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Design and Validation of a Complex Loading Whole Spinal Segment Bioreactor

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Master of Science

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ABSTRACT

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Intervertebral disc (IVD) degeneration is a prevalent health problem that is highly linked to back pain. To understand the disease and tissue response to therapies, *ex-vivo* whole IVD organ culture systems have recently been introduced. The goal of this study was to develop and validate a whole spinal segment culturing system that loads the disc in complex loading similar to the *in-vivo* condition, while preserving the adjacent endplates and vertebral bodies. The complex loading applied to the spinal segment was achieved with three pneumatic cylinders. The pneumatic cylinders were rigidly attached to two triangular alumni plates at each corner, comprising the loading mechanism. By extending or compressing the pneumatic cylinders, three modes of loading were achieved: flexion-extension, bi-lateral bending, and cyclic compression. The cylinders were controlled via microcontroller, and the entire system was fully automated. The culture container, which housed the spinal segment during culturing, was a flexible silicone container with an aluminum base and lid. The culture container attached to the loading mechanism allows for loading of the spinal segment. It had a vent attached to the aluminum lid that allowed for gas exchange in the system. The dynamic bioreactor was able to achieve physiologic loading conditions with 100 N of applied compression and approximately 2-4 N-m of applied torque. The function of the bioreactor was validated through testing of bovine caudal IVDs with intact endplates and vertebral bodies that were isolated within 2 hours of death and cultured for 14 days under a diurnal cycle. The resulting IVD cell viability following 14 days of loading was approximately 43% and 20% for the nucleus pulposus and annulus fibrosus respectively, which was significantly higher than the unloaded controls. The loading system accurately mimicked flexion-extension, bi-lateral bending, and compression motions seen during daily activities. Results indicate that this complex dynamic bioreactor may be appropriate for extended pre-clinical testing of vertebral mounted spinal devices and therapies.

Keywords: bioreactor, intervertebral disc, cell culture, mechanical loading

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1 INTRODUCTION

1.1 Problem Statement

The objective of this research was to design, build, and validate a dynamic bioreactor to keep intervertebral discs alive outside the body while preserving the vertebral bodies. The design of the dynamic bioreactor was then validated using *ex-vivo* organ culture tests of bovine tail intervertebral discs using static bioreactor tests as a control.

1.2 Significance

Back pain is a leading cause for visiting orthopedic surgeons in the United states [1], with an estimated $100 billion spent annually on the condition [2]. Disc degeneration is one of the primary causes of back pain [3, 4], yet there are limited treatments available for the disease.

Developing a bioreactor that is able to keep intervertebral discs (IVDs) alive outside the body for an extended period of time, while maintaining disc mechanics, will allow clinicians to further research disc degeneration and develop new therapies and treatments. Conducting long-term tests on living tissue that is not inside a living patient will broaden the horizon of research possibilities and can potentially reduce the instances of chronic back pain.

1.3 Overview

This thesis describes the development and testing of a dynamic bioreactor. The bioreactor was tested for two weeks with a bovine IVD and then imaged for cell viability. The results are compared to two static controls whose cell viability is also calculated.

1.4 Summary

Chapter 2 includes a literary review of topics including cell culture, IVD nutrition, and whole organ IVD bioreactors. Loading of the disc is valuable for retaining disc health and aiding in nutrient transport. Several whole organ IVD dynamic bioreactors have previously been presented, however the bulk of them only load the disc axially, and only one retains adjacent bone.

Chapter 3 is a presentation of the materials, methods, and short discussion of this project. It represents the bulk of the thesis work. Bovine caudal spinal segments were used to validate the dynamic bioreactor by undergoing a 14-day test. The validation of the complex loading dynamic bioreactor proved successful, and a resultant cell viability of 20%-43% was seen. The work in this chapter will be submitted for publication shortly with Anton E. Bowden and Laura C. Bridgewater as co-authors.

Chapter 4 takes a more detailed look at the work that went into this thesis. It includes a synopsis of the design troubleshooting process. An overview of non-selected designs is described, as well as the pneumatic system design and bioreactor programming.

Chapter 5 concludes this work and presents suggestions for furthering the work on this project. While the design of the bioreactor was successful in achieving the functional requirements, it could be improved by switching the culture container material from aluminum to titanium and shortening the incubation time in the fluorescent dye bath.

2 BACKGROUND

The following sections will outline IVD nutrition, anatomy, mechanics, and past work on IVD bioreactors. In order to understand the functional requirements of a dynamic bioreactor for use on IVDs, it is necessary understand these topics and review past literature on bioreactors to see how they are lacking and can be improved upon.

2.1 Functional Spinal Unit Anatomy and Nutrition

The basis of the dynamic bioreactor revolves around the needs of a functional spinal unit (FSU), therefore it is pertinent to understand the anatomy and nutrition of an FSU.

An FSU consists of an IVD and the surrounding endplates and vertebrae, or vertebral bodies (Figure 2-1). The cartilage endplates are very thin layers of hyaline cartilage that are crucial for the nutrition of the disc [5]. They are rigid, porous plates that are one of the main pathways for nutrients to be supplied to the disc [5]. The cartilage endplate distributes pressure from the disc to the bone to prevent the disc from bulging into the vertebral body [6]. As we age, the cartilage endplate thins and increases in diameter to cover the surface of the disc [6]. By the age of 18, the bony endplate has formed between the cartilage endplate and the vertebral body [6].

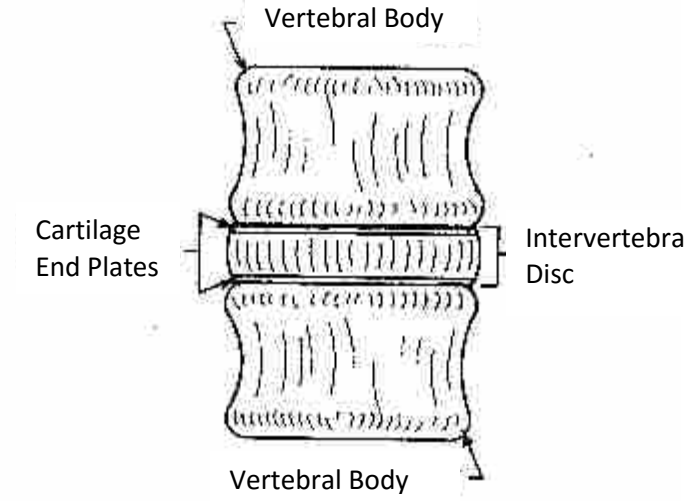

Figure 2-1: Anatomy of a functional spinal unit. Image from Coltman et al.[7].

The vertebral body is the cylindrical portion that makes up the largest part of the vertebra. It has a thin outer shell made of compact cortical bone and an interior of porous, vascularized cancellous bone[8]. The human spine has 24 vertebrae plus the sacrum and coccyx (tailbone). The 7 cervical vertebrae are the smallest and highest up on the spinal column. 12 thoracic vertebrae make up the mid-portion of the spine, and 5 lumbar vertebrae are the largest and make up the lordotic curve of the spine at the lower back [8]. The sacrum connects the spine to the hip bones, and the coccyx provides attachment of ligaments and tendons from the pelvic floor[9].

IVDs are pads of soft tissue located between vertebral bodies in the spine. They allow limited movement of the spine, and distribute loads to the surrounding endplates[10]. The outer region of the disc, namely the annulus fibrosus (AF), consists of a network of collagen fibers obliquely organized between the vertebrae, while the inner region, the nucleus pulposus (NP) is an amorphous region made of proteoglycan, water gel, and a loose network of irregularly oriented collagen fibers (Figure 2-2)[10, 11].

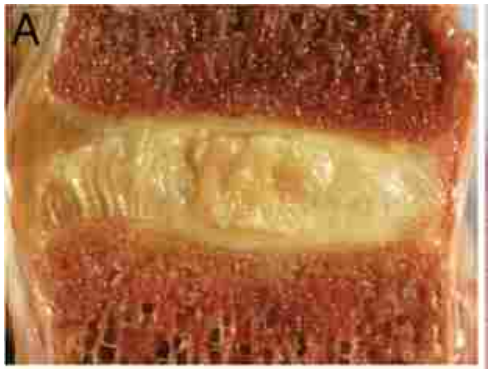

Figure 2-2: A cross sectional view of a human intervertebral disc and surrounding bone. Taken from Lotz et al.[6].

Discs experience cell death and degeneration when there is a lack of nutrient supply [5, 10, 12-14]. In adults, the disc is largely avascular, therefore most of the nutrient transport occurs by diffusion from blood vessels surrounding outer AF, diffusion through the endplate of the surrounding vertebrae [5, 10, 12], and through bulk fluid flow into the disc[15].The disc undergoes a diurnal cycle of loading which results in roughly 25% of the disc's fluid being lost and regained daily [16]; this loss and gain of fluid is a contributing factor in nutrient transport to the cells in the disc [17]. Diffusion is the primary mode of nutrient movement for small nutrients, such as oxygen and glucose, while fluid flow highly influences the movement of large nutrients [15].

The IVD cells make up 1% by volume of the disc, but are responsible for producing and breaking down the extracellular matrix[16]. When the cells cannot synthesize as quickly as they breakdown, the matrix begins to degrade which leads to structural failure [16], and therefore degeneration. Matrix synthesis and repair decline with aging, thus decreasing the health of the disc, and are partially due to decreased cell density [10]. Keeping the cells in the IVD alive and

healthy is vitally important in maintaining a healthy disc and maintaining a balance of synthesis and degradation of the matrix.

An additional mode of degeneration is through aging. When humans age, the cartilaginous endplates begin to calcify, which impedes the passage of nutrients to the disc [18]ultimately contributing to degeneration. Aging also results in decreased water and proteoglycan content in the disc, and increased collagen content and cross linking [10]. These characteristics inhibit the synthesis of disc matrix which leads to reduced IVD strength [10]. All of these factors contribute to a higher probability of structural failure and degeneration of the disc [10].

It has been found that isolated discs with mild degeneration are able to experience repair and improvement when loaded under physiological conditions [19]. Additionally, Kuo et al. determined that “traction treatment is effective in enhancing nutrition supply and promoting disc cell proliferation of the degraded discs.”[20]

The present study retrieved bovine tail discs for use in testing the dynamic bioreactor. Bovine tail discs are an excellent choice for this study because they have similar creep and compressive stiffness as human IVDs [21, 22]. Out of 8 animal models tested, bovine tail discs were also found to have the closest height to human discs[23], which is beneficial for comparison purposes.

In summary, an FSU is made up of an IVD with surrounding cartilage endplates, bony endplates, and vertebral bodies. The disc receives nutrients through diffusion and bulk fluid flow, which is aided by physiologic loading, and bovine caudal discs are appropriately similar to human discs, making them an appropriate choice for use in the dynamic bioreactor.

2.2 Disc Segmental Mechanics

Physiologic loading, which aids in bulk fluid flow and nutrient flow, is broken down into three motions: flexion-extension (FE), lateral bending (LB), and axial rotation (AR) (Figure 2-3). These are the three motions a disc is subjected to, however the disc also experiences a constant compression load from the weight of the head and torso.

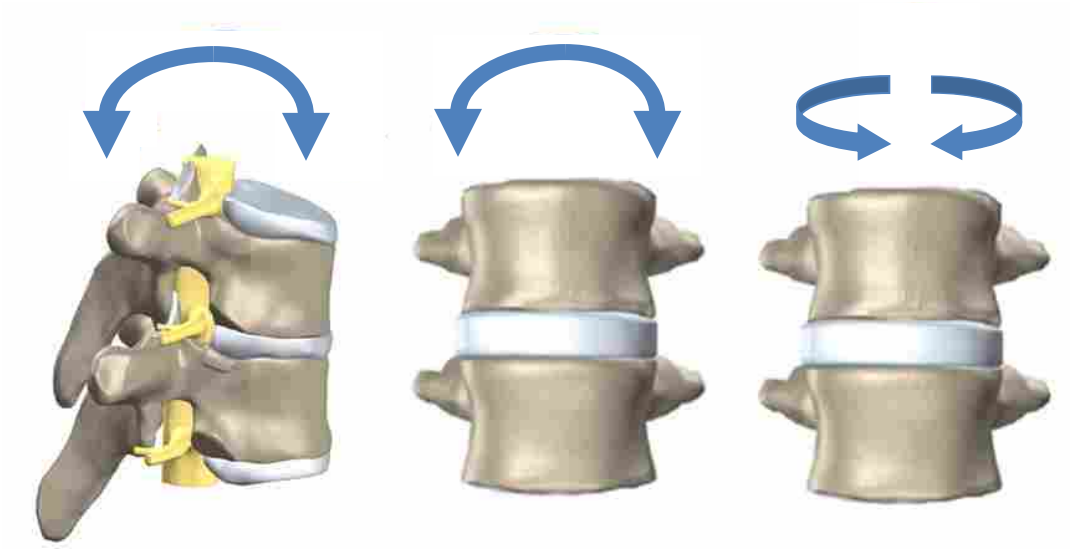

Figure 2-3: From left to right, flexion-extension, lateral bending, and axial rotation movements of a spinal segment. Image retrieved and modified from Medical MultiMEDIA Group.

The range of motion of a single lumbar spinal segment is ±1.5-2° in AR, ±2-5° in LB[24-26], and ±3-6° in FE[25, 26], however, this decreases significantly with degeneration of the disc [27]. Cervical spine segment range of motion is ±4° in AR, ±6° in LB, and ±7.5° in FE [25, 26].

The compressive loads seen by IVDs range from 100-1000 N [28-30], and the intradiscal pressures range from 0.5-1.7 MPa depending on the activity [31]. The average torque applied to the disc from FE or LB is roughly 3-8 N-m [25, 26]. The compressive load felt from the weight

of the body from standing up is 0.5 MPa [31], which correlates to roughly 55 N for a bovine caudal disc, which was used in the research for this thesis.

These are loads seen during every day activities, however at night while lying horizontally they can nearly disappear, and intradiscal pressure drops down to 0.1-.015 MPa [31].

The stiffness of the disc ranges from 0.2-1.2 N-m for FE and LB, and is about 8 N-m for AR [32]. Disc degeneration reduces the stiffness of the disc in AR, but increases the stiffness in FE and LB [32]. Degeneration also decreases the range of motion in all modes of loading [32].

FE, LB, AR, and compression play a large role in nutrient transport to the disc, and in the prevention of disc degeneration. Physiologically loading a whole IVD is a crucial factor in studying it outside the body, and this can be done using bioreactors.

2.3 Bioreactors

Bioreactors play a vital role in biological research. Bioreactors are the avenue through which cell culture is practiced. Cell culture is common in molecular biology for providing models that allow scientist to study the physiology and biochemistry of cells [33]. Whole organ bioreactors allow for a larger spectrum of research to be conducted by retaining the structural components in addition to the cells, and whole FSU bioreactors allow for study of vertebra-attaching spinal devices to be tested simultaneously with disc health.

2.3.1 Cell Culture

Cell culture is the isolation of cells from a plant or animal and their growth inside a controlled environment that is favorable for proliferation. The cells are cultured inside a vessel called a bioreactor, which has culture medium inside of it. Culture medium is a fluid that has all

the essential nutrients needed for cell growth, such as amino acids, carbohydrates, vitamins, minerals, growth factors, hormones, and phosphate buffers [33]. Most commonly, bioreactors are placed inside and incubator which controls the temperature and CO_2 content of the air. The combination of CO_2 content and phosphate buffer in the culture medium is what maintains a suitable pH for the cells.

Cell culture is the means by which scientists can study cells, such as in the following ways[33]:

- metabolic studies
- aging
- the effects of drugs and toxic compounds
- mutagenesis and carcinogenesis
- drug screening and development
- large scale manufacturing of vaccines and therapeutic proteins

Cell culture is ideal for these applications because of the consistent and reproducible results that are achievable[33].

The first bioreactors used on IVD cells were studying the metabolism of the cells and what they respond to in an attempt to understand disc degeneration [34-37]. Studies have been done on IVD tissue and cells under a large range of conditions since the late 1980's to help scientists understand the disease.

Various studies have found the following: values for matrix and cell density, cells respond to cyclic load [16, 38, 39], collagen II composition can be altered depending on the type of bioreactor [40, 41], optimization of induced degeneration[42], and production of a tissue-engineered IVD[39].

Cell culture has been very useful in the understanding of IVDs and their metabolism, however it is unable to give us any information about the mechanics of the disc or allow for any preclinical testing of whole IVD therapies. For this reason, whole organ IVD bioreactors have been studied.

2.3.2 Whole Organ IVD Bioreactors

Whole organ IVD bioreactors are the implementation of cell culture on entire IVDs without the isolation of cells or small amounts of tissue. This allows scientists to study the mechanics and biology of the disc simultaneously, leading to larger advances in the knowledge of IVDs, as well as the option for preclinical testing without harm to patients.

Whole organ IVDs began with static culture (no load or static load) [43-47], but after 7 days of culture, there was often dismal cell viability within the disc. In order to preserve cell viability and disc mechanics, many research labs turned to dynamic bioreactors. The idea behind dynamic bioreactors was that a dynamic load would stimulate the cells and cause bulk fluid flow into the disc, aiding in large nutrient flow. Ultimately, the goal was to preserve disc viability and mechanics for as long a period of time as possible to study various aspects of the disc.

In the last decade the standard design for a dynamic bioreactor became cyclic diurnal uniaxial compression of the disc with or without cartilaginous endplates [4, 48-52], however, more recently, flexion-extension (FE)[53] and torsion [51] have been implemented (Figure 2-4).Under these conditions multiple items could be examined: gene expression, metabolism, stiffness and creep of the disc, disc height, and others.

The Table 2-1 is a summary of the past dynamic bioreactors shown in Figure 2-4.

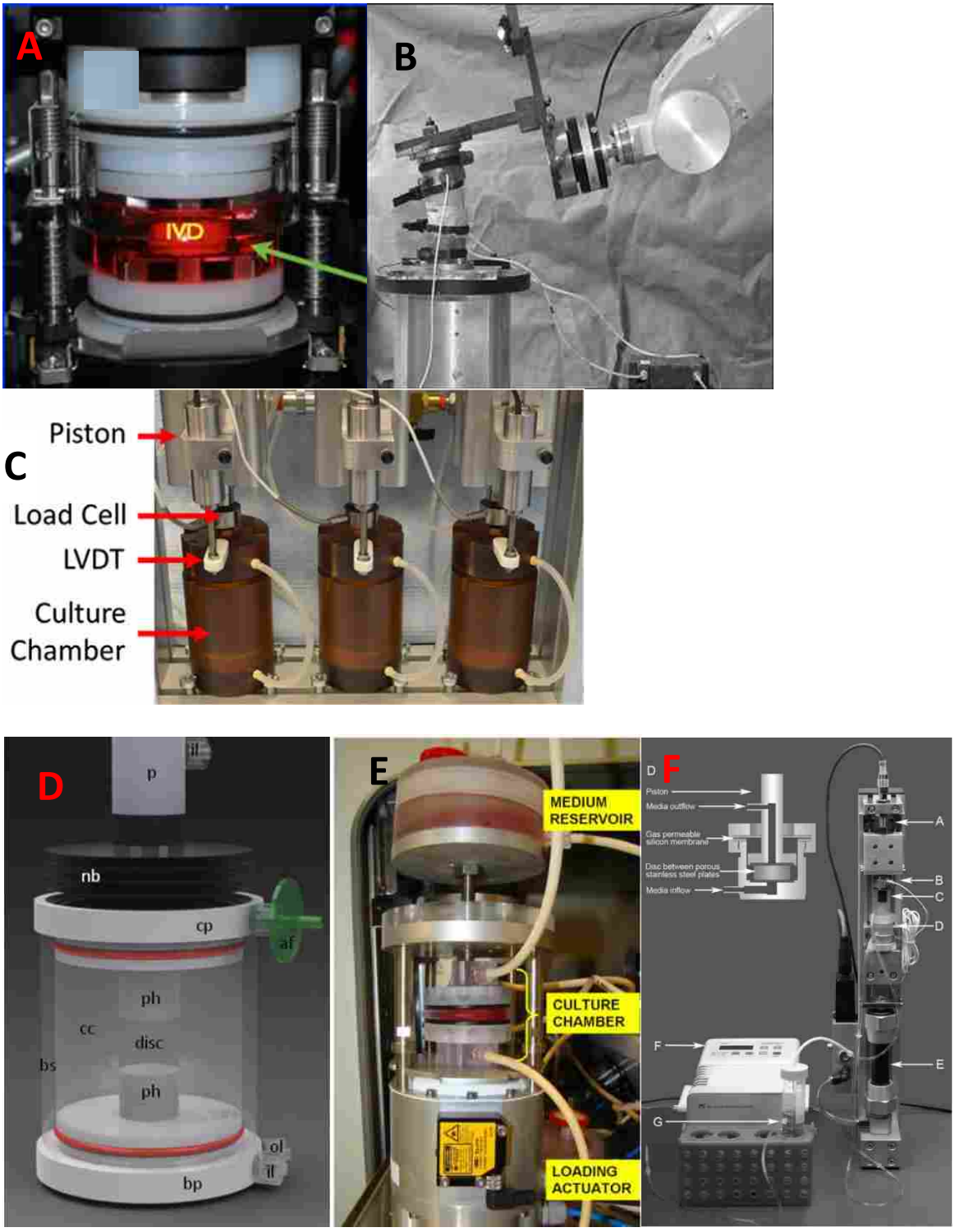

Figure 2-4: A) Chan et al. torsional load bioreactor[51], B) Hartman et al. with FE bioreactor [53], C-F) Axial compression bioreactors from Walter et al., Haglund et al., Paul et al., Illien-Junger et al. [48-50, 52].

Table 2-1: Previously published dynamic bioreactor designs [48-54]

Specification	Chan	Haglund	Walter	Paul	Illien-Junger	Hartman
Loading -Flexion-extension -Lateral-bending -Compression -Torsion	 n/a n/a 87-174 N (calculated) n/a	 n/a n/a 32-850 N(calculated) n/a	 n/a n/a 200-500N(reported) n/a	 n/a n/a 40-80, 40-240 N (calculated) n/a	 n/a n/a Undisclosed n/a	 0.2-0.5 N-m n/a n/a n/a
Motion -Flexion-extension -Lateral-bending -Torsion	 n/a n/a ±2°	n/a	n/a	n/a	n/a	 7-15° n/a n/a
Culture Medium Volume	40 mL	120 mL	Undisclosed	40 mL	50 mL	200 mL
Frequency of medium changing	2-3 days	3-4 days	2-3 days	2 days	2-3 days	n/a (1.25 mL/min perfusion)
Sterilizability	Yes	Yes	Undisclosed	Yes	Yes	Undisclosed
Gas permeability (for CO_2 regulation)	Yes	Yes	Yes	Yes	Yes	Yes
Cycle length	15 days	28 days	7, 14, 21 days	0, 7, 14, 21 days	14 days	30 hours
Loading Frequency	0.2 Hz	0.1 Hz	0.1 Hz	0.1-0.2 Hz, 1 Hz	0.2 Hz	Undisclosed (0.1 mm/sec)
Self-Contained	Yes	Yes	Yes	Yes	Yes	Yes
Vertebral Endplates	Yes	No	Yes	Yes	Yes	Yes
Diurnal Cycle	Yes	Yes	Yes	Yes	Yes	No
Cell Viability	11-77%	80-88%	87-89%	Low (undisclosed)	82-94%	Undisclosed

The majority of the bioreactors in Table 2-1 used low frequency loading (0.1-0.2 Hz) with low loads (50-400 N), which has been found to be most beneficial for cell life [51]. Additionally, they share the trait of culturing for 14-28 days and changing the culture medium every 2-3 days.

