Visualizing the complex 3D geometry of the perfusion border zone in isolated rabbit heart

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Myocardial infarction, caused by a major blockage of a coronary artery, creates a border zone (BZ) between perfused and nonperfused tissue, which is believed to be the origin of fatal cardiac arrhythmias. We used a combination of optical clearing and polarization-sensitive optical coherence tomography to visualize a three-dimensional organization of the BZ in isolated rabbit hearts (n = 5) at the microscopic level with a high spatial resolution. We found that the BZ has a complex three-dimensional structure with nonperfused areas penetrating into perfused tissue with finger-like projections. These “fingers” may play an important role in the initiation and maintenance of ventricular arrhythmias. © 2012 Optical Society of America

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1. Introduction

Myocardial ischemia is a medical condition in which the blood flow to the heart is partially or fully obstructed in one of the major coronary arteries. This can be a devastating condition, affecting 785,000 people annually in the United States alone [1]. If the blockage is severe, the region of tissue downstream of the obstruction site will not receive blood from this artery and its capillaries, creating a region of nonperfused tissue, known as myocardial infarction (MI). In these hearts, a border zone (BZ) between the perfused and nonperfused regions is created due to differences in the perfusion levels. Electrophysiological changes that develop at the MI BZ are known to be the substrate for serious or fatal arrhythmias [2–4]. Although it has been demonstrated that the BZ itself plays an important role in the initiation of these arrhythmias [5–7], very little is known about its three-dimensional (3D) geometry.

Imaging the 3D geometry of the BZ has been achieved using conventional imaging modalities such as contrast enhanced echocardiography [8–10], magnetic resonance imaging (MRI) [11–15], and histology [16–20]. Contrast-enhanced echocardiography has the ability to precisely register myocardial perfusion, function, and geometry at the MI BZ [9]. However, the quantification of MI may result in an overestimation of its size, due to the fact that the infarct size is determined from multiple layers in the transmural direction [21]. Cardiac MRI is another nondestructive imaging technique that allows for the identification and quantification of irreversibly damaged myocardium on the surface as well as the assessment of the transmural infarct. However, the main drawback of using MRI is the limited spatial resolution (1 mm) [11,12,21,22], which prevents discerning the BZ in fine detail. The third conventional imaging technique is histology, which uses 2,3,5-triphenyltetrazolium chloride (TTC) to show viable and nonviable tissue [18,19]. Although histology provides a very high spatial resolution (less than 10 μm), its destructive nature does not provide for the most accurate 3D reconstructions of the MI BZ. All three techniques have been used to determine the 3D structure of the MI BZ, and the results revealed
that the BZ changes not only on the epicardial surface but through the depth of the myocardium [12,23,24]. The precise 3D geometry of the MI BZ is still unknown due to the limitations of these imaging modalities. Therefore, there is a need for an imaging technique that overcomes the spatial limitation of echocardiography and MRI while avoiding the destructive nature of conventional histology to resolve the 3D BZ geometry.

Recently, we adopted a novel optical clearing technique [25], which render specimens transparent by matching the intracellular and extracellular refractive indices and therefore allows for deeper light penetration due to a reduction of light scattering. It has been demonstrated that up to 4 mm of transparent cardiac tissue can be imaged instead of less than 100 μm in nontransparent tissue [26].

In the present study, we combined our optical clearing technique with optical coherence tomography (OCT) [27] to nondestructively determine the complex 3D geometry of the BZ at a very fine spatial resolution (~15 μm and ~28 μm in the axial and lateral directions, respectively). We created a BZ in the isolated rabbit heart by individually perfusing the left circumflex artery (LCX) with the clearing solution, thereby creating a border between the LCX (perfused region) and the rest of the heart (nonperfused region). By using a polarization-sensitive OCT system [28], we found that nonperfused tissue protruded into the perfused region in a finger-like fashion; the specific geometry of these “fingers” may provide the anatomical substrate to facilitate ventricular arrhythmias in MI hearts.

