

Video Article

Design of a Biaxial Mechanical Loading Bioreactor for Tissue Engineering

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Abstract

We designed a loading device that is capable of applying uniaxial or biaxial mechanical strain to a tissue engineered biocomposites fabricated for transplantation. While the device primarily functions as a bioreactor that mimics the native mechanical strains, it is also outfitted with a load cell for providing force feedback or mechanical testing of the constructs. The device subjects engineered cartilage constructs to biaxial mechanical loading with great precision of loading dose (amplitude and frequency) and is compact enough to fit inside a standard tissue culture incubator. It loads samples directly in a tissue culture plate, and multiple plate sizes are compatible with the system. The device has been designed using components manufactured for precision-guided laser applications. Bi-axial loading is accomplished by two orthogonal stages. The stages have a 50 mm travel range and are driven independently by stepper motor actuators, controlled by a closed-loop stepper motor driver that features micro-stepping capabilities, enabling step sizes of less than 50 nm. A polysulfone loading platen is coupled to the bi-axial moving platform. Movements of the stages are controlled by Thor-labs Advanced Positioning Technology (APT) software. The stepper motor driver is used with the software to adjust load parameters of frequency and amplitude of both shear and compression independently and simultaneously. Positional feedback is provided by linear optical encoders that have a bidirectional repeatability of 0.1 μm and a resolution of 20 nm, translating to a positional accuracy of less than 3 μm over the full 50 mm of travel. These encoders provide the necessary position feedback to the drive electronics to ensure true nanopositioning capabilities. In order to provide the force feedback to detect contact and evaluate loading responses, a precision miniature load cell is positioned between the loading platen and the moving platform. The load cell has high accuracies of 0.15% to 0.25% full scale.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50387/>

Introduction

We have designed a loading bioreactor that is capable of applying uniaxial or biaxial mechanical strain to a tissue engineered biocomposites fabricated for transplantation. This device is primarily designed as a bioreactor for engineered replacements for articular cartilage; it could also be used for other load-bearing tissues in the human body. Our motivation in this bioreactor design stems from Drachman and Sokoloff¹, who made the seminal observation of abnormal formation of articular cartilage in paralyzed chick embryos due to absence of motion. Similarly, physical exercise is essential for development of normal muscle and bone. In keeping with this concept, many research groups have investigated how different modes of physical stimuli during *in vitro* cultivation modulates the biochemical and mechanical properties of cell-biomaterial biocomposites and tissue explants²⁻⁷. The concept of functional tissue engineering involves the *in vitro* use of mechanical stimuli to enhance the functional properties of tissues, *i.e.* the mechanical properties that enable the tissue to withstand the expected *in vivo* stress and strain^{8,9}. Numerous studies report the use mechanical loading in terms of shear and compression to stimulate engineered cartilage constructs for articular joints. Mauck *et al.*¹⁰ suggest that mechanical loading alone can induce chondrogenesis of mesenchymal stem cells even in the absence of growth factors that are considered vital. Application of intermittent mechanical loading such as compression or shear during tissue cultivation has been shown to modulate cartilage and bone formation, however the optimum dosimetry of loading differs with cell and tissue properties¹¹.

The most important function of articular cartilage is the ability to withstand compressive and shear forces within the joint, therefore it has to have high compressive and shear moduli. The lack of functional mechanical strength and physiological ultrastructure in engineered cartilage has resulted in the breakdown on neo-cartilage *in vivo* and the failure of cartilage replacement strategies in joints. Although compression and shear have been commonly demonstrated to modulate and improve mechanical strength of articular cartilage biocomposites, a combination approach is rare^{6,12-15}. Wartella and Wayne¹⁶ designed a bioreactor that applied tension and compression to produce meniscal cartilage replacements. Waldman *et al.*¹⁵ designed a device to apply compression and shear to chondrocytes cultured in a porous calcium polyphosphate substrate.

Bian *et al.*¹⁷ demonstrated mechanical properties matching native cartilage with the *in vitro* cultivation of adult canine chondrocytes in gels and application of biaxial mechanical loading (compressive deformational loading and sliding contact loading).

The biaxial mechanical loading bioreactor was originally designed by Danielle Chu in our laboratory with the overall goal to induce morphological adaptations in tissue engineered cartilage constructs resulting in higher compressive and shear moduli than currently available¹⁸. We believe this research will significantly increase our broader understanding of how mechanotransduction can be modulated to engineer clinically relevant tissues.