Through studies such as these, it was found that cartilaginous endplates are an integral part of maintaining disc health *ex-vivo*[46, 55], that cell viability remains high under lower strains rather than higher strains [52], and that torsional loading significantly decreases cell viability [51]. Length of culture duration has a tremendous effect of cell viability in unloaded and low dynamic loads, but according to Paul et al. it does not affect the case of physiologic loading [50].

The types of genes expressed have been found to be highly dependent on type of load [50], and clotted blood on endplates causes cell death [55]. Additionally, complex over-loading of spinal segments can result in the fracture of cartilaginous endplates which reduces pressure inside the disc and can lead to degeneration [56].

Only one previous study kept the vertebral bodies attached to the IVD, however cell viability went un-reported [53]. They studied rabbit FSUs loaded under FE for 30 hours to examine the effects on multiple spinal tissues, including facet cartilage and ligamentum flavum, and determined that FE significantly affected the gene expression of the facet cartilage and annulus fibrosus.

Much information has been gained from the aforementioned dynamic bioreactors, however, no publications thus far have implemented flexion-extension, lateral bending, and compression, which are three of the main movements the disc *in-vivo*. More physiologic conditions could potentially lead to longer cycle times while maintaining cell viability which

would aid in disc degeneration therapy and treatment research. Additionally, maintaining the adjacent cartilage endplates and vertebral bone could provide a significant advantage by allowing testing of pre-clinical spinal devices that attach to the vertebral bodies. The following chapter will describe the development and testing of a bioreactor that implements all three modes of loading on a whole FSU in an attempt to achieve higher cell viability and/or disc mechanics than previous publications and provide an avenue for pre-clinical spine devices to be tested.

3 DEVELOPMENT OF COMPLEX DYNAMIC INTERVERTEBRAL DISC BIOREACTOR

3.1 Introduction

Back pain is a highly prevalent health condition that contributes to a majority of chronic pain cases, has a 54-98% lifetime prevalence [57, 58], and is the leading specific cause of years lived with disability [59]. Intervertebral disc(IVD) degeneration is highly associated with back pain [2, 60, 61] and occurs when there is an inadequate supply of nutrients to the disc [10, 62, 63]. The IVD is an avascular organ, therefore nutrients and metabolites enter the disc in two ways: 1) transport from blood vessels surrounding the disc through the outer portion of the annulus fibrosus, or 2) transport through the vertebral endplate [63, 64].

As a result of daily activities and body weight loading of the IVD, up to 25% of the disc's fluid is lost and subsequently regained during sleep [63]. The resultant fluid motion significantly increases the rate of nutrient and metabolic waste transport. Spinal motion during daily activities is complex, and initiates a correspondingly complex flow pattern in the nutrient-laden fluids in the IVD. For example, flexion-extension (FE) and lateral bending (LB) are modes of loading that force a portion of the disc into tension while the opposing side is in compression, and vice versa. This drives fluid into the tensioned half of the disc and increases large nutrient flow [15]. Without this transport the disc cells gradually die, and the mechanical properties of the disc are altered.

Since the cells in the disc are robust due to their relatively avascular environment, there is potential to utilize a whole organ, *ex-vivo* bioreactor as a test bed for evaluating both the degenerative processes and potential treatments. Previous work in developing such bioreactors has identified cyclic disc loading as being a key component in maintaining cell viability for extended (multi-week) periods of time [48-53].

The present work describes the design and functional validation of a dynamic whole organ intervertebral disc bioreactor intended to keep disc nutrition, cell life, and disc mechanics similar to that seen *in-vivo* for an extended period of time. Previous studies have loaded the disc in only cyclic compression (CC) alone [48-50, 52], FE alone [53], or torsion with compression [51], generally without maintaining the adjacent vertebral bone [53]. The present work differentiates itself by 1) including a complex multi-modal cyclic loading paradigm representative of the *in-vivo* condition, and 2) retaining the vertebral bodies adjacent to the disc. This study focuses on the design and validation of the device and its ability to load a spinal segment under physiologic loading rather than cell viability results.

3.2 Materials and Methods

3.2.1 Bioreactor Design

There are several design requirements that the dynamic bioreactor must meet in order to closely resemble *in-vivo* conditions. These include applied load/torque, applied angles, cycle length, robustness, and several requirements related to cell culture (Table 3-1). The listed requirements are based on cervical spine *in-vivo* values and general cell culture practice. The goal of the dynamic bioreactor is to control the torsional and compressive loads applied to an

IVD to match *in-vivo* loading while still meeting the appropriate motion and culture requirements.

The final design of the dynamic bioreactor consisted of two components: 1) the culture chamber and 2) the loading mechanism secured to the top of culture chamber. To achieve FE, LB and CC, three pneumatic cylinders (RP 106x1.500-DAD, Norgren Inc., USA) were utilized in the loading mechanism in a three-axis parallel loading platform design (Figure 3-1and Figure 3-2). Each actuator was rigidly attached to a top plate (aluminum) and a ball joint that was subsequently attached to the bottom plate. The top and bottom plates were triangular in shape, with one actuator at each corner.

Loading in FE, LB, and CC was achieved by activating the appropriate combination of linear actuators. A complex, dynamic loading cycle was developed based on published pre-clinical spinal device testing standards (ISO 18192-1:2011 standard [25, 26]) and the published literature [65].The final duty cycle consisted of 25 seconds of flexion, 25 seconds of extension, 25 seconds of right lateral bending, 25 seconds of left lateral bending, and 50 seconds of cyclic compression followed by 150 seconds of unloaded rest. This 5-minute cycle was repeated for 16 hours, followed by 8 hours of rest to simulate *in situ* loading of the spine. Automated actuator control was provided through an integrated, programmed microcontroller (ArduinoUno Rev 3, Arduino, Italy), which was housed in a custom 3D printed protective housing.

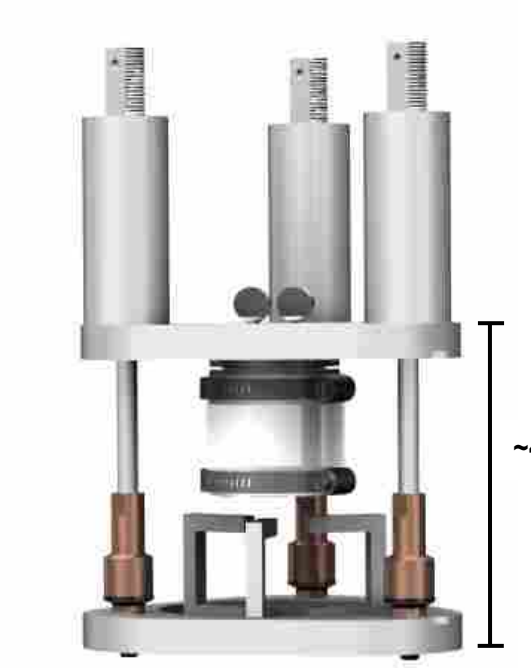

Figure 3-1: CAD rendering of the loading mechanism with risers.

Figure 3-2: Complex loading dynamic bioreactor loading mechanism and culture container.

The bovine functional spinal unit is placed between the top and bottom plates inside the culture chamber. To accommodate a broad range of segmental sizes, spacers were used below the segment. The culture chamber is a cylindrical container with flexible sides made of 1/16" thick silicone rubber which is securely clamped to the aluminum lid and base with hose clamps and silicone caulk. The lid, when unscrewed, had an opening large enough to fit the spinal segment into the container and had a vent protruding out the side with 1/8" PVC tubing attached to allows gas transport into the system and prevent excessive hypoxia. The end of the vent tube was covered in a HEPA foam filter to prevent bacteria from entering and contaminating the specimen.

When the actuators were contracted they applied a load to the disc, and when they were fully extended, the culture chamber was suspended from the top plate of the loading mechanism with no load being applied to the functional spinal unit.

3.2.2 Design Verification Testing

The actual applied load provided by the dynamic testing was measured by placing a mechanical load cell in lieu of the spinal segment. Pressures from 5.0 psi to 60.0 psi were applied to the pneumatic cylinders at a rate of 0.2 Hz and the corresponding output forces were recorded. Applied torques were calculated based on the force data.

The range of applied motion provided by the bioreactor was measured using a magnetic bevel box. The box was secured to the moving top plate of the bioreactor while it was running and the angles were displayed, as seen in Figure 3-3. The applied angles for FE and LB could be adjusted ±4° by threading the actuators in or out of the ball joints.

In order to verify the ability of the dynamic bioreactor to meet the specified functional requirements, a test was conducted with a bovine caudal FSU. The FSU was placed inside the dynamic bioreactor for 14 days under the loading conditions specified above. Two control discs placed in the static bioreactors (no applied load) were tested at the same time. At the end of the 14-day period, all three discs were fluorescently dyed and imaged to determine cell viability.

Figure 3-3: Magnetic bevel box used to measure angle displacement of bioreactor.

3.2.3 Tissue Isolation and Culture

Bovine tails were retrieved from a local abattoir within 45 minutes from the time of death. Skeletal muscle and anterior processes and facets were removed from the FSU using a band saw and scalpel dissection. The spinal segment used consisted of an IVD attached to two vertebrae that were transversely cut to a height of roughly 1.0 cm. The cancellous bone in the center of the vertebral bodies was then removed down to the level of the cartilage endplate using a Dremel tool with an 1/8” end mill bit. Since the main mode of nutrient transport is through the

cartilage endplate [63], the central reaming of the vertebrae allowed for maximum nutrition flow into the disc while still maintaining cortical bone for spinal device attachment.

Four spinal segments were prepared for the following purposes: 1) testing in the dynamic bioreactor for 14 days, 2) and 3) controls to be placed in static bioreactors for 14 days, and 4) for initial live/dead cell staining and imaging. All four segments were thoroughly rinsed with sterile saline solution and placed in Dulbecco's Modified Eagle's Medium (DMEM) within two hours from the time of death. All segments were then transferred to a laminar flow tissue culture hood and their surfaces sterilized.

The culture medium for the bioreactor was prepared using 500 mL of high-glucose DMEM (4500 mg Glucose/L + 0.584 g L-glutamine/L + 3.7 g $NaHCO_3$/L), 100 mL FBS, 12.5 µL HEPES, 12.5 µL L-ascorbic acid, 1.0 mL Pen/Strep, 500 µL Gentamycin, 3mL Fungizone, 55 mg Sodium Pyruvate, 1403 mg NaCl, and 12.5 mL of Icterine color concentrate. Ingredients were combined in a sterile environment inside a laminar flow tissue culture hood and filtered through a cellulose nitrate membrane filter unit.

For the static controls, 30 mL of the prepared medium was placed in sterilized 35 mL Nalgene containers along with the spinal segments. The lid of each container was loosened to allow for gas exchange then placed in an incubator at 37°C and 5% CO_2 for 14 days.

For the dynamic bioreactor validation testing, 30 mL of culture medium was placed into the dynamic bioreactor culture container along with the spinal segment. The entire dynamic bioreactor assembly was placed in the same incubator as the static controls, where subsequent dynamic loading of the disc occurred for 14 days.

The culture medium in the static and dynamic bioreactors was drained and replaced under sterile conditions every 3-4 days to and maintain a high level of nutrients. The medium was

regularly checked for contamination and pH levels to verify they were within an appropriate range for cell culturing. Appropriate and consistent pH levels were seen throughout the 14-day testing period.

At the end of the 14-day period, discs from the static and dynamic bioreactors were fluorescently dyed in a solution of 30 mL DMEM, 6 µL CellTracker™ Green CMFDA (which is retained in living cells), and 30 µL propidium iodide (which stains dead and dying cells red) for 40 minutes. The disc was then removed from the vertebrae, frozen in liquid nitrogen, and sectioned transversely at 10-14 µmusing a cryostat. Fluorescent microscopy was used to image the disc and determine cell viability. For comparison purposes, on spinal segment was similarly stained and sectioned at the start of the 14-day validation test.

3.3 Results

3.3.1 Requirements and Outcome

The dynamic bioreactor was able to accurately mimic physiologic loading for indefinite periods of time. It met all the requirements that were specified for cervical spines (Table 3-1), and the air pressure supplied to the pneumatic cylinders can be adjusted to account for lumbar spine values.

Table 3-1: Requirements for dynamic bioreactor and current ability to meet those requirements.

Specification	Requirement	Actual
Loading		
-Flexion	4 N-m*[28]	2.2 N-m
-Extension	4 N-m*	4.4 N-m
-Lateral-bending	4 N-m*	1.9 N-m
-Compression	100.0 N*	100.0 N
Motion		
-Flexion-extension	≥15°* [26, 28]	20° maximum
-Lateral-bending	≥6°*	15° maximum
Medium volume (to submerge spinal segment)	30-80 mL	30 mL
Frequency of media changing	3 days	3 days
Sterilizable	yes	yes
Gas permeability (for CO_2 regulation)	yes	yes
Cycle length	2+ weeks	Indefinite
Duty cycle	4-6 minutes	5 minutes
Robust	yes	yes
Size	Fits in incubator	Fits in incubator
Self-Contained	yes	yes

* These are values generally seen in the cervical spine.

The motion achieved by the complex loading dynamic bioreactor was larger than the requirement, however it should be noted that this was the largest range of motion of the device with no segment inside the culture container. When a segment was inside the culture container the motion was limited to the range of motion of the segment itself.

3.3.2 Cell Viability

The dynamic bioreactor was able to achieve higher cell viability in the disc than either the one-week or two-week static bioreactors (Table 3-2 and Figure 4-3). The cell viability in the nucleus pulposus directly following specimen acquisition was more than 98%, and after two weeks in the dynamic bioreactor was approximately 43%. The static controls demonstrated a cell viability of approximately 33% and 0% after one and two weeks of incubation respectively.

The cell viability in the annulus fibrosus at the beginning of the testing period was 12.4%, but was approximately 20% at the end of two weeks in the dynamic bioreactor, compared to 0% viability after two weeks in a static bioreactor. The low initial viability of annulus fibrosus compared to nucleus pulposus cells may be due to the harshness of the dissection or surface sterilization procedures, or to the extended incubation in propidium iodide that was required for dye to diffuse into the nucleus pulposus[66]. The two-week incubation in the dynamic bioreactor produced no additional reduction in viability of annulus fibrosus cells.

The cell density for the test specimens was between 27,808-47,630 cells/mm^3.

Table 3-2: Cell viability with standard deviation in IVDs under dynamic or static conditions.

	Beginning of test	Dynamic after 2 weeks	Static after 1 week	Static after 2 weeks
Average Viability (Nucleus)	98.7%±1.3%	42.9%±14.8%	33.5%±11.5%	0%
Average Viability (Annulus)	99.5%±1.3%	19.69%±15.8%	33.8%±29.2%	0%
Average Cell Density	36,599/mm^3	37,420/mm^3	24,258/mm^3	27,808/mm^3

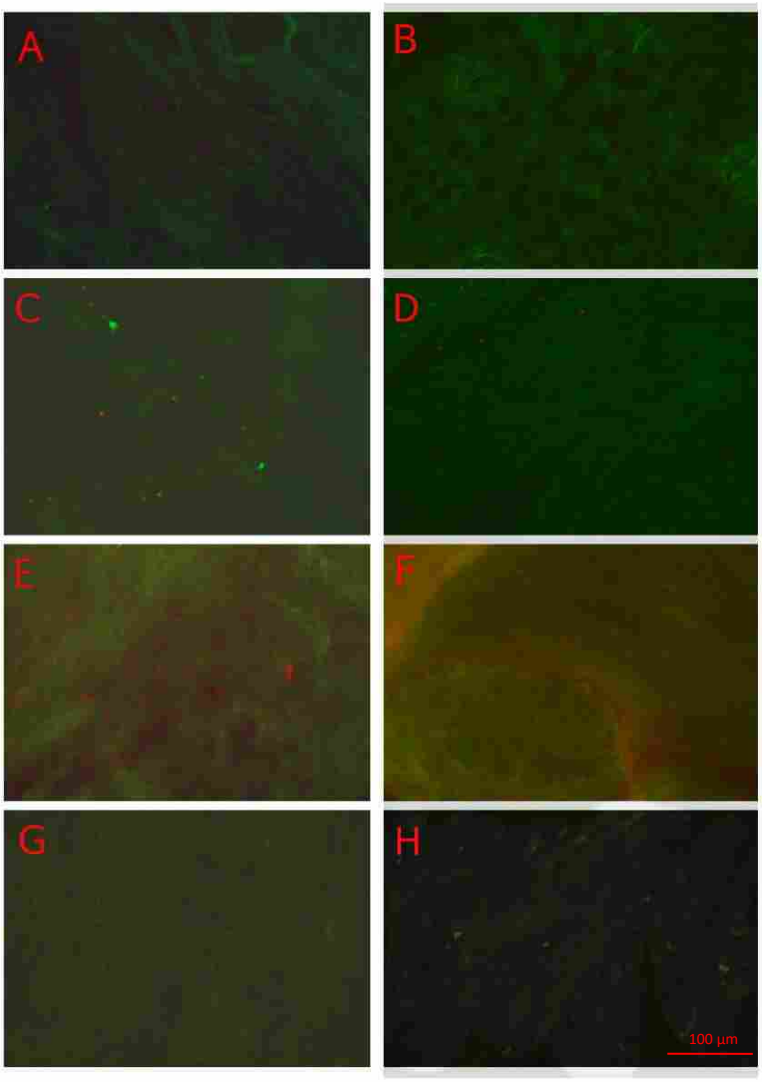

Figure 3-4: Fluorescent imaging of A) and B) disc nucleus pulposus and annulus fibrosus at the beginning of testing, C) and D) disc nucleus pulposus and annulus fibrosus from dynamic bioreactor after two weeks, E) and F) 7-day control disc nucleus pulposus and annulus fibrosus from static bioreactor, G) and H) 14-day control disc nucleus pulposus and annulus fibrosus from the static bioreactor.

3.4 Discussion

This study developed and validated a system for dynamically loading FSUs in FE, LB, and CC with intact cartilage endplates and vertebral bodies for extended periods of time. Bovine caudal spinal segments with intact endplates and vertebral bodies were cultured for 14 days under physiologic loads. The current bioreactor design was built on previously published work

[48-50, 52, 53], but introduced two non-trivial improvements: 1) complex physiologic loading with FE, LB, and CC, and 2) preservation of cartilage endplates and vertebral bodies adjacent to the IVD. These changes can provide a way for extended pre-clinical testing of vertebral mounted spinal devices and therapies.

One previous study utilized whole functional spinal units for organ culture in a dynamic bioreactor, however, they cultured rabbit FSUs which are markedly different than human spinal segments[53]. Rabbit IVDs are small enough in size that nutrient transport is almost solely controlled via diffusion[67], which is not true of human discs. Additionally, rabbit IVDs retain notochordal cells through adulthood[68], which is contrary to human IVDs. Lastly, the study only cultured the disc for 30 hours, which is not a sufficient length of time for testing cell viability since it is known that discs remain viable in an unloaded environment for up to 7 days[69]. For these reasons it is believed that the present study provides a significant step forward than the previously published whole organ IVD culturing system that preserved the adjacent vertebral bone during culture.

Bovine caudal discs were used for their availability and similarity to human discs. They are similar in diameter and height to human discs and are inexpensive and readily available [52]. They have similar creep and stiffness as human discs [21, 22], and have the same nutrient diffusion processes [4], making them suitable for organ culture.

The cell viability in the nucleus pulposus after the two-week loading cycle in the dynamic bioreactor was 43%, which was lower than other axially loaded dynamic systems have reported [48], but higher than the values reported after torsional loading [51] bioreactor protocols. It has been shown that medium and high loads on the IVD induce cell death [52]; however, since the

cell viability in this study was significantly higher than those reported under high or medium loads, it can be reasonably concluded that the load applied to the disc was not excessively high.

The lower cell viability was potentially exacerbated by the aluminum fixtures in the culture container. Aluminum is not highly biocompatible, meaning prolonged contact with culture medium may lead to contamination and cell death [70]. To mitigate these effects, the culture medium was replaced and the entire system was cleaned and re-sterilized every three days. However, there was an unknown buildup in the vent of the container that sealed it off sometime between days 12 and 14 of the testing cycle. This would have prevented gas exchange in the system, likely contributing to cell death.

Lower cell viability in the annulus fibrosus may have resulted from excessive incubation in propidium iodide, which can be toxic to cells after an extended period of time [66]. Long incubations were used in this study to allow time for the dye to diffuse through the extracellular matrix and reach the nucleus pulposus. Shorter incubation times could yield higher cell viability, but could also inhibit the dye from reaching all the cells in the IVD. It is also possible that the dissection or surface sterilization procedures contributed to the low viability of cells in the annulus fibrosus before discs were placed in culture. Failure to completely remove the bony endplate around the annulus fibrosus could have resulted in less nutrient flow into the disc in that area and lower cell viability than the nucleus pulposus, which was contrary to previous work [48]. Future efforts will be made to optimize these procedures.

The average cell density was much higher than in humans. The average human IVD has a cell density of roughly 6000 cells/mm^3 [13], which is less than 1/6 of what was found in the bovine discs. Cell density is often significantly higher in animal models than in human IVDs, however, so this was not unexpected[22, 71, 72].

The strengths of this dynamic bioreactor are that it is able to achieve complex physiologic loading of the disc with the endplates and vertebral bodies intact. It used bovine caudal discs, which are similar to human IVDs; this provides a way to study and test spinal devices and therapies on bovine discs and relate the data to human discs. The limitation of this study is the fairly low cell viability at the conclusion of the 14-day test.

In summary, the complex load dynamic bioreactor described here yielded a cell viability of 20% in the annulus fibrosus and 43% in the nucleus pulposus. Future optimization and testing could improve these results. Specifically, manufacturing the culture container out of titanium rather than aluminum and optimizing the dissection and staining techniques could improve the viability of the cells.

The current system was a successful design that made large improvements over the static control specimens. The IVD isolation and preparation with intact endplates and vertebral bodies has proven to be a successful process. This physiologically loading dynamic bioreactor has the potential to achieve high cell viability for long periods of time, aiding in pre-clinical testing for both spine spinal devices and IVD therapies.

3.5 Conclusion

This study described the design and validation of a complex loading dynamic bioreactor that is, to the author's knowledge, the first whole organ IVD bioreactor to load an IVD in FE, LB, and CC while maintaining cartilaginous endplates and vertebral bodies. The combination of complex physiologic loading and preserving of cartilaginous endplates and vertebral bodies provides increased utility in the context of preclinical testing of vertebral mounted spinal devices.

4 DISCUSSION

4.1 Bioreactor Design Optimization

A large amount of work went into designing and creating a complex loading dynamic bioreactor that fulfills all the requirements for cell culturing a whole FSU. This section will go through the design process of arriving at the final design.

4.1.1 Functional Specification

The functional specifications for a complex loading dynamic bioreactor were briefly outlined in Table 3-1, however, it is helpful to go through the requirements in more depth.

The main purpose of a dynamic bioreactor is to facilitate cell proliferation and viability while preserving the adjacent vertebral bone. This is done through complex loading of the disc in a reservoir of culture medium. Refer to Appendix A for a detailed protocol of the preparation and staining of the spinal segment for culture. The IVD loading initiates large nutrient transportation into the disc, aiding in high cell viability. The most important requirements for the bioreactor relate to the loading of the disc. The following loading requirements are based on cervical, thoracic, and lumbar IVDs:

- Able to load a bovine tail in FE, LB, and CC
- Achieve loads of 75 N – 1000 N [28-30] and 2-10 N-m [25, 26]
- Able to run for extended periods of time (2+ weeks)

- Load IVD at 0.1-0.2 Hz [51]
- Motion of at least ±2-6° in LB [24-26], and ±3-15° in FE [25, 26]

Cervical IVDs experience the largest range of motion, but the smallest *in-vivo* loads, while lumbar IVDs experience a smaller range of motion but larger loads. Thoracic IVD motion and load values are between cervical and lumbar values. Because different areas of the spine experience different motion and loading, the functional specifications for the entire spine span a large range.

