Published in final edited form as:

Ann Biomed Eng. 2016 May ; 44(5): 1436–1448. doi:10.1007/s10439-015-1465-6.

Analyzing Remodeling of Cardiac Tissue: A Comprehensive Approach Based on Confocal Microscopy and 3D Reconstructions

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Abstract

Microstructural characterization of cardiac tissue and its remodeling in disease is a crucial step in many basic research projects. We present a comprehensive approach for three-dimensional characterization of cardiac tissue at the submicrometer scale. We developed a compression-free mounting method as well as labeling and imaging protocols that facilitate acquisition of three-dimensional image stacks with scanning confocal microscopy. We evaluated the approach with normal and infarcted ventricular tissue. We used the acquired image stacks for segmentation, quantitative analysis and visualization of important tissue components.

In contrast to conventional mounting, compression-free mounting preserved cell shapes, capillary lumens and extracellular laminae. Furthermore, the new approach and imaging protocols resulted in high signal-to-noise ratios at depths up to 60 μm . This allowed extensive analyses revealing major differences in volume fractions and distribution of cardiomyocytes, blood vessels, fibroblasts, myofibroblasts and extracellular space in control versus infarct border zone.

Our results show that the developed approach yields comprehensive data on microstructure of cardiac tissue and its remodeling in disease. In contrast to other approaches, it allows quantitative assessment of all major tissue components. Furthermore, we suggest that the approach will provide important data for physiological models of cardiac tissue at the submicrometer scale.

Keywords

cardiac tissue characterization; myocardial infarction; fibroblasts; myofibroblasts; blood vessels; myocardial laminae; modeling; segmentation; remodeling

Introduction

Cardiac muscle (myocardium) is a composite material of various cell types and extracellular components including the extracellular matrix and interstitial fluids. The predominant cell type is the cardiac muscle cell (cardiomyocyte), which underlies contractile function and electrical signaling in the heart. Other cell types important for cardiac structure include fibroblasts, endothelial and smooth muscle cells. The primary function of fibroblasts is to synthesize and maintain the extracellular matrix, providing a scaffold and mechanical support for other cells and structures.³ Endothelial cells form the inner layer of capillaries and larger blood vessels, while smooth muscle cells form the muscular layer around arterioles and coronary arteries. Remodeling of cellular and extracellular components and their arrangement within the myocardium is associated with many types of heart diseases, for instance, myocardial infarction (MI), hypertrophic cardiomyopathy and heart failure.^{15, 21, 39} An example for cellular remodeling is differentiation of fibroblasts into myofibroblasts with consecutively increased production of extracellular matrix in response to MI.⁴² It is well established that disease-associated remodeling of cardiac cells and tissue leads to heart failure and arrhythmia, which can result in sudden cardiac death.⁴¹ Imaging-based methods for characterization of myocardial microstructure are essential to better understand the relationship between structural and functional remodeling. These methods are important not only in research, but also for clinical diagnosis.^{1, 17} Many methods apply transmitted light microscopy to thin sections of cardiac biopsies stained with hematoxylin and eosin, which leads to two-dimensional images. Confocal microscopy has evolved as an approach for three-dimensional (3D) characterization of cardiac microstructure. Confocal microscopy is based on fluorescent dyes excited by a laser source within a defined focal plane. Although imaging depth is limited, a major advantage of confocal microscopy is that it can provide 3D images from objects without the need for sectioning.

Others and we established approaches based on confocal microscopy for 3D reconstruction and analyses of myocardium at micrometer and submicrometer scale.^{22-24, 29-31, 33, 45} This led to important insights into normal and pathological tissue microstructure, but several limitations need to be acknowledged that render it difficult to apply these approaches. A general limitation of confocal microscopy is related to the small depth of imaging due to absorption and, more importantly, scattering. Scattering occurs at the transition between two media with differing refractive indices. In tissue, this happens mainly at the transition between lipid bilayers, e.g. cell membranes, and aqueous solutions, e.g. cytosol or interstitial fluids.²⁸ In principle, the resulting attenuation of fluorescent intensity can be corrected with mathematical methods. However, this leads to low signal-to-noise ratios in the depth of the tissue, impeding analysis and interpretation of image stacks. Promising new methods to reduce light attenuation and thus overcome this limitation are based on clearing of biological tissues. Several methods for tissue clearing have been recently developed.^{4, 13, 19, 36, 38, 40, 44} Many of these methods are based on reducing heterogeneity of refractive indices within the tissue, for instance, by removing lipids, which primarily reside in the membranes of cells and organelles, or by replacing water with compounds having a refractive index similar to other tissue components. The application of these clearing methods together with microscopic imaging led to major breakthroughs in our understanding of the three-

dimensional microstructure of the brain. However, these methods often require elaborate preparation protocols and few have been demonstrated to work in cardiac tissue. Furthermore, the degree to which they preserve microstructure at submicrometer scale and are compatible with methods of immunofluorescence is still unclear.

