High-Resolution 3-Dimensional Reconstruction of the Infarct Border Zone

Impact of Structural Remodeling on Electrical Activation

Sally L. Rutherford,* Mark L. Trew,* Gregory B. Sands, Ian J. LeGrice, Bruce H. Smaill

Rationale: Slow nonuniform electric propagation in the border zone (BZ) of a healed myocardial infarct (MI) can give rise to reentrant arrhythmia. The extent to which this is influenced by structural rather than cellular electric remodeling is unclear.

Objective: To determine whether structural remodeling alone in the infarct BZ could provide a substrate for re-entry by (i) characterizing the 3-dimensional (3D) structure of the myocardium surrounding a healed MI at high spatial resolution and (ii) modeling electric activation on this structure.

Methods and Results: Anterior left ventricular (LV) infarcts were induced in 2 rats by coronary artery ligation. Three-dimensional BZ volume (4.1 mm$^3$ and 5.6 mm$^3$) were imaged at 14 days using confocal microscopy. Viable myocytes were identified, and their connectivity and orientation were quantified. Preserved cell networks were observed in the subendocardium and subepicardium of the infarct. Myocyte tracts traversed the BZ, and there was heavy infiltration of collagen into the adjacent myocardium. Myocyte connectivity decreased by $\approx 65\%$ over 250 $\mu$m across the BZ. This structure was incorporated into 3D network models on which activation was simulated using Luo–Rudy membrane dynamics assuming normal cellular electric properties. Repetitive stimulation was imposed at selected BZ sites. Stimulus site-specific unidirectional propagation occurred in the BZ with rate-dependent slowing and conduction block, and reentry was demonstrated in one substrate. Activation times were prolonged because of tract path length and local slowing.

Conclusions: We have used a detailed image-based model of the infarct BZ to demonstrate that structural heterogeneity provides a dynamic substrate for electric reentry. (Circ Res. 2012;111:301-311.)

Key Words: arrhythmia ■ computers ■ conduction ■ infarct ■ ventricles

Healed myocardial infarcts provide a substrate for potentially life-threatening reentrant arrhythmias. Structural and electric remodeling in the infarct border zone (BZ) can give rise to slow conduction, unidirectional block, and rate-dependent electric instability after myocardial infarction (MI), which increases the probability of electric reentry. Factors that have been implicated include (1) strands of surviving myocytes surrounded by dense fibrosis that provide tortuous conduction pathways within the infarct BZ, (2) decreased lateral connections between myocytes and altered gap junction distributions in the peri-infarct region, and (3) changes in transmembrane ion channel expression adjacent to the healing infarct that reduce conduction velocity and delay repolarization during infarct healing. Cardiac magnetic resonance (CMR) has been used to characterize the extent of the peri-infarct region and the distributions of preserved myocytes and fibrosis within it. The former is a powerful independent predictor of mortality after MI, while it is argued that heterogeneous organization of myofibers and fibrosis within the BZ provides a further indication of arrhythmic risk. The 3-dimensional (3D) topology of surviving myofiber tracts adjacent to healed human infarcts has been reconstructed at a relatively coarse scale from serial histological sections. However, experimental investigation of the effects of this structural anisotropy on electric activation in the infarct BZ is problematic: surface recordings provide little direct information on intramural electric propagation, while the spatial resolution that can be achieved with plunge needle arrays is limited.

Image-based computer modeling provides a powerful means of investigating the mechanisms by which structural anisotropy influences electric activity in the infarct BZ.
Two-dimensional computer models have been used widely for this purpose and show that nonuniformly distributed inexcitable regions can give rise to electric instability and reentry. To date, though, comparable 3D models have only investigated the influence of infarct and BZ geometries acquired with relatively low-resolution CMR imaging.

In this paper, we describe a study in which the myocytes and connective tissue adjacent to healed rat infarcts have been imaged and reconstructed in 3D at high resolution. This has enabled us to analyze and quantify the topologic characteristics of surviving myocyte strands in the infarct BZ more comprehensively than has previously been possible. These data have also been used to develop image-based computer models of electric activation in the infarct BZ, which demonstrates how structural heterogeneity in the peri-infarct region provides a substrate for unidirectional propagation, rate-dependent regional slowing, and conduction block.

**Methods**

These methods are described in more detail in the Online Supplement.

**Surgical Procedures and Tissue Preparation**

All surgical procedures were approved by the Animal Ethics Committee of the University of Auckland and conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85–23). An MI was surgically induced in 2 adult male rats by permanent ligation of the LAD artery near the origin. Fourteen days postinfarction the hearts were excised, fixed in 4% formaldehyde (Sigma-Aldrich), and embedded in methyl methacrylate (MMA) resin. The specimens were sliced into 1-mm equatorial rings, dehydrated, and embedded in MMA. Each heart was serially sectioned at 10-μm intervals and stained with tetrachrome (Biebricher). Sections were imaged using a high-resolution confocal microscope (MRC 1024; Bio-Rad) with a 100× oil immersion objective. Tissue sections were mosaiced to generate a 3D volume of the entire heart. The 3D volume was then subjected to automated segmentation using an algorithm that separated surviving myocytes from nonmyocytes (ventricular myocardium, connective tissue adjacent to healed rat infarcts have been imaged and reconstructed in 3D at high resolution. This has enabled us to analyze and quantify the topologic characteristics of surviving myocyte strands in the infarct BZ more comprehensively than has previously been possible. These data have also been used to develop image-based computer models of electric activation in the infarct BZ, which demonstrates how structural heterogeneity in the peri-infarct region provides a substrate for unidirectional propagation, rate-dependent regional slowing, and conduction block.

**Image Acquisition**

Extended volume confocal images of the transmural infarct BZ were acquired at 1 μm voxel dimension using a purpose-built system. These data were processed and aligned as described by Sands et al.

**Image Segmentation and Network Construction**

The image volumes were down-sampled to 2 μm voxel dimension for image segmentation and subsequent image processing. Key morphological features were identified in the epicardial tangent planes. Normal myocytes, collagen, extracellular space, and necrotic tissue were segmented on the basis of intensity, texture, and continuity (Figure 2). Local myocyte volume and tissue connectivity indices were calculated at each voxel by applying filters of radii 5 voxels. Myocyte voxels not connected to the main tissue region were removed (~1% total myocyte volume). The interface between BZ and normal myocardium was defined by applying a fuzzy C-means clustering algorithm that separated these regions on the basis of connectivity and myocyte volume distributions (Figure 3B). The total volume of viable connected myocardium in the infarct BZs were 1.07 mm³ and 1.73 mm³. Network descriptions of the structures were generated using 3D masks of surviving myocytes. Myofiber orientations were calculated using structure tensor analysis and mapped onto the network.

**Activation Modeling**

Activation was simulated on this network description using Luo–Rudy dynamic (LR-d) membrane dynamics, assuming normal electric properties and transverse isotropy at the cellular level. Repetitive stimulation at 300-ms base cycle length (BCL) was imposed at sites within the BZ and normal tissue. Following 10 preconditioning beats, an S2 stimulus was applied at progressively decreasing coupling intervals (CI) until complete activation block occurred. Reentry was investigated by closing the circuit around the infarct via a pathway that introduced fixed time delay, but conserved action potential (AP) morphology.

**Results**

All figures here relate to heart 2, the second of the 2 hearts imaged, but comparable information for heart 1 is presented in the Online Supplement.

Key structural features of the MI and surrounding myocardium are presented in Figure 1. Wall thinning and scar formation are evident in the short axis scout image (Figure 1A). In the 3D reconstruction of the infarct BZ (Figure 1C), dense scarring is interspersed with regions of cellular necrosis in which replacement fibrosis is still in progress. Additional features include apparently normal myocytes surrounding a large vein in the subepicardial region adjacent to the infarct and a network of myocytes surrounded with collagen on the endocardial surface. At the interface with the infarct, sheets of collagen project into the adjacent normal myocardium (also seen in Figure 2).

The distribution of surviving myocytes and fibrosis in the infarct BZ can be seen more clearly in the full resolution subsection in Figure 2A. Muscle layers distant from the infarct are separated by cleavage planes that contain perimysial collagen. Adjacent to the infarct boundary, dense bands of collagen penetrate between and within muscle layers, while myocytes, necrotic cells, and collagen are interspersed in a region that contains a plexus of open, but presumably nonpatent, blood vessels. Each of these structures was segmented throughout the image volume (see Online Supplement), and the segmented image corresponding to Figure 2A is shown in Figure 2B. At higher resolution (Figure 2C), preserved myocytes are characterized by continuity of the plasma membrane and ordered sarcomere arrangement.

