The Three-Dimensional Microenvironment of the Mitral Valve: Insights into the Effects of Physiological Loads

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Abstract—Introduction—In the mitral valve (MV), numerous pathological factors, especially those resulting from changes in external loading, have been shown to affect MV structure and composition. Such changes are driven by the MV interstitial cell (MVIC) population *via* protein synthesis and enzymatic degradation of extracellular matrix (ECM) components.

Methods—While cell phenotype, ECM composition and regulation, and tissue level changes in MVIC shape under stress have been studied, a detailed understanding of the three-dimensional (3D) microstructural mechanisms are lacking. As a first step in addressing this challenge, we applied focused ion beam scanning electron microscopy (FIB-SEM) to reveal novel details of the MV microenvironment in 3D.

Results—We demonstrated that collagen is organized into large fibers consisting of an average of 605 ± 113 fibrils, with a mean diameter of 61.2 ± 9.8 nm. In contrast, elastin was organized into two distinct structural subtypes: (1) sheet-like lamellar elastin, and (2) circumferentially oriented elastin struts, based on both the aspect ratio and transmural tilt. MVICs were observed to have a large cytoplasmic volume, as evidenced by the large mean surface area to volume ratio 3.68 ± 0.35 , which increased under physiological loading conditions to 4.98 ± 1.17 .

Conclusions—Our findings suggest that each MVIC mechanically interacted only with the nearest 3–4 collagen fibers. This key observation suggests that in developing multiscale MV models, each MVIC can be considered a mechanically integral part of the local fiber ensemble and is unlikely to be influenced by more distant structures.

Keywords—Heart valves, Ultrastructure, Valve interstitial cells, Extracellular matrix, Collagen, Elastin.

INTRODUCTION

Though the function of different connective tissue types varies widely, their general structural layout remains highly conserved whereby connective tissue cells are embedded in and surrounded by dense extracellular matrix (ECM). The composition and structural organization of the ECM, particularly collagen, the main structural component in load bearing tissues, plays a critical role in the functional properties of dense collagenous tissues (e.g., tendon, ligaments, heart valves). Of the four heart valves, the mitral valve (MV) is subjected to the greatest hemodynamic forces and its structure underscores this. MV leaflets consist of four histologically distinct layers: the *atrialis* and the *ventricularis*, both of which are thin elastin-rich layers, the *spongiosa*, which consists mainly of proteoglycans (PGs) and glycosaminoglycans (GAGs), and the *fibrosa*, the main load-bearing layer, composed of circumferentially oriented collagen fibers. Though the layers are histologically distinct, they are not functionally distinct.⁸ As such, heart valve leaflets, such as the MV anterior leaflet, can be treated as a functionally graded material with properties that vary continuously across their thickness.^{8,52}

The structure–function relationship between valve interstitial cells (VICs) and the surrounding ECM enables the valvular tissues to maintain their homeostatic state under different hemodynamic and biomechanical environments. While the ECM provides the tissue with its structural integrity and influences cellular processes through matricellular, matricrine, and mechanical processes, it is the VICs residing within the valve's four layers that maintain the structural integrity of the leaflet tissue through protein synthesis and enzymatic degradation.⁷⁰ These cells are thus critical to the remodeling demands of the valve and tissue homeostasis.^{3,50,53} In the MV, the interaction of mitral VICs

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(MVICs) with the surrounding ECM,² particularly the collagen fibrils, is fundamental in modulating cellular response (Fig. 1). The ECM can transmit external mechanical stimuli, such as stretch, shear stress, and pressure, to the cell *via* integrins and other membrane-bound protein receptors.^{1,69} We have shown that while MVICs have the same stiffness and phenotypic state across all layers, their deformations vary considerably during loading due to differences in layer-specific ECM structure,^{10,33} highlighting the importance of the VIC/ECM coupling in the mechanoregulation of these cells.

Detailed knowledge of heart valve microstructure remains essential in understanding the structure–function relationship of heart valves and their remodeling under altered stress and diseased conditions. We and other groups^{33,35,76,77} have used multiscale-modeling methodologies to understand and characterize the dynamics of healthy and diseased heart valves. Yet, such approaches are limited by the lack of detailed three-dimensional (3D) microscopic structural information, which is especially important given the heterogeneous complexity of the VIC/ECM microenvironment. To date, histomorphometry techniques have provided a wealth of information on heart valve composition.^{22,59,60,64,75,79} When combined with the characterization of the mechanical behavior, such approaches have shed light on the structure–function relationship of the major ECM components.^{10,68} Light microscopy and two-photon microscopy have also provided important insights; yet, these imaging modalities are limited by their penetration depth and resolution. In these cases, complex features can easily be misinterpreted from the two-dimensional (2D) images. Moreover, detailed assessments of the microenvironment made from histological sections are

prone to inaccuracies due to assumptions that must be made, leading to unclear descriptions of heart valve microstructure. 3D methods are thus, an essential requirement for a full understanding of the valvular microenvironment.

