Osteochondral Transplant Delivery System

Final Report

Biomedical Engineering Design 301
Department of Biomedical Engineering
University of Wisconsin
May 3, 2017

Team Members:
Rodrigo Umanzor (Leader)
Eduardo Enriquez (Communicator)
Nicholas Zacharias (BSAC & BWIG)
Bilin Loi (BPAG)

Client: Dr. Brian Walczak, DO
Department of Orthopedics and Rehabilitation
University of Wisconsin - Hospital

Advisor: Dr. Krishanu Saha, PhD
Department of Biomedical Engineering
University of Wisconsin - Madison

Abstract

Current osteochondral graft surgical procedures repair articular cartilage defects on the femoral condyle using impaction for insertion of the allograft into the recipient site. The forces associated with impaction has a detrimental affect on the chondrocyte viability within the cartilage layer and can cause long-term failure of the allograft. Therefore, we proposed a novel design that utilized threading of the allograft and the recipient site for delivery through rotational means rather than impaction. A live/dead stain and confocal microscopy were used to assess cell viability across untreated, threaded, and impacted groups throughout two rounds of testing with porcine tissue samples. Using ImageJ, percent viability values for each sample were calculated at varying distances away from the surface. Average percent viability values for the impaction and threaded groups were used in a 2-sample Welch t-test resulting in p-values of 0.387 and 0.458 for the first and second round, respectively. Hence, the statistical analysis does not show that there is a difference in viability between the impaction and threaded groups. Furthermore, the large variance in viability can be accredited to the negative effects that the extraction procedure alone had on the health of the cartilage. The tool used for threading the bone samples required the application of various magnitudes of possibly detrimental torsional forces and was also observed to damage the cartilage on the outer diameter of the samples. The observed damage to cartilage during threading does not allow us to recommend this novel procedure.

Table of Contents

Introduction

Motivation

Articular cartilage defects in the knee are commonly detected in roughly 60% of patients undergoing knee arthroscopies [1]. Disease or blunt trauma to the knee induces a degenerative cascade of articular cartilage that can ultimately result in osteoarthritis (*Figure 1*). Symptoms experienced by individuals with osteoarthritis include varying degrees of pain and loss of anatomical movement or altered function of the diseased bone. Current treatment for osteoarthritis defects involve preventative care and surgical procedures that aim to replace the diseased cartilage, such as osteochondral allografts. However, osteochondral allograft surgeries exhibit a 75-80% success rate as mentioned by the client on account of current methods used to deliver the graft. Current methods have detrimental effects on the long term survival of the cartilage due to the mechanical loads associated with graft delivery [2]. As of now, there are no clinical procedures that are consistent and reliable in regenerating cartilage. Hence, there is a need for a novel procedure that will minimize the magnitude of mechanical loads required for proper delivery of the graft to reduce adverse effects in transplanted cartilage.

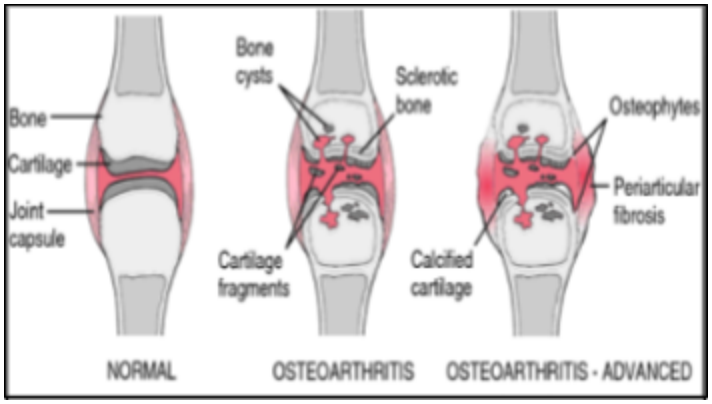


Figure 1: A diagram depicting differences between healthy bone and various cases of osteoarthritis

Existing Devices/ Current Methods

The current procedure involves placing an appropriately sized Lesion Gauge up next to the defect and driving a guide pin through the gauge into the bone. The Lesion Reamer is used along the guide pin to drill down to a section of good bone to create the recipient site. Measurements are taken of this site, and are used for fabrication of the allograft. The lesion gauge is placed into the GraftStation™ to find an appropriate surface on the donor tissue, so that the best fit is obtained in all planes of the articular surface, and the 12 O'Clock position is marked. The GraftMaker™, which is a tool that extracts cylindrical samples of bone is used after locking it into the GraftStation™ and cylindrical graft is cut. This locking prevents horizontal movement of the GraftMaker™ as movement is inhibited in the x and y plane. This cylindrical portion is removed by cutting at the lower aspect perpendicular to the cylindrical cut. The cylindrical graft is measured and appropriate modifications are made to match the recipient site.

Finally, the graft is inserted by gentle impaction using the tamp tool [3]. A calibrated dilator [4] or bevel of the bone in the recipient site [5] can be utilized as well to obtain a press fit by hand, but often impaction is required to obtain a tight fit and appropriate seating into the recipient site [4]. The cartilage surface of the graft is impacted until it is flush with the articular surface surrounding the recipient site. *Figure 2* provides a schematic for this procedure.

The impaction required to insert the graft frequently causes a decrease in the viability of the chondrocytes, which are the cells in cartilaginous tissue. This can ultimately cause associated cartilage disorders due to the lack of viable tissue. For example, fibrocartilage can form during implantation. Fibrocartilage is a scar tissue that forms due to the limited regenerative capabilities of cartilage. It also has a higher coefficient of friction, which can lead to early degeneration of the tissue [6]. In order to limit these effects, the implanted tissue should contain greater than 70% chondrocyte viability postoperatively [7]. However, this is an estimated value, and an acceptable procedure should allow for the long term success of the graft by reducing the mechanical stimuli necessary for graft insertion to limit cell death that could induce associated complications such as fibrocartilage. If after transplantation the graft is not inserted so that it is flush with the articular surface the surgeon must drill into the plug, remove it from the recipient site and make sizing adjustments of the recipient site or graft as necessary. This causes further damage to the cartilage layer as well and decreases cell viability, and is an inherent risk associated with this procedure.

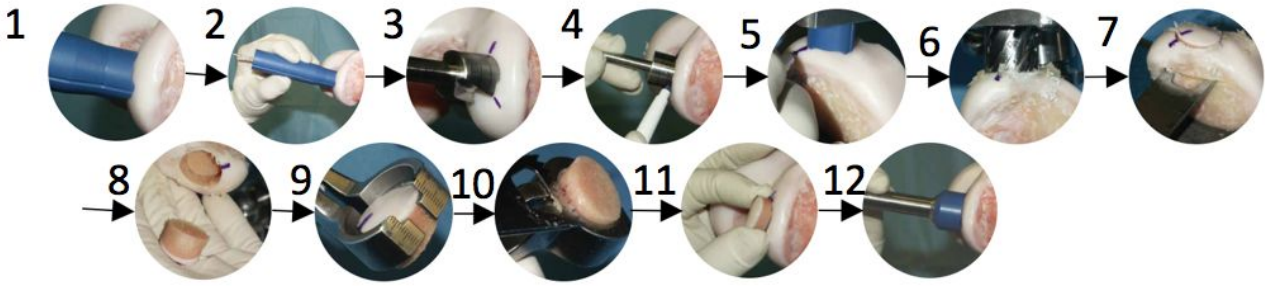


Figure 2: Current clinical procedure used for an osteochondral allograft (ACT Allograft method).

Problem Statement

Osteochondral allografting is a common procedure performed on patients that have defects in their articular cartilage. Current methods of implantation require the application of compressive forces that detrimentally affect the chondrocytes on the graft cartilage. Maximizing the amount of viable tissue during and after the surgery is a crucial factor for the success of the procedure. Hence, the client requests a delivery system that will reduce the amount of mechanical forces required to securely implant the graft into the recipient site.

Background

Client Information

Dr. Brian Walczak, a doctor of osteopathic medicine and orthopedic surgeon, was seeking a novel delivery system for osteochondral allografts that would reduce the compressive loads and in theory increase success rates of the surgical procedure.

Relevant Biology & Physiology

Unlike the majority of other connective tissue found in the human body, articular cartilage is composed of a single cell type, the chondrocyte [8]. While these are the only cell type found in cartilage, they are present at relatively low densities. Chondrocytes are responsible for producing the collagenous extracellular matrix (ECM) that makes up the avascular and aneural network of cartilage. The lack of blood supply in combination with the natural sparse presence of chondrocytes contribute to the poor regenerative properties of articular cartilage. Consequently, any significant damage done to the chondral surface of a knee will experience little to no regeneration. The injury could cause sustained cartilage degeneration from imposing detrimental stresses on the defect or following fibrocartilage formation and eventually lead to osteoarthritis.

The poor regenerative properties of articular cartilage emphasize the significance of minimizing chondrocyte death during osteochondral allograft procedures. Due to the functional importance of chondrocytes in producing and maintaining the ECM of articular cartilage, any damage done to them can jeopardize the success of a procedure intended to replace diseased cartilage. The relevance of chondrocytes in these procedures has been further investigated in studies exploring the effect of donor chondrocyte viability on overall success of osteochondral allografts performed in canines [7]. In the study by Cook et al., procedural success was defined by successful graft integration and no cartilage disorder six months post-implantation. The study found that successful grafts had at least 70% viable donor chondrocytes at the time of transplantation. Grafts with less than 70% chondrocyte viability were unsuccessful following the six-month period and experienced infiltration from fibroblasts into the surrounding host tissue. Similarly, the quantity of viable chondrocytes in human osteochondral allografts has an integral role in the overall success of the procedure.

Product Design Specifications

This design must meet specific standards in order to be a viable option for osteochondral allograft procedures. The most important criterion must be an increase in the percent chondrocyte viability from the current impaction method, which is approximately 70% mentioned by Cook et al. [7], as well as the client. This will prospectively be achieved through a reduction of mechanical forces on the articular cartilage during insertion. More specifically, the normal forces applied to the articular cartilage for proper insertion should not exceed 165 N, the force value measured by Dr. Walczak during impaction procedure. Another criterion is that the novel procedure will not cause chipping or fragmentation of the bone plug. These two specifications along with proper fitting of the graft into the recipient site will ensure success implantation success with proper graft integration into native tissue, proper maintenance of

hyaline cartilage, lack of associated cartilage disorder, and a lack of significant fissuring, fibrillation, or fibrous tissue infiltration [7]. These factors should in theory improve upon the current success rate mentioned by the client of 75-80%.

Additionally, the tools used for the novel procedure should be compatible for use in the operating room as well as easily sterilizable. Any necessary plastic or polymeric components used in the delivery system will be designated for single use only and all metals will need to be both sterilizable and reusable if desired by the surgeon while complying with FDA standards for surgical devices [9]. The delivery system should be able to accommodate a plug and recipient site at mm increments ranging from 5 - 20mm. Furthermore, the procedure should not prolong the operating time above the current standard of five hours as mentioned by the client. Compliance of these specifications and testing should be made with an estimated budget of $250. See Appendix I for the full project design specifications.

Preliminary Testing Designs

As a novel procedure has been devised for insertion of the bone graft this procedure must be tested to determine its functionality when compared to the current procedure. In order to assess efficacy of the graft chondrocyte viability measurements are imperative to determine changes in potential surgical success. To accurately assess chondrocyte viability, three preliminary testing methods were considered. While the imaging techniques vary amongst these methods, they all use the same calcein acetoxymethyl (AM)/ ethidium homodimer-1 (EthD-1) stain to mark the live and dead cells, respectively. Calcein AM is permeable to the cell membrane and it is a precursor to fluorescent calcein. When the cell is alive this conversion will be made intracellularly by the enzymatic action of esterases, a process not possible in a dead cell. Calcein can then be a fluorescent marker as it is well retained in the cells and will illuminate as green. On the other hand, EthD-1 does not enter intact cell membranes, however if the membrane is damaged it can enter and bind to nucleic acids, which promote its red fluorescence [10]. The approaches considered for imaging are wide-field fluorescence microscopy, confocal microscopy and flow cytometry.

Testing Alternative 1

Wide-field fluorescent microscopy shines ultraviolet light up to the specimen using a dichroic mirror [34] (*Figure 3*). Fluorescent microscopes use this ultraviolet light to display fluorescence within cells. For live cells stained with Calcein AM, the green fluorescence can be observed under the FITC channel. For dead cells stained with ethidium homodimer-1, the red fluorescence can be observed using the TRITC channel. These filters and stains can be utilized to determine the viability of chondrocytes. In order to adequately image cells through the extent of the tissue, samples must be cryosectioned to view the horizontal plane at multiple depths. Transverse sections could also be made by sectioning perpendicular to the articular surface. This is required as only the superficial layer of the tissue can be imaged in this method.