Protocol

1. Biaxial Loading Bioreactor Design

1. The bioreactor employs two stages manufactured by Thor-labs (Newton, MA) for precision-guided laser applications for applying uniaxial or biaxial mechanical strain to engineered tissues, with great precision of loading dose (amplitude and frequency) and application to a wide variety of tissue culture conditions from single to 24 well plates (**Figure 1**).
2. Bi-axial loading is accomplished by two TravelMax stages (LNR50SE). These stages are mounted orthogonally in an XZ configuration. The horizontal stage provides dynamic shearing motions by oscillating along the X-axis. The vertical stage provides dynamic compressive loading by oscillating along the Z-axis. These stages have a 50 mm travel range and are driven independently by stepper motor actuators (DRV014), controlled by a closed-loop stepper motor driver (BSC102) that features micro-stepping capabilities, enabling step sizes of less than 50 nm.
3. The device is mounted on a rigid 25 cm x 30 cm x 12.5 mm aluminum base plate that is used as a platform for assembly of machine components and for mounting of tissue culture plates. Adjustable kinematic stops are used to lock tissue culture plates in place on the aluminum base plate. These kinematic stops have fine adjustment screws to allow for precise alignment that is otherwise not achievable by hand. The modular design of the base plate allows for flexible placement of these kinematic stops to accommodate plates of different sizes and shapes (Petri dishes vs. multi-well plates).
4. A custom-machined polysulfone loading platen is coupled to the bi-axial moving platform via a precision-machined right angle bracket. Polysulfone material was chosen due to its biocompatibility, ease of machining, and ease of sterilization.
5. Movements of the stages are controlled by Thor-labs' Advanced Positioning Technology (APT) software. The stepper motor driver is used in combination with the software that permits the adjustment of load parameters of frequency and amplitude of both shear and compression independently and simultaneously.
6. Positional feedback is provided by linear optical encoders that are attached to each moving platform and are integrated with the software. The encoder system has a bidirectional repeatability of 0.1 μ m and a resolution of 20 nm, translating to a positional accuracy of less than 3 μ m over the full 50 mm of travel. These encoders provide the necessary position feedback to the drive electronics to ensure true nanopositioning capabilities and a direct readout of the absolute position.
7. In order to provide the force feedback necessary for detecting contact between platen and samples and evaluate loading responses, a precision miniature load cell (Honeywell Model 31) is positioned between the loading platen and the moving platform (**Figure 2**). The load cell has high accuracies of 0.15% to 0.25% full scale. The display unit (SC500) for the load cell can also provide load measurements of up to 5 decimal places. Additionally, it has an RS-232 port to allow for data collection on a computer.

2. Cell-Seeded Agarose Constructs

1. Prepare 4% agarose: Add 0.8 g agarose to 20 ml DMEM (no additives) in a 50 ml flask, boil, and then keep in 70 °C oven until use.
2. Adjust volume of cell suspension for double amount of the desired cell seeding density. This suspension is mixed with equal volume of 4% w/v agarose to create a 2% agarose gel at the desired seeding density.
3. Place both the cell suspension and one 10 ml pipette in the incubator.
4. Set up gel casting system. One 1.5 mm and one 0.75 mm spacer plate should be put together to create a 2.25 mm thick gel. Other size spacer plates may be used to create different gel thicknesses. The gel casting system is not large enough to hold these plates together, so they must be securely taped to prevent leaking.
5. The next step is to quickly mix the cell suspension with the previously prepared agarose and pipette into the gel mold before the agarose solidifies. Remove the liquid agarose from the oven and place a sterile thermometer in it. The agarose must cool to 42-43 °C before mixing with cells. Warm up the cell suspension to 37 °C. Once the agarose hits 43 °C, quickly pipette up the desired amount and then immediately pipette the cell suspension up and down a few times to mix. Then immediately pipette the entire mixture into the gel mold.
6. Allow the gel to solidify for 10-15 min and then carefully tilt it to a horizontal position.
7. Remove the top glass plate and punch disks with the biopsy punch. Disks can be picked up with a small sterile spatula. In our experience, a 9 ml gel was large enough to make more than a hundred 5-mm-diameter discs.

3. Culture the Disks

1. Place one disk in each well of a 24 well non-tissue culture treated plate.
2. Add 2 ml of serum-free chondrogenic differentiation medium to each well.
3. Put plates in incubator (37 °C, 5% CO₂).
4. For media changes, replace 1 ml per well every 2-3 days.

4. Immobilization of Samples for Mechanical Loading

1. Prepare 4% agarose (no cell suspension added) and the gel should be thinner than the samples themselves (to prevent interference during loading). The recommended thickness is 1.5-1.9 mm (for 2.25 mm thick samples).

2. Once gelled, punch 16 mm diameter disks for 24 well plates. In each disk, punch one 5 mm hole for the sample to be placed in. If desired, punch another 5 mm hole on the edge of the disk for the pipette to be placed during media changes.
3. Once agarose wells have been made, place in 24 well plate, as shown in **Figure 3**.
4. Once the agarose wells are in the 24 well plate, press fit samples in each well. The sample should protrude from the top of the agarose well.

