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Integrating Structural Heterogeneity, Fiber Orientation, and Recruitment in Multiscale ECM Mechanics

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Abstract

Extracellular matrix (ECM) plays critical roles in establishing tissue structure-function relationships and controlling cell fate. However, the mechanisms by which ECM mechanics influence cell and tissue behavior remain to be elucidated since the events associated with this process span length scales from the tissue to molecular level. Entirely new methods are needed in order to better understand the multiscale mechanics of ECM. In this study, a multiscale experimental approach was established by integrating Optical Magnetic Twisting Cytometry (OMTC) with a biaxial tensile tester to study the microscopic (local) ECM mechanical properties under controlled tissue-level (global) loading. Adventitial layer of porcine thoracic artery was used as a collagen-based ECM. Multiphoton microscopy imaging was performed to capture the changes in ECM fiber structure during biaxial deformation. As visualized from multiphoton microscopy images, biaxial stretch induces gradual fiber straightening and the fiber families become evident at higher stretch levels. The OMTC measurements show that the local apparent storage and loss modulus increases with the global biaxial stretch, however there exists a complex interplay among local ECM mechanical properties, ECM structural heterogeneity, and fiber distribution and engagement. The phase lag does not change significantly with global biaxial stretch. Our results also show a much faster increase in global tissue tangent modulus compared to the local apparent complex modulus with biaxial stretch, indicating the scale dependency of ECM mechanics.

Keywords

extracellular matrix; multiscale mechanics; fiber orientation; fiber recruitment; heterogeneity; optical magnetic twisting cytometry; biaxial loading

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Introduction

Extracellular matrix (ECM) provides the principal avenue for mechanical and biochemical communications between tissue and cells. Cells actively sense the external mechanical forces applied through the ECM and also their surrounding local stiffness (Throm Quinlan, et al., 2011). External mechanical forces and ECM stiffness have been shown to play important roles in regulating fundamental cellular morphology (Wells, 2008) and functions, such as proliferation (Wells, 2008) (Schrader, et al., 2011), differentiation (Engler, et al., 2006) (Even-Ram, et al., 2006) and mobility (Pelham & Wang, 1997) (Zaman, et al., 2006). Many pathological conditions involve significant alterations of ECM mechanical properties (Baker, et al., 2009) (Janmey & Miller, 2011) (Lu, et al., 2012) (Engler, et al., 2008) (Chaturvedi, et al., 2010) (Georges, et al., 2007) (Wells, 2008), (Shkumatov, et al., 2015) (White, 2015). Understanding the translation of mechanical forces from the tissue to cellular level is crucial and a requisite bridge between research in the fields of cell and tissue mechanics. However, the force translation in ECM is inherently a multiscale process ranging from the macroscopic to microscopic level. The mechanical properties of ECM, known to have a 3D hierarchical structure, have been shown to be highly dependent on the scale of measurement (Aifantis, et al., 2011) (Li, et al., 2017). As a result, the distribution of mechanical force in the ECM is highly dependent on the hierarchical structure and the local mechanical properties of the ECM (Pizzo, et al., 2005) (Mow, et al., 1994).

The macro- and microscopic mechanical properties of ECM have been broadly studied. To investigate the macroscopic mechanical properties of collagen matrix, various experimental methods, such as uniaxial tension (Roeder, et al., 2002) (Feng, et al., 2003) (Duan & Sheardown, 2006) (Van Oosten, et al., 2016), biaxial tension (Jhun, et al., 2009) (Sander, et al., 2009) (Xu, et al., 2011), and rheological tests (Vader, et al., 2009) (Xu, et al., 2011) (Kurniawan, et al., 2012) (Li & Zhang, 2014) (Motte & Kaufman, 2012) have been performed. In microscopic mechanical measurements, controlled mechanical forces were applied using a variety of techniques including indentation (Wenger, et al., 2007) (Aifantis, et al., 2011) (McKee, et al., 2011), laser tracking microrheology (Velegol & Lanni, 2001) (Parekh & Velegol, 2007) (Sun, et al., 2004) (Shayegan & Forde, 2013), magnetic twisting cytometry (Leung, et al., 2007), and optical magnetic twisting cytometry (OMTC) (Li, et al., 2017). These studies on the mechanical properties of ECM are focused on a single measurement scale. Advances in imaging techniques promote the integration of multiscale mechanical loading and imaging modalities to provide perspectives on load induced changes in ECM microstructure, cellular morphology and alignment (Bell, et al., 2012) (Throm Quinlan, et al., 2011). An integrated mechanical loading-confocal microscopy system was developed and used to study three-dimensional (3D) mechanical strains in collagen matrix at multiple length scales in response to uniaxial (Voytik-Harbin, et al., 2003) (Roeder, et al., 2004) (Roeder, et al., 2009) and biaxial stretching (Bell, et al., 2012). To obtain local mechanical properties, local strains were measured, and the macro-scale mechanical properties of a substrate are usually used in the calculation of local traction forces (Franck, et al., 2011) (Toyjanova.J, et al., 2014).

To date, there has been limited understanding of multiscale ECM mechanical properties. Several previous studies have attempted to measure the local mechanical properties of ECM

and showed that the local mechanical properties of an interconnected network do not always match its global properties. Microscopic mechanical properties of ECM measured by laser trap microscopy (Velegol & Lanni, 2001) and AFM (Gautreau, et al., 2006) (Throm Quinlan & Billar, 2012) yielded consistently greater stiffness measurements than those obtained from macroscopic rheometry and uniaxial tensile tests. Significant differences in the mechanical properties of ECM were shown ranging from the macro to nano scales as $E_{\text{tendon}} < E_{\text{fiber}} < E_{\text{fibril}}$ (Aifantis, et al., 2011). The microscopic mechanical properties were found to be less sensitive to changes in collagen concentration, as compared to the macroscopic mechanical properties (Li, et al., 2017) (Baniasadi & Minary-Jolandan, 2015). The scale of measurements was found to play an important role in multiscale ECM mechanical properties. The differences in multiple ECM mechanical properties emerge when the probe size is comparable to or smaller than the average pore size of the sample (Costa, et al., 2003).

