

DEVELOPMENT AND VALIDATION OF A SYSTEM FOR STUDYING
CHONDROCYTE MECHANOTRANSDUCTION WITH
PRELIMINARY METABOLOMIC RESULTS

by

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ABSTRACT

Osteoarthritis (OA) is a degenerative disease currently affecting over 46 million Americans. OA is most commonly characterized by breakdown of articular cartilage within the joint resulting in abnormal loading, loss of motion, and pain. Articular cartilage is the tough, flexible, load-bearing material that allows for joints to articulate smoothly *i.e.* running with relative ease. Currently there is no cure for this disease and the exact causes remain relatively unknown. Chondrocytes are the only cell type found in cartilage and are responsible for all biological maintenance and repair. Previous studies have shown that chondrocytes respond to mechanical load by cellular mechanotransduction, the process by which cells convert mechanical stimuli into biochemical activity. The aim of this thesis is to study the effects that mechanical loads have on human chondrocyte metabolism to better understand OA. To study chondrocyte mechanotransduction it was vital to develop a machine that could simulate *in vivo* loading of chondrocytes within the human knee joint. A bioreactor was designed, built, and validated that can simulate physiological loading in a tissue culture environment. This bioreactor was then used to characterize the mechanical properties of a viscoelastic material (agarose) capable of maintaining viable 3-Dimensional cell cultures. Inside the body chondrocytes are surrounded by a pericellular matrix (PCM), which provides a unique stiffness much less than the stiffness of cartilage. The mechanical property tests performed on agarose allowed for an accurate representation of this cellular microenvironment. Agarose gel concentrations were found that can model both healthy PCM and OA PCM stiffness. Methods were then developed to encapsulate living chondrocytes within these physiologically stiff gels. Utilizing these newly developed gel constructs and custom built bioreactor, 3-Dimensional chondrocyte suspensions were subjected to dynamic compression simulating normal physiological loading *i.e.* walking. It was hypothesized that moderate dynamic loading would promote changing in the central metabolism pathways, such as glycolysis. To study this hypothesis, mass-spectrometry techniques were utilized to identify metabolites present in each sample, and if the amount of each metabolite changed due to dynamic compression. These results provide a robust foundation for understanding cellular mechanotransduction.

INTRODUCTION

Background

Osteoarthritis (OA) is the most common form of arthritis affecting millions of people every year. OA, commonly called the wear-and-tear arthritis, occurs when the protective cartilage on the ends of bones wears down over time. This disease most commonly affects the knees and hips, but can occur in any joint of the human body. It causes pain, swelling, and can reduce the overall motion of the joint. OA is characterized by progressive degenerative changes in the morphology, composition, and mechanical properties of the articular cartilage, subchondral bone, synovium and other joint tissues (Guilak, 2011). Without healthy articular cartilage present in the joints bones rub together and over time this can lead to serious joint damage. Currently, there is no cure for this disease and the most common treatment for severe cases is surgery.

Even though OA affects the whole joint (e.g. bone, cartilage, synovium, etc.) scientific studies usually focus on just one component, seemingly the problem becomes more complex the more variables get added to it. It has been shown under advancing OA cartilage can change immensely. Macroscopically cartilage can begin to soften and erode. Microscopically, cartilage layers can begin to disappear and cellular necrosis can occur. It has been shown that cartilage cells undergo phenotypic changes in the presents of OA tissues. Phenotype is the makeup of an organism's observable traits such as: morphology, biochemical and physiological properties, behavior, and development. Changes to any one of these characteristics greatly affect the overall health of cartilage.

Structure of Cartilage

Cartilage is a non-vascular tissue that is found throughout the human body. There are three types of cartilage: hyaline cartilage, fibrocartilage, and elastic cartilage.

Cartilage found in human joints is of the hyaline variety (Gray, 1973). Articular cartilage is a tough, flexible, load-bearing connective tissue that is found on joint surfaces of bone. It is a specialized tissue with unique mechanical properties that provide a very smooth and wear resistant surface for joints to articulate on. Articular cartilage can be broken up into four different zones: the superficial zone, the middle zone, the deep zone, and a region of calcified cartilage (Pearle et al., 2005).

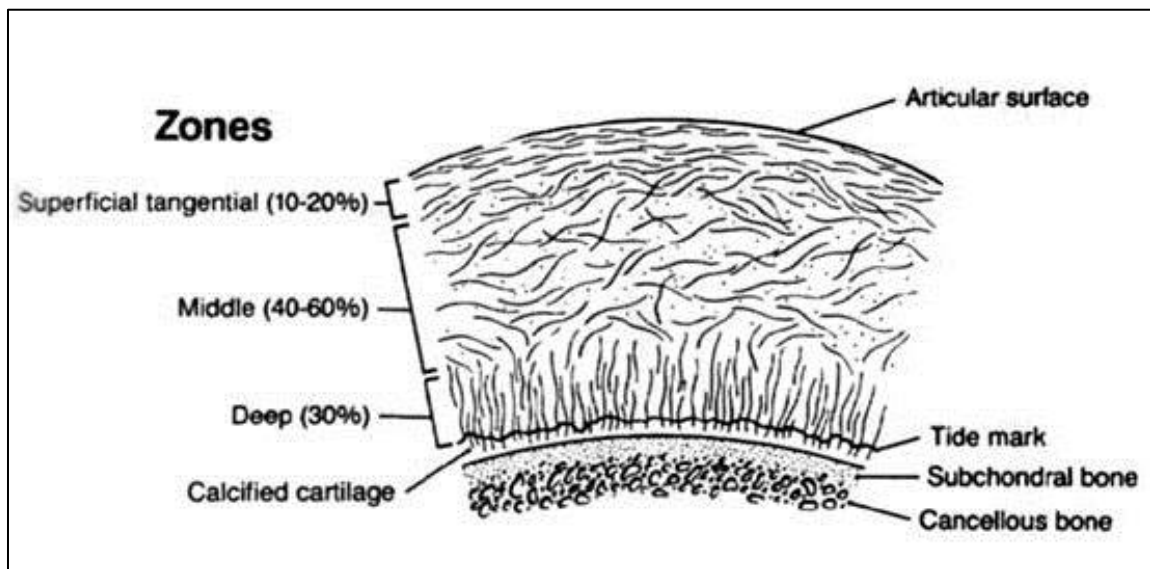


Figure 1: Articular Cartilage Cross-section

<http://www.sbes.vt.edu/freeman/images/research/cartilage-tiss-eng.jpg>

The superficial zone makes up 10 to 20 percent of the overall thickness. It has the highest content of collagen fibers, which are densely packed and tangent to the articulating surface. This zone is in contact with synovium and provides cartilage with

the ability to resist loads caused by articulation. The middle zone makes up about 40 to 60 percent of the cartilage, the collagen fibers are not as organized in this region and provide a stiffer compression modulus than that of the superficial zone. The deep zone makes up approximately 30 percent of the cartilage and provides added stiffness to the overall structure. Collagen fibers in this region are oriented perpendicularly to the articular surface making them more resistant to compressive loads. This region also has the least amount of water and contains the highest percentage of proteoglycans. The final zone contains calcified cartilage, which sits in direct contact with subchondral bone. Calcium salts begin to develop in the matrix and this region provides the needed bone to cartilage interface (Pearle et al., 2005). This layered structure, coupled with the dense extracellular matrix provides cartilage, with a structure not unlike some engineering materials such as composites. Its innate ability to distribute intense loads with relative ease is due to the composition of its extracellular matrix (ECM).

Extracellular Matrix: The ECM provides a physical microenvironment for cells to exist in. It provides a substrate for cells to anchor onto in addition to various other important tasks. The ECM is one of the defining features of all connective tissue found in the human body. The extracellular matrix is also responsible for the transmission of environmental signals to the cells that are in contact with it, which affects cell proliferation (growth or production), differentiation (less specialized cell becoming a more specialized cell), and cell death. The ECM is primarily composed of water, collagen, and proteoglycans. Other molecules are present such as noncollagenous proteins, lipids, phospholipids, and glycoproteins (Sophia Fox et al., 2009). Water makes

up most of the weight of articular cartilage, one of the contributing reasons articular cartilage has viscoelastic mechanical properties. Insoluble collagen fibers are the most abundant molecule in cartilage. Collagen is a triple-helical protein that forms fibrils of great tensile strength (Lodish H, 2000).

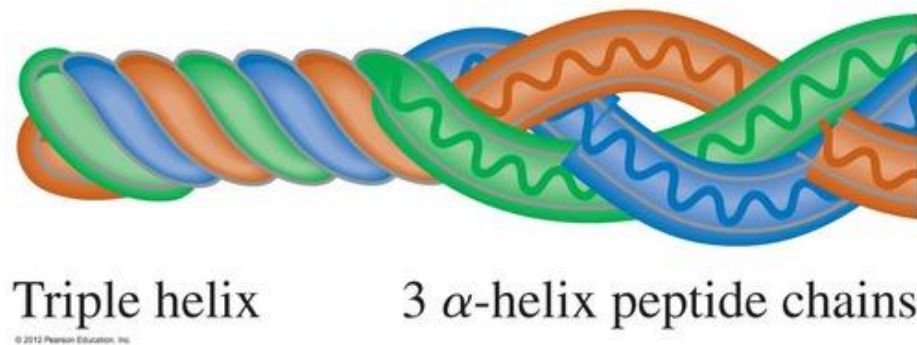


Figure 2: Structure of Collagen
<http://o.quizlet.com/zZyMrBw0SpiUI54M2oNKw.jpg>

Collagen can be found throughout the human body in all connective tissues. There are a number of different types of collagen, but type II collagen accounts for about 90% of the collagen found in the cartilage ECM. Type II collagen fibers provide the ECM with strength and resistance to tensile and shear loads. Proteoglycans are the second most abundant molecule found in articular cartilage. The largest and most significant is aggrecan. Aggrecan possesses a great number of keratan sulfate glycosaminoglycan and chondroitin sulfate chains formed around a central protein core. The bottle brush structure of aggrecan binds to hyaluronan molecules forming long chains that intertwine with collagen fibers (Ng et al., 2003). Aggrecan provides articular cartilage with ability to resist compressive loads. Found throughout the ECM are living cells called chondrocytes.

Articular Chondrocyte: Chondrocytes are the only cells found in cartilage, making it their responsibility to maintain, degrade, and synthesize the ECM. Chondrocytes originate from mesenchymal stem cells and make up about 10% of the total volume of articular cartilage. They can vary in size and shape depending on which structural zone they reside in. Since articular cartilage is a non-vascular tissue, the chondrocyte must rely on diffusion for nutrient and metabolite exchange (Archer and Francis-West, 2003). The chondrocyte is a very slowly proliferating cell and adult chondrocytes may not proliferate at all. This low turnover rate is one of the reasons cartilage has a very limited capacity for healing in response to injuries. This has led to the belief that chondrocyte metabolism plays a key role in the health of articular cartilage.

Chondrocyte metabolism consists of any chemical processes involved with survival, growth, matrix synthesis, etc. In general metabolism is the management of material and energy resources utilized by a cell (Campbell et al., 1999). The chondrocyte metabolism is heavily dependent on glycolysis. This is because of the low level of oxygen found in cartilage (Archer and Francis-West, 2003). Glycolysis is a metabolic pathway where glucose is broken down into pyruvate, during this process free energy is released (Campbell et al., 1999). Each chondrocyte is responsible for its immediate surrounding and very rarely do chondrocytes form cell-to-cell contacts for signal transduction and intercellular communications (Sophia Fox et al., 2009).

Each chondrocyte cell is surrounded by a thin membrane called the pericellular matrix (PCM), which provides an interface between the ECM and the chondrocyte cell (Darling et al., 2010). A schematic of the chondrocyte, PCM, and ECM is shown in

Figure 3. The blue ring identifies the chondrocyte cell membrane, the red ring represents the PCM, and the peach color is the surrounding ECM.

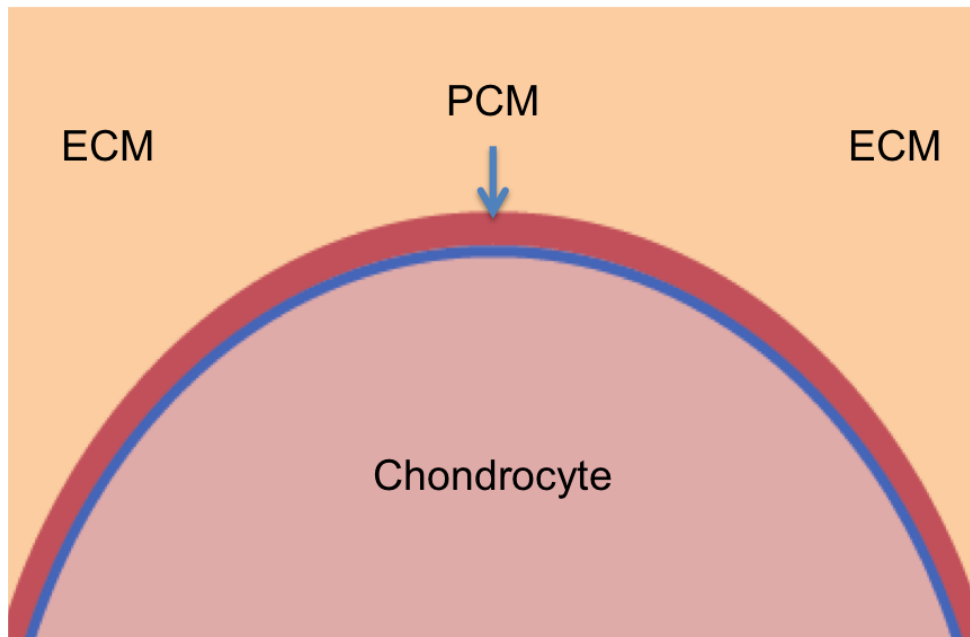


Figure 3: Diagram of ECM and PCM

The PCM contains proteoglycans, glycoproteins, as well as other non-collagenous proteins. It is believed that the PCM serves as a transducer of physical signals within the chondrocyte's microenvironment, therefor playing a very important role in chondrocyte mechanotransduciton (Alexopoulos et al., 2005a) and the overall metabolism of cartilage.

Mechanotransduction

Mechanotransduction describes the processes by which cells translate mechanical stimuli into biochemical responses. Sensory neural cells do this all the time: for example, when someone smells something or touches a hot plate, there is a mechanical stimuli (smell or touch) that is converted into a biochemical signal. The mechanical stimuli can

be just about anything. The process of mechanotransduction provides cells with the unique ability to adapt to their physical environment (Jalouk and Lammerding, 2009). Because of its location throughout the structure of articular cartilage the chondrocyte is subjected to an array of different mechanical stimuli, in the form of mechanical loads. For example as a result of normal human activity (e.g. walking), cartilage, and therefore chondrocytes, experience mechanical loading which varies with both time and spatial position in the joint (Harris et al., 2012). These varying loads can induce compressive, tensile, and shear forces along with hydrostatic pressure changes. Figure 4 provides a nice example of three different forces chondrocytes can experience.

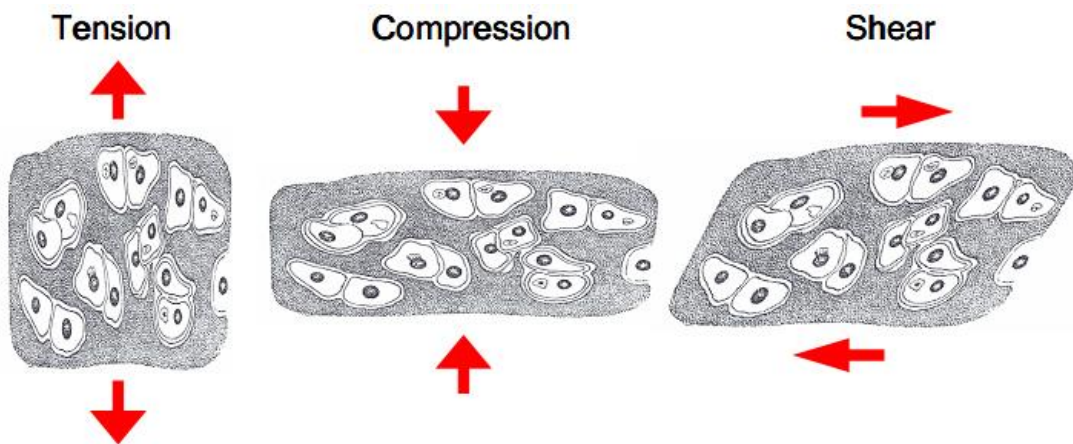


Figure 4: Forces Experienced by Chondrocytes
http://upload.wikimedia.org/wikipedia/commons/9/9e/Cartilage_forces.jpg

Mechanical stimuli can have both anabolic and catabolic effects on articular cartilage (Farnsworth et al., 2013). Anabolic processes are defined as the synthesis of more complex molecules from less complex molecules; this process is very energy intensive and can be shown by increased levels of adenosine triphosphate (ATP) within

the chondrocyte. Catabolic processes are defined as breakdown of complex molecules into simpler ones often releasing energy in the process. With respect to cartilage, anabolic processes can be thought of as synthesis of the PCM and ECM molecules, where catabolic processes can be thought of as the breakdown of PCM and ECM molecules. Chondrocytes that reside in healthy tissue (distinct from OA tissue) maintain a balance between these two processes, which leads to a healthy turnover rate of the PCM and ECM. Experiments have shown that chondrocyte metabolism is not only affected by mechanical stimuli, but also sensitive to different types of loads.

Under physiologic conditions, joints are subjected to millions of cycles of loading, which can result in forces up to 10 times the person's body weight (Guilak, 2011). It has been shown that under physiological static loading cartilage can compress up to 45% of its original thickness (Herberhold et al., 1999). Normal physiological loading conditions such as walking can produce contact pressures inside the knee joint as high as 5-6 MPa at a frequency of 1 Hz. Physiological loading conditions such as running or jumping can produce loading rates as high as 140 Hz with contact pressures up to 18 MPa (Morrell et al., 2005). Joint motion and variable loads are important to maintain normal articular cartilage function and health.

Unfortunately, not all joint motion and therefore joint loading is a good thing. Clinical and animal studies have shown that abnormal loads can lead to alterations in the composition, metabolism, structure, and mechanical properties of articular cartilage (Guilak, 2011). Abnormal loading can be caused by a variety of different factors such as immobilization, joint instability, obesity, overuse or trauma (Guilak, 2011). It has been

hypothesized that joints subjected to abnormal loading can cause and/or increase the progression of OA. The well-known Framingham Osteoarthritis Study showed that obesity plays a key role in OA (Felson et al., 1988). Yet, it is still not fully understood if OA is a reaction to the increased load on joints caused by obesity or if obesity promotes biochemical changes in metabolism of the joint. In order to understand which biological and biomechanical mechanism are involved in the onset and progression of OA tissue scientists have developed new models for studying chondrocyte mechanotransduction.

Scientific Studies

Since *in vivo* studies on articular cartilage are usually more expensive and harder to control, scientists have created *in vitro* studies that mimic normal and abnormal physiological environments. One method is to remove cartilage samples surgically from animals or human donors and mechanically stimulate them in the laboratory. This method is very beneficial when studying cartilage mechanics and ECM properties. Another method is to remove chondrocytes from the cartilage, culture them, and study the effects of mechanical stimuli on the cell directly. It has also become popular to encapsulate chondrocytes in hydrogels resulting in 3D cell suspensions, which allows for the *a. priori* usage of standard molecular biological techniques such as gene delivery via lentiviral transduction. For relevance, these gel constructs must mimic the physiological environment of the *in vivo* chondrocyte. All of these methods have proven to be beneficial when studying mechanotransduction. For example, exogenous loading has been shown to change Superficial Zone Protein expression (Neu et al., 2007), RhoA

activation (Haudenschild et al., 2008), and induce transcription of ECM genes (Fitzgerald et al., 2008).

By applying lower levels of oscillatory strain to cartilage explants or 3D cell-cultures, one can simulate walking. It has been found that cyclic loading at mild frequencies (less than 5 Hz) will promote metabolic changes in chondrocytes and therefore the surrounding matrix. Dynamic loading performed directly on cartilage samples has been shown to increase [^{35}S] sulfate and [^3H] proline uptake suggesting stimulation proteoglycan and protein biosynthesis (Jin et al., 2001). Cyclic compression at low frequency (less than 1 Hz) over the course of a month performed on cell-seeded hydrogels has been found to promote gene expressions such as MMP-13, which is a marker for catabolic changes in the ECM (Nebelung et al., 2012). As little as 5 minutes of dynamic compression has been seen to activate TGF- β signaling (Bougault et al., 2012a), a well-known growth factor that plays a role in cell proliferation and differentiation. Expression of various genes can be a direct result of the activation of intracellular pathways. Shear and compressive forces have been shown to activate MAPK pathways. MAPK pathways are heavily involved in extracellular stimulation, and have been hypothesized to regulate anabolic and catabolic functions of chondrocytes (Fitzgerald et al., 2008). All of these studies have led to the discovery that low levels of dynamic mechanical loading, usually result in positive physiological changes in chondrocytes and articular cartilage. In fact, dynamic loading has even been reported to increase stiffness of cell-seeded hydrogels (Ng et al., 2006).

Studies on bovine articular cartilage samples, that mimic trauma or injury using impact or large-magnitude static compression, can induce chondrocyte apoptosis, while increasing these loads can stimulate matrix degradation (Loening et al., 2000). In the early 2000's B. Kurx et al. discovered that once cartilage was subjected to an injury chondrocytes had a hard time maintaining healthy tissue (Kurz et al., 2001). The lack of matrix synthesis led to lower overall dynamic and equilibrium moduli for articular cartilage. Injury or trauma experiments are not the only form of mechanical stimuli proven to be detrimental to chondrocytes. Cell death has been observed when applying long-term static compressive strain to bovine cartilage samples (Chahine et al., 2007). When comparing loads that simulate injury or inactivity to dynamic stimulation experiments it is easily seen that chondrocyte mechanotransduction is heavily influenced by the kind of load articular cartilage is being subjected to.

Experimental studies have also been performed on OA tissues. The PCM of chondrocytes found in OA tissues is significantly less stiff when compared to that of healthy chondrocytes (Alexopoulos et al., 2005b). Cells that reside in OA tissue express different genes than those that reside in healthy tissue. Through evaluation of mRNA expression it has been determined that there are distinct metabolic changes between healthy and OA cartilage (Fukui et al., 2008). OA cartilage explants have been found to express p53R2, a tumor suppressor protein, which is then increased when put under 5% tensile strain. By inhibiting p53R2 an increase in matrix synthesis was observed through Akt phosphorylation. Akt also plays a role in cell proliferation. This suggests that down

regulation of p53R2 might have an anabolic effect on human cartilage (Kawakita et al., 2012).

Many of these studies have been able to identify specific gene or protein expression and even identified possible mechanosensitive metabolic pathways. The mechanisms that modulate these cellular responses have also been studied. Primary cilia have been hypothesized to act as mechanosensory organelles that transduce mechanical forces acting on chondrocytes into biochemical signals (Shao et al., 2012). It has been shown that if the primary cilia is disrupted, IHH gene expression gets aborted (Shao et al., 2012). IHH gene expression is related to signaling molecules involved in cellular mechanotransduction. Other studies have shown that primary cilia are required for ATP-induced Ca^{2+} signaling in reaction to mechanical stimuli (Wann et al., 2012). These studies identify the primary cilia as one of the possible transduction pathway for articular chondrocytes.

Usually mechanotransduction studies performed on chondrocytes are focused on small subunits of the metabolism. Many of the experiments examine gene transcription or protein phosphorylation. While these types of experiments can lead to huge scientific breakthroughs, they are unable to predict and understand interactions between molecular players. The whole picture being, how the entire metabolism of a cell is likely affected by mechanical stimuli. To address this, one objective of this study is to use systems metabolomics to study chondrocyte mechanotransduction.

Metabolomics

Metabolomics is the comprehensive analysis of small molecules in a biological system (Adams et al., 2013). These small molecules are known as metabolites.

Metabolites are products of the cellular metabolism, which can be greatly influenced by environmental conditions. These metabolites can transfer energy, control growth, and provide maintenance to a biological system. The comprehensive analysis of these metabolites provides a “top down” view of biochemical processes in cells, tissues, and complex organisms (Nicholson and Lindon, 2008). “Top down” has been coined to describe metabolomics, because analysis takes place at the end of a biological process. In engineering language, the concentration profile of metabolites represents a functional array of state variables describing the system performance. This is a much different approach than classical molecular biology, where analysis typically starts at gene or protein expression.

One problem with metabolomics is that it generates so much information; one environmental stimulus can generate thousands of metabolites. So, highly specialized machines and data analysis programs have to be implemented into experiments. One of those machines is called a mass spectrometer. Mass Spectrometry (MS) is a technique to measure the masses of the molecules comprising a sample of material. A mass spectrometer has the ability to measure the masses of individual metabolites, which then can be identified.

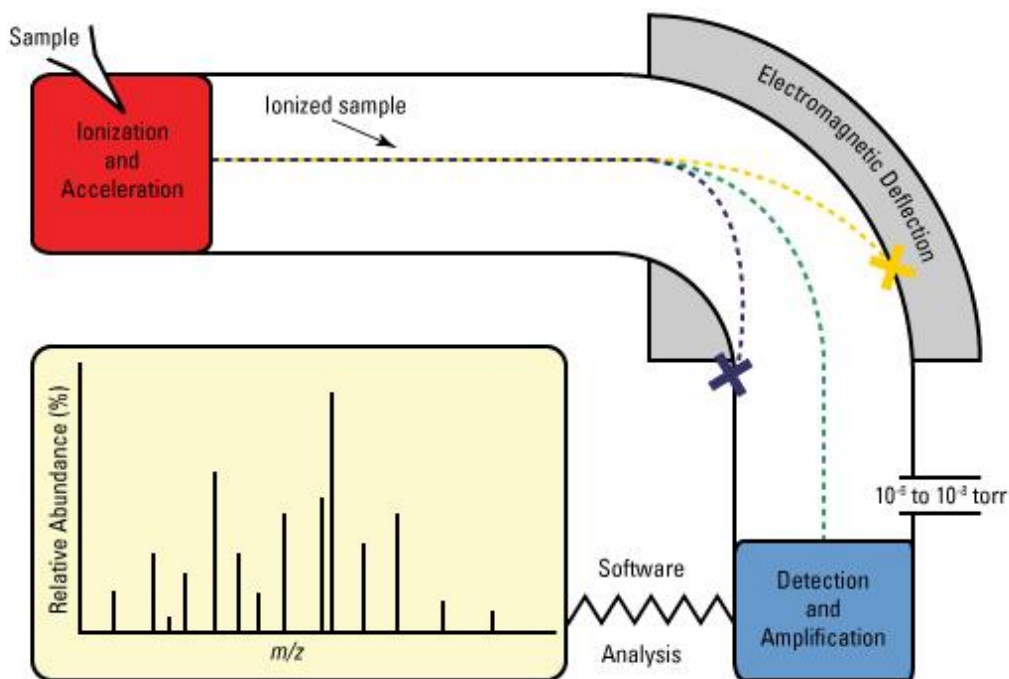


Figure 5: Basic Schematic of Mass Spectrometer

<http://www.piercenet.com/media/Mass%20Spec%20Diagram%20540px.jpg>

A mass spectrometer works by identifying and quantifying masses. Figure 5 shows a basic schematic of a mass spectrometer. Liquid samples are first vaporized and ionized by an ion source. Each molecule in the sample receives a charge and is accelerated into a chamber under an extreme vacuum. The ions are then deflected by an electric and/or magnetic field based on their mass and charge. The magnetic or electric field is then adjusted to focus the ions on to a detector. The mass and relative abundance can then be analyzed. As the ions are formed from the sample they may fall apart or fragment, resulting in an array of different masses. Isotopes can also be detected. These two factors can cause problems during metabolite identification so usually a separate

computer program is used to match the identified mass to the molecular weight of a molecule.

Thesis Outline

The over-arching goal of the research described in this thesis is to better understand the metabolic changes that chondrocytes exhibit when subjected to mechanical stimuli. Environmental factors play key roles in any mechanotransduction experiment, so controlling them was extremely important. A bioreactor capable of applying well defined static and dynamic loads was designed, manufactured, and validated. The next step was to define and characterize a material capable maintaining viable 3-dimension cell suspension in a physiologically stiff environment, this lead to the mechanical analysis of a viscoelastic material. Once, this was completed it was then possible to apply biomechanical loads to large populations of chondrocytes in a medium that allowed for metabolite extraction. The final step was to identify possible metabolites involved in the central metabolism of chondrocytes; that are being affected by mechanical stimulation.

MACHINE DESIGN

Description of Bioreactor

Various bioreactors are used to study the effects that mechanical stimuli have on chondrocyte mechanotransduction (Bougault et al., 2012a; Knight et al., 1998; Lee and Martin, 2004). A bioreactor can be any manufactured or engineered device that supports or simulates a biological environment. In order to move forward with mechanotransduction experiments a machine capable of simulating physiological loading environments had to be designed, manufactured, and validated. This chapter will go through the design process, identify key parts and components, and present validation data. The final concept design and delivered design are shown in Figure 6.

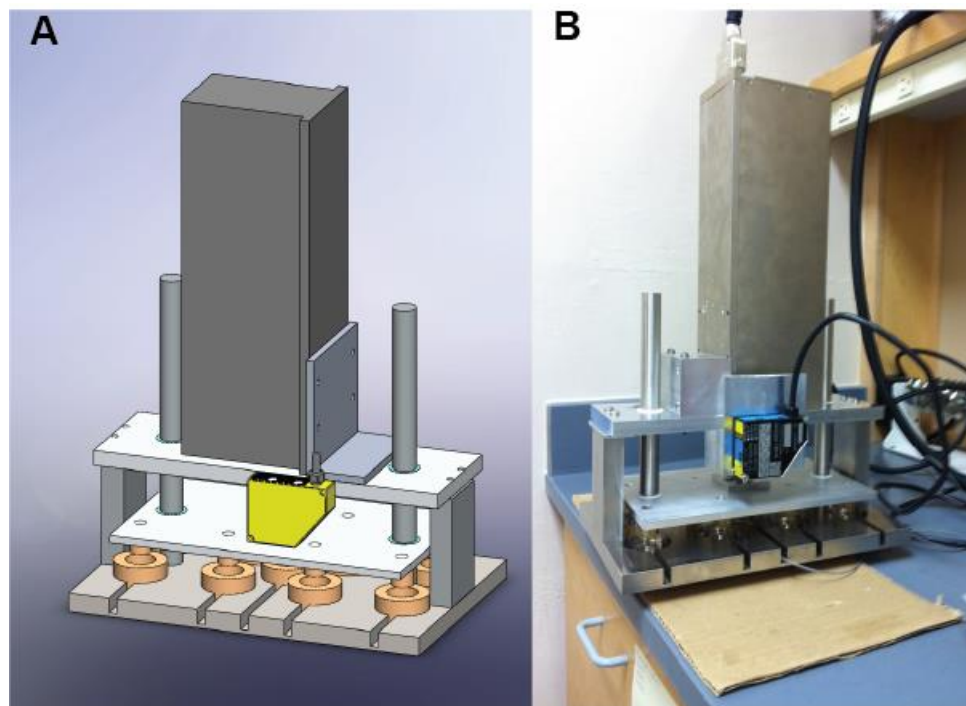


Figure 6: Bioreactor Design

Design Requirements

The basic purpose of this particular bioreactor was to apply well-defined compressive deformations to soft samples in a tissue culture setting. The following requirements were implemented after background research on past mechanotransduction experiments.

1. The system had to be able to apply both dynamic (cyclic) and steady state displacement loading over long periods of time with accuracy down to the single .01 mm.
2. Must be capable of applying compressions with high repeatability to 9 samples simultaneously, and have the ability to measure individual load responses on all 9 samples.
3. Allow for easy removal of samples
4. Fit inside standard tissue incubator
5. All parts had to be able to be autoclaved
6. Allow for various sample sizes (5 to 15 mm in height and 3 to 15 mm in diameter)
7. Have the ability to generate both load and displacement files documenting experimental results
8. Keep costs low

The best way to describe this machine is that it is a vertical press. The design of this machine can be broken up into a two different parts. The first being the loading apparatus and the second being the control system. The loading apparatus encompasses

all of the machined parts and components that go inside the incubator. The control system involves all of the electrical components, motion control, and data acquisition hardware. Some preliminary design information was needed before any design work could be done.

Preliminary Design Information

The most important component of this machine was going to be the vertical motion. It was decided that all of the generated motion was going to come from an electronic actuator, so finding one that met the requirement was very important. The actuator had to have a stroke of 15 mm, but the force requirements were not known. Building on a past in vivo experiment (Alexopoulos et al., 2005b) it was determined that the majority of samples we would be compressing had a stiffness of 55 kPa. There was never going to be a time when more than 25 % compressive strain was needed. So with a stiffness value known and a strain value known, induced stress was calculated to 13.75 kPa. Since stress is a function of force and area, the required force to compress 9 samples at once was calculated. It was determined that an actuator capable of applying 90 N would be sufficient for all future experiments. After much consideration the SMAC LAR95-015-72M was chosen.