Additional functional requirements relate to cell culture and the health of the disc:

- Hold 30-80 mL of culture medium without leaking
- Hold the FSU and keep it in a vertical position
- Keep the disc submerged in culture medium
- Be able to fit inside an incubator
- Gas permeability for pH control and oxygen for the cells in the disc
- Ability to not get contaminated from the air
- Autoclavable culture container for sterilization (withstand 249° F [121° C] for at least 30 minutes)

The remaining functional requirements are for ease of use:

- Be self-contained
- Allow for easy changing of culture medium
- Simple to set up
- Be powered from a wall outlet

These requirements guided the concept generation stage of the design process.

4.1.2 Concept Generation and Selection

The concept generation phase of the design process had two major considerations: 1) loading mechanism, and 2) culture container. The loading mechanism design was straightforward, while the culture container design required several iterations based on unanticipated challenges.

4.1.2.1 Loading Mechanism

The loading mechanism was required to load the FSU in FE, LB, and CC while staying as simple and minimalistic as possible. The concept for being able to load in all three modes of loading was a triangular platform; a simplified version of a Stuart Platform. The design required two triangular plates attached at each corner by a linear motor. The spine would rest in between the two plates for loading.

The first concept was to use threaded rods attached to motors to induce loading (Figure 4-1). As the motors spin, the threads spin also, drawing the two plates together.

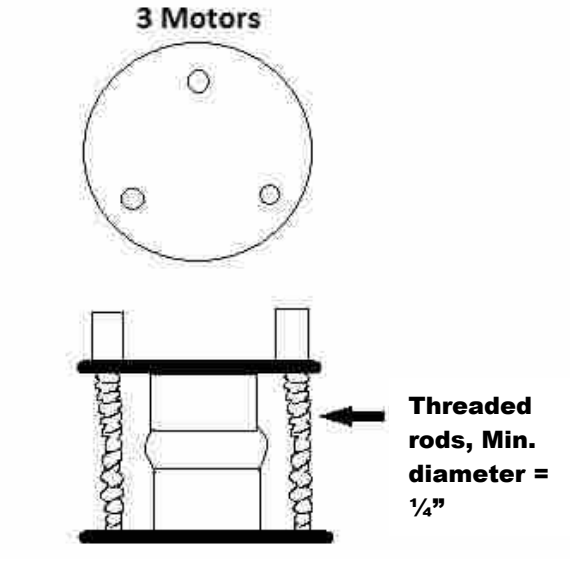

Figure 4-1: Sketch of initial design of bioreactor with threaded motors.

The other concept generated was to use pneumatic linear actuators. The two plates would allow for FE and LB either with very loose tolerances (Figure 4-2) or with ball joints attached to the bottom plate to allow for movement.

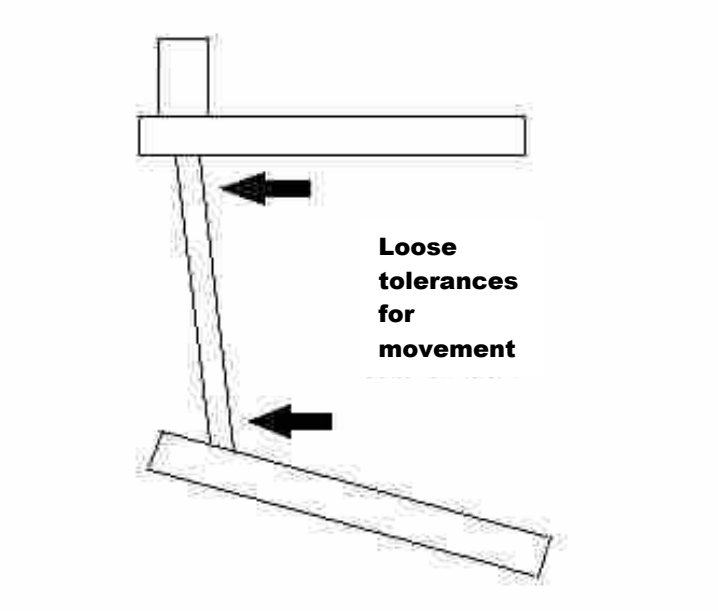

Figure 4-2: Sketch of loose tolerances.

Out of the designs described, the chosen design was using linear actuators with ball joints. This was the simpler and more reliable method to achieve all the modes of loading. Once the loading mechanism was conceptualized, the culture container concept design was initiated.

4.1.2.2 Culture Container

The culture container had significantly more functional requirements than the loading mechanism, and thus far more concepts were generated.

One concept included a silicone sheet secured to the top and bottom aluminum plates of the loading mechanism with O-ring seals pinched by aluminum rounds. Culture medium would flow in through the top aluminum plate and leave out the bottom plate (Figure 4-3). In this

design the FSU is held in place with L-brackets screwed to the bottom plate and into the vertebrae.

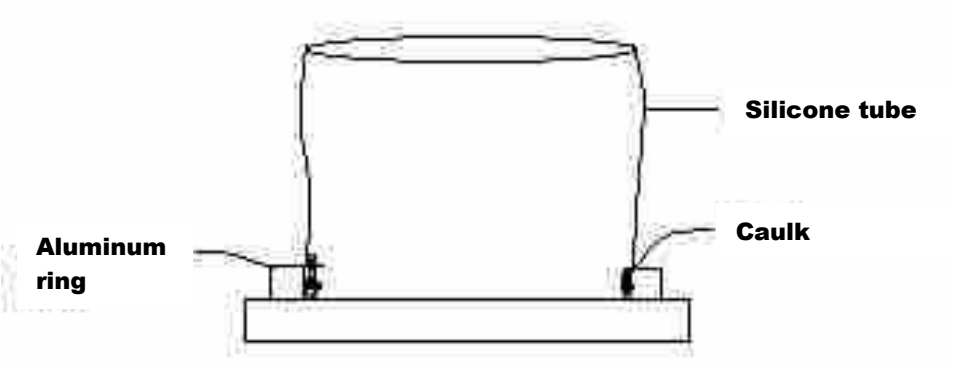

Figure 4-3: Culture container design with O-ring seal.

An additional concept generated was a beaker filled with culture medium that the FSU would be submerged in. Refer to Figure 4-4. The FSU would be secured using spiked L-brackets so that screwing holes into the vertebrae would not be necessary. The top plate would apply a load to the spinal segment, and the culture medium would be open to the air, so no silicone would be used.

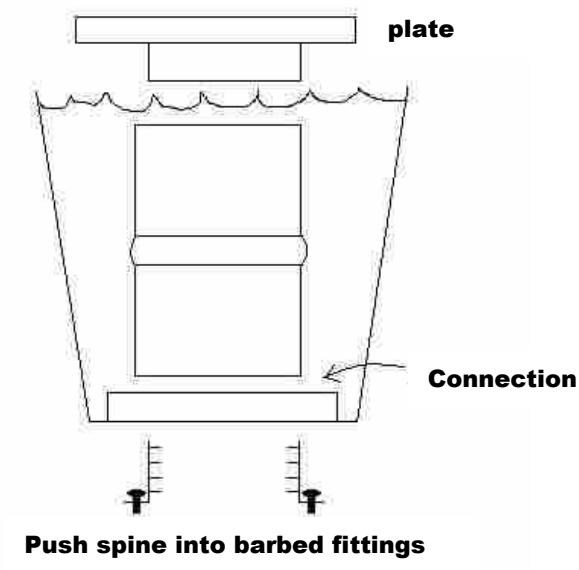

Figure 4-4: Beaker container design open to the air.

The next conceptual design was a silicone tube, sealed at the bottom to hold the culture medium. The FSU would be inside the tube, and the tube would be resting inside a beaker. Silicone rubber stoppers would be used to wedge the FSU in place, and the top of the silicone tube would be twisted over on itself to allow for gas to enter the container without contamination (Figure 4-5).

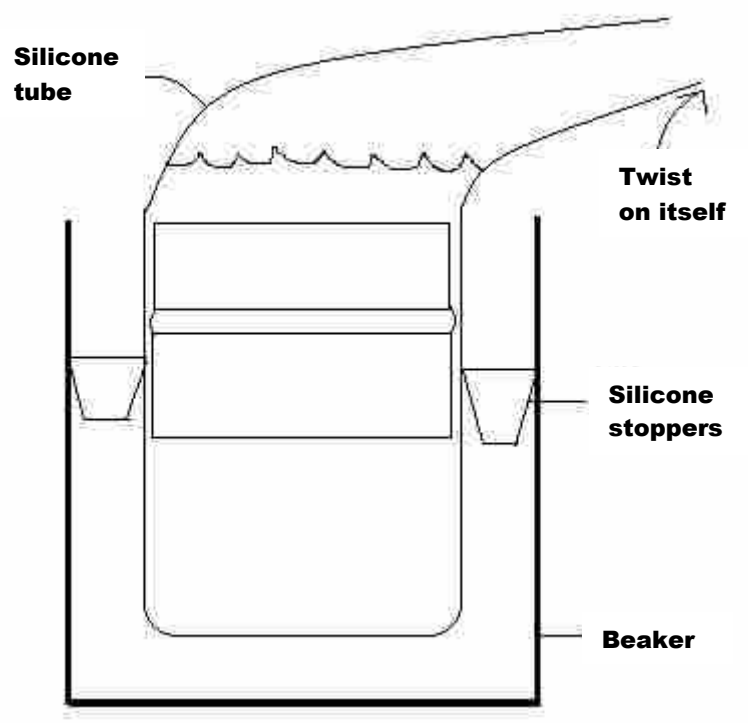

Figure 4-5: Concept design of culture container using a beaker and silicone stoppers to hold the FSU in place.

Another version of the same design included sealing the top of the silicone tube with a CamelBak bladder clip (Figure 4-6), and inserting a Gore valve to the silicone tube. Gore valves allow for air to pass through, but not liquid, so this would allow for gas permeability to the container without leaking or contamination. The FSU would be secured with ¼-20 thumb screws

entering through the sides of the beaker and tightening on to the bottom vertebra (**Figure 4-7**). The screws would have silicone on the tips, so as not to puncture the silicone container.

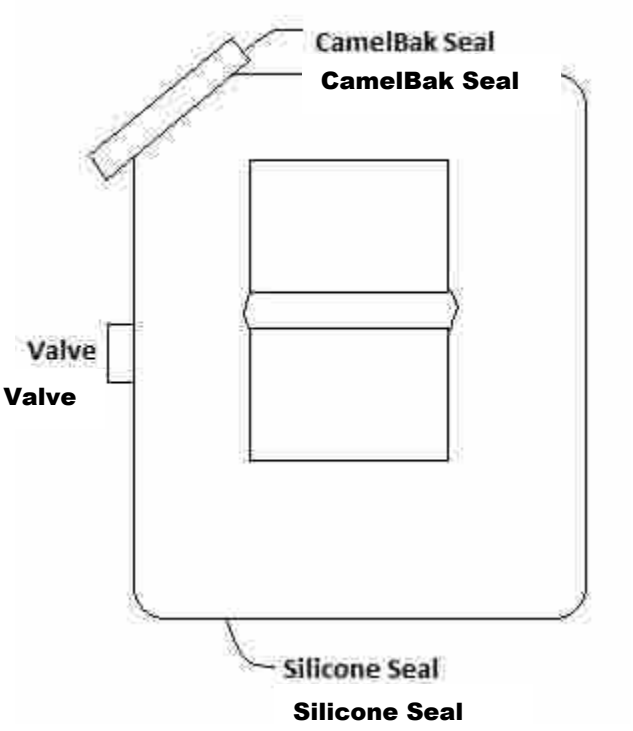

Figure 4-6: Sketch of CamelBak clip concept.

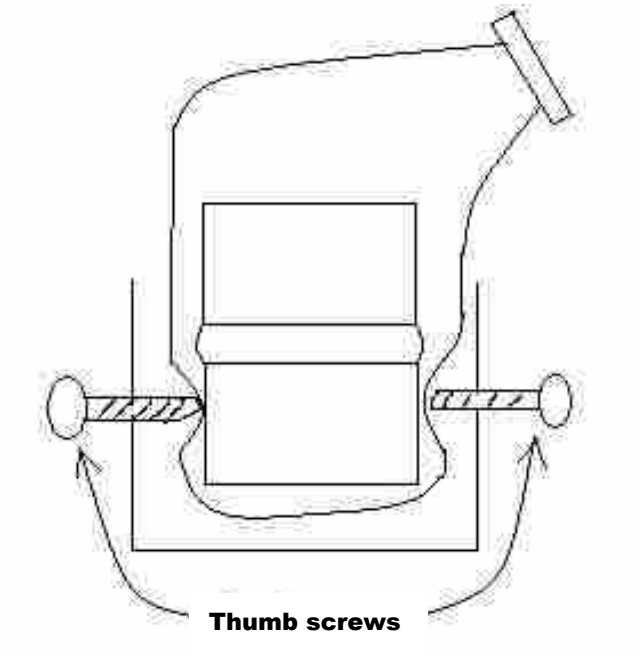

Figure 4-7: Sketch of thumb screws used to hold the FSU in place.

Another concept implemented reinforcement of the silicone bag with fiber-reinforced adhesive tape. The seal at the top of the bag was a slanted seal where the silicone was pinched between two pieces of plastic, and a vent would be protruding from the top for gas permeability (Figure 4-8). For this design, risers would be used to transfer the load from the bottom plate to the FSU.

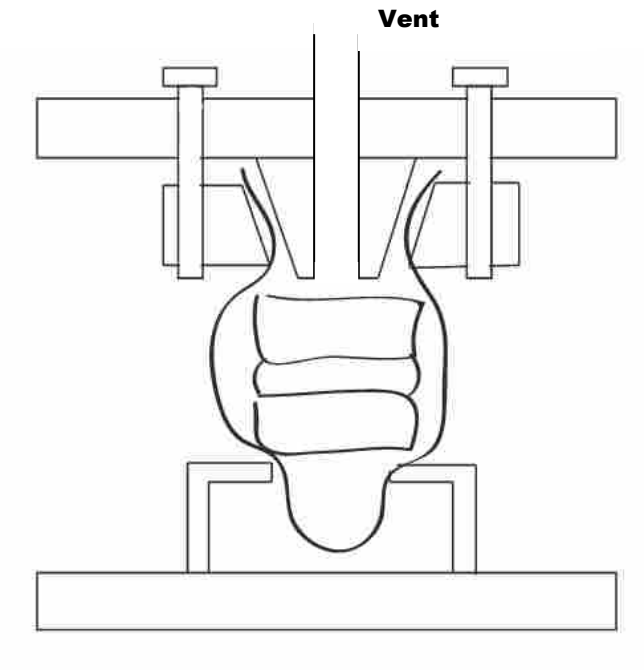

Figure 4-8: Sketch of the slanted plastic sealing mechanism.

The last concept design generated was a cylindrical container with silicone sides and aluminum top and bottom. The top was a lid that could screw shut and sealed with an O-ring, and the silicone sides would attach to the lid and bottom with silicone caulk and hose clamps. A vent would protrude out the side of the lid for gas permeability (
Figure 4-9 and Figure 4-10). Vertical rods would be attached to the bottom plate to keep the FSU centered.

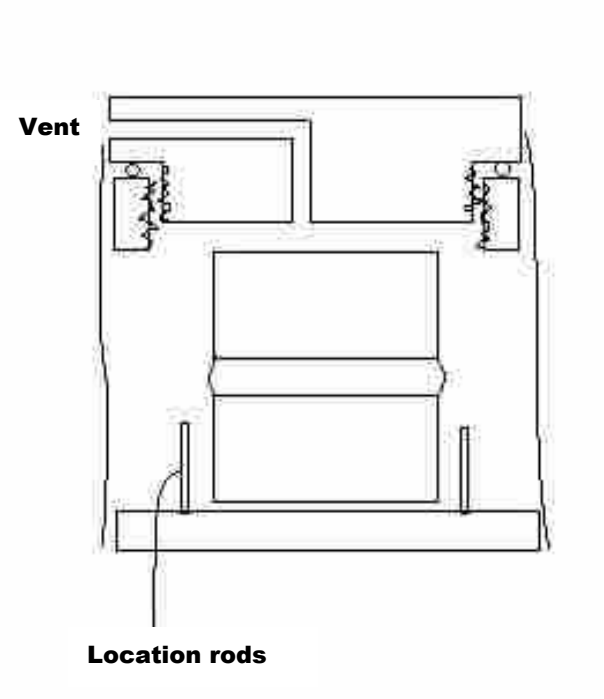

Figure 4-9: Concept sketch of container design with aluminum lid.

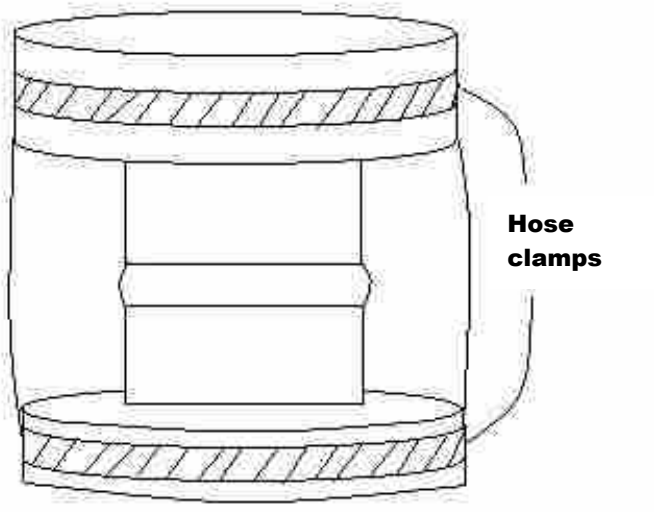

Figure 4-10: Concept sketch of container design with aluminum lid.

Once the concepts were designed and solidified, the prototyping phase was entered. Out of the 6 designs conceptualized, 4 were chosen and manufactured.

The first concept (with aluminum rings and O-ring seals) was disregarded because of the contact with the loading mechanism. The culture container was not self-contained, but rather the spine and culture medium had open contact with the top plate of the loading mechanism. Because of the electrical components that needed to be attached to the loading mechanism, autoclaving was not possible and the chance for contamination was high.

The second concept described was disregarded because of the possibility of contamination. The design of this container had the FSU and culture medium open to the air, which increased the risk of contamination largely. For this reason, it was not moved on to the prototype phase as the other concepts were.

4.1.3 Prototyping and Testing

The chosen designs were prototyped and tested for final design selection. The manufacturing of the loading mechanism was done with both a CNC Mill and a manually operated milling machine. After testing the loads, angles, and torques, the design was deemed sufficient for use in the final product.

The culture container prototypes were all tested with the loading mechanism using a bovine tail FSU and water in place of the culture medium.

The first culture container design prototyped was the silicone tube that was twisted at the top to allow for gas permeability (Figure 4-11). During testing of this design the silicone stoppers were unable to keep the FSU suspended from the bottom, resulting in tipping over of the FSU within minutes of the start of the test. The top of the silicone tube allowed water to escape when the FSU was being loaded, and ultimately would have led to contamination if tested with culture medium.

Figure 4-11: Semi-sealed container design with silicone stoppers.

The CamelBak seal and locating screws design was manufactured and tested with Gore valves (Figure 4-12 and Figure 4-13). When the FSU was loaded, the Gore valves became impinged between the top plate of the loading mechanism and the beaker, which resulted in fracture of the beaker within 24 hours of starting the test (Figure 4-15).

The culture container design implementing the slanted seal with a vent out the top was manufactured (Figure 4-16 and Figure 4-17), and during testing was performing very well. Part of the slanted seal was manufactured with aluminum, rather than plastic, fur durability (Figure 4-18). This container was successful in testing for up to three days, but ultimately the reinforced silicone tube wore through causing catastrophic leaking.

Figure 4-12: CamelBak bladder clip used to seal the silicone bag.

Figure 4-13: Thumb screw setup for securing the FSU.

Figure 4-14: Gore valve causing the acrylic beaker to break.

Figure 4-15: Shattered acrylic from the Gore valve.

Figure 4-16: Image of the slanted seal design with vent out the top.

Figure 4-17: The silicone tube reinforced with fiber-reinforced adhesive tape.

Figure 4-18: An aluminum version of the slanted seal with the vent protruding from the top.

The final concept put into the prototype phase was the cylinder with an aluminum lid and silicone sides (Figure 4-19 through Figure 4-22). During the testing of this device, the liquid was able to leak out through an insufficient silicone caulk seal. When the seal was re-made, this container was able to perform well during a 10-day test with no complications or failures. Because the top and bottom of the container were rigid and horizontal, there were no issues with the FSU tipping over or staying centered during loading, thus the locating rods from the concept design were removed.

Figure 4-19: Final culture container concept bottom piece.

Figure 4-20: Final culture container design bottom piece.

Figure 4-21: Final culture container design lid with vent.

Figure 4-22: Final culture container design prototype.

At the conclusion of the prototyping and testing phases, the final selection was made and subsequent validation testing was conducted.

4.1.4 Final Selection and Validation Testing

The design chosen for use in the final testing phase was the last design described above, which uses an aluminum plate for the base, with flexible silicone sides, and an aluminum lid that can screw closed. This design was selected because of its superior performance during testing; it was the only prototype able to meet all the functional specifications.

To verify that the culture container and loading mechanism could meet all the functional requirements in tandem, a 14-day test was conducted using culture medium and a fresh bovine caudal FSU. Two static control FSUs were used for comparison purposes. At the end of the 14 days, all three specimens were examined for cell viability, and the dynamic bioreactor was able to keep 20-43% of the cells alive.

The final two-week test was a validation that the culture container and loading mechanism was able to meet the requirements necessary to a complex loading dynamic bioreactor.

4.2 Design of Pneumatic System

The ideal design of the pneumatic system consisted of high pressure air from the wall splitting three ways and going to three automated pressure regulator valves which control the three pneumatic actuators. This would allow variable pressure supply to the actuators to achieve the most ideal physiologic loading. Due to budget constraints, this was not an option.

The valves chosen were simple solenoids that changed the direction of air flow. Because high pressure (for dynamic loading) and a low pressure (for the compressive load representing head/torso weight) were desired, pressure regulators were necessary. After much deliberation and discussion, we were able to arrive at a simple design, as seen in Figure 4-23. The compressed air from the wall or from an air compressor would immediately split through a y-

connection. Pressure from both directions would go through pressure regulators. One regulator would be set to high pressure and one regulator would be set to low pressure. Both of these are attached to one of the valves, where one of the outlets of the valves has a plug on it. This allows high pressure to go through when the valve is switched one direction, and low pressure to go through when it is switched the other direction. The outlet of the first valve goes to a three-way manifold which outputs to three more valves. These valves control whether the air extends or compresses the pneumatic cylinders. This system allows for two distinct air pressures to go to the pneumatic cylinders for high and low pressure loading and fulfills its purpose.

