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Sequential Multimodal Microscopic Imaging and Biaxial Mechanical Testing of Living Multicomponent Tissue Constructs

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Abstract

Understanding relationships between mechanical stimuli and cellular responses require measurements of evolving tissue structure and mechanical properties. We developed a 3D tissue bioreactor that couples to both the stage of a custom multimodal microscopy system and a biaxial mechanical testing platform. Time dependent changes in microstructure and mechanical properties of fibroblast seeded cruciform fibrin gels were investigated while cultured under either anchored (1.0:1.0 stretch ratio) or strip biaxial (1.0:1.1) conditions. A multimodal nonlinear optical microscopy-optical coherence microscopy (NLOM-OCM) system was used to delineate noninvasively the relative spatial distributions of original fibrin, deposited collagen, and fibroblasts during month long culture. Serial in-culture mechanical testing was also performed to track the evolution of bulk mechanical properties under sterile conditions. Over the month long time course, seeded cells and deposited collagen were randomly distributed in equibiaxially anchored constructs, but exhibited preferential alignment parallel to the direction of the 10% stretch in constructs cultured under strip biaxial stretch. Surprisingly, both anchored and strip biaxial stretched constructs exhibited isotropic mechanical properties (including progressively increasing stiffness) despite developing a very different collagen microstructural organization. In summary, our biaxial bioreactor system integrating both NLOM-OCM and mechanical testing provided complementary information on microstructural organization and mechanical properties and, thus, may enable greater fundamental understanding of relationships between engineered soft tissue mechanics and mechanobiology.

Keywords

Biomechanics; NLOM-OCM imaging; Fibrin; Engineered tissue

INTRODUCTION

Many soft connective tissues, ranging from skin to arteries, experience multiaxial mechanical loading *in vivo* and exhibit diverse responses to changes in these loads,

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DISCLOSURE

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including growth and remodeling, injury, and repair. These responses are mediated by resident and recruited cells through proliferation, migration, and apoptosis as well as through changes in matrix traction, organization, deposition, or degradation, all of which may alter the cellular mechanical environment and, in turn, elicit further cellular responses. This dynamic and reciprocal interaction between adherent cells and matrix has been proposed to establish, maintain, or restore a mechanobiological equilibrium or homeostasis (e.g., tensional⁴). Indeed, mechanical conditioning of tissue engineered constructs has been used to promote seeded cell responses that generate tissues having more physiological properties than static loads alone, with the ultimate goal that these constructs emulate and integrate within native tissues.⁵ Yet, understanding relationships between mechanical environments and cellular responses (i.e., the mechanobiology) and using these relationships to guide tissue morphogenesis in culture remain challenging.

One approach to understanding relationships between tissue mechanics and cellular responses is simply to study responses of cell-seeded tissue constructs cultured under different mechanical conditions. Different platforms have been developed that can characterize tissue mechanical properties uniaxially or biaxially as well as the underlying microstructural organization.^{10,21,25,28–32,34} Several microscopy techniques have been used to characterize tissue construct microstructure. For example, laser scanning confocal microscopy utilizing image stacks has provided three-dimensional renderings of tissue architecture with micrometer resolution, but relies on fluorescently tagged protein(s).^{12,15} Confocal reflectance microscopy¹⁶ and even electron microscopy³³ have also yielded insight into the microscopic organization of cultured gels. Yet, the need for exogenous labels or the lack of chemical specificity can complicate serial measures of tissue microstructure and the interpretation of microscopic events, such as deposition of new collagen matrix within an existing fibrin network.³⁶

To characterize mechanical properties of constructs *via* mechanical testing, applied loads are obtained from standard transducers and resulting deformations are typically monitored by tracking multiple fiducial markers. The requisite calculation of stress from such data need not be straightforward, however. Stress is defined as an applied force acting on an undeformed (the first Piola–Kirchhoff stress) or current deformed (the Cauchy stress) oriented area. To calculate area, it is the measurement of engineered tissue construct thickness that emerges as a significant challenge, particularly if it is to be measured serially during an extended experimental protocol. For nearly incompressible native tissues, thickness may be predicted under mechanical testing. Nevertheless, even with well approximated strain distributions, calculated material properties of nearly incompressible native tissues have been found to depend strongly on the assumption of incompressibility.⁷ In contrast, for soft engineered tissue, especially at early stages, the solid fraction of fibers is low and the fluid volume fraction is high, thus resulting in more ability for fluid movement out of the tissue construct during testing and therefore more compressibility. Several methods have been reported to measure thickness, typically at the end of an experiment in conjunction with biochemical and ultrastructural analyses. These methods include the use of a low force probe³³ or laser displacement system in which a small reflective disk is placed on the sample and its height recorded.³ Longitudinal measures of tissue construct thickness

have included common bright field microscopy, which suffers from poor resolution especially for measuring changes in thickness of tens to hundreds of micrometers.³⁶