In the present study, we created a multiscale experimental approach that combines optical magnetic twisting cytometry (OMTC) and biaxial tensile testing techniques to study the changes in local ECM mechanical properties with controlled global biaxial mechanical loading. The biaxial tensile test considers the multi-axial loading state under physiological conditions and has shown promises in fully characterizing the anisotropic nature of soft biological tissues (Sacks & Sun, 2003) (Nerurkar, et al., 2010) (Bell, et al., 2012). By oscillating microscale ferromagnetic beads bound to collagen fibers, OMTC was used to measure the local mechanical properties of collagen matrix. OMTC allows the measurements of a population of beads and can effectively eliminate unbound or loosely bound beads (Fabry, et al., 2001). Porcine adventitia layer was used as an ECM equivalent due to several reasons: 1) Porcine adventitia is the outmost layer of the artery and consists primarily of type I collagen fibers. 2) It is semi-transparent so it is possible to make optical measurements using OMTC. 3) Compared to collagen gel, it is more robust to work with when biaxial loading is applied. Multiphoton microscopy was also used to capture the structural changes of collagen fibers under tissue-level biaxial loading.

Materials and Methods

Sample preparation

Descending thoracic aortas of porcine (12 – 24 month old) were harvested at a local abattoir and transported to the laboratory on ice. Before experiments, the aortas were cleaned of adherent tissues and fat and rinsed in deionized water. All samples were taken from approximately the same longitudinal region of the aorta to avoid changes in mechanical properties along the longitudinal anatomic position (Zeinali-Davarani, et al., 2015). The adventitial layer was carefully peeled from the porcine artery and cut into squares of about $2 \text{ cm} \times 2 \text{ cm}$ ($n = 5$).

Streptavidin coated ferromagnetic beads (Spherotech Co.) with an average diameter of $4.7 \mu\text{m}$ were used. The primary constituents of adventitia layer are thick bundles of type I collagen fibers (Chow, et al., 2014). Tight binding between streptavidin and collagen is formed via bio-conjugation (Chen, et al., 2012) (Guo & Kaufman, 2007). A bead solution of 0.4 mg/ml was poured onto the adventitia surface and then the sample was incubated at

37 °C for ~ 40 min to allow beads to bind to collagen. The sample was then washed twice with deionized water to remove unbound beads.

Integrated multiscale testing system

A multiscale experimental methodology was developed by integrating a one-dimensional OMTC (EOL Eberhard, Switzerland) with a biaxial tensile tester, as shown in Figure 1a. The OMTC was custom built to fit the configuration of the microscope and the biaxial tensile tester setup. The integrated magnetic tensile-twisting device was mounted on a Zeiss inverted microscope (Axio Observer D1), which was placed on a vibration isolation table. To apply biaxial loading, sandpaper tabs were glued to the sides of the adventitia sample. The tissue dimensions between the sandpaper tabs were approximately 1cm × 1cm. Sutures were looped through the sandpaper and connected to the linear positioners of the biaxial tensile tester. The local and global mechanical properties were then measured using OMTC and the biaxial tensile tester, respectively.

Tissue-level mechanical property measurements

Four linear positioners (MX80S, Parker Hannifin Corporation, Irwin PA) connected to a four-axis motion controller (DMC-4040, Galil Motion Control, Rocklin CA) were positioned so that the ECM network can be loaded biaxially at the tissue-level and maintained at the center of the field-of-view (Figure 1b). The movements of the linear positioners were controlled by a LabVIEW program (National Instruments, Austin, TX). Load cells (250 g Model 31 Low, Honeywell, Golden Valley, MN) mounted in both the circumferential (x_1) and longitudinal (x_2) anatomical sample directions were used to measure the load applied to the sample. Tissue stretch was measured by tracking four carbon marker dots glued to the sample using a custom Labview program (National Instruments, Austin, TX). At first, a preload (3g) was applied to straighten the sutures. The preloaded marker positions were used as the reference state for later stretch calculations. Preconditioning was performed by loading the sample equibiaxially at 60g until a repeatable material response was achieved. After preconditioning, 8 cycles of equibiaxial tension with a load of 100g were applied to the tissue sample. The biaxial response from the last cycle was used for further data analysis. Assuming the adventitial layer is incompressible, the Cauchy stresses are calculated as:

$$\sigma_1 = \frac{F_1 \lambda_1}{h L_{02}} \sigma_2 = \frac{F_2 \lambda_2}{h L_{01}} \quad (1)$$

where σ_i ($i = 1, 2$) is Cauchy stress, F_i and λ_i ($i = 1, 2$) represents the load and stretch in the x_i direction; h and L_{0i} ($i = 1, 2$) are the undeformed tissue thickness and side length, respectively.

Local mechanical property measurements

The local mechanical properties of adventitia were measured using the OMTC, which is based on measuring the lateral displacement of ferromagnetic beads bound to collagen fibers when the beads are subjected to magnetic twisting (Trepap, et al., 2004). The ferromagnetic

beads were first magnetized by a brief (0.1ms), strong magnetic pulse ($>0.1\text{T}$) applied in the horizontal plane. The circumferential direction of adventitia was aligned with the direction of the magnetizing field. Subsequently, a homogeneous, oscillatory magnetic field ($<0.01\text{ T}$) applied vertically induces a mechanical torque that causes the beads to rotate (Fabry, et al., 2001). During twisting, specific twisting torque $T(t)$ (mechanical torque per unit volume with a dimension of Pa) applied to each bead can be calculated by:

$$T(t) = C_{bead} * C_{coil} * I(t) \quad (2)$$

where C_{bead} (Pa/G) is the bead constant, C_{coil} (35.3G/A) is the magnetic twisting coil constant, and $I(t) = I_0 \sin \omega t$ is the applied sinusoidal current with magnitude $I_0 = 2.5\text{A}$ and oscillating frequency $\omega = 1\text{Hz}$. The bead constant $C_{bead} = 1.14\text{Pa/G}$ (Spherotech Co) is related to the intrinsic property of ferromagnetic beads.