The LAR95 is both a linear and rotational actuator. It was purchased for future experiments that might incorporate a rotational component, but this bioreactor only utilizes its linear moving core. The LAR95 has an onboard linear encoder with a resolution of .1 microns. It is programmable in force, velocity, and position control via a

single axis controller. Another reason why this actuator was chosen was that it can withstand corrosive environments and has been implemented in similar systems before.

Two more important components that influenced design were the load cell and displacement sensor. There were several options when it came to load and displacement sensors. Typical vertical compression machines implement an inline load sensor that can measure both tension and compression; this was not an option in consideration of the 9 separate samples. Since there was a height restriction a low profile load button sensor was chosen. Futek produced the lowest cost sensor with highest accuracy; factory specifications are presented in Appendix A. Multiple options were explored for displacement sensors from LVDTs to ultrasonic devices to laser displacement sensors. None of which possessed the necessary precision to verify the onboard linear encoder of the actuator at a low enough cost. In the end a laser displacement sensor was found to be the best option and an Acuity laser measurement sensor was purchased.

With the onboard non-manufactured components chosen it was possible to begin the design the loading apparatus. Materials were selected based on their resistance to corrosion and ability to be autoclaved. 6061-T6 was chosen for a few parts because of its resistance to corrosion, cost, and machinability. Type 316 stainless steel was utilized for strength, corrosion resistance, and ability to hold a tight tolerance. Type 304 stainless steel was used for its machinability and corrosion resistance. Polysulfone was used for its machinability, autoclavability, and non-reactive characteristics. An initial design was proposed (Figure 7) that met all of the level 1 requirements. It had eight parts that needed to be machined. As one of the design requirements it was extremely important to keep

costs low for this design project; so all machined parts were made in house using manual and CNC machining techniques. Before any sort of manufacturing took place design drawing were created for each individual part seen Appendix B. It was important to have a record of design drawing in case a part has to be remade in the future.

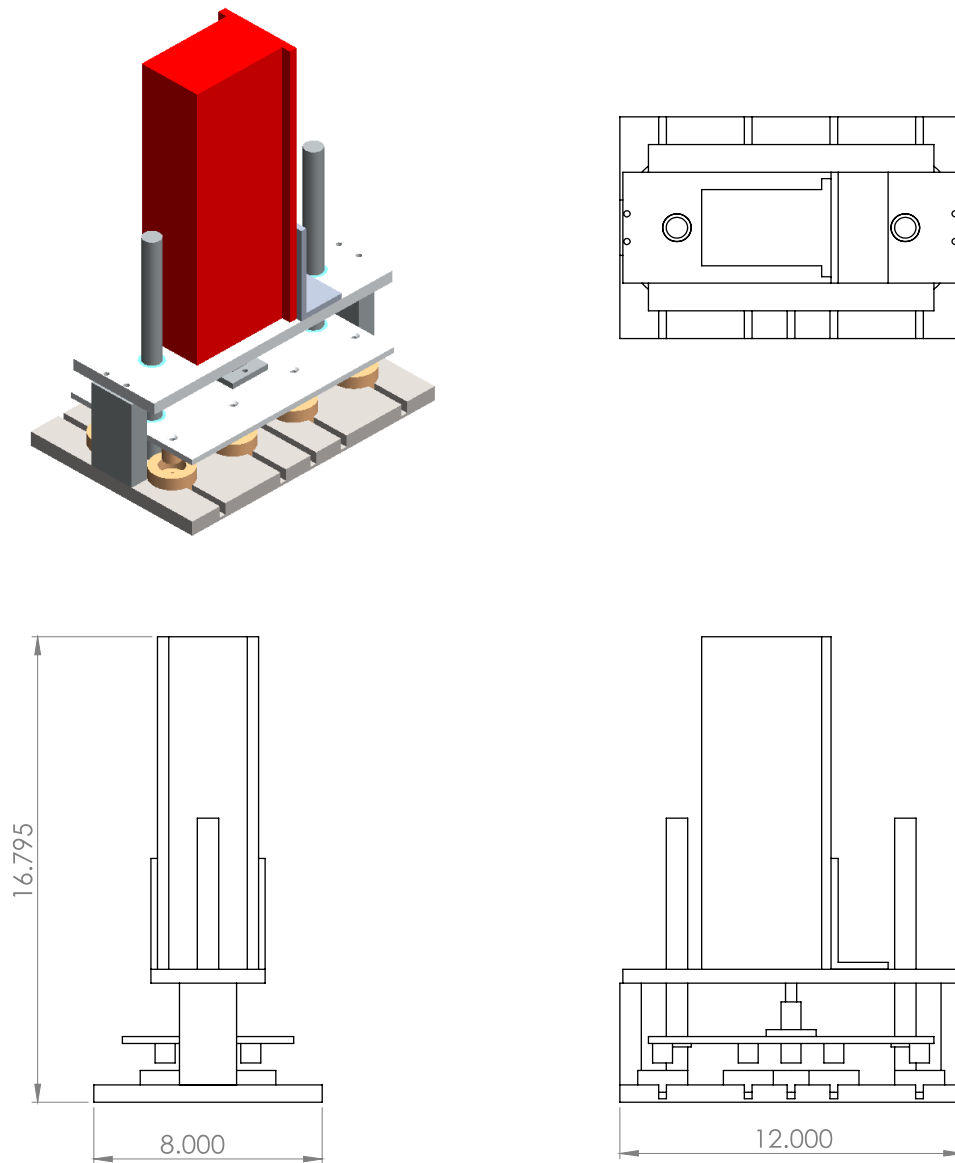


Figure 7: Initial Bioreactor Design

Critical Parts

A vital characteristic of this machine are its critical dimensions, which are in the vertical direction. It is extremely important to control the horizontal surfaces of any compression operation. Without well-controlled horizontal datums experiments can become inconclusive. To get this desired control some critical parts had to be machined.

The *Base Plate* see in Figure 8 is the cornerstone of the entire machine; it is made of ultra-corrosive resistant stainless steel providing a long service life inside of an incubator. The stock material was precision ground with a flatness of .005 millimeters and had the ability to hold tight tolerances. Nine cylindrical wells were machined into the top surface; each well can support one Futek subminiature load button load cell and one polysulfone specimen cup. A 6.35 millimeter wide channel was machined from the outer edge to the center of each well, this provided a place for the load cell wire to go. When assembled the specimen cup sits directly on top of the load cell, this allows for inline measurement of load. Also machined into the top surface are two counterbored guide holes. These guide holes allow for two vertical guide rods to be rigidly fixed to the baseplate. Built up from the baseplate is a frame that supports the actuator. The frame is composed of two gauge blocks and top plate parallel to the base plate.

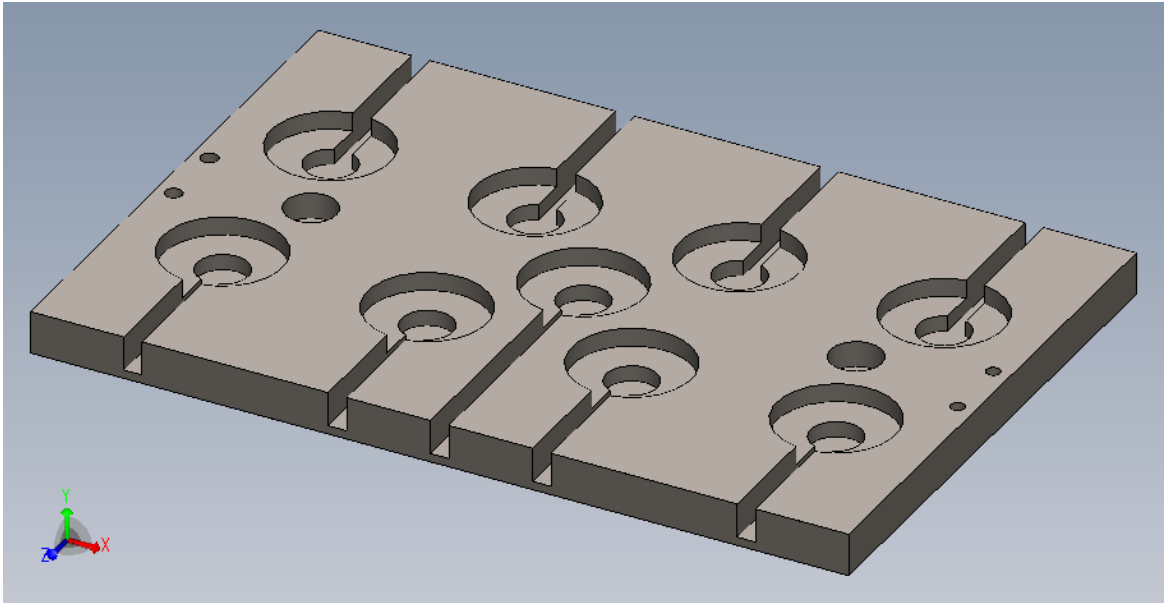


Figure 8: CAD Model of Base Plate

The *Top Plate* shown in Figure 9 is a half inch thick piece of aluminum with three pockets machined through it. The Top Plate supports the SMAC LAR95 actuator, the rectangular center pocket allows the actuator shaft to pass through the plate without any interference, while the other two circular pockets help align the vertical guide rods. A Teflon bushing was press fit into each circular pocket allowing for easy assembly. Also shown in Figure 9 are two gauge blocks that hold the top plate in place the proper distance from the baseplate.

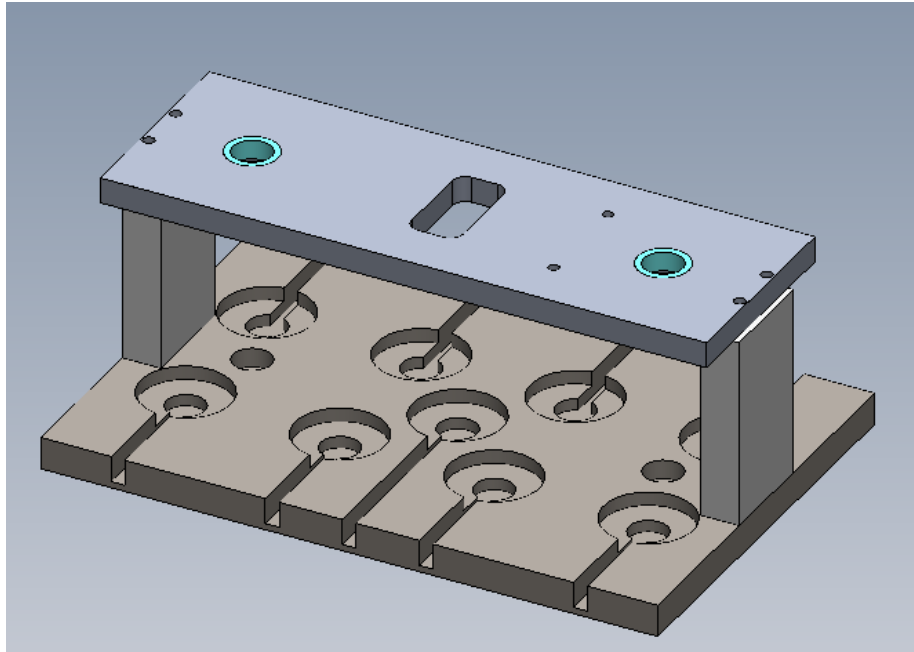


Figure 9: CAD Model of Loading Frame

The *Gauge Blocks* are made from stainless steel and provide a fixed distance of 3.67 inches from the baseplate. Both Gauge Blocks were machined using precision CNC machines, cutters, and techniques. Each Gauge Block is mounted to the baseplate and top plate with four stainless steel machine screws. Spacers (not shown) can be added to the top of the gauge block to change their stand-off distance. The spacers are precision-machined 7000 series aluminum for added hardness compared to normal aluminum. The spacers provide desired height changes to the Gauge Blocks they can be added at any time.

The *Vertical Guide Rods* allow for vertical translation in a consistent and repeatable manner. There are two vertical guide rods in this design, which can be seen in Figure 10. They are also made out of precision ground stainless steel, which provides a long surface life and a smooth surface finish. The vertical guide rods are pressed into

two counterbored holes located on the surface of the base plate and held in place with stainless steel fasteners. Having two vertical guide rods prevents rotation during compression experiments.

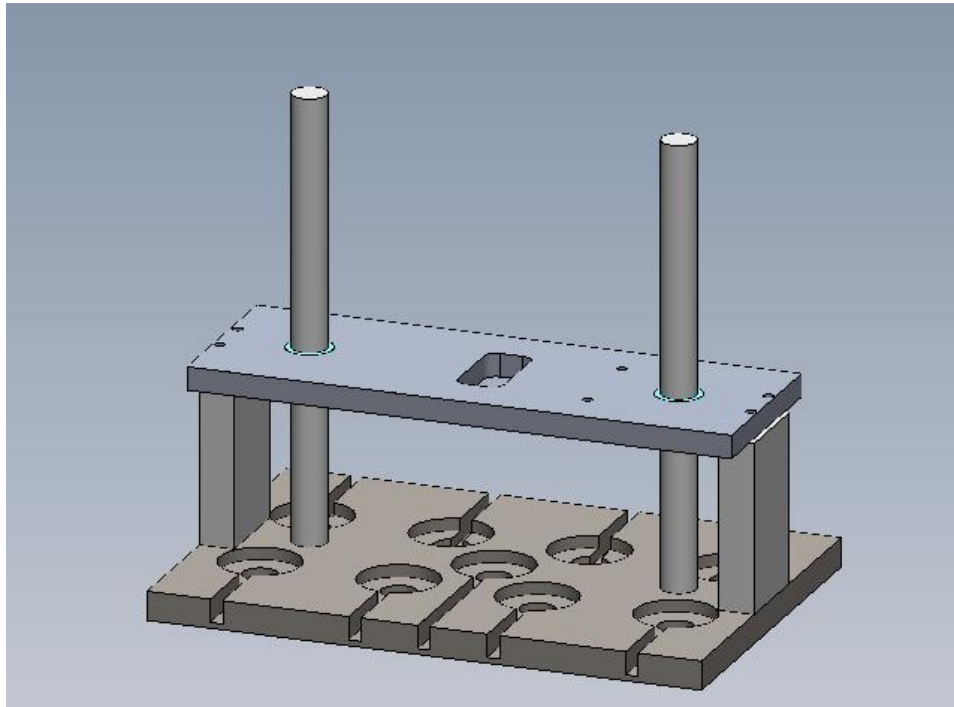


Figure 10: CAD Model of Vertical Guide Rods

All of the parts mentioned so far are fixed and rarely need to be removed. They provide a rigid structure capable supporting dynamic experiments. The next two parts are attached to the actuator and are considered moving mass.

The *Forcing Plate* highlighted by a yellow box in Figure 11 is made of aluminum and allows for multiple samples to be tested at the same time. It has a large surface area to attach nine polysulfone plungers to its bottom surface. The plungers are attached to the forcing plate by stainless steel machine screws. The forcing plate also has two large circular pockets that were machined to provide enough space for two more Teflon

bushings that were press fit into the top plate. These Teflon bushings are press fit into the forcing plate and contact the vertical guide rods. The bushings allow the forcing plate to move easily up and down along the vertical guides. Attached to the top surface is the mounting bracket.

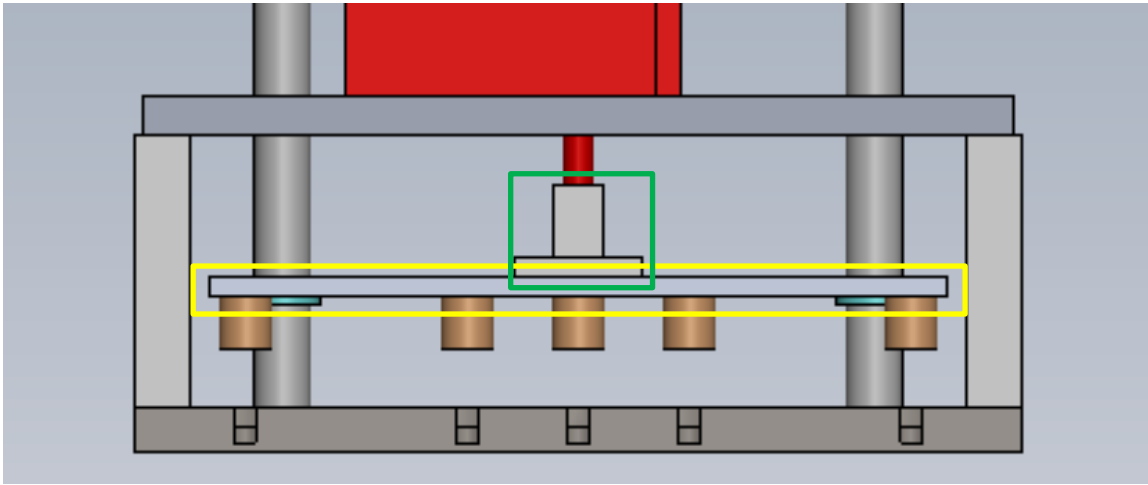


Figure 11: CAD Model of Forcing Plate

The *Mounting Bracket* highlighted by a green box in Figure 11 attaches the forcing plate to the SMAC LAR95 actuator. It is made of stainless steel, which provides a rigid connection between the forcing plate and the actuator shaft. It is mounted with four stainless steel cap screws and has a threaded hole through its center to allow for easy removal from the actuator.

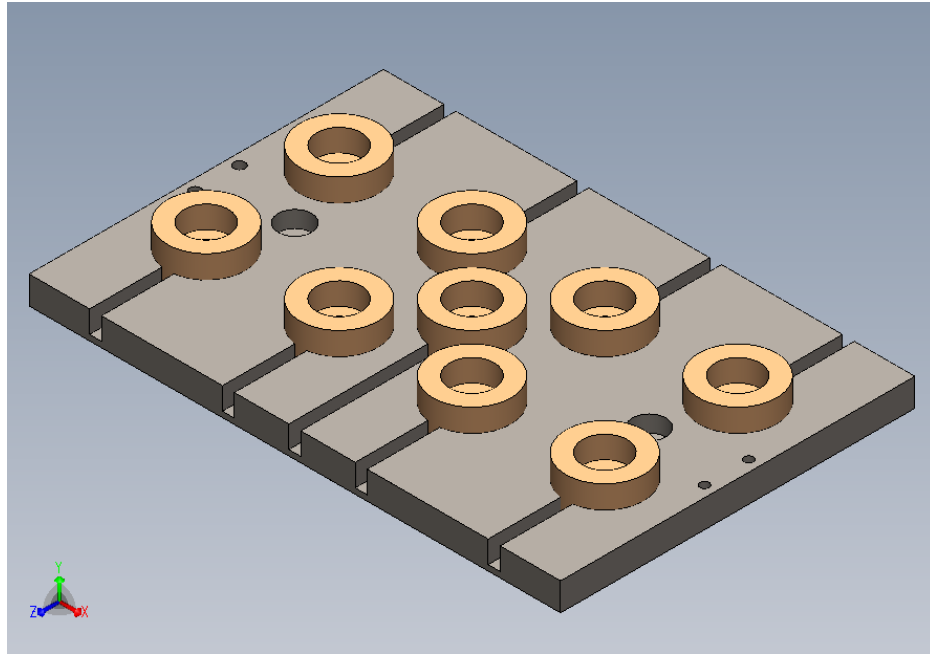


Figure 12: CAD Model of Base Plate with Polysulfone Cups

The *Polysulfone Cups* and *Polysulfone Plungers* are the only parts of the machine that come in contact with the test specimens. As the name implies they are made out of polysulfone. Polysulfone was chosen as the material because of its resistant to corrosion and its abilities to withstand high temperatures and pressure. As mentioned previously the polysulfone cups rest inside the wells of the Base Plate (Figure 12). These cups provide enough volume to completely submerge a fifteen-millimeter specimen in solution (PBS, DMEM, etc.). The cups can be easily removed and are interchangeable between wells. During testing symmetry must be maintained, if only one side of the machine has testing specimens it could create an unbalanced system. The polysulfone plungers attach to the forcing plate with stainless steel machine screws. The plungers contact and compress the tissue samples. They are small cylinders 17.8 millimeters in length and diameter.

Design and Manufacturing Challenges

The largest design and manufacturing challenges were the tight tolerances necessary for this bioreactor. All horizontal surfaces required a surface flatness of 0.025 millimeters. Standard mechanical engineering practices were utilized to determine specific fits. For example the interference location fit between the base plate and vertical guide rods. CNC machining techniques were utilized to overcome these challenges.

CNC stands for computer numerical control, so instead of a person operating a tool/cutter the machining process is automated. All critical parts were machined on a HURCO 4-axis mill with carbide cutters. Carbide cutters were utilized because of their ability to machine harder materials. Using CNC machines allowed for complex geometries to be machined at a relatively fast rate. Some examples of the CNC codes used during the manufacturing process can be seen in the Appendix B.

When using CNC machines a tool library has to be created and loaded in the machine. A cutting tool is what does all of the material removal and shaping of the part. Seven different tools were used to manufacture all of the parts for this bioreactor. The first three tools were drills a center-drill, a $\frac{1}{4}$ inch drill, and a $\frac{1}{2}$ inch drill. The last four tools were various different endmills: a 4-flute 1-inch carbide endmill, a 6-flute $\frac{1}{2}$ inch carbide endmill, a 4-flute $\frac{1}{4}$ inch carbide endmill, and a $\frac{1}{4}$ inch roughing endmill.

Even with the use of CNC machining techniques there were still machining challenges that had to be overcome. Stainless steel is a very hard material, which results in a lower machinability. When machining parts made from stainless steel it was extremely important to ensure there was no rubbing, caused by mechanical vibration or

tool chatter. If a machine tool dwells too long on one location stainless steel can work-harden during the machining process. If this happens cutters can wear out extremely fast or even break. To make sure this did not happen tools were inspected between runs and machining paths were used that only engaged the material.

Another manufacturing challenge was to produce a high number of identical polysulfone cups. A prototype cup was machined on a manual lathe and the process took a little over an hour. It was determined that making multiple cups the exact same dimension was going to be a daunting task, so a tombstone was designed that allowed five cups to be manufactured at once. A tombstone is a fixture that holds multiple parts during a machining process. This is a very common practice among machine shops, when high part volumes are needed.

This particular tombstone was designed to hold 44.45 millimeter polysulfone cylinders. 50.8 millimeter polysulfone round stock was turned down to 44.45 millimeters and then sectioned into 1 inch pieces. Each piece was then placed into the tombstone. The tombstone was then loaded into the CNC machine where all five cups were cut during the same program. Figure 13 shows CAD model of the tombstone. The finished product was made from 6061-T6 Aluminum. Each cup was held in place by a set screw throughout the machining process.

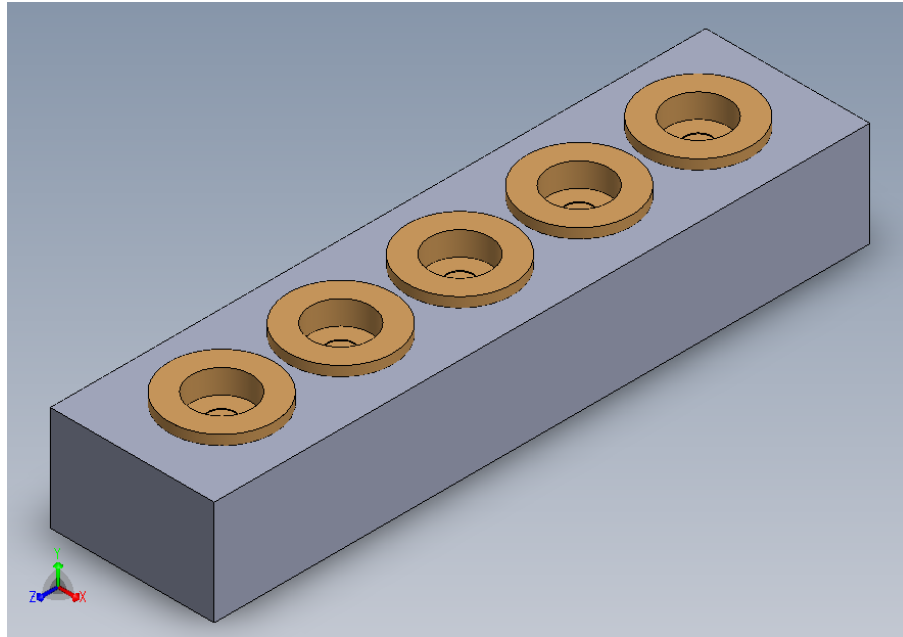


Figure 13: CAD Model of Tombstone

A major design challenge was determining what material to use for the forcing plate. The heavier the forcing plate the more moving mass the actuator had to deal with. Minimizing the moving mass of the system was a concern because of actuators recommended 40 % duty cycle. Original designs suggested titanium as an option but it was just too expensive. In the end aluminum was chosen and the system never exceeded its 40 % duty cycle limit. The only drawback of using aluminum is that the system could reach higher speeds if the forcing plate was lighter, but it achieves all necessary experimental velocities.

The last major design and manufacturing challenge became apparent during the assembly process. Due to the height constraints placed on the loading apparatus the overall design was very compact. This resulted in the assembly and disassembly of the machine to be non-trivial. There is a sequence that should be followed anytime the

loading apparatus needs to be taken apart. Appendix C has a step-by-step process for this purpose.

Incorporated Hardware and Software

Hardware:

- SMAC LAR95-15 Linear/Rotary Actuator with .1 Micron Resolution
- SMAC LAC-1 Single Axis Controller
- SMAC LAH-RED-03 Cables
- Acuity AR200-6 Laser Displacement Sensor
- Futek 10 lbs. Subminiature Load Button Load Cell
- Nation Instruments NI 9237 Bridge Analog Input Module
- Nation Instruments NI cDAQ-9171 USB Chassis
- Mean Well 300 Watt Power Supply
- Dell Latitude D810 Laptop

Software:

- Nation Instruments LabVIEW 2011
- Nation Instruments Measurement and Automation
- Windows Hyper Terminal

In addition to manufactured parts, purchased parts played a key role in the system. The basic wiring diagram for the control system is shown in Figure 14. It was desired to utilize only one independent power source. A Mean Well 300 Watt 48-volt power supply powers the laser displacement sensor and the LAC-1 controller. The LAC-1 controller draws three amps directly from the 48-volt power supply. In order to utilize the same

power supply a switcher circuit was built to step the forty-eight volts down to twenty volts necessary for the laser displacement sensor. Figure 15 shows the custom-built switcher circuit design for this system.

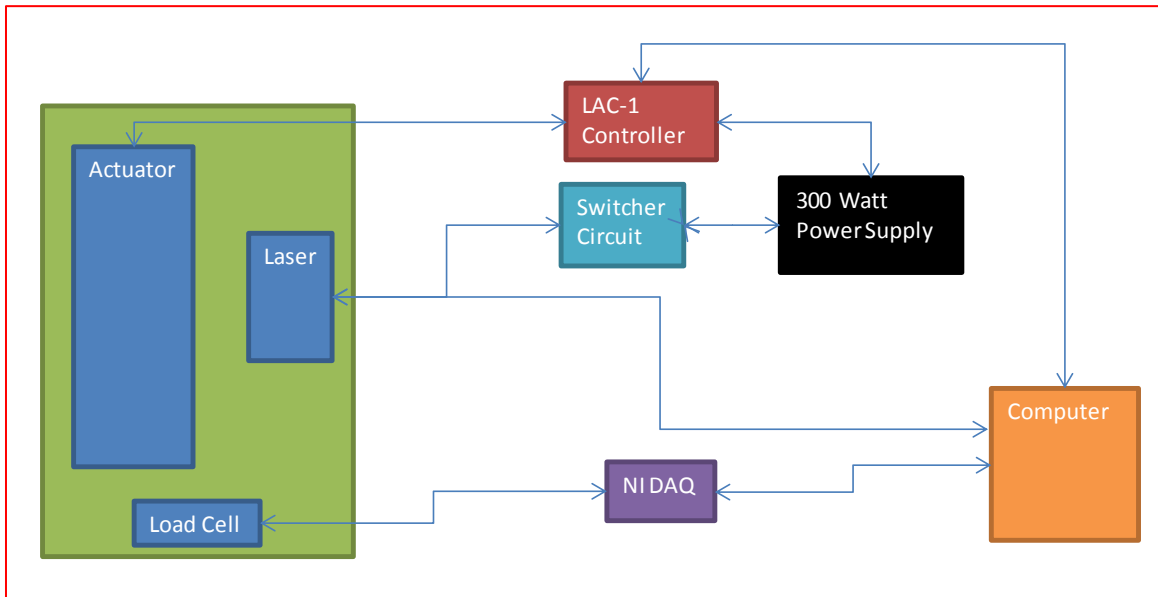


Figure 14: Basic Wiring Diagram

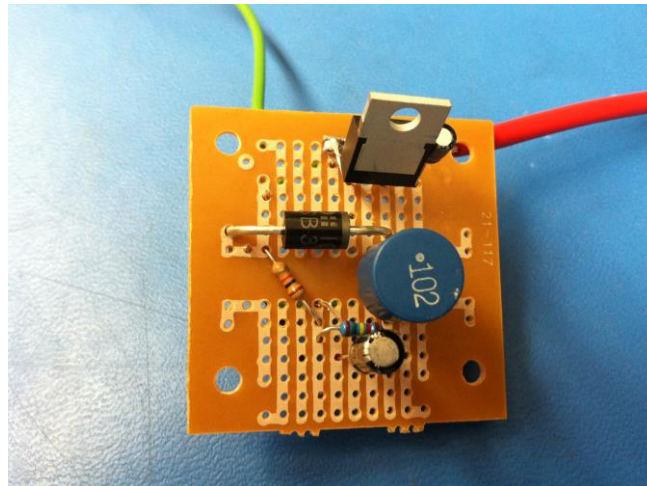


Figure 15: Switcher Circuit

As mentioned before, the only movement is in the vertical direction which allows for the actuator to be controlled by a simple single axis controller the SMAC LAC-1. The controller interface is through Windows HyperTerminalTM, which is a text based control system. The LAC-1 controller can hold over 250 lines of code in its non-volatile memory, it is in this on board memory where programs can be loaded and called on for execution. One computer is used to control both the actuator and data acquisition system.

The Dell Latitude Laptop was chosen to controller this system in part because of availability; this laptop previously existed in the June Lab. It was also chosen because it provided the necessary input ports for all of the components to plug into. The actuator/controller communicates through a serial interface and so does the laser displacement sensor. There are not many laptops that provide two serial interfaces, and most new computers do not even have one. The data acquisition system, which controls the load cell, communicates through a standard 2.0 USB port. This USB port provides enough power for the data acquisition system.

One of the design requirements was the ability to measure force and displacement independently from the actuator's linear encoder. This provided the ability to validate the actuator's accuracy and allows for experiments to generate larger data files. In order to measure displacement and force, two key instruments were built into the design of this machine; a laser displacement sensor that has the ability to detect distances on the micron level and a load cell that can measure the force that the actuator is applying to a given sample.

A laser displacement sensor (Acuity AR200-6) is a low cost device with relatively high accuracy. The AR200-6 has a span of 6.35mm and has a resolution of .03% of full span, which is better than of other displacement sensors in the same price range. The AR200 uses triangulation to measure distance. The laser is projected on to a target and then reflected back to a collection lens. The lens then focuses an image of the spot on a linear array camera. The position of the spot image on the pixels of the camera is then processed to determine the distance to target. The laser only has a span of 6.35 millimeters and a standoff distance of about 21 millimeters. The laser could only be mounted on to the side of the top plate and displacements reading are measured off of the forcing plate (see Figure 16). The laser is fully configurable; everything from sampling rate to units. It can either be configured by a manual function button on the side of the housing or through serial communication. All of the data generated by the AR200 sensor is sent over the computer's serial port. A LabVIEW program was then used to communicate between the laser and computer. For quick experiments where displacement data does not need to be recorded or for calibration tests, the AR200 can be used in conjunction with Windows HyperTerminal. It should be noted that only one device can be operated by HyperTerminal at a time, so it is not possible to control both the laser displacement sensor and the actuator under these settings.