Here, we describe a biaxial bioreactor which was designed to culture cruciform-shaped constructs under well-defined mechanical stretch. With this bioreactor we performed longitudinal nondestructive force–extension tests on these constructs and characterized both overall thickness and microstructural composition and organization noninvasively using a multimodal nonlinear optical microscopy-optical coherence microscopy (NLOM-OCM) system.³⁷ Fibroblast seeded fibrin gels were investigated through 31 days. NLOM-OCM was used to delineate relative spatial distributions of collagen, fibrin, and fibroblasts noninvasively using endogenous contrast. Additionally, with the appropriate objective, the integrated imaging system complemented biaxial mechanical testing by measuring construct thickness as a function of stretch as needed to compute Cauchy (true) stress.

MATERIALS AND METHODS

Cell Culture and Tissue Constructs

Neonatal human dermal fibroblasts (NHDF) from ATCC (Manassas, VA) were transfected with GFP-vinculin plasmid.²³ Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% bovine serum and 1% antibiotic–antimycotic solution (Sigma). Cells were passaged near confluency and harvested for experiments from passages 5 to 12.

Fibrinogen stock solution was formed with bovine fibrinogen (Sigma) placed in DMEM, supplemented with 1 mg/mL of ϵ -amino caproic acid (ϵ -ACA; Sigma), an inhibitor of plasmin activity (which degrades fibrin).²⁴ Fibrinogen solution was filtered, then mixed with 5% fetal bovine serum (FBS) and thrombin (25 U/mL; Sigma) at a ratio of 1:0.02:0.002. The solutions were then mixed thoroughly with a suspension of cells and 100 μ m diameter black polyethylene microspheres, which served as fiducial markers for strain measurements (Cospheric, CA). The final densities of fibrin and cells were 5 mg/mL and 2×10^6 /mL, respectively. All mixtures were poured into a cruciform silicone mold placed within a glass Petri dish. Customized bars of porous polyethylene (Small Parts, FL), each punctured with two holes by which the gel was anchored in the bioreactor (see description of tissue culture device), were positioned at each end of the four arms of the cruciform mold (end-to-end length and arm width of 73 and 20 mm, respectively) before adding gel solutions. Cell seeded fibrinogen-FBS-thrombin solution was added to the mold and left undisturbed for 60 min in a humidified CO₂ incubator at 37 °C during which suspended microspheres sank to the gel solution bottom. Following gelation, the construct was flipped and coupled to the load arms within the bioreactor *via* the four embedded porous polyethylene bars. The density of the microspheres was 3/mm² and limited to the surface of the tissue construct. One of two static biaxial stretches ($\lambda_1:\lambda_2$) was applied to individual constructs, 1.0:1.0 stretch (anchored) or strip biaxial at 1.0:1.1 stretch. Each stretch protocol was performed on three constructs ($n = 6$ specimens total, each for 31 days). Central regions of the constructs were characterized by NLOM-OCM over the month long culture. On days of NLOM-OCM imaging, tissue constructs were also tested biaxially. Bioreactors were kept in a humidified CO₂ incubator at 37 °C except during imaging and mechanical testing. Medium supplemented with 10% fetal bovine serum, L-ascorbate, and TGF- β (BD Biosciences) was

used to support collagen synthesis and deposition.¹⁴ On Day 31, the last day of culture, after biaxial mechanical testing, medium was replaced by deionized water to decellularize the constructs and mechanical testing repeated to delineate the contributions of resident cells.

