Three-dimensional transmural organization of perimysial collagen in the heart

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Pope AJ, Sands GB, Smaill BH, LeGrice IJ. Three-dimensional transmural organization of perimysial collagen in the heart. Am J Physiol Heart Circ Physiol 295: H1243–H1252, 2008. First published July 18, 2008; doi:10.1152/ajpheart.00484.2008.—There is strong support for the view that the ventricular myocardium has a laminar organization in which myocytes are grouped into branching layers separated by cleavage planes. However, understanding of the extent and functional implications of this architecture has been limited by the lack of a systematic three-dimensional description of the organization of myocytes and associated perimysial collagen. We imaged myocytes and collagen across the left ventricular wall at high resolution in seven normal rat hearts using extended volume confocal microscopy. We developed novel reconstruction and segmentation techniques necessary for the quantitative analysis of three-dimensional myocyte and perimysial collagen organization. The results confirm that perimysial collagen has an ordered arrangement and that it defines a laminar organization. Perimysial collagen is composed of three distinct forms: extensive meshwork on laminar surfaces, convoluted fibers connecting adjacent layers, and longitudinal cords. While myolaminae are the principal form of structural organization throughout most of the wall, they are not seen in the subepicardium, where perimysial collagen is present only as longitudinal cords.

connective tissue; perimysium; myocardium; laminar architecture

THE ORGANIZATION AND FUNCTION of the myocardium is highly dependent on the cardiac extracellular matrix (ECM). ECM comprises fibrillar proteins such as collagen, elastin, and fibronectin, signaling molecules and enzymes, all surrounded by ground substance. Not only does the ECM provide for structural organization and force transmission, but, more recently, it has come to be seen as a dynamically active entity, continually changing in response to local cell signaling. Collagen is the predominant structural component of the ECM and has been classified into three components (36): epimysium, surrounding the entire muscle; endomysium, surrounding and interconnecting individual myocytes and capillaries; and perimysium, surrounding and interconnecting groups of myocytes. It is the latter component that we have focused on in this work as it defines the three-dimensional (3-D) organization of cardiac myocytes and thereby plays a central role in the mechanical performance of the heart.

There has been continuing development of the understanding of the muscular anatomy of the heart. The ventricular myocardium has been described as 1) discrete muscle bundles that follow a complex helicoidal pathway through the ventricular wall (31, 34, 45); 2) a continuum in which myocyte orientation varies smoothly across the ventricular wall from the subepicardium to subendocardium (42); and 3) more recently, as an ordered arrangement of muscle layers or myolaminae (27). This laminar model of the ventricular myocardium views cardiac myocytes as being organized in branching layers surrounded by an extensive perimysial collagen network. The 3-D arrangement of myocardial laminae accommodates the changing fiber orientation across the ventricular wall, and the regular discontinuities that have been observed widely in the ventricular myocardium (12, 21, 41) lie along cleavage planes between laminae.

The laminar model has been challenged of late. The view that the ventricular myocardium is composed of discrete helical muscle bundles has been revisited in the form of the helical ventricular muscle band (26, 44), whereas other researchers (2, 29) have disputed the existence of any ordered laminar arrangement of ventricular myocytes. However, consistent transmural distributions of ventricular myolaminae have been reported for various species in fresh and processed myocardial tissue with different microscopic techniques (10, 19, 53) and also in vivo hearts using diffusion tensor MRI (DT-MRI) (7, 17).

There is accumulating evidence that myolaminae are a key element of myocardial structure and that they play an important functional role in the heart. It has been demonstrated experimentally that the left ventricular (LV) myocardium has significantly different bulk electrical conductivities associated with both fiber and laminar orientation (20). Whole heart studies have demonstrated that the large shear deformations that occur during ventricular filling and ejection are aligned along myolaminal laminae (10, 28, 41). Furthermore, examination of excised tissue blocks from the LV free wall has shown that the myocardium shears preferentially in directions parallel with the laminar structure (11).