In this study, we introduce an integrated approach for enhancing imaging depth and reconstructing cardiac tissues and cells in 3D at the submicrometer scale. The approach fills a gap between imaging at nanometer scale and microscopic imaging at the micrometer scale. In particular, our approach is well suited for characterizing cell-cell and cell-structure relationships as well as features of the extracellular space. For this purpose, we developed a new method for compression-free mounting with tissue clearing media that proved to preserve tissue structures and cell shapes. Furthermore, we designed imaging protocols to improve imaging depth in compression-free mounted samples. We applied the methodology in order to image immunostained normal cardiac tissue and tissue from the MI border zone with confocal microscopy. We processed the acquired imaging stacks and segmented them into myocytes, fibroblasts, myofibroblasts, blood vessels and extracellular spaces. We demonstrate that the resulting 3D reconstructions yield comprehensive data on tissue composition, the distribution of cells and cell-cell relationships as well as the spatial arrangement of vessels and the extracellular space in normal and diseased myocardium.

Materials and Methods

Animal Model

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. In this study, we used four New Zealand White rabbits of 2.5 to 3 kg. In one animal we produced MI as described previously.¹⁶ Briefly, the animal was anesthetized with an intramuscular injection of ketamine and xylazine and then underwent thoracotomy to induce MI by ligation of the left circumflex coronary artery. Tissue was harvested 21 days (d) after surgery. Hearts were excised after a lethal injection of sodium-pentobarbital and perfused for 5-10 min with a modified Tyrode's solution (containing in mM: 92 NaCl, 4.4 KCl, 5 MgCl₂, 1.15 NaH₂PO₄, 11 glucose, 12.5 NaOH, 24 HEPES, 20 taurine, 5 creatine, 5 Na pyruvate). For preparations of living tissue, we dissected tissue from the left ventricle. For preparations of fixed tissue, the hearts were perfused with 2.5% paraformaldehyde solution for 10 min.³³

Fixed Tissue Preparation and Fluorescent Labeling

Left ventricular biopsies with a diameter of 5mm were taken from the border zone of the MI and from a matched region of a control heart. In the infarcted heart the center of the biopsy needle was positioned on the border between infarcted and normal tissue. The biopsies were then put into a 30% sucrose solution for 2 hours (h) to avoid tissue damage during the subsequent freezing process. Before cryosectioning, biopsies were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands). Sections parallel to the epicardial surface of 100 µm thickness were cut at -24 °C using a cryotome (CM 1950, Leica AG, Wetzlar, Germany). Only mid-myocardial slices, 0.4 to 1.2 mm below the epicardium, were investigated. After cryosectioning, OCT

remnants were removed by rinsing the tissue slices three times with phosphate buffered saline (PBS, containing in mM: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, 1.8 KH₂PO₄). The sections were rinsed after each subsequent staining step. Incubations were conducted at room temperature (20 °C) on a laboratory platform rocker. At first, blocking solution containing 5% bovine serum albumin, 5% goat serum and 0.25% Triton X-100 in PBS was applied for 1 h to reduce unspecific staining. To label vimentin and alpha smooth muscle actin (α -SMA), we used anti-vimentin IgG₁ (V6630, Sigma-Aldrich, St. Louis, MO, USA) and mouse monoclonal anti- α -SMA IgG_{2a} (V5228, Sigma-Aldrich), respectively. Primary antibodies were diluted 1:200 in blocking solution and incubated for at least 6 h. As secondary antibodies, we applied goat anti-mouse IgG₁ AF555 (A-21127, Life Technologies, Carlsbad, CA, USA) and goat anti-mouse IgG_{2a} AF633 (A-21136, Life Technologies) diluted 1:100 in blocking solution for at least 6h together with 16.7 μ g/ml 4', 6-diamidino-2-phenylindole (DAPI, D3571, Life Technologies) to stain nuclei. Finally, CF488A wheat germ agglutinin (WGA, 29022, Biotium, Hayward, CA, USA) was applied at a concentration of 40 μ g/ml in PBS for at least 6 h.

Mounting of Tissue Slices

After labeling, we mounted the tissue slices free of compression by placing them on a cover slip of thickness 1. Fluoromount-G (#17984-25, Electron Microscopy Science, Hatfield, PA, USA) was used as mounting medium. Fluoromount is a phosphate-buffered aqueous solution of glycerol and polyvinyl alcohol, similar to other mounting media.¹¹ Additionally, we mounted some tissue slices in a conventional way, embedding them in Fluoromount between a glass slide and a cover slip. All mounted samples were dried for at least 24 h at room temperature in a humidity chamber with relative humidity between 20% and 100%. Afterwards, we sealed samples with varnish (Liquid Vinyl, Vyna-Kote Products, Westminster, CA, USA) to avoid desiccation. Imaging was performed within 14 d using the setups shown in Figure 1A and 1B.