Micro- and nano-scale 3D visualization has always been a challenge in the life sciences. Though multiple strategies have been used, electron microscopy (EM) has played a prominent role. While transmission electron microscopy (TEM) provides sufficiently high resolution, it results in a 2D representation. Scanning electron microscopy (SEM) provides surface characteristics and morphology, but cannot provide information on the 3D microenvironment of the sample that is imaged since the focus is solely on surface topology. As such, TEM has been used to assess the cellular and sub-cellular structures of heart valves in 2D, whereas SEM provides adequate surface morphology of the valve leaflet.² Serial sectioning TEM techniques can be used for 3D reconstructions; however, these techniques are labor-intensive and time-consuming. Artifacts arising from stain contamination, knife marks, or loss of sections are ubiquitous and can obstruct or conceal features of interest, thereby severely hindering reconstruction quality.¹⁹

Previous studies on the VIC microenvironment have been primarily two-dimensional, using light and confocal microscopy, and 2D TEM. These studies focused on the geometry of the nucleus, and were not able to capture the complexity of the surrounding fibrous microenvironment.^{10,26,33,70} The accurate visualization and quantification of valvular microstructure will allow us to have a better understanding of heart valve micromechanics and its role in VIC mechanobiology. This is especially important in the incorporation of the

FIGURE 1. (a) Transmission electron microscopy of the mitral valve interstitial microenvironment. The arrows represent specific components of the microenvironment, black: collagen fibrils, blue: amorphous elastin, red: mitral valve interstitial cell. (b) Scanning electron microscopy of the collagenous microenvironment highlighting the interconnection between valve interstitial cells and the collagen fibrils. *T* Transmural, *C* Circumferential, *R* Radial. Scale bars: 1 μm .

heterogeneous valvular microstructure into computational models that truly replicate the native valvular microenvironment.

The desire to understand the 3D relationships of meso-scale hierarchies has led to the development of advanced microscopy techniques, such as focused ion beam scanning electron microscopy (FIB-SEM) and serial-block face SEM, that have made it possible to acquire high-resolution 3D ultrastructural information.^{15,45} FIB-SEM is a powerful microscopy tool that enables the acquisition of serial images of a specimen surface with minimal sample movement: the gallium ion beam mills into the bulk of a specimen, exposing the deeper regions of the material, which are then imaged with an SEM backscatter or secondary electron detector. Automation of these steps yields large stacks of images that can be reconstructed into 3D renditions.¹⁹ Though this imaging modality has been used for circuit edits and mask repair in the semi-conductor industry for decades, its benefits were not exploited in biological tissue applications until more recently.⁹ When equipped with a high-performance SEM where osmium-stained structures can be visualized, FIB-SEM can be a promising tool for 3D reconstructions of cellular architectures.^{14,31,37} Several groups have made use of this tool to gain insights into lamellar bone,⁵⁴ osteocyte lacuna-canalicular networks,^{62,63} the aortic medial lamellar unit,⁴⁵ atherosclerotic tissue,²⁴ bone and dental implants,¹⁹ human dentin,⁴³ infected erythrocytes,⁴⁰ and cell/scaffold interactions.³¹

In this work, we utilized FIB-SEM to examine the 3D mitral valve microenvironment, at a level not achieved in previous studies, to identify major structures and how they change under physiological loads. This approach allowed us to gain subsequent insight into the role of the changes in MVIC microenvironment with MVIC deformation and to have a better understanding of how tissue-level deformations are transduced to individual cells in the collagenous microenvironment.

RESULTS

Three-dimensional (3D) reconstructions from serial FIB-SEM images allowed us to quantify the MVIC

microenvironment while shedding light on its highly complex 3D nature. We observed that MVICs were not neatly arranged within the microenvironment as ellipsoid, but are rather intimately connected to the surrounding ECM structures through cytoplasmic protrusions and elongations. These volume reconstructions also revealed interconnections of MVICs with the collagen, as well as elastin's complex structure. To quantify changes that occur in the microstructure at physiological load levels, we determined the respective volume fractions of the MVICs, elastin, and collagen in the unloaded state and in the estimated peak physiological load states (0 and 150 N/m equibiaxial membrane tension, respectively). We observed that while the field-of-view volume fraction of collagen increased in the loaded configuration (43–72.7%), volume fractions of VICs and elastin decreased, from 7.81 to 5.54 and 4.57 to 1.25% respectively (Table 1). This analysis indicates that there is increased collagen fiber compaction under loading.

FIGURE 2. Representative 2D image from the serial stack of images acquired from the focused ion beam scanning electron microscope. This image highlights the quality and contrast that can be obtained from this imaging modality. Individual collagen fibrils are discernible, as well as the elastin, VIC cytoplasm, and VIC nucleus. *T* Transmural, *R* Radial. Scale bar: 1 μm .

TABLE 1. Characterization and quantification of the mitral valve interstitial cells and their surrounding microenvironment.