Despite the demonstrated importance of the laminar structure, there have been no systematic attempts to characterize either the relationship between 3-D perimysial collagen organization and the laminar arrangement of myocytes or the way in which this architecture varies across the ventricular wall. Light microscopy and scanning electron microscopy have been used to characterize the arrangement of endomysial collagen, which interconnects adjacent myocytes, in considerable detail (6, 35) and also to visualize features of the perimysial collagen network (6, 27, 30, 35, 36). However, such studies have been limited to tissue volumes too small to capture the extensive nature of myolaminar architecture. High-resolution imaging in much larger volumes is required to fully characterize the 3-D organization of perimysial collagen across the LV wall. This has been made possible by the recent development, in our laboratory, of an automated system for imaging extended tissue volumes (13, 40).

In this study, we addressed two hypotheses that arose from the considerations above. The first hypothesis, which follows...
from the well-established transmural variations in deformation (10, 28), is that laminar architecture, and the perimysial collagen that defines it, changes across the LV free wall. The second is that reproducible secondary or tertiary levels of myocyte arrangement and perimysial collagen organization cannot be obtained from two-dimensional (2-D) images unless they are referred to appropriate local structural axes.

We used extended volume confocal microscopy to reconstruct the 3-D organization of perimysial collagen across the LV wall in normal rat hearts and demonstrated that the perimysial collagen network is more complex than has previously been realized. We identified three distinct forms of perimysial collagen that constitute an ordered structural framework, and variations in this arrangement result in differences in myocyte organization across the wall. Laminar structure is evident throughout the subendocardium and midwall but not the subepicardium, where perimysial collagen is composed of regularly spaced cords. In the subendocardium and midwall, perimysial collagen is present mainly in the form of extensive sheets.

**METHODS**

This study was approved by the Animal Ethics Committee of the University of Auckland and conforms to the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 85-23, Revised 1996).

**Animals and tissue preparation.** Adult male rats were anesthetized with 5% halothane, and hearts were exposed through a thoracotomy. Heparin (100 IU/kg) was injected into the LV and allowed to circulate with 5% halothane, and hearts were exposed through a thoracotomy.

**METHODS**

**Animals and tissue preparation.** Adult male rats were anesthetized with 5% halothane, and hearts were exposed through a thoracotomy. Heparin (100 IU/kg) was injected into the LV and allowed to circulate for 1 min before hearts were quickly excised and immersed in chilled saline. Hearts were immediately suspended on a gravity-driven Langendorff apparatus and perfused with cardioplegic solution (containing (in mM) 118 NaCl, 60 KCl, 1.18 MgSO4, 1.18 KH2PO4, 24.8 NaHCO3, 10 d-glucose, 0.25 CaCl2, and 30.2,3-butanedione monoxime) oxygenated with a mixture of 95% O2-5% CO2 and warmed to 37°C. While still on the Langendorff apparatus, the heart was then perfusion fixed with Bouin’s solution and perfusion stained for 2 h with picrosiris red dye (0.1% Sirius Red FSBA in picric acid). The stained heart was left in Bouin’s solution for 5–7 days, after which 2-mm-thick equatorial rings were cut through the ventricles and small transmural blocks were cut from the lateral LV free wall of these rings (53). These tissue blocks were dehydrated in a graded ethanol series and then in propylene oxide, embedded in an agar resin (PROCURE 812, ProSciTech, Queensland, Australia), and polymerized for 48 h at 60°C.

**Image acquisition.** Tissue blocks were imaged from one Wistar and six Wistar-Kyoto (WKY) rat hearts using a purpose-built system that has been described in detail elsewhere (40). Briefly, the resin-embedded tissue block was mounted on a high-precision three-axis stage, which, together with a confocal laser scanning microscope, was used to acquire extended images at a series of focal planes from the surface of the block to a depth of 35 μm. The surface was then milled to remove 30 μm, and the process was repeated. Image processing was subsequently employed to reduce noise and blur and to enhance 3-D registration. Overlapping image subvolumes were assembled to form the complete 3-D image, and histogram equalization was used to correct for attenuation of intensity with imaging depth. The WKY volumes were ~4 × 1 × 0.3 mm with isotometric voxels of size (1 μm)3, and the Wistar heart was imaged at (1.22 μm)3 over a dimension of 4 × 1 × 1 mm. A higher-resolution image [560 × 420 × 330 μm, (0.4 μm)3 voxel size] was also acquired from the midwall of one WKY heart. Volume images were rendered and visualized using Voxx (http://www.nephrology.iupui.edu/imaging/voxx/).