2. Materials and Methods

A. Optical Clearing of the Heart

All experiments conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996) and the University of Minnesota guidelines regulating the care and use of animals. New Zealand white rabbits (Bakkom Rabbitry, 1.3 to 2.0 kg, n = 5) were first heparinized (550 Units/100 g) and then anesthetized with ketamine and xylazine (35 mg/kg and 5 mg/kg, respectively). The heart was excised and placed in ice-cold cardioplegia solution [29]. Immediately following excision, the heart was fixed on the cannula via the aorta and perfused by warm (37 ± 1°C), oxygenated Tyrode’s solution [29] until all blood was washed out of the heart.

To create the BZ, the LCX was identified and perfused with clearing solution as described previously [25]. Specifically, the LCX was selectively perfused with a graded ethanol series (vol. % ethanol:cardioplegic solution: 50, 70, 75, 90, 95, and 100%), followed by a 1:2 benzyl alcohol:benzyl benzoate (BABB) solution. The rest of the heart, that is, the tissue supplied by the right coronary and left anterior descending arteries, was not cleared, and thus remained in a healthy physiological condition. As a consequence, a BZ developed between the region perfused by the LCX and the nonperfused regions of the left anterior descending and the right coronary arteries. The BZ created by the abovementioned procedure is similar to what is seen in clinical in vivo MI [30–36]. The entire procedure lasted approximately 45 min. Five samples were created to ensure that the BZ has a complex 3D geometry in all hearts. For presentation purposes, we only show one sample of the BZ.

B. Optical Coherence Tomography (OCT)

OCT imaging of the BZ was performed three times: before the LCX was perfused with ethanol, after the LCX was perfused with ethanol, and after the clearing procedure.

Imaging of the BZ under all three conditions was performed using a previously described polarization-maintaining fiber-based swept-source polarization-sensitive OCT system [28]. The system, operating at the 1300 nm region, provided axial and lateral resolutions of approximately 15 μm and 28 μm, respectively. Two types of images were obtained simultaneously from the recorded data set: (1) reflectivity image and (2) phase retardance image. Reflectivity is the conventional OCT contrast, and our imaging system has a sensitivity of 106 dB for reflectivity measurements. The phase retardance, resulting from tissue birefringence, is an additional contrast provided by polarization-sensitive OCT. Details on the description and operation of the imaging system can be found in [28]. A two-dimensional (2D) cross-sectional image containing 1000 A-lines (depth profiles) was acquired as the beam was laterally scanned (B-scan) over the heart by using a galvanometer scanner. A second scanner was utilized to scan (C-scan) the beam along the other lateral direction, so that OCT data for a volume was obtained by stacking 300 cross-sectional images. Information in the 3D data set is presented by several cross-sectional (2D) images whose lateral locations are indicated on an en face image that is also obtained from the OCT data set. The en face image is calculated by integrating the cross-sectional images along the depth direction; therefore, its top-view-like image is derived from subsurface structures. We also stacked all of the cross-sections for a 3D visualization of the BZ using V3D software [37].

3. Results

A representative picture of a rabbit heart before and after clearing is shown in Figs. 1(A) and 1(B), respectively. Before clearing [Fig. 1(A)], light could not pass through the heart due to scattering. After clearing [Fig. 1(B)], the region supplied by the LCX became partially transparent (amber color) as the refractive indices of tissue constituents and media (clearing solution) were matched. In contrast, the tissue supplied by the other arteries (left anterior descending and right coronary arteries) remains unchanged as it was not perfused with the clearing solution. Therefore, the BZ [see arrow in Fig. 1(B)] divides the regions of perfused and nonperfused tissue in
a finger-like manner, which can be easily seen on the epicardial surface of the heart. Note that the positions of the heart are different in Figs. 1(A) and 1(B) in order to better demonstrate the BZ.

To examine the geometry of the BZ both epicardially and transmurally, we performed OCT scans as depicted schematically in Fig. 2. Figure 2(A) illustrates the situation when the fast lateral scan (B-scan) was performed on the epicardial surface of the heart (see the black line and letters N and P for nonperfused and perfused regions, respectively). Several cross-sectional OCT images were acquired from apex to base. Selected images from these scans are shown in Figs. 3, 4, and 6. To perform transmural OCT scans, we made two transverse slices through the heart as illustrated in Fig. 2(B) to obtain a 3 mm thick slab of cardiac tissue; this slab was then imaged. The OCT B-scans producing cross-sectional images were performed from epicardium to endocardium (see black line and Epi to Endo in Fig. 2(B) for directional information), simultaneously providing depth information from base to apex. Multiple cross-sectional images were acquired by scanning the beam along the perfused and nonperfused regions. Note that the C-scan in this configuration started approximately 2 mm to the left of the BZ and ended 2 mm into the perfused region, thereby capturing all of the BZ. Data from these scans are shown in Figs. 5 and 7.