	Volume fraction per field of view (%)			Surface area to volume ratio		% total elastin	
	VIC	Collagen	Elastin	Cytoplasm	Nucleus	Sheet-like elastin	Elastin strut
Unloaded	7.81	43.0	4.57	3.68 ± 0.35	1.68 ± 0.11	58.0	39.2
Loaded	5.54	72.7	1.25	4.98 ± 1.17	2.17 ± 0.23	100	0
<i>p</i> value	–	–	–	0.0146	0.0246	–	–

FIGURE 3. A 3D reconstruction of the mitral valve interstitial cell microenvironment highlighting distinctive collagen fibers consisting of tightly packed fibrils with closely intertwined elastin structures. Reconstruction of 30 serial sections (total thickness = $1.50\ \mu\text{m}$) with a slice thickness of $50\ \text{nm}$ and a horizontal field width of $20.7\ \mu\text{m}$. Scale bar: $0.5\ \mu\text{m}$.

FIGURE 4. 3D reconstruction of the collagen microenvironment in both the unloaded and loaded configurations. In the unloaded configuration, collagen fibrils are organized into distinct bundles, also referred to as collagen fibers. In this reconstruction, we can clearly see two collagen fibers. The fibrils lose this organization in the loaded configuration and instead combine into a large fibrillar population. Scale bars: $0.5\ \mu\text{m}$.

Collagen Fiber/Fibril Characterization

Various studies have quantified the effect of physiological loads on valvular collagen.^{4–6,59,71} These studies, however, focused on changes that occur at the meso-scale, defined as less than $100\ \mu\text{m}$. A closer examination of the collagen network, however, suggested that the collagen and elastin fiber populations were structurally more complex and require more detailed analyses of their fibrillar components (Fig. 1). To characterize the fibrillar microenvironment and

gain a deeper understanding of its behavior under physiological loads, we developed an electron microscopy protocol that allows us to acquire consecutive high-resolution images (Fig. 2) of the MV ECM, including collagen and elastin and create three-dimensional reconstructions (Fig. 3). A novelty of this work thus lies in the ability to discern and visualize in 3D the individual MV collagen fibrils (Figs. 2 and 3). In line with previous work on soft collagenous tissue,^{17,18,55,56,74} collagen fibers in MV anterior leaflets

were found to be highly aligned in the circumferential direction, with tightly packed parallel fibrils that form fibers. In the unloaded configuration, collagen fibers had an average surface area of $227 \pm 47 \mu\text{m}^2$. Each fiber in turn consisted of an average of 605 ± 113 fibrils, with an average fibrillar diameter of $61.2 \pm 9.8 \text{ nm}$. The inter-fibrillar distances allowed us to quantify the fibril packing density in each collagen fiber. In the unloaded configuration, inter-fibrillar distance is equal to $79.0 \pm 11.6 \mu\text{m}$. In the loaded configuration, the collagen microenvironment appears to consist mainly of fibrils, whereby the fiber/fibrils are

so packed that they cannot be distinguished (Figs. 4 and 5).

Elastin: First Evidence of Structural Subtypes

Though many studies have focused on the characterization of valvular microenvironment, including the microstructure of the elastin population,^{65,66} the detailed ultrastructure of elastin in heart valves remains elusive. It is important to note that although elastin may not be prominent in the fibrosa layer, it has been observed in small amounts in this layer.^{2,33} In the

FIGURE 5. Volume rendition of the 3D collagen microenvironment in the loaded and unloaded configurations. This reconstruction highlights the packing of the collagen fibrils under physiological loading. Reconstruction of 77 serial sections (total thickness = $15.4 \mu\text{m}$) with a slice thickness of 200 nm and a horizontal field width of $53.6 \mu\text{m}$. Scale bar: $5 \mu\text{m}$.

FIGURE 6. 3D reconstruction of the elastin population highlighting its distinct ultrastructure in the valvular leaflet. In the unloaded configuration, valvular elastin appears to exist in two sub-types, long sheets resembling lamellae (sheet-like/lamellar elastin) and circumferentially-oriented elastin struts. Yellow arrows highlight pores within the sheet-like/lamellar elastin. Scale bar: $0.5 \mu\text{m}$.

present study, we found that the elastin volume fraction in the field of view was significantly lower than that of the collagen. Only 4.57% of the volume consists of elastin the unloaded configuration, whereas collagen takes up 43%. Interestingly, the 3D volume renditions of the elastin population allowed us to identify two distinct elastin structures (Fig. 6): (1) sheet-like elastin that resembles lamellar elastin and (2) circumferentially-oriented elastin struts.

Sheet-like elastin forms smooth continuous sheets that extend along the length of the radial direction with some protrusions that turn into circumferentially oriented elastin struts. These sheets are fibrous in nature with some containing intricate pore-like indentations through their thickness. In the current data set, no VICs protrude through the pores present in the elastin. The circumferentially oriented elastin struts are thick chord-like structures that branch from the long sheet-like elastin present in the microenvironment. In the unloaded configuration, 58% of the elastin consists of sheet-like elastin and 39.2% consists of the circumferentially oriented struts. In the loaded configuration, the elastin population loses the pores that exist in the sheet-like elastin as well as the circumferentially oriented structural subtypes and instead, forms one large, continuous sheet of elastin (Fig. 7).