**Image analysis.** Software custom written in LabView (National Instruments, www.ni.com) allowed the user to digitally resample extended tissue volumes on arbitrary planes. To characterize the transmural variation of structural parameters, it was necessary to define a transmural axis, normal to the epicardial surface and extending to the origin of papillary muscle or endocardial trabeculae. The transmural variation of the fiber angle was determined from a series of image planes resampled perpendicular to this axis. Each image plane was divided into subregions in which the local structural orientation was computed using an intensity gradient technique (47). The fiber angle for each plane was determined to be the contrast-weighted average of subregion orientations.

To determine collagen density, image subvolumes were extracted at uniformly spaced intervals along the transmural axis defined above. Collagen was segmented from each subvolume using techniques that identify voxels that are bright relative to the local background. Where there were large contiguous volumes of collagen, a clustering-based thresholding technique was used to segment collagen. In the high-resolution image where nuclei were abnormally bright, a region growing algorithm was used to identify collagen on the basis of its connectivity.

Flat transmural cutting planes show varying projections of myocardial structure due to the transmural rotation of the fiber orientation. To make consistent comparisons across the wall, it is useful to view the structure from the same relative orientation. We defined a curved transmural cutting plane that is perpendicular everywhere to the local myofiber axis (defined as the cross-fiber plane), enabling the structure to be visualized across the LV wall independent of fiber rotation. Changes in perimysial collagen organization were computed on this plane using a measure of local collagen alignment. For each collagen pixel, segmented on the cross-fiber plane, the fraction of collagen was computed along a 120-μm-long line centered on the pixel. This was repeated for 1° steps over 180°, and the maximum fraction and corresponding orientation were recorded. Distributions of these data were generated over defined regions, and the normalized variance of the maximum aligned collagen fraction was determined. Figure 1 shows two examples of collagen organization that can be differentiated by the normalized variance of collinearity (σr/μ). In Fig. 1A, collagen (left) has the punctate appearance typical of areas with little collagen alignment in the viewing plane, and collagen colinearity was clustered about a low mean (middle) with a normalized variance of 0.016. The scattered organization of collagen is additionally shown by the broad distribution of the angle of maximum alignment (Fig. 1A, right). Figure 1B shows aligned collagen (left) with a greater normalized variance (0.059; middle) and a predominant angle clustered ~50° to the vertical axis (right).

**Statistical analysis.** Evidence of variation in sample measurements was tested using two-factor ANOVA with significance demonstrated for P < 0.05. Variation was subsequently explored using two mutually orthogonal vectors of post hoc contrast coefficients.

**RESULTS**

The complex organization of the ventricular myocardium is shown in Fig. 2, where transmural image volumes from the LV free wall are shown for one WKY heart (A) and the Wistar heart (B). High-intensity collagen is bright, whereas lower intensities correspond to myocyte autofluorescence. Cleavage planes between adjacent bundles of myocytes are evident, particularly in the midwall. Virtual erosion of myocytes revealed an extensive 3-D collagen network with qualitatively similar transmural variation in density and organization for WKY (Fig. 2C) and Wistar (Fig. 2D) rat hearts. Both volumes have papillary muscles and trabeculae at endocardial surfaces, and collagen surrounding a large subepicardial blood vessel was evident in Fig. 2D.
In Fig. 3, the transmural changes in myocyte orientation (A) and collagen density (B) were quantified across the image volumes shown in Fig. 2, omitting the region encompassing the large subepicardial vessel in the Wistar heart. In both Fig. 3, A and B, myocyte orientation changes from around \(-80^\circ\) (base-apex direction) at the epicardium (0%) to \(-60^\circ\) at the subendocardial interface with trabeculae or papillary muscle (100%) but remains relatively constant across endocardial trabeculae and papillary muscle. Transmural fiber rotation was \(-140^\circ\) and ranged from 100 to 150\(^\circ\) across all seven hearts. The collagen volume fraction (Fig. 3B) was relatively uniform across most of the ventricular wall, with an apparent reduction at \(\sim 70\%\) wall position. However, there was a sharp increase in the collagen fraction at the epicardium (wall position <5%) and considerable variability near the endocardium (wall position >100%). Table 1 shows the transmural variation of collagen fraction across all hearts. Two-factor ANOVA showed no significant difference \((P = 0.42)\) in the collagen fraction sampled at the five wall depths and, similarly, no differences between hearts \((P = 0.1)\).