5. Mechanical Loading

1. Sterilize platen (**Figure 2**).
2. Secure aluminum plate to load cell. Secure loading platen/load cell/aluminum plate assembly to the stage.
3. Turn on stepper motor controller (switch in back).
4. Turn on PC and open "APT User" program (**Figure 4**).
 1. Left screen controls horizontal stepper motor. Right screen controls vertical stepper motor. In each screen, "Graphical Control" tab allows for manual positioning and "Move Sequencer" tab allows for automation. All units are mm.
5. Go to "Graphical Control" tab on both screens and press "Home/Zero" button. Both stepper motors have a range of 50 mm. Pressing "Home/Zero" will send both stepper motors to the zero position (top- and right- most positions).
6. Prepare the samples in the 24 well plate for loading by removing some media from each well. No more than 1 ml of media should be left in each well to prevent overflow during loading. Be sure that enough media is left in the well to keep the sample covered.
 1. Note that the incubator is kept in low-humidity conditions to prevent instrument failure.
7. Place 24 well plate in bioreactor and carefully line up with the platen.
 1. The plate is secured to the bioreactor using four adjustable kinematic locators. To make it easier to line up the platen, the two left locators have been pre-positioned. Tighten the two right locators so that the plate is secure. Make sure to line the plate up flush with the front of the bioreactor base.
 2. In "Graphical Control" tab, a specific stepper motor position can be manually entered by clicking on the position box. Use this capability to slowly lower the platen and move it horizontally to line up with plate.
8. Once the platen is close to coming in contact with samples, start to bring the platen down in very slow increments (0.1 mm) until you reach the predetermined starting position (see part 6).
9. Once the starting position is reached, go to "Move Sequencer" tab and load the desired move sequence by pressing "Load." Then press "Run" to start. (**Figure 5**) See part 7. Writing a Dosing Protocol.
10. When finished loading, manually raise the platen. If any samples are stuck to the platen, carefully put them back into the appropriate well using a sterile spatula.
11. Remove 24 well plate from bioreactor and replace the media.
12. Carefully remove the platen from the load cell and then turn off instruments.

6. Calibrating Loading Platen

To ensure that the proper strains are applied to samples, each platen must be carefully calibrated prior to starting an experiment.

1. Perform steps 5.1 to 5.8.
2. Manually put horizontal stepper motor at the 25 mm position.
3. Carefully lower platen until it just barely comes in contact with the base of the bioreactor. Load cell will show increased loads at this point. Take note of the exact position of the vertical stepper motor (be as precise as possible, as all compression strain measurements will be computed from this value).
4. Record the position. This value will be used to write a dosing protocol based on sample dimensions and desired strains.

7. Writing a Dosing Protocol

1. The bioreactor is capable of applying both compressive and shear strain, either simultaneously or individually. Three main parameters must be decided: tare compressive strain, dynamic strain amplitude, and loading frequency.
2. A tare strain is applied in order to prevent liftoff of the platen from the sample.
3. Sample dynamic strain amplitude and loading frequency are chosen.

In this study we define compressive and shear strain as follows:

$$\begin{aligned} \% \text{ Strain (Compression)} &= \frac{\text{Change in Vertical Position}}{\text{Sample Starting Thickness}} \cdot 100\% \\ \% \text{ Strain (Shear)} &= \frac{\text{Change in Horizontal Position}}{\text{Sample Starting Thickness}} \cdot 100\% \end{aligned}$$

Example Biaxial Dosing Protocol

Sample thickness: 2.25 mm

Tare Strain (Compression): 10% of the sample thickness (0.225 mm)

Dynamic Strain Amplitude (Compression): 10% (+/- 5% of the sample thickness)

Frequency (Compression): 1 Hz

Dynamic Strain Amplitude (Shear): 25% of the sample thickness (0.5625 mm): Shear stress is

applied to the sample by the platen moving horizontally.

Frequency (Shear): 0.5 Hz

Typical dosing protocol is 3 hr of loading per day.

In this example, dynamic and shear loading is applied simultaneously rather than sequentially. We believe this pattern better mimics the complex loading environment in the human knee.

4. Once a dosing protocol has been selected, a compression move sequence program must be written.
5. The move sequence is exactly what it sounds like, a list of positions that the stepper motor will move to at a specified acceleration and maximum velocity.
6. Compute desired vertical positions based on dosing protocol and platen calibration value (from part 6).
7. Example computations for Platen 1 are provided below:

	Difference from Calibration Value	Vertical Position
Platen Calibration Value (touches bottom of bioreactor)	0 mm	29.7700 mm
Platen makes contact with Sample (2.25 mm sample)	4.4140 mm	25.3560 mm
Strain (5% Thickness)	4.3015 mm	25.4705 mm
Strain (10% Thickness)	4.1890 mm	25.5810 mm
Strain (15% Thickness)	4.0765 mm	25.6955 mm

8. Once positions are computed, experiment with acceleration and maximum velocity values to get the correct frequency. The number of cycles should be chosen, accordingly (e.g. 10,800 cycles for 3 hr at 1 Hz).
9. Example Dynamic Compression Move Sequence Program (10% tare compression, 10% dynamic strain amplitude, 1 Hz) (**Figure 5**)
10. Dynamic shear move sequence program: The number of cycles should be chosen according to the desired frequency and duration (e.g. 5,400 cycles for 3 hr at 0.5 Hz).
11. Example Dynamic Shear Move Sequence Program (10% tare compression, 0.5625 mm (25% of the thickness) dynamic shear strain amplitude, 0.5 Hz) (**Figure 5**).