Mitral Valve Interstitial Cells

3D volume renditions from FIB-SEM provide new insights on VIC morphology and their interaction with the surrounding microenvironment. The reconstructions that includes both cell and microenvironment show that MVICs are tightly intertwined with both the collagen as well as elastin populations (Fig. 8). Moreover, it is clear from the 3D reconstructions that the shape of the MVIC is not a simple ellipsoid, as it is often assumed,^{10,26,33,70} but rather a much more complex shape with protrusions and extensions (Fig. 8). Based on the 2D images and the volume renditions, the nucleus is much more regular in shape than the cytoplasm/cell membrane. The cytoplasm weaves in and around the collagen bundles to different levels, whereas the nucleus has a shape that is more regular in both the loaded and unloaded configurations. When loaded, the volume fraction of the VIC dropped from 7.81 to 5.54% (Fig. 9) and the collagen takes over the field of view as the tissue is strained (Fig. 9a). Measurements of the surface area to volume ratio for both nucleus and cytoplasm provided insights into cell mechanotransduction and cell response to mechanical stimuli (Fig. 10). The surface area to volume ratio of both the cytoplasm and nucleus increased significantly under physiological loading: for the cytoplasm, the ratio increases from 3.68 ± 0.35 in the unloaded con-

FIGURE 7. Elastin ultrastructure changes under physiological loading as shown by these volume renditions of the mitral valve anterior leaflet in the unloaded and loaded configurations. Sheet-like/lamellar elastin and circumferentially-oriented elastin struts appear to have distinct structures in the unloaded configuration. The elastin in the loaded configuration is present as a long sheet-like structure and loses the sub-structures that exist in the unloaded configuration. Scale bars: 5 μm .

FIGURE 8. 3D reconstruction of the valve interstitial cell and its surrounding microenvironment capturing the previously unrecognized complexity of the interconnection between the cell and the surrounding extracellular matrix with a high level of detail. Reconstruction of 77 serial sections (total thickness = $15.4\ \mu\text{m}$) with a slice thickness of $200\ \text{nm}$ and a horizontal field width of $53.6\ \mu\text{m}$. Scale bar: $5\ \mu\text{m}$.

FIGURE 9. 3D reconstruction of the loaded ($150\ \text{N/m}$) mitral valve anterior leaflet microenvironment (a) with the collagen, elastin, and mitral valve interstitial cell nucleus and cytoplasm, and (b) with only the VIC nucleus and cytoplasm included in the rendition. Reconstruction of 160 sections (total thickness = $40\ \mu\text{m}$) with a slice thickness of $250\ \text{nm}$ and a horizontal field width of $50.9\ \mu\text{m}$. This reconstruction highlights the packing of the collagen fibrils into one large fibrillar population under loading instead of distinctly organized collagen fibers. Scale bars: $2\ \mu\text{m}$.

FIGURE 10. Mitral valve interstitial cell deformation under physiological loading. Volume renditions of the cytoplasm and nucleus in the unloaded and loaded configurations underscore that changes that occur in the microenvironment are sensed by the cell, which deforms accordingly. Scale bars: 1 μm .

figuration to 4.98 ± 1.17 in the loaded configuration ($p = 0.0146$). For the nucleus, the surface area to volume ratio increased from 1.68 ± 0.11 to 2.17 ± 0.23 ($p = 0.0246$).

DISCUSSION

Light microscopy and histology represent the current state-of-art techniques used to characterize soft tissue in general, and heart valve microstructure in particular. These techniques resolve micro-scale features, such as collagen fibers, MVIC nuclei, and the overall distribution of microenvironment components. Prior work has focused largely on quantifying VIC deformation using light and confocal microscopy techniques.^{3,10,26,33,53,70} However, little is known about the ultrastructure of the VIC microenvironment, particularly the elastin. This is, to the best of our knowledge, the very first time that the VIC microenvironment has been quantitatively assessed in 3D at the ultrastructural scale using the FIB-SEM modality.

The techniques utilized in this work allowed us to capture previously unknown MVIC geometry and surrounding microenvironment with a high level of detail. FIB-SEM fully preserves the powerful function of 2D electron microscopy techniques, such as TEM and SEM, and extends them into the third dimension. An advantage of serial electron microscopy such as FIB-SEM over single section TEM and other microscopy techniques is that it allows us to track individual collagen fibrils. The combination of TEM, SEM, and FIB-SEM allowed us to speed up the milling process

and focus the data acquisition on more relevant regions of the tissue, for example, regions that contain both collagen and elastin along with the MVIC. Furthermore, the high resolution allows us to recognize collagen fibrils and to delineate cytoplasm and MVIC nuclei. The FIB-SEM volume renditions presented in this work substantiate previous findings that use histology and confocal microscopy, but also reveal new knowledge, such as the existence of different elastin structures and emphasize the highly complex collagen/MVIC coupling. This study highlights the previously unrecognized complexity of the interconnection between VICs and ECM fiber networks and enables us to develop more structurally accurate computational models that incorporate the heterogeneous complexity of the mechanobiologically stimulated microenvironment (Videos 1 and 2).