Segmented preserved myocytes were analyzed to quantify the relative surface area of nearest-neighbor cells in physical contact (connectivity) and the fraction of total tissue volume occupied by myocytes. Figure 3A is a reconstruction of myocytes showing the normalized connectivity field. The layer of preserved myocardial cells surrounding the large...
epicardial coronary vessel is evident, and the subendocardial network of surviving cell tracts adjacent to the infarct is more apparent when the surrounding collagen is removed. Sparsely connected networks of myocytes penetrate the infarct BZ and strands, as fine as 1 cell thick (Online Figure XIII), pass through it, connecting adjacent normal myocardium to surviving subendocardial and subepicardial cell layers (subvolumes 2 & 3).

The connectivity field distant from the infarct (subvolume 1 in Figure 3A) shows layers of highly connected myocytes (indicated by bands of red) 3 to 4 cells thick that are separated by cleavage planes. We observed limited coupling across cleavage planes, although adjacent layers branch and interconnect. Within the BZ, this laminar structure is lost and coupling between cells in surviving muscle tracts is reduced (subvolumes 2 and 3). Figure 3C summarizes the connectivity between adjacent myocytes in relation to distance from the interface between normal myocardium and BZ tissue (Figure 3B). In the former, connectivity is typically high, with lower values reflecting reduced lateral coupling in the cleavage planes between layers. Connectivity drops markedly over 200 to 300 μm at the interface of infarct BZ and normal tissue (Figure 3C). Scatter within the BZ is due to structural heterogeneity and, in particular, preserved cell tracts surrounded by CT. The connectivity does not reflect the decrease in myocyte volume directly, as connectivity is only represented for volumes containing myocytes. Over the same distance across the BZ interface, the total viable myocyte volume fraction (Figure 3D) decreases abruptly from ≈0.8 to ≈0.1. Near-identical findings were obtained for heart 1 (Online Figure XV).

Myocyte orientation is also altered in the BZ. In Figure 4, we present myofiber orientation with respect to a midwall circumferential plane. The ordered transmural myofiber rotation characteristic of normal myocardium is progressively disrupted across the BZ. Within the infarct, fiber angles are dominated by surviving subepicardial and subendocardial myocytes, which maintain orientations ≈90°.
to the circumferential plane. Corresponding distributions for heart 1 are very similar (Online Figure XVI).

The extent to which structural remodeling in the infarct BZ affects electric propagation was investigated by simulating activation on a network representation of the preserved myocardium using LR-d membrane dynamics. Uniform, axially anisotropic electric properties (anisotropy ratio 2.3:1) were assumed. Figure 5 demonstrates the effect of stimulus site on 3D propagation in the infarct BZ for the 10th stimulus at a BCL of 300 ms (Figure 5A). Two activation pathways through the BZ between midwall and subepicardium are identified (Online Videos I and II). With subendocardial stimulation (Figure 5B), activation blocked at an abrupt tissue dilation in region 1 and propagated into the subepicardial BZ via region 2. With stimulation from the subepicardial BZ (Figure 5C), conduction blocked in region 2, but propagated into the midwall through region 1. These observations demonstrate that tracts through the BZ provide a substrate for stimulus site-dependent unidirectional block. Similar features were observed for heart 1, although fewer complete tracts traversed the BZ (Online Figure XVII). Average CV for both was 0.4±0.2 ms⁻¹ for all stimulus sites.

Figure 6 shows representative activation pathways and times for the 2 stimulus protocols in Figure 5. For subendocardial stimulation (Figure 6A), propagation through the BZ was convoluted with relatively uniform spread in the adjacent midwall. Similar behavior occurred with subepicardial stimulation (Figure 6B), but propagation spread along a different pathway. These processes are reflected in the volume of tissue depolarized as a function of time, shown in Figure 6C. Three distinct phases are evident for subendocardial stimulation. The first reflects activation of the subendocardial BZ (∼7 ms), the second activation of normal myocardium adjacent to the infarct, and the third activation of the subepicardial BZ (∼5 ms); a total of 21 ms. For subepicardial stimulation, activation times followed a similar pattern in the
reverse direction. In this case, the time to traverse the subepicardial BZ was relatively long (~15 ms), giving a total activation time of 26 ms. Activation path lengths for subepicardial and subendocardial stimulation were 6.01 mm and 6.88 mm, respectively. Total activation time was greater for subepicardial than subendocardial stimulation, despite the fact that path length was shorter. This demonstrates that the prolonged activation times seen with stimulation in the infarct BZ were a result of both tortuous activation pathways and time delays along those paths. Cross-sectional areas transverse to the fiber direction were calculated along the activation paths, and these data show that block was associated with rapid increases in tract cross-section (Online Figure XIV).

Susceptibility to rate-dependent activation delay and block within the infarct BZ was investigated by challenging the model with a single S2 stimulus immediately after 10

Figure 4. Myofiber organization adjacent to the infarct. A, Fiber angle distributions at sites in the normal zone (NZ), border zone (BZ), and infarct zone (IZ). B, Transmural fiber angle plotted as a function of relative wall thickness. C, Location of sampled transmural sites 1 to 6, with the midwall circumferential plane indicated. The length of the transmural samples reduces with wall thickness.

Figure 5. Electric activation in the infarct border zone. A, Ten cycles of paced activation at base cycle length 300 ms. Results below are from the final cycle. B, Activation time for a subendocardial stimulus (red sphere), viewed from the basal (left) and apical (right) sides. C, Activation time for subepicardial stimulus in the same views. Activation time is saturated at 24 ms and contours are separated by 1 ms. Total activation time is 21 ms and 26 ms for subendocardial and subepicardial stimulus, respectively. Lines of block are represented by red and white lines. The marker ● indicates the basal subepicardium and is used as a fiducial reference.
preconditioning beats at BCL=300 ms. The coupling interval (CI) between S1 and S2 was progressively reduced until block occurred (Figure 7). For subepicardial stimulation, there was a pronounced time delay at CI=300 ms across a region of less than 100 μm (∼1.6 mm from the stimulus site; Figure 7A and 7B). As CI was reduced, delay in this region increased and block occurred at CI=185 ms. There was less pronounced slowing at 2 other points: at the junction of the BZ and normal midwall tissue (at ∼3 mm), and within the subendocardial BZ (∼5.4 mm). With subendocardial stimulation, activation followed the green path. Regional slowing was again evident at CI=300 ms, but it was spread over a wider region at the interface between subendocardial BZ and normal myocardium (between 1 and 2 mm from the stimulus site; Figure 7A and 7C). The delay increased with reducing CI, and conduction block occurred at CI=156 ms. Total activation time for the cycle immediately preceding block increased to 35 ms and 31 ms for subendocardial and subepicardial BZ stimulation, respectively. Propagation failure occurred at CI=147 ms with stimulation from normal midwall myocardium adjacent to the infarct (not shown).

APD dispersion for the 10th cycle of activation at BCL=300 ms was minimal; ∼7 ms and 13 ms for subendocardial and subepicardial BZ stimulation, respectively. Beat-to-beat APD alternans were not observed, and the longest APDs (129–135 ms) occurred in regions where activation delays were most pronounced. APD decreased with reduced CI, but APD dispersion increased markedly in the regions most susceptible to rate-dependent conduction slowing and block (Online Figures X and XI).
We investigated conditions under which unidirectional block and conduction delays provide a substrate for reentry. An extended model (with a fixed transmural time delay) was paced with a subepicardial stimulus train at reducing CI until block occurred. Sustained reentry required a time delay of 120 ms at CI/H11005 157 ms (Figure 8). Collision and block (cycles 1 & 2) were followed by reentry (cycle 3), which approached steady state from cycle 5 (Online Video III). At a time delay of 110 ms, block occurred in the subendocardial BZ and there was no reentry.

The structural data sets presented here will be made available to other researchers via a repository operated by the Cardiac Atlas Project (http:www.cardiacatlas.org).