Three-Dimensional Confocal Microscopy of Fixed Tissue

For confocal microscopy we used a Zeiss LSM 5 Duo microscope (Carl Zeiss, Jena, Germany) with a 40 $\times$ oil lens (numerical aperture 1.3). We applied a dual-track imaging protocol with two laser lines and acquisition channels per track. Tracks were switched at each optical section. In the first track, we used the 364 nm and 543 nm laser lines for concurrent excitation of DAPI and Alexa Fluor 555 (anti-vimentin). In the second track, we applied the 488 nm and the 633 nm laser lines for concurrent excitation of CF 488A (WGA) and Alexa Fluor 633 (anti- α -SMA). Spectral separation of fluorescent light was achieved by a set of appropriate beamsplitters and filters (Online Table 1). Voxel dwell time was kept constant at 1.6 μ s throughout all image acquisitions.

We acquired 3D image stacks with typical sizes of 1024 $\times$ 1024 $\times$ 300 voxels and a voxel size of 0.2 $\times$ 0.2 $\times$ 0.2 μ m. Accordingly, a tissue volume of 204 $\times$ 204 $\times$ 60 μ m was imaged. The z-axis was defined parallel to the laser beam. The image plane closest to the coverslip was defined as $z_0=0$ μ m as a reference for the discrete depth z [μ m]. To reduce depth-dependent attenuation of the fluorescent signal, we used a linear increase in laser power (Online Table 2) and introduced the following model for each imaging channel c :⁸

$$I_c(z) = \gamma_c (A_c + zB_c) e^{-C_c z} + D_c \quad (1)$$

with $I_c(z)$ the recorded fluorescence intensity at depth z measured in arbitrary units [AU], γ_c [AU/mW] the intensity yield per laser power, A_c [mW] the laser power at z_0 , B_c [mW/ μm] the linear increase in laser power with depth, C_c [μm^{-1}] the attenuation coefficient, and D_c [AU] an additive constant reflecting the digital offset. The parameter γ_c reflects quantum yield, fluorophore density and imaging gain. C_c [μm^{-1}] approximates the exponential decay in intensity due to scattering and absorption of both the excitation and emission light.^{6, 32} A_c and B_c were adjusted for each channel to achieve high signal-to-noise ratios while avoiding signal saturation (Online Table 1).

Preprocessing of Acquired Images

Noise was reduced in WGA and other images by filtering with a Gaussian kernel ($5 \times 5 \times 5$ voxels, $\sigma = 0.2 \mu\text{m}$) and mean filtering ($3 \times 3 \times 3$), respectively. Subsequently, deconvolution was performed by application of the Richardson-Lucy algorithm (8 iterations) with measured point spread functions as described previously.^{22, 23, 33}

To correct depth-dependent changes in signal intensity resulting from attenuation and laser power adaptation, we calculated the mean fluorescence intensity $\bar{I}_c(z)$ per slice. $\bar{I}_c(z)$ was then approximated based on the attenuation model in equation 1. While parameters A_c and B_c were known from the laser settings, γ_c , C_c and D_c were estimated using a curve fitting function implemented in Matlab R2014a (fit, Mathworks Inc., Natick, MA, USA). We used the Levenberg-Marquardt algorithm and specified initial and boundary values within the possible physical range of the parameters. Intensities of each $I_c(x, y, z)$ were corrected by inversion of equation 1 leading to the corrected intensities $\bar{I}_c^*(x, y, z)$.

Image Segmentation, Analyses and Visualization

After preprocessing, we segmented all major tissue components. Images were converted into binary images by applying histogram-based threshold. For this purpose, the mode and standard deviation (σ) were determined for each channel of an image stack. Threshold levels were set to $\text{mode} + \sigma$ for the WGA channel and $\text{mode} + 2\sigma$ for the DAPI, vimentin and α -SMA channels. We used a lower weighting factor for σ to threshold WGA channels because the high ratio of voxels with WGA signal ($\sim 20\%$) led to a relatively higher standard deviation versus other channels. Images were then filtered with a binary median filter (radius 1). The segmented WGA signal was used to segment each cardiomyocyte by means of a watershed-based method as described previously.³⁴ This method created labeled segments for each volume enclosed by WGA signal. At first, we identified myocytes. Subsequently, vessel lumens were identified within the non-myocyte segments. Vessel lumens were then dilated with a radius of $1 \mu\text{m}$ to include the vessel walls and endothelium. Possible overlap with the myocyte space was subtracted after the dilation. Cardiomyocyte and vessel volumes were then subtracted from the segmented WGA, DAPI, vimentin and α -SMA volumes. The remaining DAPI was identified as non-cardiomyocyte nuclei and merged with the remaining vimentin and α -SMA volumes. Using distance maps, we classified voxels of the merged volume closer to vimentin than to α -SMA as fibroblasts, while other voxels were classified

as myofibroblasts. Finally, remaining non-classified segments from the watershed algorithm were merged with the remaining WGA volume and classified as extracellular space.