A cross-fiber plane was used to analyze structural detail in a consistent orientation relative to the fiber axis. A two-dimen-

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**Fig. 1.** Measures of linearity for two regions representing punctate (A) and aligned (B) perimysial collagen. In A, there was a low, clustered distribution of maximum alignment, with a normalized variance \((\alpha/\mu)\) of 0.016 and a spread of random alignment angles. B shows a broader range of maximum alignments \((\alpha/\mu = 0.059)\) and a dominant alignment angle \(-50^\circ\) to the vertical axis.

**Fig. 2.** Three-dimensional (3-D) volume renderings extracted from images of Wistar-Kyoto (WKY; A) and Wistar (B) rat left ventricles (LVs). C and D: lower intensity tissue was made transparent to reveal collagen structures. The rendered volume size was \(-4 \times 1 \times 0.25\) mm, with \((1 \mu m)^3\) (A and C) and \((1.22 \mu m)^3\) (B and D) voxel sizes. Note the large subepicardial vessel to the left in B and D and papillary muscles and trabeculation on both endocardial surfaces.
sional image was extracted from the transmural volume on a thin curvilinear section defined to be normal everywhere to the local myofiber axis (Fig. 4C). One such cross-fiber plane was flattened, as shown in Fig. 4A, and it was evident that there were substantial differences in myocyte and collagen organization in different transmural regions. Throughout most of the wall, the predominant features were the discontinuities between groups of cells. These cleavage planes were evident because they opened during tissue processing and revealed two approximately orthogonal populations of myocardial laminae. The corresponding organization of perimysial collagen is shown in Fig. 4B, where myocytes have been removed. This view revealed an ordered and extensive arrangement of perimysial collagen. In the midwall, collagen was arranged in a parallel array of sheets with a predominant orientation around $-40^\circ$ to the transmural axis, although there was a further subpopulation of orientations that were approximately perpendicular to this. In the subendocardial region, there were no openings between groups of myocytes; however, collagen appeared to form dense septae with a uniform orientation. In contrast, there were no planes of discontinuity in the subepicardial region, nor was there any evidence that perimysial collagen was arranged in sheets.

We extracted subvolumes from the Wistar dataset (Fig. 2B) in subepicardial, midwall, and subendocardial regions to investigate the transmural variation in 3-D collagen organization more fully (Fig. 5). In the subepicardial region, perimysial collagen formed a dense array of long wavy cords that were sparsely interconnected and ran parallel with the myocyte axis (Fig. 5A). In the midwall, sheets of collagen surrounded groups of cells (Fig. 5B), giving rise to the cleavage planes shown in Figs. 2 and 4. Longitudinal collagen cords were also present in the midwall, but these appeared to be more sparsely distributed. Perimysial collagen in the subendocardial region (Fig. 5C) had a similar organization to that seen in the midwall but appeared to be more concentrated into sheets.