Figure 3 shows representative examples of reflectivity and retardance en face images [Figs. 3(A) and 3(C), respectively], and reflectivity and phase retardance cross-sectional OCT images [Figs. 3(B) and 3(D), respectively] of the epicardial surface of the cleared rabbit heart. In addition to the reflectivity, the form birefringence is also expected to reduce due to refractive index matching in the clearing process. At this stage, this should be seen as supportive evidence, as we are not comparing these two contrasts extensively. The bright color in the reflectivity images [Figs. 3(A) and 3(B)] indicates the nonperfused tissue that exhibits more scattering than the cleared tissue (black color). Because the nonperfused tissue is highly scattering, muscle fibers in this region can be seen; whereas in the perfused region, the refractive index is matched and fibers cannot be observed. Note that the reflectively en face image [Fig. 3(A)] displays anatomical information (i.e., vessels) and more importantly illustrates the complex structure of the BZ. In the retardance en face image [Fig. 3(C)], areas of dark color indicate small birefringence, which might be reduced because of the clearing effect on birefringence. The area on the right

![Fig. 1. (Color online) Rabbit heart before and after clearing to show the surface BZ. (A) Rabbit heart in the air before dehydration and clearing. The white arrow shows the LCX. Scale bar = 1 cm. (B) The same heart rotated clockwise to show the BZ after dehydration and clearing with 1:2 (vol.%) BABB. The heart shows distinct regions of perfused and nonperfused tissue. The arrow indicates the BZ between these two regions. Inset shows an enlarged view of the perfused (amber color, right), the BZ, and nonperfused (normal colored, left) tissue. Scale bar = 0.50 cm.](image1)

![Fig. 2. (Color online) Schematic of the heart preparation for OCT imaging. (A) Longitudinal view of the BZ. For these scans, the heart was not sliced. The cleared region of the LCX is depicted by the amber color. The BZ is shown by the dotted line. The black line shows the region in which the 3D scan was performed. The letters N and P indicate the orientation of images shown in Figs. 3, 4, and 6. (B) The short-axis view of the imaged BZ. Two transmural slices were made, which resulted in a 3 mm thick slab, which was then imaged. The black line (Epi to Endo) shows how the region was scanned. The letters show the orientation of the images shown in Figs. 5 and 7.](image2)
includes noise caused by the signal-to-noise ratio reduction due to refractive index matching in the perfused region. This could be seen in the reflectivity [dark color; Fig. 3(B)] and retardance [noise pattern; Fig. 3(D)] images. The border between these two regions can be clearly distinguished [see BZ suggested by the white dotted line drawn manually in Figs. 3(A) and 3(C)]. In the retardance images, the color change from dark to bright (epicardium to endocardium) represents higher birefringence typically in the nonperfused region. All the images demonstrate that the cleared tissue penetrates into the uncleared region in a so-called “finger-like” projection.

Figure 4 depicts representative reflectivity and retardance en face images [Figs. 4(A) and 4(C), respectively] as well as 2D OCT reflectivity and phase retardance images [Figs. 4(B) and 4(D), respectively] of uncleared heart. These images are taken from the same area as in Fig. 3 just before clearing. Note that the heart was rotated slightly from Fig. 3 to capture more of the BZ. The reflectivity and retardance en face images in Fig. 4 illustrate heart anatomy and explicitly show no signs of a BZ. In both Figs. 4(B) and 4(D), only the epicardial surface of the tissue can be observed due to the high level of light scattering in the tissue. A supplemental movie that shows 300 sequential 2D cross-sectional slices (Media 1) shows that there is no indication of the BZ from apex to base at any depth.

To determine whether the ethanol affects the penetration depth of the tissue during OCT imaging, we imaged the BZ after ethanol treatment. Representative 2D OCT reflectivity and phase retardance images are shown in Figs. 4(E) and 4(F), respectively. Note that only the epicardial surface of the heart is visualized in both OCT images, similar to Figs. 4(B) and 4(E). Therefore, the BZ that we observe in Fig. 3 is due to the difference in perfusion with the BABB solution and not due to any effects of the ethanol.