We determined volume ratios of all tissue components. Also, we calculated the number of neighboring myocytes by selecting complete cardiomyocytes, which were not truncated at image borders. We generated distance maps from the complete myocytes and then identified and counted all myocytes within a distance of 0.5 μm to the outer sarcolemma.

For 2D visualizations of images we used customized software. For 3D visualizations we applied Paraview (Kitware, Clifton Park, New York, USA), VolView (Kitware) and customized software.

Living Tissue Preparation, Fluorescent Labeling and Imaging

For co-labeling with WGA and a volume marker of the extracellular space, we used sections of left ventricular tissue from a control heart and incubated them in modified Tyrode's solution containing 40 $\mu\text{g/ml}$ WGA AF555 (W32464, Life Technologies) for 5-10 min. Subsequently, we placed the tissues in a bath with modified Tyrode's solution and 5mg/ml dextran (molecular weight: 10 kDa) conjugated to fluorescein isothiocyanate (FD10S, Sigma-Aldrich). We used a cotton swab to gently press the endocardial surface of the tissues onto the cover slip at the bottom of the bath.²³ The tissues were then imaged through the cover slip using similar approaches as described above.

For evaluation of our approach for vessel reconstruction, a control heart was perfused with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DIL, D-282, Life Technologies) directly after perfusion with modified Tyrode's solution, following an established protocol.²⁶ A tissue section was taken from the right ventricle and labeled with 40 $\mu\text{g/ml}$ WGA CF488A (29022, Biotium) in PBS before compression-free mounting on a cover slip and confocal microscopy.

Analysis of Co-localization in Images

As a measure of co-localization of intensities in two image channels c_1 and c_2 we used Pearson's correlation coefficient:

$$P = \frac{\text{cov}(c_1, c_2)}{\sigma_{c_1} \sigma_{c_2}} \quad (2)$$

with cov the covariance and σ the standard deviation.

As a measure of co-localization of segmented signals we used the Dice coefficient:

$$D = \frac{2|c_1 \cap c_2|}{|c_1| + |c_2|} \quad (3)$$

Results

Preserved Tissue Morphology with Compression-Free Mounting

First, we compared 20 images acquired from tissue sections mounted either conventionally, i.e. embedded in mounting media between two glass slides, or using the new compression-free method (Figure 1A versus B). Conventional mounting resulted in deformed cardiomyocytes and compressed blood vessels (Fig. 1C). Since in these images cardiomyocytes were aligned with the y-axis, they appeared flattened in the cross-sectional views with a large width (x-axis) and a small height (z-axis). Capillaries and their lumens were not visible. To quantify the degree of compression, we measured the thickness at the center of two WGA-labeled tissue slices by determining the maximum range of fluorescence signal with a 20 $\times$ lens. The slices were cut with the cryotome set to 100 μ m. One day after mounting, slice thickness was only 65 μ m in the conventionally mounted sample, but 102 μ m in the sample mounted with the new method.

Compression-free mounting led to cardiomyocyte shapes with similar width and height (Fig. 1D). Capillaries were abundant in the tissue and their lumens were open. Furthermore, the images presented regions of weakly labeled extracellular space.

Reducing Depth-Dependent Signal Attenuation

We next investigated the effects of relative humidity during drying of the mounting medium (Online Figure 1). Imaging directly after mounting or after keeping samples for 3 d at 100% humidity revealed pronounced signal attenuation and high noise levels already at 20 μ m depth. However, signal intensities improved with decreasing humidity with the optimum reached near 40%. Further drying of the mounting medium below 35% humidity deteriorated WGA signals. For all subsequent preparations, we therefore used the compression-free mounting approach and dried samples at 40% humidity for at least 24h. Subsequent sealing with liquid varnish efficiently avoided desiccation of the mounted sample or potential re-absorption of water. This ensured stable imaging quality for at least 4 weeks.

To further improve signal-to-noise ratios at high imaging depths, we linearly increased laser powers with increasing distance from the tissue surface. This avoided signal saturation while counteracting depth-dependent attenuation. We compared images obtained with and without laser power increase (Fig. 2A and B). After image acquisition and deconvolution, mean signal intensities in axial direction were calculated. As expected, constant laser power produced an exponential decrease in signal intensity, whereas increasing laser power resulted in roughly constant signal intensity (Fig. 2C and D). Curve fitting based on the model described in equation (1) produced good approximations for both constant and increasing laser powers (Fig. 2C and D, Online Table 3). We inverted the fitted curves to correct mean signal intensities, an important step for subsequent thresholding. Comparison of images after correction (Fig. 2E and F) revealed that increasing laser power improved the signal-to-noise ratio particularly in depths >40 μ m. Images of the XY plane at 60 μ m depth are provided in Online Figure 2. With the described improvements, we acquired image

stacks within a depth of ~60 μm containing several layers of cardiomyocytes without deformation.