Representative Results

The device was tested by using agarose gels seeded with 20 million cells/ml chondrocytes and cultivated in the presence of uniaxial (compression) or biaxial (compression and shear) mechanical loading. Primary porcine chondrocytes were isolated from the articular cartilage of 2-4 month old pigs. 5 mm diameter and 1.5 mm thick samples were cultured in 2 ml of defined chondrogenic culture medium (High glucose DMEM, 1% ITS+ Premix, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 2.5 µg/ml amphotericin B, 50 µg/ml ascorbic acid, 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline,) in 24-well plates at 37 °C, 5%CO₂. 10⁻⁷ M dexamethasone and 10 ng/ml TGF-β1 were supplied for the first 10 days of culture. The samples were loaded for 3 hr/day between days 10-30. Uniaxial loading consisted of 10% compression peak-to-peak amplitude, 1 Hz and biaxial loading consisted of 0.15 mm (10% thickness) compression and 0.075 mm shear peak-to-peak amplitude, 1 Hz. The dynamic strain amplitude and loading frequency are chosen based on published studies^{17,19}. At the end of 30 days biochemical and mechanical properties of engineered cartilage were assessed.

This study employed three groups: 1- No loading control, 2- Uniaxial (compressive) loading, 3- Biaxial (compressive and shear) loading. The DNA contents and the wet weights of constructs remained similar in the three groups after 30 days of cultivation ($p > 0.05$). The GAG content was highest in the group that was subjected to biaxial loading (group 3, $p < 0.001$ compared with the control group), followed by the uniaxial loading group (group 2, $p < 0.05$) (**Figure 6**). The GAG contents of groups 2 and 3 correspond to 48% and 50% of native cartilage, respectively. Group 3 resulted in significantly higher amount of collagen than groups 1 and 2 ($p < 0.01$). Group 2 also had thicker constructs than group 1 ($p < 0.01$). Surprisingly, the equilibrium compressive Young's modulus was the highest in group 2 (uniaxial loading, $p < 0.01$) and there were no significant differences between group 3 and 1. The Young's modulus of group 2 corresponded to 60.1% of native porcine cartilage.

The histological analyses indicated positive and homogeneous staining for glycosaminoglycans (alcian blue, safranin O) and type II collagen (**Figure 7**). All groups stained negative for type I collagen (not shown).

In summary, these preliminary results suggest that this bioreactor successfully applied compression and biaxial (compression and shear) mechanical loading during long term cultivation of engineered tissues. In this study biaxial loading was shown to the proteoglycan and collagen deposition and the thickness of the tissue engineered cartilage samples. Uniaxial compression increased both the proteoglycan deposition and the Young's modulus.