To determine if the “fingers” are present transmurally within the heart, we sliced the heart transversely as shown in Fig. 2(B). Representative reflectivity and retardance en face images [Figs. 2(A) and 2(C), respectively] and 2D OCT reflectivity and phase retardance images [Figs. 2(B) and 2(D), respectively] of the cleared heart are depicted in Fig. 5. These images demonstrate a clearly distinguished border between the perfused and nonperfused regions of the heart (BZ suggested by the white dotted line drawn manually in Fig. 5). Therefore, the BZ has a complex 3D geometry, both epicardial and transmurally, that is comprised of “fingers” of perfused tissue that protrude into the nonperfused region.

The cross-sectional OCT reflectivity and retardance images of the epicardial surface of the cleared heart
at different locations are illustrated in Figs. 6(A) and 6(B), respectively. The image slices start on the apical surface (0.03 mm) and progress to the base (9.09 mm). The blue lines on the en face image denote where on the surface the slices were taken [Fig. 6(A)]. The nonperfused region is on the left, which can be seen due to the highly scattering nature of the tissue; the perfused region is on the right and can be seen through the depth due to the lack of scattering within the tissue. The “fingers” of the BZ can be clearly distinguished at each location through the tissue. For instance, there are distinct “fingers” on the apical surface (0.03 mm), and near the base (5.50 mm and 7.33 mm). However, the absence of visible “fingers” at 1.82 mm and 3.65 mm indicate that the number and size of the “fingers” change, supporting the complex 3D geometry of the BZ. A movie showing the BZ from these 300 cross-sectional slices is shown in Media 2.

To illustrate that the “fingers” are present not only on the epicardial surface but transmural as well, we provide snapshots of 2D OCT reflectivity and retardance images in Figs. 7(A) and 7(B), respectively. The blue line on the en face image denotes where on the surface the slices were taken [Fig. 7(A)]. Similar to Fig. 6, “fingers” can be distinguished at 3.65 mm and 7.33 mm. Again, the number and size of the “fingers” vary at different locations; this is further supported by Media 3.

To visualize the “fingers” of the BZ, we made a 3D reconstruction of the BZ using the reflectivity images. Figure 8 shows that the perfused region is invisible because of the clearing, leaving the nonperfused region visible. The “fingers” of the BZ are readily observed. Media 4 through Media 6 show the 3D BZ at different orientations (Media 4), sliced from perfused to nonperfused (Media 5), and sliced from epi- to endocardium (Media 6).
4. Discussion

By using the combination of optical clearing and OCT, we were able to observe, with high resolution, the 3D geometry of the MI BZ of the rabbit heart with a 15 μm axial resolution. In this manner, we have determined that the BZ is not a sharp transition between perfused and nonperfused tissue but is rather comprised of “fingers” of perfused tissue protruding into nonperfused regions. The number and size of the “fingers” change both epicardially and transmurally, indicating a complex 3D geometry of the BZ.

To our knowledge, this is the first study to combine optical clearing with OCT to determine the complex 3D geometry of the BZ. OCT has emerged as a powerful technique to noninvasively image biological tissue [38]. The principle of OCT is based upon low-coherence reflectometry and excels in depth penetration of a few millimeters depending on the tissue and center wavelength of the source. In transparent tissue (via optical clearing), penetration depths of 3 mm can be expected. In low-coherence reflectometry, reflected or backscattered light from inside the specimen is interfered with light that has traveled a known reference path [39], which provides information on the time-of-flight delay from the reflective boundaries in the specimen. The signal is detected when the reflections of the sample and reference are nearly matched (within a coherence length) [27]. There is an inverse relationship between the bandwidth of the light source and the axial resolution. Cross-section images are, therefore, generated by scanning an optical beam across the tissue and measuring the intensity of the backscattered light [39]. Even in uncleared cardiac tissue (up to 2 mm) [40-43], OCT is beneficial because the fibrous tissue and fat create an optical contrast within the myocardium. However, to image deeper internal structures of the heart using OCT (more than 3 mm), the tissue should be made transparent by a technique called optical clearing. Optical clearing is a technique that makes tissue transparent by matching the refractive index of the intra- and extracellular space. For imaging studies, matching of the refractive index allows for imaging deeper within the tissue; this is due to a reduction in light scattering within the tissue. By adapting the optical clearing technique to create a BZ, we can see the border not only on the epicardial surface [Fig. 1(B)] but also through the depth of the myocardium (Fig. 6 and Media 2) at a high resolution using OCT. It is important to note that the dark voids that we see in reflectivity en face images [Figs. 3(A), 4(A), and 5(A)] are most likely vessels, which may provide more information on how the tissue is perfused during MI experiments.