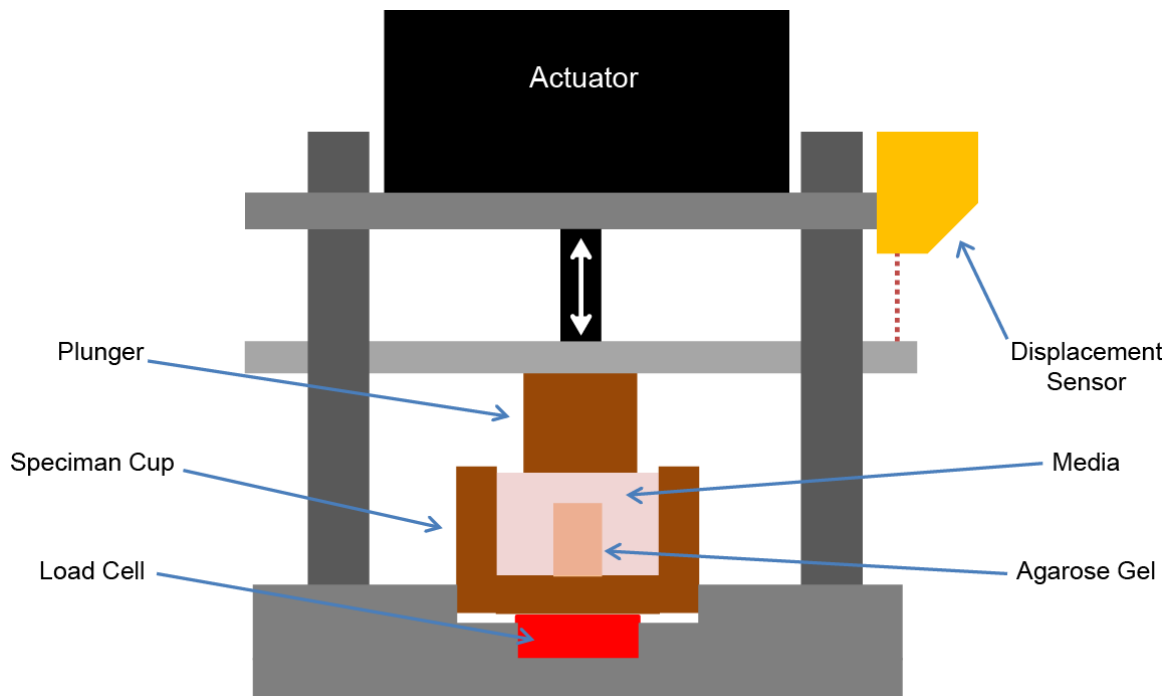


Figure 16: Schematic of Mechanical Testing System

The load cell (LLB300) sensor is built for inline compression testing (Figure 16). It is constructed out of 17-4 stainless steel, with an outside diameter of approximately 19 mm. It has an operating temperature of -60 to 250 °C. It has very low deflection characteristics of approximately .05 millimeters under full load. The sensor has a nonlinearity of .2%. Two sensors were purchased for testing they have ranges of 0-10 pounds (44.5N) and 0-25 pounds (111N). The sensor has a rated output of 2mV/V and requires an excitation voltage of 1 to 18 Volts. In order to use these sensors a data acquisition system (DAQ) had to be purchased. The DAQ had to be able to supply the necessary excitation voltage and have a built in amplifier to read the sensor's signal. The DAQ includes a National Instruments NI 9237 Bridge Analog Input Module and a

National Instruments NI cDAQ-9171 USB Chassis. This DAQ communicates to the computer by use of the same LabVIEW program.

Programming

All programming takes place through the Microsoft HyperTerminal™ interface. HyperTerminal is a simple computer communication program that sends commands over the laptop's serial port. HyperTerminal is not offered with newer windows operating systems so Windows XP was installed on the laptop. In order to communicate the actuator HyperTerminal needs to be opened and a new communication program has to be selected. Within HyperTerminal certain parameters need to be selected. Various programs have been designed, and the communication program necessary is labeled "Actuator".

Immediately after the system is powered on the LAC-1 controller can receive commands. To verify the system is communicating correctly press the ESC key, this should cause the greater than sign ">" prompt to appear on HyperTerminal command window. Commands sent to the LAC-1 controller should be standard ASCII characters and followed by a return to execute. At any time before the command line is executed, mistakes can be corrected through use of the backspace key. Also, if a command needs to be cancelled simply hit ESC and a new prompt sign will appear (Marks, 2005).

The system has the capability to operate under simple "command and execute" protocol and under pre-written programs. Command and execute protocols are useful when positioning the actuator or running simple tests. Pre-written programs are used

when the actuator is required to perform multiple moves in a row or execute different acceleration, etc.

All commands should follow the same syntax:

Command[Argument]<CR>

or...

Command[Argument], Command[Argument],
Command[Argument], Command[Argument], Command[Argument],...etc.

The majority of commands are two text characters followed by the argument. The argument will be a number with a range depending on the specific command for example:

MA10000,SV10000 <CR>

This command would tell the actuator to move to an absolute position of 1millimeter (MA10000) at a specific velocity (SV10000).

There are over 100 different commands broken up into different groups: parameter commands, reporting commands, motion commands, register commands, sequence commands, learned position commands, macro commands, register commands, and input/output commands. Definitions for each command can be found in the LAC-1 user's manual.

There are three different programming modes: force mode, velocity mode, and position mode. Force mode is open loop, which uses no feedback from the encoder. A desired force can be programmed and then executed by the actuator. Velocity mode allows for the actuator rod to move at a desired velocity, acceleration, force, and direction. Position mode allows for the actuator to move to various positions along its stroke at specified velocities and accelerations. All of the programs that have been written for this device are in position mode.

Position is determined by counts inside the linear encoder. The more accurate the encoder the more counts it has. Since the actuator purchased for this system had a linear encoder accurate to the .1 micron there are 150000 counts long the 15 mm stroke of the actuator. This means for every millimeter there are 10000 counts. For example if a desired position was 7.5 millimeters away that would be programmed as MR750000, with MR meaning move relative. This allows for very precise movements theoretically the actuator could move in increments of .0001 millimeters.

The LAC-1 controller allows for pre-written programs to be downloaded and stored in the controller's memory up to 255 command lines can be stored and called on later. Programs are organized by command line is called Macros and should be numbered in ascending order for example:

MD10,MN,PM,SV20000

MD11,MA150000,GO,WS300

MD12,MR4000,GO

MD13,GH,EP

MD stands for macro definition once downloaded to the controller it is possible to call on the macro later and execute the commands that followed it. Once a macro has been executed it will immediately begin executing the next macro in the sequence this is how programs can be developed. Programs do not have to be written in HyperTerminal, but rather an array of different software (notepad, word, excel, etc.) Appendix D has sample programs use for both material testing protocols and mechanotransduction experimental protocols.

Most controllers have certain parameters that can be adjusted to aid in optimization of a system the LAC-1 is no different. The LAC-1 controller uses a PID control algorithm to reduce errors in velocity, acceleration, and position. The PID controller is described by P-Proportional, I-Integral, D-Derivative these terms each represent a basic mathematical function. The Proportional gain reduces the majority of the error in the system. The Integral gain reduces the final error in the system. The Derivative gain provides damping and stability to the system, which helps with overshoot. These parameters can be changed at any time, although optimized PID values have already been found and should be utilized. Programing PID values is simple and follows the same command [argument] syntax as previously discussed.

Data Acquisition System

The data acquisition system for this machine has two main components; the National Instruments NI 9237 Bridge Analog Input Module, which is necessary for the load cell to operate and the serial interface for the laser displacement sensor. It was important that both sensors generate their own data file; this allows them to run

independently or at the same time. LabVIEW was used to compile all of the data from the sensors, control any noise in the system, and provide a user-friendly interface during experimentation.

This section will discuss the LabVIEW programs created for experimental testing. The programs created will be utilized for a variety of mechanotransduction experiments. Three programs were created: (1) for only the load cell, (2) for only the displacement sensor, and (3) for a combination of the load cell and the laser displacement sensor. The first program discussed will be the laser displacement sensor program. The AR200 laser displacement sensor communicates over a standard serial port interface. The default serial mode is RS-232, and data can be transferred at baud rates from 300 to 115.2K baud, in binary or ASCII formats.

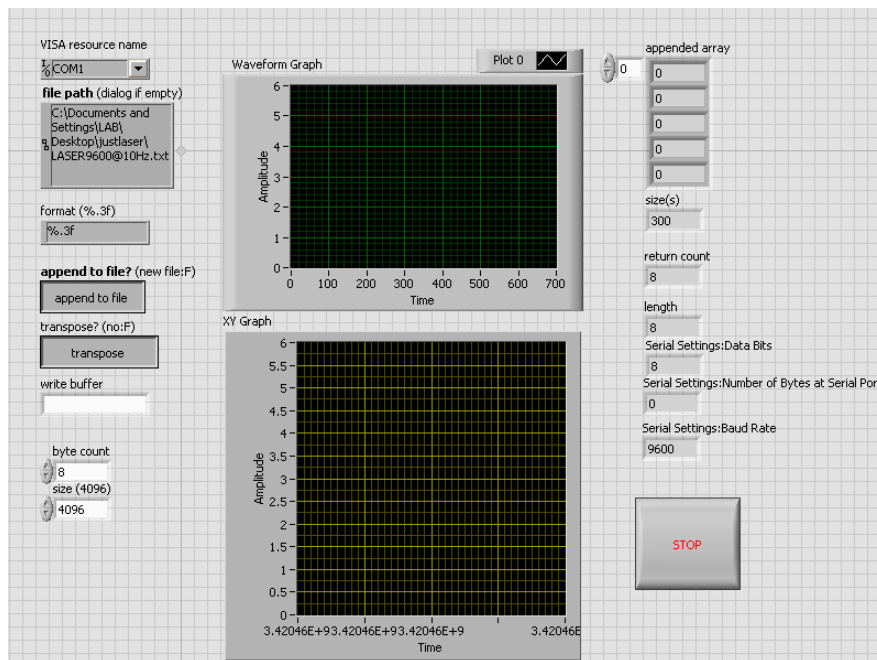


Figure 17: Front Panel for Laser Displacement Sensor Program

Shown in Figure 17 is the front panel for the laser displacement sensor program. It is here that program inputs are typed and the outputs from the program can be read. The front panel is organized so the inputs are on the left side and the outputs are on the right side. There are standard inputs that correspond to writing the data to a text file and there are inputs that are unique to serial communication.

The box titled *VISA Resource Name*, should always correspond to the com port that the laser displacement sensor is plugged into, which 99% of the time will be com 1. The *Write Buffer* input is for communication with laser if a setting needed to be changed on the device for example, sampling rate, this box is used. The *byte count* and *size* inputs specify the byte count expected and the size of the buffer. There are two charts that will graph the data as it is being gathered. The top graph has amplitude on the Y axis (mm) and counts on the X axis. The lower graph has amplitude compared to a built- in time stamp.

The output titled *laser data* will display the displacement reading in real time. The *array size* box displays the size of the array that is being built with the data. The *length*, *serial settings: Data Bits*, *Serial Settings: Number of Bytes at Serial Port*, *Serial Settings: Baud Rate*, and *return count* boxes are in place to help monitor the system. The data gathered is written to a text file that is specified in the *file path* box. The front panel also contains a *STOP* button; this is how all programs should be stopped. If a program is canceled by another means it is possible to lose data. The *STOP* button also has the fastest reaction time.

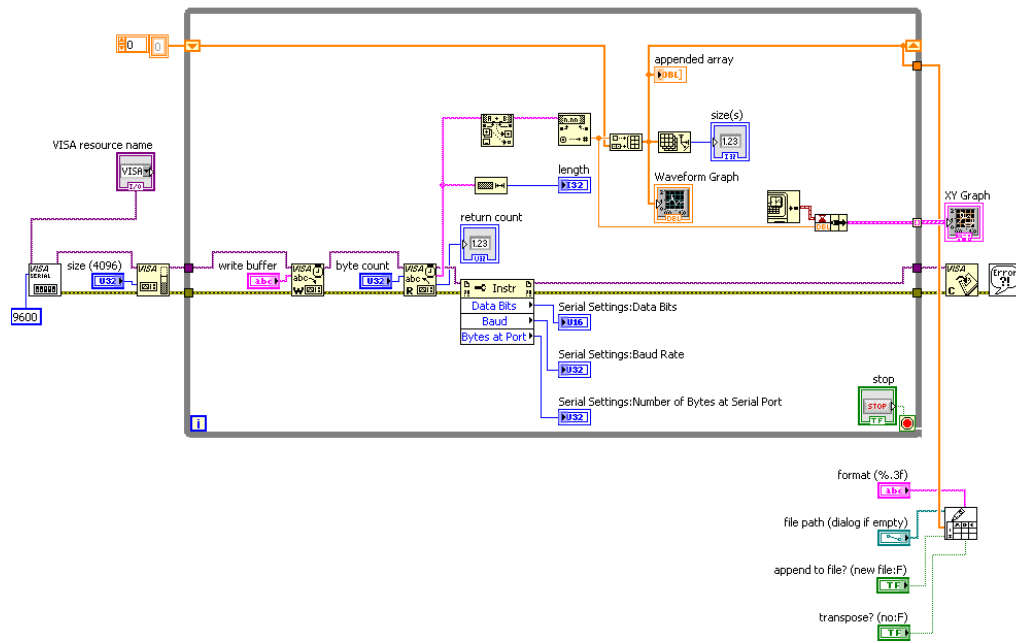


Figure 18: Block Diagram for Laser Displacement Sensor Program

Shown in Figure 18 is the block diagram for the same laser displacement program. This is a graphical representation of the program code. Key features of this diagram are the VISA Serial VI's and string to number conversions. The first VISA is the *serial configuration VI*, which simply configures a given serial port to except the data being transmitted. Most of the options are set to default and will not need to be changed. The next VI shown establishes the buffer size, this should not need to be changed either. The first 2 VI's inside the loop allow for the read and write functions of the program. Without the read function data would not be gathered. However the program can be run without the write function and can be removed if need be. The read function produces a string (pink line) that is first stripped of its tokens and then is converted into a number (orange line). Once the string has been converted an array is built using shift registers.

This array is then written to a text file, using the *write to spread sheet* VI. The final VI is the *close serial* function that allows for the com port to be used by other programs when the *STOP* button has been pressed.

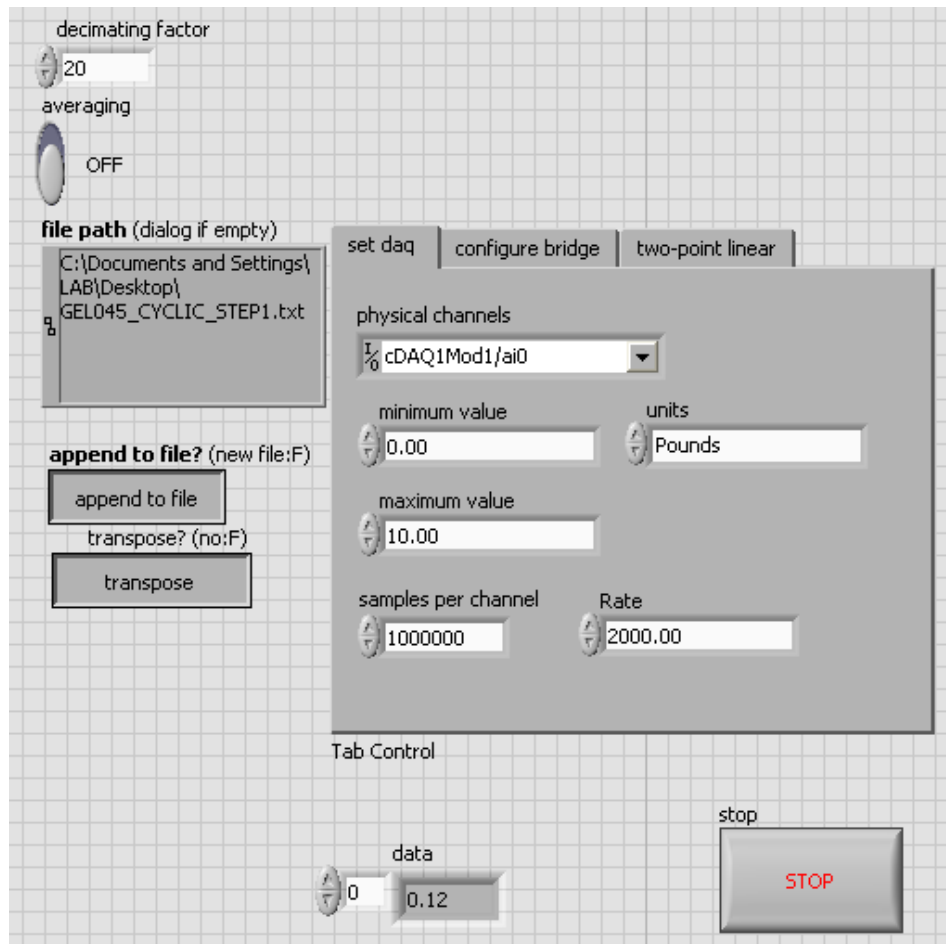


Figure 19: Front Panel for Load Cell Program

Shown in Figure 19 is the front panel for the load cell program. Like the laser program inputs are required for the program to run. (Note: In both programs the most recent inputs are saved as the default values, which saves the operator some time) Just like the laser program a file path needs to be created in order to store data. The data

acquisition system used has four channels so the correct channel has to be selected for the program to run. All of the data acquisition parameters can be changed from the front panel, but should never need to be. The only inputs that should be changed from test to test are the *Rate* and *samples per channel*. The rate specifies the number of samples to be taken in one second. The minimum value is 1600. Since this program is set up to take continuous samples until the *STOP* button is pressed the *samples per second* box just specifies the buffer size. One of the most important inputs for this program is called the *Decimation Factor* (top left), this allows for data to be sampled at a slower rate. For example if sampling rate is set to 2000 s/s and a decimation factor of twenty is used actual sampling rate would now be 100 Hz. The outputs from this program are displayed in the top right corner of the front panel. The important ones here are the time stamp show in the *current time* box and the *data* box. The data box shows the load in real time. It is important to keep an eye on this number, since the load cell has a maximum value which should never be exceeded.

Shown in Figure 20 is the block diagram for the load cell program. This program uses DAQmx VI's to configure the data acquisition equipment and generate usable data from the load cell's voltage outputs.

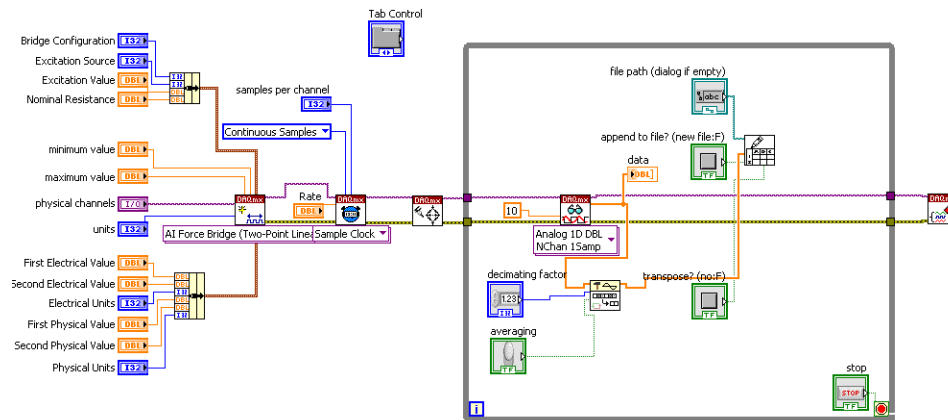


Figure 20: Block Diagram for Load Cell Program

The first shown is the *Create Virtual Channel VI*, this allows for the proper testing application to be chosen. The application for this program is a two point linear force bridge. All of the bundles and other indicators are the inputs from the front panel. The next VI is the *Sample Clock*. The *sample clock VI* is very important because it indicates whether the data will be sampled continuously or if it will sample a finite amount of points. It also sets the buffer size and the sampling rate, both of which can be changed from the front panel. The first VI inside the loop is the *DAQmx Read VI*. From here the data can be read many different ways and put into many different formats (*e.g.* waveform, number, dbl, etc.). This program utilizes 1D DBL format, which then can be put into an array and written to a text file. The last DAQ mx VI in this program is *DAQmx Clear Task*. The whole point of this VI is to avoid allocating unnecessary memory to the program after it has been finished.

Shown in Figure 21 is the combination program front panel. This program combines the laser displacement sensor and the load cell programs into one. The block diagram is simply these two programs in parallel, with one STOP button. The loops start at the same time and write to two different text files. Displayed on the front panel are the inputs and output just like the other two programs. The inputs are organized into tabs, which can be cycled through to either the laser inputs or the load cell inputs.

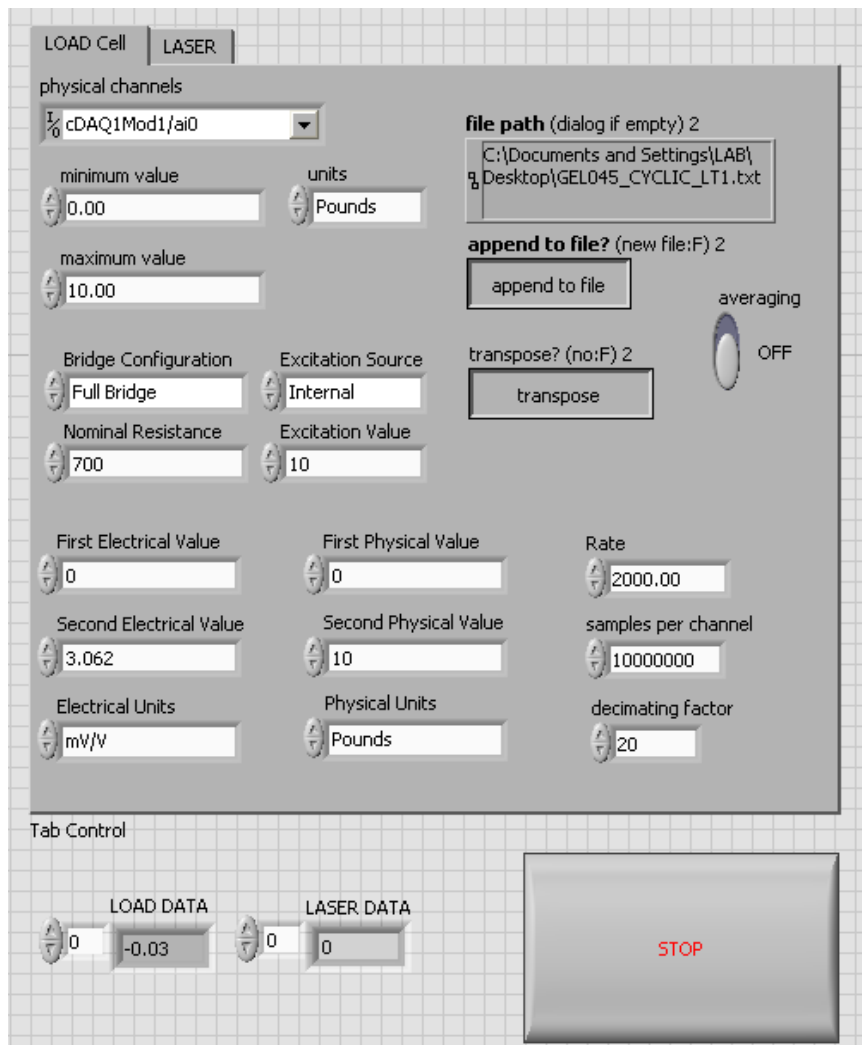


Figure 21: Front Panel for Combination Program

These LabVIEW programs allow for future experiments in mechanotransduction to be well controlled and well documented. One problem that arose during experimentation with the combination programs was that the data files would get so large the computer would crash. It was determined that when running both sensors at the same time a sampling rate of 100 Hz should not be exceeded. A newer laptop with at least two processing cores would be recommended to avoid this problem in the future. After the DAQ programs were finished it was possible to run validation tests on the newly designed system.

Validation of Bioreactor

Validating the Bioreactor was extremely important. Without confirming the capabilities of the machine, any experiment done with it could be thrown out or not trusted. Also with any mechanotransduction experiment controlling the mechanical stimuli as best as possible should be a number one priority. The first step was to identify proper PID control parameters to ensure stable motion control. Once this had taken place initial validation tests were performed on system to assess static and dynamic motion capabilities. Subsequent tests were performed six months later to revalidate the bioreactor's capabilities.

PID Control Parameters

SMAC provided initial PID values of (SG5, SI10, SD120) that were used as a starting point for determining motion control parameters that could be used for a variety of different motion profiles *e.g.* sinusoidal motion. As mentioned in the programming

section PID controllers utilize three parameters to stabilize the system: proportional gain, integral gain, and derivative gain. In order to test PID values the actuator was programmed to move to a given location and stop. The entire displacement curve was recorded by the onboard linear encoder and collected in the HyperTerminal command window. From the data collected the position error and stability could be determined.

The initial values provided by SMAC produced a very stable system, but with a position error of approximately 1-5% of proscribed displacement. To reduce this position error the proportional gain (SG) of the system had to be raised. By incrementally raising the SG parameter the overall position error was reduced to $< .5\%$. The position error was further reduced by incrementally increasing the integral gain (SI) until the over error was $< 1\mu\text{m}$ no matter the proscribed displacement amount. It was determined that an SG40 and an SI50 would produce motion profiles with very low position error, but the tradeoff was a system that was not stable at higher velocities or under sinusoidal motion. When the system becomes unstable the actuator begins to oscillate uncontrollably, which could result in damage to the loading apparatus. The derivative gain parameter (SD) was used to maintain stability. It was determined that a parameter setting of SD320 maintains stability through all motion profiles tested.

Initial Validation

To validate and test the repeatability of the bioreactor, dynamic and static testing protocols were repeated. The system first was given prescribed displacement ramp rates of 0.1 mm/s and 10 mm/s which were followed by ramp to position tests (RTP) with displacements of 1mm, 4.99mm, and 5mm. These tests evaluated both ramp velocity and

steady-state position control. Dynamic sinusoidal motion tests (DSM) were also performed at rates of 0.5 Hz, 1 Hz, and 5 Hz with amplitudes of 50 microns up to 1mm. These tests allowed for validation of possible physiological loading experiments.

The system was found to have the ability to repeatedly apply load very slow at a rate of $99.7 \pm 0.2 \mu\text{m} / \text{s}$ and very fast at a rate of $9.83 \pm 0.14 \text{ mm} / \text{s}$ across the entire range of the machine Table 1 displays data compiled analyzing ramp velocity for over 60 tests.

Table 1: Ramp Velocities

Proscribed Displacement (mm)	Slow Ramp Velocity ($\mu\text{m/s}$) n=10	Fast Ramp Velocity (mm/s) n=10
1	$99.4 \pm .03$	$9.78 \pm .05$
4.99	$99.7 \pm .004$	$9.69 \pm .29$
5	$100 \pm .005$	$10.02 \pm .03$

Confirmation of steady-state capability was found by looking at the RTP test. Figure 22A shows a representative RTP test that exhibited a standard deviation of less than $.3 \mu\text{m}$. Most other RTP test exhibited average standard deviation less than 0.437. Steady state conditions have since been evaluated for 2-hour RTP tests with no distinct variation from these numbers. Figure 22B shows results from twenty RTP tests for a displacement of five millimeters plotted against a proscribed velocity. The blue X's show measured ramp velocities for a proscribed velocity of 0.1 mm/s and the red X's show the measured ramp velocities for 10 mm/s. In most cases the standard deviation is so small the X's appear right on top of the proscribed velocity line. Figure 22C shows a similar

chart, but for proscribed ramp displacements. Blue X's indicate measured displacements from a 1mm proscribed ramp displacement, while red X's indicate measured displacements from a 5 mm proscribed ramp displacement.

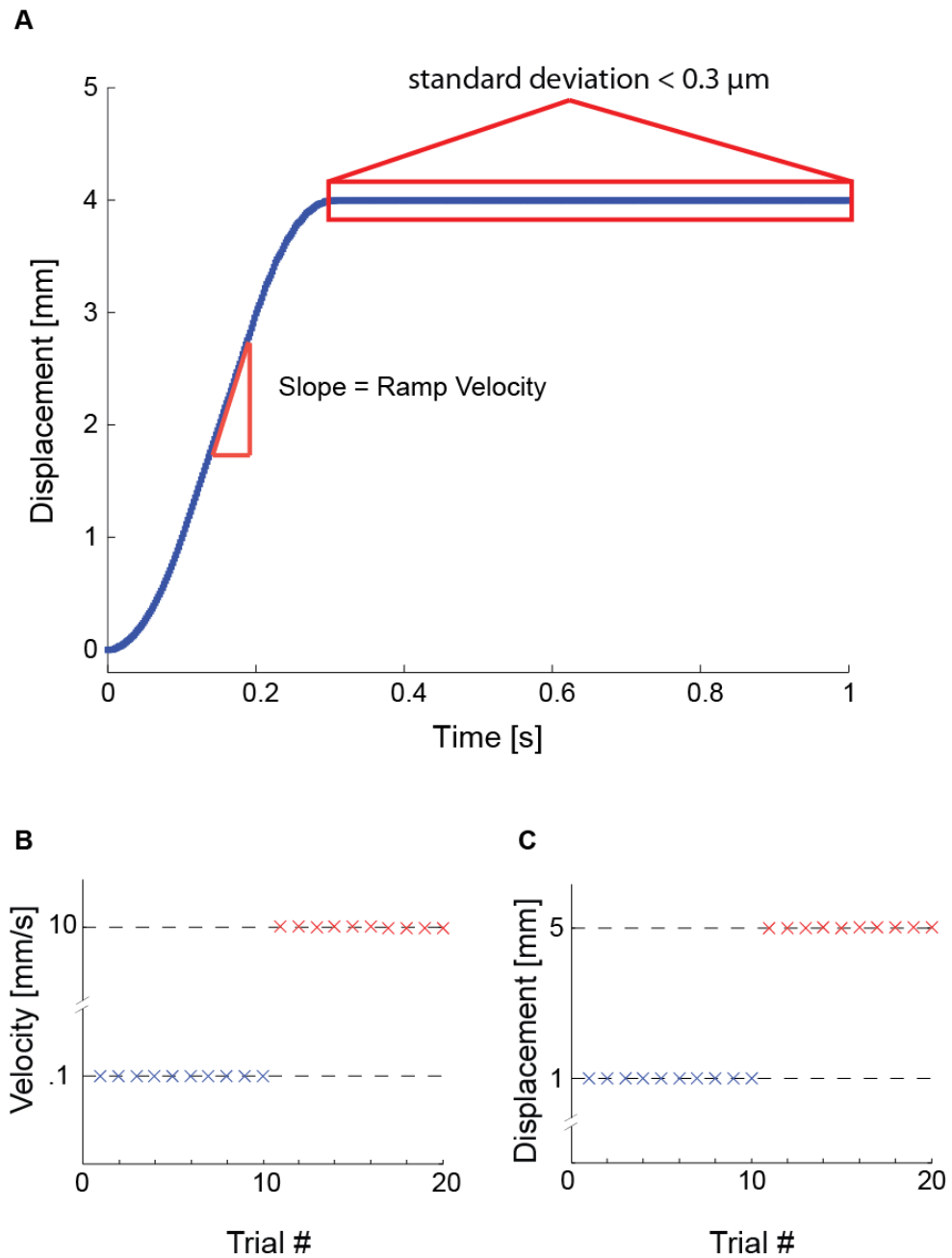


Figure 22: Representative Ramp to Displacement Test

DSM tests were validated first by comparing the laser displacement sensor to the linear encoder built into the actuator and second by comparing amplitude peak values over multiple cycles at the specified frequencies (Figure 23). Student's t-test revealed no statistical differences between mean peak values for laser and encoder. Validation data was compiled from over 140 experiments. For a controller input of 1 mm amplitude: the system produced the following amplitudes $998 \pm 0.167 \mu\text{m}$ at 0.5 Hz, $999 \pm 7.71 \times 10^{-2} \mu\text{m}$ at 1 Hz, and $1000 \pm 0.170 \mu\text{m}$ at 5 Hz.

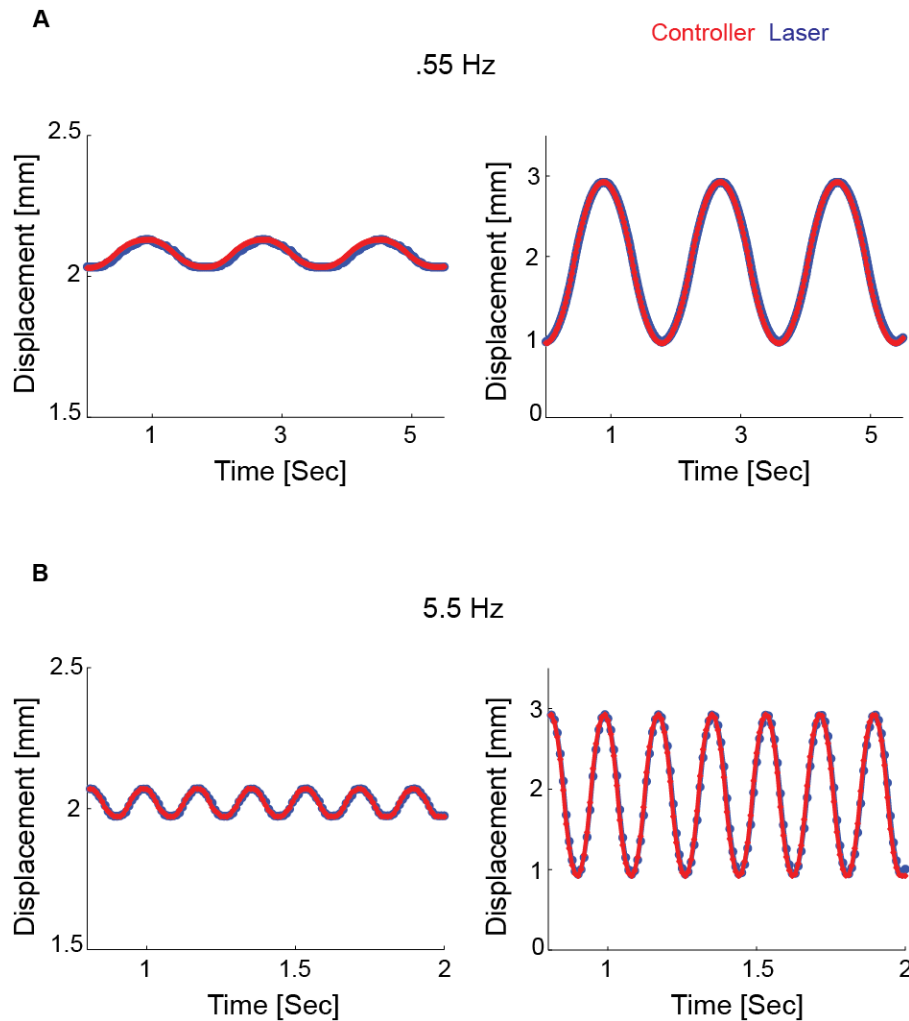


Figure 23: Sine Wave Verification

Subsequent Validation Tests

RTP test were performed at proscribed velocities of 0.1 mm/s and 10 mm/s for 1mm and 5 mm displacements. DSM tests were performed at 0.5 Hz and 1 Hz with amplitude of 1 mm. From these tests ramp velocity was determined along with steady state displacements and sinusoidal amplitudes. Table 2 shows the results from the ramp velocity tests. Bioreactor again displayed the ability to achieve very slow ramp rates as well as very fast ramp rates. These results were consistent with previous measured ramp rates.