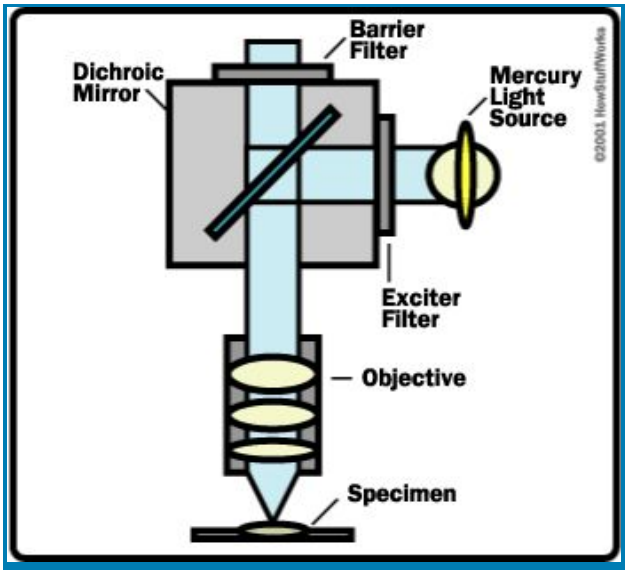


Figure 3: Light path of an epifluorescence microscope [31]

Test Alternative 2

Confocal Laser Scanning Microscopy allows for imaging using fluorescence at different depths. This allows for viewing of structures that are difficult to section since the samples do not need to be relatively thin. The Confocal Laser Scanning Microscope (CLSM) functions by passing a light source through a slit which is then focused onto a small area of the sample by an objective lens (*Figure 4*) [11]. An image of the illuminated sample is then constructed through the collection of emitted photons in the sample. The CLSM is based on the conventional optical microscope, however it substitutes the lamp light source for a laser. Any light exposed to fluorescent areas is reflected back to the optical lens, and only light that is in the confocal plane with the pinhole aperture located in front of the detector is recorded. Thus, any light that is not in the focal plane is rejected, which reduces excess signal commonly experienced in fluorescence microscopy. An image is then displayed on the screen of a monitor after processing. Reflected light will have a low intensity and is amplified by the photomultiplier tube. The laser beam creates a standing image and as the laser moves, the system constantly detects this information and creates images as if it were a real-time image. This method allows viewing at multiple depths and allows for cross sectional images by solely changing depth or solely changing width, so as to view the entire sample at one depth. CLSM is paired with dies for staining live cells and dead cells, such as calcein-AM for live and ethidium homodimer-1 or CYTOX blue for dead. This allows the imaging software to output photos of the sample with the fluorescent regions clearly illuminated, indicating the live and dead cells based on the color of their fluorescence.

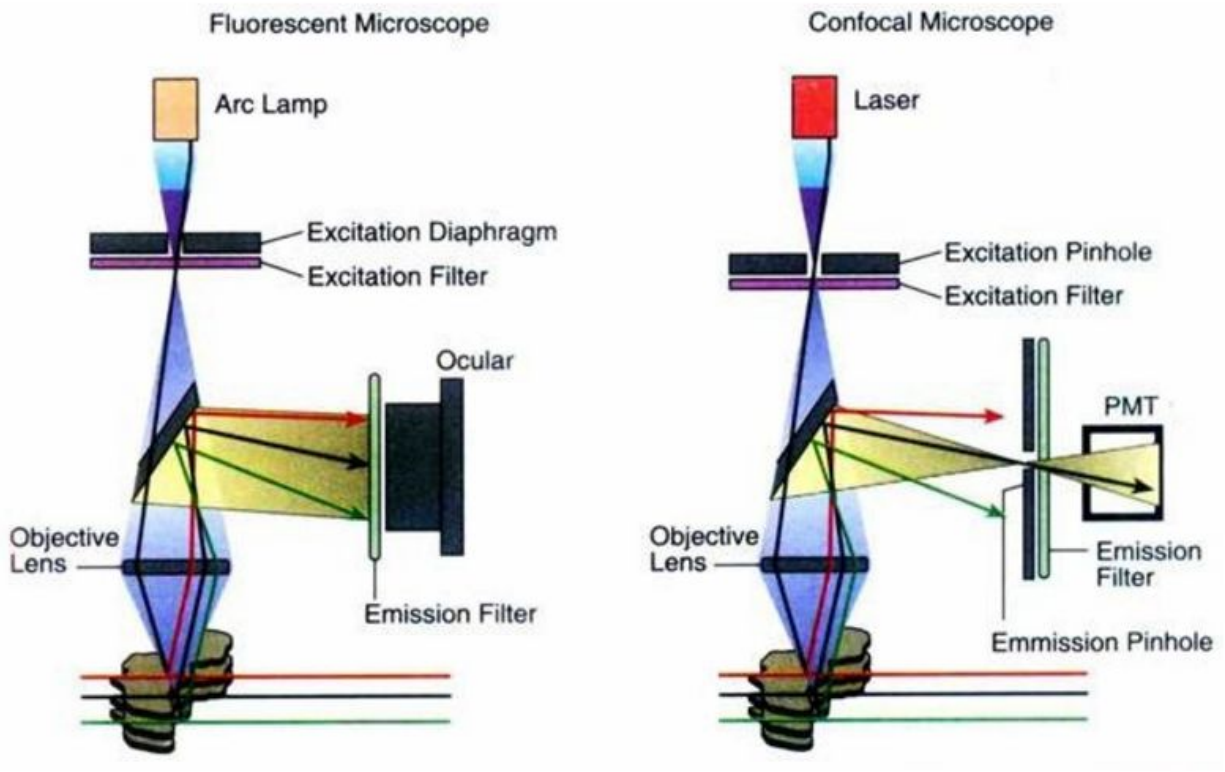


Figure 4: Fluorescent and Confocal Microscope-similarities and differences [32]

Testing Alternative 3

Unlike the other two imaging approaches, flow cytometry requires the disintegration of the tissue sample so that the cells can freely flow through the optical instrument [12]. Typically a collagenase reagent is used because of its ability to break the peptide bonds present in collagen, the main structural protein found in the extracellular matrix of tissues. Without the presence of intact tissue sections, this imaging approach requires a fluid component for the transport of the cells. The fluid column allows for analysis of individual cells as they flow one at a time through the laser component of the instrument (*Figure 5*). The fluorescence and light scattering properties of each cell flowing through the optical component of the cytometer is then used to collect data regarding the size of the cell. Such a characteristic can allow for the separation of cells in a tissue sample that contain multiple types of cells. Further application can involve the use of stains with fluorochromes to assess cell viability. Typically, one stain is used to mark a dead cell and a different stain to mark a live cell. The stain used would cause the cell to emit a fluorescence intensity corresponding to the respective stain and this allows each cell to be registered as alive or dead. Flow cytometer technology also creates a visual representation of the data collected for the cell sample. Such a feature eliminates the factor of user error during data analysis.

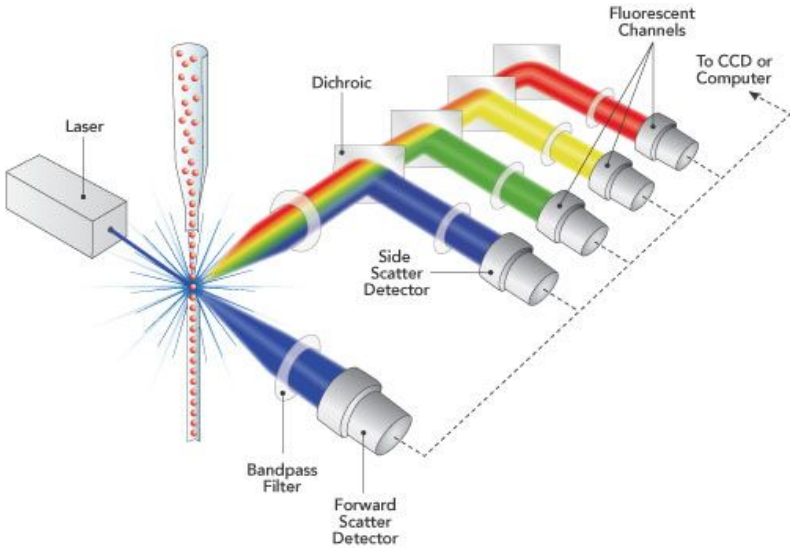


Figure 5: Structure and main components of a flow cytometer [33]

Preliminary Design Evaluation

Table 1: Design matrix used to evaluate the three microscopy methods according the set criteria. The values in parenthesis next to the criteria represent the relative weight given to it out of a total of 100. The highlighted sections correspond to the design with the highest score for the respective criteria.

Criteria	Fluorescent Microscopy		Flow Cytometry		Confocal Microscopy	
Accuracy (35)	(3/5)	21	(4/5)	28	(5/5)	35
Cost (30)	(5/5)	30	(1/5)	6	(4/5)	24
Ease of Use (20)	(5/5)	20	(2/5)	8	(3/5)	12
Tissue Section Prep (10)	(3/5)	6	(5/5)	10	(4/5)	8
Procedure Length (5)	(3/5)	3	(2/5)	2	(5/5)	5
Total	80		54		84	

Legend: Blue squares correspond to the design with the highest score for the respective criteria.
Scoring on a system of 5 points, with 5 being the highest and these scores weighted based on the relative importance given to each criterion.

Wide-field fluorescent microscopy, flow cytometry, and confocal microscopy were evaluated to determine the best imaging method to test cell viability. The design matrix criteria (*Table 1)* were chosen and weighted based on importance from the criteria in the PDS. The goal was to determine the most efficient imaging approach to assess the viability of the cartilage samples after experimentation. Thus, the accuracy criteria was weighted at 35. Provided that confocal microscopy does not require the use of a potentially detrimental collagenase and its imaging is not limited to the superficial layer of a sample, it was given the highest value for accuracy. Flow cytometry was given the second highest value for accuracy because of its requirement for the use of collagenase to disintegrate the tissue membrane. The collagenase may damage cells and result in altering the viability of the cells present in the tissue sample after experimentation. Since fluorescent microscopy is limited to collecting data of the cells on the superficial layers of a sample it was ranked the lowest with regards to the accuracy of the results that it would produce.

The cost of the imaging technique was weighted at 30 because the procedure must be affordable in order to image multiple times throughout the semester while staying within the $250 budget. Reagents required for the different imaging techniques in addition to the cost per use of the instrument contributed to the overall ranking of the considered designs. Flow cytometry was ranked the lowest because the cost per use of a flow cytometer is significantly above the indicated budget. Confocal microscopes are available for use with a fee of $30 per use. Fluorescent microscopes are available for use without any associated fees per use. Hence, fluorescence microscopy was ranked the highest.

Ease of use was weighted at 20, on account of the amount of experience and skill required to use the imaging instrument. The complex setup of optical filters and beam splitters required for flow cytometry it was ranked the lowest. In order to rent the confocal microscopes we must receive training, but we all have prior experience using a fluorescent microscope. The prerequisite for the use of the confocal microscope led to it being ranked below the fluorescent microscope in this category.

Tissue section preparation was rated at 10, as it is a relevant consideration because the constraints regarding the tissue sample vary amongst the three imaging approaches. Flow cytometry only requires the use of collagenase to disintegrate the extracellular matrix of the tissue to allow for the cells to flow in the cytometer. This procedure was given the highest value because it requires the least amount of skill to prepare the sample for imaging. While both fluorescence and confocal microscopy require that sectioning of the tissue samples into thinner slices, the use of fluorescence microscopes requires much thinner slices because they are only able to image cells at the superficial surfaces. Confocal microscopes, however, allow the user to image at various depths within a tissue section. Due to the imaging constraint of fluorescent microscopes, it was ranked the lowest since it would require a larger time and skill investment in obtaining the proper tissue sections.

Finally, procedure length was weighted at 10 on account of our time constraint throughout the semester. A tentative testing protocol calls for at least three tissue samples for each of the three conditions at different time points. This would result in a significant amount of time spent imaging to obtain the overall results of chondrocyte viability for each condition. Hence, confocal microscopy was ranked the highest in this category as it is expected to take the

least amount of time invested in imaging each tissue sample. Our prior experience working with a fluorescence microscope and a speculation based on background research on the time required to set up a flow cytometer led to giving fluorescence microscopy a higher ranking in this category and flow cytometry the lowest rank.

Proposed Final Testing

Consideration of the aforementioned criteria for each of the imaging techniques resulted in a very close overall score for confocal and fluorescence microscopy with values of 84 and 80, respectively. We proposed the use of confocal imaging methods for assessing chondrocyte viability after experimentation. The confocal microscope was the preferred imaging instrument because of its ability to image a various depths of a tissue section. It also required no further sectioning of the tissue. Thus, allowing the cartilage to be maintained in its native state.

Fabrication/Development Process

Materials

The operating procedure must be recreated to test cell viabilities post delivery using confocal microscopy. To test this materials were obtained from many sources to follow an extraction and testing procedure similar to that used in the operating room. The extraction protocol used discarded animal tissue and was initially attempted on bovine tissue due to the sizeable knee area and ease of access, but ultimately fresh porcine tissue were experimented on as the bovine tissue was frozen prior to our acquisition. Fresh porcine tissue was obtained from Dr. Tim Hacker, Director of Cardiovascular Physiology at the Clinical Science Center (CSC) Facility located in the UW Hospital. For creation of the recipient site and plugs as well as threading of both, tools were obtained from the UW Student Shop located in the Engineering Centers Building (ECB) of UW-Madison. These tools included a cordless power drill, drill bits, a hole saw, tap and die, hacksaw, a vice, and material to fabricate the guide for the hole saw.

To test the aforementioned plugs for cell viability, the testing protocol required materials that were obtained from the client, Dr. Walczak, our advisor, Dr. Saha, as well as the ECB Biomedical Engineering (BME) Department Tissue Laboratory. D-MEM media, 1X PBS, 24 well plates, and 2 μM Calcein AM and 4 μM Ethidium Homodimer were used to culture and stain the cartilage disks from the bone plugs. Sterile scalpels obtained from the BME Department Tissue Laboratory (Engineering Centers Building, Madison) were used to remove cartilage from bone and section the cartilage for imaging. Training and access was provided by Mr. Lance Rodenkirch for the Nikon A1RS confocal microscope in the UW-Optical Imaging Core (UWOIC) located in the Wisconsin Institute for Medical Research (WIMR) in Madison, WI. Detailed protocols can be found in Appendix II and III.

Methods

Porcine tissue was acquired approximately four hours following euthanasia of a 4 month old Landrace X pig that had undergone removal of cardiovascular organs and complete exsanguination. All results discussed in this study were obtained from testing with porcine tissue.