Biaxial Tissue Culture Device

To enable serial in-culture mechanical testing and imaging without compromising sterility, our previous bioreactor¹⁷ was refined with several modifications that follow our initially proposed design.¹⁹ First, an immersible 50 g force transducer (Honeywell) was integrated in line on each axis by a polycarbonate adapter (Fig. 1). All parts were assembled, immersed within 70% ethanol, and air dried before use. In this way, serial mechanical tests were performed directly within the sealed bioreactor during culture, avoiding possible damage or contamination by transferring to a separate mechanical testing device. Second, a polycarbonate lid with a center hole sealed with optical quality glass was used during tissue culture and for viewing tissue embedded microspheres during mechanical testing. Third, a different, interchangeable polycarbonate lid fitted with a window assembly was used during in-culture microscopic imaging (Fig. 2). The window assembly consisted of a plastic bushing, glass tube with o-ring and coverslip bottom, and glass adapter (Fig. 2a). The position of the glass tube with the coverslip bottom was adjusted to contact the tissue construct during microscopic imaging. The completed assembly is shown in Fig. 2b. Compression of the glass tube o-ring formed a seal with the glass adapter, and the bushing was glued into the lid to complete the seal. The lids were sealed with silicone rubber gaskets and secured on top of the bioreactor chamber by screws and switched under aseptic conditions in a class II biosafety cabinet. Two Millex filter units (0.2 μm pore size) were incorporated into the polycarbonate lid for sterile air exchange. Finally, silicone rubber was used to seal the sleeve bearing through which stainless steel rods traversed the bioreactor wall and coupled with the tissue construct. This additional layer of silicone rubber was enough to prevent sample contamination previously occurring following repeated mechanical testing. With these key improvements, tissue constructs could be cultured for at least a month and subjected to repeated microscopic imaging and mechanical testing without contamination.

Integrated NLOM-OCM Imaging System

Our custom NLOM-OCM system has been described previously.^{2,37} Briefly a titanium:sapphire oscillator generating sub-10-femtosecond pulses with central wavelength at 800 nm and 130 nm full-width-at-half-maximum (Femtosome, Femtolasers) was used as a common source for both NLOM and OCM. The integrated NLOM-OCM system utilized a Michelson interferometer configuration where one arm was directed to the tissue construct for both NLOM and the sample arm of OCM while the other was used as the reference arm for OCM. NLOM signals generated from the sample were directed to a two-channel detector for image rendering at two different wave-lengths for second harmonic generation (SHG) in collagen and two-photon excited fluorescence (TPF) in cells. Backscattered laser light from the sample was combined with the return beam of the reference arm and directed to a custom spectrometer for Fourier-domain OCM. Integration of NLOM and OCM enabled microscopic tissue characterization using simultaneously acquired imaging signals that are both constituent specific (nonlinear optical signals) and non-specific (backscattered laser

light). For thickness measurements, a low numerical aperture objective provided a long depth of focus to render cross-sectional images by optical coherence tomography (OCT).

Representative SHG and OCM images of acellular collagen gels (3.5 mg/mL) are shown in Fig. 3a, acquired from 50 and 1500 μm below the surface. At 50 μm , co-registered SHG and OCM images revealed collagen fibers with high contrast relative to background. Images acquired at 1500 μm displayed greater loss of signal in SHG compared with OCM. This loss of signal was evident when plotting normalized (signal) intensity as a function of depth from the surface (Fig. 3b). In this plot, SHG and OCM intensity images at each depth were summed and normalized to total intensity at surface.

Image Processing and Analysis for Collagen Deposition in Fibrin Gel

During imaging, three NLOM-OCM image stacks were acquired from the central region of engineered constructs on Days 1, 3, 10, 17, 24 and 31. Fiber orientation was analyzed by a Matlab routine based on a 2D fast Fourier transform (FFT) algorithm. The angular distribution was obtained by polar coordinate analysis of the filtered power spectrum image. Briefly, the relative intensity (RT) for angles between Θ and $\Theta + 4^\circ$ was calculated by,

$$RT(\Theta) = \frac{\sum_{\theta \geq \Theta}^{\theta < (\Theta + 4)} g(r, \theta)}{\sum_{\theta \geq 0}^{\theta < 180} g(r, \theta)},$$

where $g(r, \theta)$ is the gray-scale level of a pixel at the polar coordinate in the filtered power spectrum image. An alignment index (AI) was then used to quantify distributions of collagen fiber orientations and to enable statistical comparisons among different treatments, that is, the fraction of collagen fibers lying within 20° of the predominant direction normalized by that of a random distribution (= 0.22); values of AI range from 4.55 for strong alignment (i.e., parallel fibers) to 1.00 for random alignment.¹⁷