The lateral bead displacement in response to oscillatory twisting torque is tracked through images captured by a progressive scanning black and white CCD camera (AVT Pike F-100b, Advanced Vision Technology Co.) mounted on a Zeiss inverted microscope (Axio Observer D1) with an acquisition frequency of 10Hz. 10 images ($714\mu\text{m} \times 714\mu\text{m}$ with a $10\times$ objective) are captured every twisting cycle by the CCD camera controlled by a custom Labview (NI, Austin, TX) program (Trepate, et al., 2004). Ferromagnetic beads are selected by an image-processing algorithm based on a series of bead acceptance criteria including the size, shape and the distance between adjacent beads (Trepate, et al., 2003). The positions of the selected beads were tracked following a bead-tracking algorithm (Trepate, et al., 2003). Lateral bead displacement can be obtained by eliminating drift and noise using a moving average filter between two adjacent oscillating cycles. Beads with erratic and irreproducible motions are rejected in post analysis.

Discrete Fourier transformation was performed at the fundamental frequency of 1Hz on both specific twisting torque $T(t)$ and lateral bead displacement $d(t)$ to calculate the apparent complex modulus G_d^* , which is a function of oscillating frequency ω :

$$G_d^*(\omega) = \frac{T(\omega)}{d(\omega)} = G'_d(\omega) + iG''_d(\omega) \quad (3)$$

where G'_d and G''_d represent the apparent storage and loss modulus, respectively, and have a unit of Pa/nm. A dimensional quantity δ with unit of rad can be calculated to characterize the phase angle between the twisting torque and bead displacement response:

$$\delta = \arctan \frac{G''_d(\omega)}{G'_d(\omega)} \quad (4)$$

where $\delta = 0$ corresponds to an elastic material and $\delta = \frac{\pi}{2}$ corresponds to a viscous material. The ferromagnetic beads were remagnetized after each increase of tissue-level mechanical loading to ensure bead magnetization was kept aligned throughout the experiment. Before biaxial loading, the sample was preconditioned by loading the sample equibiaxially to 30g with a 10s half cycle time for eight cycles. The sample was then subjected to equibiaxial loading of 5, 10, 20, 30, 40, 60, 80g with a 10s half cycle time and held for OMTC measurement. At each loading level, local mechanical properties were measured at three or four different locations, and the average apparent storage and loss modulus was used to represent the local mechanical properties. In order to keep the magnetizing direction of the beads circumferential, once the tissue sample was loaded, ferromagnetic beads were remagnetized before OMTC measurements.

Multiphoton imaging

Multiphoton imaging was performed to assess the collagen fiber network structure in adventitia during mechanical loading. A multiphoton microscope (Carl Zeiss LSM 710 NLO microscope with Coherent Chameleon Vision-S laser set to 800 nm) illuminates the collagen via second harmonic generation passed through an emission filter at 417 nm with 80 nm width. Samples were imaged at 1, 1.05, 1.1, 1.15, and 1.2 equibiaxial grip-to-grip stretches using a custom-made biaxial loading device (Chow, Turcotte, Lin, & Zhang, 2014). All samples were aligned so that 0° and $\pm 90^\circ$ represent the aorta's circumferential (C) and longitudinal (L) directions, respectively. For each sample, images of $425 \times 425 \mu\text{m}^2$ at five locations were recorded. At each location, z-stack images were acquired in $2 \mu\text{m}$ increments. Each z-stack imaging took about 40 seconds. Imaging of all five locations on a sample took a little under five minutes including the time to move between locations. Maximum intensity projection of the z-stack images were used for imaging analysis.

Adventitial collagen fiber waviness was defined by a straightness parameter, $P_s = L_o/L_f$, where the end-to-end distance (L_o) and total fiber length (L_f) were traced with NeuronJ (imagescience.org/meijering/software/neuronj). As collagen fibers become less wavy, P_s increases until being equal to 1 for a straight line. In addition, fiber orientation was quantified with the directionality plug-in in FIJI (fiji.sc/Fiji, Ashburn, VA) using two-dimensional fast Fourier transform analysis (FFT).

Results

Tissue-level mechanical properties and ECM structure

Figure 2a shows the average Cauchy stress vs. stretch curves from biaxial tensile testing of the adventitia tissue sample, which is highly nonlinear with the longitudinal direction stiffening slightly earlier than the circumferential direction. To better compare the mechanical properties in the longitudinal and circumferential directions, tangent modulus was obtained by differentiating the Cauchy stress-stretch curves in Figure 2a as $d\sigma/d\lambda$. As shown in Figure 2b, a prominent increase in tangent modulus was observed when the stretch is beyond 1.1. The large error bar at higher stretches is due to the variations in tissue stiffening among tissue samples.

Representative multiphoton images under equibiaxial stretching are presented in Figures 3a-e. Collagen fibers have a wavy configuration at the undeformed state (Figure 3a). As the stretch increases, the collagen fibers are gradually straightened, resulting in an increase in straightness parameter of the collagen fibers, as shown in Figure 3f. When the stretch increases to beyond 1.15, the fibers are almost totally straightened with the straightness parameter approaching 1.

Figure 4 shows the normalized average collagen fiber distribution as biaxial stretches vary from 1 to 1.2. Collagen fibers in the adventitia exist in multiple fiber families. However these fiber families are obscured, i.e., not visible, at low tissue stretches of 1 and 1.05. When the fibers are straightened at higher stretches of 1.15 and 1.2, multiple prominent peaks appear, revealing the existence of multiple collagen families.