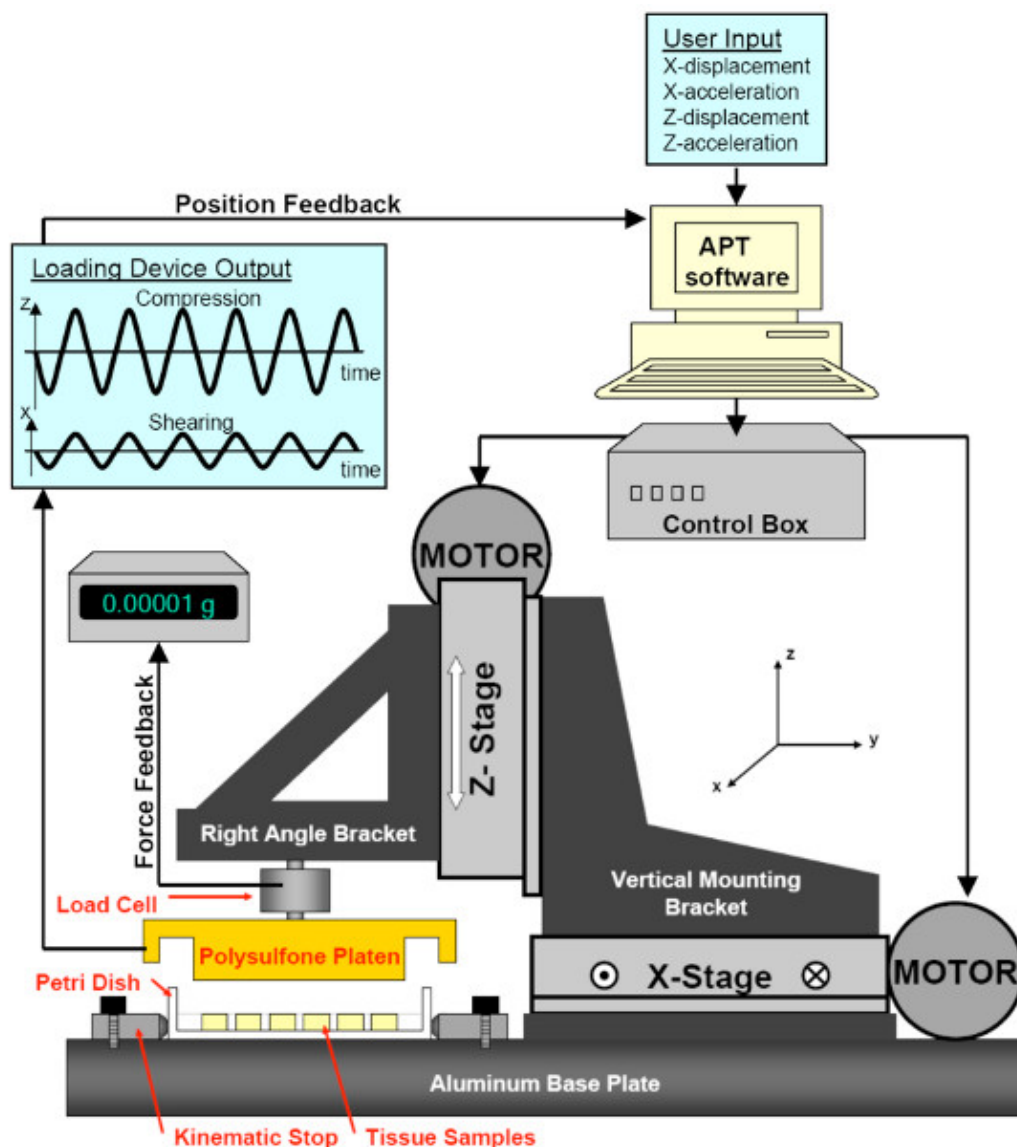


Figure 1. Biaxial loading is accomplished by the X-stage (shearing) and the Z-stage (compression). The figure shows a custom-made loading platen attached to the stages to load samples in a 24-well plate. The loading parameters are controlled with a computer connected to the stepper motors¹⁸.

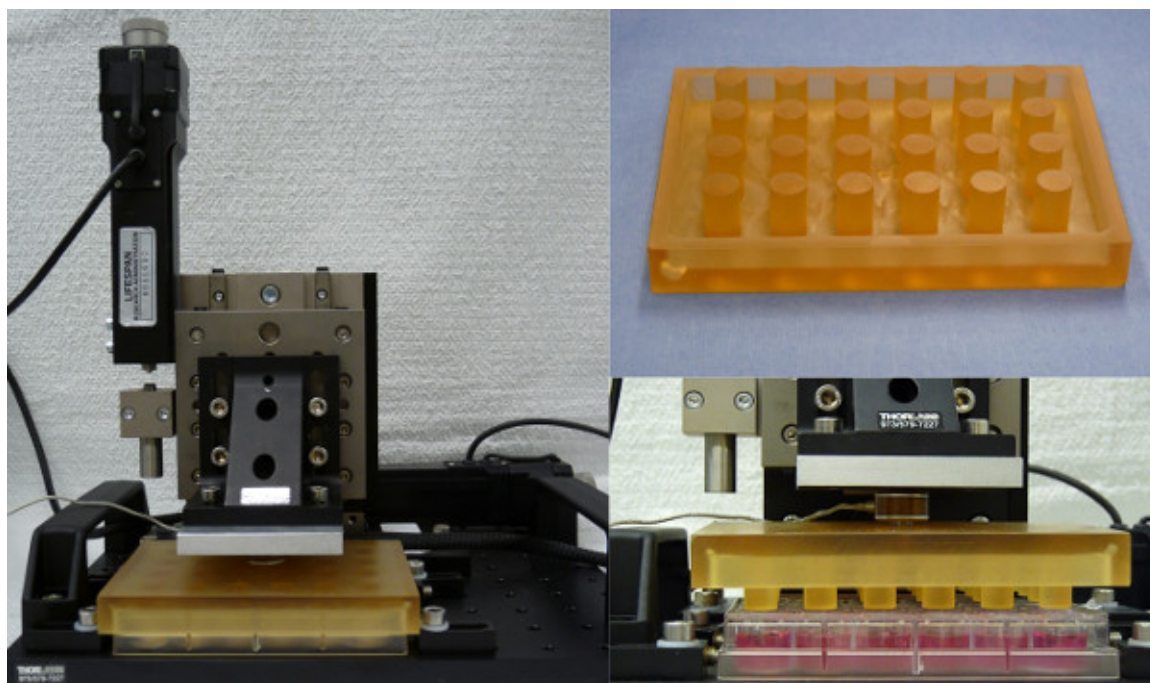


Figure 2. Left: The polysulfone loading platen designed for 24-well plates. Right: The loading platen attachment to the biaxial loading bioreactor.