Bovine tissue was also utilized because of the extensive surface of articular cartilage that allows for multiple plug extractions and is readily available. However, FDA regulations required that all animals slaughtered for future consumption to be stored at freezing temperature for multiple days following slaughter. It was likely this freezing and the handling of the tissue following slaughter that resulted in cell viabilities that could not be used for comparison between graft insertion methods because they were too low and varied significantly throughout the tissue. Thus, fresh porcine tissue was investigated as an alternative because it could be obtained fresh, immediately following euthanasia. Tissue was acquired by complete removal of both the forelimbs and hindlimbs near the shoulder and hip respectively. The porcine limbs were transported on ice to the BME Department Tissue Laboratory (UW Madison, Engineering Centers Building) where working surfaces and tools were thoroughly washed with 70% ethanol. Due to budget constraints and a lack of tool compatibility with more efficient sterilizing techniques (e.g. autoclaves and ethylene oxide) this was the only form of sanitization for the tools and working surfaces. However, it must be noted that all tools can be adapted for use in an operating room with a sufficient budget. Following sanitization working surfaces were covered with laboratory countertop paper. The limbs were then cleaned of all muscles, ligaments and tendons using sterile scalpels and taking utmost care to avoid damaging articular cartilage surfaces.

Due to the size of the porcine bone and limited surface area, the guide was unable to be fixed to the bone and so a posterior approach was utilized for graft retrieval. After securing the tissue in a vice covered with laboratory countertop paper, a hole saw was used to cut starting from a proximal surface moving distally through the extent of the articular cartilage (5-6 in *Figure 6*). This approach was utilized as it prevented damage to the cartilage experienced from "walking" of the hole saw experienced when trying to start the cut. The hole saw was then detached from the cordless power drill and the graft pushed outwards using the flat end of a nail. This extraction from the hole saw resulted in no compressive loads onto the articular cartilage as the cartilage would be pushed outwards from the back and the articular cartilage would be the first to emerge from the modified hole saw. Additional plugs were obtained in an identical fashion utilizing all available tissue except that which was used for recipient holes.

Three plugs at least 15mm in length were placed in a vice with the cartilage (lateral end) facing outwards and threading was started from the cartilage layer inwards using a 7/16-14 die (7-8 in *Figure 6*) appropriately sized to the inner diameter of the 9/16 inch hole saw. Recipient holes were created using a 11/32 drill bit (1-2 in *Figure 6*) and a cordless power drill and later threaded using a 7/16-14 tap (3-4 in *Figure 6*) matching the aforementioned die. The threaded bone plug was manually screwed into the recipient site by turning the plug clockwise into the hole (9 in *Fig. 6*); slight pressure was placed on the cartilage during the final turns when insufficient bone was available for grip. The graft was then screwed out of the recipient site and placed in media until further analysis. The impaction method was tested by impacting the graft with a hammer to a depth that allowed removal of the graft by gripping the bone with a pliers due to limited recipient sites, and the plug was placed in media until further analysis. Using a sterilized scalpel, the cartilage was removed from the threaded grafts following insertion, the impaction grafts after supplied loads, and an untreated condition in which slices were taken directly from the cartilage surface of one of the limbs, due to an insufficient amount of tissue available for plug retrieval, during the experiment one. During experiment 2 plugs were retrieved, but no further treatment was made in the untreated condition. These methods are detailed in Appendix II.

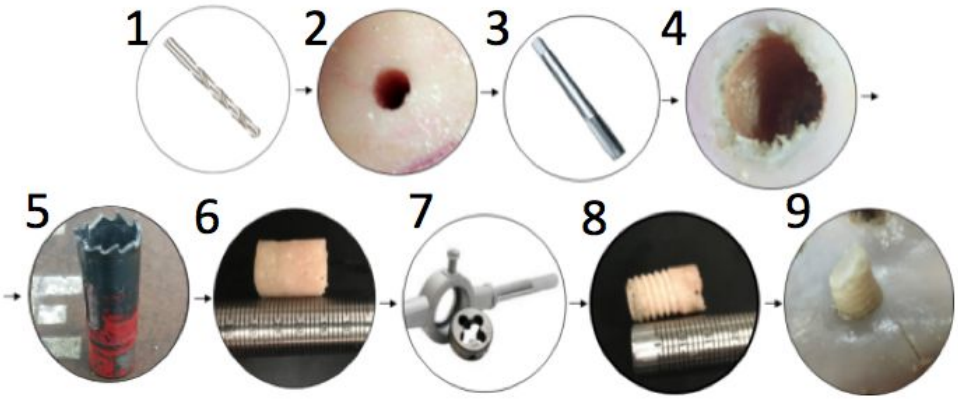


Figure 6: Flow chart outlining the steps in the proposed delivery method

Final Prototype

The final prototype includes the 10mm bone plug and recipient site. These are created using the methods outlined above and have been tested using the protocols below (*Figures 7-9*).

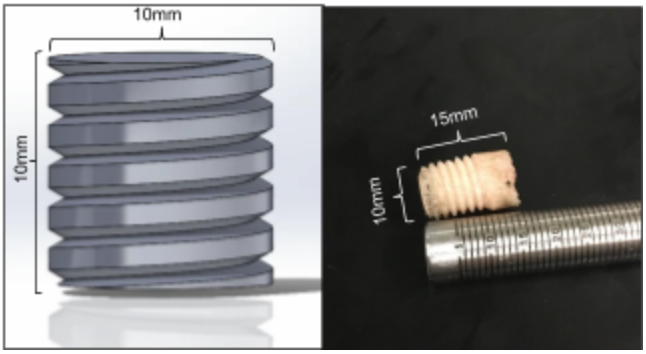


Figure 7: On the left, 10mm x 10mm plug representation modeled in Solidworks. On the right, a 10mm x 15mm bone plug created from a bovine condyle.

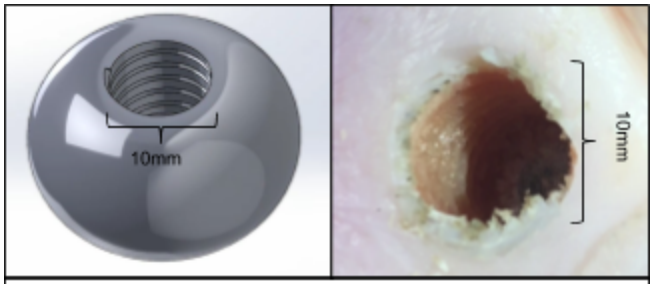


Figure 8: On the left is a Solidworks model of the threaded recipient site. On the right is a threaded recipient site on a condyle bovine femur

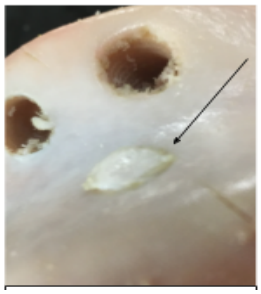


Figure 9: The threaded plug with intact cartilage properly screwed into the recipient site

Experimentation

The purpose of experimentation was to compare chondrocyte viability of the threading and impaction methods. This factor was prioritized due to its correlation to the overall success rate of the procedure. Although testing was limited to a non-sterile environment, the described procedures were conducted under the same conditions, these conditions expected to impose a large variability for both methods. Hence, a greater maintenance of cell viability would show promise for future testing and result in a greater success rate in an operating room setting.

Using the methods outlined above, three osteochondral plugs for each condition were obtained from porcine tissue in the UW College of Engineering, Student Shop area, located in the basement of the ECB. Due to a lack of familiarity with pig anatomy tissue for round 1 experimentation was extracted from the antebrachiocarpal joint of the forelimbs as well as from the tarsocrural joint in the hindlimbs, these joints are structurally characteristic of the pig ankle. These were severed at a distal aspect to the elbow and the knee respectively. In the second round of experimentation, tissue was extracted from the elbow joint and the femorotibial joint--structurally analogous to the knee--removed at a distal aspect to the shoulder and hip bones respectively. The porcine appendicular skeleton is illustrated in *Figure 14* for reference. The extraction procedure was carried out along with the threading and impaction procedures and the cartilage surfaces were then removed from each graft using a surgical scalpel. The cartilage disks were placed into an appropriately labeled 24 well plate and cultured in MEM-C media. Tissue samples were taken from the disks at 1 hour and 24 hour time points to determine the long term success of the graft as a progression of greater cell death at later timepoints is noted in previous study [2]. Three biological replicates for the three conditions were compared at the same time point to provide an indication of the error predicted by in each condition. Measurements at one hour provided a useful representation of the success of our transplant method. The control condition served as a baseline to demonstrate the extent of cell death caused by the treatment conditions.

Testing was completed using two stains to detect live and dead cells. Live cells were stained green using Calcein AM while dead cells were stained red with Ethidium Homodimer-1 (EthD-1) obtained from a LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific). Each disks was submerged in 50 µL of calcein AM/EthD-1 stain solution at a concentration of 2µM calcein AM and 4µM EthD-1 in 1X PBS; an additional 50 µL of 1X PBS were added to these samples to ensure full submersion in the stain solution. Calcein AM/EthD-1 was selected for LIVE/DEAD assay on account of its accessibility and visibility under the confocal microscope. Staining was done for a 20 minute time period during which the cartilage

sections were incubated in the stain solution. Sections were then washed in 1X PBS to allow for removal of excess stain.

The cartilage sections were then imaged on the Nikon A1RS confocal microscope at the UWOIC allowing visualization at variable depths of the tissue to acquire a correlation between chondrocyte viability and tissue depth in each treatment groups. It was previously shown that chondrocyte viability increased at greater depths into the tissue following a surface load [2]. A 495 nm and 528 nm wavelength laser was used to excite the Calcein AM and ethidium homodimer-1, respectively, and a 505-530 nm bandpass filter and a >560 nm laser will be used to capture the images, respectively [15]. For data analysis, surface depth zero was set as the location in which a number of cells could first be imaged. Each subsequent image was then subtracted from this reference to obtain the relative depth from the surface. Images were taken at random intervals depending on changes in both visualization of cells and cell locations and saved with their true depth for finding relative depth later. These images were then used to define the various depths of visualization and the cell viability at each depth. Each image was saved in Tiff format with separate channels to maximize cell count accuracy and some images were saved in PNG format for viewing sample images.

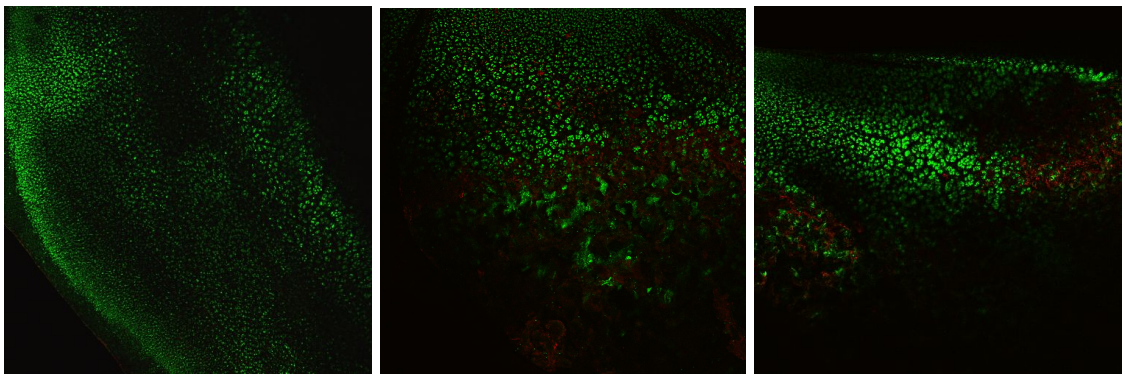


Figure 10: Untreated, Impaction, and Threaded sample round 1, 1-hour images, respectively.

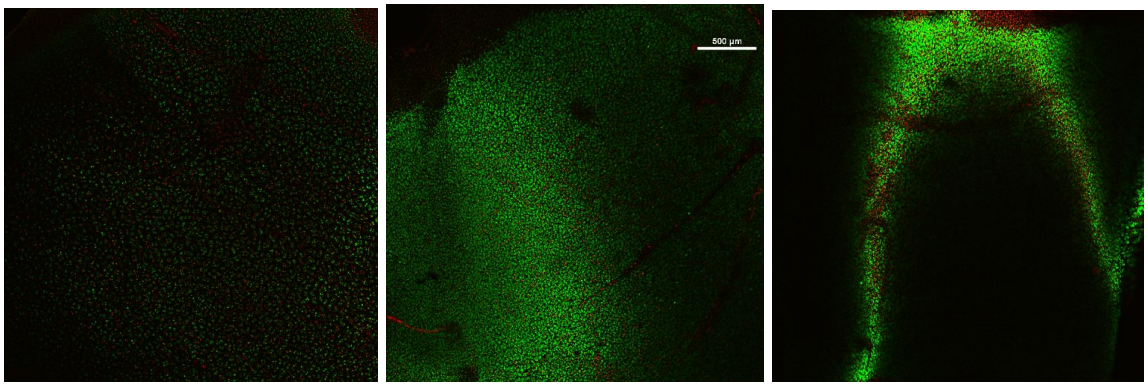


Figure 11: Untreated, Impaction, and Threaded sample round 1, 24-hour images, respectively.