The form of perimysial collagen organization at different transmural locations was quantified on cross-fiber planes in all seven hearts using the measure of local collagen alignment outlined in METHODS. Collagen was segmented on each cross-fiber plane, with collagen surrounding vessels or on heart surfaces removed from consideration. The normalized variance of collagen colinearity ($\sigma/\mu$) was measured in three regions on each plane: one in the subepicardial region, one in the midwall, and one in the subendocardial region (Table 2). Collagen colinearity was substantially greater in subendocardial and midwall regions than in the subepicardial region. Moreover, alignment angles were clustered in preferred directions in subendocardial and midwall regions but were randomly distributed in the subepicardial region. These results are consistent with an ordered arrangement of perimysial collagen on
cross-fiber planes across most of the LV free wall but suggest that perimysial collagen in the subepicardial region is principally in the form of longitudinal collagen cords (see Fig. 1A). Two-factor ANOVA confirmed the regional variation of collagen colinearity ($P = 0.002$), and the data were further examined using a matrix of orthogonal post hoc contrast coefficients. $F$-tests demonstrated no differences between midwall and subendocardial regions ($P = 0.91$) but significant differences between the subepicardial region and the other two regions ($P = 0.003$), which corresponds to the differences shown in the collagen arrangement shown in Fig. 1. ANOVA also demonstrated a variation in the organization between hearts ($P = 0.014$).

The relationship between myocardial laminar organization and perimysial collagen structures was investigated in further detail (Fig. 6) by imaging a further block of tissue from the midwall of the WKY heart at higher resolution [voxel size (0.4 $\mu$m)$. In the front face of Fig. 6A, myocytes and capillaries are seen in cross section with their boundaries surrounded by fine endomysial collagen. Perimysial collagen formed an interconnected network (Fig. 6B) in which extensive sheets of collagen were found on the surfaces of muscle layers, as shown in Fig. 5B. The spaces or cleavage planes between layers were not evident in fresh tissue, where on a cut surface under a dissecting microscope, the laminae appeared as stacks of cards with no space between. However, the histological processing shrinkage artifact provides the advantage of opening these potential spaces, revealing the collagen networks that would otherwise be hidden between adjacent myocardial layers.

Figure 7 shows subvolumes extracted from Fig. 6 that revealed three distinct forms of perimysial collagen associated with myolaminae. The subvolumes shown in Fig. 7, B and E, show that collagen within the muscle layers was primarily in the form of long, relatively large-diameter (2–5 $\mu$m), branching collagen cords. These intralaminar cords were approximately aligned with the myocyte axis and ran adjacent to myocytes and capillaries. Fine processes originated from the cords and ran obliquely for short distances. The other subvolumes (Fig. 7, A, C, and D) were chosen to reveal the organization of perimysial collagen on and between muscle layers. The high-resolution volume images revealed the complexity and extensive nature of the collagen that surrounds and defines muscle layers. This meshwork (Fig. 7A) consisted of fibers with orientations predominantly oblique to the myocyte axis and a wide range of dimensions and cross-sectional shapes. In addition, adjacent layers were connected by long, convoluted fibers that integrated with surface collagen at each end (Fig. 7D). Although interconnections between these fibers were sparse, multiple fibers appeared to aggregate at layer edges to form thick collagen tendons (Fig. 7C). All three forms of perimysial collagen (long cords within layers, meshwork on the surface of layers, and collagen between layers) were structurally distinct; however, they were all interconnected, forming a coupled network.

**DISCUSSION**

We have provided a systematic description of 3-D myocyte arrangement and collagen organization across the LV free wall in a normal rat heart that is both more comprehensive and at higher resolution than previously available. This has been made possible through the use of a novel imaging system that enables confocal microscopy to be extended over large tissue volumes with preservation of image registration (40) as well as visualization tools that allowed us to standardize views with respect to local tissue microstructure and to separate perimysial collagen from surrounding myocardial tissue. We quantified perimysial collagen density, alignment, and connectedness across the LV free wall in normal rat hearts and also provided detailed structural information that reveals the arrangement of three different forms of perimysial collagen. These data strongly support the view that myolaminae are the principal intermediate-scale functional units in the LV. However, they also demonstrate that laminar architecture, and the perimysial...
collagen that defines it, changes across the LV free wall, despite the fact that the collagen fraction is relatively unchanged transmurally. We argue that this variability reflects changing local deformation across the LV wall and plays a crucial role in ensuring efficient pump function in the normal heart.