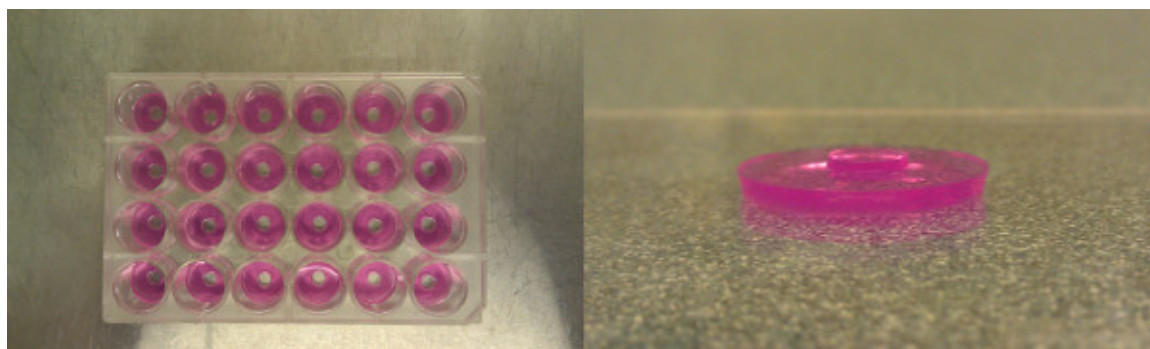


Figure 3. Preparation of agarose wells for immobilizing samples during shear loading. The immobilized construct placed in the agarose well for mechanical loading. This figure shows a 1.5 mm thick agarose well and a 2.25 mm thick sample.

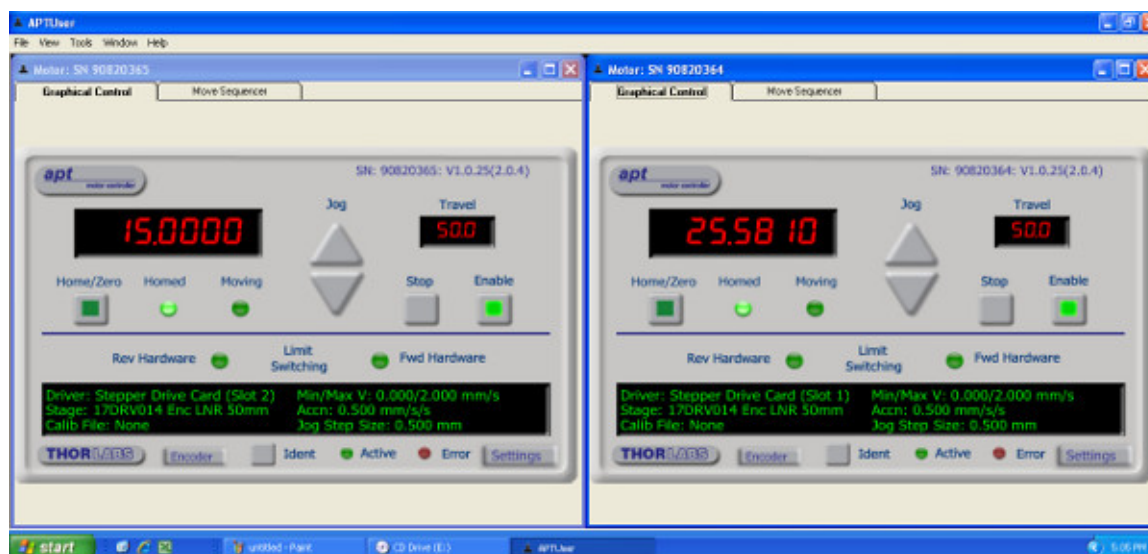


Figure 4. The graphical user interface to control the biaxial loading device. [Click here to view larger figure.](#)

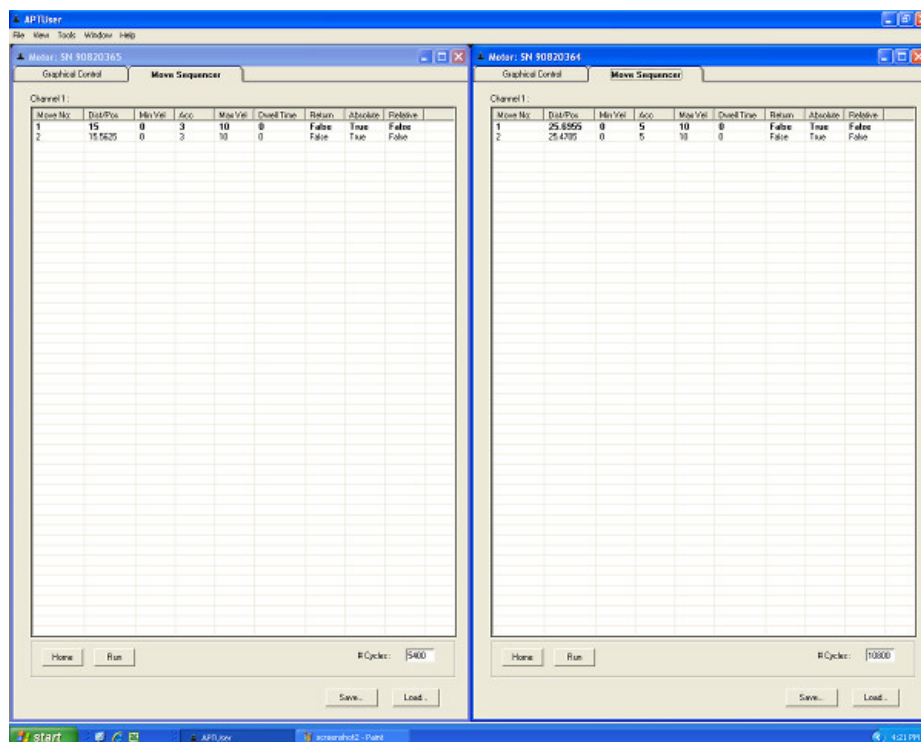


Figure 5. Example Biaxial Loading Move Sequence Program Graphical User Interface: Dynamic Compression Move Sequence Program (10% tare compression, 10% dynamic strain amplitude, 1 Hz) and Dynamic Shear Move Sequence Program (10% tare compression, 25% dynamic shear strain amplitude, 0.5 Hz). [Click here to view larger figure.](#)