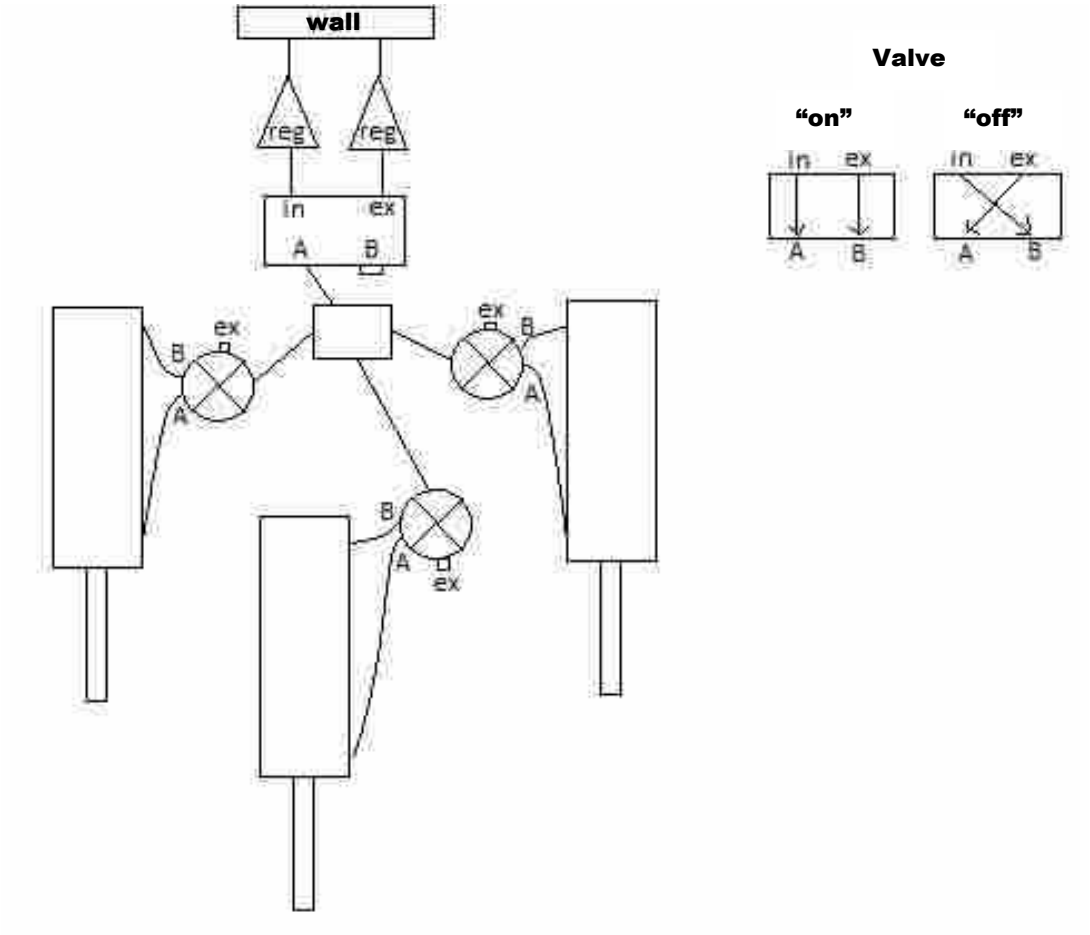

Figure 4-23: Pneumatic system design.

The pneumatic cylinders chosen were Norgren RP series cylinders with 1 1/16" bore size, 1 ½" stroke, and a double acting end mount. The compressive loads achievable by the pneumatic cylinders were calculated for various input psi using the cylinder bore size and verified using a load cell placed in the center of the loading mechanism where the culture container was subsequently placed. The calculation to determine the compressive load was done by multiplying the psi input by the combined piston area for the three cylinders. Refer to Appendix B for graphs of the verification output loads of the dynamic bioreactor for numerous input pressures. 100 N of pure compression was desired to imitate cervical spine *in-vivo* values. 100 N of compression in the center of the loading mechanism was achieved with roughly 8 psi of compressed air distributed to each pneumatic cylinder. A total of 100 N of compression indicates ~33 N of force from each cylinder since there are three cylinders. The applied torque for FE and LB was calculated by taking the force of the cylinders used to achieve that load, and multiplying it by the distance to the center of where the spinal segment was. For example, in right LB, only one cylinder is putting a load on the spine, so right LB torque is calculated by taking 33 N and multiplying by the lateral distance to the center of the spine, which is ~0.06 m. This yields a right LB torque of 1.9 N-m. Similar calculations were carried out for the remaining torques.

Because the 14-day test was conducted in a building that does not have high pressure air from the wall in the cell culture room, an air compressor was necessary. The air compressor had to be large enough to handle constantly outputting at least 10 psi continuously for 2 weeks. The compressor used (Figure 4-24) was able to handle the loads necessary for a 14 day validation test.

Figure 4-24: Air compressor used to supply air to the system.

A feedback loop in this system would have been able to verify the output forces of the system. In a clinical bioreactor this would likely be necessary; however, for the purpose of this research the forces have proven to be accurate enough (Appendix B) without a feedback loop. The system is simple enough that a feedback loop can be avoided.

4.3 Programming of Bioreactor

The complex loading dynamic bioreactor was powered by an Arduino Uno. The electrical components were initially connected using a bread board (Figure 4-25), but later the joints were soldered to prevent disconnection of the valves or the power supply.

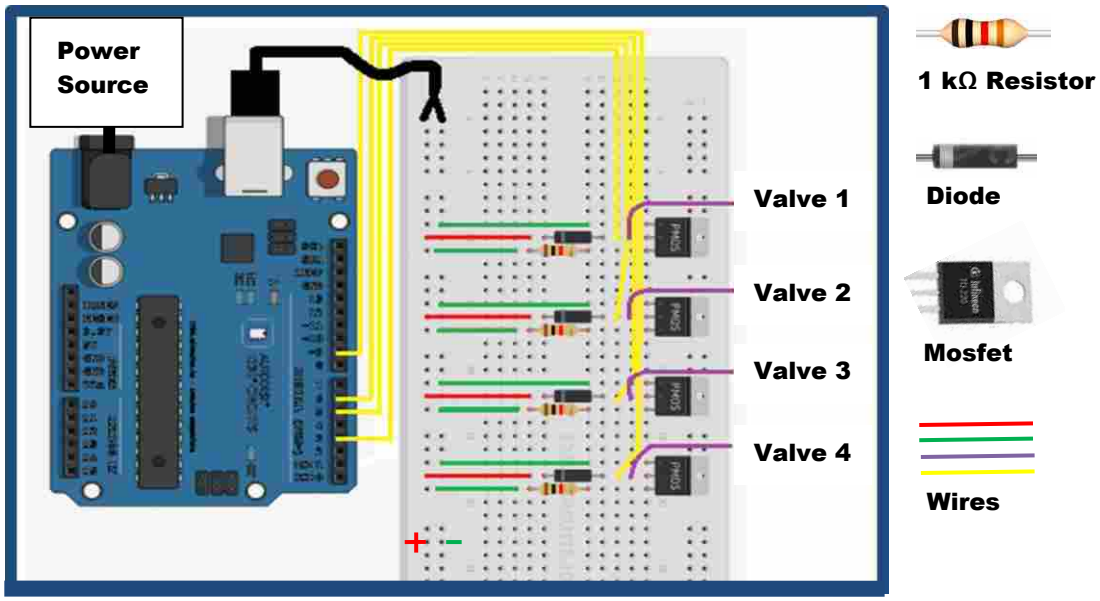

Figure 4-25: Electrical components and breadboard.

The bioreactor was programmed with a dynamic loading frequency of 0.2 Hz (Appendix C). This was suggested by Chan et al as being "the best [frequency] in preserving disc metabolism" [51]. The cycle time (16 hours) and rest time (8 hours) were implemented to closely resemble physiologic conditions. The method of combining of FE, LB and CC is a modified ISO 18192-1:2011 [44] standard that is used for testing implants for surgery, and deemed appropriate for this study.

Figure 4-26 represents the layout of the three pneumatic cylinders. FE, LB, and CC were accomplished using the following combinations:

- Flexion: cylinder 1 compressed, cylinders 2 and 3 extended
- Extension: cylinder 1 extended, cylinders 2 and 3 compressed
- Left lateral bending: cylinder 2 compressed, cylinders 1 and 3 extended
- Right lateral bending: cylinder 3 compressed, cylinders 1 and 2 extended
- Compression: cylinders 1, 2, and 3 compressed

- No load: cylinders 1, 2, and 3 extended

Because FE and LB torques are induced by separate compression and extension of actuators, the pivot point of the bottom plate doesn't occur at one of the ball joints, but rather centered between two sets of ball joints. The FE pivot point is in the center of the bottom plate, while the LB pivot point is in between cylinders 1 and 2.

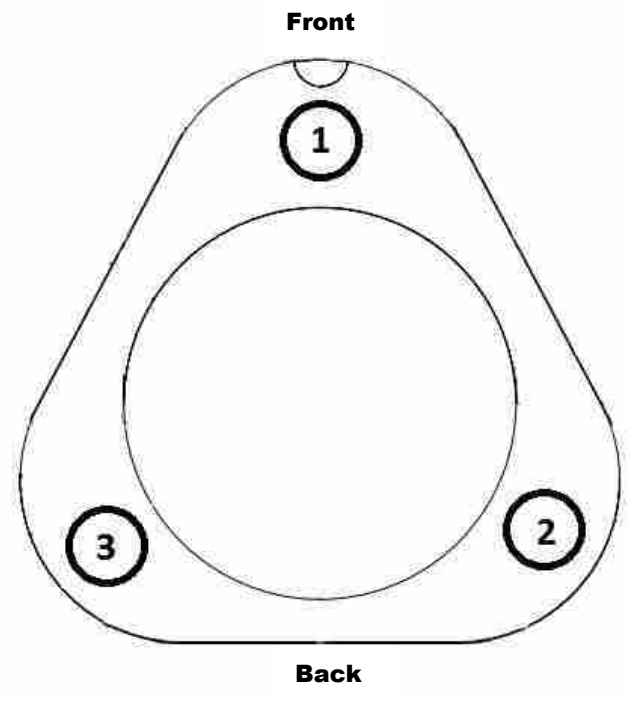

Figure 4-26: Layout of the three pneumatic cylinders.

A case for the Arduino Uno was 3-D printed to protect all the soldered joints (Figure 4-27).

Figure 4-27: 3-D printed Arduino Uno case.

This final bioreactor design was effective in long-term tests with no leaking and no unplugging of electrical components. It was also easily autoclaved for sterilization purposes.

5 CONCLUSIONS AND FUTURE WORKS

5.1 Contributions

The whole FSU complex loading dynamic bioreactor described in this thesis is the first to be developed to my knowledge. It is the first organ culture to retain the vertebral bodies attached to the IVD while inducing FE, LB, and CC on the disc.

The work in Chapter 3 is prepared for publication and will shortly be submitted to the Journal of Biomechanical Engineering. An initial prototype of the bioreactor has been presented at the Emerging Ideas in Biomedical Engineering conference—a biomedical engineering research conference held at Brigham Young University—and received the 1st place honor.

This dynamic bioreactor has potential to help scientists develop new treatments for disc degeneration and back pain. With a bioreactor as described here, living discs with vertebrae attached can be tested for extended periods of time, and the results could more closely resemble *in-vivo* results than previous organ culture systems. With minor revisions, this dynamic bioreactor could allow progression of disc therapies and spinal devices that could potentially reduce instances of back pain.

5.2 Future Work

The current study was the development of a physiologic loading dynamic bioreactor. In verifying that the product worked properly, one caudal bovine IVD was tested for two weeks

inside the dynamic bioreactor and cell viability was then compared to caudal bovine IVDs placed in static bioreactors. Based on our results, we identified several paths for follow-on work that could further refine the bioreactor design.

The described bioreactor was constructed from 6061 Aluminum alloy, which provided several advantages in terms of machinability and cost. However, aluminum ions are known to have detrimental effects on some biological tissues. Titanium exhibits a higher biocompatibility that may increase the viability of the cells, while still meeting the functional requirements for mechanical properties[70]. In addition, specific research and testing should be conducted on culture medium contamination explanations and the effects of metal ions on intervertebral disc cell function and longevity.

The goal of the present work was to design, build, test, and validate the mechanical performance of the segmental bioreactor. Thus, only a single extended FSU test was conducted using a bovine tail segment. However, in order to evaluate the effects of the mechanical environment on cell viability with statistical significance, it will be necessary to utilize the bioreactor to measure cell viability on a larger sample group under multiple loading paradigms. Likely this testing will include multiple bioreactors acting simultaneously. It would also be beneficial to test cell viability at various stages during the test (e.g. at 5 days and 10 days).

To help improve cell viability, it would be beneficial to develop a new dissection protocol that ensures the vertebral bone is hollowed out down to the thin cartilage endplate rather than hollowing it out by look and feel. The transition from cancellous bone to bony endplate to cartilage endplate in a spinal segment is very smooth and uniform. When hollowing out the vertebral body with an end mill this makes it difficult to determine whether the bony endplate or cartilaginous endplate is exposed. The goal was to hollow it out down to the cartilage endplate,

which is very thin, without breaking through and damaging the disc. The bony endplate is thicker and makes nutrient transport to the disc more difficult. Because of the difficulty in determining how for to mill out the bone, there is a possibility that excess bony endplate around the annulus fibrosus caused a decrease in cell viability. Additionally, the endplate has a slight curvature to it, make the transition from bony endplate to cartilage endplate even more difficult to identify when working from the top of the bone with limited visibility. Previous studies have reported higher cell viability in the annulus than the nucleus of the disc [48], which is contrary to the results of this bioreactor. This supports the theory that the bony endplate was not fully removed. Developing a new protocol to ensure removal of the bony endplate could improve the viability of the disc.

It would also be extremely interesting and valuable to monitor the degree to which nutrient transport in the intervertebral disc is enhanced by the mechanical motion. There has been considerable controversy in the literature regarding the relative importance of bulk fluid flow versus diffusion in providing cellular nutrition. A follow-on study utilizing a colored dye of the same approximate molecular weight as glucose and oxygen molecules could potentially resolve this controversy. Similarly, more work needs to be done with regards to the influence of extended time in a bioreactor on the mechanical properties of the intervertebral disc.

There were several potential design improvements that became of interest based on observation of the function of the final bioreactor prototype. Using electric actuators in the future could provide benefit to the bioreactor by eliminating the need for an air compressor and allowing for variable output forces. This would allow testing to be done with multiple loading configurations so load magnitudes could be optimized for cell viability. Electric linear actuators would also allow the system to achieve exactly the torques desired in conjunction with the exact

compression force desired without the need to change dimensions or locations of the linear actuators. Another design improvement would be to adapt the media container to support a continuous fluid flow through an inlet and outlet in the container. This would allow for a larger pot of culture medium which could supply more nutrients to the disc. It would also allow for elimination of the vent and HEPA filter on the container, which possibly contributed to the contamination. Overall, having a flow of culture medium into and out of the culture container would likely improve the cell viability of the IVD.

In conclusion, the dynamic bioreactor presented here was successful at loading the IVD in physiologic loading conditions for an extended period of time. This dynamic bioreactor is a first generation testing mechanism, and a considerable amount of optimization remains to be done. However, the bioreactor design shows considerable promise. Not only was it able to provide a physiologic loading regimen, it was able to do so unattended for a considerable length of time (2 weeks) comprising approximately 30,000 cycles of complex loading (flexion-extension-compression-lateral bending-rest). It was able to achieve higher cell viability than the static controls it was compared to, while preserving the adjacent vertebral bodies for attachment of medical implants for preclinical testing.

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APPENDIX A. PROCEDURE FOR CULTURING INTERVERTEBRAL DISC TISSUE

The following sections outline the procedure for procuring discs, preparing them for culture statically or in the dynamic bioreactor, preparing the culture medium, and LIVE/DEAD® cell staining.

A.1 FSU Retrieval

Materials:

PBS-Solution
Dissection Mats
Latex/Nitrile Gloves Safety Glasses
Face-Shield
Dissection Gown &booties
Band saw or sawzall
Dremel® w/ wheel blades
Scalpels: handles & blades

Immediately following the time of donor "death" (TOD), and obtaining the cervical spine

> *Note: Time of death may refer to the time that the animal/donor dies, or the time that a tissue sample was removed from a living donor. However, this must be exclusive for all samples in the test group (i.e., all the tissue samples must come from living donors, or all the samples must come from a donor following death) in order to maintain consistency in the supporting environment of the cell/tissue.*

1. In lab notebook, mark the TOD of animal.
2. Thoroughly wet the entire cervical spine with PBS solution.
3. Locate the IVD of interest using palpation techniques.

4. Using a saw (band saw, sawzall, or Dremel® w/ wheel blade), make a mid-transverse cut through the superior and inferior vertebra, adjacent to the IVD of interest, and remove the vertebra-IVD-vertebra section from the rest of the tissue.
5. Use the band saw or scalpel to remove any peripheral soft-tissue that may contribute stiffness to the IVD joint.

The FSU is now clean and ready for Flexibility Studies or see additional prep for culture and/or staining.

A.2 IVD Preparation for Static Culture or LIVE/DEAD® Staining

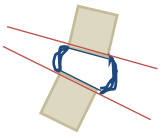
Materials:

Latex/Nitrile Gloves Safety Glasses
Face-Shield
Dissection Gown &booties
Band saw or sawzall
Dremel® w/ wheel blades and end mill bit
Scalpels: handles & blades
Beaker (2)
DMEM
Pen/Strep
Forceps
Flame

1. Using a saw (band saw, sawzall, or Dremel ® w/ wheel blade), remove posterior vertebral bone.
2. Place the IVD in a beaker with a solution of DMEM+Pen/Strep (antiseptic) and transport to a clean facility.
3. Drop the IVD into a small beaker containing ethanol and retrieve using the long forceps.
4. Transfer the IVD into the large, laminar flow tissue culture hood.
5. Tap off the excess ethanol before passing the disc over a flame (with long forceps) to burn off all impurities and contaminants.

The disc is now clean and must not leave the sterile zone (i.e. laminar flow tissue culture hood)

6. Clean off all musculature and bony endplate whilst in the hood using a sterile scalpel.
    a. Make a transverse cut through the superior bony vertebral endplate.
        i. Using a various sterilized cutting tools (e.g., scalpel, Dremel® with end mill bit), remove as much of the bony endplate as safely possible leaving only the cartilage endplate. **



b. Make a transverse cut through the inferior bony vertebral endplate.
    i. Using a various sterilized cutting tools (e.g., scalpel, Dremel® with end mill bit), remove as much of the bony endplate as safely possible leaving only the cartilage endplate.**

7. Use sterile saline to rinse off shavings/debris from cuts.
8. **The disc is now ready for culture or staining**
    a. **For static culture…** Place the disc into the IVD culture medium and into 5% CO_2 and 37° C incubator.
    b. **For LIVE/DEAD® staining…**Place the disc into the LIVE/DEAD® stain solution.

*****It is advised that the cartilage endplate be left intact as much as possible to discourage swelling of the disc***

******All lids and openings of the static culture container must be tightly closed while transported out of the laminar flow tissue culture hood, once within the incubator lids must be loosened to allow for flow of Co_2 into the container.***

9. Change the medium in the static culture container approximately every 3 days to continue optimal nutrient flow through the disc.

A.3 IVD Preparation for Dynamic Bioreactor Culture

Materials:

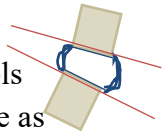
Latex/Nitrile Gloves
Safety Glasses
Face-Shield
Dissection Gown &booties
Band saw or sawzall
Dremel® w/ wheel blades and end mill bit
Scalpels: handles & blades
Beaker (2)
DMEM
Pen/Strep
Forceps
Flame
PBS

1. Using a saw (band saw, sawzall, or Dremel ® w/ wheel blade), remove posterior vertebral bone.

2. Place the IVD in a beaker with a solution of DMEM+Pen/Strep (antiseptic) and transport to a clean facility.
3. Drop the IVD into a small beaker containing ethanol and retrieve using the long forceps.
4. Transfer the IVD into the large, laminar flow tissue culture hood.
5. Tap off the excess ethanol before passing the disc over a flame (with long forceps) to burn off all impurities and contaminants.

The disc is now clean and must not leave the laminar flow tissue culture hood.

10. Clean off all musculature whilst in the hood using a sterile scalpel.
11. Using the Dremel® End-mill tool (sterilized), remove the anterior and posterior vertebral bodies (caudal and cephalic). Using this access point
    a. Using the Dremel® End-mill tool (sterilized), or other various cutting tools (sterilized) (e.g., scalpel, Dremel®), remove as much of the bony endplate as safely possible**



It is advised that the cartilage endplate be left intact as much as possible to discourage swelling of the disc

12. /Using the PBS-solution (sterile) clean the IVD to remove any blood, and bone-fragments

****You should now have a whole, single IVD.***

13. Once cleaned, the disc can be placed onto the stand within the Dynamic Bioreactor and covered with culture medium.
14. Remove bioreactor from the laminar flow tissue culture hood and transport to a 5% $CO_2$and 37° C incubator.
15. Change the medium in the bioreactor approximately every 3 days to continue optimal nutrient flow through the disc.

A.4 Culture Medium Preparation

Ingredients

- 500 mL high-glucose DMEM (w/ 4500 mg Glucose/L + 0.584 g L-glutamine /L + 3.7 g NaHCO3 /L)
- 100 mL FBS (20%)
- 12.5 mL HEPES
- 12.5 uL ascorbate (L-ascorbic acid)
- 1.0 mL Pen/Strep
- 500 uL Gentamycin

- 3 mL Fungizone
- 55mg Sodium Pyruvate
- 1403 mg NaCl
- 12.5 mL Red Icterine Color Concentrate

Sterile Requirements

Note: It is important that the medium remain sterile throughout this process so the final product and various chemicals involved stay free from contaminants.

- Containers and instruments must be autoclaved before use and not opened until within the laminar flow tissue culture hood to keep sterile.
- All chemicals should be mixed within a sterile laminar flow tissue culture hood in the clean room
  - Anything exposed to air within the lab, or clean room is considered unsterile, so everything must be sprayed with 70% ethanol before brought into the laminar flow tissue culture hood.
  - The person mixing the medium must wear gloves that will be sterilized with ethanol before brought to work under the hood.
- Although it is considered clean within the laminar flow tissue culture hood, care must be taken in case contaminants are present, pipette tips must be replaced before using a different chemical, and again if it has accidentally touched anything during pipetting.
- The pipette-er must take great care not to touch the pipette tip to anything throughout the process to ensure the tip remain sterile
- If contact is made to potentially unsterile objects, change the pipette tip.
- Avoid the lips of containers when retrieving chemicals as that is the most likely place for contaminants to reside (It may help to tip bottles in order to access the chemicals without inserting the pipette fully into the container).

Mixing Procedure

Strictly observing the sterile requirements (above), mix all ingredients within the DMEM container

1. *Thaw FBS, Pen/Strep, Fungizone
   a. Place in zip-loc bags to avoid direct contact with water
   b. Submerge chemicals in a 37° incubation bath until the chemicals have thawed completely
2. Using the large (25 mL) pipette, transfer 100-mL FBS to DMEM
3. Using the large (10 mL)pipette, transfer 12.5 mL HEPES to DMEM
4. Using the 20 uL pipette, transfer 12.5 uLascorbate to DMEM
5. Using the 1000 uL pipette, transfer 1 mL pen/strep to DMEM

6. Using the 1000 uL pipette, transfer 500 uL gentamycin to DMEM
7. Using the 1000 uL pipette, or the larger (5 mL) pipette, transfer 3 mL fungizone to DMEM
8. Once all the sterile chemicals have been mixed into DMEM, remove container from the sterile laminar flow tissue culture hood to add the potentially unsterile chemicals to the solution
    a. Measure 55 mg of sodium pyruvate and 1403 mg NaCl on an accurate weight scale, using clean weigh boats and spatulas to transfer and weigh chemicals to avoid excess contaminants
    b. Add chemicals into DMEM outside of the laminar flow tissue culture hood and carefully swill the bottle to dissolve the powders
    c. Using the large (10 mL) pipette, transfer 12.5 mL of Red Icterine Color Concentrate to DMEM.
9. Sterilize the DMEM and filter package with ethanol before bringing into the laminar flow tissue culture hood
10. Assemble the filter and attach the vacuum pump under the laminar flow tissue culture hood (making sure all containers on the filter are tight to allow for a tight vacuum and fast filtration)
11. Pour the medium into the top container and turn on the vacuum pump from beneath the laminar flow tissue culture hood to begin filtration
12. Once the medium has been pulled through the filter into the container below, remove filter and screw on the sterile lid contained within the filter package

Parafilm the seam around the lid of the container and label the bottle BABEL with the date prepared before storing in the fridge until needed.