Collagen and Elastin Microenvironment

There are extant data on the collagenous microenvironment of heart valve leaflets, most of which emphasize that collagen fibers exhibit a microfibrillar sub-structure.^{49,57} Our group has used small angle x-ray scattering (SAXS) to study collagen fibril structure and deformation under loading.³⁶ These measurements are restricted to collagen fibrils only and are not affected by the presence of elastin fibers and other tissue components. In this work, we found that the collagen fibrils are tightly bounded and deform as a single fiber-level unit and that at the homogenized tissue-level scale of ~ 1 mm, the collagen fiber network in the MVAL deforms according to an affine kinematics model.³⁶

Collagen fibers are oriented parallel to the loading direction in the highest loaded physiological structures, such as tendon and ligaments, highlighting the importance of parallel arrangement in high loading conditions. In heart valves, collagen is oriented mainly in the circumferential direction to support applied loads. Although the existing data provides important information on valvular microstructure, little information exists on collagen/VIC interactions and fibrillar dimensions.

In this work, we characterized the collagen microenvironment and concluded that the VIC does not sense all collagen fibers, but rather only a small subset of fibers (Fig. 11). This finding provides evidence that the cell is only sensing the local fiber ensemble^{33,79} and can be used to improve the way that both the VIC and collagen are represented in computational models. In some of our more recent work,^{7,16,27–29,51,58} we showed the profound impact of incorporating internal tissue microstructure in developing high-fidelity computational models of the MV. As such, the characterization of the microstructure of the MV and other valves is necessary to create models that can predict tissue- and cell-level behavior. The quantification of the fibril/fiber populations that was performed in this study will allow us to more accurately depict the VIC microenvironment in our current and future computational modeling efforts.

Though some groups have characterized valvular elastin by enzymatic isolation,^{65,66} its structure and

micromorphology in heart valves remains poorly understood. A more recent study has characterized elastin structure in the aortic lamella, particularly the existence of different elastin subtypes: lamellar elastin, elastin struts, and interlamellar elastin fibers.⁴⁵ Similar to the aortic medial lamellar unit, valvular elastin exists in two main structures: sheet-like/lamellar elastin and circumferentially oriented elastin struts. Although some structures in the volume renditions resembled the interlamellar elastin fibers of the aortic medial lamellar unit that were characterized by O'Connell *et al.*⁴⁵ using serial block-face scanning electron microscopy, the structures observed in our work are most likely artifacts of image segmentation and smoothing, and not actual representations of this particular sub-type of elastin.

MVICs

Our measured FIB-SEM VIC volume agreed well with previous studies with relatively consistent proportions of cytoplasm and nuclei and a nuclear aspect ratio that is ~ 2.5 in the unloaded state.^{10,33} The findings from this study provide important insights on VIC mechanotransduction mechanisms. The elongation of the nucleus and increase in surface area to volume ratios of both nucleus and cytoplasm suggest that forces applied to the cytoplasm are transferred to the nucleus. Since the cytoplasm of the cell contains cytoskeletal matrices that transmit forces from the

FIGURE 11. Mitral valve interstitial cell immediate environment. Top: 2D transmission electron micrographs, Bottom: tracings of MVICs and collagen bundles (dark red: MVIC nucleus, red: MVIC cytoplasm, blue: collagen fibers). This data ($n = 4$ cells) underscores that each MVIC is micromechanically influenced by the nearest 3–4 collagen fibers. Scale bars: 1 μm .

ECM to the nucleus, the stress on the surface of the cell's cytoplasm is most likely linked to the cell's biosynthetic responses.

The FIB-SEM modality allowed us to preserve the significant features of conventional 2D electron microscopy techniques, such as TEM and SEM, while extending to 3D results. This work thus provides a new perspective of heart valve microstructure and provides the first high resolution 3D image of this tissue type. The characterization of these images clarified the complex interrelated architecture of collagen, MVICs, and elastin. As such, this work emphasizes that structure is important to the overall material performance of heart valves in a mechanically demanding environment.

