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Creating Homogenous Strain Distribution Within 3D Cell-Encapsulated Constructs Using a Simple and Cost-Effective Uniaxial Tensile Bioreactor: Design and Validation Study

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ABSTRACT: Mechanical loading bioreactors capable of applying uniaxial tensile strains are emerging to be a valuable tool to investigate physiologically relevant cellular signaling pathways and biochemical expression. In this study, we have introduced a simple and cost-effective uniaxial tensile strain bioreactor for the application of precise and homogenous uniaxial strains to 3D cell-encapsulated collagen constructs at physiological loading strains (0–12%) and frequencies (0.01–1 Hz). The bioreactor employs silicone-based loading chambers specifically designed to stretch constructs without direct gripping to minimize stress concentration at the ends of the construct and preserve its integrity. The loading chambers are driven by a versatile stepper motor ball-screw actuation system to produce stretching of the constructs. Mechanical characterization of the bioreactor performed through Finite Element Analysis demonstrated that the constructs experienced predominantly uniaxial tensile strain in the longitudinal direction. The strains produced were found to be homogenous over a $15 \times 4 \times 2$ mm region of the construct equivalent to around 60% of the effective region of characterization. The strain values were also shown to be consistent and reproducible during cyclic loading regimes. Biological characterization confirmed the ability of the bioreactor to promote cell viability, proliferation, and matrix organization of cell-encapsulated collagen constructs. This easy-to-use uniaxial tensile strain bioreactor can be employed for studying morphological, structural, and functional responses of cell-embedded matrix systems in response to physiological loading of musculoskeletal tissues. It also holds promise for tissue-engineered strategies that involve delivery of mechanically stimulated cells at the site of injury through a biological carrier to develop a clinically useful therapy for tissue healing.

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Introduction

Musculoskeletal tissues exist in a dynamic in vivo environment where they experience various kinds of mechanical strains including tensile, compressive, and shear on a daily basis (Basso and Heersche, 2002; Carter et al., 2004; Powell et al., 2002). Uniaxial tensile forces, particularly relevant to bone, tendon, ligament, and skeletal muscles are hence known to induce specific biological responses in resident mesenchymal cells (Schache et al., 2012; Verbruggen et al., 2012; Wang, 2006). It has been observed that the strained cells respond by altering their size, morphology, proliferation rate, and extracellular matrix gene expression that direct changes in tissue structure, composition, and mechanical properties (Plunkett and O'Brien, 2011; Riehl et al., 2012; Trumbull et al., 2016). Thus, uniaxial tensile bioreactors that can simulate the mechanical microenvironment of musculoskeletal cells have emerged as a valuable tool to investigate the biochemical expression and signaling pathways underlying the cellular behavior to mechanical stimulation.

Bioreactors capable of stretching three-dimensional (3D) cellular constructs are considered to be good biomimetic models due to their ability to mimic the complexities of the cellular microenvironment along with mechanical cues (Bono et al., 2016; Riehl et al., 2012). Current uniaxial tensile strain bioreactors for loading 3D cellular constructs include the commercially available Flexcell® Tissue Train® Culture system, STREX 3D Cell Stretching system, and CellScale MechanoCulture system, along with a few custom-built tissue bioreactors (Berry et al., 2003; Birla et al., 2007; Butler et al., 2009; Cook et al., 2016; Govoni et al., 2014; Heher et al., 2015;

Moon et al., 2008; Wang et al., 2012a; Zimmermann et al., 2000). Flexcell®, in particular, has been widely used for applying uniaxial strains due to its well-characterized strain profile and varied modes of loading (Geest et al., 2004; Matheson et al., 2006). These bioreactors that stretch soft biomaterials (predominantly collagen) require innovative techniques to grip the construct without compromising its structural integrity. Most of such bioreactors employ nylon mesh or foam anchors as grips onto which the collagen solution is poured and allowed to polymerize to minimize the risk of construct disintegration during mechanical loading (Birla et al., 2007; Garvin et al., 2003; Riehl et al., 2012). Also, they are often coupled with a pneumatic non-contact actuation system to deform the membrane onto which the cell-seeded collagen constructs are anchored at the ends (Kamble et al., 2016; Matheson et al., 2006; Nirmalanandhan et al., 2008). However, this actuation and gripping system can typically lead to non-homogenous strain distribution within the collagen construct where the strain experienced by the cells inside the construct vary significantly based on their spatial location. It is observed that only a narrow uniform strain region near the center of the construct is obtained, while wide variation in strain magnitudes are seen near the ends of the construct (Riehl et al., 2012; Wang et al., 2012b; Youngstrom and Barrett, 2016). Thus, there is a persistent demand for uniaxial tensile strain bioreactors that can produce enlarged area of homogenous strain distribution within 3D cellular constructs along with the minimal risk of construct disintegration.

In this study, we have developed a simple and cost-effective uniaxial tensile strain bioreactor to apply homogenous cyclic strains to 3D cell-encapsulated collagen constructs over a substantial proportion of its length without compromising its structural integrity. In addition, it is also easy to setup, operate, and maintain, and compact enough to fit into a standard cell culture incubator. The bioreactor uses silicone-based loading chambers specifically designed to effectively stretch the 3D collagen constructs without direct gripping, thus eliminating the abnormally high strain produced at either end in case of traditionally gripped constructs. It is driven by a precise and versatile stepper-motor ball-screw actuation system to apply consistent and reproducible uniform uniaxial strains in the range of physiological loading frequencies of musculoskeletal tissues. The bioreactor performance was first evaluated by characterizing the spatial strain profiles experienced by the 3D constructs under static and cyclic loading conditions through Finite Element Analysis. Next, the uniaxial tensile strain bioreactor was biologically characterized to investigate its effect on cell viability, proliferation, and matrix organization within 3D cell-encapsulated collagen constructs.