**Discussion**

In this study, discrete transmural infarcts in the 2 rat hearts were imaged using extended-volume confocal microscopy at voxel dimensions of 1 μm³. As a result, we have been able to reconstruct the 3D arrangement of viable myocytes and collagen in the infarct BZ at much higher resolution than has previously been reported. Structural analysis confirms the existence of extensive tracts of viable cardiomyocytes in the infarct BZ. Networks of preserved cells, heavily surrounded by fibrosis, extended across the endocardial surface of the infarcts, while layers of surviving subepicardial cells were observed at the infarct margins and surrounding patent superficial veins. Finally, tracts of myocytes penetrated the infarcts, some forming connected pathways. Lateral coupling of cells within these tracts and smooth transmural myofiber rotation were both progressively reduced within the BZ. The infarct BZ from the first heart imaged was initially used to develop techniques. While it did not provide as comprehensive a data set, morphological features were consistent across both (see Online Supplement). We have simulated the spread of electric activation using a network model of the connected myocytes, with normal cellular electric properties. The results demonstrate rate-, path-, and direction-dependent activation delays, as well as unidirectional conduction in the infarct BZ. We argue that structural remodeling alone in the infarct BZ provides a sufficient substrate for reentrant arrhythmia.

The structural remodeling revealed here in the infarct BZ is consistent with previous qualitative observations. Studies in infarcted human and dog hearts have shown that the Purkinje fiber network is preserved on the endocardial surface of the infarct,20 together with an underlying layer of ventricular myocytes.2 Layers of preserved myocytes adjacent to the cavity surface and surrounding intramural blood vessels have been reported in infarcted human papillary muscle.3 Peninsulas of viable tissue surrounded by dense collagen have been observed within the BZ of both human and sheep infarcts and tortuous strands of myocytes that extend through the BZ to link separated regions of viable myocardium adjacent to the infarct have also been noted.21,22 Preservation of myocytes in these regions is presumably due to diffusive pathways for oxygen delivery either from the LV cavity or from patent circulatory components that traverse the BZ.

A key feature of this study is that we have quantified the arrangement of viable myocytes within and adjacent to the...
infarct. In order to do so, we have developed new tools that enable us to characterize the 3D topology of connected cell tracts in the BZ and the extent of physical coupling within those tracts. Myocyte volume fraction and connectivity both dropped abruptly over \( \approx 250 \) \( \mu \text{m} \) at the interface of normal and BZ tissue (see Figure 3). Myocyte connectivity was determined for viable myocytes only and is therefore not simply related to volume fraction. Instead, the reduction in connectivity reflects decreased lateral coupling as a consequence of structural remodeling. In the normal LV, ventricular muscle cells are arranged in branching layers\(^{23} \) with extensive transverse coupling between myocytes, whereas preserved myocytes in the infarct BZ form thin strands surrounded by collagen in which transverse coupling may be limited to 1 or 2 adjacent myocytes (Online Figure XIII). These findings are consistent with data from the BZ of healed canine infarcts,\(^4 \) which showed that the length profile of cells connected via intercalated disks altered anisotropically, with side-to-side connections reduced by around 75% but relatively little change in end-to-end connections.

Marked changes in myofiber orientation were another feature of the BZ. The transmural variation of fiber orientation in the myocardium adjacent to the infarct was consistent with previous studies in normal hearts.\(^{24} \) However, there was increasing disorder in fiber orientation at the border of the infarct, with 2 distinct populations identified in the BZ that coincide with subepicardial and subendocardial fiber orientations in the adjacent myocardium. This may be due to the fact that myocytes in these regions are more likely to be preserved and, after wall thinning, occupy a relatively greater fraction of the BZ. In this study, local myofiber orientation was estimated from spatial gradient variation. Some uncertainty must be attached to the orientations estimated in the midwall BZ because there were few viable myocytes in this region. However, our observations are comparable with those of Sosnovick et al.\(^8 \) using diffusion spectrum MRI, and the higher resolution of our imaging method has allowed us to reconstruct the spatial separation of the 2 fiber orientation populations more precisely.

There is compelling evidence from in vivo experiments, computer modeling, and patterned cell culture studies that the structural anisotropy quantified here provides a substrate for slow propagation, conduction block, and electric instability. Myocyte volume fraction and connectivity both dropped abruptly over \( \approx 250 \) \( \mu \text{m} \) at the interface of normal and BZ tissue (see Figure 3). Myocyte connectivity was determined for viable myocytes only and is therefore not simply related to volume fraction. Instead, the reduction in connectivity reflects decreased lateral coupling as a consequence of structural remodeling. In the normal LV, ventricular muscle cells are arranged in branching layers\(^{23} \) with extensive transverse coupling between myocytes, whereas preserved myocytes in the infarct BZ form thin strands surrounded by collagen in which transverse coupling may be limited to 1 or 2 adjacent myocytes (Online Figure XIII). These findings are consistent with data from the BZ of healed canine infarcts,\(^4 \) which showed that the length profile of cells connected via intercalated disks altered anisotropically, with side-to-side connections reduced by around 75% but relatively little change in end-to-end connections.

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There is compelling evidence from in vivo experiments, computer modeling, and patterned cell culture studies that the structural anisotropy quantified here provides a substrate for slow propagation, conduction block, and electric instability. Such studies have shown that (1) dense fibrosis and tortuous strands of viable myocytes in the infarct BZ give rise to slow “zig-zag” conduction,\(^3 \) (2) “source-sink” mismatches due to multiple branching tissue strands\(^{25} \) or sudden tissue expansion\(^{26} \) can cause substantial conduction slowing and unidirectional block, and (3) anatomic obstacles and abrupt changes in fiber direction can generate rate-dependent “vortex shedding” and re-entry.\(^{27} \) Recent modeling studies have also shown that fibrosis can cause activation delays and rate-dependent electric instability, increasing the likelihood of re-entrant arrhythmia.\(^{16} \)

Less clear is the extent to which factors such as electric remodeling at a cellular level, altered gap junction expression, and interactions between myocytes and fibroblasts may contribute to these processes in healed myocardial infarcts. In the BZ of subacute canine infarcts, peak sodium, calcium, and potassium currents are reduced, producing decreased CV and APD prolongation.\(^{6,7,28} \) If replicated in the chronic infarct, such changes would exacerbate reductions in CV and increase the probability of block, as demonstrated in a modeling study by Decker and Rudy.\(^{29} \) However, most studies have reported normal resting membrane potential and AP upstroke in the BZ of chronic infarcts, with varied results for APD.\(^{30–32} \) Changes in the distribution of the gap junction protein Cx43 have been observed at all stages of infarct healing. In normal ventricular myocardium, Cx43 is located only at the intercalated disks, but in subacute infarcts it is dispersed across the lateral cell membrane. In the dog heart, end-to-end coupling between myocytes was reduced by 25% 3 to 10 weeks postinfarction, but side-to-side connections declined by 75%.\(^4 \) However, Cx43 expression remains lateralized in healed human and sheep infarcts.\(^{22,33} \) The direct effects of the changes in gap junction distribution in infarct BZ remain uncertain. It seems unlikely that lateralized Cx43 forms active channels because transverse CV is reduced in this region.\(^34 \)

In this study, we have tested the contributions of structural remodeling, independent of any cellular electric changes, by simulating the spread of electric activation in a structurally detailed computer model of the infarct BZ and surrounding normal myocardium, assuming normal cellular electric properties. We investigated the rate dependence of activation within this context and used LR-d cell membrane dynamics\(^{19} \) to provide a biophysically based representation of APD and CV restitution. An important outcome is the finding that structural remodeling in the infarct BZ gives rise to unidirectional propagation and stimulus site-specific activation delays. This is evident in Figure 5, in which 2 separate tracts of surviving myocytes pass between midwall and subepicardial BZ. With subendocardial stimulation, activation propagated through one of these tracts, but blocked at the other; this pattern was reversed with subepicardial stimulation. Activation delays were seen in the subendocardial and subepicardial BZ in both cases, although their extent varied with stimulus site (Figure 6C).

Tortuous conduction pathways contributed to the observed activation delays. Complex pathways through the infarct connected midwall to subepicardial and subendocardial BZ (Figure 6). In addition, surviving cell tracts along the subendocardial surface, surrounded by dense collagen, formed a network with relatively sparse lateral coupling. These gave rise to zigzag patterns of activation clearly evident in the videos provided in the supplementary data. Similar observations have been made in infarcted human papillary muscle.\(^5 \) Finally, propagation around the infarct boundary was near normal to muscle layers, which was inherently slow because electric coupling across the clefts that separate myolaminae occurs via muscle bridges.\(^{13} \) In the infarct BZ, coupling between adjacent layers was further reduced by infiltration of thick bundles of collagen projecting from the infarct into the space between myolaminae. Activation path length provides a partial explanation only of the propagation delays observed in the infarct BZ. This is demonstrated by the fact that total activation time was longer.
for subepicardial than for subendocardial stimulation, even though path length was shorter (Figure 7A). To explore the relationship between time delay, conduction block, and BZ structure, we computed network cross-sectional area transverse to myofiber direction along both pathways and compared the data with local activation times at BCL = 300 ms. For each pathway, activation times were considered for the direction in which block occurred and the direction of successful propagation (Online Figure XIV).