Local mechanical properties

Figure 5a shows the beads' lateral displacement from the OMTC measurement with the arrows representing the direction and magnitude ($\times 100$) of the displacement. A majority of the beads' lateral displacement is distributed between -40° to 40° from the circumferential direction since the magnetizing direction was always kept consistent with the circumferential direction of adventitia. The orientation distribution of bead displacement in Figure 5b shows a tri-modal feature with a central major peak and two minor peaks. To capture the tri-modal feature, the orientation distribution was fit with a probability density function (PDF), which is assumed to be the sum of three standard normal PDF as (SAS

Institute Inc, 2016): $\sum_{i=1}^3 \frac{\pi_i}{s_i} \Phi\left(\frac{x - \mu_i}{s_i}\right)$, where μ_i , s_i and π_i are the respective mean, standard deviation, and proportion for the i^{th} modal; and $\pi_1 + \pi_2 + \pi_3 = 1$. Function Φ is a standard

normal PDF, and is defined as $\Phi(x) = \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}}$. Figure 5c shows the distribution of bead displacement orientation with increasing equibiaxial stretch. While the portion of beads moving along the circumferential direction (twisting direction) decreases with stretch, there is an increasing trend of beads moving with $+70^\circ$ or -70° degree deviation from the circumferential direction. This can be further verified by a decrease in the fitting parameter π_2 , which represents the proportion of beads moving along the circumferential direction (Fig 5d).

The apparent complex moduli from OMTC measurements located within the major and minor peaks of the bead displacement orientation distribution were plotted separately in Figure 6. We can see that the modulus measured within the major peak shows a stronger linear dependency on stretch with a higher coefficient of determination. The moduli measured within the minor peaks, however, are more dispersed. Also, the discrepancy between the results from major and minor peaks increases at higher stretch levels.

Changes in local mechanical properties with tissue-level stretch and stress

More than 100 beads are tracked during each OMTC measurement. To study the heterogeneity in local mechanical properties, distributions of apparent complex modulus at different loading levels are shown in Figure 7. The red dashed line indicates the position of

the median, which is used as a measure of the local mechanical properties in the OMTC experiment. The position of the median moves toward a higher modulus as the tissue level stretch increases (Figures. 7a-c). The median absolute deviation (MAD), defined by Equation (5), is calculated at different stretch levels to characterize the variability of the dispersed data (Rousseeuw & Croux, 1993).

$$\text{MAD} = \text{median} (|X_i - \text{median} (X_i)|) \quad (5)$$

In Equation (5), X_i is the measured apparent complex modulus from all beads, $\text{median} (X_i)$ represents the median of X_i . Figure 7d shows that the MAD increases with the global equal stretch levels for all samples, suggesting increased heterogeneity in local mechanical properties with global loading.

The local apparent modulus consistently increases with tissue stretch up to about 1.2–1.3 (Figure 8a). A similar trend is observed in the local apparent modulus vs. stress plots, however this trend seems to plateau when stresses are higher than 100kPa (Figure 8b). The phase angle provides a measure of the dissipated energy relative to the stored energy. Resulting from a proportional increase for both local storage and loss modulus, the phase angle measured from OMTC does not change significantly with tissue stretch (Figure 8c).

Both the tissue tangent modulus and the local apparent complex modulus increase with mechanical loading, however the tissue tangent modulus increases at a much faster rate. It can be seen from Figure 9 that as the global tissue stretch increases from 1 to 1.15, the tissue tangent modulus in the circumferential direction increases nonlinearly for about 6 times, with a more gradual trend of increase at the beginning followed by a more rapid increase when the stretch is higher than about 1.07. The local apparent complex modulus doubled with a relatively linear trend of increase.

Discussion

In this study, a new experimental methodology was developed to study the changes of microscopic ECM mechanics under macroscopic loading. Specifically, OMTC was used to measure the local mechanical properties of the collagen network when subjected to controlled global biaxial mechanical loading. Our study suggests that it is important to consider the complex interplay among ECM fiber orientation, fiber engagement, and the tissue-level mechanical loading when understanding the scale dependency of ECM mechanics.

Collagen fiber orientation may play an important role in OMTC measurements of local mechanical properties. In the OMTC measurements, when the collagen fiber orientation coincides with the bead magnetizing direction, bead rotation tends to stretch and straighten the fibers. On the other hand, when the collagen fiber orientation is not aligned with the bead magnetization direction, bead rotation tends to bend and twist the fibers. This latter scenario often results in a relatively large lateral bead displacement, which was interpreted as lower stiffness. However, in most cases, it is likely that both scenarios play a role in OMTC

measurements, and thus contribute to the heterogeneity in local stiffness measurements (Figure 7). Interestingly, while majority of the beads rotation is along the circumferential magnetizing direction, a minor portion of ferromagnetic beads move along ± 70 degrees (Figures 5b and 5c), resembling the multiple fiber family distribution in adventitial collagen (Figures 3 and 4). Furthermore, measurements from beads moving along the directions under the major peaks show a stronger linear dependency on tissue stretch (Figure 6). Such results further suggest that the complex interaction between ferromagnetic beads and collagen fiber orientation distribution contribute to the heterogeneity of local ECM mechanical property measurements.

As tissue is deformed, the gradual engagement of collagen fibers results in higher local ECM stiffness. At low equal biaxial stretch levels (under 1.05), most collagen fibers are wavy and do not take much mechanical load (Figure 3). These wavy “floppy” fibers have little restriction to the rotation of the magnetic beads. As seen in Figure 7a, the local stiffness is relatively small at no loading or low stretch levels, represented as a dominant peak distributed between 0.05 – 0.1 Pa/nm. As the global stretch increases, more collagen fibers are gradually engaged to bear the load, which can be seen at 1.1 and 1.15 stretch levels that consists of a network of both stretched and wavy fibers (Figure 3). Ferromagnetic beads bound to straighter fibers will be more difficult to rotate and result in stiffer local mechanical properties. Therefore, the portion of measurement with low stiffness decreases and the median moves toward the stiffer region (Figures 7b and 7c).

