, we demonstrated that corneal fibroblasts cultured in the reactor would populate a loaded collagen substrate and generate an aligned corneal cell layer on the top and the bottom of a collagen substrate. The experiments showed that corneal fibroblast uniformly were oriented at an angle (135 and 45 degree) with respect to load direction and would continuously thrive in the reactor for up to 14 days. By the end of chapter 2, it was proven that a primary human culture system could be grown from initial seeding through matrix production while providing minute-by-minute high magnification observations and permitting simultaneous mechanical stimulation. Thus, the ability to carry out the two goals of the dissertation had been established: to potentially observe matrix deposition and control directly and to test a mechanobiological hypothesis.

In chapter 3, we extended our initial work by culturing nucleus-stained PHCFs in the bioreactor and observing them simultaneously on DDCS substrates exposed to a controlled uniaxial mechanical load and on glass coverslips. The design of the mechanobioreactor permitted in situ staining of the nucleus of the PHCFs. The live nuclear stain also permitted quantitative evaluation of the migration behavior of the cells
which were imaged at 6-minute intervals (DIC microscopy) and one hour intervals (fluorescent microscopy). The combination of live DIC and fluorescence microscopy allowed us to extract and quantify dynamic migration and morphology information from the PHCFs such as orientation, alignment, velocity and cell-cell interactions. The combination of the live imaging with post-experiment immunohistochemistry also permitted correlation of cell behavior with matrix organization and the direction of mechanical load.

With regard to the mechanobiological hypothesis, the data from FFT and SAF examination of the cell culture showed that cell alignment on the coverslip (i.e. no orientational cues) was only locally correlated while cell alignment was both locally and globally correlated on loaded DDCS samples both after the first and second week. The 6% strain (strong guidance cue) applied during the second week of fibroblast culture on DDCS did not significantly change the cell alignment or orientation. This was a surprise. What we found was that the small preload (0.01N) had significantly altered cell alignment even though it was applied prior to cell seeding. To ensure that this result was due to the applied mechanical tension, a control series with no preload was run and confirmed that the tension was causative. Thus, our hypothesis that mechanical force is a driver of cell organization was supported. Interestingly, it had not been shown before that a small, static load could control fibroblast organization. The DDCS sample was under only a 0.01N load (~ 50KPa, 1% strain in x-direction; 2% strain in y-direction), which suggests that PHCFs are very sensitive to small static tension. While we are not sure how such a small load was sensed by the cells, we cannot rule out that there may have been a stiffness differential created by the uniaxial load through the non-linear modulus of the
DDCS. We estimate that there was an induced difference in stiffness between the x and y directions of approximately 7kPa. It is possible that the cells are responding to this difference. It is also possible that they are attempting to avoid both the tensile and compressive loading induced by the applied strain (which is somewhat consistent with the 135° degree preferential alignment). The small difference in fibril alignment and binding site density induced by the ~1% strain (tensile) in the x-direction and the ~2% Poisson compression in the y-direction was unlikely to change cell behavior. Further, we do not understand why the symmetry in the system was never broken such that the top layer of cells was 45° and the bottom layer was 135°. It was always the other way around. With regard to the migratory behavior of the PHCFs, we found that cell velocity was initially fast but slowed down to a steady-state (6-10 μm/hr) following confluence and through matrix production. We also found a rapid convergence of the cell velocity direction with the cell orientation on the loaded DDCS. We surmise that the packing of the cells resulted in their slowing down, but cannot prove this definitively. Also, the rapid co-alignment of the velocity direction and orientation prior to confluence raised an interesting question and lead us to the conclusion that the mechanical state of the matrix was driving PHCF organization. Finally, the ability to directly observe cell migration dynamics with the fluorescent nucleus stain showed an unusual pattern on the DDCS where the cells are all moving on the same line, but in opposite directions. We were unable to identify this kind of pattern in any other neural crest cell system.

With regard to matrix production, we found that on both the coverslip and the DDCS that the cells produced a non-fibrotic matrix similar to the corneal stroma. However, on the loaded DDCS the collagen was co-aligned with the cells which were globally oriented
by the applied tension. On the glass coverslip, the organization of the cells and matrix were also co-aligned, but only locally organized. We concluded that the matrix follows the cells which obey the mechanics.