The block along these 2 pathways for activation entering and leaving the subepicardial BZ occurred in regions where there was a transition from a sparsely connected myocyte network to a larger well-connected volume. This was due to current source-load mismatch, a mechanism that has been extensively studied by other workers and is inherently unidirectional in its effects. Activation delays were seen in specific regions with successful propagation in the other direction (Figure 7). Differences in activation dynamics at these sites provide further insight into mechanisms of source-load mismatch in the BZ. With subepicardial stimulation (7B), there was a long activation delay between points 100 μm apart in the subepicardial BZ, where the tract narrowed to form a single strand of myocytes and cross-sectional area was abruptly reduced. The AP in this region was characterized by a slow rising foot, indicating a focal deficit in the inward current necessary to bring downstream myocytes to threshold. This contrasts with delays observed at the interface between subendocardial BZ and adjacent normal myocardium (Figure 7C). Here delay was distributed more widely across a region of network expansion.

Rate-dependent block occurred at the sites where delay was most evident. The increased probability of block in regions with low safety factor is predictable since, due to CV and APD restitution, current supply decreases as CI is reduced. However, the delay and block along both pathways exhibited different characteristics. In the subepicardial BZ (Figure 7B), delay increased progressively at the same point as CI was reduced, but the AP upstroke remained rapid. Conduction block occurred at CI = 185 ms when cells within the tract failed to reach threshold. In contrast, in the subendocardial BZ (Figure 7C), the region of delay broadened with decreasing CI, and there was a progressive reduction in AP upstroke velocity.

Minimal but increasing APD dispersion was observed with decreasing CI. This was most pronounced in regions of activation delay, such as the networks of surviving myocytes in the subendocardial BZ (Online Figures X and XI). There is evidence that rate-dependent APD dispersion and APD alternans give rise to electric instability in the presence of patchy fibrosis. However, neither contributes to the rate-dependent block observed in this discrete infarct.

We have demonstrated that structural remodeling in the infarct BZ can produce the conditions necessary for electric re-entry. These include stimulus site–dependent unidirectional conduction with long activation delays and rate-dependent block. We have observed delays of up to 35 ms in our image-based model along 6- to 7-mm path lengths through a very small BZ section. Finally, we have shown that these features can give rise to sustained reentry when the circuit around the infarct was closed and a further time delay of 120 ms was introduced (Figure 8). This is equivalent to a wavelength for reentry of 27 to 31 mm if it is assumed that the observed delays were replicated around the full circuit, or ~60 mm assuming CV through the auxiliary circuit to be normal. It should be noted that the wavelength for reentry is directly related to the effective refractory period and, for the well-established LR-d membrane model that we elected to use, this is significantly longer than in rat or mouse hearts and shorter than in human hearts. However, the activation delays and unidirectional block observed here reflect source-load mechanisms that involve cellular architecture and apply across species. On this basis, we hypothesize that structural remodeling can provide a dynamic substrate sufficient to support electric reentry in the absence of cellular electric remodeling. Changes in gap junction distribution and the cellular electric remodeling that occurs in heart failure, for instance, would certainly amplify such effects. Furthermore, the processes that trigger rate-dependent block and reentry (early and late afterdepolarizations, autonomic dysfunction, etc.) cannot be explained by structural remodeling alone.

Limitations
This study was carried out in relatively small transmural segments, comprising healed infarct, BZ, and surrounding normal myocardium, from 2 rat hearts only. Specimen dimensions, number of hearts studied, and species were influenced by the time taken to acquire and process volume images at such high spatial resolution and to model electric activation on them, but each introduced limitations. The dimensions of the rat heart prevented us from mapping intramural electric activity in the BZ, and we were therefore not able to compare model predictions with corresponding experimental data. Specimen dimensions and artificial boundaries also precluded initiation of reentry wholly within the image volume and likely reduced the numbers of possible alternative pathways around sites of block. Despite this, we have been able to demonstrate reentry by extending the image-based activation model, and the examples of directional and rate-dependent delay and block reported here did not occur on, or near, the artificial boundaries. That said, it will be necessary to study a larger number of hearts before robust statements can be made about the probability of observing structural substrates for reentry in the infarct BZ. A further limitation is the animal model used and the extent to which the results translate to large animal hearts and, in particular, to human heart disease. We reiterate that the structural mechanisms responsible for delay and block in this study likely apply across species, and it seems reasonable to expect that their potential contribution to reentry would be greater with increased heart size and infarct dimension. The occlusion infarct studied here was more discrete than the reperfusion infarcts that are most commonly observed clinically. A final technical issue is that image quality was degraded by flare produced by picrosirius red fluorescence in areas of dense collagen. While we were able to identify myocytes, the volume of preserved strands of myocytes running through the BZ was likely underestimated in the
segmentation process. Error introduced by this artifact was mitigated by using a topology-preserving dilation operator that added an additional voxel at most to myocyte tracts (Online Figures II and III).

Conclusions
In this study, we have produced unique data sets, in which tissue structure in the BZ and normal myocardium adjacent to a healed MI have been reconstructed in 3D at 2 μm³ voxel dimensions. The infarct BZ was characterized by tracts of preserved myocytes with markedly reduced lateral coupling that, in some cases, form continuous pathways across it. We have used image-based modeling to demonstrate that these pathways exhibit direction- and rate-dependent delay and block. Activation delays are not uniformly distributed along these pathways, but instead are associated with specific regions in which there are rapid changes in tract cross-section. We conclude that these mechanisms together with tortuous activation pathways provide a dynamic substrate for reentry.

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Disclosures
None.

References
What Is Known?

- The border zone (BZ) surrounding a healed myocardial infarction (MI) is a region in which the normal organization of cardiac cells is disrupted.
- The infarct BZ can provide a substrate for reentrant electric activity, giving rise to propagation delays and unidirectional conduction block.

What New Information Does This Article Contribute?

- We have imaged the 3-dimensional (3D) arrangement of myocytes and collagen in the BZ and normal myocardium adjacent to healed rat infarcts at higher spatial resolution and across larger tissue volumes than previously reported.
- We have quantitatively determined the topology and physical coupling of myocytes from these images and interrogated the functional impact of BZ structure on electric activation using computer models.
- We have demonstrated that in the BZ following MI, the 3D cellular organization alone is sufficient to provide the necessary conditions for sustained reentrant electric activity.

Regional changes in the 3D arrangement of cardiac myocytes, cell-to-cell gap junction expression, and cell activation properties adjacent to an MI increase the risk of heart rhythm disturbance. However, mechanistic understanding of electric propagation in the BZ of a healed MI remains incomplete. In part, this reflects the difficulty of mapping intramural electric activity at sufficiently high spatial resolution to address this issue experimentally. This article presents results showing a new and detailed understanding of the 3D arrangement of surviving myocytes in the BZ. Preserved myocyte tracts provide transmural pathways showing propagation delay and unidirectional conduction. Under appropriate circumstances these pathways provide a substrate for sustained reentry. They are the result of regional structural change alone and occur primarily where there are abrupt changes in tract geometry. The use of high-resolution image analysis and computer modeling has enabled us to probe the effects of tissue structure on electric activity in the infarct BZ, in a way that has not been achieved previously. Our approach provides a basis for interpreting electric mapping studies and, as noninvasive imaging modalities develop further, may assist in stratifying the risk of reentrant arrhythmia associated with specific BZ tract topology.
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Supplemental Material

High Resolution 3D Reconstruction of the Infarct Border Zone: Impact of Structural Remodeling on Electrical Activation

Sally L. Rutherford, BSc Hons1,2, Mark L. Trew, PhD1*, Gregory B. Sands, PhD1, Ian J. LeGrice, MD, PhD1,2, Bruce H. Smaill, PhD1,2

1Auckland Bioengineering Institute and 2Department of Physiology, University of Auckland, Auckland, New Zealand.