Biaxial Mechanical Testing

A custom computer-controlled biaxial loading platform was used to mechanically test tissue constructs within the bioreactor.¹⁹ During testing, the bioreactor was placed on the loading platform and each of the four load bars was attached to a high-resolution (0.01 μm) stepper motor (Parker, OH) located at a corner of the square platform. Stepper motors were controlled using a high-speed Labview interface *via* feedback from either the load cells (for load-controlled tests) or positions of microspheres embedded within the (surface of) gel (for strain-controlled tests). To compute strains, a CCD camera mounted above the bioreactor and positioned over the glass sealed central hole of the bioreactor lid was used to monitor positions of at least four locally embedded microspheres. Images were captured at 30 Hz *via* a video frame grabber board, which digitized the image in a 512×512 pixel matrix. For each capture (i.e. every 1/30 s), a search algorithm based on pixel intensity located the approximate centroid of each marker. The software computed components of the 2-D deformation gradient tensor by comparing current marker positions, given by pixel coordinates, to reference positions and by using bilinear isoparametric interpolation. Force and displacement data were recorded along with corresponding video images of the constructs. The constructs were tested quasi-statically. For each step, the change of strain

was ~0.5% over 5 s, with more than 15 s required to image and track beads, compute deformations, and process feedback control before the next step. Mechanical testing data from the bioreactor are shown in Fig. 4 for a fibroblast seeded collagen-fibrin construct preconditioned through 3 cycles of 15% equibiaxial stretch followed by three different stretch protocols (Fig. 4a), namely, (1) *Y*-direction strip biaxial, (2) equibiaxial, and (3) *X*-direction strip biaxial stretch. The orthogonal direction to applied stretch in cases (1) and (3) was fixed during testing. The load-stretch curves of strip biaxial (stretch protocol 1) and equibiaxial testing (stretch protocol 2) are shown in Figs. 4b and 4c, respectively.

Cross-Sectional (B-Scan) Images of Tissue Constructs by OCT

The intrinsic optical sectioning capability of sub-10-fs pulse ultrashort coherence length was used also to render cross-sectional images for measurements of tissue thickness. OCM en face image acquisition was switched to cross-sectional (B-scan) OCT with use of a low numerical aperture objective (1× Achroplan, 0.025 NA) to provide a long depth of focus of 1.5 mm. Cross-sectional OCT images were rendered from a sequence of axial reflectance depth profiles (A-line) by laterally scanning the laser focus across the tissue.²² Each A-line was acquired in a single shot in the Fourier domain (FD)⁹ without scanning a reference mirror, thus eliminating a source of noise and greatly increasing imaging speed. In FD-OCT, all scatterers in an A-line are measured simultaneously and contribute to the interference signal. This interference signal was measured in the FD by a custom-built spectrometer. Primary components of the spectrometer were a diffraction grating (NT43–211, Edmund Optics, Barrington, NJ) and a 2048-pixel line scan camera (L104–2k, Basler, Exton, PA).³⁷ Each A-line contributed to a B-scan image by Fourier transform of the spectrum of the interferogram. Image data were acquired and averaged five times for increased signal-to-noise ratio (SNR).

Construct thicknesses were measured *via* OCT at different stretches immediately following the cyclic mechanical tests. That is, constructs were stretched to preset biaxial values, fixed in the deformed configuration by set screws on the loading arms, transferred to the microscopy stage for OCT imaging, placed back on the mechanical testing rig, and returned to the original configuration before stretching to the next biaxial value for thickness imaging.

Representative cross-sectional images from a construct are shown at select stretch ratios in Figs. 5a–5d. Average thicknesses obtained from the OCT cross-sectional images are shown in Fig. 5e as a function of stretch ratio. Construct thickness exhibited significant deviation from the incompressible limit (i.e., $\lambda_3 = 1/\lambda_1\lambda_2$; where 3 is the out of plane or *z* direction), which is shown for reference (dotted line). The deviation in thickness from the incompressible limit was exhibited by all tissue constructs. Thus, Cauchy stress was calculated herein using measured thicknesses as a function of stretch without assuming construct incompressibility.

Stress Calculation

Though “load” (g) is an important measure of mechanical responses, stress is a preferred derived quantity that can reveal tissue mechanical properties such as material stiffness. Two

definitions of stress are particularly useful, the first Piola–Kirchhoff (or nominal) and the Cauchy (or true) stress. The former is useful experimentally and the latter is useful analytically. The first Piola–Kirchhoff stress, P , is a measure of applied force acting on an undeformed oriented area and is, therefore, convenient to measure. Assuming that loads are transferred uniformly to the central region of the cruciform-shaped gel, the first Piola–Kirchhoff stress is given by¹⁸

$$P_{11}^{\text{central}} = \frac{f_1}{L_2 H}, \quad P_{22}^{\text{central}} = \frac{f_2}{L_1 H},$$