Materials and Methods

Uniaxial Tensile Strain Bioreactor

The schematic of the uniaxial tensile strain bioreactor for mechanical stimulation of 3D collagen constructs is depicted in Figure 1. All mechanical components were designed using SolidWorks 3D CAD design software (SolidWorks, Watham, MA). The individual components were manufactured and assembled at a high-precision local machine shop (OBARS Machine and Tools Company, Toledo, OH). The parts of the strain bioreactor are made

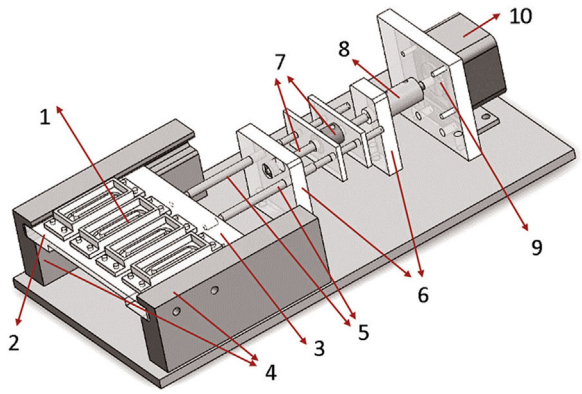

Figure 1. Schematic of the uniaxial tensile strain bioreactor. (**1**) Silicone loading chambers containing the 3D collagen constructs. (**2**) Fixed plate. (**3**) Moving plate. (**4**) Guiding sleeves for supporting plates. (**5**) Connecting rods to transmit motion to the moving plate. (**6**) Bearing supports to hold the ball-screw mechanism. (**7**) Ball screw mechanism to produce precise linear motion. (**8**) Coupling connecting the screw and the motor shaft. (**9**) Motor support. (**10**) A 2-phase high torque stepper motor connected to programmable controller to produce controlled stretch.

of polycarbonate, aluminum, or stainless steel (McMaster-Carr, Elmhurst, IL), suitable for operation in the cell culture environment. The loading chambers made of silicone are supported by a polycarbonate base consisting of fixed and moving plates that have aluminum pins fitted on one of their ends to secure the loading chambers. The plates are supported by polycarbonate guiding sleeves on either side to allow translation motion of the moving plate, while the fixed plate is screwed into the guiding sleeves to render it stationary. Two connecting rods screwed into the free end of the moving plate are responsible for transferring the motion from the driving mechanism to the silicone loading chambers. A precision ball screw assembly (Thomson Linear Motion Systems, Radford, VA) coupled with a two-phase high torque stepper motor with driver-controller (Lin Engineering, Morgan Hill, CA) constitutes the actuation system of this bioreactor. This system ensures high efficiency in the transfer of rotational motion of the ball screw to translational motion of the ball nut linked to the connection rods that displace the moving plate, which in turn produces stretching of the silicone loading chambers containing the collagen constructs. The entire assembly is placed on a polycarbonate base, into which the fixed components of the bioreactor are secured, and a polycarbonate lid is used to cover the loading chambers during mechanical stimulation.

Silicone Loading Chamber

A unique part of this uniaxial tensile strain bioreactor are the silicone-based loading chambers. They are specifically designed to maximize the mechanical load transferred to the 3D collagen constructs through minimizing the relative movement (sliding) of collagen constructs during mechanical loading, without the use of direct grips. The main features of the loading chamber are shown in Figure 2. The cellular collagen construct is added within the groove of the loading chamber that comprises of two linear strips connected by circular sections on either side. The dimension of one

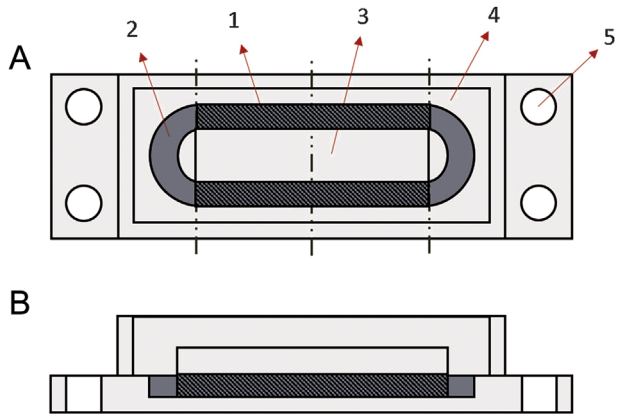

Figure 2. Schematic of the silicone loading chamber: (**A**) Top view (**B**) Front view. (**1**) Groove into which cellular collagen constructs are polymerized comprises of linear strips (shaded) with dimensions of 25 × 4 × 2 mm (LxWxT) indicating the effective region for characterization and (**2**) semi-circular strips at either ends linking the two linear strips of the construct. (**3**) Island at the center provides support to the constructs during loading. (**4**) Well to hold sufficient media for cell survival, proliferation, and differentiation. (**5**) Pin holes through which the supporting base plates are inserted in order to apply loading.

linear strip is 25 × 4 × 2 mm (LxWxT) which is considered as the region of characterization of the construct. Collagen is supported by an island at the center to eliminate direct gripping at the ends and to increase the homogeneity in strain distribution across the linear part of the construct. The groove and island are enclosed within a well to hold culture media for the cell-encapsulated 3D constructs. The well is flanked by flaps having pinholes through which aluminum pins from the supporting base of the bioreactor system is inserted in order to produce stretching of the loading chambers. A mold corresponding to the geometry of the chamber is used to fabricate the silicone loading chambers. Silicone rubber components (Dragon Skin® 10, Smooth On) is mixed in 1:1 ratio, poured into the mold and allowed to polymerize to obtain the chambers.