Limitations

The FIB-SEM imaging modality and post-processing pipeline used in this study are based on well-established approaches and their accuracy has been demonstrated by several groups.^{39,44,46,72,73} The effective resolution and has been reviewed in detail,^{25,30,42,44,61,72,73} whereby 10 nm-sized gold particles and quantum dot particles with 7 nm-sized cores were detected in single cross-sectional images (6 nm lateral, with a z-slice thickness of 20 nm). Though FIB-SEM has provided us with significant insight on the MV microenvironment, it does have limitations. To work around the tradeoff that exists between volume of interest (VOI) and resolution, we acquired two datasets: 1) a high-resolution dataset that focused on the collagen fibrils, and 2) a lower-resolution data set that captured the entire MVIC in 2D as well as the surrounding microenvironment. Due to the tradeoff between the size of the VOI and the resolution, the FIB-SEM volume renditions were not complete reconstructions of the full MVIC and its surrounding microenvironment, but rather a portion of it. Due to this limitation, we were not able to use absolute volume as a metric to characterize VIC size and instead used volume fractions to quantify relative amounts of each constituent. System instabilities, such as beam shift, sample drift, changes in focal depth, and irregular slicing step, are usually accentuated with larger VOIs and longer slicing/milling cycles.⁶² These drawbacks can reduce image resolution and thus, hinder the automatic segmentation. Furthermore, biological materials, such as soft tissue, are usually more prone to charging, leading to reduced image quality and incorrect milling positioning. Like other soft tissues, heart valve leaflets are heterogeneous materials, whereby the ECM is denser in certain areas and less dense in others. Due to the heterogeneous nature of these samples, milling rate tends to vary from cycle to cycle and can

thus, lead to inconsistent slice thickness. It is important to note that this technique, including both tissue preparation and the FIB-SEM milling/imaging cycles, can be quite lengthy. 3D information of a specific cell/region can be obtained by performing a series of slice-and-view cycles, which can take up to 20 h depending on the size of the VOI. This said, it remains a significant improvement from the conventional ultramicrotomy methods that are much more labor intensive and more prone to error. Clearly, such morphological studies must be carried out on many samples to obtain statistically reliable microstructural information, which may be logistically challenging using the FIB-SEM modality. Though limited by the small sample size, we view our work as an important step to more detailed quantitative studies on the heart valve microenvironment in the future.

Conclusions and Implications

This work provided key insights into the MV microenvironment under physiological loading. We demonstrated that, as in other dense connective tissues, MV collagen is organized into large fibers, with an average surface area of $227 \pm 47 \mu\text{m}^2$, each consisting of an average of 605 ± 113 fibrils with a mean diameter of 61.2 ± 9.8 nm. Interestingly, we observed that elastin was organized into two distinct structural subtypes (sheet-like lamellar elastin and circumferentially oriented elastin struts) and revealed that MVICs intimately integrated within the surrounding collagen fibers and elastin interlamellar networks through their cytoplasmic extensions. Even at this very local level, a predominantly circumferential orientation of the collagen, elastin, and MVICs was noted, correlating well with reported values of physiological stress, collagen recruitment, and MV leaflet biomechanical behavior.

Perhaps our most interesting finding was that each MVIC was micromechanically directly in contact only by the nearest 3–4 collagen and elastin fibers, and is unlikely to be influenced by more distant structures (Fig. 11). Thus, each MVIC can be considered a mechanically integral part of a single fiber ensemble, which is defined as a collection of fibers sharing a common orientation,³² and has been used in MV tissue^{36,79} and multiscale models.^{33–35} Such approaches can provide key insights into the relations between alterations in organ-level stresses and MVIC mechanotransduction. Such approaches will form the basis for accurate computational models of MV remodeling due to disease and therapies.

Our findings can also be extended to various clinically relevant scenarios, such as surgical repair and myxomatous valve disease. In surgical repair, for example, under-loading or over-loading of the leaflets

can lead to surgical repair failure over time.^{3,20} This is largely due to cellular response and increased VIC biosynthetic activity.^{12,13,38} Degenerative diseases, on the other hand, are known to cause an increase in PGs and GAGs.^{11,21,47,48} Though the technique used in this work does not identify these specific ECM structures, the results do emphasize the importance of elastin and collagen in VIC response to loading, suggesting that in degenerative valves, alterations in collagen and elastin structures would lead to different mechanoregulation by the VICs. These findings can also be applied to tissue engineering of heart valves; results from this work are vital for understanding native tissue microstructure and can provide valuable information to emulate the native heart valve structure-function.

EXPERIMENTAL PROCEDURES

Biaxial Stretch Device Development

Current cyclic stretch bioreactors prescribe strains in one direction only^{4,5,41,59}; however, this does not fully replicate the micromechanics of the *in vivo* MV environment, which impose strains on both radial and circumferential directions.²³ To fully elucidate MV response to stress overload under an *in vitro* setting, we developed a tissue culture system that imposes biaxial loading to replicate physiological *in vivo* valvular strains. In brief, a biaxial stretch device was developed to replicate the complex physiological biaxial strains of the MV. The design consists of four linear actuators and two load cells, one on each axis. A four-pillared stacking system in the middle of the system holds the specimen chamber, which is made of wear-resistant and temperature-stable polysulfone. A pin attachment system is used to mount the tissue inside the specimen chambers. The system can be used as either a strain- or load-controlled device and allows the operator to modulate strain, strain rate, and the membrane tension along each axis.