where f_1 and f_2 are measured applied forces in the x and y directions, L_1 and L_2 are the lengths of the central region in the undeformed configuration over which the forces act (here, $L_1 = L_2 \approx 2$ cm), and H is the original thickness (~ 460 μm). Cauchy stress, t , is a measure of applied force acting on a current deformed oriented area. If one can assume incompressibility during transient loading, which holds for many native tissues,⁷ calculation of Cauchy stress is straightforward. In tissue engineered constructs, however, calculation of Cauchy stress, t , in planar samples requires the more problematic measurement of tissue thickness, which may evolve due to cell-induced compaction or change with load-induced stretch as shown in Fig. 5. Thus, Cauchy stress calculated for tissue constructs incorporated actual stretch dependent thickness measurements (current deformed thickness). In the compressible regime, Cauchy stress may be calculated as,

$$t_{11}^{\text{central}} = \frac{f_1}{\lambda_2 L_2 h}, \quad t_{22}^{\text{central}} = \frac{f_2}{\lambda_1 L_1 h},$$

where λ_1 and λ_2 are in-plane stretch ratios, and h is the current deformed thickness retrieved from OCT images.

RESULTS

NLOM-OCM images are shown in Fig. 6 for the central region of anchored constructs on Days 1, 3, 10, 17, 24 and 31. TPF images (Fig. 6, column a) showed initially round fibroblasts (Day 1) that quickly developed an elongated shape and extended processes (Day 3). Note, too, that the number of cells gradually increased in the field of view coinciding with an increase in cell density. Images rendered from SHG (Fig. 6, column b) showed a deposition of collagen that accumulated over time. Overlay images of TPF and SHG (Fig. 6, column c) are false colored green and red, respectively, to reveal the relative distribution of cells and deposited collagen, much of which appeared to be aligned with the cell bodies. OCM images (Fig. 6, column d) suggested distinct fibrin fiber and cell morphologies on Day 1, but at later times, OCM image contrast was dominated by high fibrin density resulting from gel compaction.

NLOM-OCM images from the central region of constructs cultured under a strip biaxial stretch (1.0:1.1) condition are shown in Fig. 7. The primary stretch axis was oriented vertically in the images. Consistent with the anchored constructs shown in Fig. 6, TPF

images (Fig. 7, column a) revealed that cell morphology was initially rounded on Day 1, but elongated with extended processes by Day 3. Of note, the elongated cells appeared to align preferentially with the direction of principal stretch. SHG images (Fig. 7, column b) indicated an accumulation of collagen preferentially oriented along the axis of principal stretch. Overlay images of TPF and SHG images (Fig. 7, column c), false colored green and red, respectively, again revealed that collagen deposition aligned with cell bodies. Also, similar to anchored gels (cf. Fig. 6), OCM images (Fig. 7, column d) were dominated by the dense fibrin matrix (over the course of 24 h, the gels thinned from ~1 to ~0.1 mm, regardless of boundary conditions).

NLOM-OCM images from three stacks from the central region of the constructs acquired at six different days during month long culture were analyzed to quantify evolving matrix fiber orientation. Distributions of angles are shown in Fig. 8 for anchored (column a) and strip biaxial stretched constructs (column b). Recall that OCM images were used to calculate fibrin fiber orientations whereas SHG images were used to calculate deposited collagen fiber orientations. Because the OCM images revealed isotropic distributions of fibrin from Days 1 to 31, data are shown only for Day 1.

Alignment indices on Day 1 were 1.0948 and 1.1132, for the anchored and strip biaxially stretched constructs, respectively. In the anchored constructs, fibrin fiber isotropy was mirrored by an isotropic (random) cell orientation that also resulted in an isotropic distribution of collagen fibers. The latter was reflected by small AI values (AI ~ 1.2179) (see Fig. 8a). In contrast, in strip biaxial stretch cultures, the 10% stretch in one direction was enough to induce cell alignment and a consequent anisotropic collagen deposition as early as Day 3, with AI = 1.2933 (predominant fiber angle coincident with principal stretch axis at 0°) even though fibrin fiber angles remained isotropically distributed on each day. This anisotropic distribution of collagen fiber angle distribution sharpened with culture time along the principal stretch axis, with increasing AI = 1.5638 and 1.7431 on Days 10 and 17, respectively. This high collagen anisotropy remained from Day 17 to 31 with AI values ranging from 1.6366 to 1.7601. Results from all samples cultured under anchored ($n = 3$) and strip biaxial conditions ($n = 3$) are summarized in Table 1 listing mean thickness and AI with standard deviation.