A.5 LIVE/DEAD® Cell Staining

Stain Ingredients:

30mL DMEM
6 µL Cell Tracker green
30 µL Propidium Iodide (Red)

Staining Procedure:

1. Spray the bioreactor and wipe off the excess ethanol with kimtech wipes before bringing under the sterile hood.

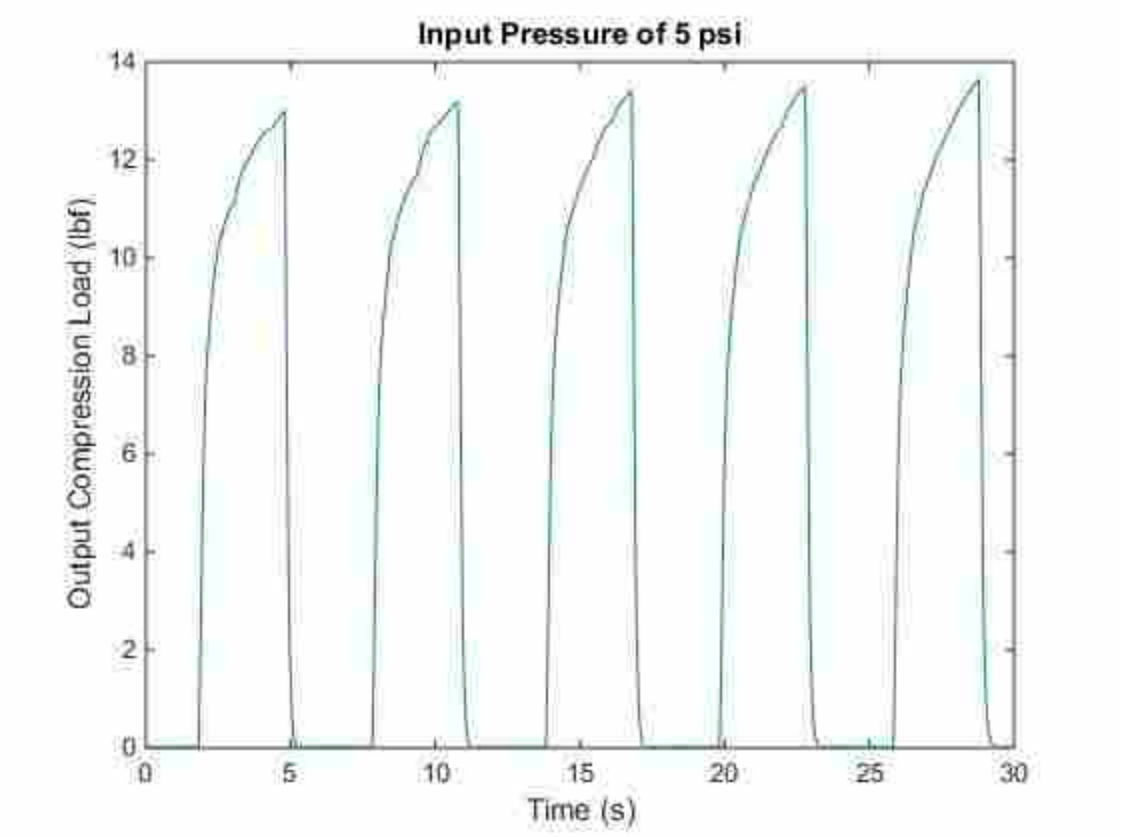
Tighten the lids of the bioreactor before removing from incubator

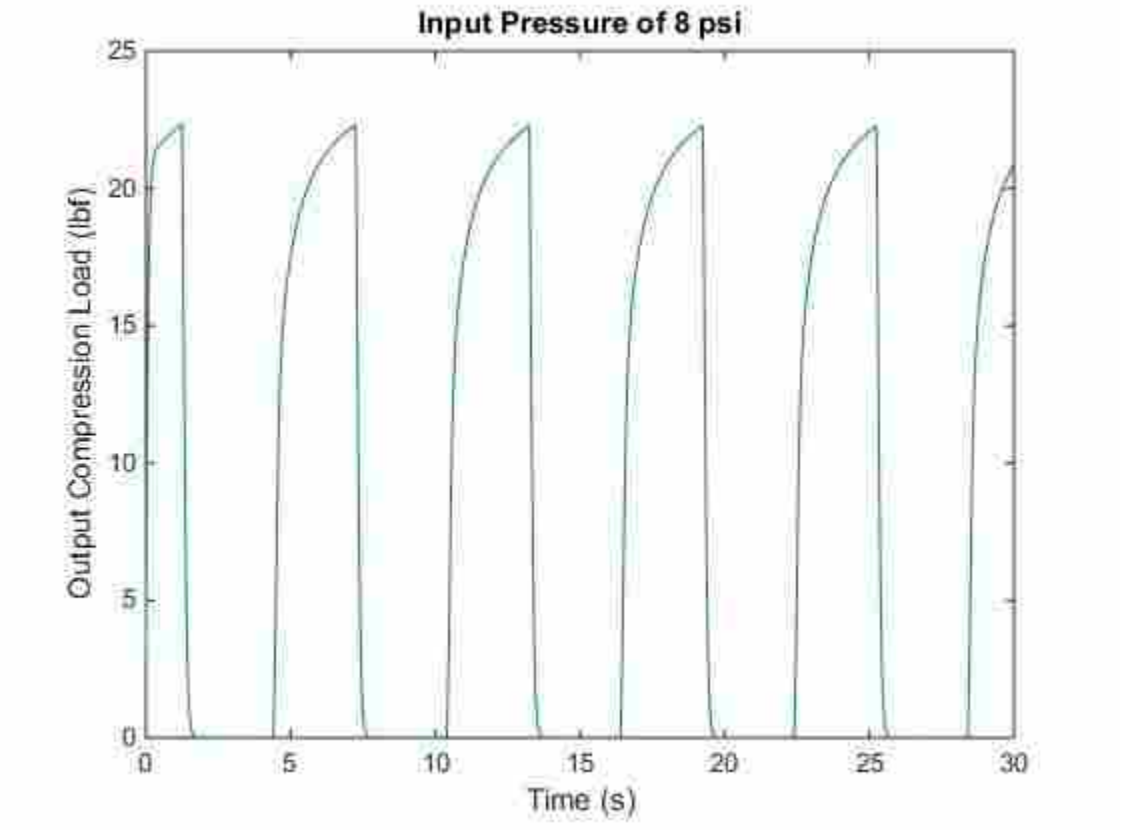
2. Under the hood, remove disc from the bioreactor using sterile forceps and transport into the beaker of dye previously prepared under the hood
3. Remove the beaker from the laminar flow tissue culture hood (at this point contamination is of no concern)
4. Pump the disc within the medium for 5 minutes before incubating; induce loading in both lateral bending and flexion extension ranges of motion for even distribution of dye.
5. Cover the entire beaker with aluminum foil to keep light from disrupting the proteins within the dye
6. Transport to a 37˚ incubator for 45 minutes
7. After incubating wash, the disc briefly with PBS solution to remove excess dye; induce loading in both lateral bending and flexion extension ranges of motion for even washing
8. Wrap in aluminum foil
9. Flash freeze the disc in liquid nitrogen for 10 seconds by placing the disc in a plastic box within the storage racks, and submerging within the liquid nitrogen.
10. Once flash froze the disc can be bound to a cryostat using OCT tissue solution.
11. Samples from the disc can now be taken using the cryostat and placed on slides by gently pressing the slide on top of the sample cut from the disc within the cryostat.
12. View samples under the fluorescent microscope in the RIC facility on the 3rd floor of the Life Sciences Building.

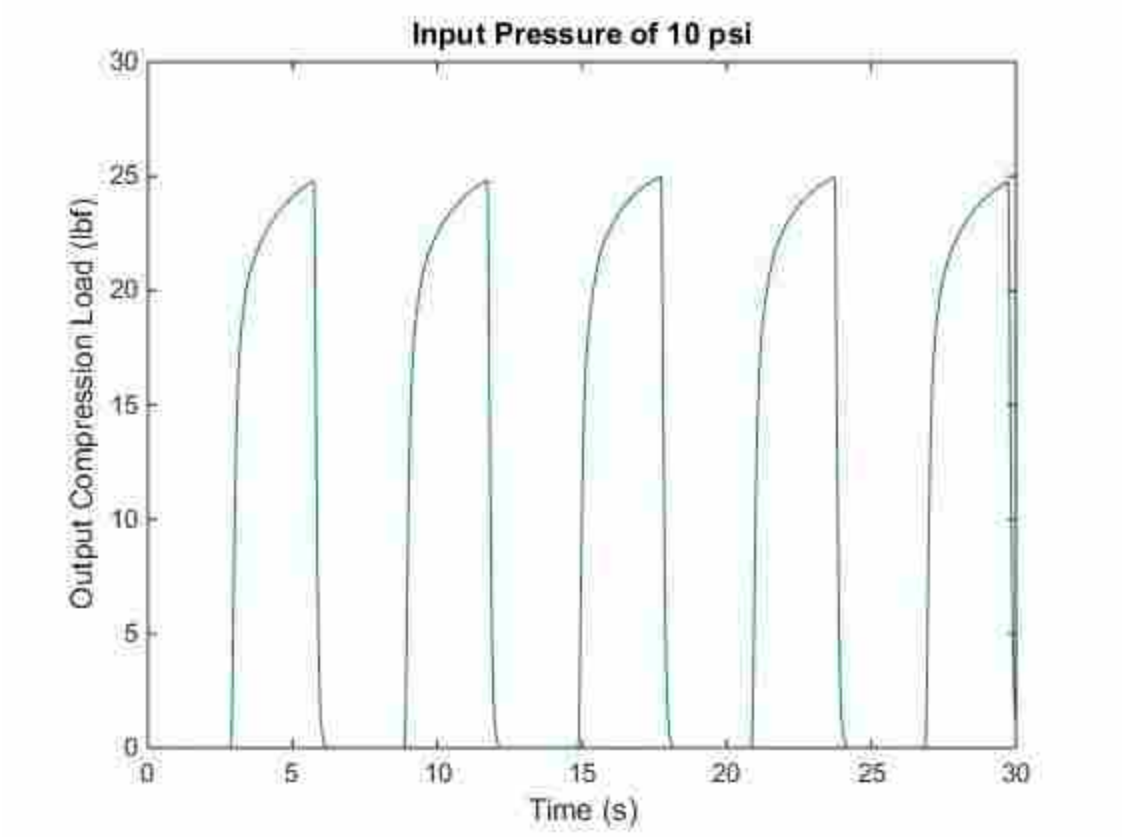
APPENDIX B. LOAD OUTPUT GRAPHS OF BIOREACTOR

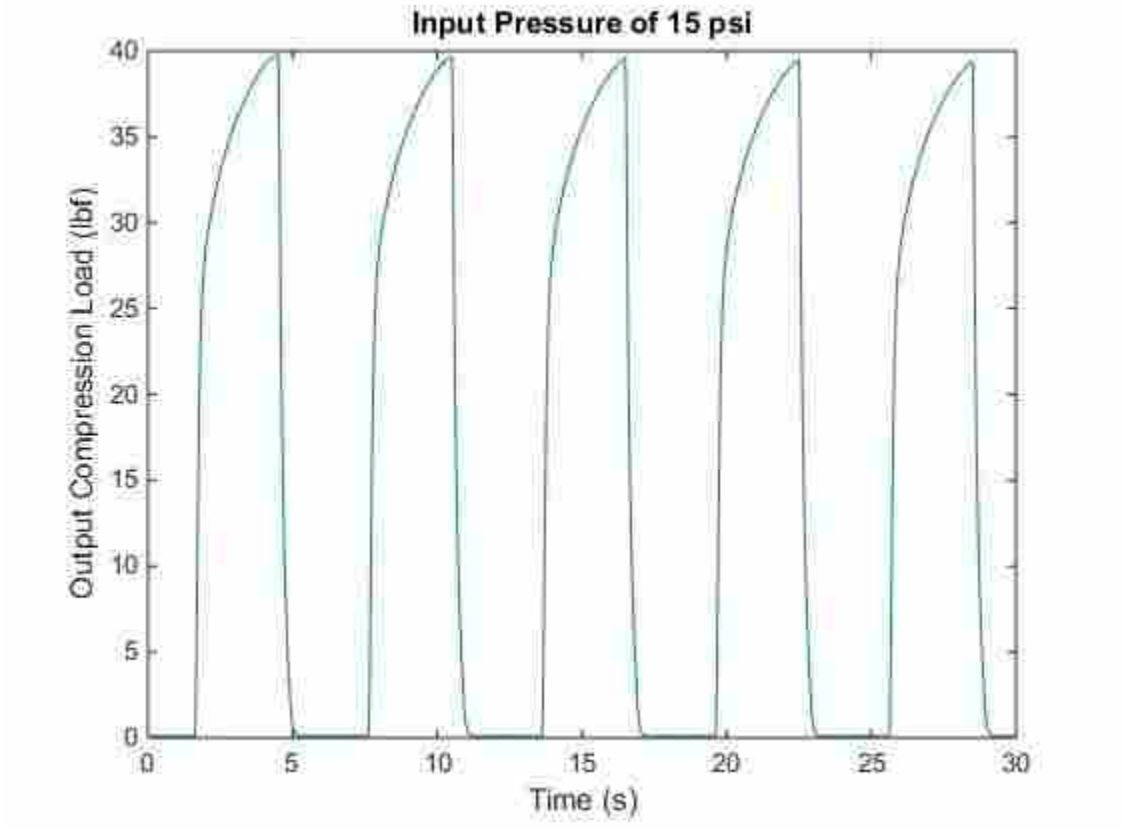
The load cells used to retrieve the output loads of the dynamic bioreactor were 1000 N and 400 N. The noise was filtered out of the 1000 N load cell data with a low-pass first order Butterworth filter with the following Matlab code:

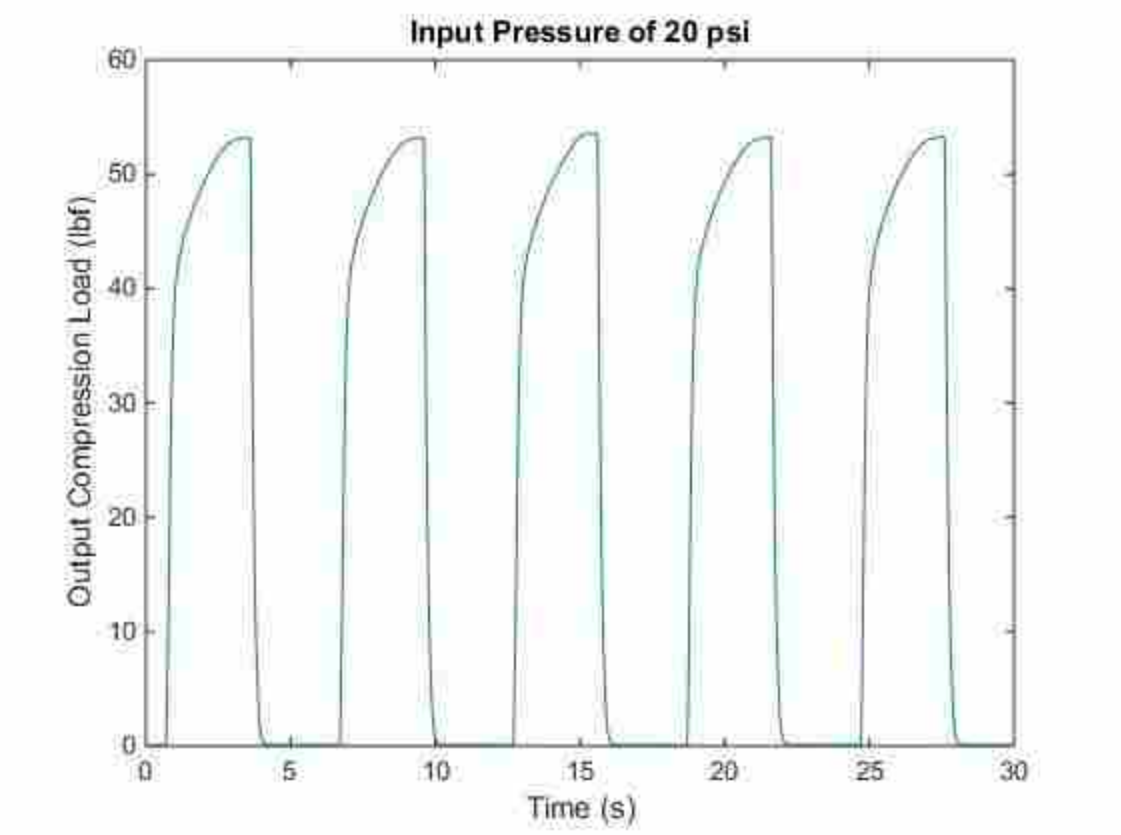
```
clc;
clear;
filename = '60 psi 1k.xlsx';
time = xlsread(filename,'A2:A1502'); %1502 is where 30 seconds ends
load = xlsread(filename,'J2:J1502'); %The load data length where 30 seconds
ends
[b,a] = butter(1,5/51,'low'); % first order Butterworth low pass filter. 5/51
= normalized cutoff frequency
dataIn = load;
dataOut = filter(b,a,dataIn);

plot(time,abs(dataOut))
title('Input Pressure of 60 psi')
ylabel('Output Compression Load (lbf)')
xlabel('Time (s)')
```

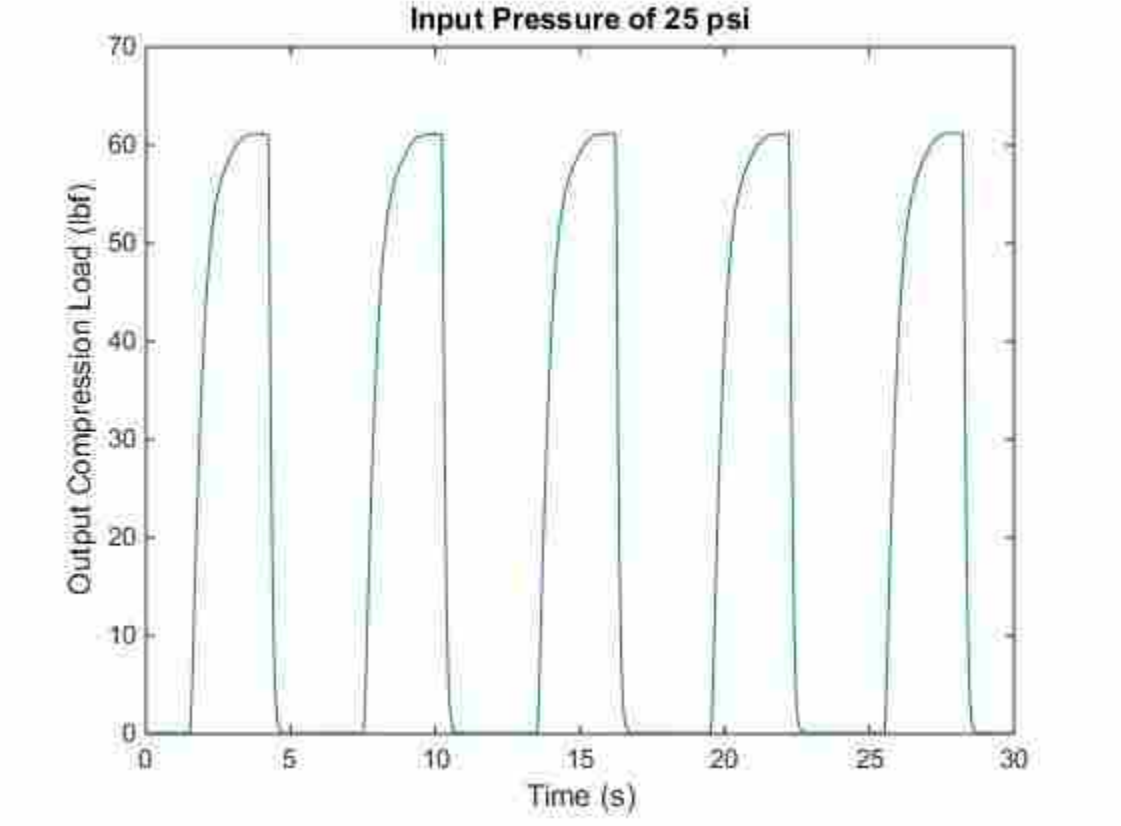

Input Pressure of 5 psi
Output Compression Load (lbf)
Time (s)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30

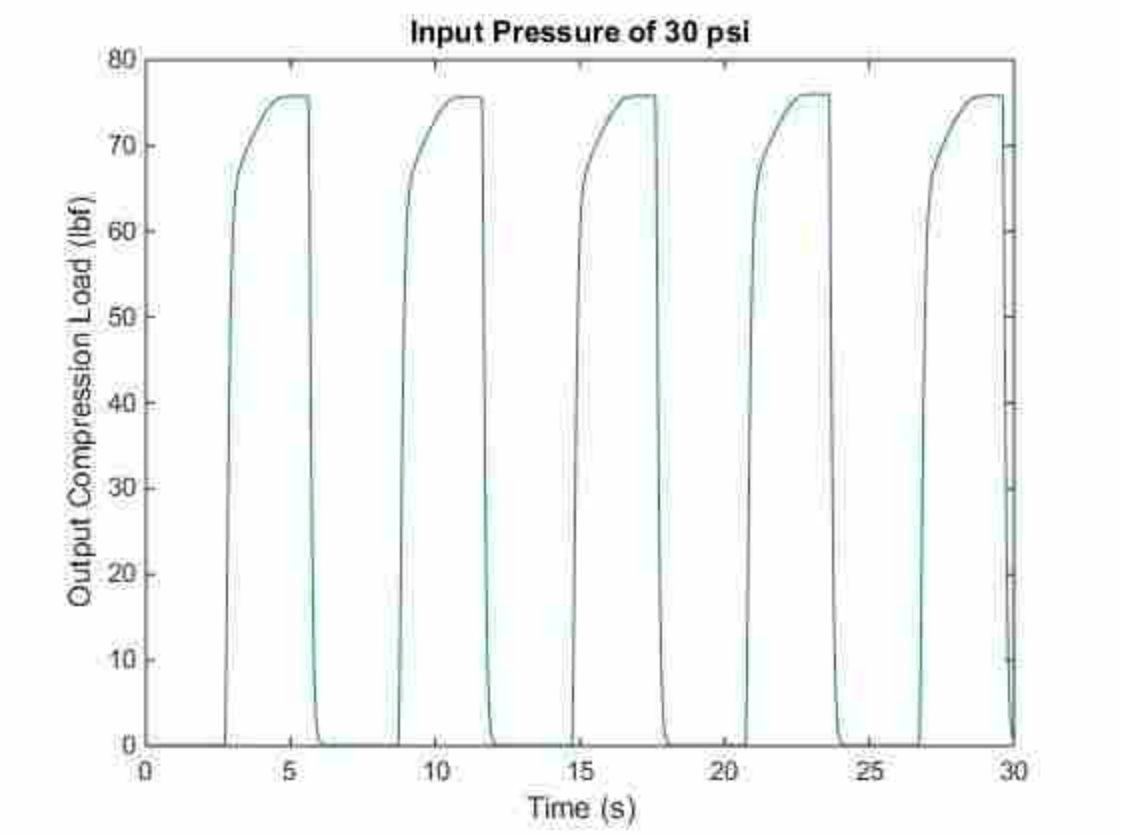

Input Pressure of 8 psi
Output Compression Load (lbf)
Time (s)
0
5
10
15
20
25
0
5
10
15
20
25
30