The slow increase in local modulus in Figure 9 is likely related to the lower strain experienced at the microscopic level. It was shown that the individual collagen fibril strain is considerably lower when compared to overall tissue strain (Fratzl, et al., 1997) (Puxkandl, et al., 2002) (Screen, et al., 2004). The lower strain at the microscopic level is largely due to the hierarchical structure of collagen fiber bundles. Deformation including intra- and inter-fibril sliding, and fiber-interstitial fluid interactions may occur simultaneously upon tissue level deformation (Fratzl, et al., 1997) (Puxkandl, et al., 2002) (Screen, et al., 2004). Deformation at these structural sublevels regulates the multiscale transfer of deformation from macro- to micro- scale. Nonaffine fiber rearrangement has also been shown to contribute to the lower fiber level strain (Screen, et al., 2004) (Roeder, et al., 2009) (Chandran & Barocas, 2005) (D’Amore, et al., 2014). Fibers reorient to the loading direction gradually, resulting in an increasing number of load bearing elements and a reduction of strain experienced by each fiber. Since equibiaxial tension was used in the present study, it is likely that some fiber reorientation occurred, but it may not play a dominant role.

Interestingly, the heterogeneity in local mechanical property measurements increases with global tissue deformation, as shown in the increasing median absolute deviation with tissue stretch (Figure 7d). The wide range distribution of stiffness in Figure 7 is associated with the inhomogeneous distribution of thick and wavy collagen fiber bundles in the adventitia (Figure 3). It has been shown that the modulus of porcine adventitia has a distribution ranging four orders of magnitude due to its nature of structural heterogeneity from atomic force microscope measurements (Grant & Twigg, 2013). Our study suggests that the engagement/straightening of collagen fibers results in a redistribution of local ECM stiffness. At higher stretch levels, the proportion low stiffness in the OMTC measurements

gradually decreases, but still exists. In the meantime, the higher stiffness region starts to emerge (Figures 7b and 7c), and result in a more spread distribution of the local modulus and increased MAD (Figure 7d).

The macroscopic mechanical properties of biological tissues are often related to their microscopic ECM structure (Tower, et al., 2002) (Voytik-Harbin, et al., 2003) (D'Amore, et al., 2014). Adventitia shows an anisotropic hyperelastic stress-stretch relationship with the longitude being stiffer than the circumference (Figure 2a), which is associated with the preferred fiber alignment and progressive recruitment of collagen fibers (Gasser, et al., 2006). The orientation of collagen fiber families are obscured at low or no mechanical loading due to the large fiber waviness and become more evident when the fibers are straightened (Chow, et al., 2014). The appearance of multiple peaks from FFT analysis (Figure 4) is consistent with studies on either porcine (Chow, et al., 2014) (Mattson, et al., 2017) or human adventitia (Schriefl, et al., 2012). The preferred fiber distribution in the longitudinal direction explains the greater increase in tangent modulus in longitude than circumference when stretch is beyond 1.1 (Figure 2b).

We would like to point out several limitations in our study. The unit of local stiffness is Pa/nm. A geometric factor is need to covert the apparent modulus from OMTC measurements to shear modulus (Fabry, et al., 2003) (Mijailovich, et al., 2002) (Ohayon & Tracqui, 2005). The geometric factor is usually obtained from computational modeling which assumes the bead is embedded in a homogeneous and continuous matrix (Mijailovich, et al., 2002) (Ohayon & Tracqui, 2005) (Li, et al., 2017). Considering the discrete nature of collagen ECM, a discrete network model is required to better understand the bead-matrix interaction and the nonaffine matrix reorganization under mechanical loading. The bead-matrix binding was achieved by bio-conjugation between collagen and streptavidin in the present study. Tight binding between collagen and other proteins including fibronectin and collagen specific targeted antibody could be used in future work to verify the dependency of measured local stiffness on binding strength. Viscoelastic deformation could occur during the OMTC measurement, and its effect on the local mechanical property measurements is unknown. The morphology of adventitia was found to be highly nonuniform from microCT studies (Walton, et al., 2015). The effect of surface morphology on local ECM mechanical properties is unknown. In calculating the Cauchy stresses in Equation (1), the adventitial layer was assumed to be incompressible. In the current study, only adventitial layer was used. With the absence of the medial layer, the undulated collagen fiber bundles in the adventitial would straighten even without mechanical loading. Thus it is important to note that the tissue stretches in the current study underestimated the stretches experienced by the intact tissue. With the absence of tissue integrity, one should be careful when inferring the physiological relevance of the tissue stretch in this study. In physiological conditions, the arterial tissue experiences stretches of about 1.2–1.3, which result in adventitial straightness parameter of ~0.9 for intact tissue (Mattson et al., 2017). In the current study, however, at the tissue stretch of 1.2, the straightness parameter of adventitial collagen reaches 0.965, which is close to the fiber waviness value of fully straightened collagen fibers in the intact aorta at 1.4 stretch (Chow et al., 2014).

Conclusions

A novel experimental approach that allows the measurements of local ECM mechanical properties with controlled tissue-level biaxial mechanical loading was established and used to study the multiscale ECM mechanics of the collagen network in the adventitial layer of the arterial wall. This study reported several interesting findings of the role of ECM fiber network structure in determining the local ECM mechanical properties upon tissue-level mechanical loading. Our study shows that the ECM fiber orientation and fiber recruitment play important roles in determining the local ECM mechanics. As the tissue is deformed, the gradual engagement/straightening of collagen fibers results in higher local ECM apparent modulus. The fiber orientation also plays an important role in dictating the local ECM properties. Both tissue tangent modulus and local apparent complex modulus increase with tissue-level deformation, however the local apparent complex modulus increases at a slower rate than the tissue tangent modulus due to the multi-scale deformation within the ECM and the lower strain experienced locally. Furthermore, in addition to the inherent structural heterogeneity of the thick bundles of collagen fibers in the collagen network, fiber orientation and recruitment both contribute to the wide distribution in local ECM mechanical property measurements. These structural and mechanical interplays need to be considered in order to advance the current understanding of multi-scale ECM mechanics and to establish the connections between the macro- and microscopic mechanical properties of the ECM,