Table 2: Subsequent Ramp Velocities

Proscribed Displacement (mm)	Slow Ramp Velocity ($\mu\text{m/s}$) n=6	Fast Ramp Velocity (mm/s) n=6
1	$99.9 \pm .005$	$10.01 \pm .016$
5	$100 \pm .007$	$9.94 \pm .034$

Also documented during RTP tests was distance travelled. It was determined that for 1millimeter and 5 millimeter proscribed displacements the system achieves less than 0.1 % position error. This was determined by assessing the deviation between proscribed value and measured value Results from DSM test were very consistent as well. Measured amplitudes for 1 Hz sine wave were $0.99 \pm .004$ mm and measured amplitude for .5 Hz sine wave were $0.98 \pm .008$ mm. Student's t-test revealed that mean values from laser displacement sensor were not significantly different from those of the onboard linear encoder.

Machine Design Conclusions

The bioreactor system built met all of the original design requirements. One of the most important requirements was that it fit inside a tissue culture incubator. Figure 24 shows the loading apparatus inside double stack incubator. The incubator's glass door had to be replaced by a custom-made plexi-glass door, but other than that no modifications had to be made. The power and control cables fit through a hole in the new door. The DAQ, power supply, and computer all fit on top of the incubator.



Figure 24: Loading Apparatus inside Incubator

The validation experiments confirmed the ability to apply static and dynamic loads with relatively high accuracy and repeatability. All machined parts can withstand a corrosive environment of 100% humidity at 37° Celsius and can be sterilized via autoclave. Nine samples can be tested at once while being maintained in a physiological environment. Future validation test could be performed to assess the loading variability between samples upon the purchase of eight additional load cells. The design, assembly, testing, and optimization of the components described has resulted in the creation of a bioreactor capable of applying a wide range of *in vitro* loading environments to soft tissues and 3D cell cultures. It is the hope that this machine will contribute to further understanding of mechanotransduction.

CHARACTERIZATION OF PHYSIOLOGICAL STIFF MATERIAL

Development of the bioreactor allowed for in vitro studies capable of modeling physiological loading situations. The next step was characterizing a material that would provide a physiologically stiff environment, while maintaining cell viability. For mechanotransduction studies, chondrocytes are typically cultured in three dimensions using a variety of hydrogels including photo-crosslinked polyethylene glycol (Farnsworth et al., 2013), self-assembling peptides (Kisiday et al., 2009), alginate (Haudenschild et al., 2011), and agarose (Bougault et al., 2012a; Knight et al., 2006; Lee and Knight, 2004). Agarose is of primary interest because of its ability to create gels with stiffness similar to that of the cartilage PCM (Normand et al., 2000).

As mentioned before articular chondrocytes reside within a pericellular matrix (PCM), which defines their immediate surroundings.

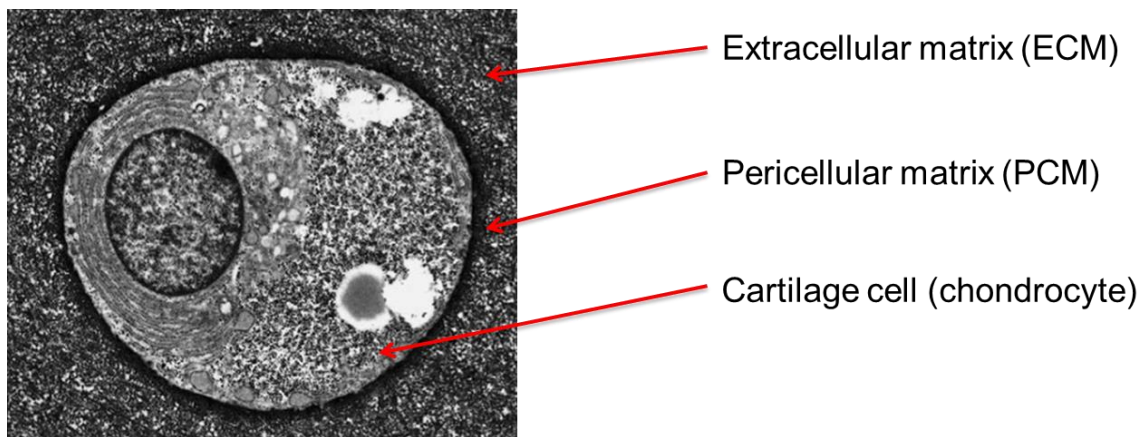


Figure 25: Typical Articular Chondrocyte within ECM

Figure 25 shows a chondrocyte (*i.e.* cartilage cell) in its microenvironment; the interface between chondrocyte-PCM and PCM-ECM can be seen. The interconnection

between the PCM and chondrocyte provides both molecular and mechanical signals. One biomechanical cue, which alters cellular phenotype and promotes cell differentiation, is stiffness. The PCM environment provides a unique stiffness for the chondrocyte. The PCM is not nearly as stiff as the ECM. Using atomic force microscopy (AFM) it has been shown that the elastic moduli for the PCM is approximately 25-200 kPa, while ECM stiffness is in the range of 130-573 kPa (Darling et al., 2010). Analysis has also compared the PCM of healthy chondrocytes to OA chondrocytes. Using micropipette aspiration, differences in PCM stiffness have been found between normal and osteoarthritic chondrons (*i.e.* extracted chondrocyte and associated PCM-like material), with Young's moduli values of 38.7 ± 16.2 kPa for healthy chondrocytes and 23.5 ± 12.9 kPa for OA chondrocytes (Alexopoulos et al., 2005b). To create a physiologically-stiff 3D hydrogel culture environment for chondrocyte mechanotransduction studies, agarose concentrations were needed that could match these stiffness values.

Agarose is a polysaccharide that when mixed with a liquid buffer forms a porous matrix capable of encapsulating living cells. It is considered to be a viscoelastic polymeric material. Under deformations viscoelastic materials can exhibit both viscous and elastic behavior. This makes the material's stiffness harder to characterize from a basic mechanics standpoint. The viscous component gives the material a time-dependent behavior, which is very common of polymeric materials. Two widely utilized experiments that characterize the modulus of viscoelastic materials are (1) stepwise stress-relaxation test (SSRT) and (2) cyclic loading test (CLT).

The SSRT is a large strain experiment, where a prescribed strain is applied to the sample (*e.g.* compress the sample to 90% of initial height), that strain level is held constant for an extended period of time. The stress in the material has an initial dynamic response, and then begins to relax over time to an equilibrium value. Two important characteristics can be determined from this experiment: a dynamic modulus and an equilibrium modulus. The dynamic modulus is representative of initial dynamic response and takes into account the peak stress, where the equilibrium modulus is a measurement of the materials stiffness after relaxation.

The CLT used in this project is a small strain (*e.g.* infinitesimal) experiment, where a sample is deformed (strained) sinusoidally. Within a few cycles of loading the stress will begin to oscillate sinusoidally at the same frequency, but will be shifted with a phase lag. A Complex Modulus can be calculated, which is representative of an in-phase elastic modulus and an out-of-phase loss modulus (Macosko, 1994). Both of these tests provided a greater understanding of agarose mechanics.

There are many different types of agarose used for a variety of laboratory tasks. Most agarose can be broken up into two categories: high gelling temperature and low gelling temperature. The gelling temperature refers to the point at which an aqueous agarose solution forms into a gelatin state upon cooling. This occurs after a solution of agarose and a liquid buffer has been heated past the melting point of the agarose powder. The gelling temperature plays a key role in chondrocyte encapsulation. It is not feasible to encapsulate living cells into high gelling temperature agarose, because gelation starts at a temperature too high for cells to survive in.

High Gelling Temperature Agarose

Preliminary experiments were performed on (Allele: AHP-CH-AGR0250) agarose, a sample product, which allowed for methodology to be tested. It was understood beforehand that high gelling temperature agarose would most likely have different mechanical properties than low gelling temperature agarose. SSRT were performed to calculate dynamic and equilibrium stiffness values for agarose gels at various concentrations.

Methods

Agarose gels were prepared with a phosphate buffered saline (PBS) solution at concentrations of 1.5, 3, 4.5, and 6 % weight per volume [w/v]. PBS is a common buffer used in a variety of biological research. Final agarose solutions were cast in a custom made anodized aluminum mold (Figure 26). This mold has the capability to cast up to nine samples at once with a height of 12.7 millimeters and with diameters of 3, 5, 7, 10, 12, and 15 millimeters. Each well was filled until the agarose/PBS solution overflows onto the top surface. The mold is then allowed to cool and the excess gel is removed via razor blade. The samples are then removed by gently pushing a glass stir rod through the hole.

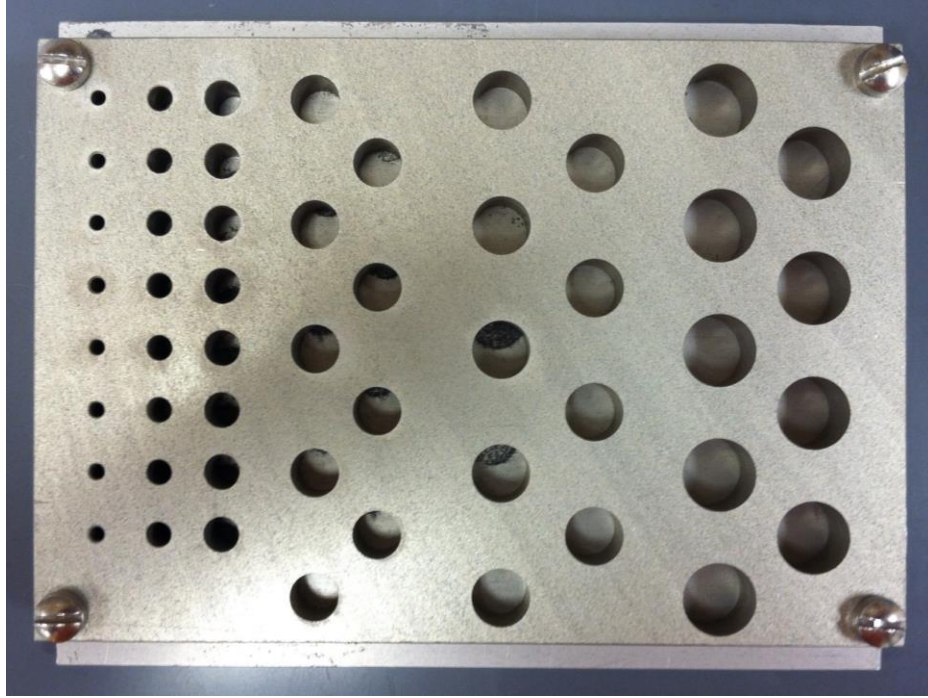


Figure 26: Agarose Mold

For these experiments the mold produced cylindrical gels with heights of $12.7 \pm .1$ mm and diameters of $7.0 \pm .1$ mm. For these tests, samples were subjected to 4 steps of 5% nominal compressive Lagrangian strain at room temperature. Each strain step was maintained for 10 minutes. Load data was sampled at 100 Hz for the duration of the test. Average stress was calculated by normalizing the load data by the sample cross sectional area (Equation 1).

$$\sigma = \frac{Force}{Area} = \frac{Load}{\pi r^2} \quad \text{Equation 1}$$

Each prescribed strain step induced a peak stress followed by relaxation to an equilibrium stress. The material's viscoelastic response is apparent in the representative stepwise stress-relaxation experiment shown in Figure 27.

MATLABTM was used to determine peak and equilibrium stress values along with dynamic and equilibrium moduli. The MATLABTM code utilized lets the user select a window around each load peak and then determines the maximum values within that window. Equilibrium values are found in a similar manner, except instead of the program determining maximums it averages surrounding data points to determine a mean equilibrium value.

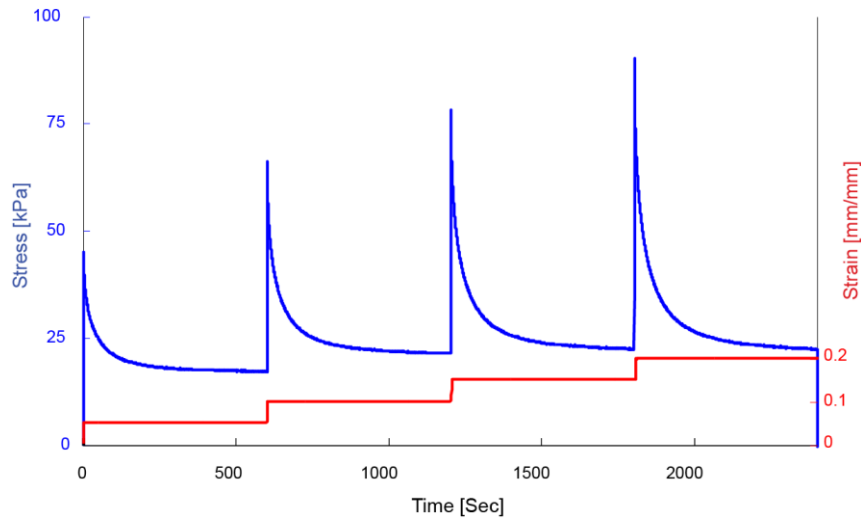


Figure 27: Load Curve Generated By Stress-Relaxation Test

Dynamic stiffness was calculated by performing linear regression on the peak stress versus strain data for each sample, and equilibrium stiffness was calculated similarly for the equilibrium stress versus strain data. Figure 28 shows an example of the linear regression fits on both dynamic and equilibrium stresses for a 3.0 % low gelling agarose gel. The slope of each line represents the calculated modulus. Each linear regression resulted an R^2 value $>.95$.

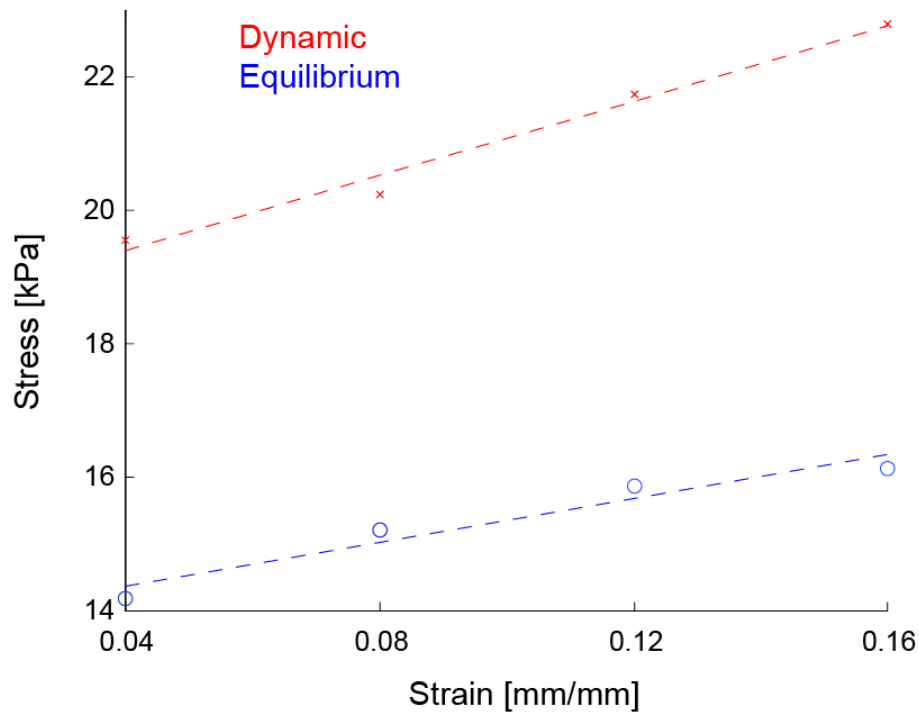


Figure 28: Linear Regression of Peak and Equilibrium Stresses

Results

It was observed that dynamic and equilibrium moduli were greatly influenced by agarose gel concentration (Figure 29). Dynamic modulus appeared to be more sensitive to concentration changes, while equilibrium moduli seemed to change less drastically from concentration to concentration. Equilibrium moduli ranged from approximately 0 to 90 kPa, while dynamic moduli ranged from approximately 20 to 560 kPa.

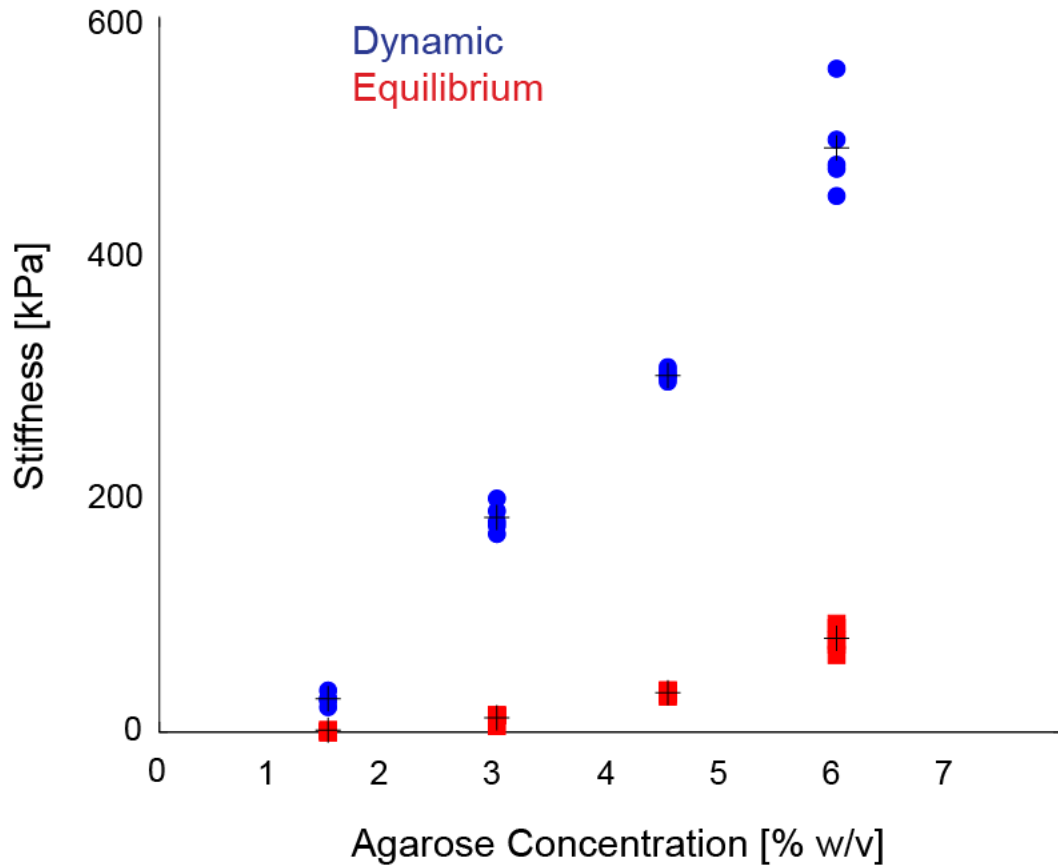


Figure 29: Moduli for High Gelling Temperature Agarose

The lowest dynamic and equilibrium values were for the 1.5% gels and the highest were for the 6% and were in the range of physiological stiffness values. Data for each test can be seen in Table 3. These values provided baseline concentration values to work from, and motivated further work using low-gelling temperature agarose, which would allow for encapsulation of living cells at physiological stiffnesses. Below 3% agarose concentration created gels too compliant for trying to match physiological stiffness.

Table 3: High Gelling Temperature Agarose Results

Concentration	1.5		3		4.5		6	
Modulus (kPa)	DM	EM	DM	EM	DM	EM	DM	EM
Trial 1	27.32	0.00	165.37	5.10	297.01	25.56	449.17	87.34
Trial 2	27.60	0.00	184.84	13.71	299.54	34.46	557.38	63.44
Trial 3	34.84	0.46	176.82	12.81	294.15	28.67	476.62	90.85
Trial 4	25.41	0.00	196.02	12.59	305.34	32.34	472.42	73.19
Trial 5	20.83	0.47	173.20	12.84	302.35	33.58	496.52	76.76

It was observed that at low concentrations some of the equilibrium moduli equaled zero. Since the modulus is the slope of a linear regression fitted to the stress vs. strain data an argument could be made that the material has dissipated all of its strain energy during relaxation in this protocol. It was also determined from these experiments that relaxation time could be greatly influenced by gel concentration. Lower concentration gels seemed to relax much faster than higher concentration gels. With this knowledge in hand tests could begin for low gelling temperature agarose.

Low Gelling Temperature Agarose

Preliminary Experiments

Taking what was learned from experimenting with high gelling temperature agarose preliminary tests were run on low gelling agarose to determine necessary relaxation times. Gels concentrations ranging from 4-6 percent agarose were made the same way and subjected to the same types of strains. Unfortunately, it was learned that low gelling agarose was more susceptible to failure (see table in Appendix F) during SSRTs: of the 29 experiments shown in this table, 10 gels failed with the majority failing

in shear. Of the 5 gel concentrations tested only 3 produced usable data sets. Table 4 shows the results from the first stress relaxation experiments performed on low gelling temperature agarose.

Table 4: Preliminary Low Gelling Agarose Results

Concentration [% w/v]	4		4.5		6	
Modulus (kPa)	DM	EM	DM	EM	DM	EM
Trial 1	156.7	40.1	193.5	48.4	309.3	111.0
Trial 2	135.6	36.7	197.2	57.6	286.8	101.0
Trial 3	137.9	32.3	183.3	52.7	295.1	104.0
Trial 4	141.1	40.6	174.8	56.5	279.9	97.1
Trial 5	126.0	31.9	178.2	52.7	305.5	110.4

It was hypothesized that the reason gels were failing was because the gels were not homogeneous and therefore unable to distribute loads evenly. When preparing gels to be cast, bubbles can form during the mixing process resulting in a non-homogenous gel. The bubbles would introduce imperfections in the gels which may have caused them to shear or buckle. The reason it did not happen in the higher gelling agarose was because that agarose is a stronger material and less susceptible to imperfections. This was remedied by de-gassing the agarose before experimentation. Agarose was either de-gassed in a water bath at 40 degrees Celsius or by a centrifuge at 600gs at the same temperature. This additional step created gels with no visible air bubbles.

It was also determined during preliminary experiments that the mechanical properties of agarose may be greatly influenced by temperature. It is well known that

polymers can exhibit changes in their mechanical properties as a result of temperature gradients (Ashby et al., 2007).

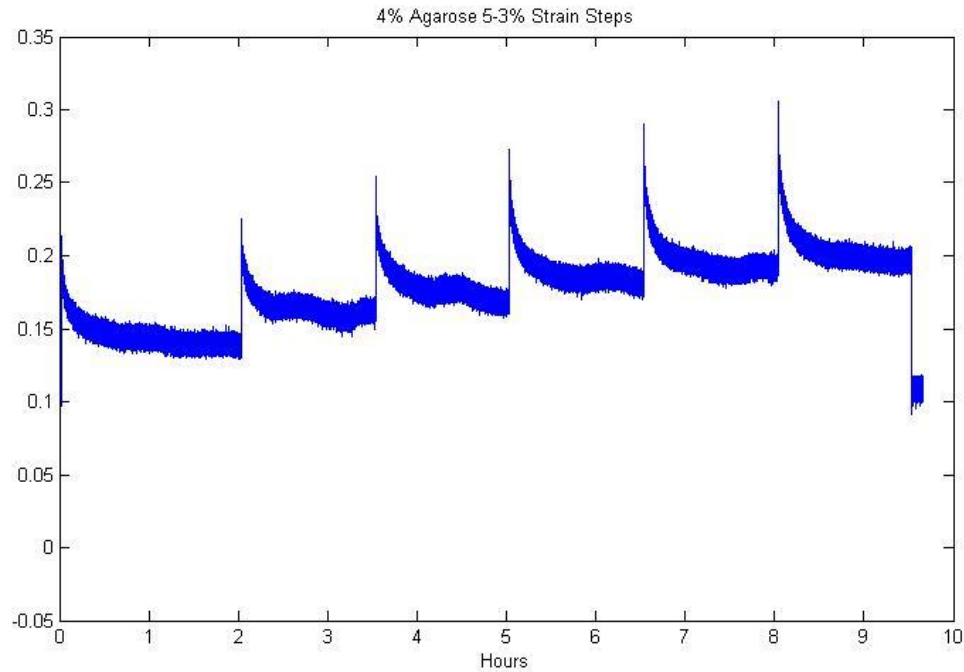


Figure 30: Possible Effects of Temperature on Agarose

Figure 30 shows a stress relaxation test that took place over a ten-hour period. A mysterious waveform seemed to propagate through the generated data. The waveform appears to have a period of approximately 1 to 2 hours. It was hypothesized that temperature fluctuation could have been causing the agarose gel to swell and therefore distort the data. It was conceivable that the HVAC system was causing small temperature gradients inside room that housed the bioreactor. Even well controlled HVAC systems cannot maintain constant temperatures, especially if loads are changing. In order to maintain comfort settings, air conditioning will cycle on and off over the course of the

day causing small temperature fluctuations (Howell et al., 2009). To remedy this problem all subsequent mechanical testing took place in an incubator at 37 degrees Celsius. Never again was this behavior observed.

Once it was decided that all material property experiments would be performed inside an incubator pilot studies were performed to look at relaxation time of various strain steps. It was determined that relaxation time for a 4% strain step would take approximately 90 minutes and 2 hours for a 5% strain step to reach equilibrium. This was determined by looking at the last ten minutes of each relaxation step in a given experiment, and determining if the absolute value of the stress-time slope was less than 10^{-7} , *i.e.* if equilibrium had been reached.

Methods

Agarose constructs were prepared using low-gelling-temperature agarose (Sigma: Type VII-A A0701). Concentrations of 3-5% (w / v) agarose were dissolved in PBS at 1.1X strength at 40°C. After ~5 minutes, dissolved agarose was diluted to 1X with 40°C PBS. (Note that this procedure is readily applied to encapsulating cells based on the methods of Lee *et al* and Bougalt *et al* (Bougault et al., 2009; Lee et al., 2000)—see below.) This final agarose solution was cast in anodized aluminum molds at 23°C. These molds produced cylindrical samples with height of 12.7 ± 0.1 mm and diameter of 7.0 ± 0.1 mm as measured using digital calipers on ~20 independent samples. This sample geometry was selected to provide uniaxial deformations consistent with spatially homogeneous strain fields (Kim et al., 1995). After 5 minutes of casting, gels were

removed and stored in PBS at 4°C for 1-2 days prior to testing consistent with standard mechanotransduction protocols (Haudenschild et al., 2008; Lee et al., 2011).

Samples were tested in the custom-built bioreactor capable of applying displacement-controlled loading with sub-micron precision. Prior to testing, samples were equilibrated in PBS at 37°C for 30 minutes. Samples were placed in polysulfone loading cups, and the bioreactor's forcing platens were lowered until sample contact was achieved as demonstrated by a load change of .089 Newtons. This load was selected as the smallest repeatable capable of being applied in this system, and is consistent with previous preloads used in the cartilage biomechanics literature (Huang et al., 2003). The sample was then allowed to relax to an equilibrium state. Based on pilot studies, complete preload relaxation was achieved in less than ten minutes. Two mechanical tests were performed in unconfined compression: stepwise stress-relaxation and cyclical loading.

Stepwise stress-relaxation tests were performed to calculate dynamic and equilibrium stiffness values for agarose gels at various concentrations. For these tests, samples were subjected to 4 steps of 4% nominal compressive Lagrangian strain. Each strain step was maintained for 90 minutes at 37°C in tissue culture conditions (humidified 5% CO₂ atmosphere). Load data was sampled at 1000 Hz for the duration of the test. Average stress was calculated by normalizing the load data by the initial sample cross sectional area.

Dynamic stiffness was calculated by performing linear regression on the strain versus peak stress data for each sample, and equilibrium stiffness was calculated

similarly for the equilibrium stress versus strain data. The transient response for each strain step of each sample was analyzed using 2 measures (June et al., 2009b). First, a model-independent parameter terms $\hat{D}$ was used to quantify the dynamics of stress-relaxation for each strain step. $\hat{D}$ is determined by calculating the area under the relaxation curve, and is analogous to a time constant. Second, the stretched exponential model shown by equation 2 (June and Fyhrie, 2013; June et al., 2011) was fit to the normalized stress-relaxation data to determine the parameters τ_{SE} and β .

$$y = \exp\left(-\left(\frac{t}{\tau_{SE}}\right)^\beta\right) \quad \text{Equation 2}$$

This stretched exponential model has been linked mechanistically to fundamental polymer physics (de Gennes, 2002) and cartilage viscoelastic behavior (June and Fyhrie, 2009; June et al., 2009a).

Cyclical loading tests were performed to assess the complex stiffness and phase lag of agarose gels. In these experiments, samples were subjected to a 5% prestrain for 2 hours followed by 100 cycles of compression from 3.1-6.9% based on the initially measured height. Samples were tested at 0.55, 1.1, and 5.5 Hz. These frequencies were selected to bound the preferred stride rates for humans (Umberger and Martin, 2007). Load and displacement values were sampled simultaneously at 100 Hz; well above the Nyquist frequency. Average stresses were calculated as described above. Cyclical loading data were analyzed to determine the complex modulus (e.g. stiffness) defined as the amplitude of the stress divided by the amplitude of the strain. The amplitudes were determined by performing a Fourier transform both the strain and stress data. The

temporal phase lag of the stress relative to the strain was determined by performing a cross-correlation function in MATLABTM.

Stepwise Stress-Relaxation Results

Stepwise stress-relaxation test reveal distinct differences in both dynamic and equilibrium moduli for the specified range of agarose concentrations. Representative load curves that demonstrate agarose time dependent viscoelastic behavior are shown in Figure 31A. It is evident that high concentration gels result in higher peak and equilibrium stresses.

It is shown that 5% agarose reached higher peak and equilibrium loads than 3% agarose under same strain. A significant relationship was found between the stiffness and gel concentration (Dynamic: $r = 0.78$ Equilibrium: $r = 0.91$ both $p < 0.001$, $n = 25$). Agarose equilibrium stiffness values were achieved as large as 51.3 kPa and dynamic stiffness values as high as 90.7 kPa. As expected 3.0% and 3.5% agarose produced the lowest equilibrium moduli of 18.9 ± 0.77 kPa and 26.0 ± 1.58 kPa respectively. At 4.0% agarose, equilibrium moduli was found to be 34.3 ± 1.65 kPa, and at 4.5% agarose, equilibrium moduli was determined to be 35.7 ± 0.95 kPa. 5% agarose produced the highest equilibrium moduli of 42.0 ± 2.88 kPa (Figure 31B).