The series of experiments in chapter 3 clearly demonstrate the substantial advantages of the relatively unique capabilities we have built to quantitatively examine the effect of mechanics on cell culture behavior in real-time and over long periods. Direct observation of the cell culture system revealed startling culture dynamics which included cell proliferation and death, increasing levels of cellular organization, multilayer formation, organizational breakdown and remodeling (healing), velocity changes and matrix production.

In chapter 4, we presented an in vitro mechanobiological traction force assay capable of directly observing the behavior of PHCF over relatively long time scales while simultaneously measuring intrinsic (cell-cell and cell-substrate) physical forces. The combination of the mechanobioreactor with the traction force microscopy method permitted direct measurement PHCF physical forces during migration and growth to confluence. The reason for adding TFM to the series of investigations was to determine whether or not the cells were able to communicate mechanically during their initial migration, which sets up the final orientation of the cell system (and ultimately the direction of matrix). Thus we were trying to observe the local mechanism of organizational control. Because the combined bioreactor/TFM system enabled dynamic, continuous and quantitative observation of the mechanobiology we anticipated gaining a more profound understanding of the role of force in controlling matrix deposition in vitro. The traction microscopy data suggest that fibroblasts develop local alignment patterns.
leading up to the formation of a cell monolayer, the strain energy in the substrate and Root Mean Square (RMS) of tractions increase, ultimately reaching a steady-state after seven days. During this period, the tractions are distributed exponentially and align spontaneously with neighboring traction vectors to form self-organized domains. The maximum forces generated by some of the PHCFs reached 150 Pa only in a punctate manner, suggesting that the fibroblasts do not form strong cell-cell contacts like epithelial or endothelial cells. However, traction maps of fibroblast share a key property observed for epithelial and endothelial cell monolayers – the exponential tails in traction distribution. The exponential tail implies that extreme values are not as uncommon as one would estimate if the distribution were Gaussian instead. Although the source of such distributions is currently unknown, its observation in our fibroblast monolayer suggests that the source may be the inability of cells to transmit forces over large distance through cell-cell junctions (because our system of cells is not “connected”). Over the period of 7 days, the tractions remain exponentially distributed, but surprisingly, the orientation of cells and the direction of tractions are strongly correlated. The ability of the PHCFs to exhibit organization without forming permanent connections is a hallmark of neural crest cell migration/streaming. The relative disconnection of the forces during their patterning provides us with interesting material for further thought.
Future work

Our data from loaded DDCS showed that a tiny load (~ 50kPa) changed the stiffness of DDCS and this amount of stress could organize human corneal cells on the front and back of DDCS substrate when they build their matrix over two weeks. The following are two suggested future extensions to our work.

First, we can culture nucleus stained PHCF’s on loaded polyacrylamide gel (~ 50kPa) for up to two weeks and measure the cell velocity, alignment and migration on polyacrylamide gel substrates (soft gels ~ 1.2kPa) as well as cell-substrate interactions. This study will show how corneal cell organize their extracellular matrix on soft gels when they are under uniaxial mechanical load and then we will compare these results with loaded DDCS samples. Based on these new results, we can study how corneal cell organize on soft substrates (polyacrylamide gels) in the presence of the tiny stress/load over long-term observation.

Second, we can culture PHCF’s on two different substrates (1- loaded DDCS and 2- polyacrylamide substrates) inside the bioreactor and then observe how corneal cells build their extracellular matrix using fluorescent microcopy. The behavior and growth of human corneal fibroblasts are tracked by imaging cells at 6-minute intervals (DIC microscopy) and one hour intervals (fluorescent microscopy) for two weeks. This study not only observes PHCFs but also evaluates and quantifies how much FITC collagen will be produced by PHCF’s over two weeks. This experiment will be long-term observations of FITC collagen produced by primary human corneal fibroblasts and will show how corneal cells produce collagen in real-time over two weeks when they are under uniaxial
mechanical load. The results will demonstrate how collagen fibrils will be produced and aligned by corneal cells.