3-D perimysial collagen organization. The ordered nature of perimysial collagen organization revealed when the effects of myofiber rotation are removed is striking (Fig. 4). Over most of the LV wall, collagen is aligned in parallel planes that are significant in extent. These have a predominant orientation around −40° to the transmural axis, with a further subpopulation approximately perpendicular to this. Figure 5, B and C, shows that this perimysial collagen forms extensive sheets that cover the surface of muscle layers and thereby define laminar architecture. However, the results shown in Fig. 5A suggest that the laminar arrangement does not extend to the subepicardial region, where perimysial collagen is mainly in the form of longitudinal cords.

The colinearity measure outlined in METHODS (and the associated distribution of maximum orientation) provides a robust means of differentiating between forms of perimysial collagen in the cross-fiber plane (see Fig. 1). When collagen in this viewing plane has the punctate appearance and scattered distribution characteristic of longitudinal collagen cords, the mean value (μ) and variance (σ²/μ) of the colinearity are both low, whereas the angle of maximum alignment has a broad distribution. On the other hand, when collagen in the cross-fiber plane has the ordered linear appearance associated with perimysial collagen that surrounds muscle layers, the mean and variance of the colinearity are both substantially greater, and the distribution of maximum alignment is clustered around predominant orientations. We used this approach in seven normal rat hearts to demonstrate quantitatively that the 3-D arrangement of perimysial collagen is consistent with an ordered laminar arrangement of myocytes over much of the LV free wall. We have also shown that there is transmural variation in the distribution of perimysial collagen forms, despite

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Table 2. Normalized variance of collagen colinearity in 3 regions across 7 hearts

|              | Subepicardium | Midwall    | Subendocardium |
|--------------|---------------|------------|----------------|
| Mean         | 0.0144        | 0.0320     | 0.0327         |
| SD           | 0.0037        | 0.0128     | 0.0161         |

Values are means and SDs of the normalized variance of collagen colinearity in 3 regions across 7 hearts. Two-way ANOVA showed evidence of variation between groups (P = 0.002). F-tests showed no significant differences between midwall and subendocardial regions (P = 0.91) but significant evidence of difference between the subepicardial region and midwall and sub-endocardial regions (P = 0.003).
the fact that the perimysial collagen density remains relatively uniform. Laminar structure is not seen in the subepicardial region, where perimysial collagen is almost exclusively in the form of long, uniformly distributed cords aligned approximately with the myofiber direction. These findings provide strong support for the first of the specific hypotheses listed for this study.

Using our extended volume imaging methods, we also reconstructed the 3-D architecture of perimysial collagen in the midwall of one rat heart at much higher resolution than has previously been reported. It is clear that the perimysial collagen surrounding myolaminae is a meshwork of collagen fibers and bundles with a wide range of sizes and orientations that are typically oblique to the fiber axis (Fig. 7A). Long cords run within the muscle layers, and these are aligned approximately with the fiber axis and appear to originate and terminate in the surface mesh. The finer oblique processes branching from these cords are likely to couple into the endomysial collagen of adjacent myocytes. Taken together, these two forms of perimysial collagen provide a collagenous skeleton that organizes the ventricular myocardium into tightly coupled units forming a discontinuous structure. The third form of perimysium is the...
networks of long convoluted collagen fibers that cross cleavage planes and connect into the surface meshwork on adjacent layers (Fig. 7D).