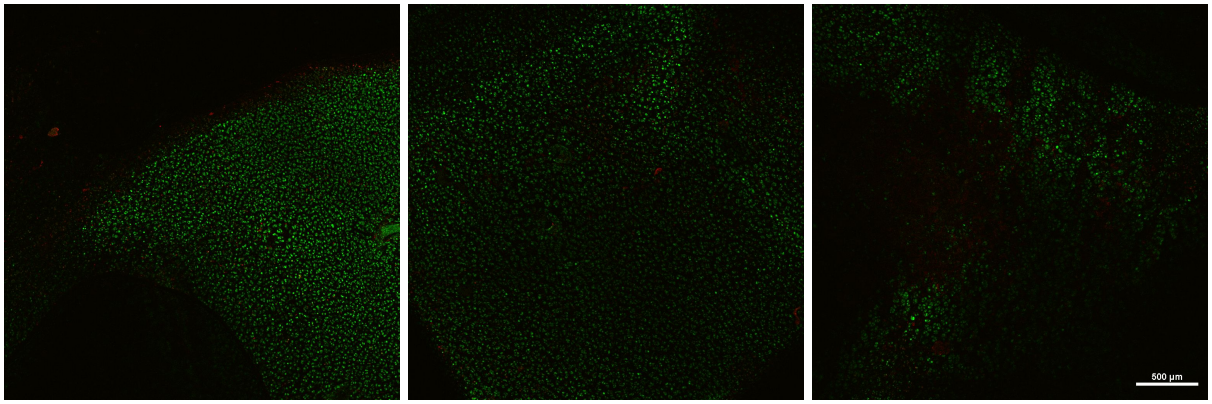


Figure 12: Untreated, Impaction, and Threaded sample round 2, 1 hour images, respectively.

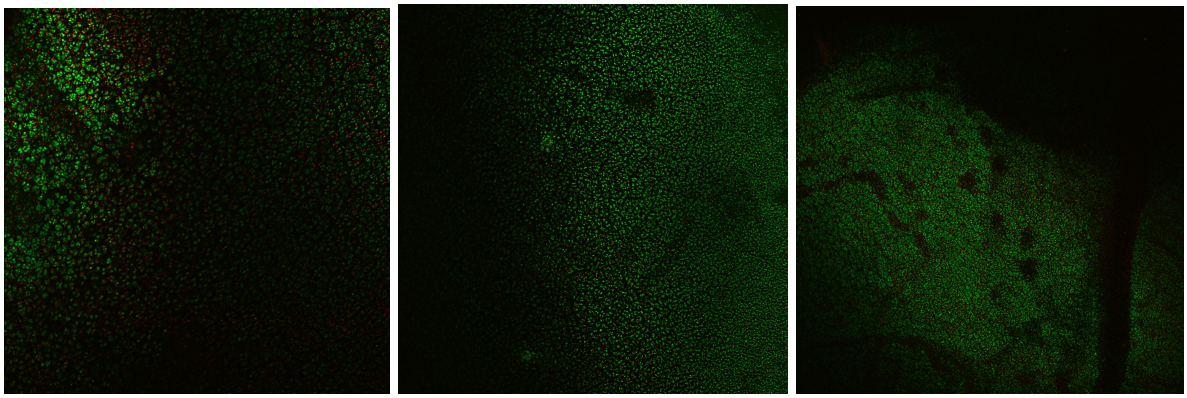


Figure 13: Untreated, Impaction, and Threaded sample round 2, 24-hour images, respectively.

Analysis of the images was completed using ImageJ. The detailed protocol can be found in Appendix III. First, the independent channel Tif files for each image were placed in stacks according to their condition and wavelength of excitation. Next, subtract background was used to remove residual staining from autofluorescence of the tissue. These were then thresholded until only cells were seen. Particles were separated using watershed. Additionally, particles were excluded by first scaling the image and then excluding all particles not in the range of 50 to 1963.5 μm^2 and within a circularity of 0.4-1 The area parameter accounts for a significant range around that expected for a chondrocyte cell, and the circularity parameter was recommended to be set at above 0.9 [29], however this excluded too much data. Analyze Particles was used to count the cells and this data was used to assess chondrocyte viability.

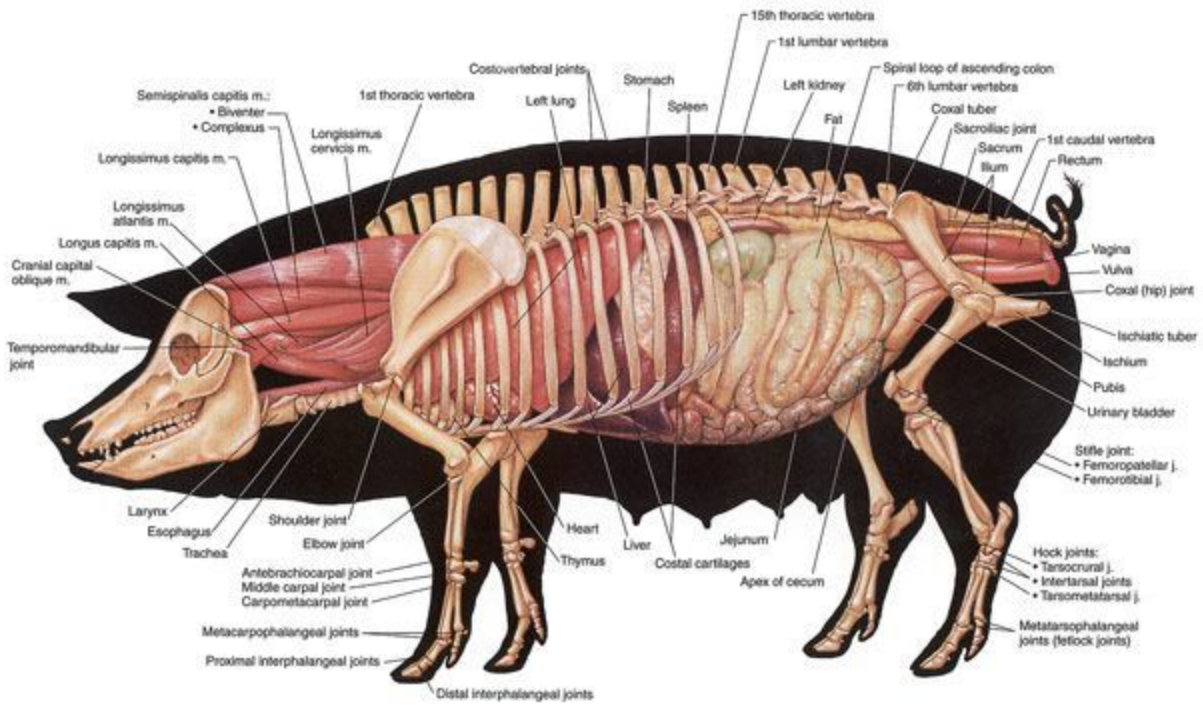


Figure 14: Porcine anatomy with major organ, muscle, and bone structures. Note the hindlimbs and forelimbs that differ greatly from humans. Image courtesy of: http://serior.info/pig-vs-human-skeleton/

Results

Using the cell count data obtained from ImageJ analysis of confocal images, averages were obtained for percent viability from each graft. Percent viability was obtained by dividing the number of live cells by total cells (i.e. the sum of live and dead cells) and this value was obtained for each image. Subsequently, all the values of percent viability from the images corresponding to one graft were averaged together to obtain the total percent viability for each given plug. *Figure 15*, shows an increase in cell viability for both 1-hour impaction and threaded treatment groups with increasing depth and no significant effect of depth on cell viability for untreated. *Figure 16*, a graph of the mean cell viabilities demonstrated the lack of differences between the two treatment groups overall cell viability while showing a decrease when compared to the untreated group. As the 24-hours progressed (*Figure 17*) the untreated group's cell viability decreased from about 95% to 60% while the impacted and threaded groups retained their viability from the 1 hour time point. However, in the 24-hour time point while both treatment groups maintained an increase in cell viability with increasing depth, threaded had a lower increase in cell viability with increasing depth as compared to impaction. The plots of the overall cell viability (*Figure 18*) show this information as well as showing the differences in variance between each group. The second round of testing (*Figure 19*), showed a different set of results with impaction demonstrating a decrease in cell viability with increasing cell depth while threading showed an increase, meanwhile controlled maintained a constant cell viability. However, this decrease in viability with increasing depth for impaction can be explained by outlier data point due to differences between individual plugs (see Appendix V) for information

about individual plugs). The averaged cell viability of the two treatment groups (*Figure 20*) were close as well as a less significant difference between control and the two treatment groups. This can be attributed to the extraction procedure being carried out on the round two untreated samples while the round 1 untreated samples were removed from untouched areas of cartilage, demonstrating the negative effect of graft extraction on cell viability.

To test whether a difference between the impacted and threaded conditions exists a null hypothesis was established. The null hypothesis states that there is no difference in chondrocyte viability between the threaded and impacted samples. Given independent data samples and unequal variances as demonstrated in *Figures 16, 18, 20* a Welch two sample t-test was used. The α-level was set at 0.05 and so the test was compared to the standard significance level of $p \leq 0.05$. The statistical analysis for Round 1 resulted in a p-value of 0.3869 and that for Round 2 in a p-value of 0.4577. Since the result for both rounds of testing had a $p > 0.05$ we fail to reject the null hypothesis. Results from this analysis performed on data from last semester is included in *Appendix VI*, which also demonstrated a failure to reject the null hypothesis.

Further statistical analysis of the untreated groups for the 1-hour imaging results from both rounds of testing showed a difference in the mean viability. Using the previously described two-sample t-test and assuming unequal variance, a p-value of 0.078 was obtained. While this p-value is not less than or equal to 0.05, it is close enough in approximation that the null hypothesis can be rejected in this test. Therefore, it can be stated that there is a possible statistical difference in the mean viability of the untreated groups at the 1-hour time point.