Tissue constructs were also subjected to equibiaxial stretch mechanical testing over the month long culture period. Cauchy stress was plotted as a function of stretch to assess evolving mechanical properties. Longitudinally measured mechanical response curves are shown in Fig. 9 for a construct cultured under strip biaxial stretch (1.0:1.1). On the last day (Day 31), an additional mechanical test was performed after removing the cells. Raw data from three cycles are shown for each axis on each plot after correcting for construct thinning with stretch. Mechanical response curves exhibited viscoelasticity (i.e., marked hysteresis, or energy dissipation), an increase in load bearing with culture time, and an initial quasi-linear response that evolved into nonlinear stress-stretch relationships that were particularly evident after 10 days in culture. As an indicator of increased load bearing with culture time, stress to induce 7.5% stretch increased an order of magnitude from 3 kPa on Day 3 (Fig. 9a) to 31 kPa on Day 31 (Fig. 9e). Removal of cells at the end of the culture on Day 31 appeared to minimally affect tissue mechanical response, suggestive of an “entrenched” remodeling

and/or deposition of matrix now bearing load (also supported by the high stresses measured), with a slight increase in mechanical anisotropy (compare Figs. 9e and 9f).

The mechanical testing data and SHG images were further analyzed to characterize the hysteresis and relative collagen content of the tissue constructs during culture (Fig. 10). Percent hysteresis, quantified as the area between the loading and unloading curves normalized to that under the loading curve, is shown in Fig. 10a as a function of days in culture. The data indicated that for both anchored and strip biaxial stretch tissue constructs, viscoelasticity decreased with culture time. Conversely, average SHG intensity of the image stacks steadily increased during the month long culture, thus indicating increased collagen density with culture time. This latter result was consistent with the increase in load required to stretch both anchored and strip biaxially stretched tissue constructs over time.

DISCUSSION

Both natural and synthetic polymers have been used to generate 3D scaffolds for seeding cells in tissue engineering, including collagen, fibrin, and decellularized tissues as well as polyglycolic acid, polylactic acid, and polyortho esters.^{1,6,11,13,20} We have previously demonstrated NLOM sensitivity to polyglycolic acid as a biodegradable scaffold in engineered tissue. The degradation of polyglycolic acid as well as collagen deposition within engineered tubular tissues under culture were revealed by our NLOM imaging system.²⁷

In this study, OCM was combined with NLOM to characterize changes in the deposition, distributions, and orientation of matrix fibers in response to mechanical stimuli over an extended period. Deviation from the incompressible limit (Fig. 5) confirmed that on-line measurement of thickness was necessary to obtain “true” stress as a function of stretch. Although multiphoton microscopy has been used within a biaxial device to measure the thickness of native tissues noninvasively (e.g., using SHG²¹), shortcomings of this approach include a limited depth of imaging with which to measure thickness, especially of dense, highly scattering tissues and, for noninvasive imaging, sole reliance on endogenous nonlinear optical signals which are inherently weak. We overcame these limitations by incorporating coherence gated microscopy and tomography (OCM/OCT). Thus, with multimodal (NLOM-OCM-OCT) microscopy, our current system can probe deeper and measure greater thickness (up to 2 mm) even in specimens whose initial predominant components (particularly fibrin) may not have nonlinear susceptibilities that generate observable nonlinear optical signals.

Due to high compliance and low strength, uniaxial tests and related similar measurements^{8,33} have often been employed to quantify mechanical responses of engineered tissues. Yet mechanical anisotropy complicates the use of uniaxial data for parameter estimation in generalized 3-D constitutive equations, which many physiological, surgical, and medical device applications require. Biaxial tests provide more complete data in terms of convenient metrics (stress or strain fields), better quantify mechanical properties of engineered tissues as well as native tissues, and yield increased insight into mechanical-stimulated growth and remodeling of tissues.^{14,15,24} Herein, three mechanical testing protocols were performed longitudinally over 31 days on single constructs; each protocol

included three cycles of loading with repeatable and stable results and a sufficient range of finite stretch for estimating material parameters in a nonlinear constitutive relation (which was beyond the present scope). Thus, our bioreactor is a sufficiently sensitive and robust tracking system for soft biomaterials testing, allowing one to compare responses from multiple combinations of multiaxial stresses and strains, including evolving contributions of deposited collagen.

A continuing challenge in tissue engineering and regenerative medicine is the characterization of evolving microstructure and properties of constructs, while maintaining sterility. In this study, force transducers were integrated into the bioreactor and sterilized before use. The bioreactor was sealed with silicone rubber gaskets and by lids. Optical access to the tissue construct within the bioreactor was facilitated by a coverslip window assembly. Microsphere displacements were recorded through a sealed transparent window during mechanical testing. It is important to note, therefore, that although the chamber was moved multiple times, it was sealed within a biosafety cabinet and tissues were secured in place during transfer from incubator to imaging stage and mechanical testing platform.