*Corresponding Author: m.trew@auckland.ac.nz

S1. Introduction

This supplemental material describes in greater detail the methods that were used to construct and analyze the three-dimensional high-resolution images of rat MI border zone presented in the paper: “High Resolution 3D Reconstruction of the Infarct Border Zone: Impact of Structural Remodeling on Electrical Activation.” Additional supporting results are presented in Section S3.

S2. Methods

Extended volume confocal imaging, image segmentation, detailed structural analysis and modeling was carried out in the infarct BZ for two rat hearts, #1 and #2. The methods described in this section are illustrated for Heart #2.

S2.1 Surgical procedures and tissue preparation

All surgical procedures were approved by the Animal Ethics Committee of The University of Auckland and conform to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23). Myocardial infarcts were surgically induced in adult male Sprague Dawley rats. The animals were anesthetized and maintained with isoflurane and oxygen using positive pressure ventilation. The heart was exposed via a left-sided incision between 4th and 5th ribs. The left anterior descending coronary artery (LAD) was ligated close to its origin with a suture passed through the myocardium around the artery. The chest was closed in layers, and the animal was allowed to recover. Local anaesthetic (Marcain) was injected into the chest wall prior to closure. A class 1A anti-arrhythmic (Lignocaine) was given every 2 hours up to 8 hours post-operatively, to reduce the risk of sudden death from ventricular fibrillation.

Fourteen days after surgery, the rats were anaesthetized with CO₂ and killed by cervical dislocation. The chest cavity was opened, and heparin (100 IUkg⁻¹) was injected into the LV, and allowed to circulate for ~1 minute. The heart was then rapidly excised with the ascending aorta intact and immersed in chilled (4°C) normal saline solution, then mounted on a gravity-fed Langendorff perfusion apparatus. The coronary circulation was perfused with room temperature, oxygenated Krebs-Henselite solution (In mM: NaCl 118; KCl 4.75; MgSO₄ 1.18; KH₂PO₄ 1.18; NaHCO₃ 24.8; Glucose 10, and CaCl₂ 2.5) until clear of blood and the heart had started beating. The heart was arrested in diastole using a high potassium modified Krebs-Henseleit solution (as above with 60 mM KCl and 0.25 mM CaCl₂). The perfusate was then switched to picrosirius red dye (PSR, Sirius Red F3BA (Pfaltz and Bauer, CT, USA) 0.1% and picric acid 99.9%) for two hours at ~0.25mlmin⁻¹. The heart was fixed with Bouin’s solution (saturated aqueous
picric acid 71.4%, formalin 23.8% and glacial acetic acid 4.8%), perfused for 15 minutes and immersed for 7 minutes.

Following fixation, the atria were removed and the ventricles sliced into 1 mm-thick equatorial rings using a microtome. Each slice was photographed at 8μm² pixel resolution (using an 8.4 Megapixel Canon 1D Mark II digital camera (Canon Inc., Tokyo, Japan) equipped with the MP-E 65 mm macro lens). The serial images were segmented, registered manually and reconstructed in pseudo 3D (see Section S3) so that infarct size and location (identified as unstained area) could be visualized.

Slices were then immersed in PSR for a further 14 days to stain the infarcted region not accessed by previous perfusion staining, dehydrated in a graded ethanol series, embedded flat in Spurr’s resin (Proscitech C035) and polymerized for 20 hours at 60 °C.

S2.2 Image acquisition

High resolution volume images were acquired using a purpose-built system described in detail elsewhere. The system consists of a modified confocal laser scanning microscope (TCS4D, Leica Microsystems AG, Wetzlar, Germany) with a Krypton-Argon Laser (Omnichrome, CA), a high precision 3-axis translation stage (Aerotech, Pittsburg, PA) and a specialized milling device (SP2600 ultramill, Leica Microsystems). All equipment was mounted on an anti-vibration table and controlled from a central computer using custom-written software. Resin blocks were fixed to the stage and a series/montage of overlapping 512x512 pixel frames were acquired at 1 μm² resolution using a 20x objective (HC PL APO 20x NA 0.70, Leica). Steps involved in the image acquisition process are illustrated in Figure 1. Initially, scout images of the upper surface of the ventricular slice were obtained (Figure 1A). A transmural region containing the infarct border was then selected, and a volume image acquired. Overlapping images of the selected region were taken in the X-Y plane (overlap 50%, 4/8 line scan) at a series of focal (Z) planes. The focal planes were separated by 1 μm spacing and acquired to a depth at which the limits of acceptable image quality were approached. The block was then moved, via the translation stage, to the ultramill and the upper surface was milled to a depth 2-3 μm less than the maximum imaging depth. This process was repeated and overlapping optical sections in the series were used to maintain Z-axis registration (1B). In normal PSR-stained myocardium, good optical sections can be obtained at depths up to 50μm below the block surface. However, dense PSR staining of the extensive fibrosis in infarct and infarct border zone (BZ) led to the rapid attenuation of fluorescent emission with depth in these regions and the maximum imaging depth was limited to between 7 and 15 μm. Image sections were acquired in the epicardial tangent plane with Z in the transmural direction. A small subvolume was also imaged at 0.5μm resolution using a 2x zoom (see Figure 2C). Total image volumes were 4.1 mm³ and 5.6 mm³ for hearts #1 and #2, respectively.

S2.3 Image processing

Initial image processing used custom-written LabVIEW (National Instruments: www.ni.com) software. Images were aligned using an automated cross-correlation procedure. Adjacent stacks in the Z plane were aligned using the closest correlation of images on 2 consecutive Z stacks and the overlapping image subvolumes were assembled to form the complete 3D image. Histogram equalization was used to correct for attenuation of intensity with imaging depth. Images were enhanced using a wavelet-based de-noising procedure that preserved key image features and 3D decovolution (for details see Sands et al (2005)). Volume images were rendered and visualized using Voxx (http://www.nephrology.iupui.edu) - see Figure 1C for Heart #2. For subsequent analysis the images were subsampled to 2 μm isotropic voxel dimensions.

The additional image processing and analysis outlined in the remainder of this section used the 2 μm voxel resolution images, but the full resolution images were used to validate segmentation techniques.
Unless otherwise stated, these procedures were implemented in MATLAB (7.1, The Mathworks Inc., Natick, MA, 2010).

**Determining structural orientations**

Local axes that represent principal structural directions were estimated throughout the image volume from the structure tensor (Jähne, 2005). The structure tensor for any voxel, \( v \), is given by:

\[
S^v = \begin{bmatrix}
g^v_{xx} & g^v_{xy} & g^v_{xz} \\
g^v_{yx} & g^v_{yy} & g^v_{yz} \\
g^v_{zx} & g^v_{zy} & g^v_{zz}
\end{bmatrix}
\]

where \( g^v_{xx}, g^v_{yy} \) and \( g^v_{zz} \) are estimates of intensity gradient for that voxel with respect to the imaging coordinate system \((x,y,z)\). The eigenvectors of this symmetric tensor characterize local principal structural directions.

We have used a 1D fast Fourier transform approach, which incorporates optimal gradient operators, to provide a robust and computationally efficient estimate of voxel intensity gradients throughout the image volume. The components of the structure tensor were constructed initially from the downsampled volume image (2 \( \mu \)m\(^3\) voxels) and were then progressively smoothed to reduce spatial frequencies to 4, 8, 16, 32 and 64 \( \mu \)m resolution. Eigenanalysis was used at each frequency scale to extract local structural directions. For each voxel, the eigenvector paired with the smallest eigenvalue (the weakest gradient direction) corresponded to the fiber orientation, whereas the eigenvector associated with the strongest gradient direction was normal to the cleavage planes that separate layers of myocytes (sheet normal).

This analysis enables us to quantify the spatial scales over which key structural features change through the image volume and provides a basis for determining the discretization necessary to capture this variation in image-based models of cardiac electrical activation. For Heart #2, Online Figure I presents the transmural variation in myofiber orientation at different 6 sites within and adjacent to the infarct BZ (see Figure 4) at spatial scales ranging from 4 to 64 \( \mu \)m, resolution. The smooth transmural rotation of fiber orientation from epicardium to endocardium that is evident at 16 \( \mu \)m resolution is preserved at the coarsest level (64 \( \mu \)m). On the other hand this is obscured by the local scatter that is present at 4 and 8 \( \mu \)m resolution.