Characterization of Tissue Structure

We applied the imaging approach to left-ventricular tissue slices from a control heart and the MI border zone three weeks after MI. Tissues were labeled with WGA and DAPI as well as for vimentin and α -SMA (Figure 3 and Online Figure 3). We acquired image stacks with a depth of 60 μm from regions without large blood vessels like arterioles or venules. Thus, control tissue showed only marginal α -SMA signal (Fig. 3C), whereas pronounced α -SMA labeling was detected in the MI border zone, indicating fibroblast differentiation into myofibroblasts (Online Fig. 3C). Vimentin was found in vessel walls and fibroblasts (Fig. 3D and Online Fig. 3D).

After preprocessing, we applied histogram-based thresholds to all image channels and then used the resulting binary images to segment and classify cardiomyocytes, fibroblasts, myofibroblasts, blood vessels and extracellular spaces. Because the watershed-based segmentation method detected vessel lumens, but not vessel walls, lumens were dilated to include these in the segmented vessels. We used co-labeling with DIL as a marker for endothelial cells to identify the appropriate dilation radius of 1 μm (Online Figure 4).

A segmented image stack of control is presented in Figure 4. Control tissue exhibited a large number of capillaries running mainly parallel to cardiomyocytes. We identified extracellular laminas of 5-10 μm thickness, oriented perpendicular to the epicardial surface between layers of 3-5 myocytes. Most fibroblasts were found in these laminas and around blood vessels. As expected, we did not find myofibroblasts in control. The MI border zone, in contrast, showed a large number of myofibroblasts and an extensive increase in fibrotic tissue and extracellular space (Online Figure 5). Figure 5 presents 3D reconstructions of the control stack from Figure 4. Including truncated cells at image borders, the stack contained 124 densely arranged cardiomyocytes in a volume of $205 \times 205 \times 60 \mu\text{m}$ (Fig. 5A) with only small amounts of extracellular space between myocytes (Fig. 5B) and a dense capillary network (Fig. 5C). Fibroblasts were densely distributed, with their flat sections partially enfolding myocytes (Fig. 5D). Extracellular laminas were extracted in Fig. 5E. In this 3D reconstruction, we detected four laminas roughly spaced at a distance of 50 μm .

Reconstructions from the MI border zone (Figure 6) show extensive remodeling of tissue structure. Cardiomyocytes exhibited severe hypertrophy and were arranged less densely than in control (Fig 6A). The images revealed extensive fibrosis and an increase in extracellular space (Fig. 6B). The number of blood vessels, however, was lower than in control (Fig. 6C). We found an increased number of fibroblasts and abundant myofibroblasts, most of them located in fibrotic areas. Extracellular laminas were enlarged and did not show the regular arrangement as in control (Fig. 6E). Detailed views of cross-sections through cardiomyocytes from control and MI border zone illustrate the remodeling that occurred after MI (Fig. 7). Remodeling includes hypertrophy of myocytes, an increase in number and volume of fibroblasts, occurrence of myofibroblasts, and a largely increased amount of extracellular space. While in control commonly only small interstitial clefts were found between adjacent myocytes and between myocytes and blood vessels (Fig. 7C and D),

myocytes in the MI border zone appeared to be encased by extracellular matrix, fibroblasts and myofibroblasts. This led to increased distances not only between adjacent myocytes, but also between capillaries and myocytes (Fig. 7G and H).

Quantification and Analysis

To quantify these findings, we calculated volume fractions of cardiomyocytes, blood vessels, vessel lumens, fibroblasts, myofibroblasts and extracellular space from segmented image stacks of control and MI border zone (Table 1). The analyses revealed the large degree of microstructural remodeling in the border zone. While the volume ratio of cardiomyocytes was reduced, volume ratios of fibroblasts and myofibroblasts were increased. We found a major increase of extracellular space, which compensated primarily for the loss of myocytes. Additionally, we determined the number of neighbors of cardiomyocytes within a distance of 0.5 μm . We considered only cardiomyocytes not truncated at the image border. The result shows that the number of neighboring cardiomyocytes was more than halved in disease.