For many tissues, including skin, ligaments, and arteries, the most important load-bearing component is collagen. It is, of course, the distribution, cross-linking, and alignment of collagen, not just its mere presence, that ultimately influences overall mechanical behavior. Several strategies have been employed to produce engineered tissues with a prescribed fiber alignment, aimed as functional replacements of diseased or injured tissues. These strategies include magnetic forces, boundary conditions on the gels, substratum topology, and mechanical loading.^{26,33,35} Consistent with above strategies, deposited collagen preferentially aligned with cells in the direction of imposed principal stretch under a strip biaxial stretch condition. Our results suggested, however, that the deposition of preferentially aligned collagen did not result in an overall strongly anisotropic tissue behavior. This counterintuitive result may have been a consequence of the static and, perhaps after adaptation, biaxially isotonic culture condition. Furthermore, this result may be consistent with the concept of tensional homeostasis,³ that cells strive to establish, maintain, or restore a mechanobiological equilibrium under any configuration. A similar experiment was reported by Sander *et al.*³³ in which two cruciform geometries with differing arm widths were examined, one with a symmetric 1:1 aspect ratio and the other with an asymmetric 1:0.5 aspect ratio. Their results appeared to suggest that higher local stresses may have induced more collagen deposition, when comparing arms of different widths, which ultimately homogenized local microenvironments. In addition, it was intriguing that mechanical anisotropy was observed in strip biaxially stretched tissue constructs on Day 31 only after decellularization, which suggested that the embedded cells had a homogenizing effect on tissue mechanical properties (Fig. 9f). A similar result was observed in tissue constructs cultured under strip biaxial stretch for much shorter duration (6 days) in which collagen fiber alignment (AI) with principal stretch increased following removal of embedded fibroblasts.¹⁷ Further work is clearly needed to define these cellular effects on matrix microscopic organization and mechanical responses.

This longitudinal study characterized and compared fibroblast seeded fibrin constructs cultured under two mechanical conditions, anchored at 1.0:1.0 stretch and strip biaxial at

1.0:1.1 stretch, over a period of 1 month. There were, however, a couple of subtle experimental limitations that may not be obvious. At this time, the mechanobiological effect of embedded microspheres on the reported results is not known, though, due to their low density, it is not expected to be significant. Also, the accuracy of measurements of tissue construct thickness in situ with OCT was subject to error from the assumed refractive index of the constructs. These OCT measurements were substantiated by the micrometer of the microscope mechanical stage using OCM to locate the tissue surfaces; measured thicknesses were found to be within 10 μm .

Though anisotropic culture conditions resulted in anisotropic orientation distribution of deposited collagen, mechanical anisotropy was not observed until after the removal of resident cells. We posit that static culture may have contributed to this observed isotropy and there is a need to impose cyclic stretch culture conditions.¹⁹ Multiple bioreactors have thus been built so that future comparative studies may investigate mechanobiological responses to multiple mechanical culture conditions simultaneously. Nevertheless, this experimental approach is not high throughput and will be aided by mathematical modeling that can predict dynamic tissue microstructural and mechanical responses that should be tested experimentally. In other words, mathematical modeling may be used to guide and inspire mechanobiological experiments, while the quantitative measurements enabled by the developed tools may be used to further refine the mathematical models. These quantitative measurements may include cell number, collagen concentration, and fiber angle distributions over time.

In summary, we validated a biaxial bioreactor suitable for month long culture of cruciform shaped 3D tissue constructs that may be coupled to a multimodal NLOM-OCM system and biaxial testing platform for collecting complementary microstructural information and characterizing mechanical properties. Microscopic organization and segmentation of multicomponent constructs may be characterized with NLOM-OCM as well as measurement of stretch dependent thicknesses. Mechanical properties of tissues were characterized by multiple multiaxial testing protocols. Multimodal microscopy and biaxial mechanical testing were performed longitudinally on individual engineered tissue constructs to provide time-dependent microstructural and mechanical properties. This integrated bioreactor platform enabled an unprecedented opportunity to correlate evolving mechanical properties with microscopic events, including matrix deposition, initial alignment, and subsequent remodeling in mechanically stimulated tissues.

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FIGURE 1.

Photograph of a cruciform-shaped gel (measuring 7.3 cm end-to-end) coupled to the four loading bars within the biaxial bioreactor; notice the single force transducer fitted on each axis. The four load arms were attached to the construct *via* two posts that projected from the bottom of polycarbonate end pieces; these posts inserted into two holes within the porous polyethylene bars positioned at the ends of the cruciform construct. Upper left corner: porous bar at the ends of the cruciform construct.