**Segmentation of viable myocardium**

Key morphologic features were identified in the imaging plane. Viable myocytes, collagen, extracellular space and regions of necrotic tissue were segmented on the basis of intensity (8-bit), texture and continuity using custom software written in LabVIEW. The results of this segmentation were viewed by an operator and corrected manually where necessary. This process is illustrated in Figure 2. Viable myocytes, identified on the basis of intermediate image intensity (50-120) are also characterized by continuous connected cell membranes, the existence of intercalated disks and, at higher resolution, by ordered striations. Fibrosis was readily identified as connected regions of high image intensity (121-255), while necrotic tissue was differentiated as relatively substantial low intensity regions (>50) containing collagen strands. These 3D segmentations were used in subsequent image based analysis.
Online Figure I. Myofiber orientations estimated by eigen-analysis of structure tensors at varying frequencies and spatial locations in Heart #2 (see Figure 4). Transmural myofiber orientations at sites: 1, in preserved myocardium adjacent to infarct; 3, in infarct BZ; and 5, in infarct (numbering following Figure 4). Analysis frequencies correspond to 4, 8, 16, 32 and 64 µm resolution.

Topology preserving dilation

Analysis of myocytes incorporated into the 3D segmentation of surviving myocardium suggests that their cross-sectional dimensions were underestimated in some cases. There are two reasons for this. Firstly, fixation and the tissue processing associated with embedding cause cumulative shrinkage. Secondly, PSR which binds principally to collagen produces intense fluorescence that "shadows" and can even obscure the lower level autofluorescence used to identify surviving myocytes. In the first instance, the artifact introduced is relatively small. On the other hand, flare due to PSR can have substantial impact in images of the infarct BZ where the cross-sectional area of surviving tracts of myocytes is small and fluorescence associated with the extensive fibrosis is very strong. Therefore, the raw 3D segmentation likely underestimates the true volume of surviving myocardium and particularly the thin strands of myocardium that characterize the infarct BZ.

A conservative, 3D topology-preserving, dilation method was used to correct for these artifacts and add at most one voxel to the boundary of the segmented myocardium. The basic algorithm for the method is illustrated for a simple 2D test problem in Online Figure II. Three sections from the segmented Heart #2 image volume are shown in Online Figure III with the effects of topology preserving dilation superimposed on them. This operation increased the volume of the segmented 3D mask by 8.0%. The cross-sectional areas of fiber tracts were assessed before and after dilation using methods outlined later in this section. Total fiber tract cross-sectional area increased from 7929 mm² to 8837 mm² (11.46%). Simulations of electrical activation (see Section S2.4) performed before and after the topology preserving dilatation showed almost identical activation sequences except through the sparsely connected networks of myocytes in the border zone transitions. In those regions, electrical activation computed using the
dilated segmented mask was able to move through the sparsely connected networks, whereas prior to the dilation block occurred. The topology preserving dilation serves to increase the functional safety of the resulting electrical activation models.

**Online Figure II.** Steps in the topology preserving dilatation algorithm to ensure that structures are not closed by the dilation. 

- **A.** Original image.
- **B.** Original image is morphologically closed using a 5 voxel diameter spherical mask.
- **C-D.** The closing is dilated twice using a 3×3×3 cross mask. The dilations ensure that no closing or bridging occurs at points where channels through the mask flare open.
- **E.** The perimeter of the original mask is found.
- **F.** The XOR combination of the perimeter, the original mask and the dilated closing is determined.
- **G.** The resulting dilation (one of several possible, i.e. using different closing and/or dilation masks) that preserves the original topology of the mask.

**Online Figure III.** Topology preserving dilation scheme applied to Heart #2 rat MI tissue. The voxels added by the dilation are shown in red. The original segmentation is in black.

- **A.** Slice 50/330.
- **B.** Slice 150/330.
- **C.** Slice 250/330.

**Computing volume and connectivity distributions**

The connectivity and volume of surviving myocytes throughout the image volume were estimated as follows. In addition to the 3D segmentation of surviving myocardium (Online Figure IV-B), a
Supplemental Material

Infarct border zone structure and activation

Segmentation consisting of surviving myocardium, connective tissue and necrotic tissue was also constructed (Online Figure IV-C). Spherical and shell summation filters (radii 5 voxels or 0.01 mm) were applied to all voxels in both 3D segmentations using a fast-Fourier technique. The filter weights were determined by over-sampling the voxel discretization at the boundaries of sphere and spherical shell (see online figures V-B and V-C).

**Online Figure IV.** A. Mid stack (165/330) confocal image of Heart #2 rat MI border zone. B. Segmentation of surviving myocardium on same layer. C. Tissue volume (myocardium and connective tissue) segmentation on same layer.

Following application of the weighted sum filters, a level 4 binomial filter was used to smooth the myocardial and tissue volume and surface area data from the original 2 µm to 4, 8 and 16 µm resolution. Myocardial to tissue volume and surface area data are shown at 8 µm resolution in Figure 3.

Connectivity is therefore a relative measure of the surface area of segmented myocytes in physical contact with neighboring myocytes. Where adjacent myocytes are separated by perimysial collagen (collagen in the clefts between layers or bundles of myocyte) or in the infarct BZ by replacement collagen, connectivity is reduced (see Figure 3).
Defining the MI border

Online Figure VI. Defining the infarct border based on the volume and connectivity fields. A. Median connectivity index of each block in 100 µm (5x5x5 blocks) subvolumes in one plane. B. Median volume of each block in each subvolume. C. Separation of Normal tissue (red) from borderzone (green) defined using a fuzzy C-Means algorithm. D. As per C with classification of indicated subregions reset. E. 3D representation of the separation of NZ from BZ for Heart #2.

The distributions of viable myocyte volume fraction and connectivity were used to define the interface between the BZ and normal myocardium (NZ). The process is illustrated for Heart #2 in Online Figure VI. The image volume was bundled into 100x100x100 µm³ subvolumes each containing 125 blocks. Median values for connected surface area (Online Figure VI-A) and volume fraction of viable myocytes (Online Figure VI-B) were computed for the blocks contained in each subvolume. The resulting values were smoothed using a 3x3x3 moving average filter. Both measures distinguish between infarct and surrounding preserved myocardium. However, a fuzzy C-Means clustering algorithm⁶ employing both measures was used as it provided a more discrete separation between the two regions than could be achieved using a simple threshold based on surface area connectivity or volume alone (Online Figure VI-C). In two isolated subregions (see circles in Online Figure VI-C), these classifications required further consideration. Connected myocytes surrounding a major epicardial blood vessel within the infarct BZ were identified as normal, while a small region within preserved myocardium at the edge of the block was classified as BZ. These were manually reclassified (Online Figure VI-D). A 3D boundary surface was defined in 3D (Online Figure VI-E) and mapped back onto the original data set. The connectivity index of each block was plotted against distance to the BZ boundary, and a smoothed fit was computed for a reduced dataset (down-sampled to ~35,000 points) using a Savitzky-Golay least-squares smoothing for non-uniformly spaced data⁷ with piecewise linear fitting over 1001-point windows (see Figure 3).
Fiber tract cross-sections

A measure of local fiber tract cross-sectional area was found at sample points throughout the tissue volume (the nodes of the network model described in Section S2.4). The 3D segmentation of surviving myocardium was resampled onto image stacks (25×159×159 voxels, 0.050×0.318×0.318 mm$^3$) centered on the sample point and transverse to the local fiber orientation in the long dimensions. The dimension 0.05 mm was chosen as being approximately half the length of a typical rat LV myocyte. The segmented voxels in the sample stack that were physically connected to the voxel at the sample point were retained and projected onto the middle 159×159 pixel plane of the stack. The effective fiber tract cross-sectional area was the sum of segmented pixels in this plane.

S2.4 An image based model of the infarct border zone

In order to probe the current loading features of the detailed border zone structure, a 3D computational model of the rat MI was constructed from the tissue images. The model was based on a network description of the myocardium and a reaction-diffusion model of the bio-electric physics.

A volume network description of myocardium

We have developed a novel technique that enables us to reduce the 3D segmentation of surviving myocardium to a 3D network of node volumes and edge connections. The image volume was tessellated into a mesh of conforming voxel units, the discrete volumes and representative nodal locations of surviving myocardium within each unit were determined, and the connection areas and representative edge locations between units were identified. Multiple discrete fiber tracts through a voxel unit were treated as independent network components. The complete topology of a network description of the MI border zone was determined from the independent components using an efficient algorithm based on multiple applications of Quicksort.