Experimental Validation of Uniaxial Tensile Bioreactor With 3D Collagen Constructs

The uniaxial tensile strain bioreactor was experimentally validated to evaluate the efficiency of the device in transmitting the applied load to the loading chamber and the 3D collagen construct within the chamber. The validation experiment was conducted by measuring the initial and final displacement undergone by the linear region of the loading chamber and the collagen construct following loading. A 3D collagen constructs were prepared with 2.5 mg/mL Collagen Type-1 solution (Corning Life Sciences, Tewksbury, MA) neutralized to pH 7∼8 with chilled 1N NaOH solution along with phosphate buffer saline and sterile water according to manufacturer's protocol. The sample solution was added into the grooves of each loading chamber and polymerized at 37°C for 1 h. For displacement measurements, embedded markers were placed at the ends and the center of the linear region of the loading chamber and collagen construct, corresponding to 0, 12.5, and 25 mm in length from the end nearest to the load application (Fig. 3A). Images of the samples were taken run at initial and final positions in triplicates for each applied load of 1–3N. The images were analyzed using an image processing software (ImageJ, NIH, Bethesda, MD), and the initial and final displacements of the embedded marker for each sample at each applied strain was measured.

Finite Element Modeling of Strain and Stress Distribution

A Finite Element Model (FEM) was generated to investigate the strain and stress profiles experienced by the collagen constructs during mechanical stimulation using the uniaxial tensile strain bioreactor. The model assembly was meshed with C3D10I; a 10-node general purpose tetrahedron with improved surface stress formulation elements using ABAQUS (6.12, Dassault Systèmes, France). Mesh sensitivity studies were conducted to obtain the most computationally efficient mesh without affecting the obtained results. The silicone loading chamber was modeled as a hyperelastic material while collagen constructs were modeled as a viscoelastic time-dependent material according to Prony series model (Supplementary Fig. S1; Tables S1 and 2). The material parameters in each case were obtained via nonlinear curve fitting of the experimental uniaxial tensile testing data following our previously employed methodology (Elsaadany et al., 2017b). The polycarbonate moving and fixed plates were modeled as an elastic material. Young's modulus and Poisson's ratio were obtained from the supplier (McMaster-Carr) and were 2.4 GPa and 0.35, respectively. The representative view of the loading assembly with the surfaces used to apply the loading and boundary conditions are shown in Figure 3B. Both the fixed and moving supporting plates bottom surfaces (1) were constrained in both X and Z axes. Surface (3) was constrained in the X axis. The load was applied on the surface (2) via kinematic coupling with a reference point constrained in all degrees of freedom except the X direction. The load was applied as a displacement-controlled boundary condition on the reference point. The surface-to-surface interaction was applied to the interface of silicone loading chamber and the polycarbonate plates and also between silicone and the collagen construct. For silicone-polycarbonate and silicone-collagen interactions, normal penalty hard contact and tangential static-kinetic exponential decay frictional contact was used.

Cell Culture, Construct Synthesis, and Bioreactor Loading Parameters

Human cardiomyocytes AC10, murine myoblasts C2C12, and murine osteoblasts OB6 were used to characterize the effect of the uniaxial tensile strain bioreactor on cell viability and proliferation. AC10s, C2C12s and OB6s were maintained in 1:1 ratio of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium (HyClone, GE Healthcare Bio-Sciences, Pittsburgh, PA) DMEM/low glucose (HyClone), and α-Minimum Essential Medium (αMEM, Life Technologies, Grand Island, NY), respectively, with each media was supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Gaithersburg, MD) and 1% Penicillin–Streptomycin solution (Life Technologies). Cellular 3D constructs were prepared by encapsulating each cell line within neutralized Collagen Type-I solution at 1×10^6 cells/mL seeding density. The collagen solutions

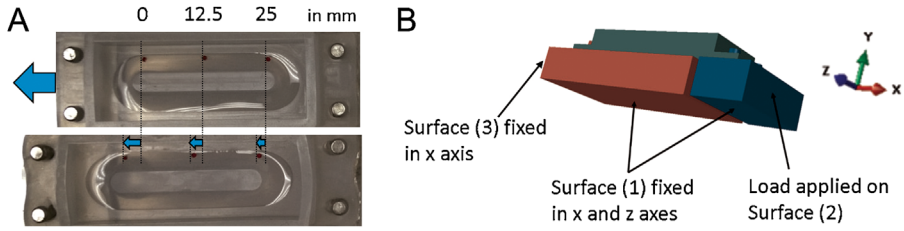

Figure 3. Experimental validation of the uniaxial tensile strain bioreactor and FEM generation. (**A**) Schematic of the placement of embedded markers and deformation measurement through image-based analysis using ImageJ for experimental validation of the uniaxial tensile strain bioreactor. (**B**) Schematic for the boundary conditions applied in order to generate the Finite Element Model for characterizing the strain and stress profiles experienced by the 3D collagen constructs when subjected to mechanical loading using the uniaxial tensile strain bioreactor.

were added into the groove of the silicone loading chambers, polymerized at 37°C for 1 h and incubated in their respective media for 48 h. The samples were then subjected to cyclic loading using the uniaxial tensile strain bioreactor at an applied load equivalent to 2% strain with 0.1 Hz loading frequency for 1 h/day for a period of 3 days. Constructs subjected to no loading (non-loaded) were used as control samples. Samples were harvested at the day 3, and the linear region of the construct was excised to conduct the biological studies.

Cell Viability and Proliferation

The cell viability within the constructs subjected to mechanical loading was assessed at the end of 3 days using Live-Dead Assay kit (Life Technologies). Calcein and Ethidium Homodimer-1 dyes at 1:2 ratio were incubated with the samples for 30 min at 37°C, and fixed with 4% paraformaldehyde (Sigma, St. Louis, MO). The constructs were subjected to confocal microscopy at 490/525 and 557/576 nm excitation/emission wavelengths for visualizing live and dead cells, respectively. DNA quantification was performed using PicoGreen ds DNA kit (Life Technologies) to indirectly determine the total number of cells within the loaded and non-loaded (control) samples. Cells were liberated from the collagen constructs by snap-freezing followed by mechanical disruption with a homogenizing pestle, resuspended in lysis buffer (50 mM Tris HCl, 1 mM $CaCl_2$, 400 μg/mL proteinase K, pH = 8) and incubated at 55°C overnight. The lysate was diluted 1:10 in TE buffer and mixed with 1:200 dilution of PicoGreen dye in 1:1 ratio and incubated at room temperature for 5 min. The fluorescence intensities of the samples were measured at 480/520 nm excitation/emission wavelengths using a microplate fluorometer (Wallac 1420). The total amount of DNA was determined using a standard curve generated with varying amount of DNA in ng and their corresponding fluorescence values.