Sample Preparation for Electron Microscopy Imaging

Fresh porcine hearts were collected from young hogs (10 months, 250 lbs.) from a local USDA approved abattoir (Harvest House Farms, Johnson City, Texas) within 30–45 min of slaughter. MV anterior leaflets (MVALs) were isolated on-site and submerged in ice-cold phosphate buffered saline (PBS) (Thermo Fischer Scientific, Waltham, MA) for transport to the laboratory. Once in the laboratory, MVALs were cut into 15 mm x 15 mm squares and mounted in the biaxial bioreactor specimen chamber while keeping track of the radial and circumferential directions. Samples were fixed in Grade II EM Grade Glu-

taraldehyde (Electron Microscopy Sciences, Hatfield, PA) for 4 h at either 0 N/m or 150 N/m equibiaxial tension ($n = 3$ per group). Samples were then prepared for TEM imaging using a modified version of the protocol by Starborg *et al.*⁶⁷ In brief, fixed samples were stained with osmium tetroxide for 4 h, dehydrated with alcohol and acetone, infiltrated in epoxy resin overnight, and cured for 48 h in a 60 °C oven. The resulting resin blocks were trimmed with a razor blade into a trapezoid block face such that the y-axis represents the transmural axis and the x-axis represents the radial axis of the MVAL.

Transmission (TEM) and Scanning Electron Microscopy (SEM) Imaging and Mapping

A test thick section (1 μm) was sectioned, stained with toluidine blue, and imaged using a light microscope to check for the presence of tissue in the area of interest and to also guide subsequent thin sectioning for TEM. The block face was then trimmed and smoothed using a Diatome Trimtool 45° (Diatome Diamond Knives, Hatfield, PA) set at 500 nm. Thin sections (70 nm) were cut using a Diatome Ultra Diamond Knife 35° (Diatome Diamond Knives, Hatfield, PA), picked up with Formvar-coated slot grids (Electron Microscopy Sciences, Hatfield, PA), and imaged with an FEI Tecnai TEM (FEI, Hillsboro, OR). To create a sample map that can be used to guide the FIB-SEM milling and imaging, TEM imaging was carried out at low-magnification and throughout the entire section (representing the *en face* surface of the block). The resin-embedded blocks were then mounted on low profile 45° mounts (Ted Pella, Inc., Redding, CA), with the longer side (radially oriented) adhered to the mount with double coated carbon conductive tape (Ted Pella, Inc., Redding, CA) and the block face facing up at a 45°. Mounts were coated with carbon paint on the corners, sputter coated with 15 nm of Pt/Pd using a Cressington 208 Benchtop Sputter Coater (Cressington, Watford, UK), and imaged with a Zeiss Supra 40 V SEM (Carl Zeiss AG, Oberkochen, Germany) to ensure that the *en face* surface is smooth for FIB milling.

Focused Ion Beam Scanning Electron Microscopy (FIB-SEM)

FIB-SEM was performed at the Rice University Electron Microscopy Center. The technique has been described in the past⁹ and used extensively in the study of soft tissue.^{14,24,31,37,45} The 45° aluminum mount that contains the sample was placed in the FEI Helios NanoLab 660 DualBeam (FEI Company, Hillsboro, OR) system for block-face milling and imaging. Based

on the questions that are asked in the study, a decision must be made between imaging a large area/volume with low resolution or a smaller area/volume with a larger resolution. To work around the tradeoff that exists between volume/area of interest and resolution, we chose to acquire two datasets for each sample: the “Microenvironment Only” (MO) group focused on the collagen fibrils at a higher resolution and thus, had a slice thickness of 50 nm and a horizontal field width (HFW) of 20.7 μm for the unloaded sample (0 N/m) and a slice thickness of 10 nm and HFW of 11.4 μm for the loaded sample (150 N/m equibiaxial membrane tension). The “Whole Cell” (WC) group captured the MVIC as well as its surrounding microenvironment: the slice thickness was equal to 200 nm and the HFW was 53.5 μm for the unloaded sample and a slice thickness of 250 nm and HFW of 49.1 μm for the loaded sample.

All samples underwent cross-section milling, also referred to as ‘rough milling’, to prepare the flat surface that will be used for serial milling and imaging. Large FIB beam currents were first used to mill this initial cross-section and smaller currents were subsequently used to polish its surface. For both the MO and WC groups, rough milling was performed for 30 min with a current of 2.5 nA and a voltage of 30 kV. The dimensions of the cross-section were as follow: $x = 50.0 \mu\text{m}$, $y = 2.0 \mu\text{m}$, $z = 10.0 \mu\text{m}$. FEI’s *Auto-Slice-and-View* (ASV) software package (FEI Company, Hillsboro, OR) was then used to automatically collect serial SEM images from the freshly milled surface after sequential FIB milling and SEM viewing, yielding a stack of images for subsequent 3D reconstructions and volume renditions. The ASV procedure has been described in detail.⁷⁸ In brief, a fiducial marker was milled into the top corner of the sample and recorded into the program to allow accurate stage positioning at each new thickness level and to decrease stage drift after each milling step. For the ion beam, a current of 2.5 nA and voltage of 30.0 kV were used. For the electron beam, the parameters were as follow: image resolution = 1536 x 1024, dwell time = 10 μs , and frames = 1. The options for Auto Contrast & Brightness, Auto Focus, and Auto Alignment were enabled.