Once the complete network topology was determined it could be visualized or analyzed. For Heart #2, Online Figure VII shows (for the indicated subregion of the rat MI image set in Online Figure VII-A); (i) the starting segmentation (Online Figure VII-B), (ii) the complete network of nodes and edges (Online Figure VII-C), (iii) the network visualized with edges and nodes scaled to reflect the relative (0-1) edge connection surface areas and nodal volumes (Online Figure VII-D), and (iv) the local fiber and sheet orientations overlaid on the network (Online Figure VII-E).
Online Figure VII. Network mesh for subregion in Heart #2. A. Location of subregion. B. 3D segmentation. C. Network model showing nodes and edges. D. Network model showing volumes assigned to each node and edges scaled to reflect relative cross-section. E. Fiber and sheet orientations projected onto network.
Modeling electrical activity in the volume network

The sequence of electrical activation through the network description of the rat MI border zone was modeled using a finite volume discretization of the reaction-diffusion monodomain equation:\(^{10}\)

\[
A_m C_m \frac{\partial V_m}{\partial t} - \nabla (\sigma \cdot \nabla V_m) = -A_m (I_m - i_s).
\] (S2.4.1)

This equation is subject to external boundary conditions (with normal \(b\)):

\[
\nabla V_m \cdot (\sigma b) = 0.
\] (S2.4.2)

Here \(V_m\) is the membrane potential, \(A_m\) the nominal cell surface area to volume ratio, \(C_m\) a specific membrane capacitance, \(\sigma\) an effective bulk electrical conductivity tensor, \(I_m\) the membrane current per unit area (this is conventionally positive for current flowing across the membrane from within the cell), and \(i_s\) a stimulating transmembrane current. Conductivities were specified in the fiber, and the sheet-sheet-normal material coordinate orientations (the principal directions or eigenvectors of the conductivity tensor). The discrete network captured the laminar discontinuities so that transversely isotropic electrical conductivities could be specified. The determination of the material coordinates was described in Section S2.3. The two independent principal components of the effective conductivity tensor were found from the effective intra- and extra-cellular conductivity tensors as:

\[
\sigma^f = \frac{\sigma^f \sigma^e_f}{\sigma^f + \sigma^e_f} \quad \text{and} \quad \sigma^{s/n} = \frac{\sigma^{s/n} \sigma^{s/n}_e}{\sigma^{s/n}_i + \sigma^{s/n}_e}.
\] (S2.4.3)

Equation (S2.4.1) can be expressed in a spatially discrete (temporally continuous) form as:

\[
M \frac{\partial V_m(t)}{\partial t} - K V_m(t) = -\frac{1}{C_m} M \left(I_m(V_m, s, t) - i_s(t)\right).
\] (S2.4.4)

The matrix \(M\) is the mass matrix and the matrix \(K\) is the stiffness matrix. The vector of states for the membrane model is given by the vector \(s\). Following integration over a discrete time step from \(t^n\) to \(t^{n+1}\), a first order split time step approximation to (S2.4.4) is:

\[
(M - \Delta t K)V_m^{n+1} = MV^n + \Delta t \hat{M} V_m^{n+1}.
\] (S2.4.5)

The predicted membrane potential arises from the solution of a system of ODEs describing cell membrane dynamics.

The entries in the mass and stiffness matrices are:

\[
M_{ii} = A_m C_m V_i
\]
\[
M_{ij} = 0, \forall i \neq j
\]
\[
K_{ii} = -\sum_j A_{ij} \frac{1}{\ell_{ij}^- + \ell_{ij}^+} \left(\sigma_{ij}^- + \sigma_{ij}^+\right)
\] (S2.4.6)
\[
K_{ij} = A_{ij} \frac{1}{\ell_{ij}^- + \ell_{ij}^+} \left(\sigma_{ij}^- + \sigma_{ij}^+\right), \forall i \neq j,
\]

with the geometric components as shown in Online Figure VIII, and the projections of the effective conductivities onto the network edges given by:

\[
\sigma_{ij}^\pm = \sigma_f (e_{ij}^\pm \cdot f_{ij}^\pm)^2 + \sigma_s (e_{ij}^\pm \cdot s_{ij}^\pm)^2 + \sigma_n (e_{ij}^\pm \cdot n_{ij}^\pm)^2.
\] (S2.4.7)
Online Figure VIII. Components of a generic network node-edge-node connection.

The network discrete monodomain equations described by (S2.4.6) and (S2.4.5) were implemented in custom written code which was parallelized and tuned for both memory and computational efficiency.

To advance the discrete equations in time, first order operator-splitting was used where one solution of the predictor membrane potential (S2.4.5) was found for each time step. This is an identical approach to what has previously been used in a finite element discretization\textsuperscript{11}. A carefully optimized CG method was written to solve (S2.4.5). The predictor membrane potential was found by solving an LR-d model\textsuperscript{12} of membrane dynamics. Using a similar approach to Rush & Larsen\textsuperscript{13}, some equations were approximated by their closed steady state form. Other equations were modified as necessary using l'Hôpital’s rule to ensure they remained bounded to their limiting values as both numerator and denominator approached zero.

Potentials and activation times were found throughout the network for various stimulation protocols. The parameters used were as follows: time step 0.005 ms, $A_m$ 200 mm$^{-1}$, $C_m$ 0.0112 μF/mm$^2$, and $(\sigma_f, \sigma_s, \sigma_n)$ (0.1368,0.0583,0.0583) mS/mm. The set of electrical conductivities was derived from Hooks et al.\textsuperscript{14} and unpublished observations from the same authors.

Activation sequence pathlines through the network

Activation in the rat MI model develops in a discrete fashion and loading effects mean that the shortest distance paths through the network typically do not represent the actual activation paths through the network. Using the activation times for each node in the network, representative activation pathlines were determined for the various stimuli.

The basic approach is summarized in Online Figure IX-A. Starting with a seed node, nodes with sufficiently similar activation times (typically within one time step of the seed node) are blocked together (Online Figure IX-A, Block 1) and the node central to the block is identified. Nodes adjacent to the block with earlier activation times are identified (Online Figure IX-A, Interface) and using both activation time and proximity new seed nodes are chosen to construct the subsequent block (Online Figure IX-A, Block 2). The representative activation sequence pathline is constructed from the sequence of block central nodes.

For visualization purposes, the activation sequence pathlines were smoothed using Savitsky-Golay filtering\textsuperscript{7} with third-order piecewise polynomials and a typical window size of 21. Activation sequence pathlines from epicardial and endocardial stimulus sites on the 10$^{th}$ beat of a 300 ms BCL sequence of stimuli are shown in Figure 6.
Supplemental Material

Infarct border zone structure and activation

12

Online Figure IX. A. Activation sequence pathlines. Blocking similarly activated nodes to form a representative activation path through the network. B. Linking together otherwise unconnected start (S) and end (E) nodes to complete a circuit for studying reentry. C. Morphologically identical but delayed action potentials along the path from S to E. D. The link from the epicardium (S) to the endocardium (E) with a delay of 120 ms used to assess reentry.

Conduction velocity

Activation time gradients at each network node were computed using a generalized finite difference approximation based on previous work. The norm of the activation time gradient vector was inverted to give the local nodal conduction velocity (CV).

Method for closing a pathway

In order to investigate the delays required for a re-entrant circuit to form following unidirectional block, an action potential connection with identical morphology and a specified delay was made between two disparate parts of the network model. The concept of the network links are shown in Online Figure IX-B and the morphologically identical but delayed action potentials are shown in Online Figure IX-C. The connection that was used to study reentry arising from subepicardial pacing in Heart #2 is shown in Online Figure IX-D. A delay of 120 ms along this path was necessary to establish a re-entrant activation circuit. The development and maintenance of reentry is shown in Figure 9 and is available as a movie M3.