Matrix Organization of 3D Collagen Constructs

The structural changes in the construct matrix due to mechanical stimulation was examined using the Scanning Electron Microscope (SEM). The cellular collagen constructs were fixed overnight with 4% paraformaldehyde; then the samples were sequentially dehydrated by incubating them for 15 min each in a series of ethanol/water gradients followed by hexamethyldisilazane/ethanol gradients ranging from 30% to 100%. The constructs were air dried overnight, sputter coated with gold, and visualized under SEM to observe the morphology and structure of the matrix.

Statistical Analysis

Four samples ($n = 4$) were used for experimental characterization of the uniaxial tensile strain bioreactor. Student t-test with a confidence interval of $P < 0.05$ was used to determine the statistically significant difference between two groups. The data is reported as ±standard deviation.

Results and Discussion

Uniaxial tensile strain bioreactors for mechanical loading of musculoskeletal cells have emerged to be an important tool for tissue engineering to study cellular biochemical responses and signaling pathways triggered by the synergistic effect of mechanical and micro-environmental cues (Berry et al., 2003; Brown et al., 1998; Seliktar et al., 2000; Skutek et al., 2001). However, most of the current custom-built and commercial bioreactors for loading 3D cellular constructs are limited by the generation of non-linear strain profiles over the length of the construct (Riehl et al., 2012; Youngstrom and Barrett, 2016). The bioreactors also tend to be complex in operation and maintenance and require handling of constructs which may risk the structural integrity of the construct (Altman et al., 2002; Garvin et al., 2003; Puk et al., 2006; Woon et al., 2011). In this study, we present a mechanical loading bioreactor that is straightforward in design and construction for the application of precise and homogenous uniaxial tensile strains to 3D cell-encapsulated constructs (Patent Application Number PCT/US17/20706). The bioreactor is programmed to operate at physiological loading strains (0–12%) and cycling frequencies (0.01–1 Hz) that mimic the in vivo environment of musculoskeletal tissues such as bone (Verbruggen et al., 2012), tendon (Wang, 2006), ligament, and skeletal muscle (Fukunaga et al., 2001) that experience uniaxial stretch on a daily basis (Trumbull et al., 2016). The main advantages of this uniaxial tensile strain bioreactor are that it is

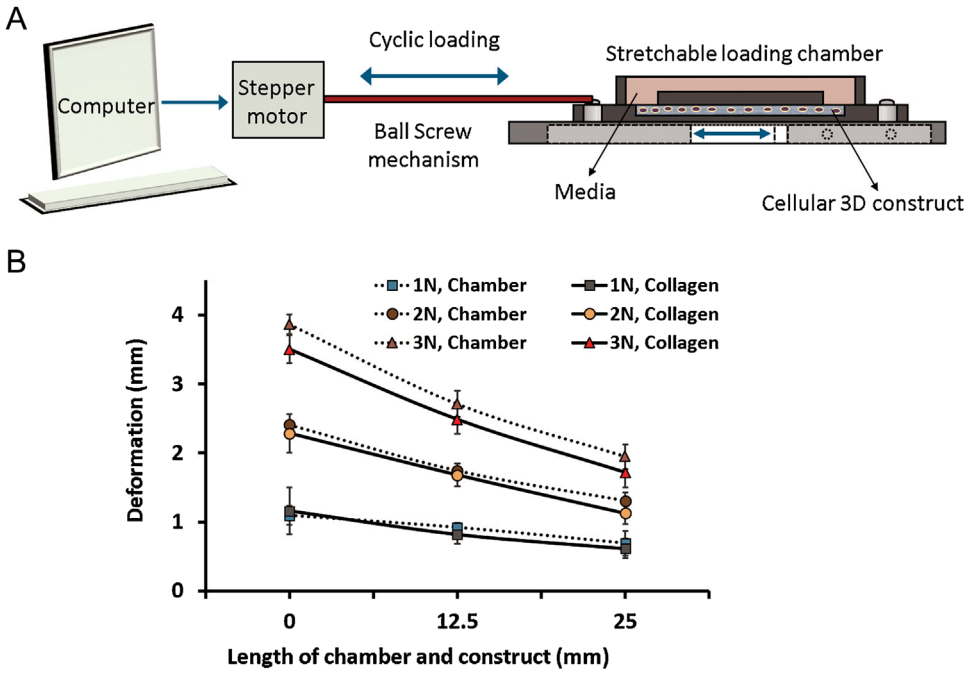

Figure 4. Operation and performance of the uniaxial tensile strain bioreactor. (**A**) Schematic of the major components involved during the operation of the bioreactor. A versatile and easy-to-use driver-controller system programmed through LabVIEW-based software produces specified rotation of the high torque 2-phase stepper motor which in turn rotates the miniature ball-screw actuation system that translates into the reciprocal movement of the moving plate, resulting in the stretching of cellular collagen constructs within the silicone loading chamber. (**B**) Experimentally determined deformation undergone by the linear region of the silicone loading chamber groove and collagen construct at the ends (0 and 25 mm) and center (12.5 mm) at applied loads of 1–3N. No significant difference seen between the deformation values obtained for silicone and collagen indicates that the applied load is being transferred effectively to the 3D collagen construct.

simple in set up, easy to operate and maintain, compact, and cost-effective when compared to existing bioreactors for mechanical stimulation of 3D collagen constructs.