Colocalization of WGA with Volume Marker

One goal of this study was to evaluate WGA as a marker of the extracellular space in cardiac tissue. WGA binds to the glycocalyx on cell membranes and other glycoproteins of the extracellular matrix, e.g. collagen, but does not directly label interstitial fluids.³³ Thus, we evaluated the co-localization of WGA with an extracellular volume marker (Online Figure 6). We used dextran conjugated to fluorescein as a volume marker in the bath solution and imaged living tissue previously labeled with WGA conjugated to AF555. Dextran diffused into the interstitial space and marked the interstitial space (Online Fig. 6A). Small interstitial clefts between neighboring myocytes and extracellular laminae were visible, but signal intensity in the t-tubular system of myocytes was weak. WGA, however, strongly labeled cell membranes and the t-tubular system, while large extracellular laminae were only weakly labeled (Online Fig. 6B). This resulted in locally variable degrees of colocalization (Online Fig. 6C). Pearson's correlation coefficient calculated from 4 image stacks was 0.59 ± 0.03 . Similar results were obtained after thresholding with mode plus one standard deviation (Online Fig. 6E-H). The Dice coefficient calculated from 4 thresholded image stacks was high (0.73 ± 0.03).

Effort

The time to complete the described process chain from tissue staining to image segmentation and analysis was 4-7 d. Staining of tissue samples required 1-2 d (see methods), subsequent drying in the mounting medium another 1-2 d. Image acquisition required ~45 min for a 60 μm stack. Automated and manual steps of image processing, segmentation and analysis required 2-3 d per image stack.

Discussion

This study introduces a comprehensive approach for 3D characterization and analysis of cardiac tissues at the submicrometer scale. Importantly, the approach allows simultaneous quantitative assessment of all major tissue components, i.e. cardiomyocytes, fibroblasts, myofibroblasts, vessels and extracellular space. We suggest that the developed approach

constitutes an important step towards automated and high-throughput 3D reconstruction of cardiac tissues. We suggest that these 3D reconstructions and their quantitative analysis will provide new insights into cardiac microstructure and structure-function relationships in normal and diseased tissues. The 3D reconstructions of tissue microstructure lend themselves to computational simulations of cardiac physiology, for instance, electrophysiology and metabolite transport. Applying numerical approaches that others and we developed it is possible to calculate parameters for models of electrical conduction from the reconstructions.^{2, 14, 33, 37} Specifically, extracellular conductivity and how it changes in remodeled tissue can be estimated from numerical calculations based on the reconstructed data. The values obtained can then be incorporated into conduction models operating on a larger scale. In our recent work,³³ we were limited by a small penetration depth and thus a small number of cardiomyocyte layers, which rendered it difficult to reliably measure conductivities along the z-axis. Here we presented a method that attenuates this limitation.

The method is based on matching refractive indices within tissues using Fluoromount and optimal drying, which considerably reduced light scattering. In combination with increasing laser power, we were able to acquire and process image stacks with an axial dimension of 60 μm , containing up to 4 layers of cardiomyocytes. Although the signal intensity gradually decreased at depths greater than 60 μm , the t-system, which is close to the resolution limit of confocal microscopy in rabbit ventricular cells, was still visible at 120 μm (Online Figure 1). Our clearing approach is based on reducing of heterogeneity of refractive indices within tissue. As with many other tissue clearing methods, water is replaced by an organic compound with a higher refractive index.^{13, 19} Advantages of our approach are that it is compatible with immunolabeling and does not alter the arrangement of structures. In particular, we did not find alterations of the spatial distribution of fluorescence associated with connexin 43 and ryanodine receptor clusters in cleared tissues versus fixed cells (data not shown). A sophisticated clearing protocol, CLARITY, has recently been developed.⁴ Lipids are removed from tissue specimens after proteins and other structures have been stabilized by generating an acrylamide-based hydrogel and by fixation. This reduces heterogeneity of the refractive index and light scattering in tissue preparations. CLARITY is compatible with immunohistochemical labeling and produces highly transparent preparations. The approach was developed for clearing brain specimens, but there are no reports of its use in cardiac tissue. Furthermore, it is a time-consuming process, taking ~8 d to clear a mouse brain, for instance. Nevertheless, we note that CLARITY and other clearing methods, including established protocols using benzyl alcohol-benzyl benzoate,^{7, 36} might be compatible with the described protocols for compression-free tissue mounting and fluorescent labeling.

An important component of the presented approach is the compression-free tissue mounting. We demonstrated that conventional mounting leads to compression of cardiomyocytes and blood vessels, whereas the new method avoids deformation of preparations and preserves morphological features of tissue. These improvements allowed us to identify extracellular laminas, which were discriminated from the interstitial space by their geometry and commonly a lower intensity of WGA signal versus the interstitial space between adjacent cardiomyocytes (Figures 3 and 4). The low WGA signal might result from lower concentrations of collagen within these laminas. We found similar laminas in living tissue

(Online Figure 6), suggesting that the laminas are not an artifact of tissue processing or the compression-free mounting. On the other hand, laminas were not detectable with conventional mounting, potentially caused by low mechanical stiffness and compression. Originally, extracellular laminas (or sheets) had been found in studies on ventricular myocardium from canine using photography and block face scanning electron microscopy, and from rat using picrosirius red labeling and confocal microscopy.^{25, 45} It is thought that laminas are important determinants of electrical and mechanical properties of cardiac tissues. We suggest that the new approach for compression-free tissue mounting will facilitate future studies of laminas and allow their inclusion into electromechanical models of cardiac microstructure.