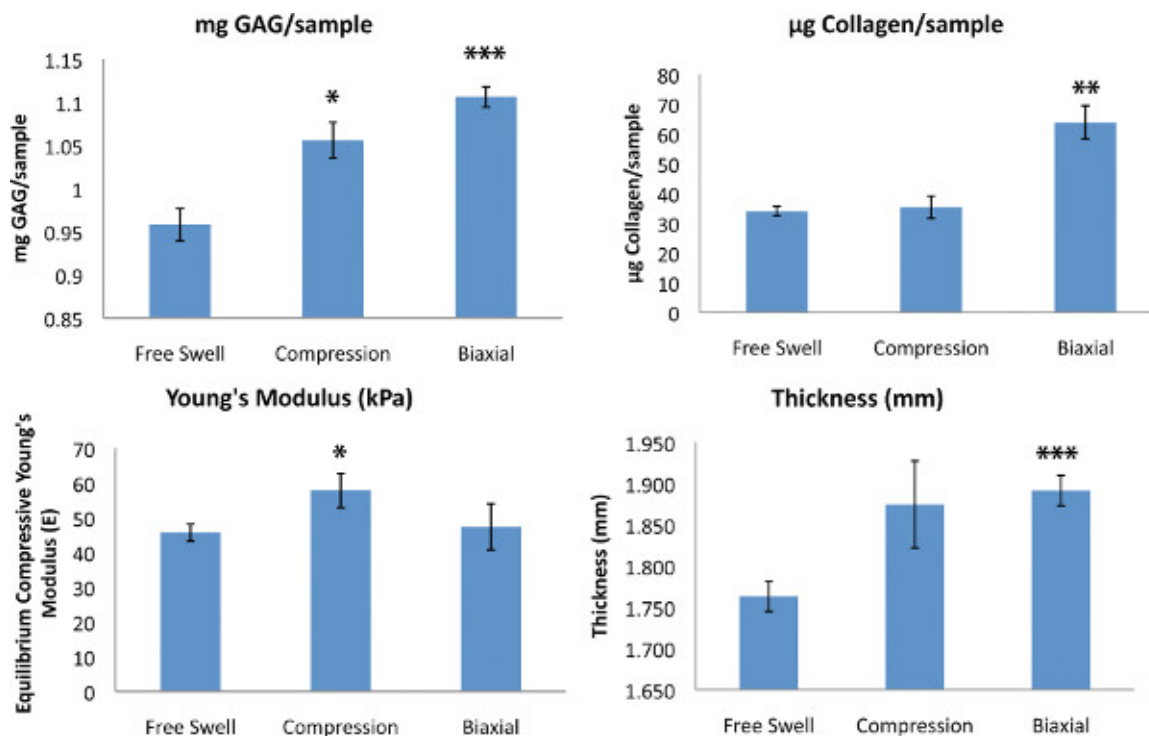


Figure 6. Biochemical and mechanical testing results (n=6). ***p < 0.001, **p < 0.01, *p < 0.05 compared with group 1 (unloaded control). Group 2: Uniaxial compressive loading, group 3: Biaxial compressive and shear loading.