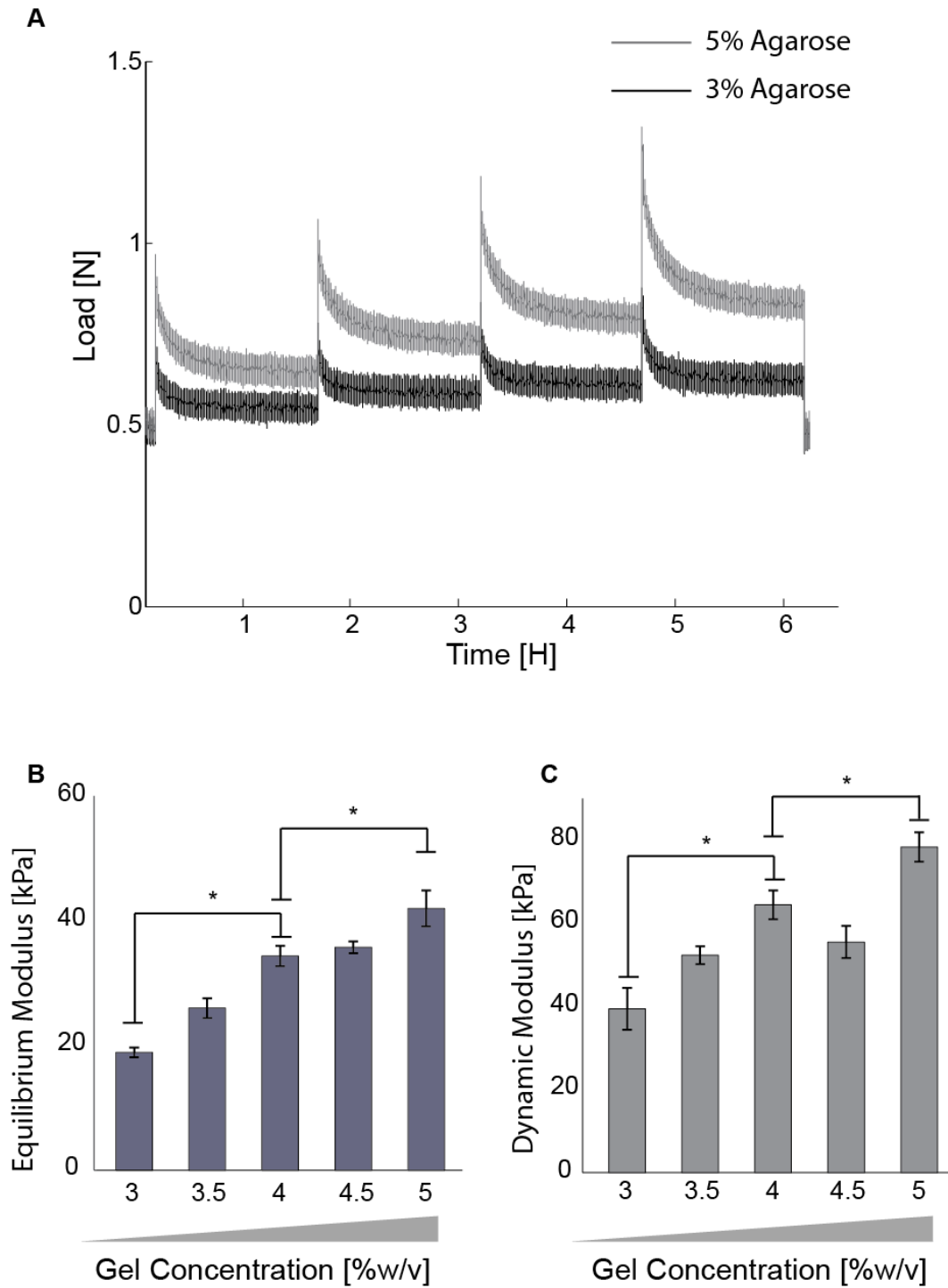


Figure 31: Concentration-Dependent Stress-Relaxation of Agarose.

Dynamic moduli were found to be approximately twice as large as corresponding equilibrium moduli (Figure 31C), with 3.0% and 3.5% yielding dynamic stiffness values of 39.4 ± 4.5 kPa and 52.3 ± 1.9 kPa respectively. 4.0% was determined to be 64.4 ± 3.1 kPa. 4.5% and 5.0% agarose were observed to have dynamic stiffness values of 55.5 ± 3.5 kPa and 78.4 ± 3.2 kPa respectively. The dynamic and equilibrium moduli for low gelling agarose express the same trend as high gelling agarose. 3, 4, and 5 percent agarose gels were found to be significantly different from each other for both equilibrium and dynamic modulus. This is depicted by the stars shown in Figures 30B and 30C.

The stretched exponential fit the stepwise stress-relaxation data well ($R^2 = 0.90 \pm .03$). Stretched Exponential parameter τ decreased with agarose gel concentrations, while β remained relatively constant with gel concentration (Figure 32A and 32B). Model independent parameter $\hat{D}$ seemed to be not effected by concentration with overall average of $8.24 \pm .05$ seconds (Figure 33). The stretched exponential model really highlights the differences in relaxations times between concentrations and also identifies the similarity among the shape relaxation curves between concentrations.

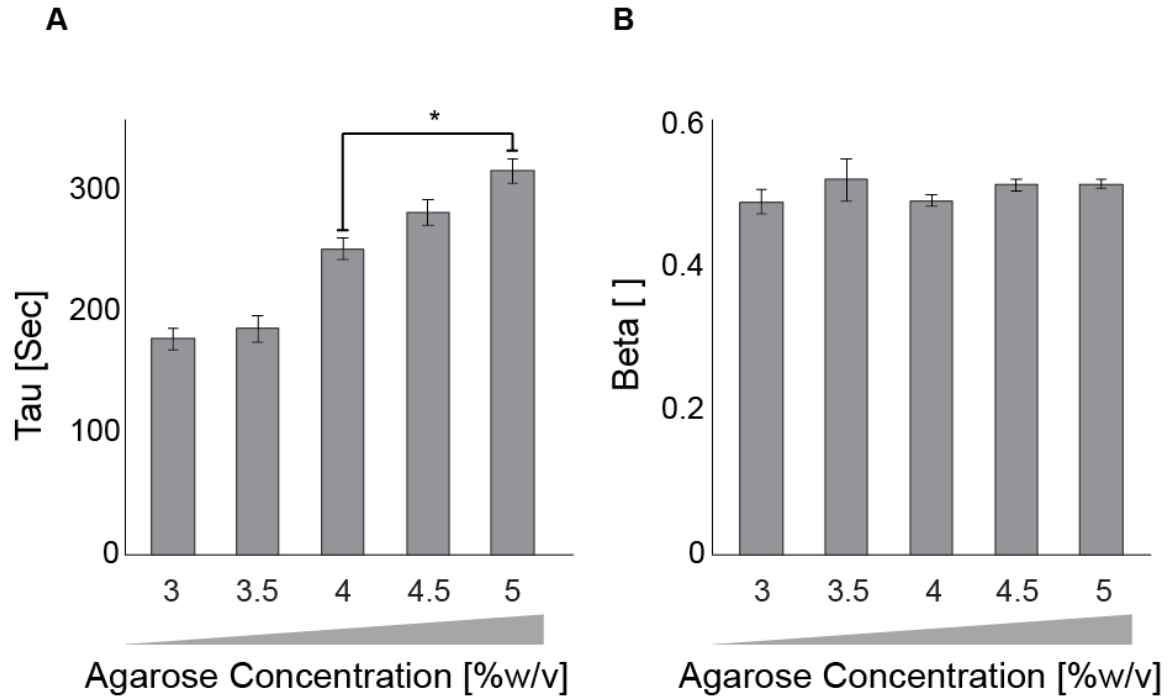


Figure 32: Stretched Exponential Parameters

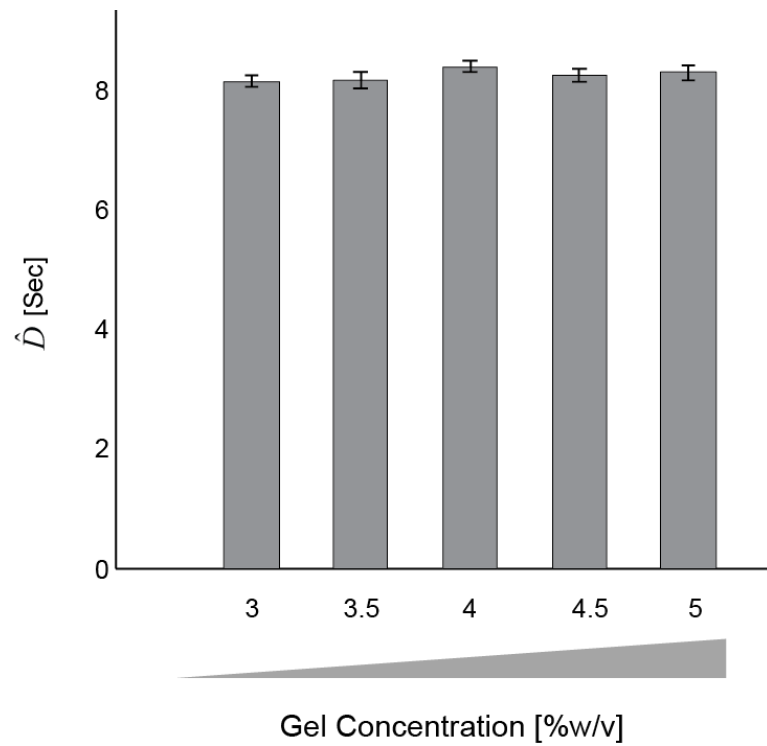


Figure 33: DHAT

Cyclic Loading Results

The sinusoidal applied strain induced sinusoidal stress profiles for all gel concentrations at all frequencies (Figure 34A). Cyclical test found complex moduli of ~100-300 kPa (Figure 32B). For a normal walking rate (~1.1 Hz) 3% and 3.5% agarose gel concentrations were found to have complex moduli of 147.6 ± 7.7 kPa and 151.4 ± 8.0 kPa respectively. 4.0% was found to have the lowest complex modulus of 119.1 ± 13.6 kPa, 4.5% and 5% agarose gel concentrations yielded complex moduli values much higher than the rest 216.5 ± 26.6 kPa and 226.1 ± 18.7 kPa respectively.

Average phase lag values were calculated at each gel concentration for frequencies of .55, 1.1, and 5.5 Hz (Figure 34B). It was observed that loading frequency drastically impacted phase lag values, but little variation was seen between agarose concentrations. Combining all gel concentrations: average phase lag for .55 Hz was $.36 \pm .003$ seconds, average phase lag for 1.1 Hz was $.11 \pm .002$ seconds, and average phase lag for 5.5 Hz was found to be $.02 \pm .02$ seconds.

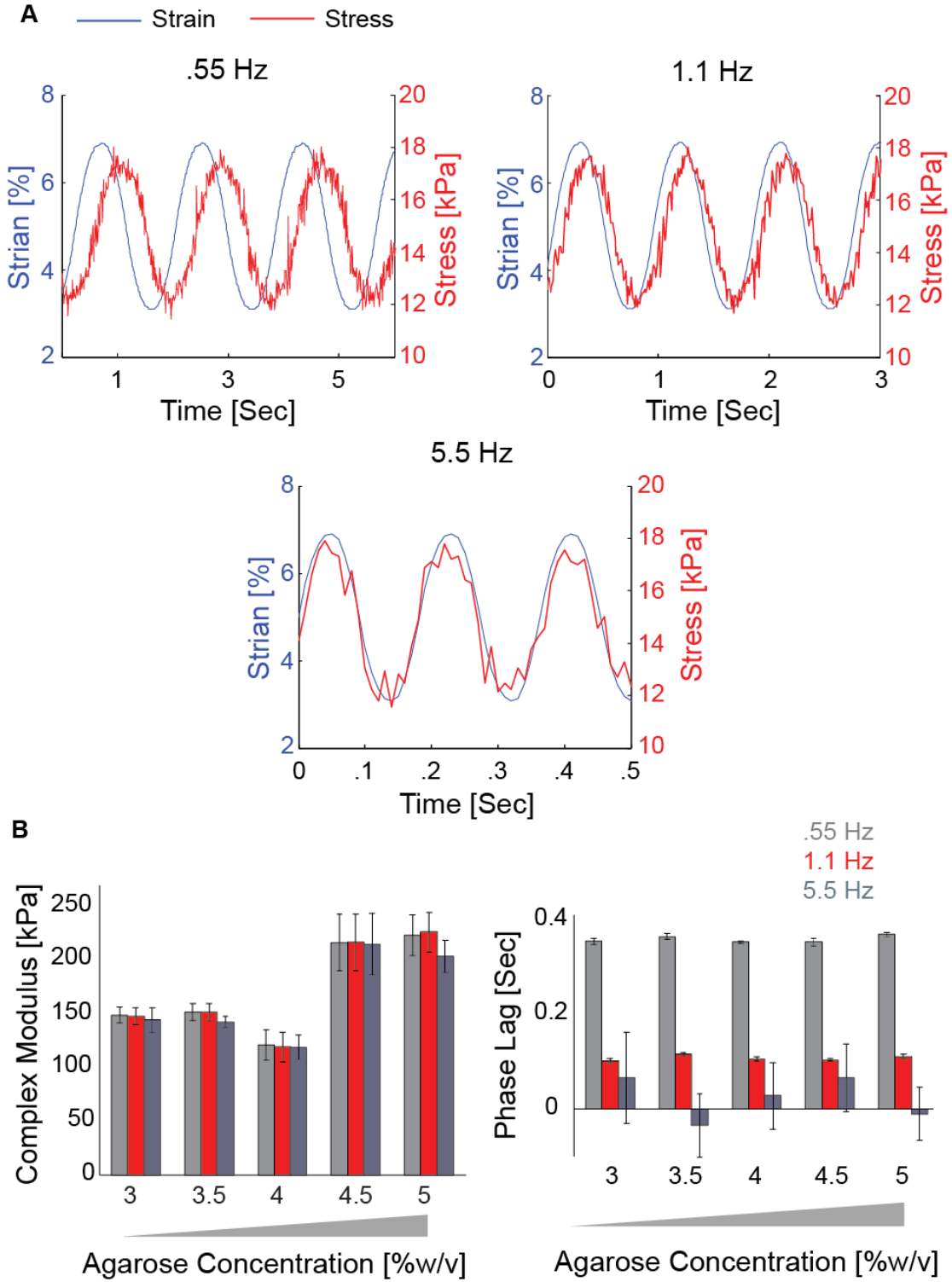


Figure 34: Results of Cyclic Loading Analysis

Discussion

Dynamic and Equilibrium Moduli were determined for an array of different agarose gel concentrations. The dynamic modulus can be thought of as an initial elastic stiffness to a rapid mechanical load *i.e.* compression. The equilibrium modulus is more representative of a relaxed, or slowly loaded, material state. Chondrocytes embedded in agarose gels will be interacting with the relaxed state of the gel for the majority of the time. The equilibrium moduli determined by the stepwise stress-relaxation experiments allowed for experimental modeling of the chondrocyte PCM. 3% agarose gels appear to be sufficient for modeling OA PCM, and 4.5-5% agarose appears sufficient to model healthy PCM (Figure 35).

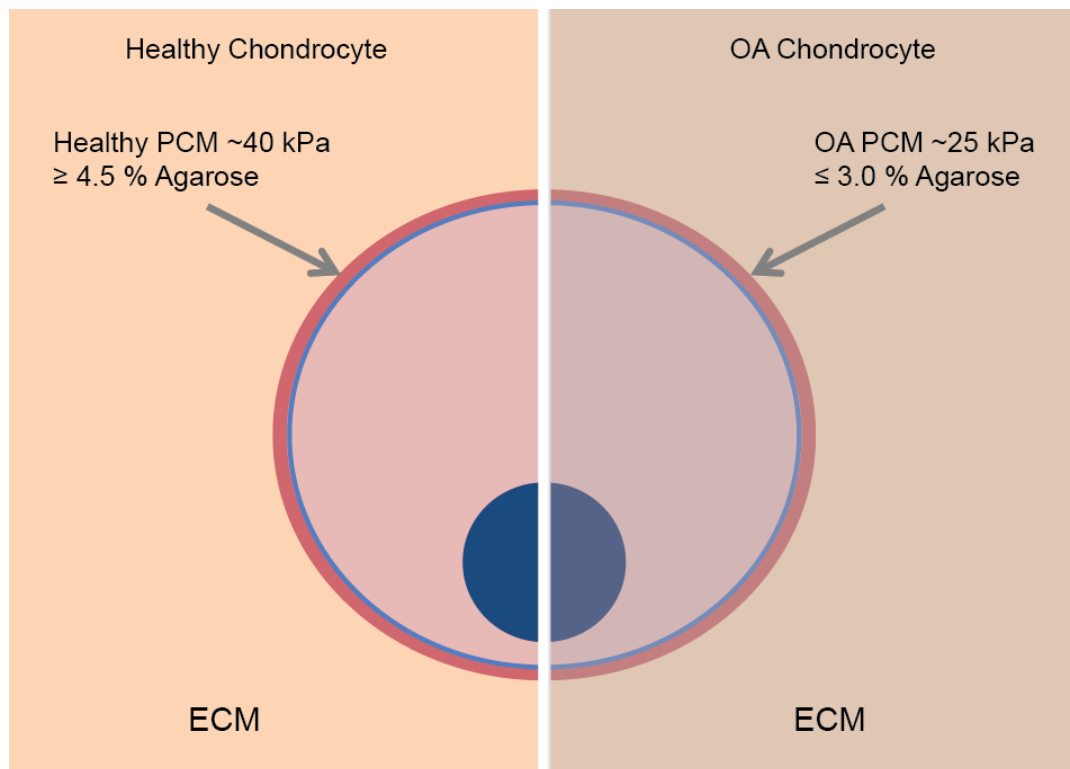


Figure 35: Physiological Stiff Agarose Concentrations

This was a significant finding because current experiments involving chondrocyte encapsulation usually use 2-3 % agarose gels (Bougault et al., 2012b; Knight et al., 1998; Lee and Knight, 2004). Gels that do not represent a physiological stiff environment could affect mechanotransduction experiments, especially experiments that are culturing chondrocytes for extended periods of time.

Mathematical Models have been used to describe the behavior of viscoelastic materials for understanding their constitutive behavior. It has become common practice to model viscoelastic behavior with either the Maxwell Fluid model or Kelvin Solid model, and sometimes a combination of both. These models usually incorporate a purely viscous dampener and a pure elastic spring. Figure 36 shows the three-parameter solid consisting of a purely elastic spring in series with Kelvin Solid that was originally purposed to model the viscoelastic nature of agarose.

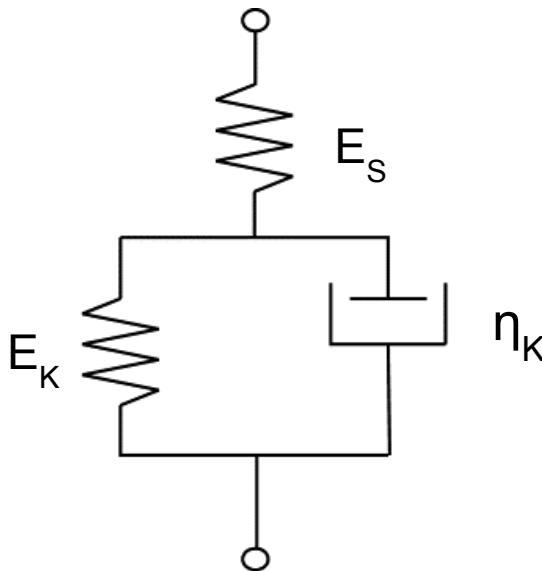


Figure 36: Three-Parameter Solid

It was thought that the spring would represent the initial elastic response and Kelvin Solid would be used to simulate the relaxation. A few assumptions had to be made about the model in order to generate a usable equation. First it was assume that stress would be evenly distributed.

$$\tau_{spring} = \tau_{Total} = \tau_{Kelvin} \quad \text{Equation 3}$$

The second assumption made was that the total strain equaled the strain of the spring plus the strain of the Kelvin Solid.

$$\varepsilon_{Total} = \varepsilon_{Spring} + \varepsilon_{Kelvin} \quad \text{Equation 4}$$

With these assumptions made it was possible to perform an analysis utilizing Laplace Transforms to generate equation of strain for the spring and Kelvin Solid. The Laplace Transforms of equation 3 and 4 are shown in equations 5 and 6.

$$\bar{\tau}_{spring} = \bar{\tau}_{Total} = \bar{\tau}_{Kelvin} \quad \text{Equation 5}$$

$$\bar{\varepsilon}_{Total} = \bar{\varepsilon}_{Spring} + \bar{\varepsilon}_{Kelvin} \quad \text{Equation 6}$$

Taking the Laplace Transform allows for easier analysis of the time depend nature of the Kelvin Solid. Equation 7 and 8 show the Laplace Transforms of the strain equations for the spring and Kevlin Solid respectively.

$$\bar{\varepsilon}_{Spring} = \frac{\bar{\tau}_{spring}}{E_s} \quad \text{Equation 7}$$

$$\bar{\varepsilon}_{Kelvin} = \frac{\bar{\tau}_{Kelvin}}{E_K} + \frac{\bar{\tau}_{Kelvin}}{\eta_K s} \quad \text{Equation 8}$$

These developed equations can then be inverted and an equation for stress in terms of prescribed strain can be written (Shames and Cozzarelli, 1997).

Equations 7 and 8 highlight the initial steps taken to generate a MAPLETM code capable for simulating stress relaxation experiments. This MAPLETM code can be seen in Appendix E along with a generated stress relaxation curve.

Maxwell Fluid and Kelvin Solid models are most popular when describing linear viscoelastic behavior, articular cartilage on the other hand exhibits nonlinearly viscoelasticity, which necessitated using the stretched exponential model (June and Fyhrie, 2013; June et al., 2011). The Stretched Exponential is also used to model polymers, which is exactly what agarose is. In pilot studies Power Law and Fractional Derivative models were used to model stress-relaxation curves, but it was determined that the data were best described by the Stretched Exponential model described herein.

The main purpose of using the Stretched Exponential model was to further understand agarose's time dependent mechanics. The model works by adjusting parameters tau and beta to minimize error of the fitted curve to the data. It was observed that the stretched exponential time constant (tau) was strongly dependent on gel concentration ($p < .001$, $n=100$). This could be the result of higher gel concentrations having more polymer entanglements, which would result in longer relaxation times. From an engineering standpoint a more densely packed polymer has a greater resistance to creep, which in essences is what is being measured with this time dependent parameter. There was not a large statistical change seen in the stretching factor β , which suggests that the distribution of relaxation dynamics is similar between gel concentrations

(de Gennes, 2002). This could simply mean that for the concentrations tested the polymer chains were roughly the same size.

Complex Moduli found in these experiments suggest a strong dependence on agarose concentration, while gel concentration appeared to not affect phase lag values. Results from cyclical loading experiments suggest that there is a fundamental change in the agarose gel properties which occurs at a concentration of ~4.0%. The average complex modulus for gel concentration less than or equal to 4.0% is 138.3 ± 4.8 kPa, while the average complex modulus for gel concentration greater than 4.0% is 216.7 ± 3.2 kPa.

The complex modulus (G^*) is a stiffness measure which combined both the in-phase, or elastic modulus (G'), and the out-of-phase, or viscous moduli (G'') (Macosko, 1994) G^* is usually represented by Equation 9.

$$G^* = G' + iG'' \quad \text{Equation 9}$$

Utilizing the phase lag (δ) it is possible to decompose the complex modulus into the elastic and viscous moduli. The relationship between phase lag and elastic and viscous moduli is shown in Equation 10.

$$\tan(\delta) = \frac{G''}{G'} \quad \text{Equation 10}$$

The magnitude of G^* is usually expressed in the form Equation 11

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad \text{Equation 11}$$

Utilizing Equation 10 and 11 an equation for G' can be derived

$$\begin{aligned}(G^*)^2 &= (G')^2 + (G'')^2 \\(G^*)^2 &= (G')^2 + (\tan(\delta) * G')^2 \\(G^*)^2 &= (G')^2 * (1 + \tan(\delta))^2 \\\frac{(G^*)^2}{(1 + \tan(\delta))^2} &= \frac{(G^*)^2}{(\sec(\delta))^2} = (G')^2 \\\frac{G^*}{\sec(\delta)} &= G'\end{aligned}$$

$$G' = G^* * \cos(\delta) \quad \text{Equation 12}$$

A similar derivation can be performed to get Equation 13

$$G'' = G^* * \sin(\delta) \quad \text{Equation 13}$$

In order to utilize Equations 12 and 13 the phase lag, which was determined in seconds it had to be converted to a usable format *i.e.* radians. Equation 14 shows the proper conversion.

$$\delta = \Delta t * 2\pi * f \quad \text{Equation 14}$$

The mechanical significance of these two properties is best stated in terms of energy. The elastic modulus (G') is representative of the amount of elastic energy stored, while viscous modulus (G'') is representative of energy lost or dissipated. As the phase lag decreases, the viscous modulus begins to get smaller, resulting in a more elastic response. Table 5 shows that this trait was observed at all gel concentrations.

Table 5: Analysis of Complex Modulus

Concentration [% w/v]	Loading frequency	Average Phase Lag [sec]	Average (G') [kPa]	Average (G'') [kPa]
3	0.55	0.354	51.1	139.4
	1.1	0.102	112.5	95.0
	5.5	0.066	129.9	-32.7
3.5	0.55	0.364	47.1	144.0
	1.1	0.116	105.4	108.3
	5.5	-0.034	133.9	8.2
4	0.55	0.352	42.0	113.1
	1.1	0.106	88.6	79.3
	5.5	0.028	111.8	-31.9
4.5	0.55	0.352	76.7	201.8
	1.1	0.104	162.5	142.9
	5.5	0.066	206.5	-42.7
5	0.55	0.368	65.0	213.2
	1.1	0.11	161.9	156.8
	5.5	-0.01	172.8	-49.3

This elastic response to higher loading rates is most evident when the agarose gels were subjected to a loading frequency of 5.5 Hz. Phase lag values calculated from 5.5 Hz sinusoidal compressions were both positive and negative with no statistical difference from zero, indicating that the agarose is beginning to behave as an elastic material at this frequency. Interestingly, for the range of frequencies tested, there was no significant effect on complex modulus (Figure 34B). These data suggest that the complex modulus of agarose is an intrinsic material property, dependent on gel concentration, but independent of loading frequency.

Encapsulation of Chondrocytes in Physiological Stiff Agarose Gels

Chondrocyte Encapsulation

Cultured chondrocytes were embedded in agarose of varying concentrations. Human SW1353 chondrosarcoma cells were cultured in 5% CO₂ in DMEM with 10% fetal bovine serum and antibiotics (10,000 I.U. / mL penicillin and 10000 ug / mL streptomycin). Figure 37 shows the cell line grown in monolayer for 2 days after a 1:10 split.

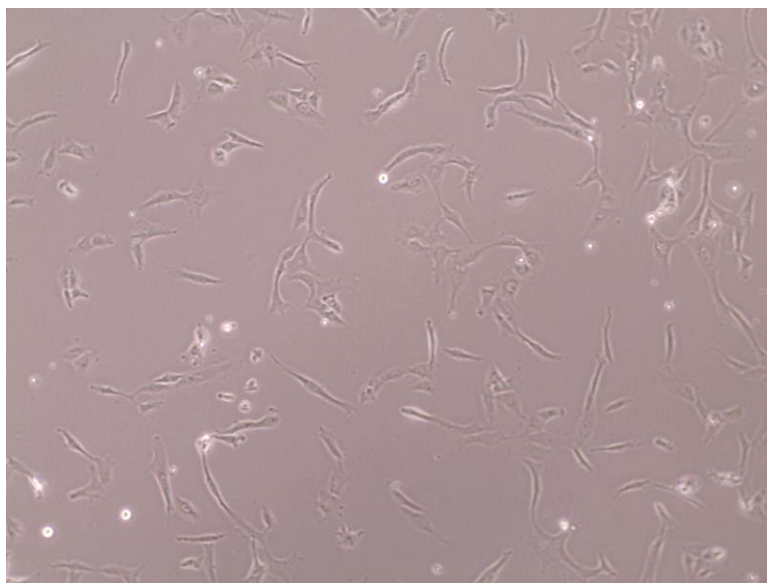


Figure 37: SW1353 Grown in Monolayer

For encapsulation, cells were trypsinized, counted, and resuspended in media at 11X. Agarose was prepared as described above. The cell-suspension was added to the agarose with vortexing to distribute the cells throughout the liquid hydrogel. Gels were subsequently cast as described above for 5 minutes at 23°C. Cell-seeded agarose

constructs were removed from the molds and placed in tissue culture for 24 and 72 hours prior to viability assay. Full experimental protocol can be seen in Appendix G.

Viability Assay

To assess effects of the encapsulation process on chondrocyte health, we assessed viability using standard methods (Ohlendorf et al., 1996). Cells were incubated in 8 μ M calcein-AM and 75 μ M propidium iodide. Following incubation, constructs were examined by confocal microscopy for calcein-AM fluorescence (excitation: 496 nm emission: 516 nm) indicating live cells via intracellular thioesterase activity and propidium iodide fluorescence (excitation: 536 nm emission: 617 nm) indicating dead cells via DNA binding indicating compromised plasma membranes. Confocal images were acquired from 6 positions within each hydrogel to assess potential spatial variability in cell viability. Images were then thresholded and the numbers of viable and dead cells were quantified.

Results of Viability Assay

Viability was assessed using confocal microscopy after 24 and 72 hours of incubation at 5 different gel concentrations. Figure 38A shows representative images taken of the encapsulated chondrocyte cells at agarose concentrations of 3, 3.5, 4, 4.5, and 5 [%w/v]. Imaging revealed high viability, which was not location-specific indicating that the methods resulted in an even spatial distribution of cells. Furthermore, we found no viability differences between the six regions tested in each gel.

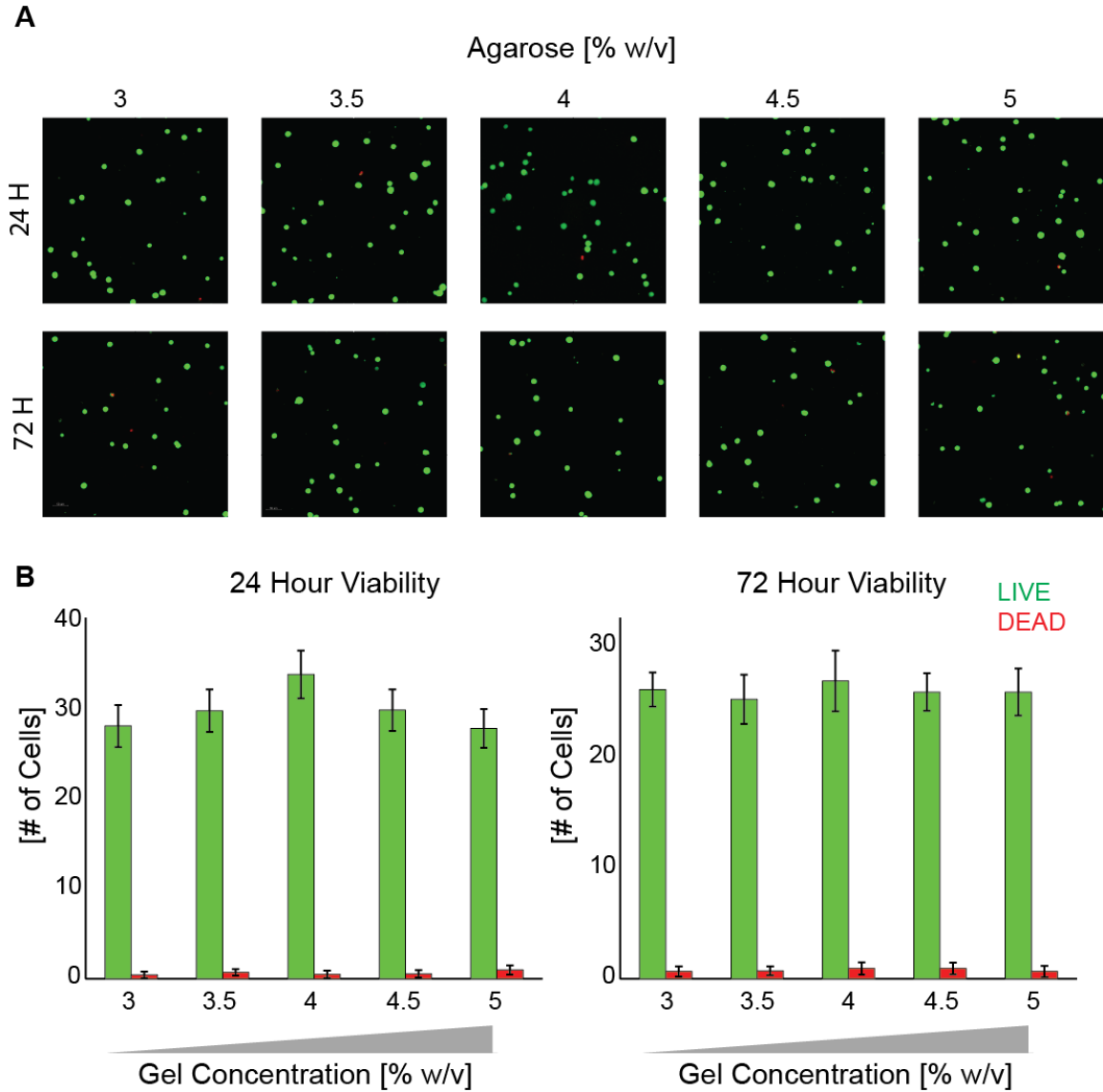


Figure 38: Viability Results

Viability for each agarose gel concentration after 24 hours of incubation were as follows: $98.4 \pm .3\%$ for 3.0%, $97.6 \pm .4\%$ for 3.5%, $98.6 \pm .3\%$ for 4.0%, $98.2 \pm .9\%$ for 4.5%, and $96.6 \pm .3\%$ for 5.0%. Viability for each agarose gel concentration after 72 hours of incubation were slightly lower: $97.5 \pm .4\%$ for 3.0%, $97.3 \pm 1.7\%$ for 3.5%, 96.6

$\pm .3\%$ for 4.0%, $96.5 \pm 1.1\%$ for 4.5%, and $97.5 \pm .4\%$ for 5.0%. Figure 38B shows the average numbers of live and dead cells quantified per image.

Conclusions

We found viability greater than 95% for all gel concentrations tested in this study. This indicates that chondrocytes grown in monolayer can be successfully encapsulated in agarose gels of variable stiffness. The pericellular matrix is the tissue directly surrounding articular chondrocytes *in vivo*. Previous studies have found that the stiffness of human PCM is in the range of 25-200 kPa. Osteoarthritic PCM appears to have a lower stiffness than healthy PCM (Alexopoulos et al., 2005b). Based upon the data and methods presented herein, it is possible to model the OA-dependent stiffness changes using chondrocytes encapsulated in agarose. It has been determined that agarose gels of 4.5% and higher represent a healthy physiological environment. In the pursuit of creating a physiological stiff microenvironment to encapsulate chondrocytes an array of mechanical properties were determined about agarose.