A further tissue component that was preserved by compression-free tissue mounting was the vasculature, primarily capillaries. Our studies indicate that conventional mounting leads to compression of vessel lumens. In fact, vessel lumens were not visible in our previous studies on fixed ventricular tissue from rat and rabbit.^{22, 33} Using compression-free tissue mounting, vessel lumens remained open and their segmentation was straightforward. However, identification of vessel walls and associated cells was hindered by the unavailability of a specific marker of endothelial cells in fixed tissues. Therefore, we dilated the vessel lumens by image processing to estimate vessel walls. We evaluated the reliability of the approach in living tissue. Previous work on rat myocardium yielded blood volumes in vessels with a diameter smaller 100 μm ranging between 4.6 and 7.4 ml per 100 g tissue¹⁸. Our analysis yielded a volume fraction of vessel lumens of 3.7% in control myocardium (Online Table 1). This is smaller than the reported value, but can be explained by our focus on imaging of regions without large blood vessels, e.g. arterioles and coronary arteries.

Similar to endothelial cells, a reliable specific marker is missing for fibroblasts. In several studies others and we used a vimentin antibody for this purpose.^{33, 46} Vimentin is expressed not only in cardiac fibroblasts and myofibroblasts, but also in vascular endothelial cells and pericytes.^{12, 20} Thus, we applied structural information to discriminate fibroblasts from other cells. For this purpose, we used α -SMA as a marker for myofibroblasts and excluded vimentin-positive cells found within 1 μm from vessel lumens in our identification of fibroblasts. Fibroblasts were found in all tissues, while myofibroblasts were only detected in the infarct border zone and associated with interstitial fibrosis. It has been suggested that this may result from sustained activation through pressure and volume overload, or cytokines.^{35, 42, 43}

While our approach relied on four fluorophores, novel confocal microscopes allow the application of up to 6 fluorescent markers in one tissue sample.⁹ Adding a specific marker of cardiomyocytes, for example immunostaining for sarcomeric alpha actinin, would allow for their automated detection. Furthermore, markers of other structures that are remodeled in cardiac disease, e.g. gap junctions, could be added. In this study, WGA served primarily as a marker of cardiomyocyte membranes and of the extracellular space. Recently, WGA labeling has been proposed as a method for detection and quantification of fibrosis, with comparable quality as the established picrosirius labeling.¹⁰ In the present study, we showed that the overlap of WGA and the dextran-labeled extracellular space was high in segmented images, as indicated by the Dice coefficient.

The increased volume fraction of extracellular space and corresponding decrease in the cardiomyocyte fraction in the MI border zone (Table 1) reflect the high proliferation of interstitial fibrotic tissue. Furthermore, the number of interconnected myocytes in this region was substantially reduced from 9.4 to 4.3 (–54%). These results are consistent with previous findings.^{27, 29} Meticulous reconstructions of serial tissue sections from normal myocardium and MI border zone tissue from canine hearts revealed 11.2 and 6.5 neighbors per myocyte, respectively.²⁷ There have also been more recent reports of decreased cardiomyocyte fraction and connectivity in the infarct border zone of rats with chronic MIs.²⁹ In the latter study, it was shown that major infarct-associated remodeling occurs within a narrow zone of 300–500 μm around the infarct. While cardiomyocyte arrangement in the border zone was reconstructed in 3D, the non-myocyte space was not dissected into different components, e.g. vessels or myofibroblasts. We suggest that the method presented in this paper will be particularly suitable for providing detail on the gradient of remodeling in the MI border zone. Our method is useful not only for precise quantification of volume ratios at the micrometer scale, but also for detailed analysis of geometrical relationships between cardiac cells. This quantification of intercellular relationships may be important in diseases like MI or heart failure where changes in tissue structure can lead to altered pathways of cardiac excitation and, thus, arrhythmia.⁵

Limitations

We acknowledge several limitations of the described approach: The applied mathematical correction of light attenuation is based on fitting a simplified model to measured z-profiles of mean image intensities. It is assumed that fluorophores are homogeneously distributed in z-direction. In case of largely inhomogeneous distributions of fluorophores the attenuation correction could be based on predetermined parameters to avoid artifacts caused by the fitting. While our approach for semi-automatic segmentation of myocytes and vessels requires only a small degree of user interaction, it is possible that errors are introduced. We used visualizations to inspect the 3D reconstructions and correct errors. In addition, rule-based methods might be useful to detect and correct segmentation errors. A further limitation is that we did not evaluate the contraction state of the heart during PFA fixation. It is known that some structural characteristics, for example the diameter of vessel lumens, are different in diastole as compared with systole.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Nora Eccles Harrison Treadwell Foundation (FBS, TS), AHA grant 14POST19820010 (TS), NIH grant R01 HL094464 (FBS), the Studienstiftung des deutschen Volkes (JCE) and Stiftung Familie Klee (JCE). The authors thank Mrs. Jayne Davis and Mrs. Nancy Allen for technical support.