FIGURE 2.

Window assembly for in culture microscopic imaging. (a) Top to bottom: plastic bushing, glass tube with o-ring and coverslip bottom, and glass adapter. Tube is positioned inside the adapter by the o-ring, which rests on the shoulder of the adapter. Bushing is screwed into the glass adapter to seal the tube by compressing the o-ring. (b) The entire assembly sits in the polycarbonate lid on top of the bioreactor and is sealed by glue.

FIGURE 3.

Depth-dependent NLOM-OCM images of collagen matrix. (a) Simultaneously acquired SHG (left) and OCM images (right) from 50 (top) and 1500 μm (bottom) below the surface. (b) Normalized intensity plot of SHG (solid line) and OCM signal (broken line) as a function of depth from surface. Intensities are normalized relative to that at the surface.

FIGURE 4.

Mechanical testing of fibroblast seeded (1 million/mL) collagen:fibrin mixture matrix (0.5:10 mg/mL). (a) Illustrative stretch histories of three separate protocols: 1. Y-direction strip biaxial stretch; 2. equibiaxial stretch; 3. X-direction strip biaxial stretch. (b) Y-direction strip biaxial stretch test of fibroblast seeded collagen:fibrin matrix. (c) Equibiaxial stretch test of fibroblast seeded collagen:fibrin matrix.

FIGURE 5.

Cross-sectional OCT images of a tissue construct within the bioreactor at 1.0 (a), 1.05 (b), 1.10 (c), and 1.15 equibiaxial stretch (d). Measured thicknesses from OCT as a function of equibiaxial stretch (e). The incompressible limit is shown as reference (dashed line).

FIGURE 6. Representative NLOM-OCM images of fibroblast seeded fibrin matrix cultured under 1.0:1.0 stretch (anchored) on Day 1, 3, 10, 17, 24 and 31. (a) Cellular fluorescence by TPF. (b) SHG in collagen. (c) False color overlay showing cells (green) and collagen (red). (d) Nonspecific OCM images

FIGURE 7.

Representative NLOM-OCM images of fibroblast seeded fibrin matrix cultured under strip biaxial stretch (1.0:1.1) on Day 1, 3, 10, 17, 24 and 31. (a) Cellular fluorescence by TPF. (b) SHG in collagen. (c) False color overlay showing cells (green) and collagen (red). (d) Nonspecific OCM images

FIGURE 8.

Analyses of initial fibrin fiber distribution on Day 1 and deposited collagen on Day 3, 10, 17, 24 and 31 in anchored (a) and strip biaxial stretch tissue constructs (b). Error bars are standard errors ($n = 60$ images each day).

FIGURE 9.

Cauchy stress as a function of stretch measured from equibiaxial testing of tissue constructs cultured under strip biaxial stretch on Day 3 (a), 10 (b), 17 (c), 24 (d), and 31 (e). Equibiaxial test of decellularized construct on Day 31 (f). Principal (minor) stretch axis of strip biaxial stretch is shown in black (gray).

FIGURE 10.

Viscoelasticity of tissue constructs decreases as shown by percent hysteresis (area between loading and unloading curves normalized by that under loading curve) as a function of culture time (a). Collagen content of tissue constructs increases as shown by average SHG signal intensity as a function of culture time (b). Measurements from anchored (strip biaxial stretch) tissue culture are indicated by filled squares (open diamonds).

TABLE 1.

Mean thicknesses and alignment indices (AI) of tissue constructs cultured under anchored (1.0:1.0) and strip biaxial stretch (1.0:1.1) conditions.

Geometry	Culture time (day)	Thickness (mm)	AI
1.0:1.0	1	1.537 ± 0.132	1.096 ± 0.052
	3	1.129 ± 0.128	1.265 ± 0.023
	10	0.549 ± 0.043	1.289 ± 0.038
	17	0.438 ± 0.064	1.321 ± 0.055
	24	0.429 ± 0.041	1.367 ± 0.052
	31	0.446 ± 0.057	1.197 ± 0.036
1.0:1.1	1	1.316 ± 0.143	1.123 ± 0.038
	3	0.979 ± 0.105	1.267 ± 0.048
	10	0.507 ± 0.083	1.596 ± 0.075
	17	0.419 ± 0.049	1.789 ± 0.111
	24	0.437 ± 0.066	1.733 ± 0.065
	31	0.422 ± 0.078	1.689 ± 0.057

Data are mean ± standard deviation from 3 tissue constructs cultured under each condition.