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References

1. Aifantis K, Shrivastava S & Odegard G, 2011 Transverse mechanical properties of collagen fibers from nanindentation. *J Mater Sci: Mater Med*, Volume 22, pp. 1375–1381. [PubMed: 21556981]
2. Baker E, Bonnecaze R & Zaman M, 2009 Extracellular Matrix Stiffness and Architecture Govern Intracellular Rheology in Cancer. *Biophysical Journal*, Volume 97, pp. 1013–1021. [PubMed: 19686648]
3. Baniasadi M & Minary-Jolandan M, 2015 Alginate-Collagen Fibril Composite Hydrogel. *Materials*, Volume 8, pp. 799–814. [PubMed: 28787971]
4. Bell B, Nauman E & Voytik-Harbin S, 2012 Multiscale Strain Analysis of Tissue Equivalents Using a Custom-Designed Biaxial Testing Device. *Biophysical Journal*, 102(6), pp. 1303–1312. [PubMed: 22455913]
5. Chandran P & Barocas V, 2005 Affine versus non-affine fibril kinematics in collagen networks: theoretical studies of network behavior. *J Biomech Eng*, 128(2), pp. 259–270.
6. Chaturvedi R et al., 2010 Passive stiffness of myocardium from congenital heart disease and implications for diastole. *Circulation*, Volume 121, pp. 979–988. [PubMed: 20159832]
7. Chen P, Herath S, Wang D & Asada H, 2012 Active Manipulation of ECM Stiffness and Its Effect on Endothelial Cell Migration during Angiogenesis. London, Proceedings of the World Congress on Engineering.
8. Chow M, Turcotte R, Lin C & Zhang Y, 2014 Arterial Extracellular Matrix: A Mechanobiological Study of the Contributions and Interactions of Elastin and Collagen. *Biophysical Journal*, Volume 106, pp. 2684–2692. [PubMed: 24940786]
9. Costa K, Ho M & Hung C, 2003 Multi scale measurement of mechanical properties of soft samples with atomic force microscopy. Florida, 2003 Summer Bioengineering Conference.

10. D'Amore A et al., 2014 From single fiber to macro-level mechanics: A structural finite element model for elastomeric fibrous biomaterials. *J Mech Behav Biomed Mater*, Volume 39, pp. 146–161. [PubMed: 25128869]
11. Duan X & Sheardown H, 2006 Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: Mechanical properties and corneal epithelial cell interactions. *Biomaterials*, Volume 27, pp. 4608–4617. [PubMed: 16713624]
12. Engler A et al., 2008 Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *J Cell Sci*, Volume 121, pp. 3794–3802. [PubMed: 18957515]
13. Engler A, Sen S & Sweeney H, 2006 Matrix elasticity directs stem cell lineage specification. *Cell*, 126(4), pp. 677–689. [PubMed: 16923388]
14. Even-Ram S, Artym V & Yamada K, 2006 Matrix control of stem cell fate. *Cell*, 126(4), pp. 645–647. [PubMed: 16923382]
15. Fabry B et al., 2003 Time scale and other invariants of integrative mechanical behavior in living cells. *Phys Rev E*, Volume 68, pp. 041914–1-041914–18.
16. Fabry B et al., 2001 Signal Transduction in Smooth Muscle Selected Contribution: Time course and heterogeneity of contractile responses in cultured human airway smooth muscle cells. *Journal of Applied Physiology*, Volume 91, pp. 986–994. [PubMed: 11457818]
17. Feng Z et al., 2003 Investigation on the mechanical properties of contracted collagen gels as a scaffold for tissue engineering. *Artif Organs*, 27(1), pp. 84–91. [PubMed: 12534718]
18. Franck C, Maskarinec S, Tirrell D & Ravichandran G, 2011 Three-Dimensional Traction Force Microscopy: A New Tool for Quantifying Cell-Matrix Interactions. *PLoS ONE*, 6(3), p. e17833. [PubMed: 21468318]
19. Fratzl P, Misof K & Zizak I, 1997 Fibrillar Structure and Mechanical Properties of Collagen. *Journal of Structural Biology*, Volume 122, pp. 119–122.
20. Gasser T, Ogden R & Holzapfel G, 2006 Hyperelastic modelling of arterial layers with distributed collagen fibre orientations. *J.R.Soc.Interface*, Volume 3, pp. 15–35. [PubMed: 16849214]
21. Gautreau Z, Griffin J, Peterson T & Thongpradit P, 2006 Characterizing viscoelastic properties of polyacrylamide gels, B.S. Project Report, Worcester Polytechnic Institute.
22. Georges P et al., 2007 Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. *Am J Physiol Gastrointest Liver Physiol*, Volume 293, pp. 1147–1154.
23. Grant C & Twigg P, 2013 Pseudostatic and Dynamic Nanomechanics of the Tunica Adventitia in Elastic Arteries Using Atomic Force Microscopy. *ACS Nano*, 7(1), pp. 456–464. [PubMed: 23241059]
24. Guo C & Kaufman L, 2007 Flow and magnetic field induced collagen alignment. *Biomaterials*, 28(6), pp. 1105–1114. [PubMed: 17112582]
25. Janmey P & Miller R, 2011 Mechanisms of mechanical signaling in development and disease. *J Cell Sci*, Volume 124, pp. 9–18. [PubMed: 21172819]
26. Jhun C, Evans M, Barocas V & Tranquillo R, 2009 Planar Biaxial Mechanical Behavior of Bioartificial Tissues Possessing Prescribed Fiber Alignment. *J Biomech Eng*, 131(8), pp. 081006–1-081006–8.
27. Kurniawan N, Wong L & Rajagopalan R, 2012 Early stiffening and softening of collagen: Interplay of deformation mechanisms in biopolymer networks. *Biomacromolecules*, 13(3), pp. 691–698. [PubMed: 22293015]
28. Li H et al., 2017 Multiscale Measurements of the Mechanical Properties of Collagen Matrix. *ACS.Biomater.Sci.Eng.*
29. Li H & Zhang Y, 2014 Modeling of the viscoelastic behavior of collagen gel from dynamic oscillatory shear measurements. *Biorheology*, 51(6), pp. 369–380. [PubMed: 25633438]
30. Lu P, Weaver V & Werb Z, 2012 The extracellular matrix: A dynamic niche in cancer progression. *J Cell Biol*, 196(4), pp. 395–406. [PubMed: 22351925]
31. Mattson J, Turcotte R & Zhang Y, 2017 Glycosaminoglycans contribute to extracellular matrix fiber recruitment and arterial wall mechanics. *Biomech Model Mechanobiol*, 16(213).