Each of the three forms of perimysial collagen has been previously reported. Both the complex weave of collagen surrounding groups of myocytes and the loose network of long convoluted collagen fibers that connect adjacent cell groups have been described in the earliest observations of cardiac collagen organization (1, 6, 24, 35, 49). Coiled or wavy collagen cords running approximately parallel with the myocyte axis have been reported in papillary muscle (36, 38), right ventricular trabeculae (16), and the LV free wall (25, 30). LeGrice et al. (27) developed a conceptual model of myocardial architecture that integrated the findings of Caulfield and Borg (6) and Robinson (35) with those of earlier, more macroscopic studies, which reported the existence of an ordered array of planar discontinuities in the myocardium (12, 21, 41). LeGrice et al. (27) showed that myocytes are grouped by perimysial collagen into branching layers approximately four cells thick and that the discontinuities, or cleavage planes, that separate adjacent muscle layers are extensive, particularly in the ventricular midwall. More recently, myolaminar structure has been observed in fresh, frozen cardiac tissue (10, 19) and in embedded cardiac tissue using extended volume imaging (13, 40, 53). Finally, both the fibrous architecture of the heart and its laminar organization have been revealed in vivo using DT-MRI (4, 7, 9, 17, 46).

These views have been challenged recently. The idea that the structure and mechanical function of the ventricles is best described as a single band of muscle coiled into two helixes has been enthusiastically promoted in the cardiothoracic surgery literature (26, 44). However, a recent review (14) concluded that the weight of evidence from 3-D imaging is against the existence of the helical ventricular myocardial band. Other investigators (2, 29) argue that there is no microscopic evidence for an ordered laminar arrangement of ventricular myocytes. They state that “histological sections taken to show the full thickness of the ventricular walls in man fail to demonstrate lamellar fibrous shelves extending from epicardium to endocardium, as was suggested by LeGrice et al.” (2). However, the ventricular myocardium is a complex 3-D structure, and its architecture cannot be understood using 2-D thin sections that do not account fully for its inherent anisotropy (14). This is shown in Fig. 8, which was extracted from the Wistar heart volume (Fig. 2B) and presents a view that is parallel everywhere to the local myofiber direction. Figure 8 shows that myofiber orientation is near parallel to epicardial and endocardial surfaces, and the perimysial collagen that defines laminar structure is not clearly seen in this view, which may explain why Lunkenheimer et al. (29) failed to observe laminar structure. In comparison, in Fig. 4, which was derived from the same 3-D data set and viewed perpendicular to the local myofiber direction, an ordered arrangement of perimysial collagen is clearly evident in midwall and subendocardial regions. The failure to demonstrate lamellar fibrous shelves extending from the epicardium to endocardium (2) is not surprising. Figure 3 shows that myolaminae do not extend across the full thickness of the ventricular walls. Muscle layers branch and interconnect and must do so to accommodate the transmural rotation of myofibers; this is clearly articulated in the original literature reporting the laminar organization of the ventricular myocardium (27, 53). The results presented in this study therefore reinforce the second of the listed hypotheses, that reproducible secondary or tertiary patterns of myocyte arrangement and perimysial collagen organization cannot be obtained from 2-D images unless they are referred to appropriate local structural axes.

Myocardial architecture and mechanical function. The cardiac connective tissue hierarchy has been viewed as a framework for maintaining spatial registration of myocytes (6, 35), for limiting the extension of myocytes during diastole (16, 30, 38), and for transmission of force (6) and storage of energy during systole (35–37, 50). Recent bioengineering analyses have provided a more complete understanding of the relationship between structure and mechanical function (22). The LV undergoes large changes in shape and dimension throughout the cardiac cycle, and the extent of this deformation is greatest at the endocardium. Although there are substantial transmural gradients of 3-D strain, myofiber extension is remarkably uniform across the LV wall in both systole and diastole (10, 43, 48), and sarcomere lengths do not exceed 2.25 μm in normal function (51). The dimensional changes experienced by the inner wall of the LV during the cardiac cycle are much greater than can be accounted for by local myocyte shortening and have been related to altered myocyte arrangement in this region (41). 3-D strain analysis has revealed substantial shearing between adjacent subendocardial layers in systole (10, 28) and diastole (43), and the LV also undergoes significant torsional deformation throughout the cardiac cycle (32). Kinematic modeling has suggested that systolic torsion and transmural myofiber rotation both contribute to the uniformity of sarcomere length across the LV wall throughout ejection (5, 33). It has been shown experimentally (28) and theoretically (3) that LV muscle layer orientations coincide with planes of maximum shear deformation.