Dynamic and Equilibrium moduli were determined to rely on agarose type as well as concentration. High gelling agarose exhibited higher dynamic modulus values, while comparable equilibrium values were observed when compared to low gelling agarose. Temperature also was observed to effect dynamic and equilibrium moduli of low gelling agarose. Samples tested at room temperature exhibit much higher dynamic moduli than comparable concentrations tested at 37° Celsius. Average dynamic modulus for a 4.5 %

low gelling agarose gel at room temperature was approximately 185 kPa while the same concentration tested inside an incubator yielded values more than 3 times less.

Complex modulus and phase lag analysis demonstrate that agarose behaves as an elastic material at higher loading rates, while viscous properties are observed at lower loading rates. At physiological loading rates *i.e.* 1.1 Hz agarose gels exhibited both viscous (G'') and elastic (G') components almost equal to each other. This characteristic was extremely important to identify. Since cartilage behaves viscoelastically when subjected to *in vitro* mechanical testing it was desired that the agarose gels maintain some viscoelastic nature as well. If a loading rate of 1.1 Hz resulted in a purely elastic response much like the 5.5 Hz loading rate did, the agarose model may not have been the best representation of the chondrocyte microenvironment.

The complex modulus expressed strong concentration dependence, especially when comparing what was determined to be an OA gel and a healthy gel. The complex modulus for an OA gel was observed to be approximately 150 kPa and 225 kPa for a gel modeling a healthy environment. It remains to be seen if this characteristic will play a role in chondrocyte mechanotransduction. What is known is that for these gel concentrations tested a fundamental change in complex modulus was observed at approximately 4% agarose concentration. To understand this property further a wider range of agarose concentrations should be tested.

METABOLOMIC STUDIES

On the cellular level mechanotransduction can be described by three steps; signal reception, signal transduction, and cellular response. This chapter focuses on the final step of that cascade: the cellular response to dynamic compression. It was hypothesized that physiological dynamic compression would result in changes in central metabolism that are consistent with increased protein synthesis. To test this hypothesis, dynamic compression was applied to chondrocytes embedded in physiologically stiff agarose for zero, fifteen, and thirty minutes. Subsequently, metabolites were extracted and identified by Liquid-Chromatography and Mass Spectrometry (LC-MS). The goal of this study was to focus on metabolites associated with glucose metabolism including glycolysis and the pentose phosphate pathway. It became apparent that focusing on metabolites associated with specific metabolism was beyond the scope of the thesis. Instead the methods and results presented herein will serve as preliminary studies that can be built upon in future studies.

Methods

No known method had been developed for extracting metabolites from cell-seeded agarose gels, so preliminary experiments were performed to determine the optimal procedures. Two different methods are described in this section. The experimental methods describe the protocols used to extract metabolites from mechanically stimulated cell-seeded gel constructs. The data analysis section describes how the raw data generated by LC-MS was converted into a usable format and how to identify metabolites.

Only one experiment is described, but the developed methods can be utilized in future mechanotransduction experiments and adapted for a variety of cell types including primary human chondrocytes.

Experimental

Human SW1353 chondrosarcoma cells were encapsulated in 5 % agarose gels as described above. Agarose gel constructs were culture in antibiotic free media for seventy-two hours in an incubator at 37° Celsius under 5% CO₂. Gels were then subjected to dynamic cyclic compression for zero, fifteen, and thirty minutes at a frequency of 1.1 Hz with a strain level of 3.1-6.9% based on the initially measured height. Through the duration of the tests, physiological conditions were maintained (37° C, 5% CO₂). To maintain proper control of each experiment 5 samples received mechanical stimulation from the bioreactor, while 5 unloaded control samples did not receive any mechanical stimulation. After each time point samples were removed from bioreactor wrapped in sterilized tinfoil and immediately frozen in liquid Nitrogen. The reason for this was to stop any metabolic activity.

Samples were then removed from the liquid Nitrogen bath and place inside individual wells of a custom-made aluminum mold called a tombstone for crushing. Figure 39 shows the tombstone utilized during the crushing process. The tombstone was kept in the -80 freeze prior to being set on ice during the crushing process. The reason for this was to keep the samples frozen.



Figure 39: Aluminum Mold Used For Crushing

From here each sample was crushed using a stainless steel platen using a ballpeen hammer. Samples were crushed to generate more surface area for easier metabolite extraction. Crushed gel particulate was then collected with a stainless steel scoopula and put into 2 mL microcentrifuge tubes. At this point samples can be kept at -80° for multiple days without detectable metabolite degradation.

Metabolites were extracted from frozen sample by adding one milliliter of a 70:30 solution of Methanol:Acetone and vortexing every few minutes for twenty minutes. Samples were then put back in the -80 freezer overnight. The liquid was then removed and dried down in a speedvac for six hours. Dried samples were then taken to the mass spec facility and resuspended in 200 μ l of a 50:50 Water:Acetonitrile solution. The samples were then run on a mass spectrometer utilizing a HILIC column to identify positively charged masses for metabolites involved with chondrocyte central metabolism.

One sample can generate tens of thousands of data points so methods were developed to analyze the data. The methods discussed herein were focused around an untargeted approach to avoid *a priori* exclusion of mechanosensitive metabolites.

Data Analysis

The first step was to upload the raw data to an online program called XCMS (Tautenhahn et al., 2012). Utilizing this online tool dynamically stimulated samples could be compared to un-stimulated samples. XCMS compares intensity levels of relative masses and determines fold changes, abundance, and statistical significance. Intensity value for each mass can then be plotted using MATLABTM. MATLABTM allows for easy identification of large fold changes between samples. Another online program called Metlin can then be used to identify possible metabolites (Zhu et al., 2013). If the metabolite is known it will receive a KEGG ID, which is usually a six digit number in the format of C#####. KEGG IDs are useful because they allow for easy identification of specific metabolites involved in different metabolic pathways. Once a specific metabolite is identified it becomes possible to track how its intensity (*i.e.* abundance) changes between loaded and unloaded samples.

Results

Cell-seeded agarose gels were subjected to loads simulating normal physiological loading *e.g.* walking for zero, fifteen, and thirty minutes. To assess metabolic changes as a result of dynamic compression, unloaded controls groups were compared to loaded groups. The cell-seeded agarose gels that received no mechanical stimulation acted as

unloaded controls, which were compared to the cell-seeded agarose gels that received fifteen minutes of dynamic compression and thirty minutes of dynamic compression. A more in-depth analysis was performed on all samples tested, which included comparisons between replicates at each time point. Results for that analysis are shown in Appendix H. Figure 40 shows a graph identifying possible metabolites determined to be significant by XCMS. The unloaded control results are plotted on the x-axis and the 15 minute dynamically stimulated results are plotted on the y-axis.

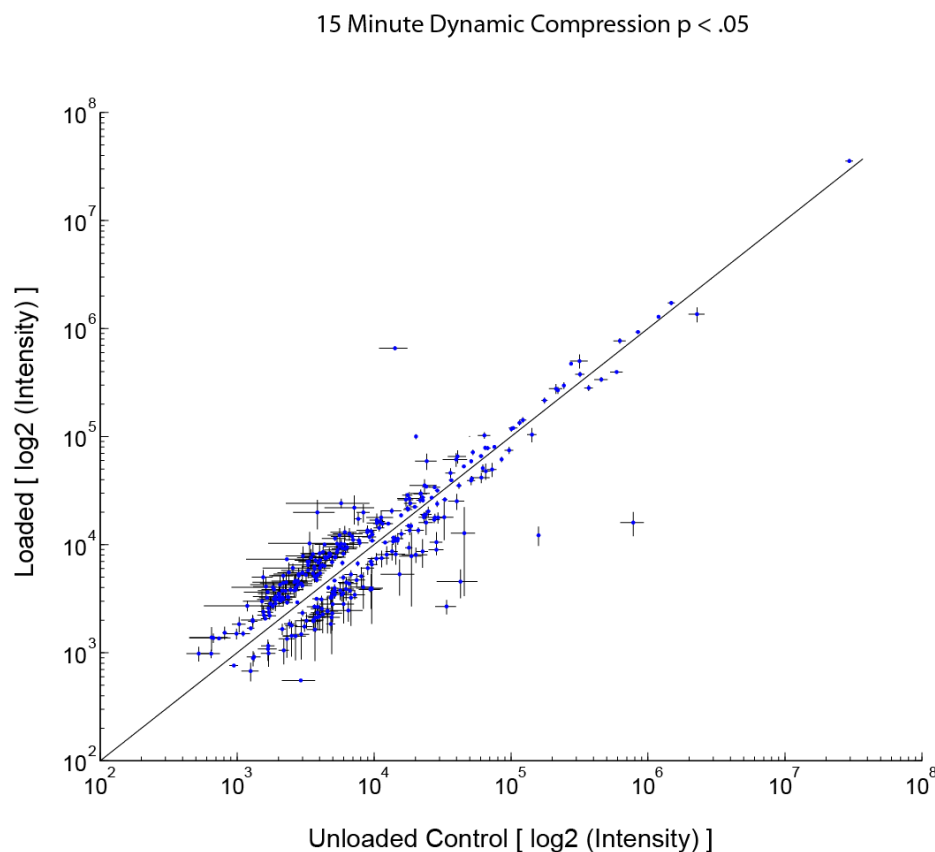


Figure 40: 15 Minute Dynamic Compression

Each data point represents the intensity of one unique mass found in all samples. Data points that fall above the $Y = X$ line are suggested to be metabolites that have been

up-regulated due to dynamic compression, while the data points that fall below the $Y = X$ line are suggested to be metabolites that have been down regulated by dynamic compression. The horizontal and vertical lines through some of the points are representative standard deviations of that mass's intensity. Figure 41 shows the same plot shown in Figure 40, but for samples that were subjected to thirty minutes of dynamic compression.

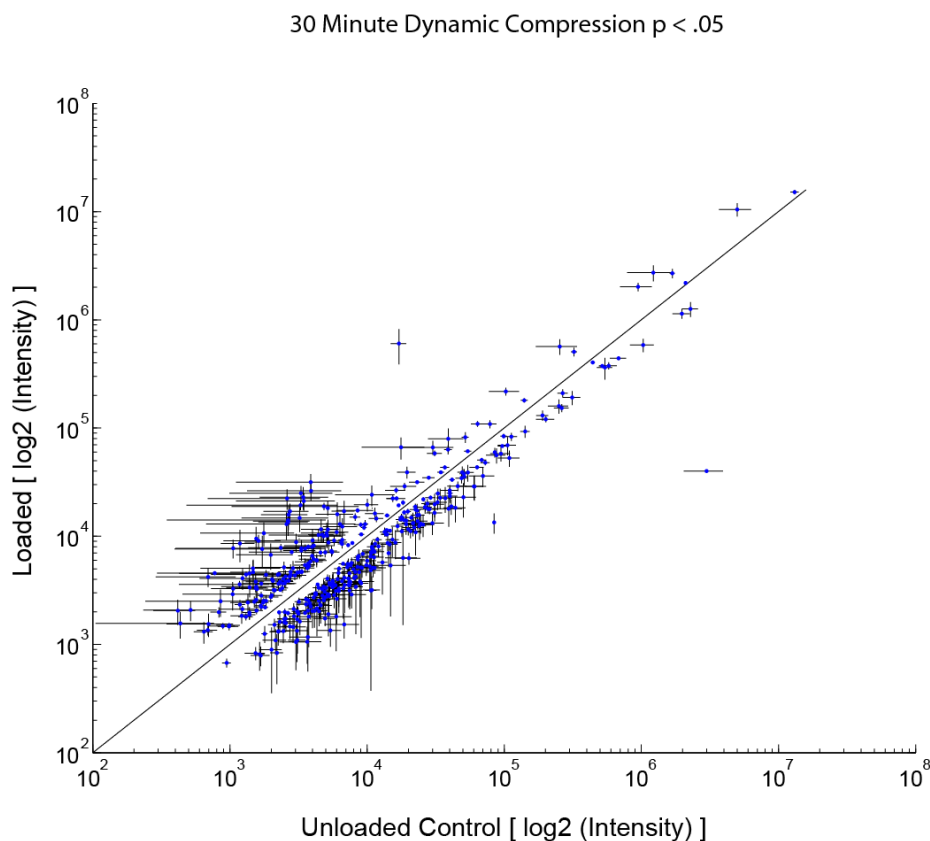


Figure 41: 30 Minute Dynamic Compression

Fifteen minutes of dynamic compression resulted in the identification of 461 metabolites with KEGG IDs, while thirty minutes of dynamic compression resulted in the identification of 769 metabolites with KEGG IDs. These two lists were then compared

to each other and it was determined that they had 164 KEGG IDs in common. From this list of 164 metabolites proline was identified as being down regulated with increased time of dynamic compression. Proline is an amino acid that has been used as a marker for ECM synthesis (Jin et al., 2001). When collagen is formed inside the cytoplasm of chondrocytes, it frequently incorporates proline and hydroxiprolin into the long α -1 amino acid chained used to create its triple helix structure. A change in proline could indicate the synthesis of collagen molecules. Figure 42 shows a plot identifying the effects dynamic compression on the relative intensity of proline at three different time intervals.

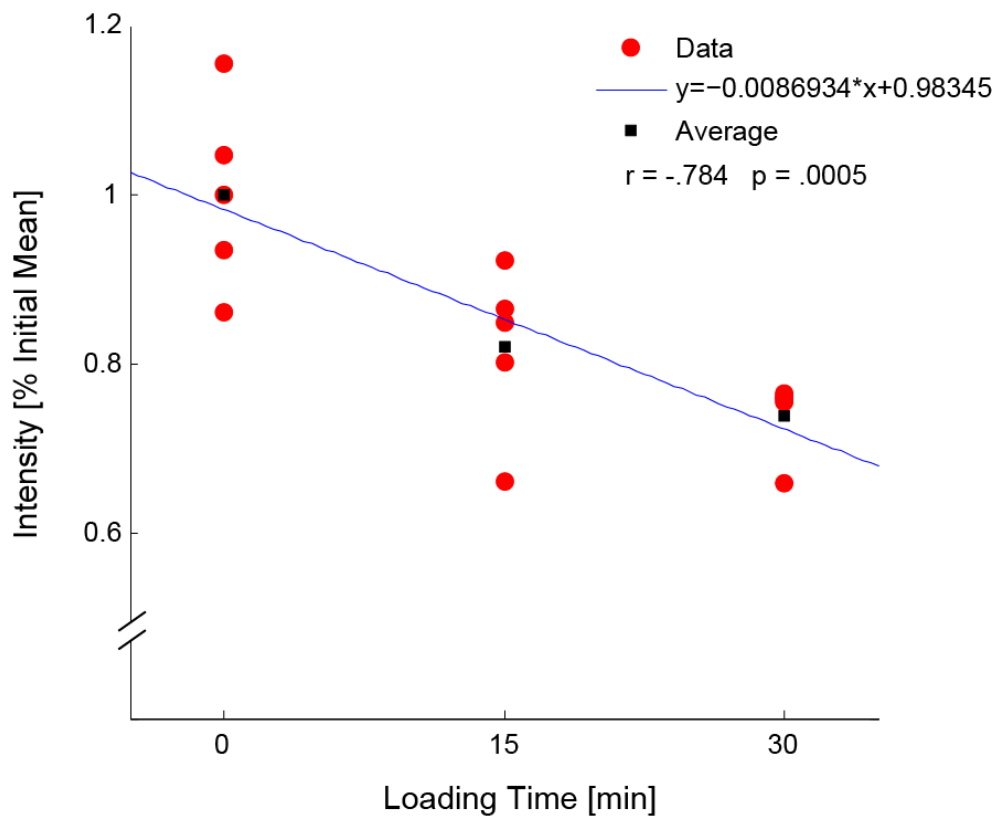


Figure 42: Proline Intensity vs. Loading Time

Proline decreased with increasing time of dynamic compression as demonstrated by a significant negative correlation ($r = -.784$ $p = .0005$). This observation is consistent with the hypothesis that dynamically stimulated chondrocytes utilize proline for *de novo* collagen synthesis. Future work will identify additional metabolites that are utilized during the creation of the collagen molecule such as glycine and lysine. Unlike proline, lysine is an essential amino acid meaning that the human body cannot produce more and must use external sources. Lysine uptake could provide directions for future experiments. With the present data it would be hard to verify the hypothesis that decreasing proline levels is an indication of collagen synthesis, but it does provide opportunities for more experiments. One way to verify the hypothesis would be to knock out the col2a1 gene and re-run the experiment. Col2a1 provides the cell with the necessary information for creation of the alpha-1 chains, that makes up the collagen triple helix molecule. If this gene is never transcribed and the observed proline trend does not change, it would indicate that proline is being utilized in some other metabolic activity.

Conclusions

Metabolomics provides a unique and powerful tool when studying articular chondrocyte mechanotransduction. These data demonstrate the feasibility of detecting metabolic changes at the cellular level as a result of mechanical stimuli. The methods described provide a solid foundation to future metabolomic studies. It was determined that extensive data analysis is needed in order to identify interesting/significant metabolites. The initial hypothesis was not answered in these preliminary studies, but these data presented allows for more scientific questions to be asked. Future experiments

will incorporate a targeted approach, which will allow for specific metabolic pathways *e.g.* glycolysis and pentose phosphate to be studied in a more direct manner. The results of the metabolomic studies coupled with the development of system for studying chondrocyte mechanotransduction provides the foundation for new and exciting laboratory research.

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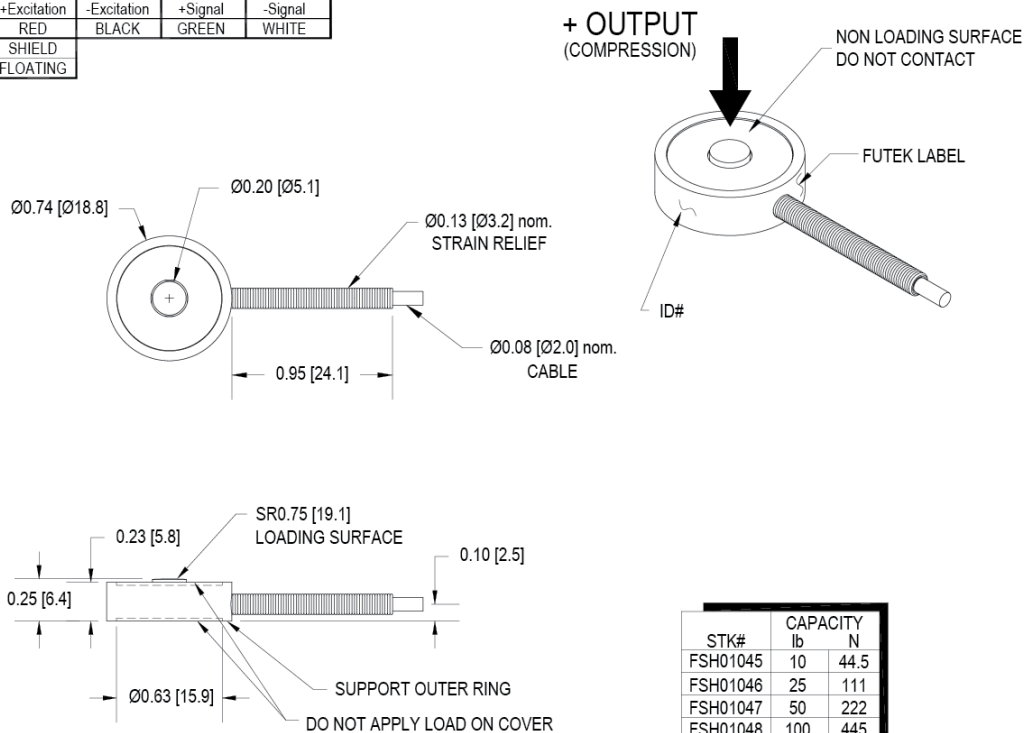
APPENDICES

APPENDIX A

COMPONENT SPECIFICATIONS

FUTEK MODEL LLB300**Drawing Number: FI1119-A****INCH [mm] R.O.= Rated Output**

WIRING CODE (WC1)			
+Excitation	-Excitation	+Signal	-Signal
RED	BLACK	GREEN	WHITE
SHIELD			
FLOATING			

SUBMINIATURE LOAD BUTTON LOAD CELL*(Previously L 1630-Q10291)*

STK#	CAPACITY	
	lb	N
FSH01045	10	44.5
FSH01046	25	111
FSH01047	50	222
FSH01048	100	445
FSH01050	250	1112
FSH01052	500	2224
FSH01053	1000	4448

SPECIFICATIONS:

RATED OUTPUT	2 mV/V nom.
SAFE OVERLOAD	150% of R.O.
ZERO BALANCE	±3% R.O.
EXCITATION (VDC OR VAC)	18 MAX
BRIDGE RESISTANCE	700 Ω nom.
NONLINEARITY	±0.5% of R.O.
HYSTERESIS	±0.5% of R.O.
NONREPEATABILITY	±0.1% of R.O.
TEMP. SHIFT ZERO	±0.01% of R.O./°F [0.018% of R.O./°C]
TEMP. SHIFT SPAN	±0.02% of LOAD/°F [0.036% of LOAD/°C]
COMPENSATED TEMP.	60 to 250°F [15 to 121°C]
OPERATING TEMP.	-60 to 250°F [-50 to 121°C]
WEIGHT	1.5 oz [43 g]
MATERIAL	17-4PH S.S.
DEFLECTION	0.002 [0.05] nom.
CABLE: #29 AWG, 4 Conductor, Spiral Shielded Teflon Cable 10 ft [3m] Long	
ACCESSORIES AND RELATED INSTRUMENTS AVAILABLE	
CALIBRATION (STD)	5 pt. COMPRESSION; 100K Ω SHUNT CAL. VALUE
CALIBRATION TEST EXCITATION	10 VDC

FUTEK
ADVANCED SENSOR TECHNOLOGY, INC.

This drawing is submitted solely for the information and exclusive use of the original addressee. It is not to be divulged in whole or in part, by any firm or individual without written permission from FUTEK.

10 THOMAS
IRVINE, CA 92618 USA
1-800-23-FUTEK (38835)

INTERNET:
<http://www.futek.com>

**Sensor Info**

Model LLB300

Item # FSH01045

S/N 469839

Capacity 10 lb

Calibration Data

Test Temp 72.5 °F (22.5 °C)

Relative Humidity 49.4 %

Excitation 10.00 (Vdc)

Input Resistance 702 (Ohms)

Zero 0.008 (mV/V)

Output Resistance 702 (Ohms)

direction: Compression

Rated Output 3.063 (mV/V)

Linearity 0.411 % of R.O.

Data Points

Load

Output

Non-Lin Error (%)

Hysteresis (%)

channel: 1

(lb)

(mV/V)

direction: Compression

0

0.0000

0.000

2

0.6187

0.202

4

1.2349

0.323

6

1.8494

0.389

8

2.4626

0.411

10

3.0625

0.000

0

0.0004

Shunt CalibrationShunt Value
(K ohm)

Output ()

Load (lb)

channel: 1direction: Compression

100

1.746737

5.704

Shunt Cal is placed across (-E)(-S)



Sensor Info

Model LLB300

Item # FSH01046

S/N 461155

Capacity 25 lb

Calibration Data

Test Temp 72.5 °F (22.5 °C)

Relative Humidity 49.5 %

Excitation 9.99 (Vdc)

Input Resistance 701 (Ohms)

Zero 0.0137 (mV/V)

Output Resistance 714 (Ohms)

direction: Compression

Rated Output 2.524 (mV/V)

Linearity -0.097 % of R.O.

Data Points

Load	Output	Non-Lin Error (%)	Hysteresis (%)
------	--------	-------------------	----------------

channel: 1

(lb)

(mV/V)

direction: Compression

0	0.0000	0.000
5	0.5031	-0.071
10	1.0073	-0.097
15	1.5125	-0.085
20	2.0182	-0.052
25	2.5244	0.000
0	-0.0004	

Shunt Calibration

Shunt Value
(K ohm)

Output ()

Load (lb)

channel: 1direction: Compression

100

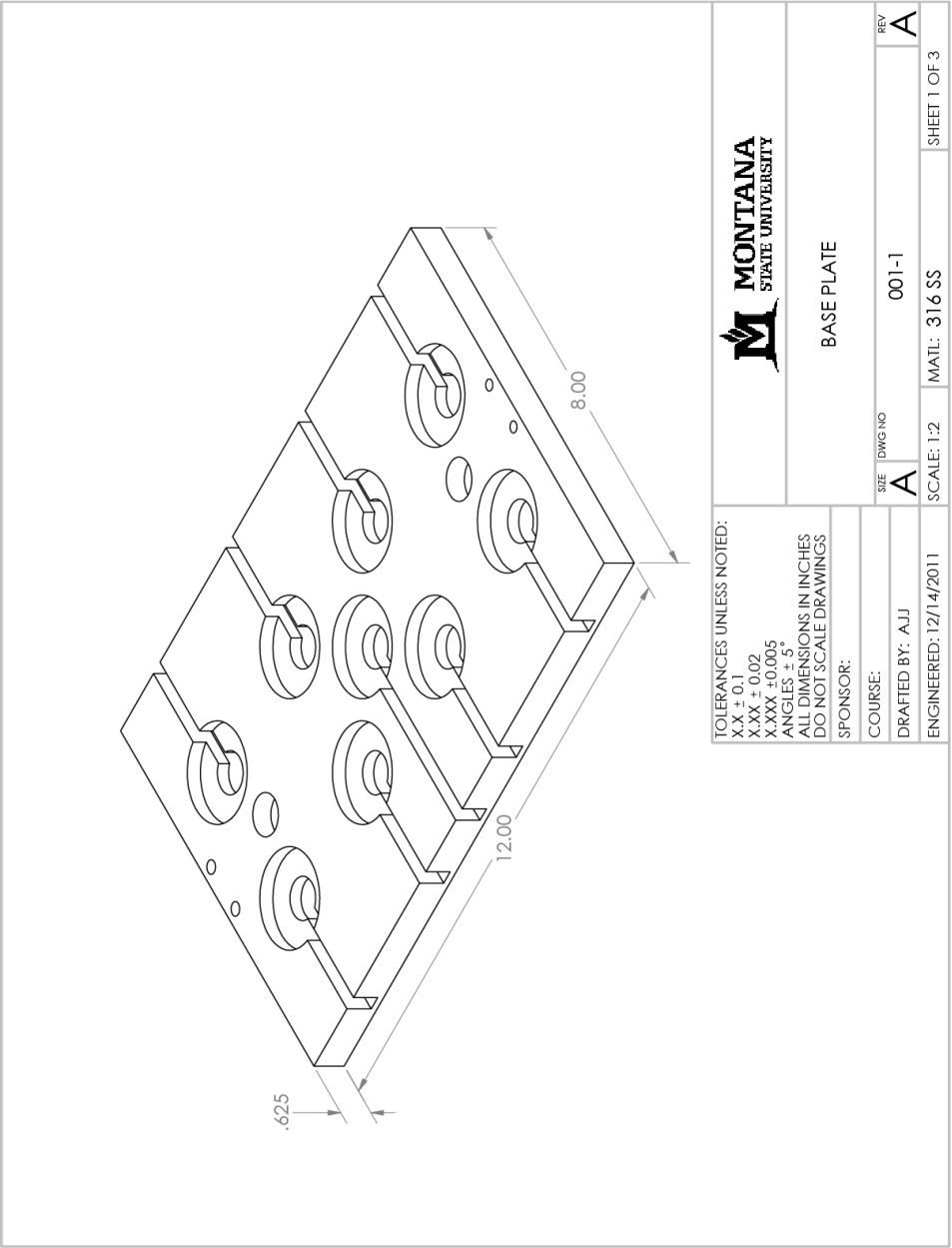
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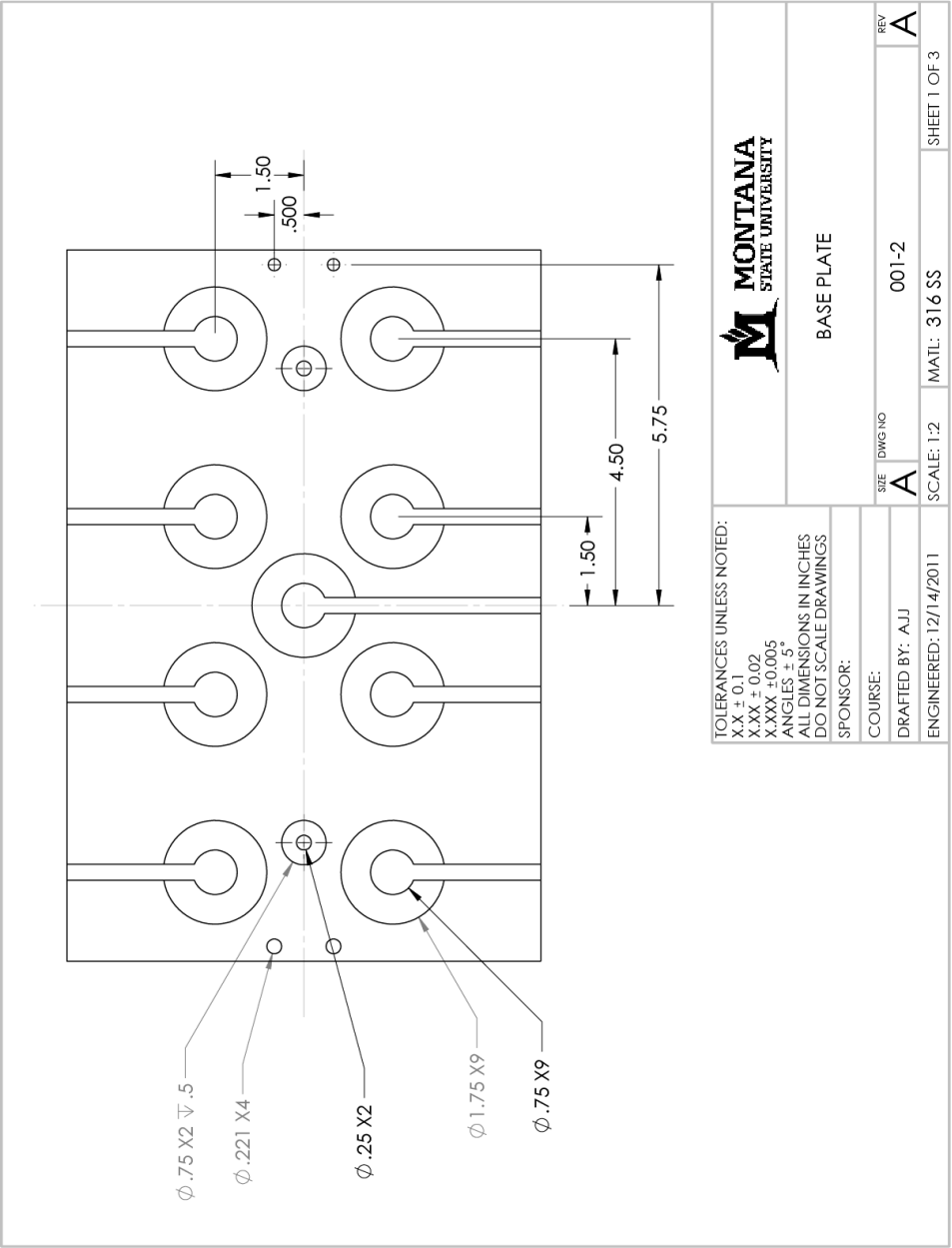
17.39