32. Mckee C, Last J, Russell P & Murphy C, 2011 Indentation Versus Tensile Measurements of Young's Modulus for Soft Biological Tissues. *Tissue Eng Part B Rev*, 17(3), pp. 155–164. [PubMed: 21303220]
33. Mijailovich S et al., 2002 A finite element model of cell deformation during magnetic bead twisting. *J.Appl.Physiol*, Volume 93, pp. 1429–1436. [PubMed: 12235044]
34. Motte S & Kaufman L, 2012 Strain stiffening in collagen I networks. *Biopolymers*, 99(1), pp. 35–46.
35. Mow V, Bachrach N, Setton L & Guilak F, 1994 Stress, strain, pressure and flow fields in articular cartilage and chondrocytes In: Mow V, Bachrach N, Setton L & Guilak F, eds. *Cell Mechanics and Cellular Engineering*. New York: Springer-Verlag.
36. Nerurkar N, Elliott D & Mauck R, 2010 Mechanical Design Criteria for Intervertebral Disc Tissue Engineering. *J Biomech*, 43(6), pp. 1017–1030. [PubMed: 20080239]
37. Ohayon J & Tracqui P, 2005 Computation of Adherent Cell Elasticity for Critical Cell-Bead Geometry in Magnetic Twisting Experiments. *Annals of Biomedical Engineering*, 33(2), pp. 131–141. [PubMed: 15771267]
38. Parekh A & Velegol D, 2007 Collagen gel anisotropy measured by 2-D laser trap microrheometry. *Annals of Biomedical Engineering*, 35(7), pp. 1231–1246. [PubMed: 17380393]
39. Pelham R & Wang Y, 1997 Cell locomotion and focal adhesions are regulated by substrate flexibility. *PNAS*, 94(25), pp. 13661–13665. [PubMed: 9391082]
40. Pizzo A et al., 2005 Extracellular matrix (ECM) microstructural composition regulates local cell-ECM biomechanics and fundamental fibroblast behavior: a multidimensional perspective. *J.Appl.Physiol*, 98(5), pp. 1909–1921. [PubMed: 15618318]
41. Puxkandl R et al., 2002 Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Phil.Trans.R.Soc.Lond.B*, Volume 357, pp. 191–197. [PubMed: 11911776]
42. Roeder B, Kokini K, Robinson J & Voytik-Harbin S, 2004 Local, three-dimensional strain measurements within largely deformed extracellular matrix constructs. *J Biomech Eng*, 126(6), pp. 699–708. [PubMed: 15796328]
43. Roeder B et al., 2002 Tensile mechanical properties of three-dimensional type I collagen extracellular matrices with varied microstructure. *Journal of Biomechanical Engineering*, 124(2), pp. 214–222.
44. Roeder B, Kokini K & Voytik-Harbin S, 2009 Fibril microstructure affects strain transmission within collagen extracellular matrices. *J Biomech Eng*, 131(3), pp. 031004–1–031004–11.
45. Rousseeuw P & Croux C, 1993 Alternatives to the Median Absolute Deviation. *Journal of the American Statistical Association*, Volume 88, pp. 1273–1283.
46. Sacks M & Sun W, 2003 Multiaxial Mechanical Behavior of Biological Materials. *Annu. Rev. Biomed. Eng*, Volume 5, pp. 251–284. [PubMed: 12730082]
47. Sander E, Stylianopoulos T, Tranquillo R & Barocas V, 2009 Image-based multiscale modeling predicts tissue-level and network-level fiber reorganization in stretched cell-compacted collagen gels. *PNAS*, 106(42), pp. 17675–17680. [PubMed: 19805118]
48. SAS Institute Inc, 2016 JMP 13 Basic Analysis. Cary, NC: SAS Institute Inc.
49. Schrader J et al., 2011 Matrix Stiffness Modulates Proliferation, Chemotherapeutic Response and Dormancy in Hepatocellular Carcinoma Cells. *Hepatology*, 53(4), pp. 1192–1205. [PubMed: 21442631]
50. Schriefl A et al., 2012 Determination of the layer-specific distributed collagen fibre orientations in human thoracic and abdominal aortas and common iliac arteries. *J.R.Soc.Interface*, Volume 9, pp. 1275–1286. [PubMed: 22171063]
51. Screen H, Lee D, Bader D & Shelton J, 2004 An investigation into the effects of the hierarchical structure of tendon fascicles on micromechanical properties. *Proc Inst Mech Eng H*, 218(2), pp. 109–119. [PubMed: 15116898]
52. Shayegan M & Forde N, 2013 Microrheological Characterization of Collagen Systems: From Molecular Solutions to Fibrillar Gels. *PLOS one*, 8(8), p. e70590. [PubMed: 23936454]
53. Shkumatov A et al., 2015 Matrix stiffness-modulated proliferation and secretory function of the airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*, 308(11), pp. L1125–L1135. [PubMed: 25724668]