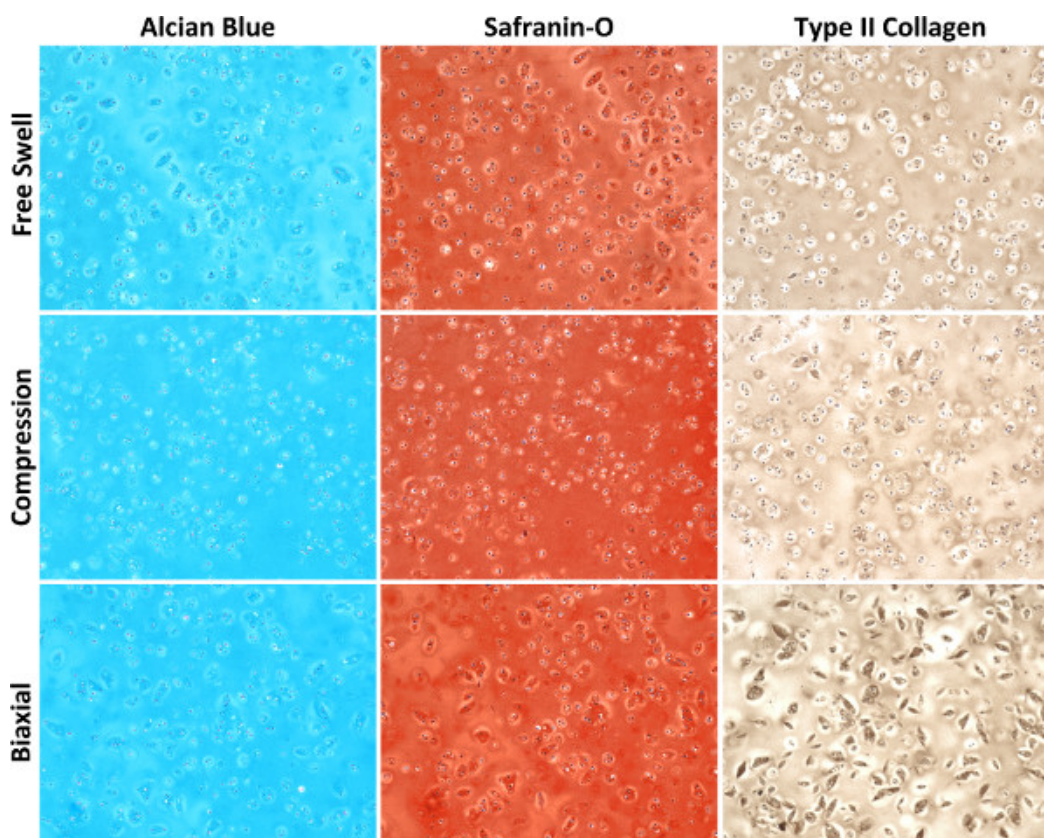


Figure 7. Histology: Alcian blue/nuclear fast red staining, Safranin O/fast green, immunochemistry for type II collagen.

Discussion

We have designed a loading device that is capable of applying uniaxial or biaxial mechanical strain to tissue engineered constructs fabricated for transplantation. The device can be used as a bioreactor for *in vitro* cultivation of engineered biocomposites or as a testing device to describe the mechanical characteristics of the native tissue or after other treatments prior to. The device subjects engineered tissue constructs to biaxial mechanical loading with great precision of loading dose (amplitude and frequency) and application to a wide variety of tissue culture conditions from single to 24 well plates.

The application of shear loading presented a set of unique challenges for the design of this system. To maximize nutrient transfer, constructs were originally unconfined in individual wells of a 24 well plate. This did not present a problem for dynamic compression, as the tare compressive strain insured that sample-platen contact was not lost. When shear strain was added to the protocol, however, unconfined samples slid along the bottom of the plate and some lost contact with the platen. Additionally, during biaxial loading protocols samples had the tendency to flip over, causing inconsistent loading. We solved this problem by creating the agarose wells to immobilize samples as described in the procedure. These agarose wells allow consistent biaxial loading of samples without limiting nutrient availability to samples.

Unlike the compression bioreactors that are very widely researched^{20,21}, our device is capable of applying precise strains on multiple axes. These axes can be independently controlled. Multiaxial loading can be applied sequentially or simultaneously. It is possible to implement a third Y-axis to provide mechanical loading in three dimensions to better mimic *in vivo* conditions.

While other multiaxial bioreactors have been developed to mimic the mechanical environment of the joint, they have large limitations compared to our system. A shear and compression apparatus designed by Frank, *et al.* allows up to 12 samples to be loaded simultaneously with load feedback, however constructs are not confined or secured⁶. During experiments involving shear strain, it is essential that constructs be secured so that they do not slide under the loading platen. Sliding will result in uneven and inconsistent shear loading of the specimen. Newer bioreactors, such as the unique "rolling ball" system^{22,23} and a biaxial stimulation device¹⁶, create a much more realistic and consistent loading environment; however, they only allow one sample to be loaded at a time. Large sample sizes are essential for performing the necessary biochemical, mechanical, and histological analyses on the constructs with a high level of confidence. Additionally, the "rolling ball" system lacks force feedback, an essential measure of construct development during long term *in vitro* cultivation. It also allows the prevention of platen-specimen non-contact and specimen overloading, which will irreversibly damage tissue engineered constructs. A sliding contact bioreactor developed by Bian, *et al.* allows up to four constructs to be loaded simultaneously, but still lacks this valuable force feedback mechanism¹⁷.

The current setup using 24-well plates allows the simultaneous loading of 24 samples; more samples are possible with modifications of the geometry of the loading platen. The loading platen offers vast flexibility to the novel design. The chosen material polysulfone is porous, can be sterilized and cultivated in the humid and warm environment of an incubator. It is easily machinable, enabling a variety of geometries and numbers of samples to be loaded simultaneously.

In conclusion, the new biaxial loading bioreactor for tissue engineering enables long term *in vitro* cultivation of tissue engineered constructs. Biaxial loading increased the proteoglycan and collagen deposition and the thickness of the tissue engineered cartilage samples but did not seem to significantly influence the mechanical properties of engineered cartilage as we hypothesized. Uniaxial compression increased both the proteoglycan deposition and the Young's modulus. We believe that the optimum dose of mechanical loading differs with cell and tissue properties. Future studies of collagen architecture and dosimetry of loading will allow us to fully evaluate the effects of biaxial loading on the development of engineered tissues.

Disclosures

The authors declare that they have no competing financial interests.

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