While electrophysiological changes at the BZ are a substrate for MI-induced arrhythmias, the 3D geometry may play a more important role in the initiation and maintenance of these arrhythmias. Indeed, a simulation study illustrated the importance of the 3D BZ during ischemia in porcine left ventricular tissue [23]. The geometry of the ischemic BZ was imaged with MRI and was found to be complex through the depth. When one perfusion region is made to be ischemic, it was found that the irregular geometry formed transmural pathways leading to...
fast reentry, facilitating and maintaining the ventricular arrhythmia. Therefore, it is imperative to determine the 3D geometry of the perfusion BZ in order to understand arrhythmogenesis during MI, which we will do in future studies. Further studies are necessary to correlate the BZ geometry with its respective electrophysiological properties.

5. Conclusion

In summary, our technique capitalizes on the properties of the optical clearing process and depth-resolved, polarization-sensitive OCT to nondestructively image the BZ with a high spatial resolution. Furthermore, this imaging process will help characterize the electrophysiological consequences of MI that lead to fatal arrhythmias by correlating BZ geometry with arrhythmogenic reentry.

6. Limitations

In most imaging techniques, fixation and subsequent dehydration of the tissue causes substantial shrinkage (15 to 35%) [44,45]. Even though there is shrinkage, this will not impact the 3D geometry of the MI BZ. Also, we found that the ethanol does diffuse through the tissue; however, it does not affect the formation or presence of a BZ. To limit any major error of characterizing the BZ, we used cleared hearts within 1 day of forming the BZ.

While it would be ideal to have histological verification of the optical clearing and OCT analysis of the BZ, the nature of the heart after clearing makes it difficult to obtain accurate histological sections. Because we do see a complex 3D geometry of the BZ, similar to what has been shown using common imaging modalities [12,23,24], we are confident that the technique is valid in imaging the BZ as would be seen in clinical in vivo MI [30–36].

It is important to note that, in this study, we can only determine the effect of perfusion on BZ geometry for a limited time interval, up to several hours. In preliminary experiments, we created a BZ at different times of perfusion mimicking acute MI (30 min, 1 h, 2 h); after staining with TTC, we found that regardless of how long it took for the BZ to be created the shape and size of the BZ did not change. Therefore, we are confident that our results are reproducible for acute cases of MI.

Appendix A

Media 1. Uncleared OCT movie from Figs. 4(B) and 4(D). The reflectivity and retardance en face images show where the slices were taken (as indicated by the moving blue line). At every depth, a BZ is not observed. More importantly, in the uncleared heart, only the epicardial surface can be seen due to the highly scattering nature of the myocardium.

Media 2. Cleared OCT movie from Figs. 3 and 5. The reflectivity and retardance en face images show where the slices were taken (as indicated by the moving blue line). At every depth, the BZ can be observed. The BZ is comprised of “fingers” that protrude from the perfused region into the nonperfused region.

Media 3. Transmural sectioning of cleared OCT movie from Figs. 5 and 7. The reflectivity and retardance en face images show where the slices were taken (as indicated by the moving blue line). At every depth, the BZ can be observed. Similar to Media 2, the BZ is comprised of “fingers” that protrude from the perfused region into the nonperfused region.

Media 4. 3D reconstruction of the BZ at different orientations. The tissue sample is rotated to illustrate the extent of BZ depth and geometry in 3D.

Media 5. 3D reconstruction of the BZ sliced from perfused to nonperfused regions. The epicardial and endocardial surfaces are indicated. Branching vasculature can be observed (dark circles), and a loss of reflectivity signal indicates the degree of clearing in the perfused tissue.
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