Operation and Performance of the Uniaxial Tensile Strain Bioreactor

The uniaxial tensile strain bioreactor comprises of specifically-designed silicone loading chambers supported by a versatile actuation system comprising a high torque stepper motor coupled with miniature ball screw assembly (Fig. 4A). The loading chamber design eliminates the necessity for clamps or hooks for direct gripping of samples and minimizes the risk of construct disintegration. This is achieved by the groove-and-island configuration of the loading chamber (Fig. 2), with the collagen constructs secured in the groove and supported by the island to ensure that they get stretched along with the loading chamber in the direction of load. The LabVIEW-based software is used to give input commands to the motor controller and can be programmed for different waveforms of loading to produce precise and reproducible movement of the moving plate through the stepper motor-ball screw actuation system. The cell-encapsulated 3D constructs within the silicone loading chambers that are securely held by pins from the fixed and moving plate get stretched along with the reciprocal movement of the moving plate.

The uniaxial tensile strain bioreactor was first experimentally validated to determine whether this design of the loading chamber is able to effectively transfer the applied loads to the collagen constructs with no substantial strain attenuation. The deformation experienced at the defined points of 0, 12.5, and 25 mm in the linear region of the loading chamber (dotted line) and collagen construct (solid line) was captured and measured through image-based analysis and depicted in Figure 4B. No statistically significant difference was observed in the deformation experienced by the loading chamber versus the collagen constructs at any location along the linear length. This indicates that even without the use of anchor tabs, hooks, or clamps the applied load is effectively being transferred from the loading chamber to the 3D collagen constructs. The groove-and-island design of the loading chamber is thus able to act as an indirect support for the construct to remain in place during mechanical loading and help preserve the structural integrity of the construct. Further, since the loading chamber is also the culture chamber for the samples, there are no procedures required to transfer the samples from culture conditions to loading fixtures, thus minimizing the handling labor, possible construct damage, and risk of contamination.

The bioreactor is capable of stretching four silicone loading chambers simultaneously in one loading regime, with two cellular constructs in each chamber. The loading chambers are mounted onto the bioreactor assembly within a laminar flow biosafety cabinet, thus ensuring aseptic culture techniques throughout the process. The uniaxial tensile strain bioreactor along with its electronic components can be placed in a

standard tissue culture incubator during operation, allowing long-term culture in a temperature and pH-controlled environment.

Bioreactor-Induced Longitudinal Tensile Strain Profile Within 3D Constructs

The performance of a mechanical loading bioreactor is largely influenced by design considerations like chamber design, clamping method, actuator system, and dimensions of the construct. Therefore, detailed characterization of its strain profile is required before it can be used for biological applications. To investigate the strain and stress profiles generated during loading with our custom-built uniaxial tensile strain bioreactor, an FE-based model was generated. The model was then validated using the experimental deformation data for the linear region of the loading chamber and collagen construct at loads of 1–3N shown in Figure 4B. The FEM-predicted values were found to be within ±10% accuracy when compared to the experimentally measured data (Supplementary Figs. S2 and S3).

The validated FEM was subsequently used to investigate the strain contours produced within the collagen construct. The longitudinal tensile strain acting parallel to the application of load was determined along the length (a–b), width (e–f), and thickness (g–h) of the linear part of the construct as shown in the schematic in Figure 5B. It is observed from Figure 5A that the predicted longitudinal tensile strain across the length of the construct is very uniform over a 15 mm length ranging from 5 mm (c) to 20 mm (d) of the length of the construct. At applied loads of 1–3N, the longitudinal tensile strain within the linear region of collagen construct (i) is relatively uniform at $2 \pm 0.11\%$, $4 \pm 0.19\%$, and $6 \pm 0.24\%$ over the length of 15 mm, respectively. The magnitude of strains from (c) to (a) and (d) to (b) are seen to increase from the uniform strain value by 1.2%, 1.5%, and 1.8%, respectively. This increase in strain at the ends, though not as amplified as direct gripping of samples, can be attributed to the change in geometry of the loading chamber and the presence of the island that acts as an indirect anchor for the 3D collagen constructs. Remarkably, the semi-circular regions of the construct (ii) do not experience abnormally high tensile strains that are usually associated with traditional gripping of constructs. Instead, the strain values go down to as low as 1% as they approach the ends of the whole construct. Figure 5C and D demonstrate that the longitudinal strain