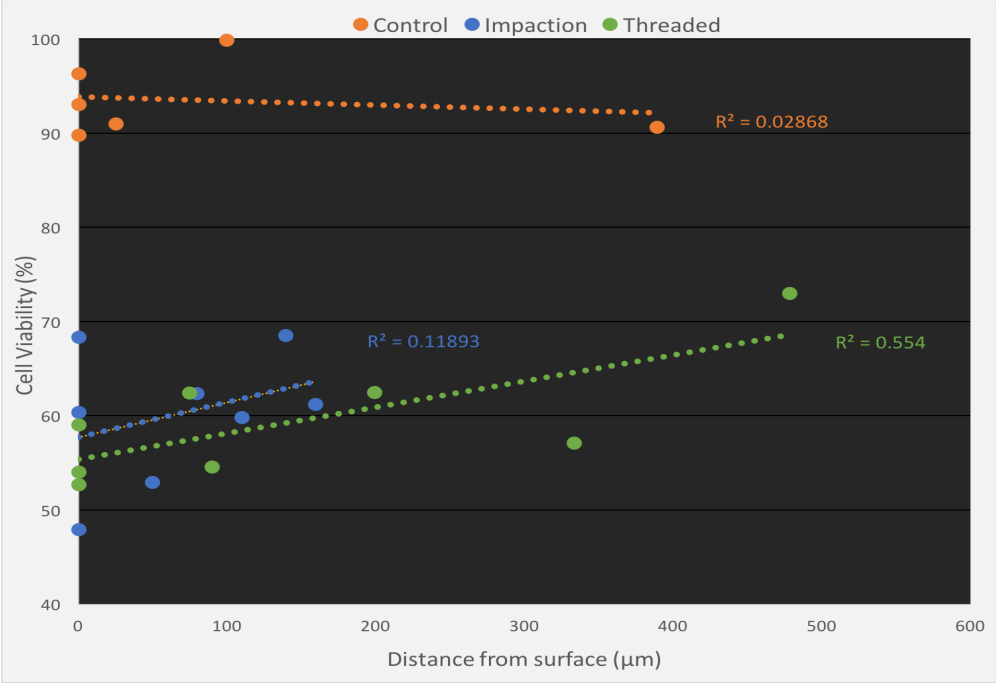


Figure 15: Round 1 Porcine Cartilage testing 1-hr

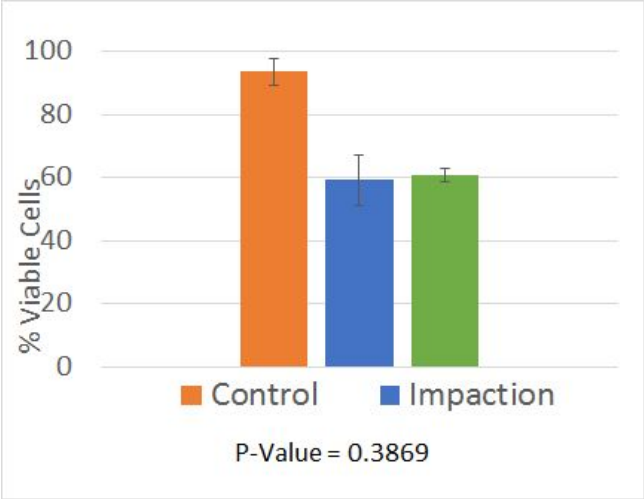


Figure 16: Round 1 Porcine Cartilage testing 1-hr

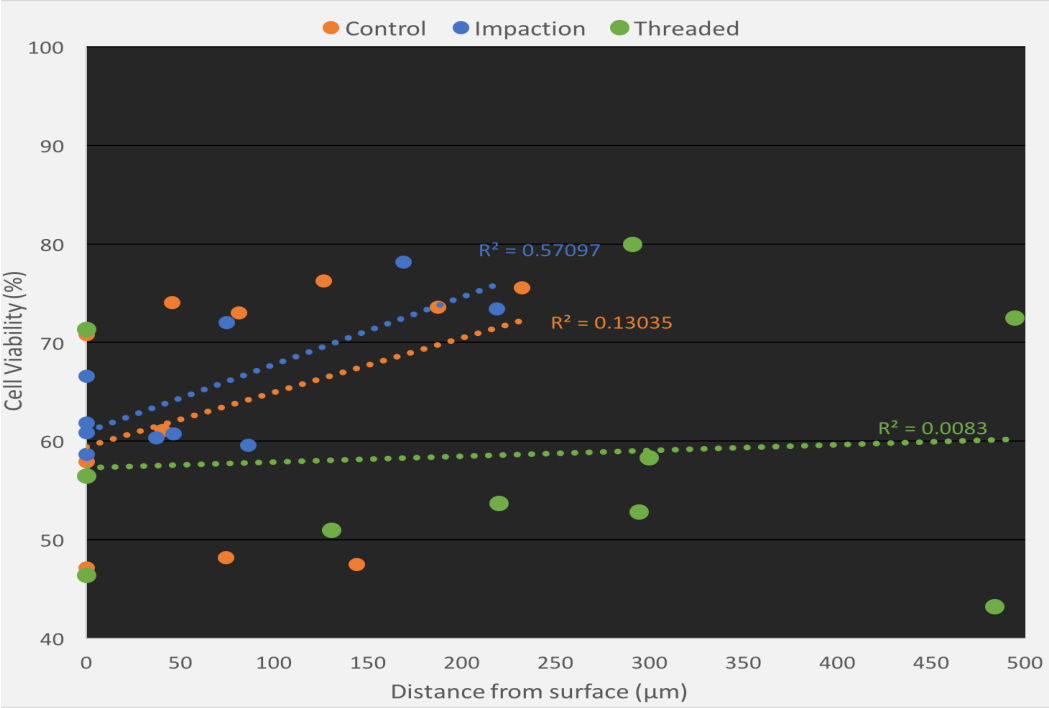


Figure 17: Round 1 Porcine Cartilage Testing 24 - hr

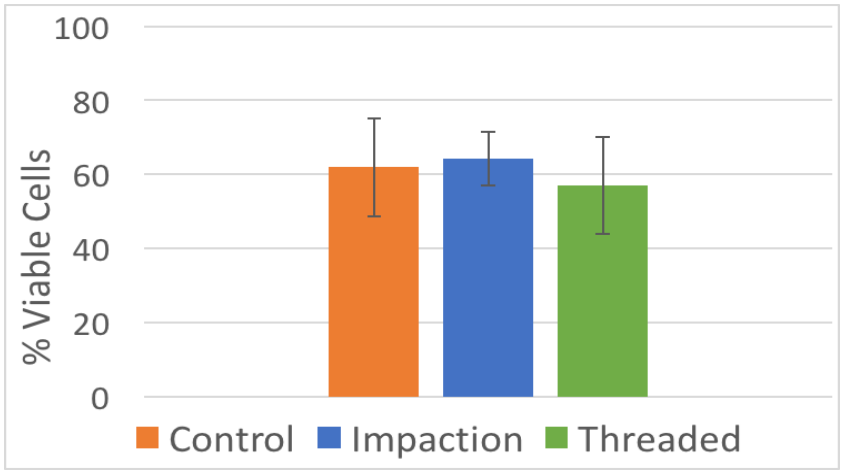


Figure 18: Round 1 Porcine Cartilage Testing 24 - hr

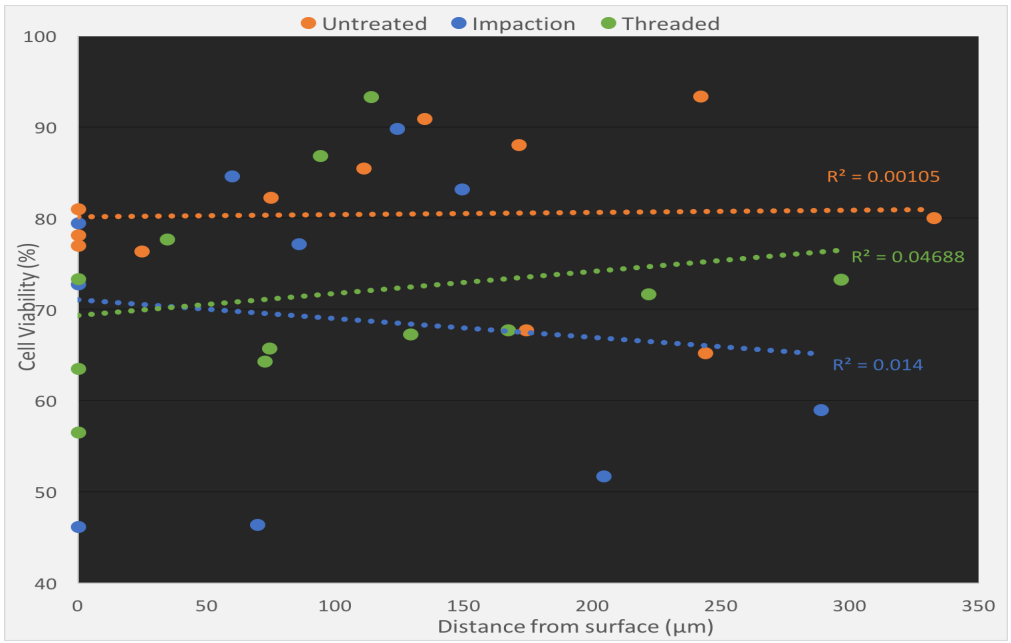


Figure 19: Round 2 Porcine Cartilage Testing 1-hr

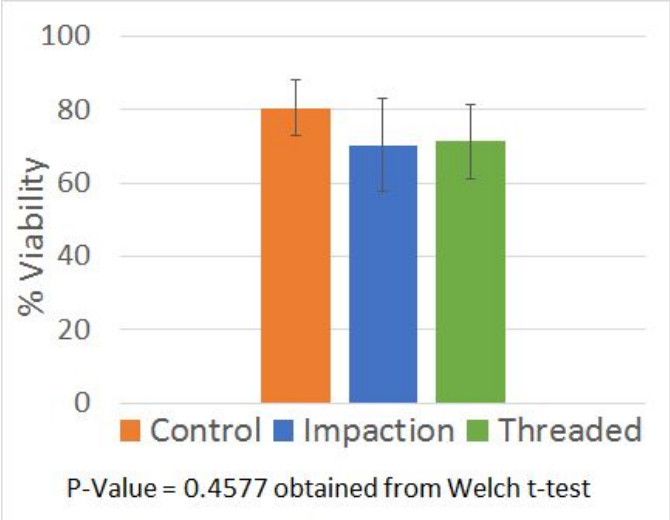


Figure 20: Round 2 Porcine Cartilage Testing 1-hr

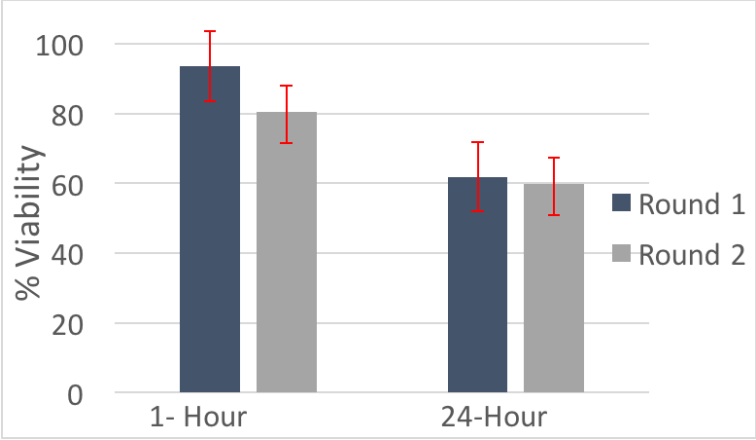


Figure 21: Control Group Viability

Discussion

The proposed experimentation aimed to demonstrate the ability of the threaded delivery system to reduce the compressive load applied to the cartilage during allograft delivery and test the effect on chondrocyte viability post-implantation. It is important to note the implications of successful allograft delivery through the proposed threaded mechanism. Current allograft procedures reliant on impaction have been shown to lose up to 21% of viable chondrocytes in the superficial 500 μm layer one hour after implantation [2]. The cylindrical allografts used in the study by Borazjani et al., were 15 mm in diameter and required approximately 10 consecutive impacts, each generating an average 2.4 kN load, for delivery. Furthermore, the articular cartilage experienced a total loss of 47% of chondrocytes in the same superficial layer 48 hours after the procedure with observed chondrocyte death in deeper layers. This increase in cell death was noted as a result of induced apoptotic pathways from the forces applied to the cartilage [16]-[18]. Hence, it is essential to minimize compressive loads to the articular cartilage during

graft delivery to avoid triggering these apoptotic pathways. The proposed delivery system reduced the need for large compressive loads to insert the graft into the recipient site. Rather, the threads on the allograft allowed for manual insertion into the delivery site through the application of relatively small torsional forces. Given previous and ongoing research, the reduction of force applied directly to the articular cartilage surface should in theory have reduced loss of chondrocytes through necrosis and apoptotic pathways.

However, the indicated results did not represent the expected theory. Rather, it was discovered that there was no statistically significant difference between the current method of impaction and the proposed threading method as the chondrocyte viability in each method was nearly identical. While the threaded delivery system demonstrated the ability to reduce compressive forces necessary for graft insertion, the inherent complications in the proposed delivery system may have contributed to the results obtained in this study. Through threading, torsional forces were introduced to the articular cartilage, as full insertion of the graft required compression and torsion generated directly to the articular cartilage. Further analysis is required to gain a full understanding of the effect of these torsional forces as a study by Moreland et al., noted a histological change in the cartilage following application of torsional load. This study and the aforementioned comparison of chondrocyte viability between the impaction and threaded conditions demonstrate that there may be an effect from torsional loading in conjunction with minor compressive loads that contribute to the diminished chondrocyte viability in the threaded grafts that is similar to impaction grafts.

Statistical analysis of the untreated groups imaged 1-hour after extraction showed a difference in mean percent viability (p-value of 0.078). This is of relevance because the untreated groups from round 1 were extracted through alternative means than those from round 2. Cartilaginous tissue available for bone plug extraction was limited during the first round of testing with the fresh porcine bone samples. Due to the limited tissue, bone plug extraction for the threaded and impaction groups were given priority. Slices of cartilage were taken from the remaining articulating surface of tissue for the control groups because there was not enough for complete bone plug extraction. In the second round of testing, however, the amount of porcine bone tissue acquired allowed for the extraction of bone plugs for all three groups. *Figure 21* shows that there is indeed a difference in the mean percent viability for the two untreated groups imaged at the 1-hour period. More specifically, the mean viability of the untreated group from round 2 was 13% less than the untreated group from round 1. Of particular significance for this delivery system, these results imply that the process of bone plug extraction alone has a detrimental effect on the health of the cartilage as it causes necrotic chondrocyte death.

Further surgical complications frequently occurred during threading of the bone graft including graft fracture and removal of cartilage from the surface. Numerous grafts exhibited fracturing along the midline as they were forced through the die and threaded due to the thin size of the graft and the amount of torsional force. Although no mechanical analysis of the forces necessary for threading was conducted in this study a theoretical limit to these forces is easily obtained for grafts using human tissue similar to those used in this study. The torsional yield strength of human bone is 3.31 MPa [27], equivalent to a torsional force of 14.606 lbs placed on the exterior of a 10mm plug. However, due to a lack of modeling it is unclear whether this theoretical limit was exceeded and thus further mechanical testing and modeling is required. Additionally it was also noted that during threading of the graft, the cartilaginous surface received extensive damage and was also threaded. This was caused due to the design of the die

which requires full threading of a cylinder for removal from the die tool as well as to allow for mating of the threads. Threading of the cartilage tissue resulted in fraying at the periphery of the graft and removal of cartilage. Due to the removal of cartilage this would not affect viability as it would completely remove the cells that it killed. However, this indentation along the periphery of the graft would then create improper mating of the normal articular cartilage and the graft articular cartilage resulting in possible complications of the surgery, osteoarthritis and the long term requirement of a new surgical procedure. Threading also caused complications with proper mating of the articular cartilage surfaces as the threads only mate in a certain manner while the graft geometry and bone geometry must also only mate in a certain manner for a flush surface. This required perfectly matched threading so that the final mating threads would allow for correct placement of the graft within the recipient site. Hence, future modifications to this design would need to eliminate the removal of cartilage as well as allow for perfectly matching threads on the graft and recipient site, both not possibilities using hand-held tools.

Sources of Error and Proposed Alterations

The devised experimentation aimed to reduce error experienced in previous testing. This was done through the creation of a procedure mimicking the surgical allograft procedure using power tools instead of a single-use manual autograft tool that experienced plastic deformation with repeated use. While this test mimicked the surgical procedure it missed vital aspects that may have resulted in decreased and variations in chondrocyte viability. None of the procedures performed in our experiments were performed in ideal testing conditions. All testing was done in the ECB on the UW-Madison campus. Most of the testing was performed in the BME Department Tissue Laboratory (Biosafety level 1) with the mechanical work done in the COE Student Shop area. The shop tables utilized are often used for greasy metal work and operation of other industrial machinery. Both environments were not sterile, and were only sanitized using 70% ethanol. However, all tools used could be easily adaptable to an operating room setting. Published research surrounding osteochondral allografts is always performed under conditions that meet the standards of an operating room. Thus, future experiments should also be performed in environments compliant with clinical standards to more accurately model these procedures.

Another source of error was the vice used for holding the femur and plugs. The vice in the COE Student Shop had large, sharp teeth meant for gripping large metal materials. A smaller, softer, and insulated vice--preferably one from an operating room--would prevent damage to the plugs during threading. The tap and die could also be redesigned for medical use. The tools in the COE shop have all been repeatedly used for non sterile industrial applications and likely contained contamination. High grade stainless steel tooling would not rust and would be more suitable for tissue. The die should be low profile, as it does not need to cut threads into metal bolts. A low profile die would allow threads to be cut into shallower plugs. The other tools such as the saw and drill bit that were used should likewise be adapted to surgical grade tools.