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Figure 1.

Mounting and imaging of cardiac tissues. (A) Conventional mounting of tissue slices between a probe carrier and a cover slip. (B) Compression-free mounting, with tissue slices sealed on the cover slip. XY, XZ and YZ sections at centers of three-dimensional image stacks from confocal microscopy of WGA-labeled cardiac tissue from rabbit with (C) conventional and (D) compression-free mounting. Capillary lumens are open and cells are less deformed when using the compression-free approach. Scale bar in (C) has a length of 40 μm and also applies to (D).

Figure 2.

Measuring and correcting depth-dependent attenuation. Cross-sectional views of image stacks acquired from WGA-labeled tissue with (A) constant laser power of 7% (0.525 mW) and (B) linearly increasing laser power from 3% (0.225 mW) to 15% (1.125 mW). (C-D) Profiles of mean intensity $\bar{I}$ in axial direction. Left y-axes apply to profiles before correction (circles) and fitted curves based on the attenuation model (crosses). Right y-axes apply to profiles after correction (squares). (E-F) Images after attenuation correction. Scale bar in (A) has a length of 40 μm and also applies to (B,E,F).

Figure 3. Central cross-sections from three-dimensional image stacks after deconvolution, noise filtering and attenuation correction. Control tissue labeled with (A) WGA, (B) DAPI, (C) anti-vimentin, and (D) anti-α-SMA. Scale bar in (A) has a length of 40 μm and also applies to (B-D).

Figure 4.

XY, XZ and YZ cross-sections from segmented image stack of control tissue. The stack has an isotropic resolution of 0.2 μm . Segmented components: cardiomyocytes (black), vessel lumens (dark red), vessel walls including endothelium (light red), fibroblasts (blue), remaining segmented WGA signal (dark green), additionally segmented extracellular space (light green). Extracellular laminas are indicated by asterisks. Dashed lines indicate sectional planes. Scale bar: 40 μm .

Figure 5.

Three-dimensional reconstructions of ventricular tissue from control rabbit. The stack size is $205 \times 205 \times 59 \mu\text{m}$. (A) Cardiomyocytes are densely arranged in the tissue. Including truncated cells, the stack contains 124 cardiomyocytes. Each cell is represented by a different color. (B) Extracellular space (green), blood vessels (red), fibroblasts (blue) and cardiomyocytes (black). (C) Blood vessels are dense and run mainly parallel to the fiber orientation. (D) Fibroblasts. (E) Extracellular laminas perpendicular to the epicardial surface between layers of 3-6 cardiomyocytes.

Figure 6.

Three-dimensional reconstructions of tissue from the MI border zone. Stack size is $205 \times 205 \times 60 \mu\text{m}$. (A) Cardiomyocytes are hypertrophied and less dense than in control. Including truncated cells, the stack contains 53 myocytes. Each cell is represented by a different color. (B) Extracellular space (green), blood vessels (red), fibroblasts (blue), myofibroblasts (orange) and cardiomyocytes (black). The extracellular space is strikingly increased versus control. (C) Blood vessels appear less in number and partly collapsed. (D) Fibroblasts and myofibroblasts form a dense network. (E) Extracellular laminas are significantly enlarged versus control.

Figure 7.

Enlarged views of regions extracted from image stacks of (A-D) control and (E-H) $\approx$ MI border zone. (A,E) Cross-sectional images from tissues labeled with WGA (green), for vimentin (blue) and α -SMA (red). Note the overlay of colors (WGA and vimentin: cyan; WGA and α -SMA: yellow; vimentin and α -SMA: magenta; WGA, vimentin and α -SMA: white). (B,F) Corresponding segmentations with extracellular space (green), blood vessels (red), fibroblasts (blue), myofibroblasts (orange), central cells (beige) and adjacent cells (gray). (C,G) 3D reconstructions of cross-sectional segments. (D,H) Adjacent cells and extracellular space were removed. Scale bar in (A) has a length of 10 μ m and applies to (B,C,E-G).

Table 1

Volume fractions of cardiomyocytes (CMCs), blood vessels including endothelium (vessels), vessel lumens, fibroblasts (FBs), myofibroblasts (MFBs) and extracellular (EC) space, average number $\pm$ SE of neighboring CMCs (neighbors) within a distance of 0.5 μ m per CMC in tissue from control and MI border zone.

	CMCs	Vessels	Lumens	FBs	MFBs	EC	Neighbors
Control	66.4%	8.0%	3.7%	1.3%	0%	24.3%	9.4 $\pm$ 0.5
MI	40.2%	3.3%	1.5%	3.1%	4.0 %	49.4%	4.3 $\pm$ 0.5