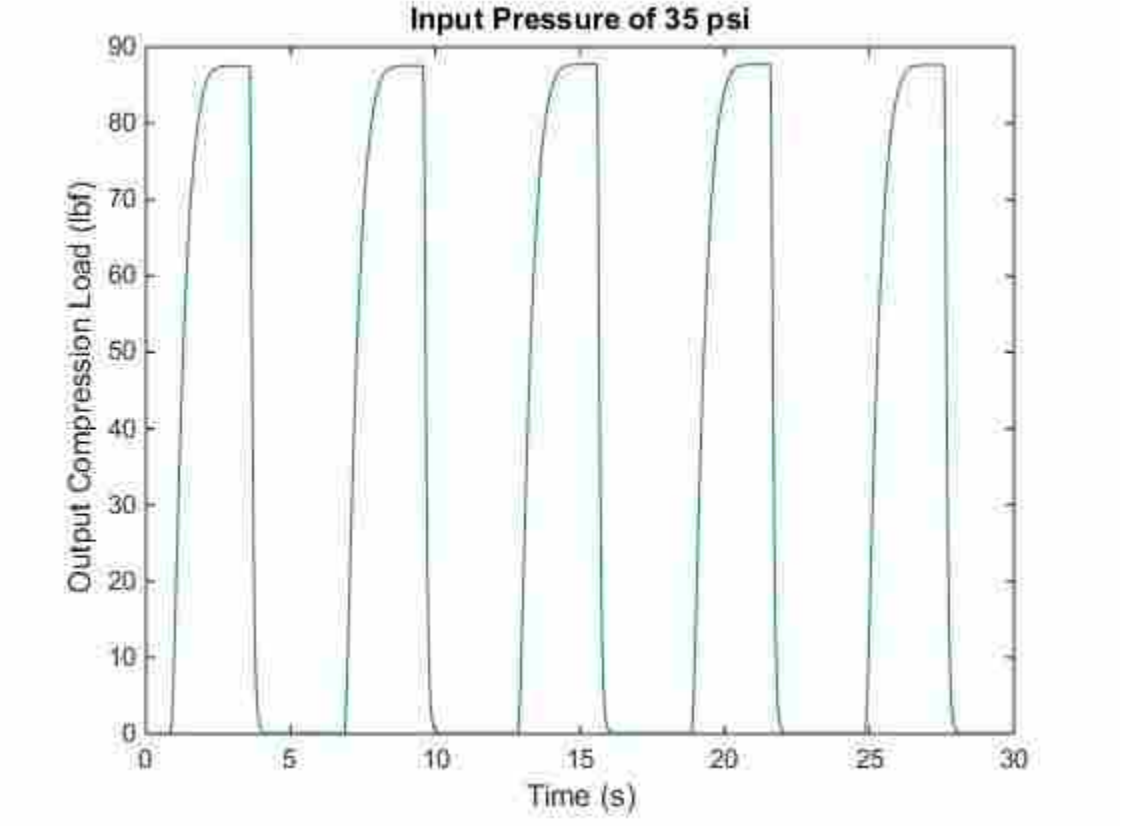

Input Pressure of 10 psi
Output Compression Load (lbf)
Time (s)
0
5
10
15
20
25
30

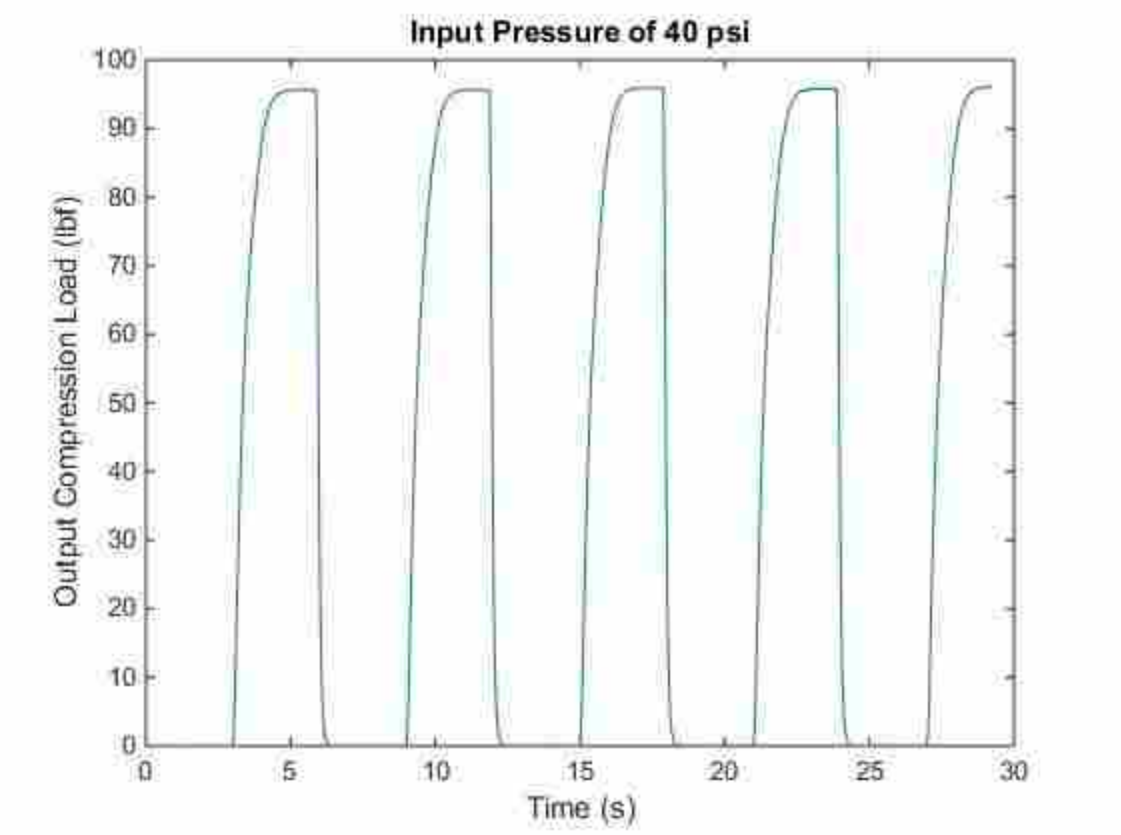

Input Pressure of 15 psi
Output Compression Load (lbf)
Time (s)
0
5
10
15
20
25
30
35
40

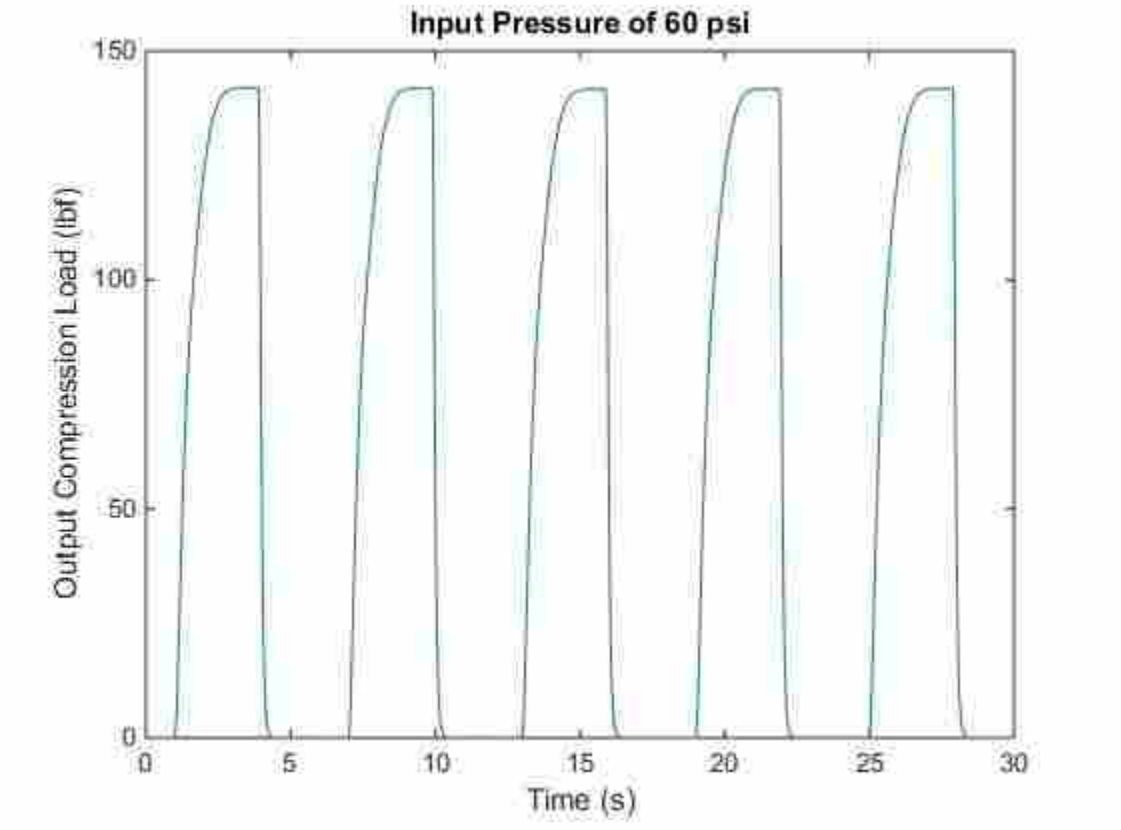

Input Pressure of 20 psi
Output Compression Load (lbf)
Time (s)
0
10
20
30
40
50
60
0
5
10
15
20
25
30


Input Pressure of 25 psi
Output Compression Load (lbf)
Time (s)
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30


Input Pressure of 30 psi
Output Compression Load (lbf)
Time (s)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30


Input Pressure of 35 psi
Output Compression Load (lbf)
Time (s)
0
10
20
30
40
50
60
70
80
90
0
5
10
15
20
25
30


Input Pressure of 40 psi
Output Compression Load (lbf)
Time (s)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30


Input Pressure of 60 psi
Output Compression Load (lbf)
Time (s)
0
50
100
150
0
5
10
15
20
25
30

APPENDIX C. ARDUINO CODE

This file is the code used for the ArduinoUno to control the three solenoid valves. The valves control which direction the air flows, and therefore whether the pneumatic cylinders are extended or contracted. The loop runs infinitely long on a 24 hour cycle.

```
//4 valves, 50% loading, 50% rest indefinitely (no 8 hour
break), .2 Hz
const int valve_1_Pin=2;
const int valve_2_Pin=5;
const int valve_3_Pin=6;
const int valve_4_Pin=9;//low pressure
int switchState = 0;//high is 1, low is 0
int var = 0; //variable for loops
int full_cycle_var = 0; //variable for the loading cycle to go
for 16 hours

//set each valve as an output
void setup(){
  pinMode(valve_1_Pin, OUTPUT);
  pinMode(valve_2_Pin, OUTPUT);
  pinMode(valve_3_Pin,OUTPUT);
  pinMode(valve_4_Pin,OUTPUT);
}
//                front (valve 1)(pin 3)
//                   /\
//                  /  \
//                 /    \
// valve 2 (pin 5) ------ valve 3 (pin 6)


void loop(){  //this loop runs indefinitely
full_cycle_var = 0;
```

```
 while(full_cycle_var <192){//this allows the entire cycle to
run for 16 hours
 //flexion extension
 //=============================================
 var=0;
  while(var < 10){ //repeat flexion/extension 10 times (each
time through takes 5 seconds)
  //flexion (valves 4 and 4 pull actuators closed)
    digitalWrite( valve_1_Pin, LOW);//front
    digitalWrite( valve_2_Pin, HIGH);//left
    digitalWrite( valve_3_Pin, HIGH);//right
    digitalWrite( valve_4_Pin, HIGH);//High pressure loading

    delay(2500);//wait 2.5 seconds

    //extension (valve 1 pulls actuator closed)
    digitalWrite( valve_1_Pin, HIGH);//front
    digitalWrite( valve_2_Pin, LOW);//left
    digitalWrite( valve_3_Pin, LOW);//right
    digitalWrite( valve_4_Pin, HIGH);//high pressure loading

    delay(2500);//wait 2.5 seconds
      var++;//repeat add one to var
  }

//pure compression
//================================================
 var = 0; //variable for loops
  while(var < 10){ //repeat pure compression 10 times (each time
through takes 5 seconds)
  //no compression
    digitalWrite( valve_1_Pin, LOW);//front
    digitalWrite( valve_2_Pin, LOW);//left
    digitalWrite( valve_3_Pin, LOW);//right
    digitalWrite( valve_4_Pin, HIGH);//high pressure loading

    delay(2500);//wait 2.5 seconds

    //compression (all three pins are pulled closed)
    digitalWrite( valve_1_Pin, HIGH);//front
    digitalWrite( valve_2_Pin, HIGH);//left
    digitalWrite( valve_3_Pin, HIGH);//right
    digitalWrite( valve_4_Pin, HIGH);//high pressure loading

    delay(2500);//wait 2.5 seconds
      var++;//repeat add one to var
  }
```

```
//lateral bending
//=====================================================
 var = 0; //variable for loops
  while(var < 10){ //repeat lateral bending 10 times (each time
through takes 5 seconds)
  //bending (valve 2 pulls actuators closed)
    digitalWrite( valve_1_Pin, LOW);//front
    digitalWrite( valve_2_Pin, HIGH);//left
    digitalWrite( valve_3_Pin, LOW);//right
    digitalWrite( valve_4_Pin, HIGH);//high pressure loading

    delay(2500);//wait 2.5 seconds

    //bending (valve 3 pulls actuator closed)
    digitalWrite( valve_1_Pin, LOW);//front
    digitalWrite( valve_2_Pin, LOW);//left
    digitalWrite( valve_3_Pin, HIGH);//right
    digitalWrite( valve_4_Pin, HIGH);//high pressure loading

    delay(2500);//wait 2.5 seconds
      var++;//repeat add one to var
  }

  //rest (for 50% rest, 50% loading cycle)
//=====================================================
 var = 0; //variable for loops
    digitalWrite( valve_1_Pin, LOW);//front
    digitalWrite( valve_2_Pin, LOW);//left
    digitalWrite( valve_3_Pin, LOW);//right
    digitalWrite( valve_4_Pin, HIGH);//low pressure-just gravity
    delay(2500);
  while(var < 150){ //rest->150 seconds
  //rest (only gravity on spine)
    digitalWrite( valve_1_Pin, HIGH);//front
    digitalWrite( valve_2_Pin, HIGH);//left
    digitalWrite( valve_3_Pin, HIGH);//right
    digitalWrite( valve_4_Pin, LOW);//low pressure-just gravity

    delay(1000);//wait one second
      var++;//repeat add one to var
  }
  full_cycle_var++;//add one to the variable so this cycle runs
for 16 hours
}
//=====================================================
 var = 0; //variable for loops
```

```
  while(var < 28800){ //rest->8 hours
  //rest (no load on spine)
    digitalWrite( valve_1_Pin, LOW);//front  //this sequence is
just to raise the cylinders for one second to see if that helps
the compression be more even amongst the three cylinders.
    digitalWrite( valve_2_Pin, LOW);//left
    digitalWrite( valve_3_Pin, LOW);//right
    digitalWrite( valve_4_Pin, HIGH);//HIgh pressure un-loading

    delay(1000);//wait one second
      var++;//repeat add one to var
  }

}
```

APPENDIX D. CELL VIABILITY

To analyze the cell viability in the IVDs tested, fluorescent microscopy was used. The discs were viewed at 20x magnification which resulted in images 447.63 µm x 335.4 µm in size.

D.1 Object Counter Matlab Code

The images were then inverted and analyzed with the following Matlab code:

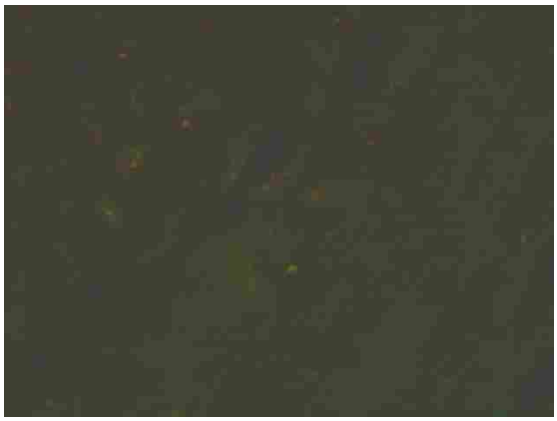
```
clc;
clear;
% Display an image.
Image = imread('Static_2_annulus_3.jpg');
Image2 = imcomplement(Image);
imshow(Image2);
holdon;
% Initialize counter.
count = 0;
message = sprintf('Click as many points as you want.\nHit return when
done.');
title(message, 'FontSize', 20);
button = questdlg(message, 'Continue?', 'OK', 'Cancel', 'OK');
drawnow;    % Refresh screen to get rid of dialog box remnants.
ifstrcmpi(button, 'Cancel')
return;
end
% Begin loop where user clicks points over display
while count < 1000 % or whatever failsafe you want.
% User clicks one point.  If user types Enter/Return, x is empty.
    [x,y] = ginput(1);
ifisempty(x)
break;
end
% Put a cross over the point.
plot(x, y, 'r+', 'MarkerSize', 15, 'LineWidth', 3);
% Increment the count.
count = count + 1
% Save coordinates (if desired).
allX(count) = x;
allY(count) = y;
end
```

D.2 LIVE/DEAD® Images

The following are the images used to determine the cell viability in the discs:

Initial (beginning of test)