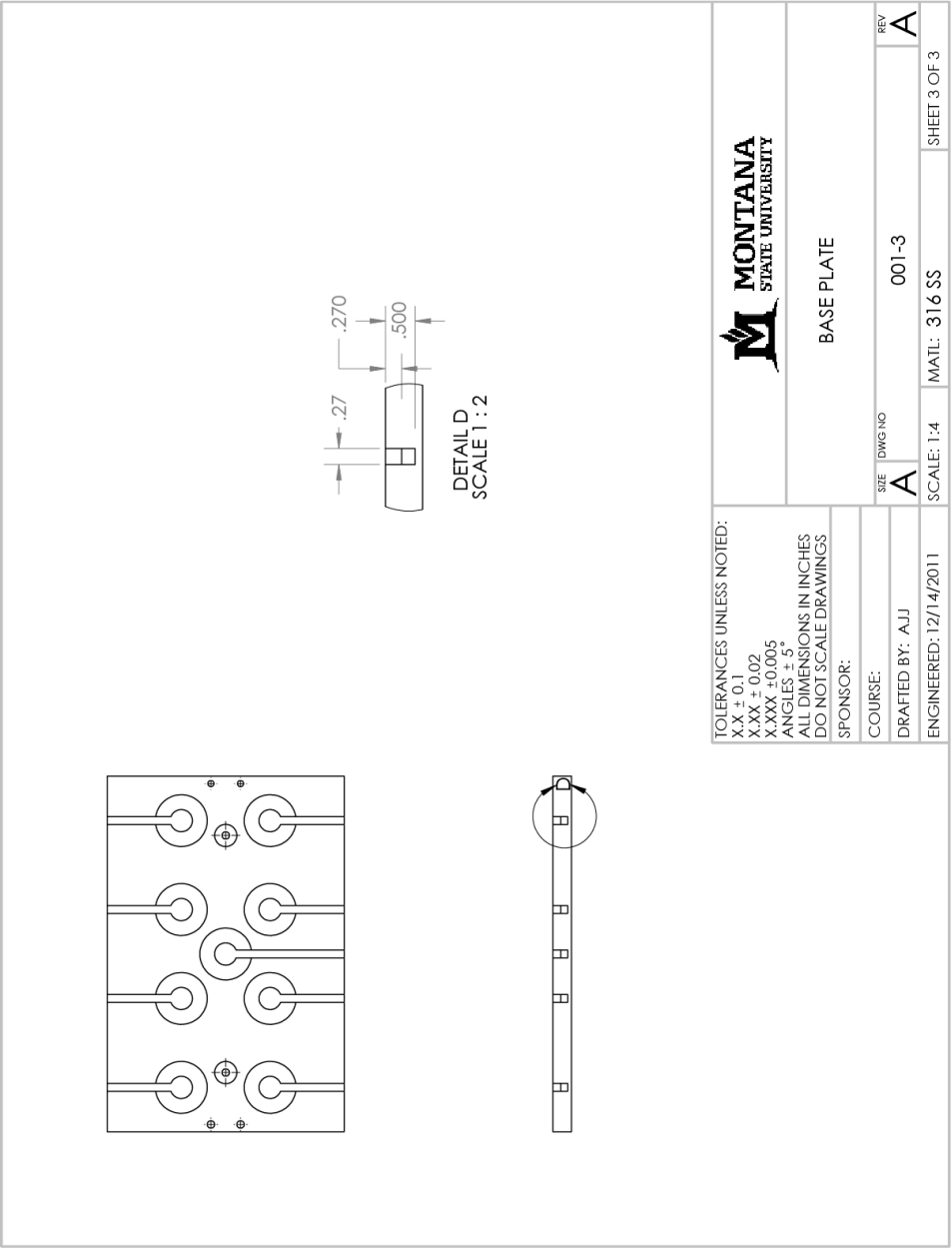
Shunt Cal is placed across (-E)(-S)

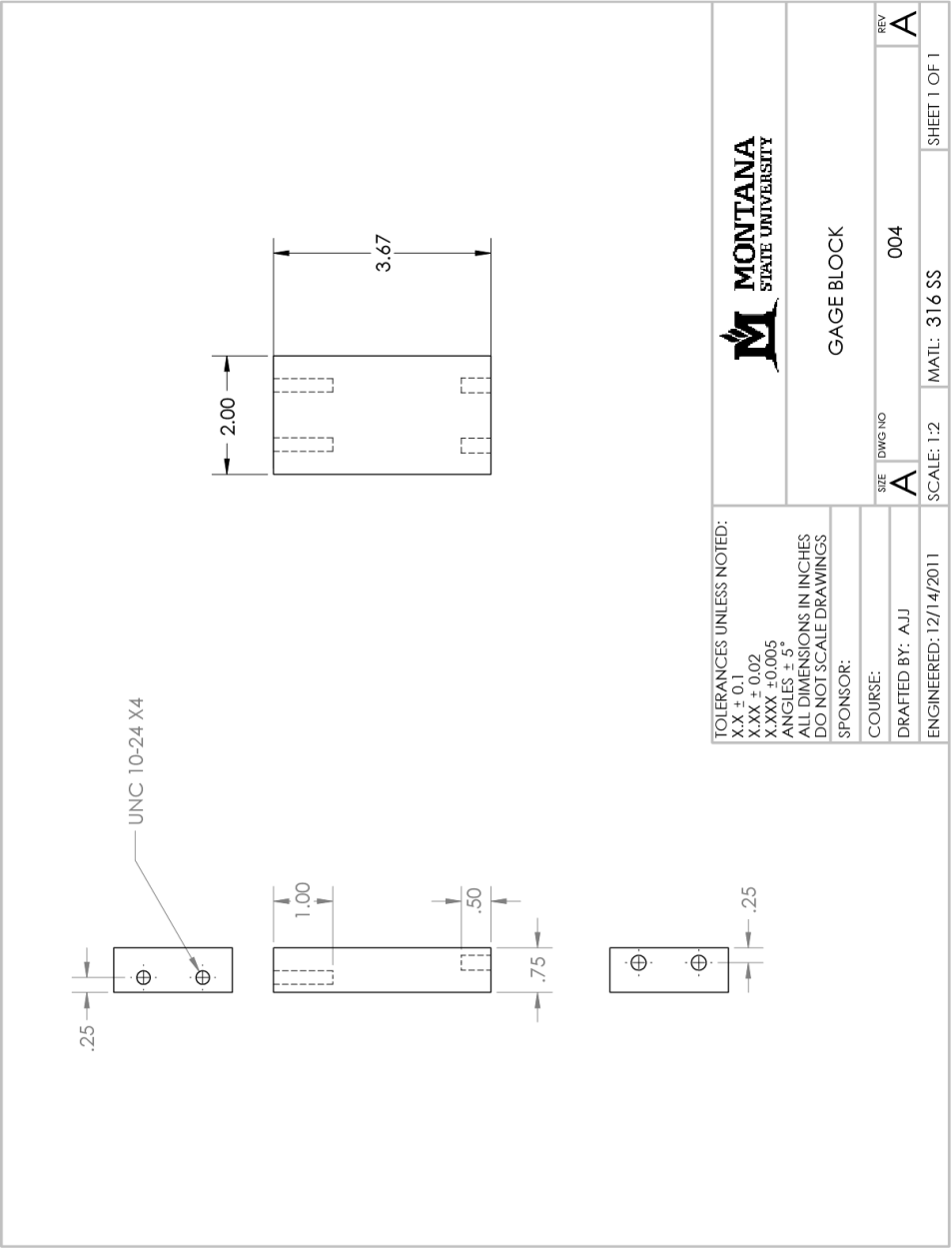
APPENDIX B

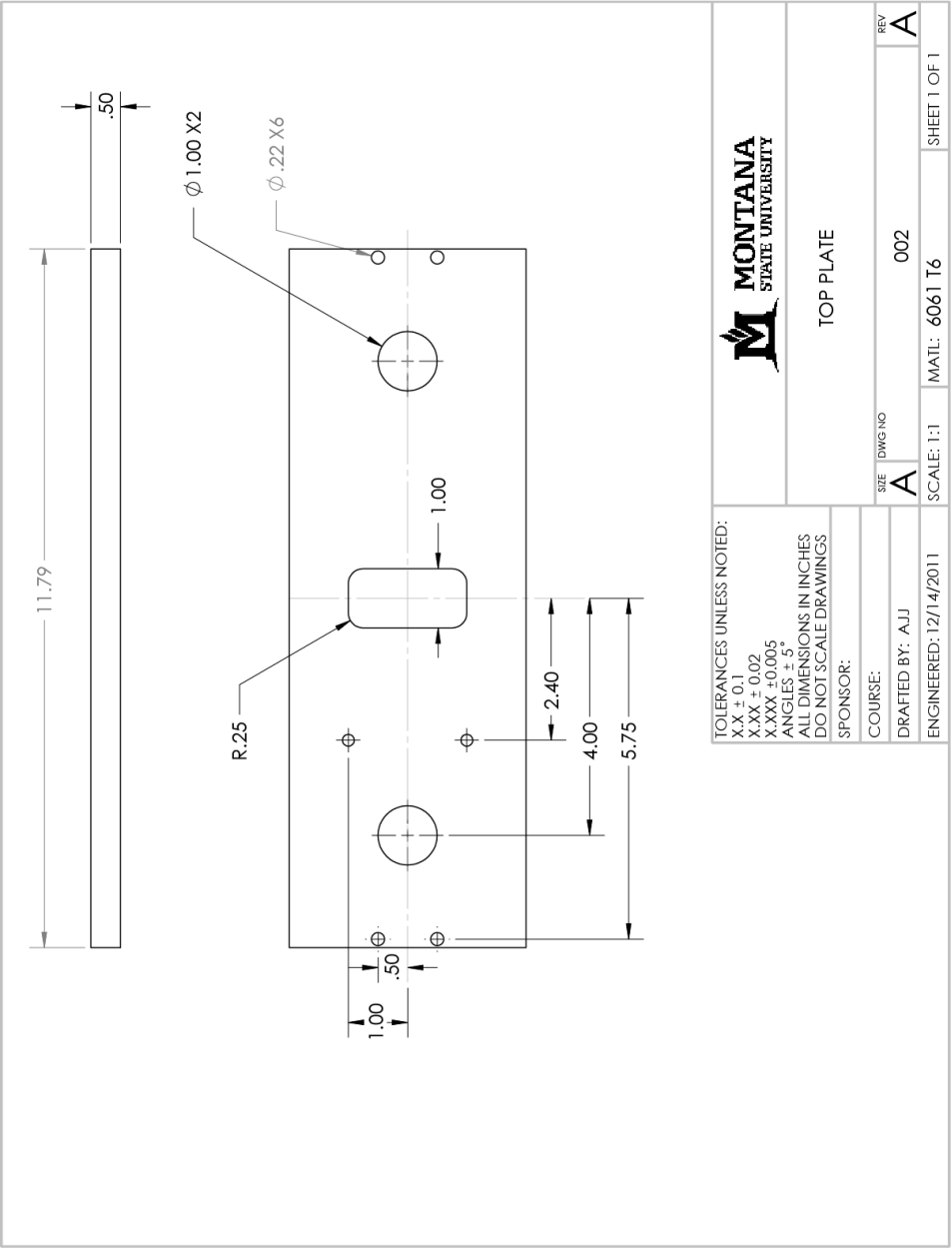
DESIGN DRAWINGS AND EXAMPLE CNC CODE

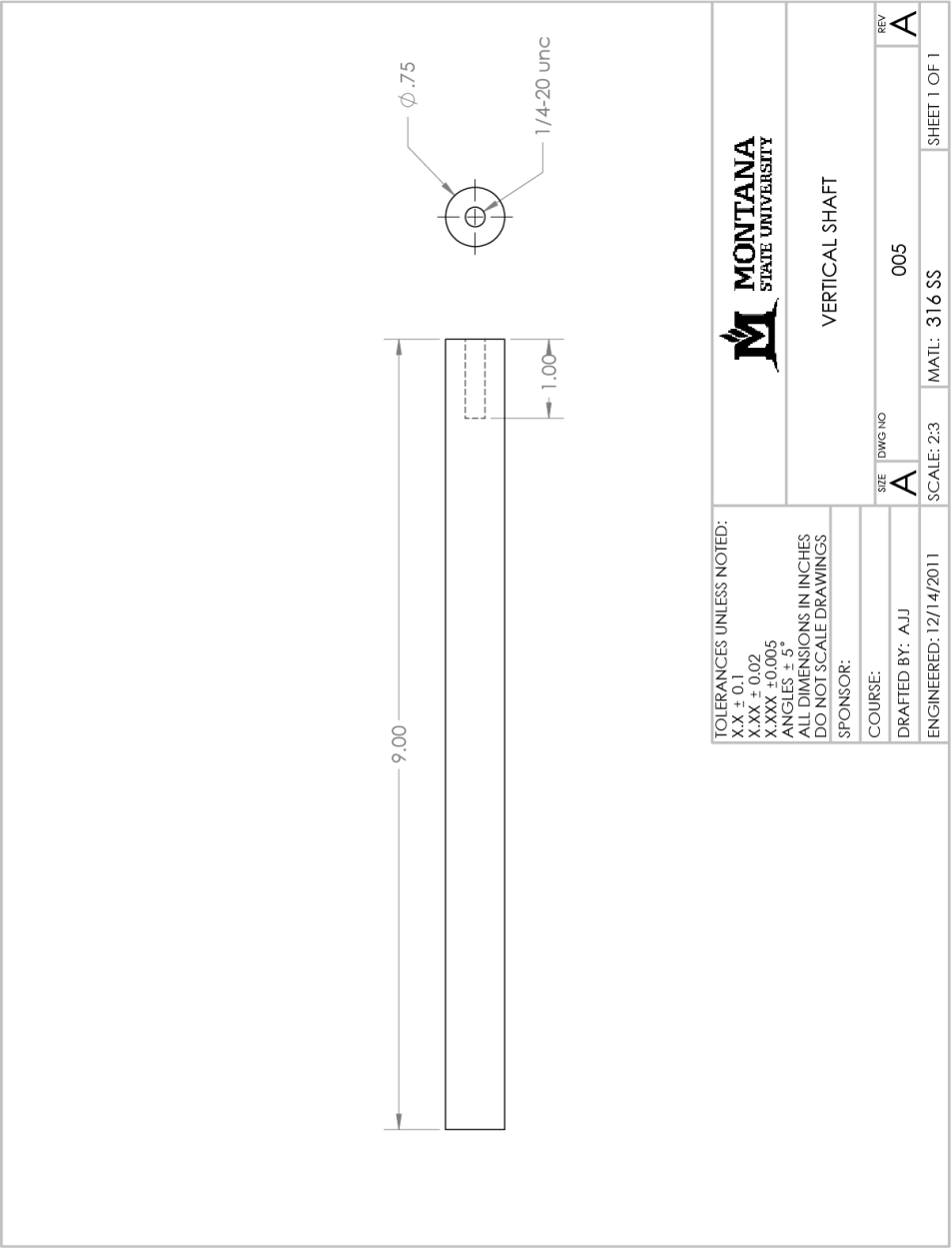


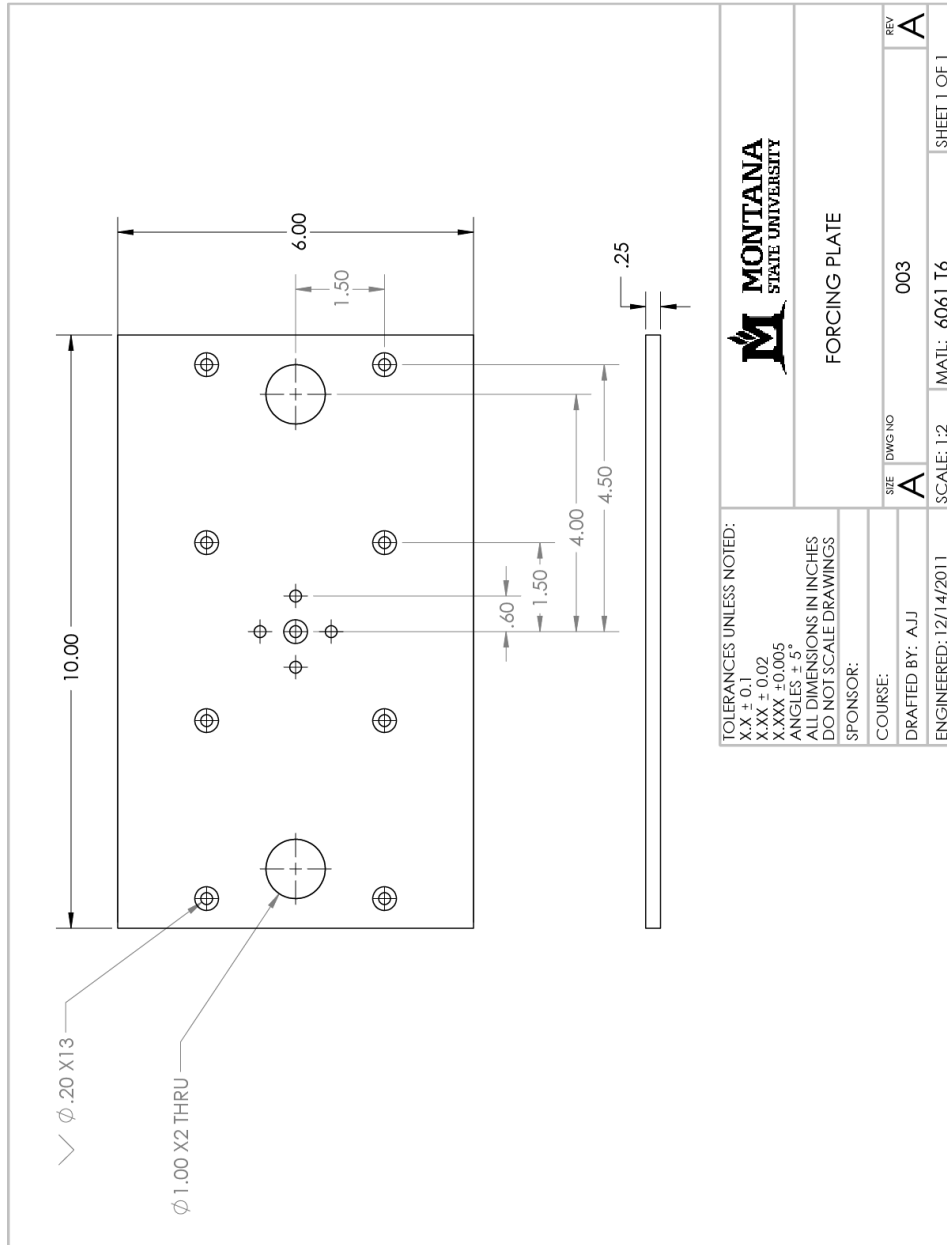


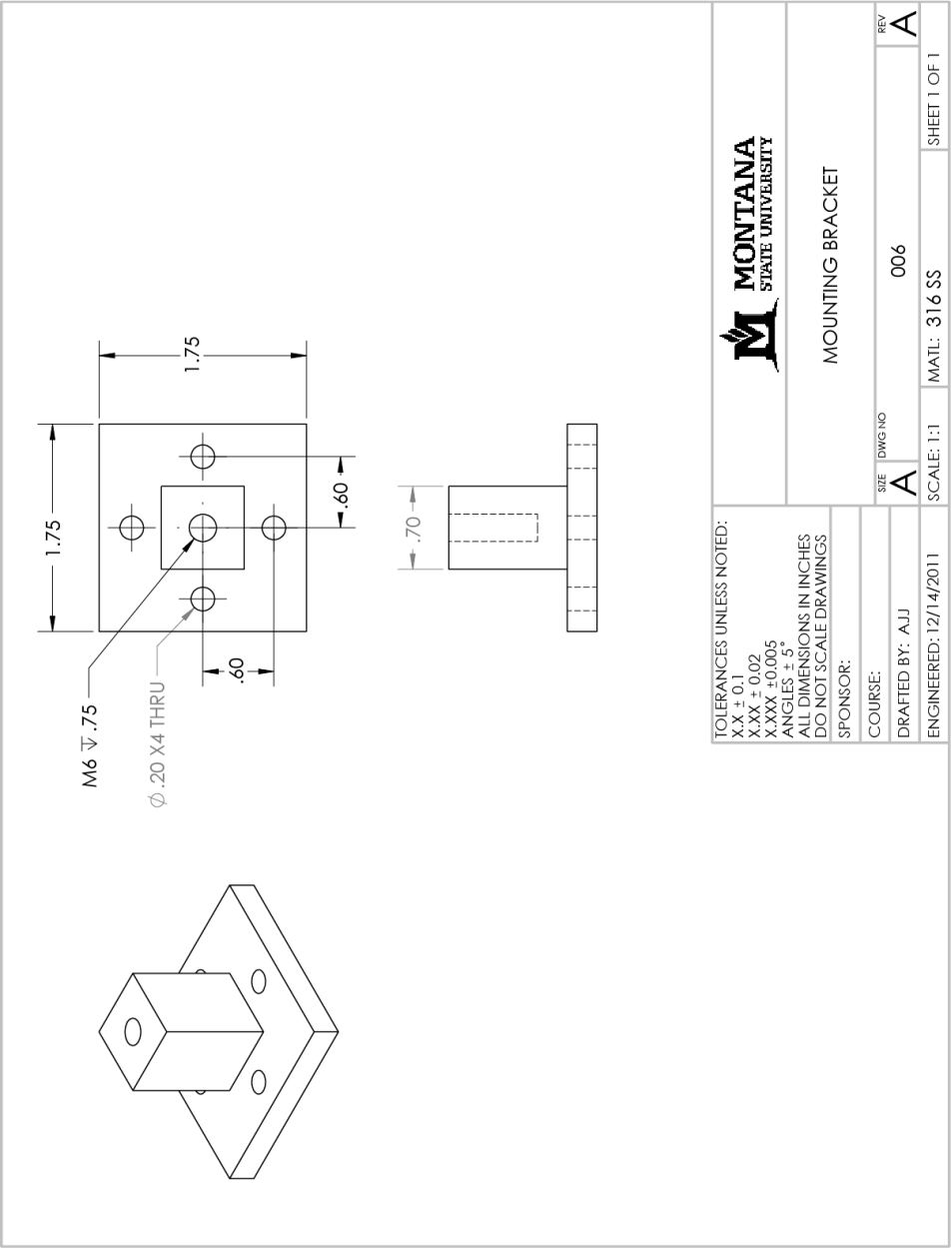


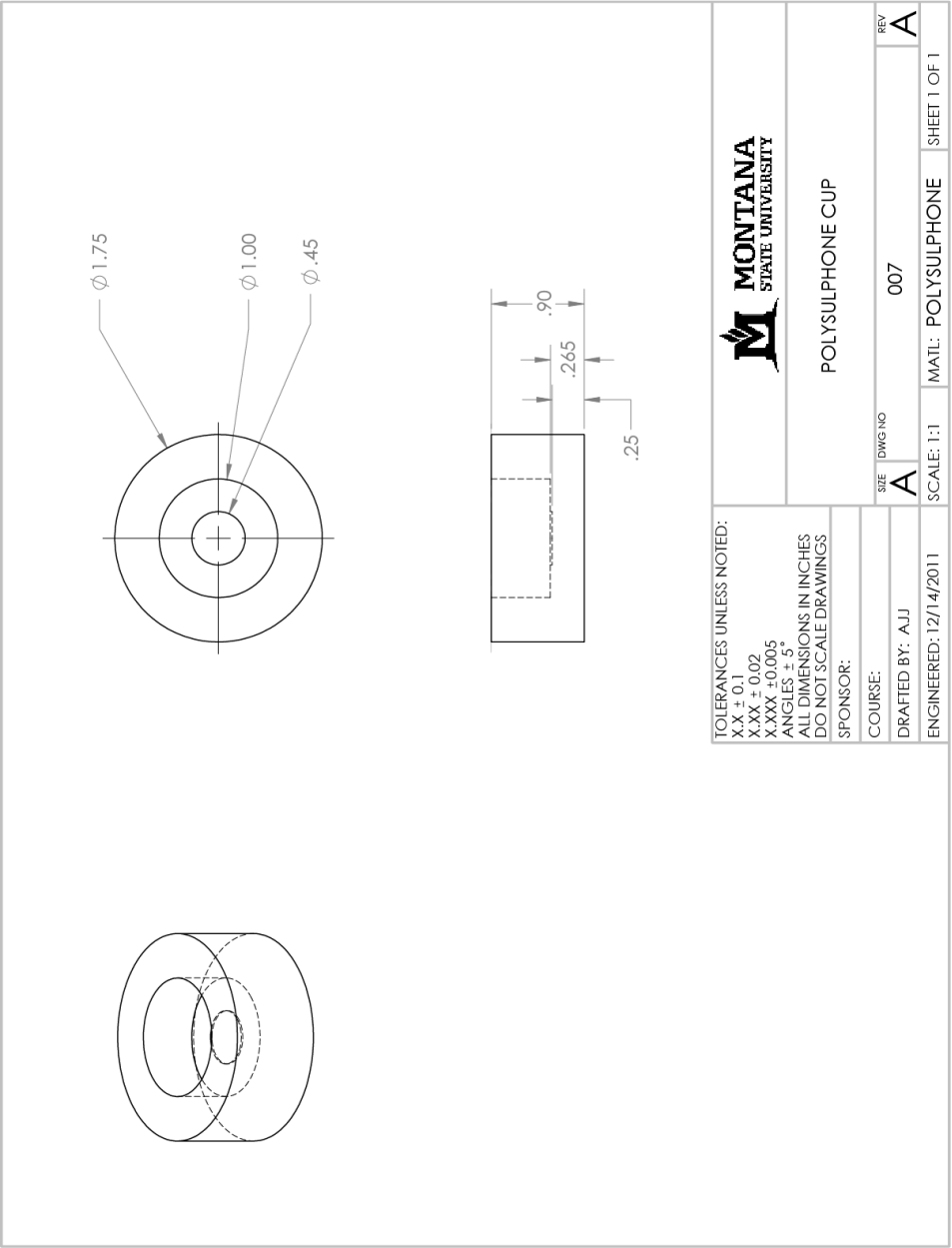


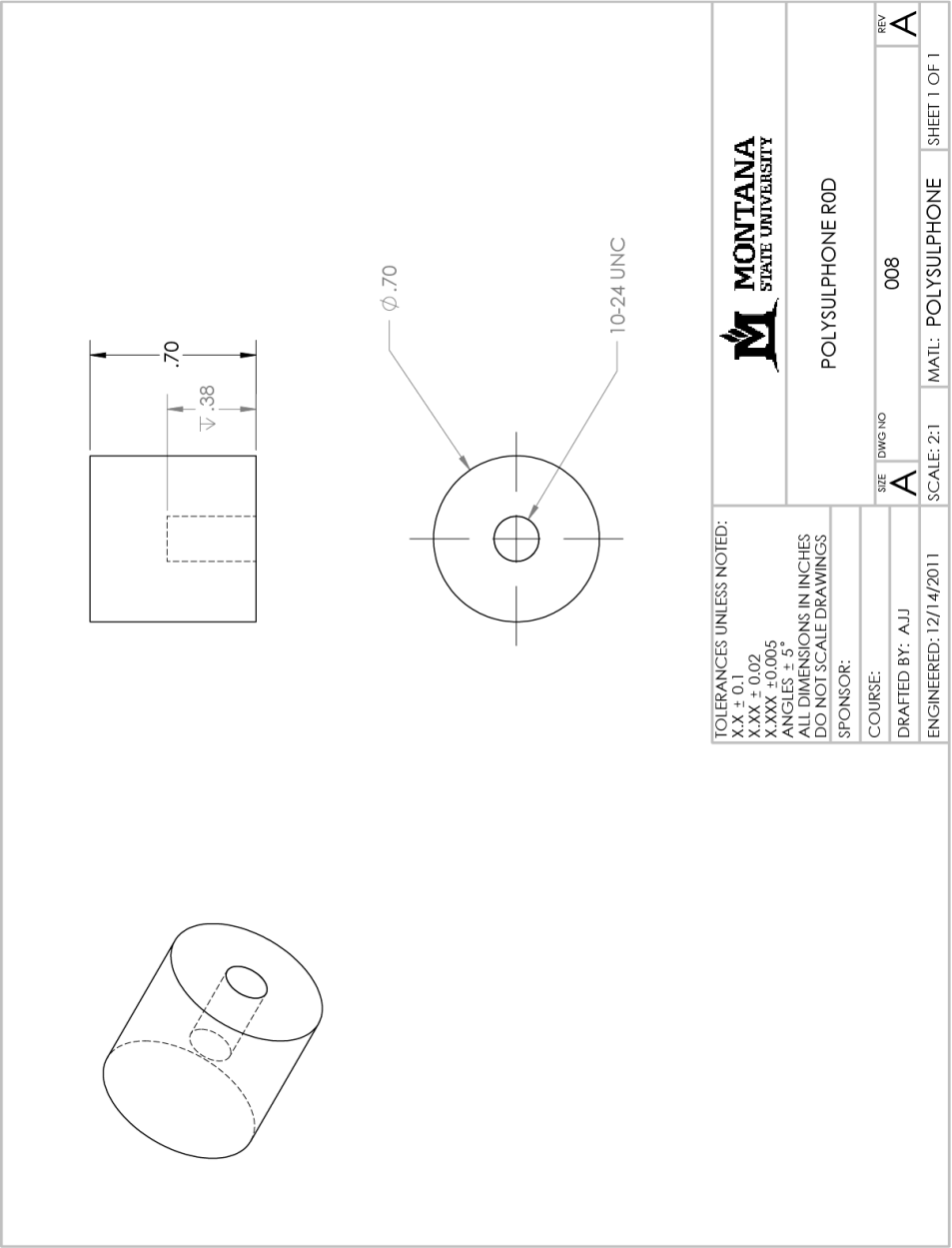












%
 (DATE=DD-MM-YY - 28-11-11 TIME=HH:MM - 11:26)
 (NC FILE - F:\MASTERCAM\FORCE!.NC)
 (MATERIAL - ALUMINUM INCH - 2024)
 (T5 | 4-FLUTE, CENTER CUTTING EM | H5)
 N100 G20
 N102 G0 G17 G40 G49 G80 G90
 N104 T5 M6
 N106 G0 G90 G54 X1.0469 Y3. A0. S3000 M3
 N108 G43 H5 Z2.
 N110 Z.2
 N112 G1 Z-.1 F5.
 N114 G3 X.9063 Y3.1406 I-.1406 J0. F10.
 N116 X.7656 Y3. I0. J-.1406
 N118 X.7799 Y2.9044 I.3282 J0.
 N120 X1. Y2.76 I.2201 J.0956
 N122 X1.24 Y3. I0. J.24
 N124 X1. Y3.24 I-.24 J0.
 N126 X.76 Y3. I0. J-.24
 N128 X.7799 Y2.9044 I.24 J0.
 N130 G1 X1.0469 Y3.
 N132 Z-.2 F5.
 N134 G3 X.9063 Y3.1406 I-.1406 J0. F10.
 N136 X.7656 Y3. I0. J-.1406
 N138 X.7799 Y2.9044 I.3282 J0.
 N140 X1. Y2.76 I.2201 J.0956
 N142 X1.24 Y3. I0. J.24
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 N148 X.7799 Y2.9044 I.24 J0.
 N150 G1 X1.0469 Y3.
 N152 Z-.3 F5.
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 N156 X.7656 Y3. I0. J-.1406
 N158 X.7799 Y2.9044 I.3282 J0.
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 N162 X1.24 Y3. I0. J.24
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 N166 X.76 Y3. I0. J-.24
 N168 X.7799 Y2.9044 I.24 J0.
 N170 G1 X1.0469 Y3.
 N172 Z-.4 F5.
 N174 G3 X.9063 Y3.1406 I-.1406 J0. F10.
 N176 X.7656 Y3. I0. J-.1406

N178 X.7799 Y2.9044 I.3282 J0.
N180 X1. Y2.76 I.2201 J.0956
N182 X1.24 Y3. I0. J.24
N184 X1. Y3.24 I-.24 J0.
N186 X.76 Y3. I0. J-.24
N188 X.7799 Y2.9044 I.24 J0.
N190 G1 X1.0469 Y3.
N192 Z-.5 F5.
N194 G3 X.9063 Y3.1406 I-.1406 J0. F10.
N196 X.7656 Y3. I0. J-.1406
N198 X.7799 Y2.9044 I.3282 J0.
N200 X1. Y2.76 I.2201 J.0956
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N206 X.76 Y3. I0. J-.24
N208 X.7799 Y2.9044 I.24 J0.
N210 G0 Z2.
N212 X9.0469 Y3.
N214 Z.2
N216 G1 Z-.1 F5.
N218 G3 X8.9063 Y3.1406 I-.1406 J0. F10.
N220 X8.7656 Y3. I0. J-.1406
N222 X8.7799 Y2.9044 I.3282 J0.
N224 X9. Y2.76 I.2201 J.0956
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N232 X8.7799 Y2.9044 I.24 J0.
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N236 Z-.2 F5.
N238 G3 X8.9063 Y3.1406 I-.1406 J0. F10.
N240 X8.7656 Y3. I0. J-.1406
N242 X8.7799 Y2.9044 I.3282 J0.
N244 X9. Y2.76 I.2201 J.0956
N246 X9.24 Y3. I0. J.24
N248 X9. Y3.24 I-.24 J0.
N250 X8.76 Y3. I0. J-.24
N252 X8.7799 Y2.9044 I.24 J0.
N254 G1 X9.0469 Y3.
N256 Z-.3 F5.
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N260 X8.7656 Y3. I0. J-.1406
N262 X8.7799 Y2.9044 I.3282 J0.
N264 X9. Y2.76 I.2201 J.0956

N266 X9.24 Y3. IO. J.24
 N268 X9. Y3.24 I-.24 J0.
 N270 X8.76 Y3. IO. J-.24
 N272 X8.7799 Y2.9044 I.24 J0.
 N274 G1 X9.0469 Y3.
 N276 Z-.4 F5.
 N278 G3 X8.9063 Y3.1406 I-.1406 J0. F10.
 N280 X8.7656 Y3. IO. J-.1406
 N282 X8.7799 Y2.9044 I.3282 J0.
 N284 X9. Y2.76 I.2201 J.0956
 N286 X9.24 Y3. IO. J.24
 N288 X9. Y3.24 I-.24 J0.
 N290 X8.76 Y3. IO. J-.24
 N292 X8.7799 Y2.9044 I.24 J0.
 N294 G1 X9.0469 Y3.
 N296 Z-.5 F5.
 N298 G3 X8.9063 Y3.1406 I-.1406 J0. F10.
 N300 X8.7656 Y3. IO. J-.1406
 N302 X8.7799 Y2.9044 I.3282 J0.
 N304 X9. Y2.76 I.2201 J.0956
 N306 X9.24 Y3. IO. J.24
 N308 X9. Y3.24 I-.24 J0.
 N310 X8.76 Y3. IO. J-.24
 N312 X8.7799 Y2.9044 I.24 J0.
 N314 G0 Z2.
 N316 X1. Y2.75
 N318 Z.2
 N320 G1 Z-.5 F5.
 N322 G3 X1.25 Y3. IO. J.25 F10.
 N324 X1. Y3.25 I-.25 J0.
 N326 X.75 Y3. IO. J-.25
 N328 X1. Y2.75 I.25 J0.
 N330 G0 Z2.
 N332 X9.
 N334 Z.2
 N336 G1 Z-.5 F5.
 N338 G3 X9.25 Y3. IO. J.25 F10.
 N340 X9. Y3.25 I-.25 J0.
 N342 X8.75 Y3. IO. J-.25
 N344 X9. Y2.75 I.25 J0.
 N346 G0 Z2.
 N348 M5
 N354 M30
 %

APPENDIX C

ASSEMBLY AND DISASSEMBLY

This manual is intended to be a reference for someone that has already been shown how to assemble/disassemble the machine.