The technique and tooling used in threading and inserting plugs should be improved upon. Measurements should be taken with greater care and precision, and cuts and threads should be made with greater stabilization to ensure proper mating of the graft with the recipient site. This was likely another source of variability. In order to maximize accuracy and efficiency, proper training by an orthopedic surgeon would be beneficial. Published papers regarding osteochondral allografts involve the expertise of senior orthopedic surgeons. This would reduce

surgical complications that may result in plug variation due to our lack of expertise and limited tissue and tool availability.

The extraction method was shown to have a significant effect on the chondrocyte viability. This may have arose from the heat produced due to a lack of irrigation as the hole saw cut through the bone. The heat was likely transferred to the chondrocytes in the tissue and would have likely caused significant damage at the exterior, which was not investigated, as well as in the interior. Another source of damage during plug extraction was noted when many of the plugs exhibited partially tearing of the cartilage from the bony surface as the hole saw cut through the articular surface during the posterior approach. This may have caused further damage that resulted in a decrease in all the conditions involving plug extraction.

As mentioned in the methods section tissue should ideally be selected from joints that are structurally similar to the human knee as these will likely exhibit similar properties. Thus, future tests should eliminate the mistake of acquiring tissue from the antebrachiocarpal joint of the forelimbs as well as from the tarsocrural joint in the hindlimbs and use the elbow joint in the forelimbs and femorotibial joint of the hindlimbs to eliminate any variations arising from structural dissimilarities.

Imaging and image analysis can also be improved upon. In our testing, a scalpel was used to cut the cartilage layer off of the plug. We attempted to keep them as similar as we could, but human error can never be overcome. Due to this error, use of a sectioning tool could obtain thinner, more precise slices which could allow for better imaging. Precise section depth would allow one to determine the ideal thickness of cartilage tissue for staining. Our sections were all quite thick, and may have made it difficult for the stain to penetrate. Future experimentation with section thickness and staining should be performed. There was also significant variability expressed in the trends when comparing the cell viability with depth for each of the individual plugs. This variability could be plausibly mitigated through the acquisition of a greater amount of images at approximately 10 μm segments through the extent of the cartilaginous tissue. This would ideally reduce variability from the current three images taken per tissue section. This would also provide further quantification of the cell viability through the extent of the graft.

The use of animal models prior to human testing is common practice. Porcine models were used because their bone and cartilage more closely resemble human tissue [16]. Ideally, *in vivo* animal models should be used to determine the long term success of the grafts. Although the design needs to be further refined and have proven efficacy on the *in vitro* models that were discussed in this study prior to *in vivo* animal studies. However, it is important to note that these results can not be definitively extrapolated to human tissue. Ultimately, human cadavers should be tested to ensure the design efficacy on human tissue prior to patient implementation.

Ethical Considerations

Osteochondral allografts were introduced decades ago and the safety of the procedure is well documented. Multiple clinical studies have shown positive results from fresh osteochondral allograft transplantation [19]. Viral and bacterial infections are rare, but potentially fatal. Prior to the implementation of a screening process allografts were implanted within 24 hours of harvest resulting in the American Association of Immunologists reporting 37 bacterial infections associated with allograft procedures in 2002. Infections most commonly occurred with bacteria within the donor tissue or bacterial contamination during procurement and/or processing of

tissue. Currently, meticulous screening of the donor is required before the operation is granted approval. On account of this screening fresh transplants are no longer used in the USA, and the FDA has banned any procedures without proper screening. Most commercial grafts in the USA are used between 15 and 35 days of harvesting; and are rarely implanted before a 7 to 10-day screening process. Studies have shown good clinical results up to 42 days after harvest although chondrocyte viability decreases with storage time. While the screening process prevents many infectious diseases, clostridium contamination risk increases with time after donor death [20, 21]. Thus, the screening process must be kept short while remaining effective. In order to keep donor tissue free of disease, there must be emphasis on aseptic harvesting, aseptic processing, and use of antimicrobials at every stage. Safety guidelines established by the American Association of Tissue Banks (AATB) advocate donor screening, serologic, bacterial, and viral testing as well as procurement and storage requirements are required until negative testing results have been received. A difficulty associated with controlling disease is the cytotoxicity associated with many sterilizing techniques. Every process that the donor tissue undergoes must avoid contamination while also keeping the cartilage alive and healthy. The procedure is performed to replace damaged cartilage with healthy, mature hyaline cartilage from the donor, so maximizing the cell viability of the donor is important.

Another important consideration would be ensuring proper surgical training in the use of threading. Since this is not a commonly used technique, consultation with surgical experts would be necessary to ensure this delivery system is applicable to the operating room. After demonstrating the surgical procedure efficacy, it must be ensured that all surgeons be able to accurately and consistently perform threading using the protocol for the prevention of graft failure during the threading process and for the safety of the patient.

In the course of this study it was important that the tenets of respect for animals commonly denoted as the three R's (reduction, replacement, and refinement) in animal research were strictly adhered too. All tissue acquired was intended for routine disposal either following slaughter or following a research study. This ensured that that no further animals were sacrificed for the purposes of this study satisfying the reduction tenet. The refinement tenet was satisfied as no further suffering or distress incurred to the animals because our experimentation followed their euthanasia. Since cell viability was a required variable for measure it was necessary to use animal tissue to demonstrate potential efficacy before using in osteochondral allograft transplants, which references the replacement. Computer simulation would not be sufficient as variability of forces cannot be accurately simulated to represent the degree of variation observed in the actual procedure.

Conclusions

Current surgical procedures for knee grafts require brute force to implant living tissue that has poor regenerative abilities. The extent of compressive loads applied to the cartilage during delivery of the graft is detrimental to the overall success of the procedure as it induces chondrocyte death through necrotic and apoptotic pathways. The proposed delivery system relies on threading the allograft extracted from the donor tissue to create a bone screw that can then be implanted into a threaded recipient site that complements the dimensions of the plug. The delivery of the allograft is accomplished through successful clockwise rotations applied manually by the surgeon until the surface is flush. The design of this transplant method was

influenced by the criteria requiring a reduction in compressive loads associated with delivery and the need to allow for retraction of the plug in the case of error during operation, such as incorrect sizing of the plug. The latter criterion is of surgical significance and was emphasized by the client, as the current procedure calls for piercing of the cartilage and retraction of the allograft in the case of over-impaction of the cartilage past the desired depth. Reduction in compressive loading for proper delivery was assumed to result in less damage to chondrocytes and subsequently higher cell viability 1 and 24 hours after the mimicked surgical procedure. Ultimately, statistical analysis of the collective images gathered from two rounds of testing with three fresh porcine bone samples per treatment group showed no difference in mean cell viability from plugs delivered using the current and proposed delivery methods.

Nevertheless, the design and its assessment can be improved. Of first priority, the tools used for bone plug extraction must be as precise as the surgical tools used in an operating room. The power tools used for tissue extraction in the clinical procedure are specifically designed for use on bone, unlike the modified hole saw and handsaw used in our protocol,both intended for woodworking applications. The priority of this modification arises from the statistical difference noted in the untreated group viability from the first and second round of testing. A threading tool that allows for complete control of threaded surface area is also recommended for future protocols as it would prevent damage to the cartilage on the outer diameter of the test specimen. Equally important, the torsional forces associated with allograft delivery through rotational means and the effects that they can have on both the cartilaginous and the osseous tissue must also be considered. Such modifications will allow for better control over the factors influencing cell death throughout the entire experimental procedure. Under ideal testing conditions, cell viability should be solely influenced by the forces associated with osteochondral allograft delivery.

References

1. W. Widuchowski, J. Widuchowski, and T. Trzaska, "Articular cartilage defects: Study of 25, 124 knee arthroscopies," *The Knee*, vol. 14, no. 3, pp. 177–182, Jun. 2007

2. B. H. Borazjani *et al.*, "Effect of Impact on Chondrocyte Viability During Insertion of Human Osteochondral Grafts," *The Journal of Bone and Joint Surgery-American Volume*, vol. 88, no. 9, pp. 1934–1943, Sep. 2006. □

3. "Allograft Cartilage Transplant Surgical Technique," MTF Sports Medicine. [Online]. Available: https://www.mtf.org/documents/PI_-43_Rev_4.pdf. [Accessed: 16-Feb-2017].

4. S. Akhavan, et al. "Cartilage Repair and Replacement: From Osteochondral Autograft Transfer to Allograft," in *SURGICAL TREATMENT OF THE ARTHRITIC KNEE: ALTERNATIVES TO TKA*, pp. 9–30. □

5. V. F. Sechriest et al. "Osteochondral Allograft Transplantation" *AANA Advanced Arthroscopic Surgical Techniques*, 2016, pp. 243-255. □

6. A. F. Steinert, et al. "Major biological obstacles for persistent cell-based regeneration of articular cartilage," *Arthritis Research & Therapy,* vol. 9 no. 213, DOI: 10.1186/ar2195, Jun. 2007 □

7. J. L. Cook *et al.*, "Importance of donor Chondrocyte viability for Osteochondral Allografts," *The American Journal of Sports Medicine*, vol. 44, no. 5, pp. 1260–1268, Feb. 2016 □

8. A. J. Fosang and F. Beier, "Emerging frontiers in cartilage and chondrocyte biology," *Best Practice & Research Clinical Rheumatology*, vol. 25, no. 6, pp. 751–766, Dec. 2011.

9. Device Advice: Comprehensive Regulatory Assistance, *U.S. FDA,* www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/default.htm, 2015

10. "LIVE/DEAD® Viability/Cytoto xicity Kit, for mammalian cells," *Thermo Fisher Scientific*, 2016. [Online]. Available: https://www.thermofisher.com/order/catalog/product/L3224. [Accessed: 09-Nov-2016].

11. C. Paul, "What is Confocal Laser Scanning Microscopy," in *Bitesize Bio*, Science Squared, 2014. [Online]. Available: http://bitesizebio.com/19958/what-is-confocal-laser-scanning-microscopy/. Accessed: Feb. 22, 2017.

12. S. Robertson, "What is flow Cytometry?," in *Life Sciences*, News-Medical, 2014. [Online]. Available: http://www.news-medical.net/life-sciences/What-is-Flow-Cytometry.aspx. Accessed: Feb. 7, 2017

13. "Confocal imaging," in *Laboratory Management*, 2017. [Online]. Available: http://loci.wisc.edu/optical-sectioning/confocal-imaging. Accessed: Feb. 22, 2017.

14. Pylawka, T. K. (2004). Studies of Cold Preservation of Allograft Cartilage for Transplantation: Anabolic Suppression of Chondrocytes. Retrieved February 9, 2017, from ProQuest, http://search.proquest.com.ezproxy.library.wisc.edu/docview/305077841

15. B. Gantenbein-Ritter, C. M. Sprecher, S. Chan, S. Illien-Junger, and S. Grad, "Confocal Imaging Protocols for Live/Dead Staining in Three-Dimensional Carriers," Mammalian Cell Viability: Methods and Protocols, vol. 740, pp. 127-140, 2011. □

16. R. W. Kang, N. A. Friel, J. M. Williams, B. J. Cole, and M. A. Wimmer, "Effect of impaction sequence on Osteochondral graft damage: The role of repeated and varying loads," *The American Journal of Sports Medicine*, vol. 38, no. 1, pp. 105–113, Nov. 2009.

17. M. S. Ashwell *et al.*, "Changes in chondrocyte gene expression following in vitro impaction of porcine articular cartilage in an impact injury model," *Journal of Orthopaedic Research*, vol. 31, no. 3, pp. 385–391, Oct. 2012. □

18. L. V. Gulotta, J. R. Rudzki, D. Kovacevic, C. C. T. Chen, D. Milentijevic, and R. J. Williams, "Chondrocyte death and cartilage degradation after Autologous Osteochondral transplantation surgery in a rabbit model," *The American Journal of Sports Medicine*, vol. 37, no. 7, pp. 1324–1333, May 2009. □

19. G. Schulze-Tanzil *et al.*, "Differing in vitro biology of equine, ovine, porcine and human articular chondrocytes derived from the knee joint: An immunomorphological study," *Histochemistry and Cell Biology*, vol. 131, no. 2, pp. 219–229, Oct. 2008. □

20. M. Demange and A. H. Gomoll, "The use of osteochondral allografts in the managementof cartilage defects," *Curr Rev Musculoskelet Med* (2012) 5:229 – 235, May. 2012 □

21. M. A. Kainer, J. V. Linden, D. N. Whaley, H. T. Holmes,W. R. Jarvis,D. B. Jernigan, L. K. Archibald, "Clostridium infections associated with musculoskeletal-tissue allografts." *N Engl J Med.*; 350(25):2564–71. 2004

22. S. Gortz and W. D. Bugbee. Fresh osteochondral allografts: graft processing and clinical applications. *J Knee Surg.;*19(3):231–40. 2006 □

23. M. T. Ghazavi, K. P. Pritzker, A. M. Davis, A. E. Gross, "Fresh osteochondral allografts for post-traumatic osteochondral defects of the knee," *The British Editorial Society of Bone and Joint Surgery;* 79-B (6) 1008-1013. July 1997 □

24. "Bone Fracture Mechanics," *Buffalo University*, 2004. [Online]. Available: http://wings.buffalo.edu/eng/mae/courses/417-517/Orthopaedic Biomechanics/Lecture 1

support materials/Orthbonemech.htm. [Accessed: 13-Dec-2016]. □

25. “Tissue Reference Group,” *US Food and Drug Administration*, 2016. [Online]. Available: http://www.fda.gov/BiologicsBloodVaccines/TissueTissueProducts/RegulationofTissues/ucm152857.htm. [Accessed: 13-Dec-2016]. □

26. “CFR - Code of Federal Regulations Title 21l,” *US Food and Drug Administration*, 2016. [Online]. Available: http://www.accessdata.fda.gov/SCRIPTs/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=888.4540. [Accessed: 13-Dec-2016]. □

27. D. Vashishth, K. E. Tanner, and W. Bonfield, "Fatigue of cortical bone under combined axial-torsional loading," Journal of Orthopaedic Research, vol. 19, no. 3, pp. 414-420, May 2001.