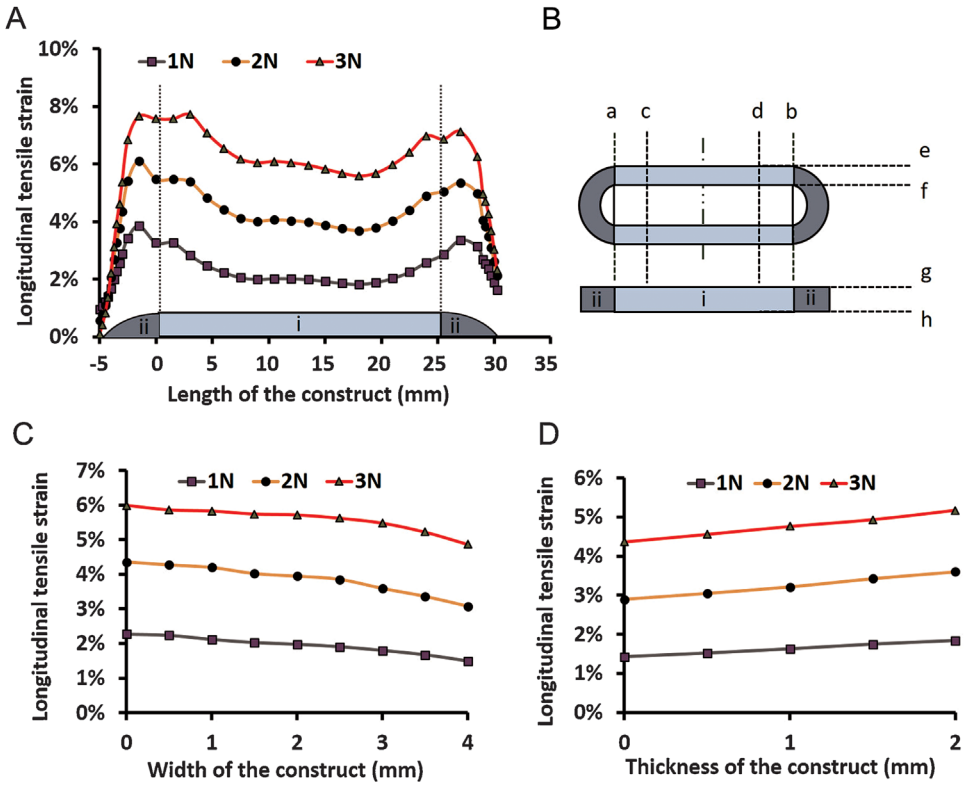

Figure 5. Bioreactor-induced longitudinal tensile strain profile within 3D constructs predicted using FEM. (**A**) Longitudinal tensile strain profiles over the total length of the construct at applied loads of 1–3N. Regions corresponding to the (i) linear and (ii) semi-circular parts of the collagen construct indicated in the schematic above the x axis. (**B**) Schematic of the direction of measurements of length (a–b), width (e–f), and thickness (g–h) across the collagen construct. (c–d) defines the homogenous tensile strain region. (**C**) Longitudinal tensile strain profiles across the width and (**D**) thickness of the collagen construct at applied loads of 1–3N. Strain experienced by the collagen construct is homogenous over the region of $15 \times 4 \times 2$ mm of the construct that is around 60% of the effective region of characterization. The applied loads of 1–3N correspond to 2%, 4%, and 6% linear strains. The strain profile near the gripped regions of the 3D construct (ii) shows decreased strain magnitudes when compared to the linear region of the construct, unlike the abnormally high strains usually generated with traditional gripping of constructs.

profile across the width and thickness of the construct is fairly homogenous for each applied load. The strain contours across both width and thickness of the construct are within ±0.2–0.3% of the uniform strain values 2%, 4%, and 6% for applied loads of 1–3N, respectively. The decrease of 0.5% observed near the line (f) for the strain magnitudes estimated across the width can be attributed to the presence of the island that resists the load. Based on these contours, it can be concluded that the region of $15 \times 4 \times 2$ mm ranging from the line (c) to line (d) achieves uniform longitudinal tensile strain profile. This region with a volume of 120 μL is estimated to be around 60% of the linear part of the 3D collagen construct and can thus be defined as the region of interest for biological studies.

Lack of focus on detailed characterization of strain profiles in the published articles employing such devices makes it difficult to evaluate the performance our custom-built bioreactor with respect to existing alternatives (Riehl et al., 2012). Nevertheless, it has been noticed that custom-built bioreactors for that use hooks or punch holes to grip collagen sponge-based constructs exhibit an uneven distribution of strain profile with a high risk of construct disintegration (Chen et al., 2010; Juncosa-Melvin et al., 2006). Flexcell® Uniflex® 2D system has its estimated homogenous strain region on the membrane to be a 140 mm^2 rectangular region at the center, which is only 47% of the effective area of characterization (Matheson et al., 2006). However, the strain profile for the Tissue Train 3D Culture system is not described in detail in the published literature (Garvin et al., 2003).

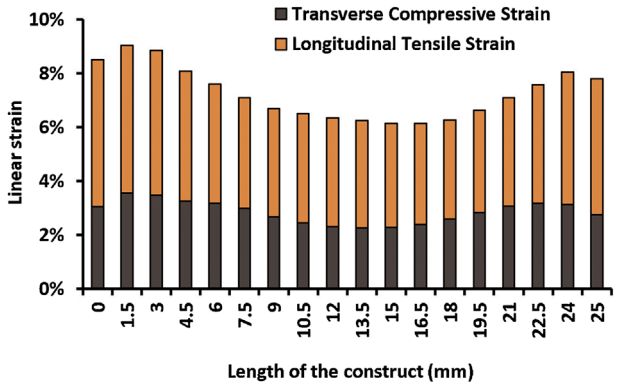

Figure 6. Bioreactor-induced tensile and compressive linear strains within 3D constructs predicted using FEM. Comparison of the longitudinal tensile strain and transverse compressive strain experienced by the collagen construct across its length on subject to a uniaxial tensile load of 2N. Transverse compressive strains account for only 33% of the total strain experienced by the construct. The homogenous tensile strain across the $15 \times 4 \times 2$ mm region is predominantly "uniaxial" in nature.

Bioreactor-induced Transverse Compressive Strain Within 3D Constructs

In addition to longitudinal tensile strains, uniaxial loading also produces a transverse compressive strain that acts perpendicular to the direction of load application due to Poisson's effect. Hence, the magnitudes of longitudinal and transverse linear strains produced by the bioreactor were identified to determine the dominant strain experienced by the 3D collagen constructs. Figure 6 shows the ratio of transverse compressive strain in comparison to the longitudinal tensile strain experienced by the length of the collagen construct at an applied load of 2N. It is evident that the transverse compressive strain is approximately $33 \pm 0.4\%$ of the total strain experienced by the construct in the region of homogenous longitudinal tensile strain. At the center of the construct with a uniform tensile strain of 4%, the predicted transverse compressive strain is 2.23%. This implies that though the strain experienced by the construct is not purely uniaxial, the transverse strains are significantly and consistently lower than the longitudinal strains along the length of the region of interest of the construct. These results indicate that the volume of $15 \times 4 \times 2\,mm^3$ of the 3D construct experiences homogenous tensile strains majorly acting in the longitudinal direction and hence can be considered "uniaxial" in nature. The bioreactor thus can be used to study the effect of uniform and predominantly uniaxial longitudinal tensile strains on viability, proliferation, differentiation, and morphological response of cells. The presence of transverse compressive strain renders the bioreactor unsuitable for studying the solo effect of tensile strains on cellular mechanobiology. However, there is evidence that fibroblasts respond similarly to pure uniaxial strain versus predominantly uniaxial strain, which suggests that low transverse compressive strains seem to have a negligible effect on cells responsive to longitudinal stretch (Lee et al., 1999).