Tools Needed:

Flathead Screw Driver

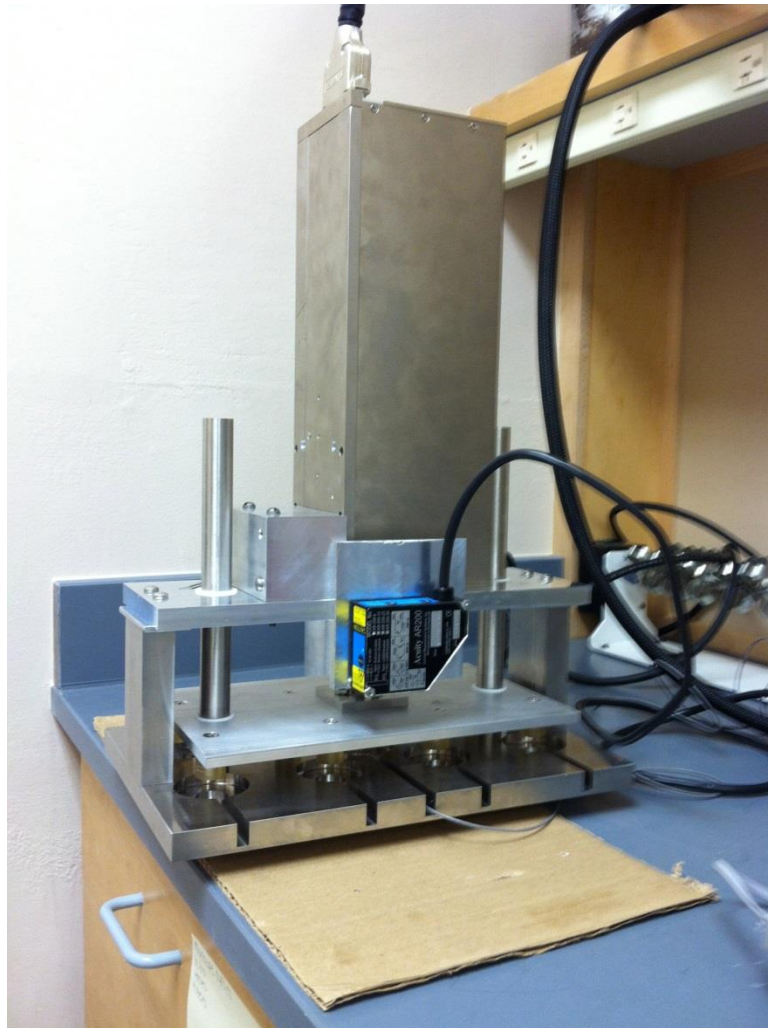
Phillips Screw Driver

5/16 inch wrench

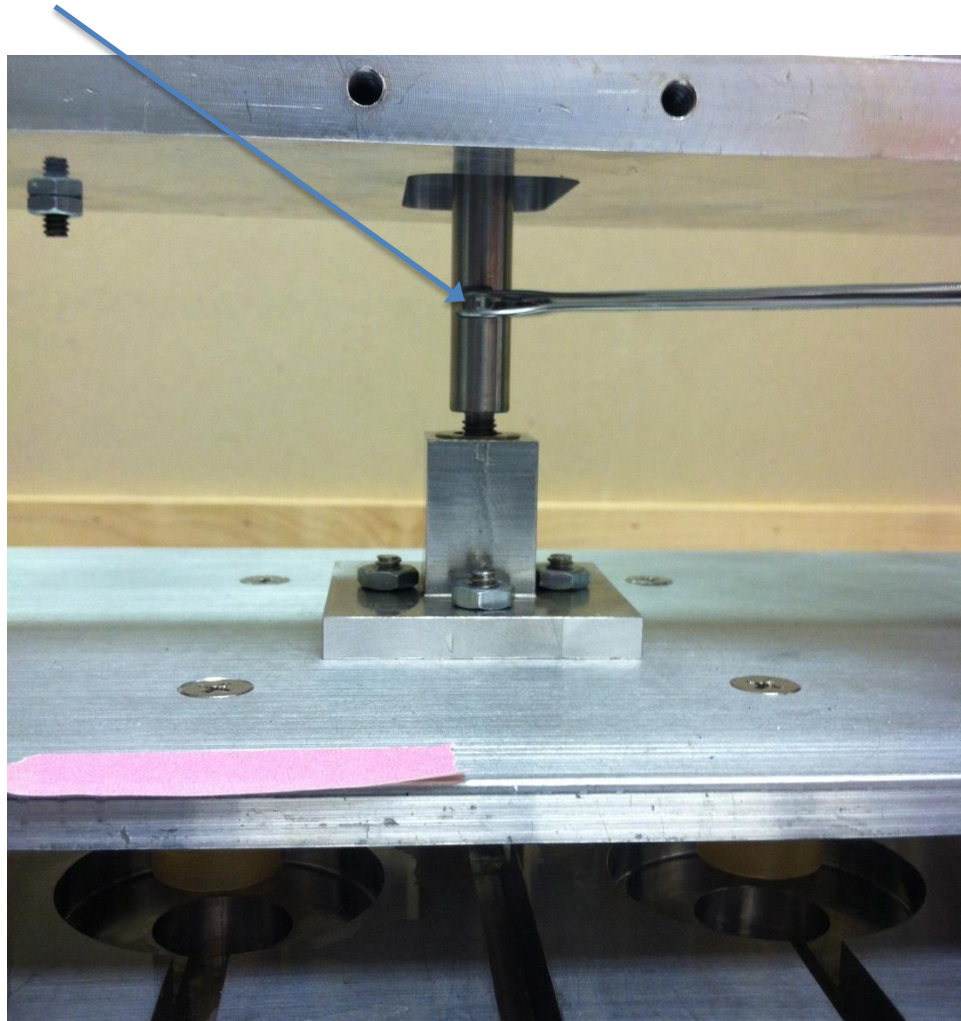
3/8 inch wrench

5/32 allen wrench

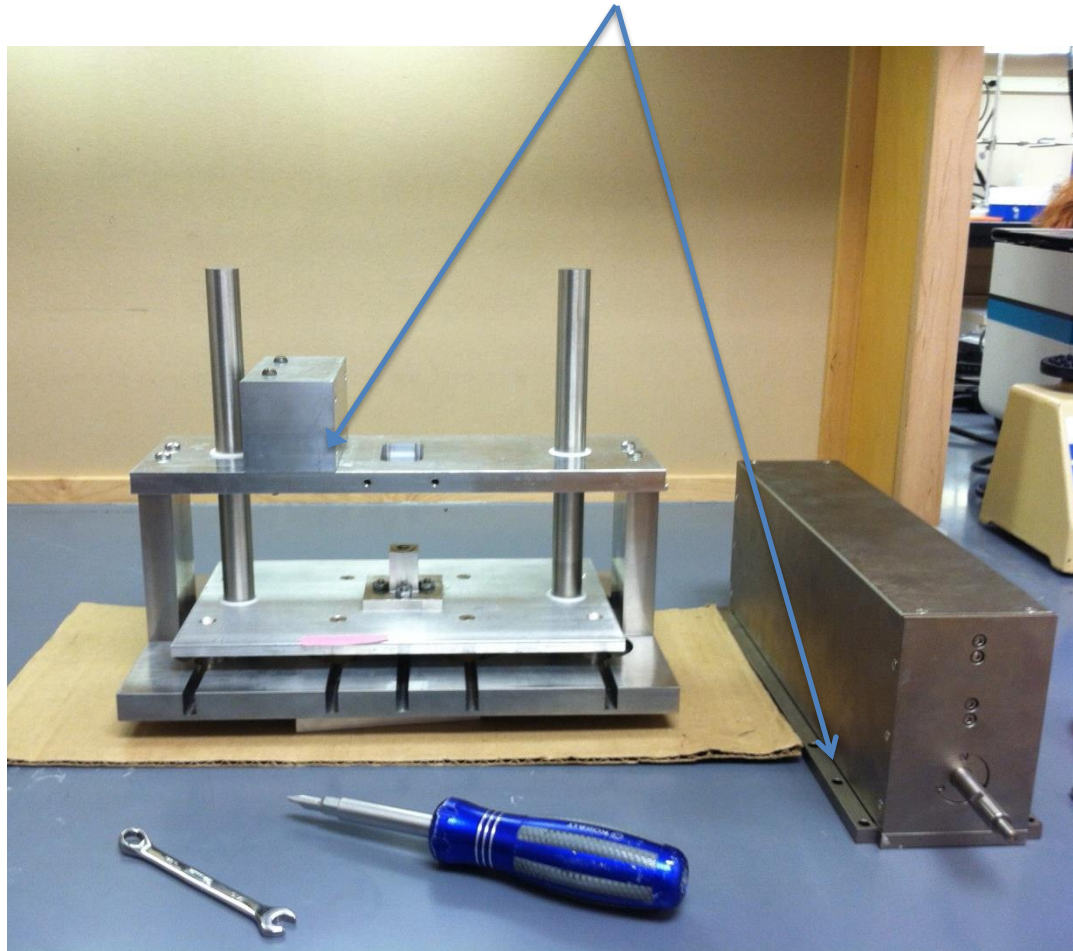
This manual provides step-by-step instructions disassembly of the loading apparatus. It is assume that the machine has already been removed from the incubator and is sitting on countertop.



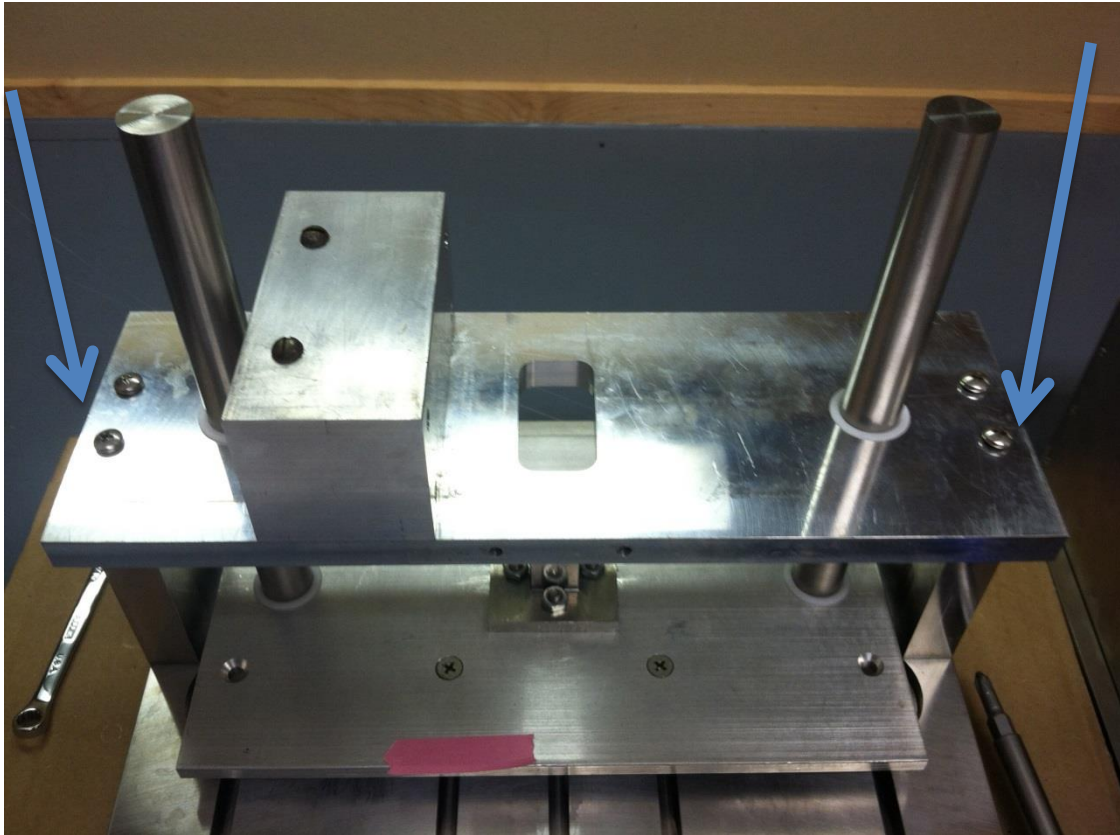
Use a small flathead screwdriver to remove control cable from top of the actuator. Next, remove load cell from whatever slot it is in with a pair of tweezers. Then remove the laser displacement sensor (two nuts hand tightened). At this point all of electrical components should be removed from the loading apparatus. Remove laser displacement sensor mounting plate and set aside (two bolts with flathead screw driver). Using a 5/16 inch wrench, turn flats on actuator shaft to unthread the actuator shaft from mounting bracket. This is a long and delicate process also make sure to support the forcing plate so it does not just smash down.



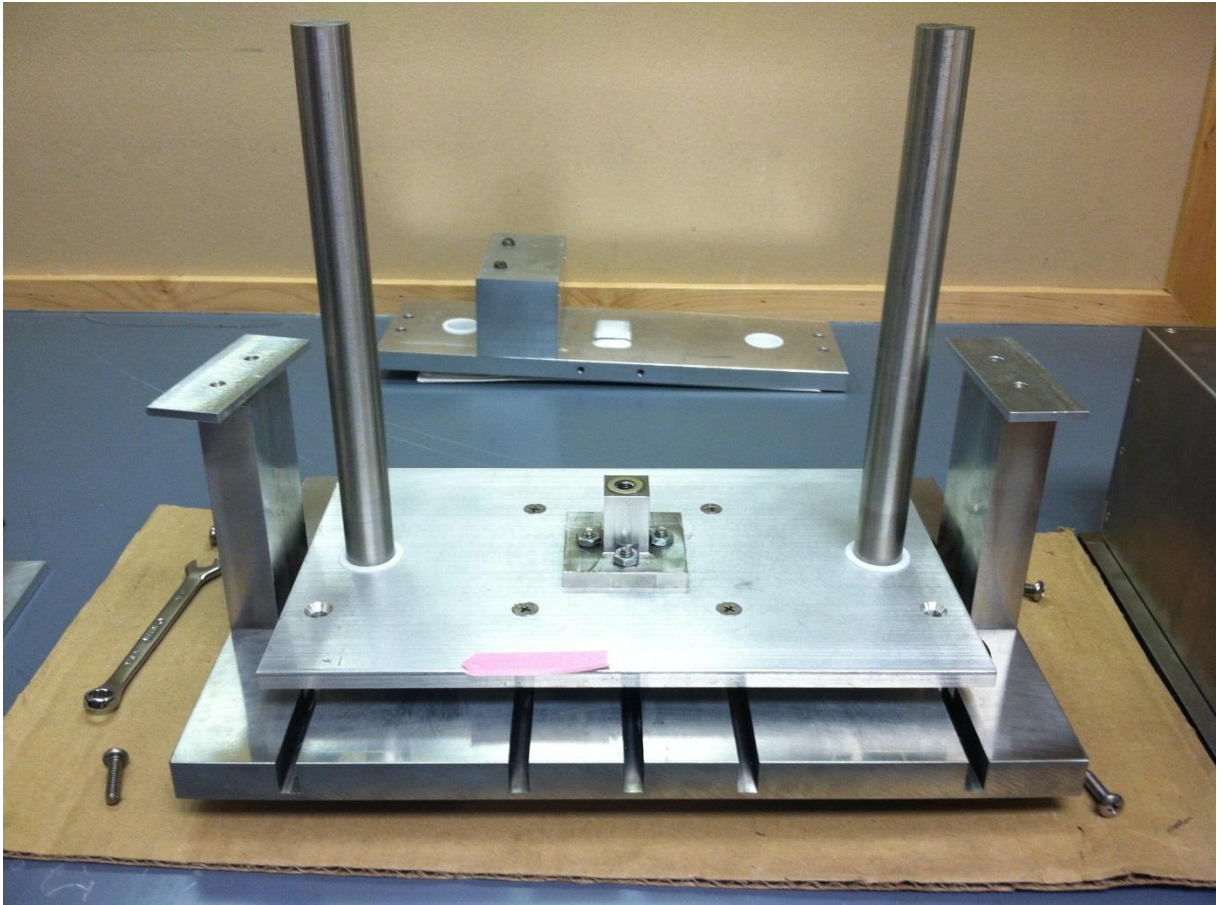
After the forcing plate has been detached from the actuator the next step is to remove the 2.5 inch horizontal bolts from the side of the actuator and lift the actuator straight up. Set the actuator aside on a flat surface away from the loading frame.



At this point there will be four machine screws holding the top plate onto the gage blocks, remove them with a Phillips screwdriver.



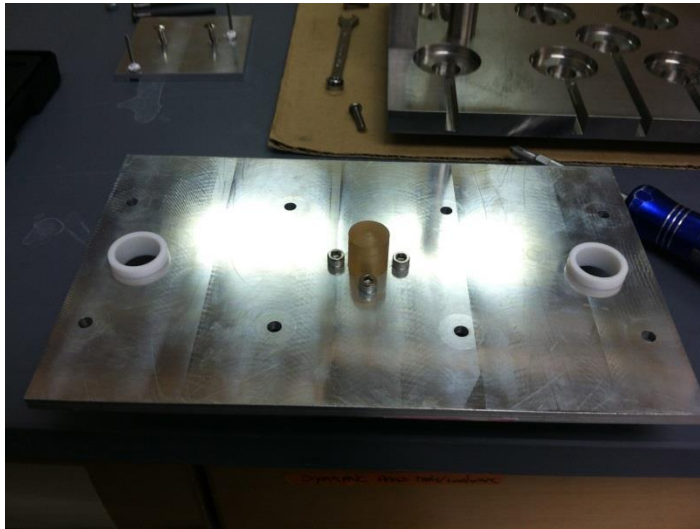
Once the top plate is removed the loading apparatus should look like this. The next step is to slide the forcing plate directly up along the vertical guide rods.



Once the forcing plate has been removed the gage blocks and vertical guide rods can be removed. Each gage block is held onto the base plate with two screws in them same configuration as the screws that held the top plate on. Carefully remove them without dropping them. The vertical guide rods can also be removed at this point. They are force fit into the base plate. In order to remove them unscrew the stainless steel fastener about a $\frac{1}{4}$ of an inch and use a hammer to tap them out. There should be no reason to remove the vertical guide rods.



With the structure of the apparatus disassembled turn back to the forcing plate. From here the polysulfone plungers can be removed and autoclaved if need. The mounting bracket can also be removed at this time. Each plunger is held onto the forcing plate with a 10-24 stainless steel screw. The mounting bracket is held onto the forcing plate with four machinist screws, use a 5/32 allen wrench remove the four screws.



At this point it is possible to sterilize all of the machine components. Wrap each part with tinfoil and autoclave them for 20 minutes. When reassembling replace the four Teflon bushing (There is supply of them in the storage cabinet). Reassemble the machine inside the biohazard cabinet in the opposite order of disassembly.

APPENDIX D

MOTION CONTROL CODES

```

;
;(MS0) = LOAD PID'S, ENCODER CHECK
;(MS20) = HOMING LINEAR
;(MS40) = POSITION MOVE
;(MS50) = SOFTLAND
;(MS60) = SINEWAVE
;
MF
RM
MD0,MJ5
;
; *****SET CHANNELS (I/O) OFF*****
MD5,CF0,CF1,CF2,CF3,MJ10
;
; *****LOADING PID'S*****
;Linear
Md10,pm,sg10,si50,sd120,il5000,ph0,fr1,ss1,se16383

; *****HOMING LINEAR*****
;First retract to hard stop
MD20,SV100000,SA10000,SQ30000,SE16383,VM,MN,DI1,GO,FI
;Look for index or forward position error
MD21,RL448,IC10,MJ23,NO,RW538,IB-500,DI0,MJ22,RP
;Look for index or retract position error
MD22,RL448,IC10,MJ23,NO,RW538,IG500,MJ25,NO,RP
;After index found, Go to position mode
MD23,AB,PM,GH,MG"LINEAR HOME",EP
;If position error occurs, motor of & end program
MD25,MG"NO INDEX",MF,EP
;
; *****POSITION MODE MOVE*****
;This line extends 2000 counts and back to 500, 5 times.
MD40,PM,MN,SV100000,SA50000
MD41,MA2000,GO,WS50,MA500,GO,WS50,RP5,EP
;
; *****SOFTLAND*****
MD50,MN,VM,SA10000,SV50000,SQ8010,DI0,GO,WA50
MD51,RW538,IG50,AB,MJ52,RP
MD52,MG"LANDED",TP,PM,EP
;

```

```

;
; *****SINE WAVE*****
MD60,CS500,AL100,WW422,AL494,WW424,AL0,WW426
MD61,PM,MN,SV7864320,SA1034,MA120000,GO,WS0
MD62,CD500
MD63,MA90000,GO,WS0,MA120000,GO,WS0,RP10,EP

;*****SINE WAVE .5hZ .2413MM AMP (2% STRAIN) *****
MD24,SV7864320,SA62,WS3000
MD25,MR2413,GO,WS0
MD26,MR-4826,GO,WS0,MR4826,GO,WS0,RP100
MD27,MR-2413,GO,WS30000,TP

;*****SINE WAVE 1hZ .2413MM AMP (2% STRAIN) *****
MD28,SV7864320,SA250
MD29,MR2413,GO,WS0,MJ30
MD30,MR-4826,GO,WS0,MR4826,GO,WS0,RP100
MD31,MR-2413,GO,WS30000,TP

;*****SINE WAVE 5hZ .2413MM AMP (2% STRAIN) *****
MD32,SV7864320,SA6243
MD33,MR2413,GO,WS0
MD34,MR-4826,GO,WS0,MR4826,GO,WS0,RP100
MD35,MR-2413,GO,WS3000,TP,GH

;*****RELAX*****
MD10,MR6350,GO,TP,ws5000
;*****SINE WAVE 1hZ .2413MM AMP (2% STRAIN) *****
MD11,SV7864320,SA250
MD12,MR2413,GO,WS0
MD13,MR-4826,GO,WS0,MR4826,GO,WS0,RP1800
MD14,MR-2413,GO,WS3000,TP
MD15,GH

```

APPENDIX E

MAPLE CODE

> restart;

This worksheet is going to explore a three parameter solid representation of a viscoelastic solid material.

Laplace transform of the three-parameter solid

> epsbar:=taubar/E[sp]+taubar/(E[k]+s*eta[k]);

$$\text{epsbar} := \frac{\text{taubar}}{E_{sp}} + \frac{\text{taubar}}{E_k + s\eta_k}$$

> simplify(epsbar);

$$\frac{\text{taubar} (E_k + s\eta_k + E_{sp})}{E_{sp} (E_k + s\eta_k)}$$

From here I will find Qbar and Pbar, which represent polynomials that fit the equation Qbar*epsbar = Pbar*taubar

> Qbar:=E[sp]*(E[k]+s*eta[k]);Pbar:=E[k]+s*eta[k]+E[sp];

$$Qbar := E_{sp} (E_k + s\eta_k)$$

$$Pbar := E_k + s\eta_k + E_{sp}$$

> with(inttrans);

[addtable, fourier, fouriercos, fouriersin, hankel, hilbert, invfourier, invhilbert, invlaplace, invmellin, laplace, mellin, savetable]

Creating the laplace transform of the creep compliance

> Jbar:=(Pbar/Qbar)*laplace(Heaviside(t),t,s);

$$Jbar := \frac{E_k + s\eta_k + E_{sp}}{E_{sp} (E_k + s\eta_k) s}$$

> J:=invlaplace(Jbar,s,t);

$$J := \frac{1}{E_{sp}} + \frac{1 - e^{-\frac{E_k t}{\eta_k}}}{E_k}$$

Create the laplace transform of the relaxation modulus

> Ybar:=1/Jbar;

$$Ybar := \frac{E_{sp} (E_k + s\eta_k) s}{E_k + s\eta_k + E_{sp}}$$

> Y:=invlaplace(Ybar,s,t);

$$Y := \text{Dirac}(1, t) E_{sp} + \frac{E_{sp}^2}{\eta_k^2} \left(-\text{Dirac}(t) \eta_k + e^{-\frac{(E_k + E_{sp}) t}{\eta_k}} (E_k + E_{sp}) \right)$$

Create a loading function representative of single step stress relaxation experiment

> load:=Strain*Heaviside(t);

$$\text{load} := \text{Strain Heaviside}(t)$$

> tau:=int(subs(t=tp,diff(load,t))*subs(t=t-tp,Y),tp=-infinity..t);

$$\tau := \text{Strain Heaviside}(t) \left(\text{Dirac}(1, t) E_{sp} + \frac{E_{sp}^2}{\eta_k^2} \left(-\text{Dirac}(t) \eta_k + e^{-\frac{(E_k + E_{sp})t}{\eta_k}} (E_k + E_{sp}) \right) \right)$$

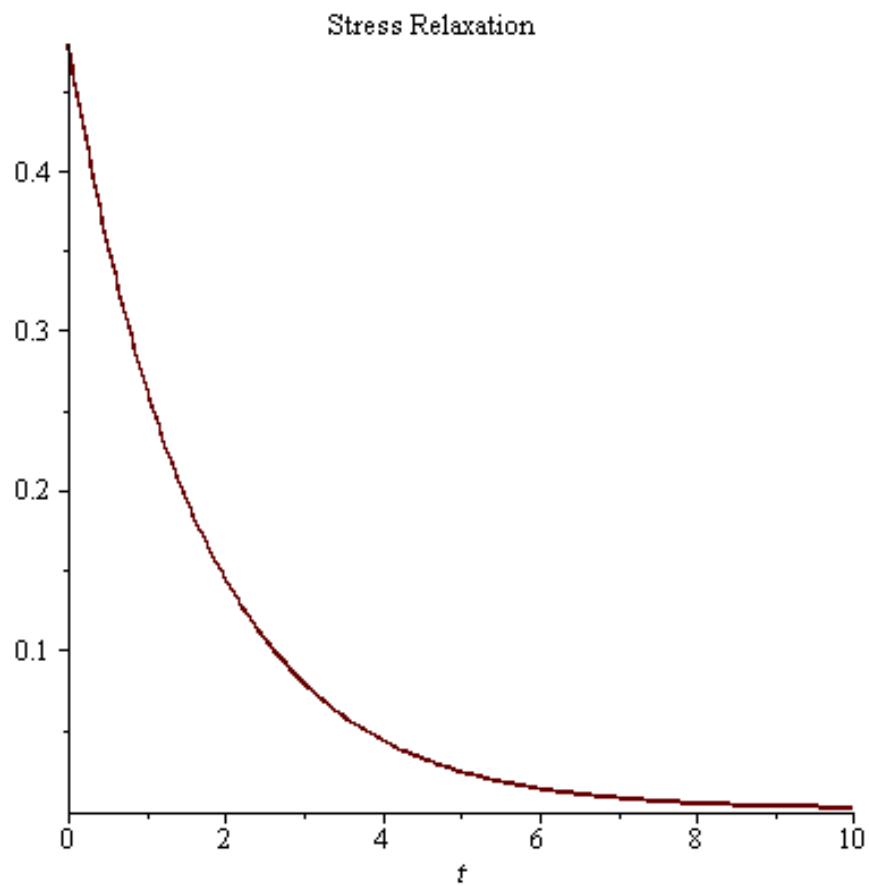
```
> data:=E[sp]=1,eta[k]=5,E[k]=2,Strain=4;
```

$$data := E_{sp} = 1, \eta_k = 5, E_k = 2, \text{Strain} = 4$$

```
> plottau:=subs(data,tau);
```

$$plottau := 4 \text{Heaviside}(t) \left(\text{Dirac}(1, t) - \frac{1}{5} \text{Dirac}(t) + \frac{3}{25} e^{-\frac{3}{5}t} \right)$$

```
> plot(plottau,t=0..10,title=["Stress Relaxation"]);
```



```
>
>
```

APPENDIX F

SUPPLIMENTARY DATA

Agarose Percent	Test #	Modulus		Notes
		Dynamic	Equilibrium	
4	1	156.6612	40.0641	
4	2	135.5795	36.6878	
4	3	137.8843	32.3495	
4	4	141.0678	40.582	
4	5	125.9644	31.8681	
4	Average	139.43144	36.3103	
	Std. Dev.	11.159464	4.12013407	
4.5	1	150.9399	39.5723	Gel Sheared. Last equilibrium point estimated. Data Error Last equilibrium step was lower than previous. Data Error. Shear.
4.5	2	177.4034	50.1616	
4.5	3	178.2221	52.7006	
4.5	4			
4.5	5	193.4894	48.4373	
4.5	6	197.1793	57.6424	
4.5	7	183.3351	52.7295	
4.5	8	174.7713	56.4894	
4.5	9	152.5339	19.9891	
4.5	10			
4.5	Average	184.06677	53.0268	Does not include data in red Does not include data in red
	Std. Dev.	9.2328346	3.54290946	
5	1	247.9056	68.1522	Gel Sheared. Last equilibrium point estimated. Gel Sheared. Last equilibrium point estimated. Gel Sheared. Last equilibrium point estimated. Gel Sheared. Data extremely skewed.
5	1A	224.4876	58.6211	
5	2	204.7878	45.3503	
5	3	77.5345	-27.3886	
5	4	212.6792	71.8043	
5.5	1	310.5428	90.1538	Gel Sheared. Last equilibrium point estimated. Gel Sheared. Last equilibrium point estimated. Does not include data in red Does not include data in red
5.5	2	297.2742	93.3392	
5.5	2A	321.2225	109.646	
5.5	3	217.1245	2.6336	
5.5	Average	315.88265	99.8999	
	Std. Dev.	7.5516883	13.7830668	
6	1	309.3324	111.0031	
6	2	286.7891	101.0101	
6	3	295.1297	104.003	
6	4	279.9237	97.1338	
6	5	305.4743	110.4403	
6	Average	295.32984	104.71806	
	Std. Dev.	12.342155	6.00056323	

APPENDIX G

EXPERIMENTAL PROTOCOLS

Gel Encapsulation

1. #'s: Mass, Volumes, etc. Pre-heat centrifuge
2. Prepare PI and Calcein staining buffers
3. Prepare Agarose at 1.1X (for example 4.4% w/v)
4. De-gas Agarose in centrifuge 5000g for 5 minutes then place in water bath
5. Prep cells: Trypsinize, pellet, re-suspend in small volume (1 ml), count
6. Dilute cells into 11X solution (for example 5.5 million cells in .5 ml) place in water bath
7. Wait 10 minutes
8. Mix cells with Agarose w/o bubbles by adding 1.1X solution with corresponding amount of 11X solution (for example 5ml of 1.1X Agarose + .5ml of 11X cell solution = 5.5ml of 5% Agarose at a cell concentration of 1M cells per ml)
 - a. Gently Vortex
 - b. Pipette
9. Cast in incubator @ 25 C for 10 minutes
10. Cut gels in half, place in 24 well plate flat side up, add staining buffer, incubate 30 – 60 minutes.
11. Remove and wash gels 3X with PBS, hydrate with 1 ml of PBS
12. Place on ice and take to confocal for imaging.

Metabolite Extraction

Items Needed:

- Approx. 2lbs of liquid nitrogen
- Tinfoil squares
- Hammer
- Micro Spatula
- Ice/Snow
- Centrifuge Tubes
- EtOH
- Tombstone
- Pounding Rods
- Insulated green tub
- Methanol:Acetone Solution (70:30); should be in -80

1. Place sterile Tombstone and Rods in freezer let cool
2. Wash gels 2X with PBS
3. Fill green tub with ice/snow, place tombstone in tub
4. Disinfect tinfoil, wrap gels then place in liquid nitrogen for minimum of 1 minute
5. Disinfect tombstone wells with EtOH
6. Remove gel, unwrap and place in well
7. Make sure gel is laying on its side then smash with rod and hammer
8. Remove smashed bits of gel with Micro Spatula and put in 2 ml centrifuge tube
9. Label sample and put in -80 freezer
10. Add 1ml of Methanol:Acetone Solution to each sample
11. Vortex every few minutes for first twenty minutes of exposure
12. Put all samples back in -80 overnight
13. Spin samples down at 4C @ 13000 rpm for 10 minutes
14. Aspirate off liquid (try not to suck up chunks) into new centrifuge tubes
15. Poke holes in lids and put in speed-VAC for approx. 6.5 hours
16. Put in freezer or re-suspend pellet in 50:50 water:Acetonitrile
17. Coordinate with mass-spec facility to run samples

APPENDIX H

METABOLOMICS DATA

To request DVD copies contact your local public or university library to place an interlibrary loan request to Montana State University. Questions call 406-994-3161.