28. M. S. Moreland, "MORPHOLOGICAL EFFECTS OF TORSION APPLIED TO GROWING BONE - AN INVIVO STUDY IN RABBITS," Journal of Bone and Joint Surgery-British Volume, vol. 62, no. 2, pp. 230-237, 1980.

29. D. Cadena-Herreraa, J. Esparza-De Larab, and N. Ramírez-Ibañezb, “Validation of three viable-cell counting methods: Manual, semi-automated, and automated,” *Validation of three viable-cell counting methods: Manual, semi-automated, and automated*, 2015. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S2215017X15000235. [Accessed: 31-Mar-2017].

30. “Pig Vs Human Skeleton,” *serior.info*. [Online]. Available: http://serior.info/pig-vs-human-skeleton/. [Accessed: 02-May-2017].

31. K. Chithanathan, “Light phase contrast and fluorescence microscopy,” *LinkedIn SlideShare*, 17-Dec-2013. [Online]. Available: https://www.slideshare.net/keerthanachithanathan/light-phase-contrast-and-fluorescence-microscopy. [Accessed: 03-May-2017].

32. “Microscopy: Principles and Specimen Preparations (With Diagram),” *Biology Discussion*, 16-Oct-2015. [Online]. Available: http://www.biologydiscussion.com/microscope/microscopy-principles-and-specimen-preparations-with-diagram/12325. [Accessed: 03-May-2017].

33. “Filters for Flow Cytometry,” *Flow Cytometry - Semrock*. [Online]. Available: https://www.semrock.com/flow-cytometry.aspx. [Accessed: 03-May-2017].

34. Freudenrich, Ph.D. Craig. "How Light Microscopes Work." *HowStuffWorks Science*. HowStuffWorks, 25 May 2001. Web. 03 May 2017. <http://science.howstuffworks.com/light-microscope4.htm>.

Appendix

I. PDS

Osteochondral Graft Delivery System

Team:
Rodrigo Umanzor (Team Leader)
Nicholas Zacharias (BSAC & BWIG)
Eduardo Enriquez (Communicator)
Bilin Loi (BPAG)

Date: February 22, 2017

Function: Osteochondral allografting is a common procedure performed on patients that require replacement of diseased bone. Current methods of implantation require the application of mechanical forces that have a detrimental effect on the live chondrocytes present on the implant. Maximizing the amount of viable tissue during and after the surgery is a crucial factor for the success of the procedure. Hence, the client requests a delivery system that will reduce the amount of mechanical forces required to securely place the implant into the donor site.

Client Requirements:

- Budget: $250
- Donor tissue must be placed into donor site with less than 165 N of force applied to the articular cartilage layer (the average impaction force for current procedures)
- Must securely fit into donor site while keeping chondrocyte cell viability >70% during and after the procedure.
- Delivery system must only require the use of sterile tools available in a surgery room, or must be made to to fit surgical standards.

Design Requirements:

- *Performance Requirements:*
    - Application of bone graft should result in approximately 70 - 90% viability of chondrocytes on donor graft.
    - Procedure must be simple enough to be done in operating room, within 5 hours (the time period of a surgery).
    - Bone graft and vice should be positioned perpendicular (relative to each other) so screws on bone graft remain straight.
    - System must be capable of decreasing the 165 N used to insert the bone graft.
    - Forces exerted on the bone by the device should not cause any bone chipping, or fragmentation and minimal damage to the articular cartilage.

- *Safety:*
    - The delivery system should not increase the chances of infection, graft dislocation, or create complications post-op.
    - Our device has failed if, postoperatively, the graft does not exhibit proper integration into the native tissue, if the hyaline cartilage is not properly maintained, if an associated cartilage disorder develops, or if significant fissuring, fibrillation, or fibrous tissue infiltration occurs.
- *Accuracy and Reliability:*
    - The delivery system should have a success rate that exceeds that of current devices (75-80% success rate)
    - The delivery system should also allow for successful graft implantation with no greater risk for post-op complications than standard practice
- *Life in Service*:
    - Expensive or specialized components should be reusable and easily manufactured components should be one time use. Both should be sterilizable. Length of time to be determined with materials chosen
    - If plastic or biodegradable materials are included in the delivery system, then these components may be one-use only
- *Shelf Life:*
    - The delivery system should be capable of storage at room temperature for 9 months unless sterility is compromised before then. No corrosion should be observed on the delivery system during its life of service and must be compliant with hospital regulations
- *Operating Environment:*
    - The delivery system has to be sterile while in use
    - Should operate in temperatures typical of an operating room (20-23 °C), with humidity of 20-60%
    - All pieces will have to withstand the forces exerted on them during operation (tension, torsion, shear, and compression).
    - Must be able to be used in conjunction with other orthopedic tools, including supports, water, and bone glue.
    - Must be able to be utilized by an orthopedic surgeon
- *Ergonomics:*
    - The delivery system should be able to be used easily by surgeon without damage during operation. Forces placed on the cancellous bone of the graft should not exceed 6.6 MPa from torsional stress, 3-20 MPa in tension, and 1.5-50 MPa in compression. Cortical bone is stronger than cancellous, so force limitations inherently include the cancellous bone. The forces applied to the articular cartilage should not exceed those at which there is less than 70% viability.
- *Size:*

    - The delivery system will be sized appropriately based on the size of the defect.
    - Range of 5mm-20mm diameter for threading bone graft
    - Height of graft must be at least 10 mm
- *Weight:*
    - Components of the delivery system are appropriately weighted for use by an orthopedic surgeon
- *Materials:*
    - Materials used in the delivery system should comply with medical standards set out by the FDA
    - Reusable materials must be sterilizable
- *Aesthetics, Appearance, and Finish:*
    - No color or aesthetics
    - Function over form

Production Characteristics:

- *Quantity:*
    - 1 final delivery system, preferably multiple testing using delivery system
- *Target Product Cost:*
    - TBD
- *Standards and Specifications:*
    - Implanted allograft should be in compliance with the FDA regulations under Section 361 of the Public Health Service Act as monitored by the Tissue Reference Group . All surgical tools should comply with the code of federal regulations under Title 21 with the FDA.

Characteristics:

- The delivery system should consist of a tap, die, vices and a bone screwdriver
- Must be made of surgery-grade material
- Various devices to stabilize tools will be necessary
- *Patient-related concerns:*
    - Completed bone graft must not cause pain
    - Allergies, immune response hemocompatibility, and biocompatibility
    - Allograft should not release unwanted fragments of bone
    - Required surgery with the delivery system should not be more invasive than current procedures
- *Customer:*
    - Orthopedic surgeons implanting an osteochondral graft will be the intended user

II. Graft Retrieval and Threading Protocol

Surgical Testing Procedure

Materials

- Large Cooler
- Porcine knuckle(s)
- Hand saw
- 1 Gallon Plastic Ziplock bag
- Cordless power drill
- Hole saw
- Cylindrical guide
- Hammer
- Long Nails
- Drill and bit
- Tap and die: 5/8-11
- 70% Ethanol
- Impaction device (PVC pipe, with calibrated heights for dropping weights that allow for loads consistent with impaction in surgery)
- Vice
- Countertop paper
- Scalpel
- 1X PBS
- MEM-C media
- 24-well plate

Procedure

1. The lower extremity of the femur will be removed and placed into a Ziploc bag containing 1X PBS solution within a cooler containing an ice bath.
2. The lower extremity of the femur will be transported to the Engineering Centers Building (on ice) where working surfaces and tools will be thoroughly washed with 70% ethanol.

Note: This will be the only form of sanitizing used for tools and working surfaces due to budget constraints and because many of the tools were incompatible to use with more efficient sterilizing techniques (e.g. autoclaves or ethylene oxide gas).

3. Working surfaces will be covered with laboratory countertop paper and the condyle will be placed onto this paper covered working surface.
4. Using a handsaw designed for wood, the knuckle will be sawed into two halves, separating the condyles from the patellar surface. This cut should allow for better access for graft retrieval and flatter surfaces for fixation within the vice.
5. To obtain the bone plugs, the condyle portion of bone will be placed into the vice with the knuckles facing upright.
6. The hole saw guide will be fixed atop the knuckle with 2 nails driven into the tissue with a hammer.
    a. If bone is too small, it will be cut to a the size of the hole saw and the cartilage plug will be extracted by entering the bone from the rear portion and exiting at the cartilage
7. PBS was pipetted onto the surface of the area for extraction to mitigate heat production due the hole saw contact with the bone.
8. The hole saw drill is removed from the middle of the hole saw and the now hollow hole saw is fitted onto the cordless drill
9. After driving the hole saw to a sufficient depth into the bone ≈ 20mm, the hole saw is removed by running it in the forwards direction and slowly pulling out.
10. The graft will be extracted by cutting perpendicular to graft with the handsaw and manually pushed out.

11. Repeat 5-9 for as many samples as needed.
12. The plugs will be placed into pre-warmed media and will then be placed into the incubator while we thread.
13. Three randomly chosen plugs will be placed in a vice with the cartilage layer (lateral end) facing outwards and threading will start from the cartilage layer inwards using a die appropriately sized to the inner diameter of the hole saw.
14. Recipient holes will be created using a drill bit, appropriately sized to the tap that matches the aforementioned die, attached to a cordless power drill.
15. The tap will be used to create mating threads that will match the threads created on the plug.
16. Repeat steps 11-13 for all three plugs in the threaded condition.
17. The threaded bone plug will be manually screwed into the recipient site by turning the plug clockwise into the hole; slight pressure will be placed on the cartilage using a gloved finger during the final turns when insufficient bone is available for grip.
18. To test the impaction condition loads will be applied to extracted plugs using the device created by Dr. Walczak that supplies loads similar to those that would be used in the operating room.
19. Using a sterile scalpel, the cartilage disks will be removed from the threaded grafts following insertion and extraction by unscrewing, the impaction grafts after supplied loads, and a control condition in which plugs will be retrieved, but no further treatment will be made.
20. Additionally, each cartilage disk will be cut into two pieces allowing for testing at 1 and 24 hour time points. These will be placed into DMEM media and incubated for the determined time point.
21. Staining will be done after incubation time period with a short incubation period for the stain to set

Live/Dead Staining Protocol
Materials

- 1 mL of calcein AM/ethidium homodimer-1 stain solution per section at a concentration of 2μM calcein AM and 4μM ethidium homodimer in 1X PBS
- Confocal Microscope
- Prepare fresh from 5 mg/ml stock by diluting 1:10,000 in water. 3μM

Protocol

1. With the tissue sections in the media, prep the Live/Dead stain.

Note: Live cells will be stained green using calcein AM. Dead cells will be stained red with ethidium homodimer-1.

2. The disks will be exposed to the stain for one hour using.
3. The sections will then be washed twice for five minutes in 1X PBS to allow for the removal excess stain.
4. The cartilage sections will then be imaged on a confocal microscope allowing visualization of the zones of the entire depth of the tissue. A 488 nm and 543 nm wavelength laser will be used to excite the Calcein AM and ethidium homodimer-1, respectively, and a 505-530 nm bandpass filter and a >560 nm laser will be used to

capture the images, respectively [15].

Note: These may be subject to change depending on the specific setting of the confocal microscope that we will gain access to.

5. 10 μm segments will be imaged through the extent of the cartilage for representative images of all of the depth zones.
6. Analysis of the images will be completed using ImageJ.

III. Image Analysis Protocol

Materials:

- Images of tissue named in order by tissue sample and picture number, specifying FITC or TRITC channel
- ImageJ

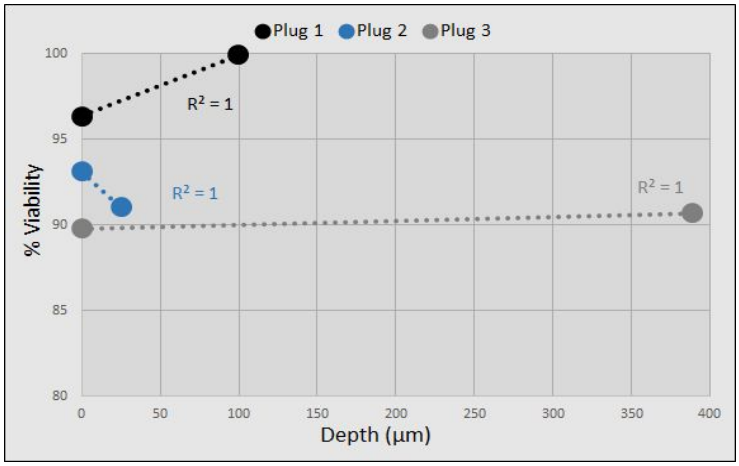
Protocol for Calculating Cell Viability

1. Using ImageJ, press File-Import-Image Sequence -(Choose Image Sequence) - (Name either FITC or TRITC to separate filters) - make it 8 bit greyscale
2. Once you have image sequence, go to Process - Subtract Background - (Rolling Ball 100)
3. Go to Image - Adjust - Threshold - Stack Histogram - (Adjust until you see no background on layers with the most cells) - Do not press apply, just exit from the threshold screen
4. Process - Binary - Make Binary
5. Process - Binary - Watershed
6. Scale image in imagej by drawing a line along the scale bar
7. Go to analyze, set scale, and set the measured pixel length to the known measurement value
8. Analyze Particles - Size (μm^2): 50-1963.5 and Circularity: 0.4-1.00- (All other settings the same)
9. Copy and paste 'summary' info into an excel sheet
10. Calculate cell viability in excel by dividing the live cells by the total number of cells or the sum of both the live and dead cells