Nucleus Pulposus

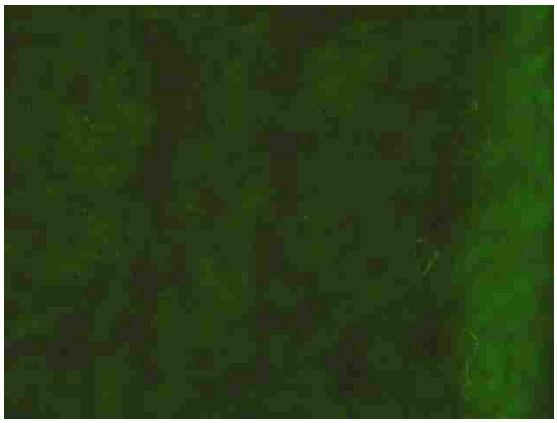


Nucleus Pulposus

Nucleus Pulposus

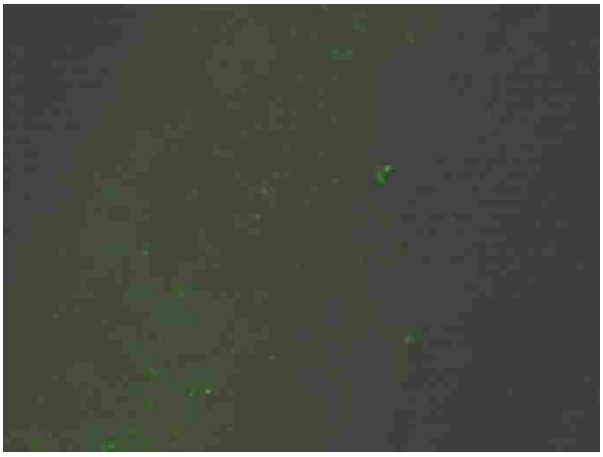
Annulus Fibrosus

Annulus Fibrosus

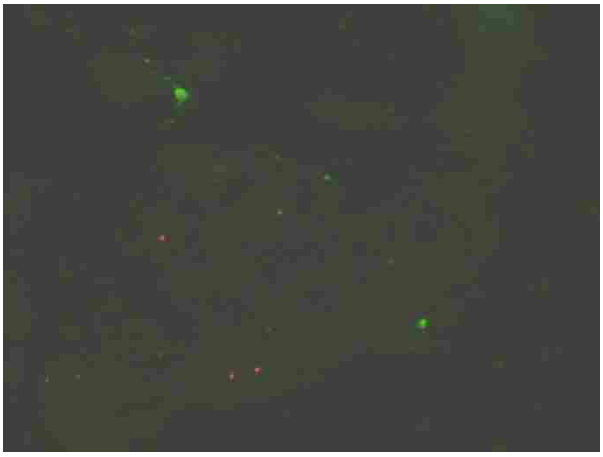


Annulus Fibrosus

Dynamic Bioreactor after 2 weeks

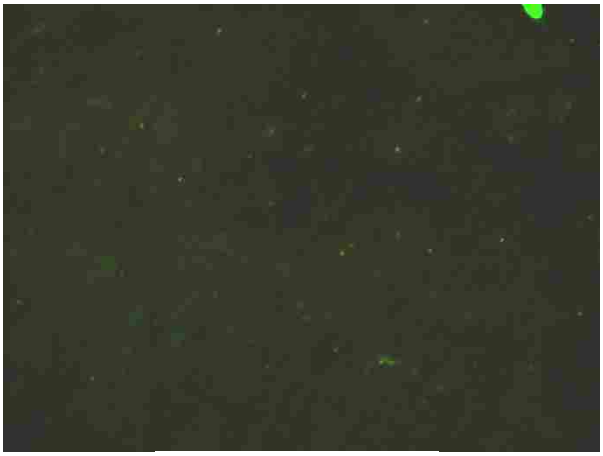


Nucleus Pulposus

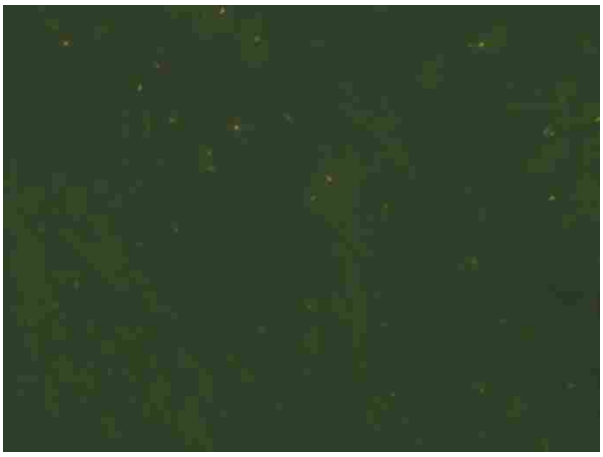


Nucleus Pulposus

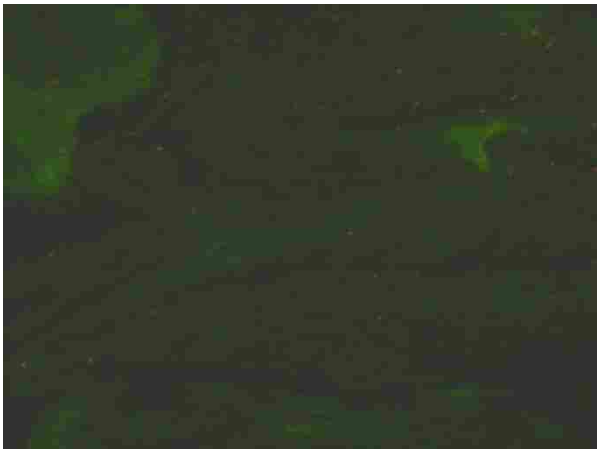
Nucleus Pulposus



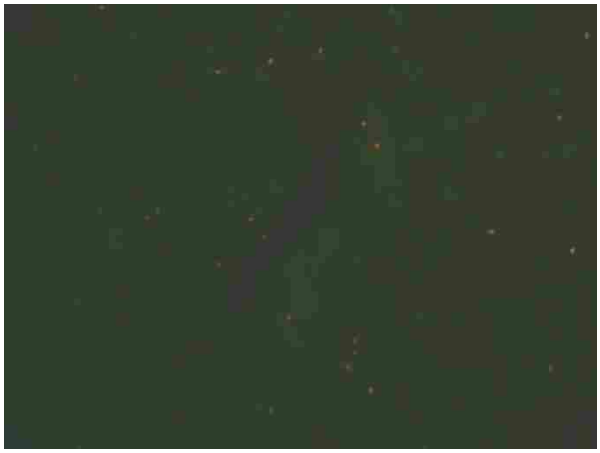
Nucleus Pulposus



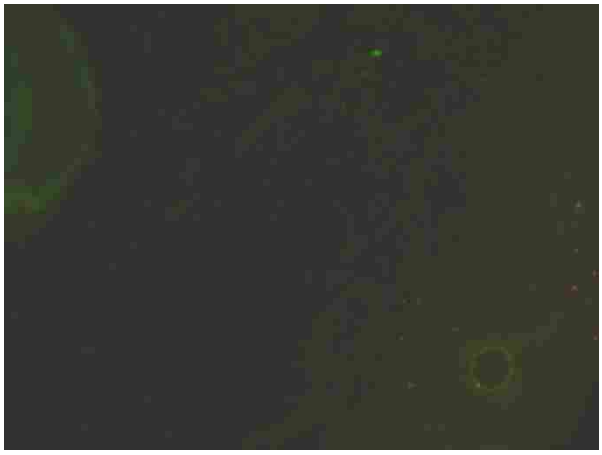
Nucleus Pulposus



Annulus Fibrosus



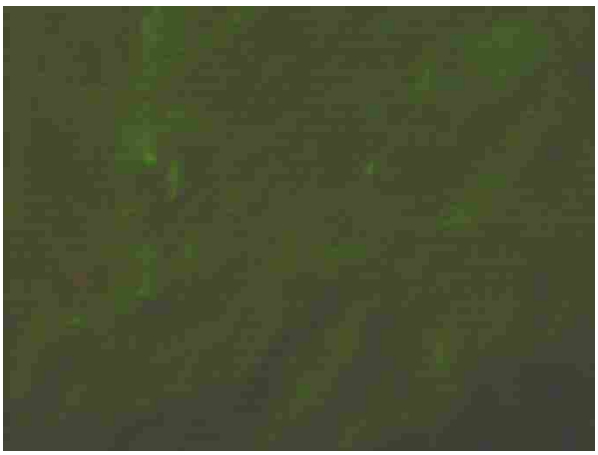
Annulus Fibrosus



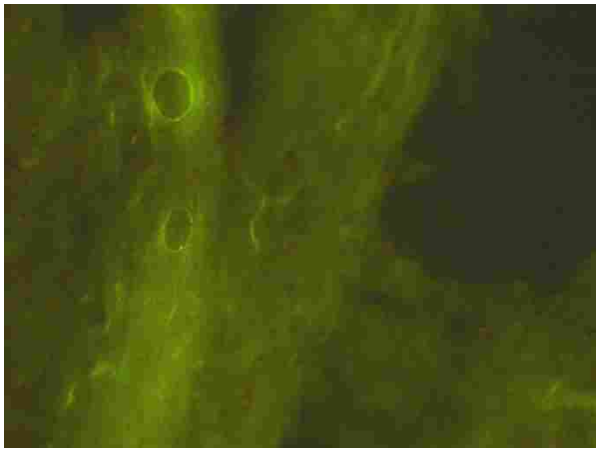
Annulus Fibrosus

Static Control after one week

Nucleus Pulposus



Nucleus Pulposus

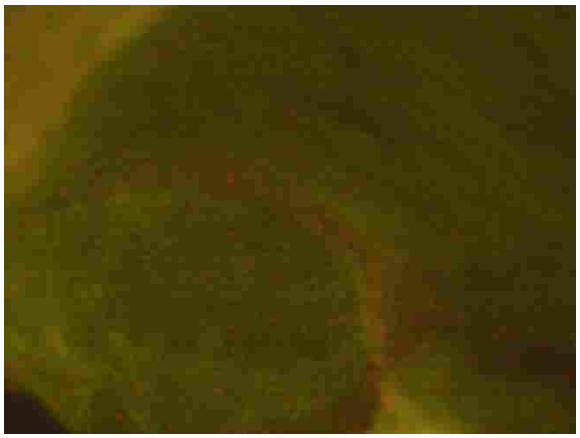


Nucleus Pulposus

Annulus Fibrosus

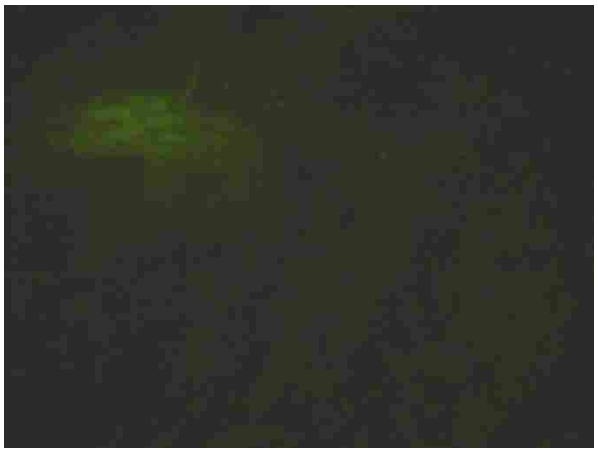
Annulus Fibrosus

Annulus Fibrosus



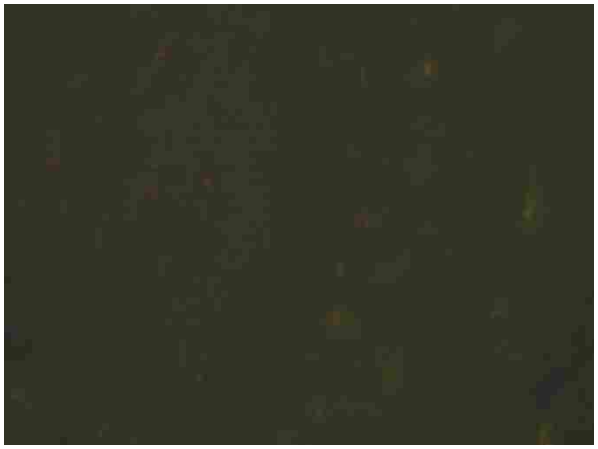
Annulus Fibrosus

Static A Control after two weeks



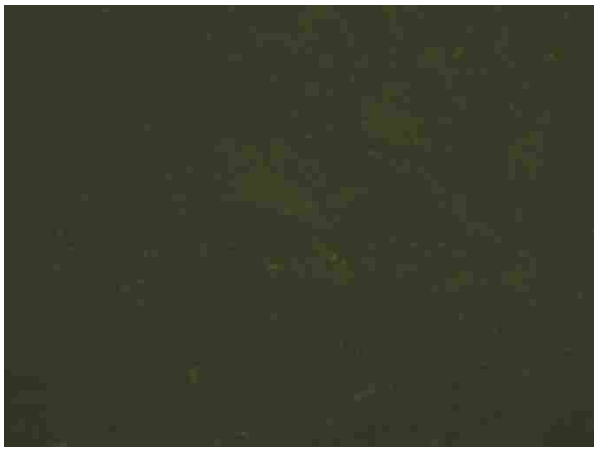
Nucleus Pulposus

Nucleus Pulposus

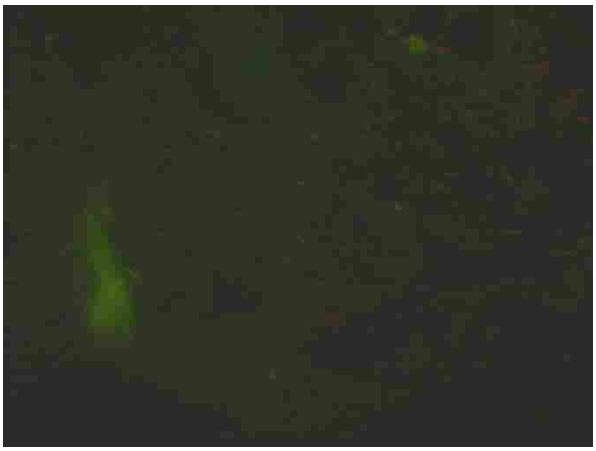


Nucleus Pulposus

Annulus Fibrosus



Annulus Fibrosus

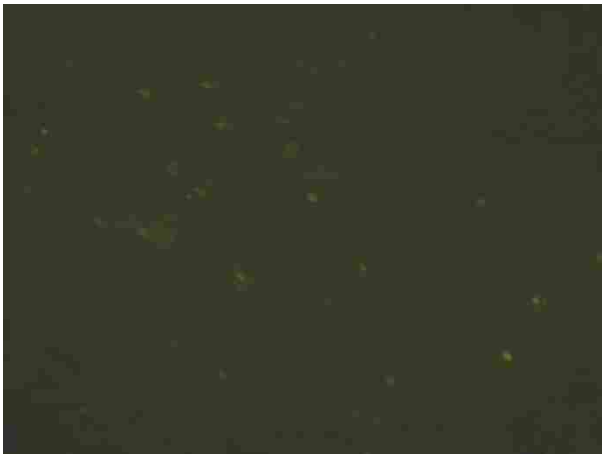


Annulus Fibrosus

Static B Control after two weeks

Nucleus Pulposus

Annulus Fibrosus



Annulus Fibrosus

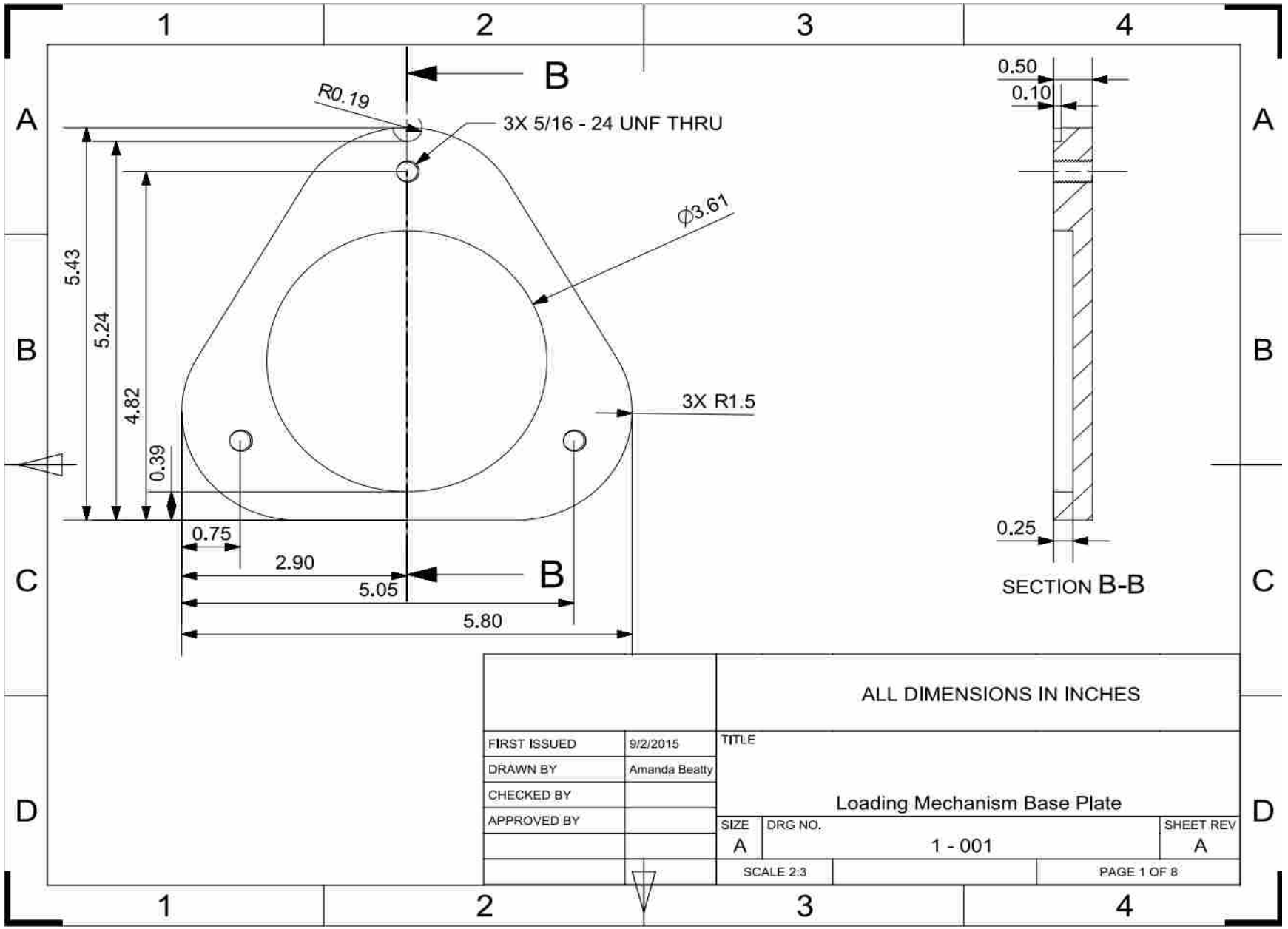
Annulus Fibrosus

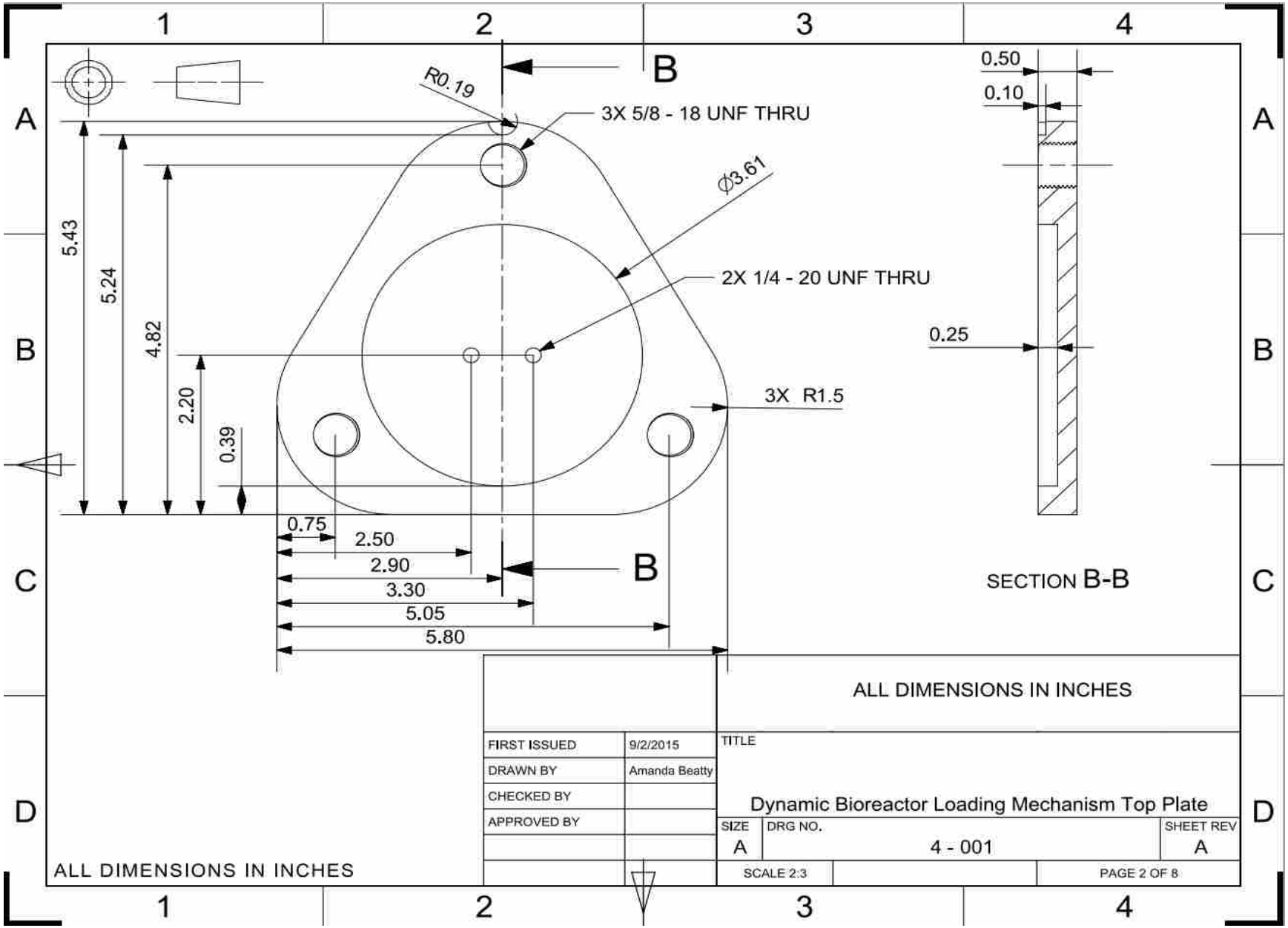
D.3 Live Dead Cell Stain Data

Disc	Area	Live	Dead	Total	Cell viability (%)	Cell Density/mm ^3	Section Thickness (um)
6/18 Control	Nucleus 1	75	1	76	98.68	36149	14
	Nucleus 2	76	0	76	100	36149	14
	Nucleus 3	76	2	78	97.44	37101	14
	Annulus 1 (Outer)	12	85	97	12.37	46138	14
	Annulus 2 (Outer)	Unreadable					14
7/2 Dynamic	Nucleus 1	67	40	107	62.62	71252	10
	Nucleus 4	37	32	69	53.62	45948	10
	Nucleus 5	22	40	62	35.48	29490	14
	Nucleus 91	20	35	55	36.36	26161	14
	Nucleus 92	19	53	72	26.39	34247	14
	Annulus 2 (Outer)	7	56	63	11.11	41952	10
	Annulus 3 (Inner)	24	33	57	42.11	37957	10
	Annulus 6 (Inner)	14	27	41	34.15	19502	14
	Annulus 7 (Mid/outer)	4	32	36	11.11	17123	14
	Annulus 8 (Mid/inner)	0	62	62	0	41286	10
	Annulus 9 (Outer)	12	58	70	17.14	46614	10
7/2 Static 1	Nucleus 4	0	49	49	0	32629	10
	Nucleus 5	0	42	42	0	27968	10
	Nucleus 6	0	41	41	0	19502	14
	Annulus 1 (Inner)	0	50	50	0	33295	10
	Annulus 2 (Inner)	0	88	88	0	58600	10
	Annulus 3 (Outer)	0	168	168	0	111873	10
	Annulus 7 (Outer)	0	104	104	0	49467	14
7/2 Static 2	Nucleus 2	0	26	26	0	17314	10
	Annulus 1 (Outer)	0	71	71	0	47279	10
	Annulus 3 (Inner)	0	35	35	0	23307	10
	Annulus 4 (Inner)	0	49	49	0	23307	14

APPENDIX E. CAD DRAWINGS

The following pages are the CAD drawings for the elements of the dynamic bioreactor that were manufactured by the author. The last drawing is an exploded view of the dynamic bioreactor including both purchased and manufactured parts.


1
2
3
4
A
B
C
D
B
R0.19
3X 5/16 - 24 UNF THRU
ϕ3.61
3X R1.5
5.43
5.24
4.82
0.39
0.75
2.90
5.05
5.80
B
0.50
0.10
0.25
SECTION B-B
ALL DIMENSIONS IN INCHES
FIRST ISSUED 9/2/2015
DRAWN BY Amanda Beatty
CHECKED BY
APPROVED BY
TITLE
Loading Mechanism Base Plate
SIZE A
DRG NO. 1 - 001
SHEET REV A
SCALE 2:3
PAGE 1 OF 8


R0.19
3X 5/8 - 18 UNF THRU
Ø3.61
2X 1/4 - 20 UNF THRU
3X R1.5
5.43
5.24
4.82
2.20
0.39
0.75
2.50
2.90
3.30
5.05
5.80
0.50
0.10
0.25
SECTION B-B
ALL DIMENSIONS IN INCHES
FIRST ISSUED 9/2/2015
DRAWN BY Amanda Beatty
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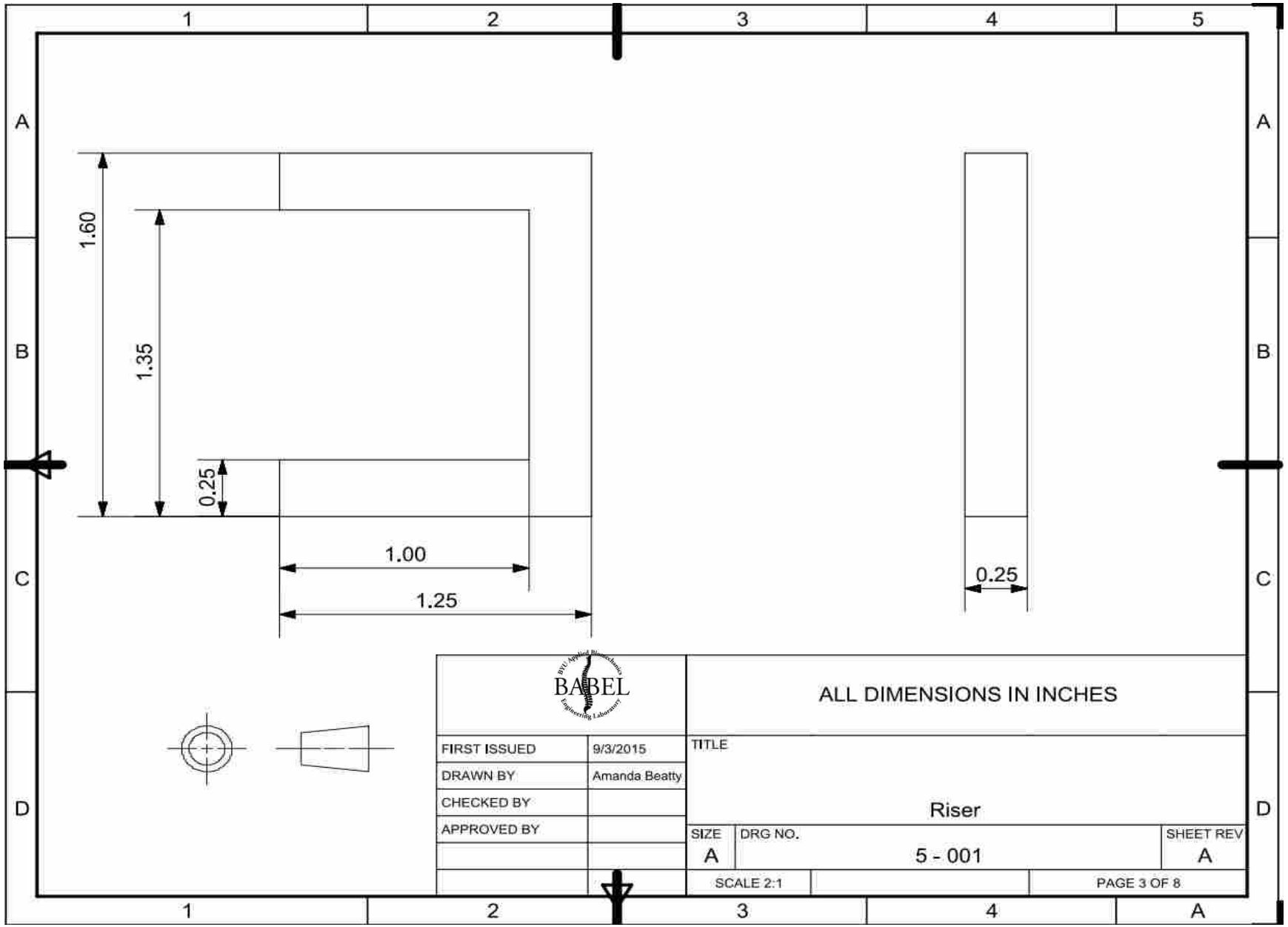
TITLE
Dynamic Bioreactor Loading Mechanism Top Plate
SIZE A
DRG NO. 4 - 001
SHEET REV A
SCALE 2:3
PAGE 2 OF 8
ALL DIMENSIONS IN INCHES