54. Sun Y, Luo Z, Fertala A & An K, 2004 Stretching type II collagen with optical tweezers. *Journal of Biomechanics*, 37(11), pp. 1665–1669. [PubMed: 15388308]
55. Throm Quinlan A & Billar K, 2012 Investigating the role of substrate stiffness in the persistence of valvular interstitial cell activation. *J Biomed Mater Res A.*, 100(9).
56. Throm Quinlan A et al., 2011 Combining Dynamic Stretch and Tunable Stiffness to Probe Cell Mechanobiology in Vitro. *PLoS ONE*, 6(8), p. e23272. [PubMed: 21858051]
57. Tower T, Neidert M & Tranquillo R, 2002 Fiber Alignment Imaging During Mechanical Testing of Soft Tissues. *Annals of Biomedical Engineering*, Volume 30, pp. 1221–1233. [PubMed: 12540198]
58. Toyjanova J, et al., 2014 High Resolution, Large Deformation 3D Traction Force Microscopy. *PLoS ONE*, 9(4), p. e90976. [PubMed: 24740435]
59. Trepas X, Grabulosa M, Buscemi L & Rico F, 2003 Oscillatory magnetic tweezers based on ferromagnetic beads and simple coaxial coils. *Review of Scientific Instruments*, 74(9), pp. 4012–4019.
60. Trepas X et al., 2004 Viscoelasticity of human alveolar epithelial cells subjected to stretch. *Am J Physiol Lung Cell Mol Physiol*, Volume 287, pp. L1025–L1034. [PubMed: 15246973]
61. Vader D, Kabla A, Weitz D & Mahadevan L, 2009 Strain-induced alignment in collagen gels. *PloS one*, 4(6), p. e5902. [PubMed: 19529768]
62. Van Oosten A et al., 2016 Uncoupling shear and uniaxial elastic moduli of semiflexible biopolymer networks: compression-softening and stretch-stiffening. *Scientific Reports*, Volume 6, pp. 19270:1–9. [PubMed: 28442746]
63. Velegol D & Lanni F, 2001 Cell Traction Forces on Soft Biomaterials. I. Microrheology of Type I Collagen Gels. *Biophysical Journal*, 81(3), pp. 1786–1792. [PubMed: 11509388]
64. Voytik-Harbin S et al., 2003 Simultaneous mechanical loading and confocal reflection microscopy for three-dimensional microbiomechanical analysis of biomaterials and tissue constructs. *Microsc Microanal*, 9(1), pp. 74–85. [PubMed: 12597789]
65. Walton L et al., 2015 Morphological Characterisation of Unstained and Intact Tissue Micro-architecture by X-ray Computed Micro- and Nano-Tomography. *Sci Rep*, 5(10074), pp. 1–14.
66. Wells R, 2008 The Role of Matrix Stiffness in Regulating Cell Behavior. *Hepatology*, 47(4), pp. 1394–1400. [PubMed: 18307210]
67. Wenger M, Bozec L, Horton M & Mesquida P, 2007 Mechanical Properties of Collagen Fibrils. *Biophysical Journal*, Volume 93, pp. 1255–1263. [PubMed: 17526569]
68. White E, 2015 Lung Extracellular Matrix and Fibroblast Function. *Ann Am Thorac Soc*, Volume 12, pp. S30–S33. [PubMed: 25830832]
69. Xu B, Chow J & Zhang Y, 2011 Experimental and Modelling Study of Collagen Scaffolds with the Effects of Crosslinking and Fiber Alignment. *International Journal of Biomaterials*, pp. 1–12.
70. Zaman M, Trapani L & Sieminski A, 2006 Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *PNAS*, 103(29), pp. 10889–10894. [PubMed: 16832052]
71. Zeinali-Davarani S, Wang Y, Chow MJ, Turcotte R, & Zhang Y, 2015 Contribution of collagen fiber undulation to regional biomechanical properties along porcine thoracic aorta. *Journal of biomechanical engineering*, 137(5), 051001. [PubMed: 25612301]

Figure 1:

(a) Picture of the integrated optical magnetic twisting cytometry (OMTC) and a biaxial tensile tester; and (b) A tissue sample that is centered within the magnetic twisting coils of OMTC and loaded biaxially to allow the measurement of local mechanical properties while the sample is subjected to biaxial tissue loading.

Figure 2:

(a) Average Cauchy stress vs. stretch curves of adventitia tissue samples from biaxial tensile testing (n=5). (b) Average tangent modulus in both the circumferential and longitudinal directions (n=5). Data is presented as average $\pm$ standard error of the mean.

Figure 3:
 (a-e) Representative multiphoton images at equibiaxial stretches of 1, 1.05, 1.1, 1.15, and 1.2, and (f) fiber straightness parameter vs. equibiaxial stretch. Images are $425 \times 425 \mu\text{m}^2$. Data in (f) is presented as average $\pm$ standard error of the mean.

Figure 4: Normalized average fiber orientation distributions with equibiaxial stretches of (a) 1, (b) 1.05, (c) 1.1, (d) 1.15, and (e) 1.2. The number of fibers in each angle direction was normalized by the total number of fibers. Fibers oriented at 0° and $\pm 90^\circ$ are in the circumferential and longitudinal directions of the adventitia, respectively.

Figure 5:

(a) The magnitude of bead lateral displacement (μm) under biaxial loading of 20g (1.15 stretch). The bead lateral displacement is oriented in the arrow direction and the magnitude of the displacement is represented by the length of the arrow ($\times 100$). C and L represent the circumferential and longitudinal direction of the sample. Note that the beads are magnetized along the circumferential direction. (b) Histogram of displacement orientation distribution of beads in (a) with tri-modal distribution fitting (solid line). (c) Tri-modal fitting of bead displacement orientation distribution at different stretch levels. (d) Parameter π_2 versus stretch levels.

Figure 6:

Apparent complex modulus vs. stretch from OMTC measurements from bead displacements located within the major and minor peaks of the bead displacement orientation distribution.

Figure 7:

Representative distributions of apparent complex modulus of sample 1 at biaxial stretch levels of (a) 1.01, (b) 1.09, and (c) 1.17. The position of the median, used as a measure of the local mechanical properties in the OMTC experiment, is marked with a red line. (d) The median absolute deviation (MAD) of locally measured apparent complex modulus with increasing biaxial stretch for all adventitia samples.

Figure 8:

Apparent storage and loss modulus vs. tissue circumferential stretch (a) and tissue circumferential stress (b), and (c) phase angle vs. tissue circumferential stretch for all samples (S1-S5).

Figure 9:

Average tissue circumferential tangent modulus and local apparent complex modulus for all samples (S1-S5) vs. tissue circumferential stretch.