Such analyses suggest that three factors enable large ejection fractions to be achieved in the normal LV despite the restricted sarcomere length range over which myocytes can shorten and develop force. These are myofiber rotation, the capacity to support substantial local shear deformations while maintaining appropriate transmural mechanical coupling (23), and strain-locking mechanisms that prevent overextension of myocytes. The different forms of perimysial collagen described in this study together constitute an integrated framework that meets these functional requirements. Myolaminae provide structural units that enable local myocyte rearrangement or shearing to occur, while the dense array of longitudinal cords distributed
throughout the LV myocardium limits passive extension along the myocyte axis. Finally, the 3-D assembly of these connective tissue components accommodates myofiber rotation and provides mechanical coupling across the ventricular wall. The fact that laminar structure is not evident in the outer 10–20% of the rat LV may reflect the relatively low shear strains in this subepicardial region. However, at least two structural constraints could also be important. First, laminae must insert into and become continuous with the epicardial surface. This may limit the capacity for relative movement between laminae and hence lessen the necessity for their presence. Second, the subepicardium must accommodate large coronary vessels and their dense surrounding connective tissue.

There can be no doubt that the 3-D arrangement of cardiac myocytes and connective tissue is intimately associated with the effectiveness with which the heart operates as a pump. This work demonstrates that despite the relatively constant collagen fraction across the wall, the 3-D arrangement of perimysial collagen varies considerably, indicating that this organization is an important determinant of cardiac function. It is clear that the changes in myocyte arrangement and perimysial collagen organization that accompany many forms of heart disease are associated with impairment of long-term ventricular function (49, 51). Features of the ventricular remodeling that occurs in hypertension, myocardial infarction, and heart failure have been characterized in extensive histological and biochemical studies. More recently, DT-MRI has been used to investigate changes in fiber orientation after myocardial infarction (8), and Helm et al. (18) have also used this approach to show that there are significant changes in myolaminar orientation in the failing heart. However, detailed information on the ways in which different cardiomyopathies alter 3-D perimysial organization across the ventricular wall is not available. The results provided here for the normal heart will provide an important frame of reference for such data in the future.

Limitations. The large volume images on which this study is based were generated by serial acquisition of individual voxels, and this is a time-consuming process. As a result, data were presented here for restricted numbers of LV sites only. As with all histological techniques, the methods used in this work introduce artifacts such as differential tissue shrinkage, which may give rise to error in measurements such as tissue volume fractions. However, our values still fell within the wide range of collagen fractions reported in the rat and other species. These varied from 2% to 20% (15, 39, 49, 52). In addition, we used a nonspecific collagen stain and were therefore not able to distinguish between different collagen types. One other limitation of the study is that it was limited to rats. There are many well-characterized rat models of cardiac disease, and the organization of myocytes and perimysial collagen in the normal rat heart is qualitatively similar to that observed in the dog (27) and pig (13). However, in these larger hearts, the extent, relative to wall thickness, of the subepicardial region in which laminar structure is not evident is less than in the rat heart.

Conclusions. We characterized the 3-D relationship between myocyte organization and perimysial collagen across the LV wall in a more systematic fashion than has previously been available. In particular, we provided detailed structural information that suggests that myolaminae are the principal intermediate-scale functional units throughout most of the LV. Myolaminae were, however, absent in the subepicardial region of the LV free wall, where longitudinal cords were the principal perimysial component.

It has long been known that changes to the ECM are associated with impaired cardiac function in a number of ventricular cardiomyopathies. Many studies have detailed the effect of altered cell signaling in these states that lead to changes in the ECM, particularly in the myocardial collagen amount, cross-linking, and type, in explaining the reduced function in these states. It is our belief that perhaps a more important consideration is the 3-D organization of the myocardium as defined by perimysial collagen. The results provided here for the normal heart provide an important frame of reference for time-course studies currently under way in our laboratory of the way in which perimysial collagen changes in hypertensive cardiomyopathy, postmyocardial infarction, and during development and aging.

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