Bioreactor-Induced Strain and Stress Profiles Within 3D Constructs During Cyclic Loading

Since tissues under in vivo mechanical loading exist in dynamic conditions, we next examined the performance of the uniaxial tensile strain bioreactor by subjecting the 3D collagen constructs to cyclic loading using the validated FEM (Supplementary Fig. S4). Cyclic loading at different frequencies was simulated using triangular waveform, and the longitudinal strain profile was recorded for an applied load of 2N (Fig. 7A). The strain contours clearly demonstrate that there is negligible difference in the strain values along the length of the construct with varying frequencies, implying that the bioreactor is stable over different cyclic loading regimes. The effect of cycle numbers on the stress and strain profiles was studied by simulating cyclic loading at 0.5 Hz for a total of 40 cycles. Figure 7B and C represent the longitudinal tensile strain and von Mises stress profiles obtained during cyclic loading, at cycle number 1 versus cycle number 40 at the applied loads of 1N, 2N, and 3N. No significant differences are observed in the homogeneity of the strain profiles between cycle 1 and cycle 40 (Fig. 7B). The strain and stress profiles at cycle 40, in fact, look more homogenous when compared to cycle 1, along with lowered stress concentration at the ends of the linear region of the construct at higher cycle number (Fig. 7C). The uniaxial tensile strain bioreactor is thus not only capable of applying homogenous strain to the linear region of the collagen construct but is also able to consistently reproduce the uniformity in strain and stress profiles during each cycle of loading.

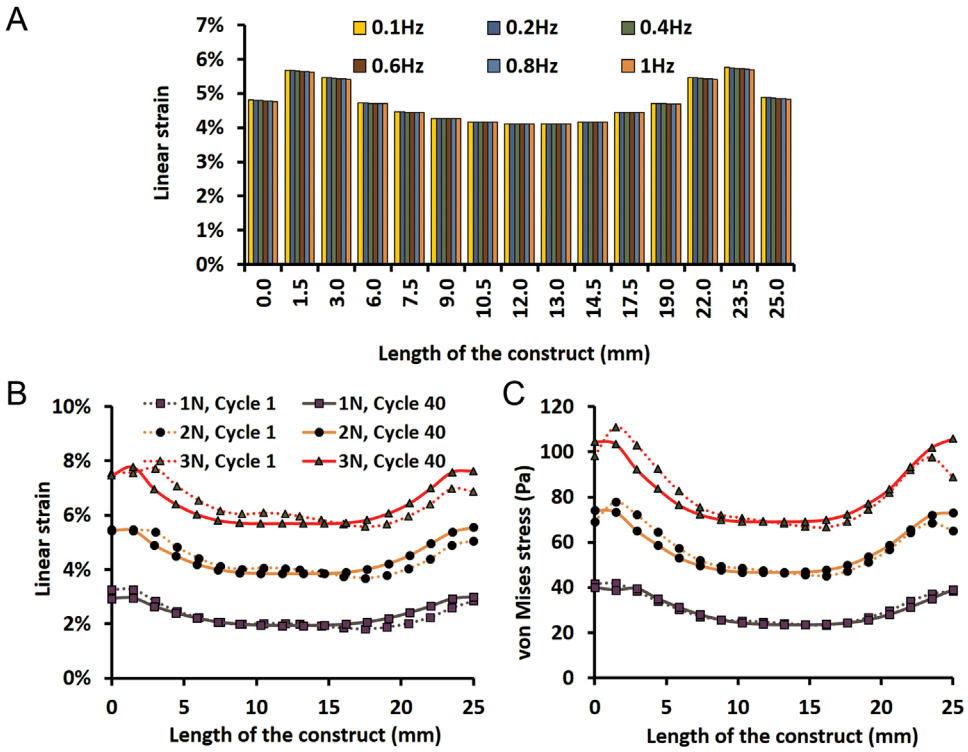

Figure 7. Bioreactor-induced strain and stress profiles within 3D constructs during cyclic loading predicted using FEM. (**A**) Strain distribution profiles generated by the bioreactor at loading frequencies of 0.1, 0.2, 0.6, 0.8, and 1 Hz across the length of the construct for an applied load of 2N. No significant difference seen in the strain magnitudes with change in loading frequencies. (**B**) Comparison of profiles at Cycle 1 versus Cycle 40 during cyclic loading of collagen constructs at 0.5 Hz loading frequency in terms of longitudinal tensile strain, and (**C**) von Mises stress, across its length at applied loads of 1–3N. The strain and stress profiles smoothen out at the ends with progression in cycle numbers, and the magnitude of both stresses and strains in the region of homogenous strain remain fairly constant.

Effect of Bioreactor on Cell Viability and Matrix Organization Within 3D Constructs

After characterizing the performance and reproducibility of the uniaxial tensile strain bioreactor through Finite Element Analysis, we performed basic biological assays to determine the effect of uniaxial tensile strains on cell viability, proliferation, and matrix structure using cell lines belonging to different musculoskeletal lineages. Figure 8A shows representative images of live-dead assay performed for loaded and non-loaded constructs seeded with OB6, C2C12, and AC10 cells using confocal microscopy. The results indicate that there is no significant difference in cell viability between the control and loaded constructs in all three sample sets (supported by Supplementary Table S3), confirming that the uniaxial tensile strain bioreactor is not causing cytotoxicity to cell seeded within the constructs. The PicoGreen DNA quantification data (Fig. 8B) shows a 2.5-fold increase in the amount of DNA for OB6, twofold increase in C2C12, and almost a fourfold rise in AC10 for both control and loaded samples compared to the initial DNA amount of 4,500 ng, establishing that not only are the loaded cells viable, but they are metabolically active and proliferating within the construct. The increased number of dead cells seen in the case of AC10 cells within both control and loaded samples can be attributed to overcrowding due to the fourfold increase in cell density that would have led to subsequent cell death.