Image Processing

We developed a pipeline to process the acquired images and in turn perform 3D volumetric characterization and analysis of the VIC microenvironment. First, the local intensity contrast in the images was improved by applying contrast limited adaptive histogram equalization. This procedure eliminated spurious brightness in regions that were over-exposed during imaging while minimally affecting regions with

uniform intensity. To eliminate noise, a bilateral filter was applied with sigma color = 0.05, sigma spatial = 15, and multichannel = False. The processed images were then imported in ImageJ (National Institutes of Health, Bethesda, MD) to register the stack of 2D images per each dataset and consolidated them into a 3D image. In brief, the StackReg functionality in ImageJ was applied to align images by minimizing the least square distance between the sequential images in each stack. Lastly, to segment the constituents of VIC microenvironment, 3D images were loaded into the ScanIP software package (Synopsys, Mountain View, CA) and *collagen*, *elastin*, *VIC cytoplasm*, and *VIC nuclei* were extracted for each dataset by empirically thresholding image intensity fields.

Collagen Fiber/Fibril Morphometry

The MO dataset was used to characterize the microenvironment. As such, the analysis focused on volume and surface areas of collagen and elastin, as well as the packing density, average number of fibrils, and diameter of collagen fibers and fibrils. The WC dataset was used for analysis of cell dimensions and interaction of the cell with the surrounding microenvironment. Collagen fibril and fiber dimensions were measured from the MO data unloaded and loaded data sets on Simpleware ScanIP software package (Synopsys, Mountain View, CA, USA). Measurements were carried out on five randomly selected 50 nm thin slices (Slices 0, 8, 16, 27 and 37) and averaged linearly. Fibrillar diameter was measured as the edge-to-edge distance passing through the center of the fibril. Measurements were carried out for $n = 5$ fibrils/fiber for $N = 3$ bundles. The fiber surface area was automatically computed on ScanIP for the different segmented masks. Areas of interest (i.e., three different collagen fibers) were chosen and then fiber surface area is computed. The surface area was measured for $n = 3$ collagen fibers. The number of fibrils per fiber were calculated by assuming that fibrils have a long, thin, cylindrical shape. The equation for the surface area of a cylinder (Eq. 1) and the measured fibrillar diameter were used to calculate the surface area of each fibril:

$$A = 2\pi rh + 2\pi r^2 \quad (1)$$

The height, h , is equal to slice thickness, in this case, 50 nm multiplied by the number of slices. To find the total number of fibrils per fiber, measured fiber surface area was divided by the calculated fibrillar surface area ($n = 3$ collagen fibers). Inter-fibrillar distance represents the distance from one fibrillar center to the next neighboring fibrillar center. Measurements were carried out for 15 different fibrils across 5 slices.

Elastin Morphometry

Volume renditions of the WC data sets for the loaded (total thickness: 15.4 μm) and unloaded (total thickness: 40 μm) configurations were used to measure elastin feature morphology in the MVIC microenvironment. Circumferential tilt angle was used to designate the elastin structures into either sheet-like/lamellar elastin or circumferentially oriented elastin struts, whereby a circumferential tilt angle that is above 60° represents sheet-like/lamellar elastin. Percentage volume of total elastin were computed directly from ScanIP by selecting regions of interest in the reconstruction that represent either the sheet/lamellae-like elastin or circumferentially oriented elastin struts. For the sheet/lamellae-like elastin, minor axis length measurements were carried out on $n = 12$ structures and circumferential tilt measurements on $n = 14$ structures. For the elastin struts, minor axis length measurements were carried out on $n = 7$ structures and $n = 16$ for the circumferential tilt.

Quantification of Valve Interstitial Cell Structure and Microenvironment

Volume fraction of different ECM constituents per field of view were measured using the WC data set volume renditions using ScanIP. VIC radial tilt measurements were done for $n = 1$ cell for both the unloaded and loaded sample. Due to limitations of the technique, only one cell was captured for each loading configuration. Since cytoplasm is much larger than the nuclei and took up more surface area, more cytoplasm were captured in the imaging. As such, the sample size for cytoplasm was larger than that of nuclei. Elastin radial tilt measurements were carried out for the sheet-like/lamellar elastin for the loaded ($n = 6$) and unloaded ($n = 8$) configurations. Surface area to volume ratios of VIC cytoplasm and nuclei were calculated directly from the surface area and volume measurements from ScanIP for both the unloaded ($n = 2$ for both cytoplasm and nuclei) and loaded configurations (cytoplasm: $n = 8$, nuclei: $n = 3$). Values are presented as mean $\pm$ standard deviation. Statistical values (P value) presented are based on a student's t test.

ELECTRONIC SUPPLEMENTARY MATERIAL

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CONFLICT OF INTEREST

None of the authors of this work, Salma Ayoub, Karen C. Tsai, Amir H. Khalighi, and Michael S. Sacks, have a conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animal studies performed by any of the authors.

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