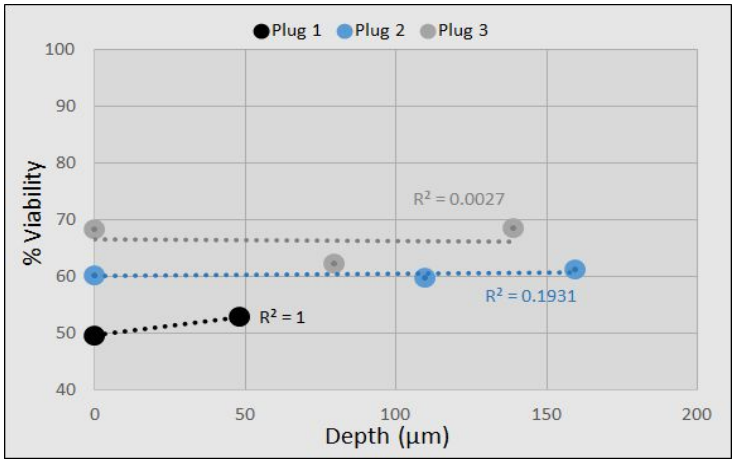
IV: Imaging Protocol

1. Login to Nikon A1RS confocal microscope using PI's login
    a. Dr. Wan-Ju Li's login was used
2. Turn on Confocal Microscope if off
    a. Turn key marked 1, and then turn on buttons 2-5 in order waiting for them to fully turn on before activating the next
    b. Procedure is on the wall
3. Open Nikon Imaging System (NIS) program on desktop
4. In NIS look for AI Compact GUI, Ti Pad, and Capture Z-Series
    a. if these are not on the configuration follow the following steps, if open go to 5.
        i. Click View: Acquisition Controls
            1. AI Compact GUI
                a. allows you to control the different laser wavelengths, intensity, voltage and other values
            2. Ti Pad
                a. allow you to change between different resolutions for the laser imaging
            3. Z-Series Setup
                a. opens Capture Z-Series
                b. allows tracking of depth as well as stepwise depth-movements
5. Within Ti Pad select the resolution at which you will image → we used 4x
    a. note: anything above 20x requires oil based imaging
6. On AI Compact GUI press EyePort
    a. focus the cartilage manually using the dials on the side of the confocal microscope
    b. turn on the filters you will use
7. Once cartilage is focused press EyePort again
    a. this takes you to the laser setting
8. press Scan
    a. this will begin scanning an image of the cartilage and displaying it on the computer
    b. Once cartilage is displayed, it will most likely not be in focus
9. Adjust focus of the image
    a. using the mouse wheel adjust the z-axis to go deeper into the tissue or farther out
    b. if the mouse has no effect on the image follow the next step:
        i. Devices → Enable Mouse Joystick Z in Live
    c. Adjust depth to see cells at different depths
10. Save Images as .tiff and .png
    a. ensure that .tiff files saves both channels separately

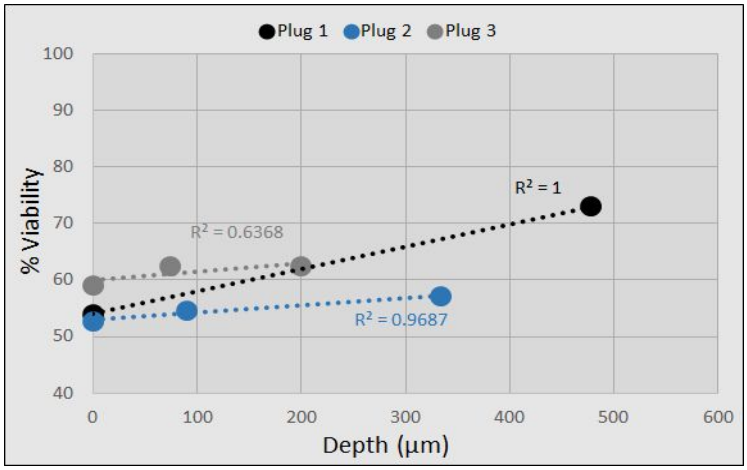
V. Results from Individual Plugs With Viability in Terms of Tissue Depth



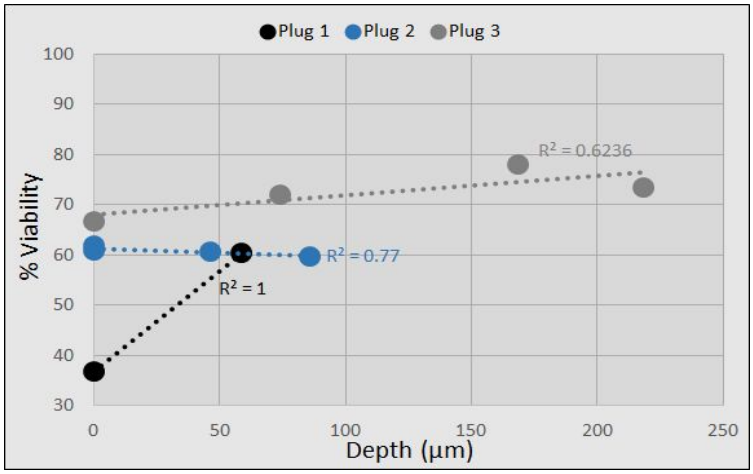
Round 1 - Untreated - 1 hr



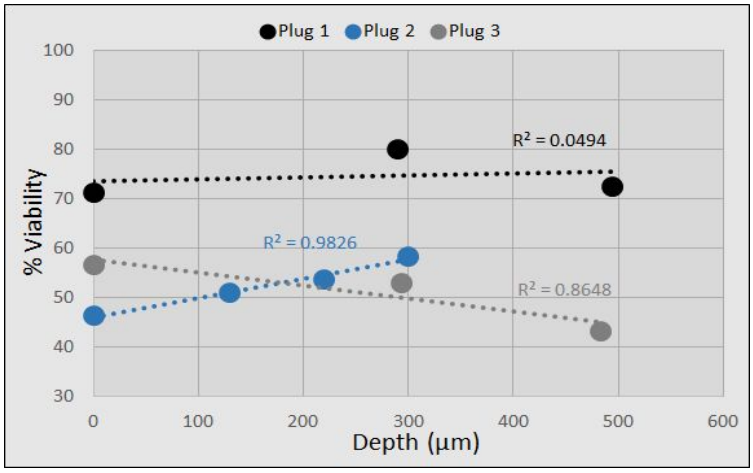
Round 1 - Impaction - 1 hr



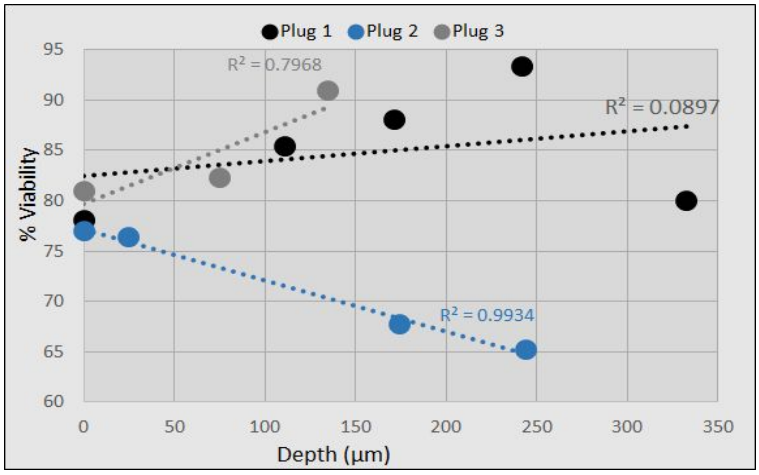
Round 1 - Threaded - 1 hr



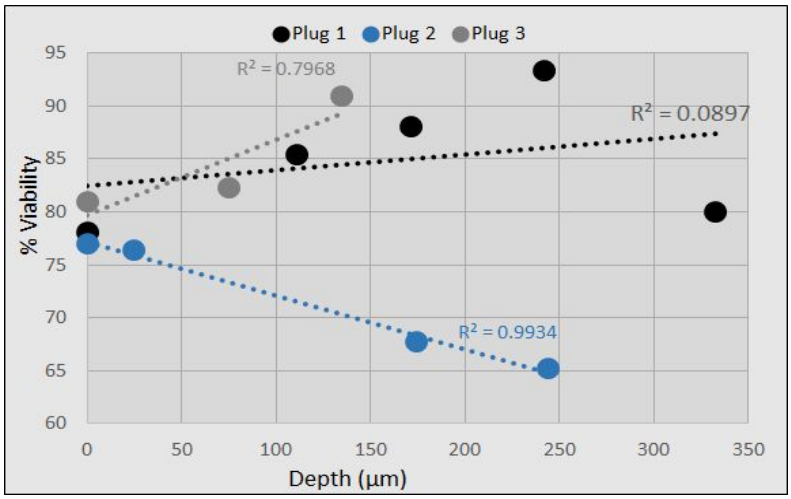
Round 1 - Untreated - 24 hr



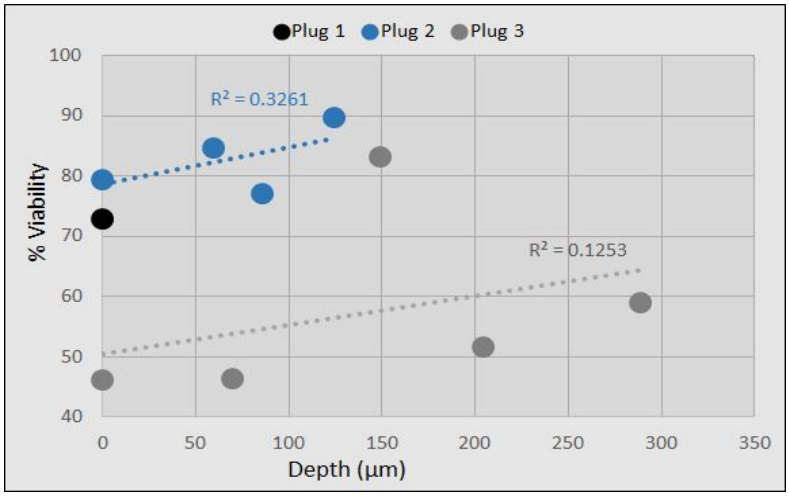
Round 1 - Impaction - 24 hr



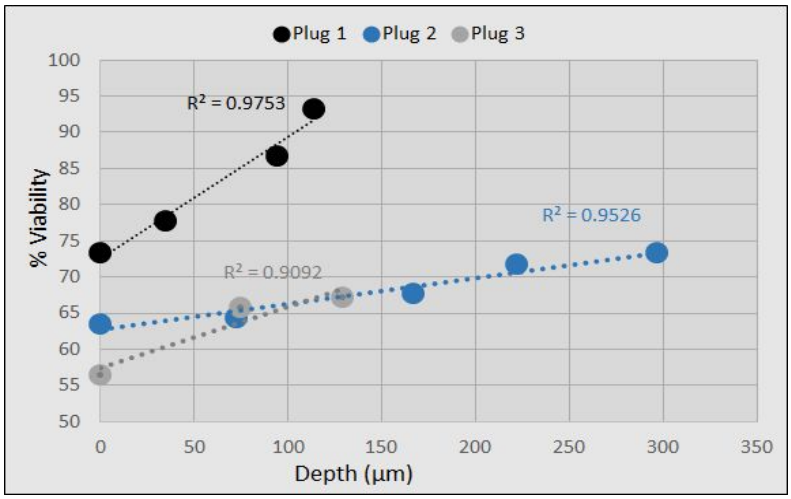
Round 1 - Threaded - 24 hr



Round 2 - Untreated - 1 hr



Round 2 - Impaction - 1 hr



Round 2 - Threaded - 1 hr

VI: Imaging Results (Preliminary)

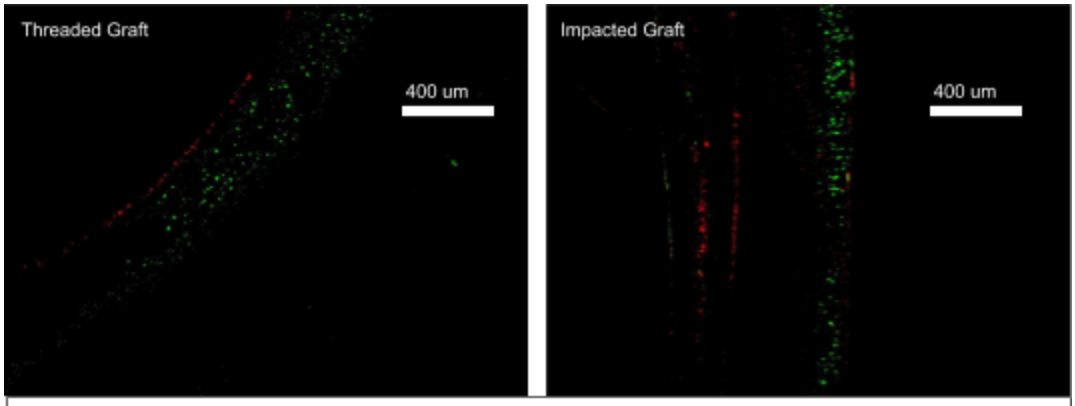


Figure 11: The left photo shows live/dead, 4X images of a threaded condition, and the left shows live/dead for an impacted condition FITC TRITC

	Viability of Threaded Plugs	Viability of Impacted Plugs
1	59.56 %	63.50 %
2	96.73 %	83.43 %
3	80.54 %	84.41 %
Average	78.94 %	77.11 %
Standard Deviation	18. 64 %	11.80 %
Standard Error	10.76 %	6. 81 %

Table 2: Summary of raw cell count data collected from the threaded and impacted conditions