The 3D cell-encapsulated collagen constructs visualized through SEM displays an increased level of matrix organization and structure within the construct (Fig. 9). Compared to the non-loaded samples, the loaded samples show a definitive orientation, with matrix organization observed to be predominantly parallel to the axis of load application in all the three groups. The aligned collagen fibers obtained for the loaded samples conform to studies that reported directionality of collagen fibers due to a combination of strain and cell-mediated matrix compaction (Elsaadany et al., 2017a; Isenberg and Tranquillo, 2003; Sander and Barocas, 2008). Interestingly, rounded cell morphology is observed in the live-dead images though the matrix shows prominent alignment (Fig. 8A). This can be attributed to a possible time lag between strain-induced matrix anisotropy and its influence on cellular orientation and morphology. Thus the period of 3 days might not have been sufficient for the cells show a response to the change in their matrix environment. Nevertheless, the biological characterization data demonstrates the ability of the bioreactor to maintain cell viability and influence matrix alignment in 3D collagen constructs encapsulated with various musculoskeletal cell lines.

In conclusion, this simple and cost-effective bioreactor can be operated at various physiological loading strains (0–12%) and frequencies (0.01–1 Hz) to apply precise and homogenous tensile strains over 60% of the effective region of the cellular 3D collagen construct, with the strain being predominantly uniaxial in the

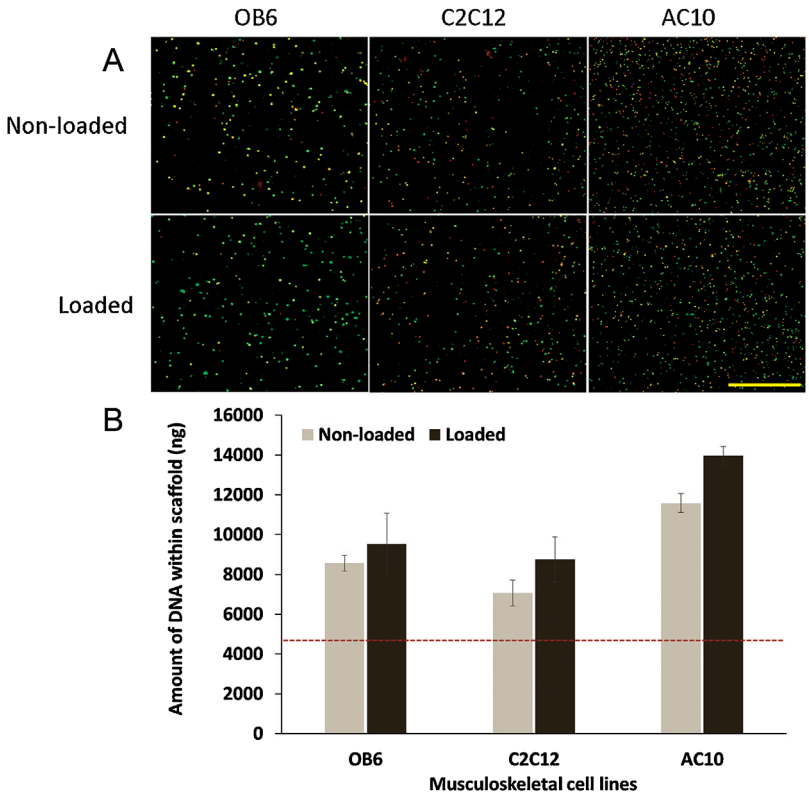

Figure 8. Effect of bioreactor on cell viability and proliferation within 3D constructs through experimental determination. (**A**) Loaded (2% strain, 0.1 Hz, 1 h/day) and non-loaded 3D collagen constructs encapsulated with OB6, C2C12, or AC10 cells were visualized under confocal microscope at day 3. Green color represents live cells while red indicates dead cells. Scale bar is 100 μm. (**B**) DNA quantification of loaded and non-loaded cells-seeded inside collagen constructs at day 3 using PicoGreen assay. The red dotted line represents the initial cell density within the scaffolds. The results show no significant differences in both the cytotoxicity and cell proliferation of loaded constructs compared to the control.

longitudinal direction. The strain profiles obtained are consistent and stable during cyclic loading conditions, and the bioreactor is able to promote cell viability, proliferation, and matrix organization of the cell-encapsulated constructs. The bioreactor presented in this study is tailored towards investigating signaling pathways, and biochemical expression underwent by cells encapsulated in a 3D microenvironment. This bioreactor, however, has the potential to be expanded to other applications including tissue engineering strategies and can be modified to load larger and longer constructs due to its excellent tensile strength of the loading chamber material,

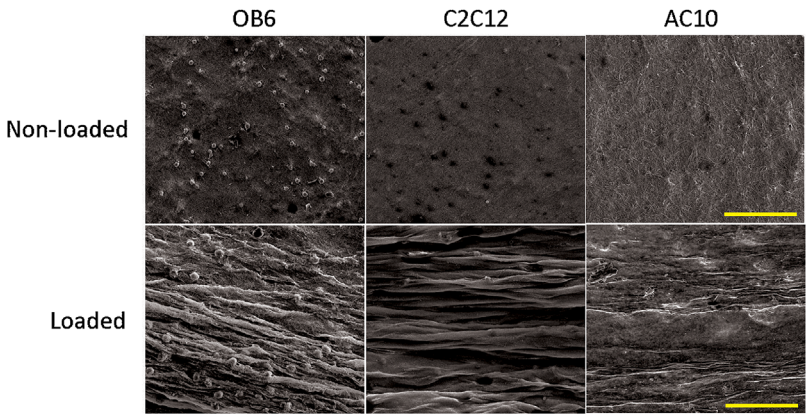

Figure 9. Effect of bioreactor on collagen matrix organization within cell-encapsulated 3D constructs through experimental determination. Scanning Electron Micrographs of the loaded (2% strain, 0.1 Hz, 1 h/day) and non-loaded cell-encapsulated 3D collagen constructs at day 3. Scale bar is 100 μm. Matrix organization is visible in all loaded samples, with the orientation of the fibers being parallel to the axis of load application.

the flexibility in the design configuration of the chamber, and performance of the driving mechanism.

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