1
2
3
4
5
A
B
C
D
1.60
1.35
0.25
1.00
1.25
0.25
BABEL
ALL DIMENSIONS IN INCHES
FIRST ISSUED 9/3/2015
DRAWN BY Amanda Beatty
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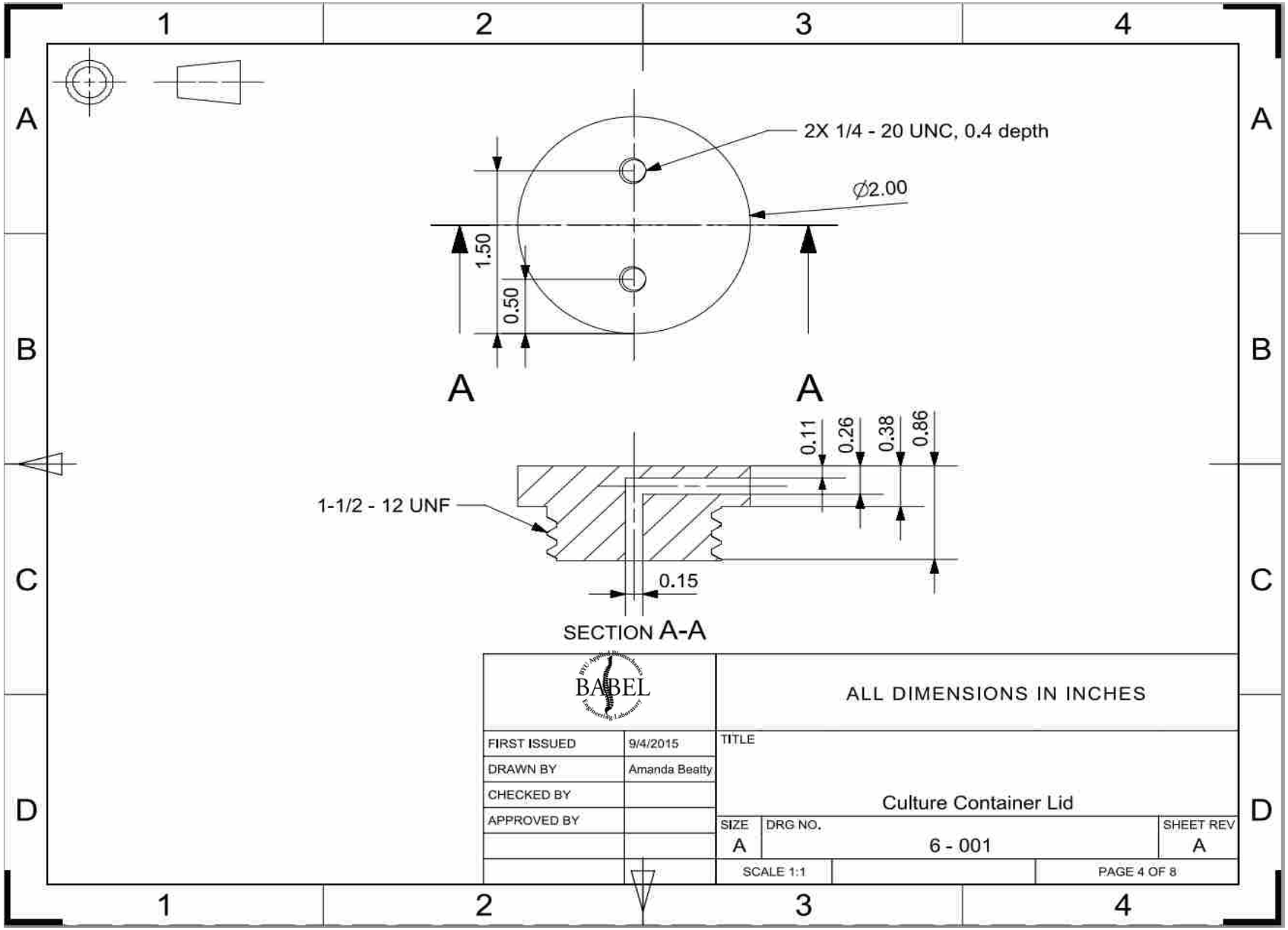
APPROVED BY
TITLE
Riser
SIZE A
DRG NO. 5 - 001
SHEET REV A
SCALE 2:1
PAGE 3 OF 8


1
2
3
4
A
B
C
D
2X 1/4 - 20 UNC, 0.4 depth
∅2.00
1.50
0.50
A
A
0.11
0.26
0.38
0.86
1-1/2 - 12 UNF
0.15
SECTION A-A
BABEL
ALL DIMENSIONS IN INCHES
FIRST ISSUED 9/4/2015
DRAWN BY Amanda Beatty
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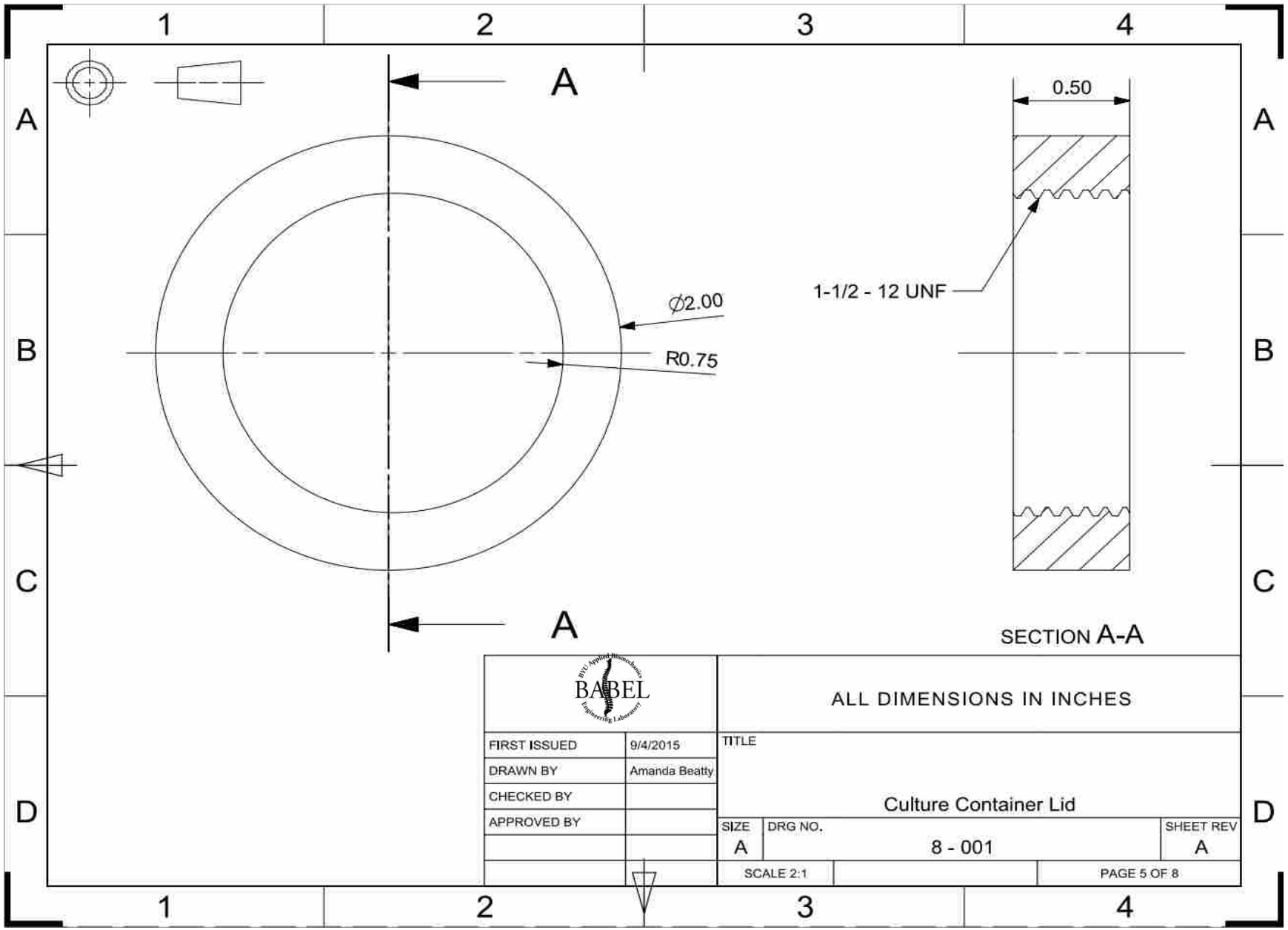
TITLE
Culture Container Lid
SIZE A
DRG NO. 6 - 001
SHEET REV A
SCALE 1:1
PAGE 4 OF 8


1
2
3
4
A
B
C
D
A
A
Ø2.00
R0.75
0.50
1-1/2 - 12 UNF
SECTION A-A
BABEL
ALL DIMENSIONS IN INCHES
FIRST ISSUED 9/4/2015
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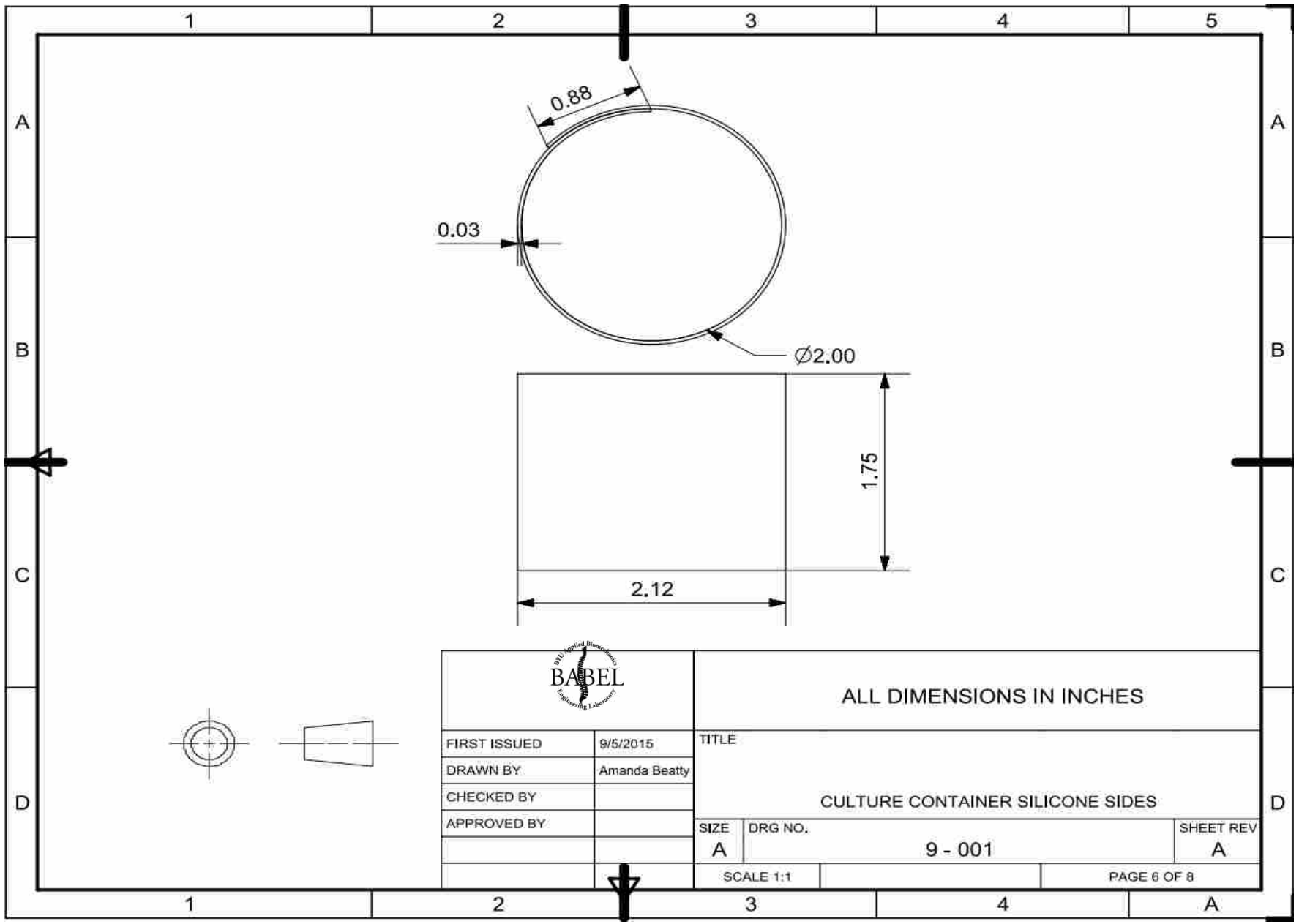
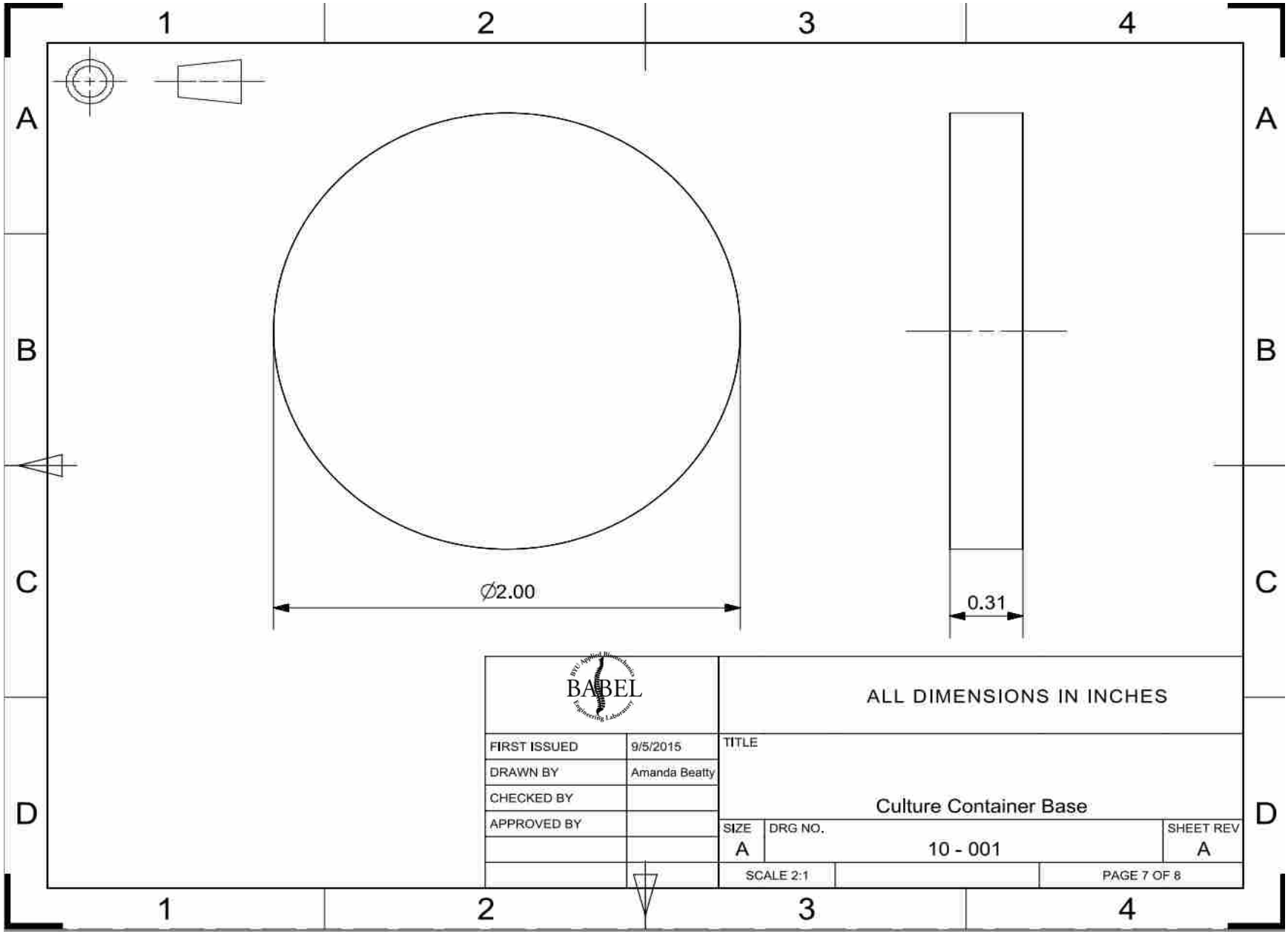
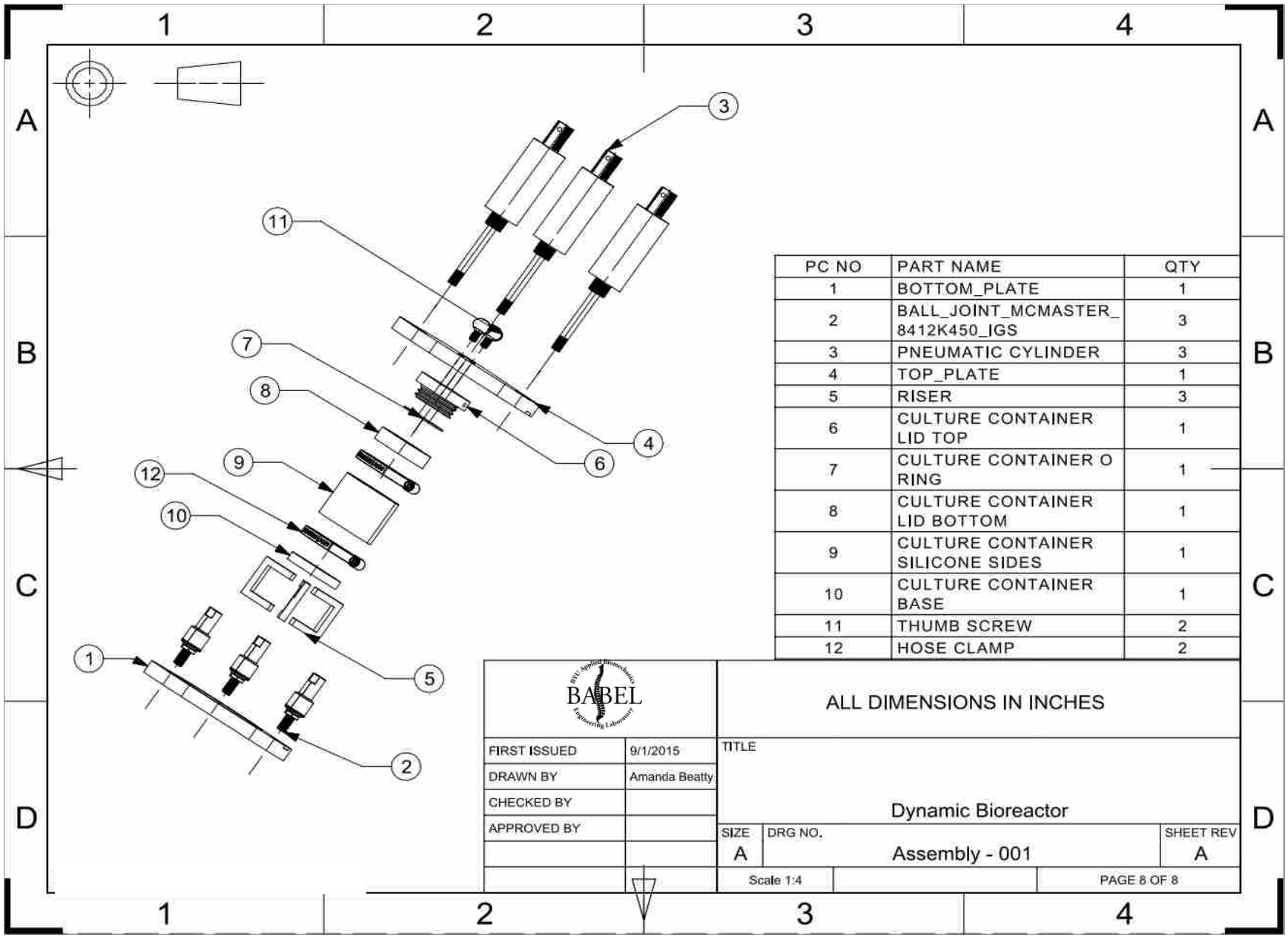
TITLE
Culture Container Lid
SIZE A
DRG NO. 8 - 001
SHEET REV A
SCALE 2:1
PAGE 5 OF 8


1
2
3
4
5
A
B
C
D
0.88
0.03
∅2.00
1.75
2.12
BABEL
ALL DIMENSIONS IN INCHES
FIRST ISSUED
9/5/2015
DRAWN BY
Amanda Beatty
CHECKED BY
APPROVED BY
TITLE
CULTURE CONTAINER SILICONE SIDES
SIZE
A
DRG NO.
9 - 001
SHEET REV
A
SCALE 1:1
PAGE 6 OF 8


1
2
3
4
A
B
C
D
∅2.00
0.31
BABEL
ALL DIMENSIONS IN INCHES
FIRST ISSUED 9/5/2015
DRAWN BY Amanda Beatty
CHECKED BY
APPROVED BY
TITLE
Culture Container Base
SIZE A
DRG NO. 10 - 001
SHEET REV A
SCALE 2:1
PAGE 7 OF 8



PC NO	PART NAME	QTY
1	BOTTOM_PLATE	1
2	BALL_JOINT_MCMASTER_8412K450_IGS	3
3	PNEUMATIC CYLINDER	3
4	TOP_PLATE	1
5	RISER	3
6	CULTURE CONTAINER LID TOP	1
7	CULTURE CONTAINER O RING	1
8	CULTURE CONTAINER LID BOTTOM	1
9	CULTURE CONTAINER SILICONE SIDES	1
10	CULTURE CONTAINER BASE	1
11	THUMB SCREW	2
12	HOSE CLAMP	2

BABEL

ALL DIMENSIONS IN INCHES

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TITLE

Dynamic Bioreactor

SIZE	DRG NO.	SHEET REV
A	Assembly - 001	A

Scale 1:4

PAGE 8 OF 8

APPENDIX F. MATERIALS LIST

Item Name	Part Number	Description	Supplier	Quantity	Assembly PC NO
Aluminum 6061-T6 Plate	n/a	0.5” x 4” plate, 12” length	Various	1	1, 4
Aluminum 6061-T6 Rectangular Rod	n/a	1.5” x 1” plate, 0.75” length	Various	1	5
Aluminum 6061-T6 Circular Rod	n/a	2” diameter, 2” length	Various	1	6, 8, 10
O-ring	9452K93	1.5” diameter, 3/32” width	McMaster-Carr	1	7
Silicone Rubber Sheet	86465K91	1/32” sheets, 24” x 36”	McMaster-Carr	1	9
Ball Joints	8412K45	5/16”-24 female shank with stud ball joint	McMaster-Carr	3	2
Thumb Screws	91745A534	¼”-20 Stainless Steel spade head thumb screw, 3/8” length	McMaster-Carr	1	11
Hose Clamps	5388K32	5/16” band width worm-drive hose clamp, 1-11/16” to 2-1/4” Diameter	McMaster-Carr	1	12
Pneumatic Valves	37A-AA0-H	Universal Solenoid Valve 1/8” NPT, up to 120 psi	MAC Valves	4	n/a
Pneumatic Cylinders	RP 106x1.500-DAD	1-1/16” Bore size, 1.5” stroke Roundline Plus stainless steel body actuators	Norgren Inc.	3	3