S3. Additional Results

APD dispersion with fixed cycle length pacing and premature stimulation in Heart #2

The network electrical activation model was paced at 300 ms BCL for subepicardial and subendocardial stimuli. Activation time distributions are shown in Figure 5 and are available for viewing as movies M1 and M2. These movies clearly show multiple activation pathways, wavefront collision, activation delays and block. The dispersion of APD was minimal, but greatest for pacing from the epicardial site (12.5 ms) compared with pacing from the endocardial site (6.9 ms). These APD distributions are shown in Online Figure X. The longest APDs occurred at sites of block or slowed activation. The shortest APDs occurred on epicardial and endocardial boundaries (distant from the site of stimulation) where electrotonic loading is less than other regions. For a subepicardial stimulus, tissue adjacent to the endocardial surface in the
NZ has shorter APDs than the same region for an endocardial stimulus, as activation progresses differently through the region in both cases. With subepicardial stimulation, activation collides with the boundaries in that region, whereas for subendocardial stimulation, activation progresses parallel to the boundary. This is best seen in the movies M1 and M2. Beat-to-beat APD alternans were not observed at either stimulus site during repeated cycles of activation at 300 ms BCL.

Premature stimuli were administered at varying CI after a preconditioning train of 10 cycles at 300 ms BCL to ascertain the interval at which block occurred. Transmembrane potential profiles during normal pacing and for premature stimuli at CIs immediately prior to block and at block are shown in Figure 7, for both subendocardial and subepicardial stimuli. Online Figure XI shows the distributions of APD for premature stimuli at CIs just prior to block. As expected, APD decreases as CI is reduced, but APD dispersion is almost twice as great immediately prior to block than at CI = 300 ms (see Online Figure X).

Online Figure X. APD distributions for 10th beat at 300 ms BCL pacing in Heart #2. A. Endocardial stimulus. B. Epicardial stimulus.
Supplemental Material

Infarct border zone structure and activation

Online Figure XI. APD distributions for a premature beat following pacing at 300 ms BCL in Heart #2. A. Endocardial stimulus with S2 CI of 157 ms. Block occurs at CI 156 ms. B. Epicardial stimulus with S2 CI of 186 ms. Block occurs at CI of 185 ms.

**Activation paths in Heart #2**

Representative activation sequence pathlines (unsmoothed) were determined for all nodes in the network. In addition, the shortest distance paths from every network node to the stimulus site were determined and the ratio of the two pathlengths determined. The ratios are summarized for subepicardial and subendocardial stimulus sites in Online Figure XII. This figure shows that all the representative activation paths are significantly longer than the shortest path through the network. This is stimulus site-dependent with the paths resulting from a subendocardial stimulus showing the greatest increase compared to the shortest path. This corresponds to what can be seen in Figure 6.

Online Figure XII. Percentage increase in actual activation path length vs shortest network path for subepicardial and subendocardial stimuli in Heart #2.
Fiber tract cross-sectional areas are rendered as a 3D colour map on the circumferential-transmural plane in Online Figure XIII-A. The effective cross-sectional area of connected myocyte bundles in the normal ventricular myocardium adjacent to the BZ is very large, whereas tracts of surviving cells within the BZ are characterized by a marked reduction in cross-sectional area which can be as little as 300 μm². Note that the cross-sectional area of human epicardial myocytes transverse to the fiber direction is on the order of 400 μm² and in the rat is on the order of 200 μm². In the BZ, therefore, there are regions where fiber tracts consists of only 1 or 2 myocytes. This is reinforced by the cross-section frequency distribution of Online Figure XIII-B. Variations in cross-section along the representative activation paths given in figures 6 and 7 are strongly related to activation delay and block, as shown in Online Figure XIV.

The data presented in Online Figure XIV correspond to stimulation at a BCL=300 ms for both anterograde and retrograde activation (that is in the directions of both block and successful propagation) for the red and green paths shown in figures 6 and 7 (through regions 1 and 2 in Figure 6). Anterograde block along both pathways occurred in regions where there was a transition from a sparsely connected myocyte network to a larger volume of well-connected myocytes (online figures XIV-A and XIV-B). With retrograde propagation through region 1 in the subepicardial BZ, time delay occurred at a point where the tract narrowed to form a single fine strand of myocytes and cross-sectional area was abruptly reduced. Elsewhere conduction velocity remained relatively uniform (c.f. activation time slope in Online Figure XIV-C). The action potential in this region was characterised by a slow rising foot (see Figure 7B) indicating a focal deficit in the inward current necessary to bring downstream myocytes to threshold. This contrasts with delays observed at the interface between subendocardial BZ and adjacent normal myocardium with retrograde activation along the second (green) pathway. Here, delay was distributed across a region where a sparsely connected network of myocytes expanded into a larger volume of well-connected myocytes (Online Figure XIV-D).

Finally, rate-dependent delay and block occurred with retrograde propagation along both pathways, but also exhibited different characteristics (Figure 7). Along the red pathway, delay increased progressively at the same point as coupling interval (CI) was reduced and block occurred there at CI=187 ms. On the other hand, the region of delay along the green pathway broadened with reducing CI.
Online Figure XIV. The impact of fiber tract cross-section (red, green) on activation delay (blue) in Heart #2. The red and green paths are those shown in figures 6 and 7. A. Activation moving from a subepicardial stimulus. Significant delay is associated with the rapid drop in cross-section between 1-2 mm. This is the point of block for a premature stimulus at a CI of 185 ms (Figure 7). B. Activation moving from a subendocardial stimulus. Delay and block are associated with rapid increases in cross-section between 1-2 mm and 4-5 mm. Retrograde activation is seen to the right of the block. C. Activation moving from a subepicardial stimulus. Block is associated with a rapid increase in cross-section between 3-4 mm. Retrograde activation is seen to the right of the block. D. Activation moving from a subendocardial stimulus. Delay is associated with the rapid increases in cross-section between 1-2 mm. This is the region of block for a premature stimulus at a CI of 156 ms (Figure 7).

Online movies

Three online movies are available. Their captions are as follows.

M1. This movie shows activation spreading from a subendocardial pacing site for the 10th beat in a train of beats at 300 ms BCL in Heart #2. It corresponds to Figure 5A. Of note is delay through the network of myocytes connecting the subendocardial BZ region to the better coupled NZ region, block at one tract connecting the NZ to the subepicardial BZ (1 in Figure 5A) but activation passage through the alternative path connecting the NZ to the subepicardial BZ (2 in Figure 5A).

M2. This movie shows activation spreading from a subepicardial pacing site for the 10th beat in a train of beats at 300 ms BCL in Heart #2. It corresponds to Figure 5B. Of note is delay and block through one network of myocytes connecting the subepicardial BZ to the NZ (2 in Figure 5B) and extended delay, but no block, through the alternate narrow tract connecting the subepicardial BZ to the NZ (1 in Figure 5B). Also noteworthy is the fast conduction pathway penetrating deep into the NZ following activation spreading through the narrow tract.

M3. This movie shows the initiation of sustained reentry through the border zone following subepicardial pacing at reducing CI in Heart #2. It corresponds to Figure 8. A virtual link was created between the
subepicardial BZ and the subendocardial BZ to complete a circuit. The link had a fixed delay of 120 ms. Subepicardial Pace 1 is the last beat prior to block and activates the tissue in the counter-clockwise direction (Basal view). Electrical activity progressing through the link from the subepicardium encounters retrograde activity from the subendocardium and collision occurs. Subepicardial Pace 2 at a CI of 157 ms blocks in both paths out of the subepicardial BZ, however, the virtual link causes activation in the refractory subendocardium and this leads to sustained reentry in the clockwise direction (Basal view). Four beats of sustained reentry are shown in the movie.

**Volume distribution, structure orientations and functional behavior in Heart #1**

Heart #1 was prepared and imaged as described in sections S2.1 to S2.3. This initial heart was imaged in an orientation which resulted in minimal visible lengths of epicardial and endocardial surfaces in the final image set. Consequently, unlike Heart #2, these images did not give a complete view of the transmural architecture. Nevertheless, the structural extent that is visible is consistent with the Heart #2 data shown in figures 3 and 4.

The volume distribution for Heart #1 is shown in Online Figure XV-A. The MI border was found using the clustering method described in Section S2.3. The volume is plotted as a function of distance from the infarct border in Online Figure XV-B. Fiber orientations were determined for the transmural extent of the image. The locations of the fiber samples and the orientations are shown in Online Figure XVI. The fiber angles moving from the normal zone (NZ) through the border zone (BZ) and into the infarct zone (IZ), shown in Online Figure XVI-C, are consistent with those shown in Figure 4.

![Online Figure XV](image)

**Online Figure XV.** Tissue volumes determined from images Heart #1. A. Distribution of tissue volume index in space. B. Tissue volume index as a function of distance from the border. The mean values (smoothed) are shown in red.
Online Figure XVI. Variation of fiber angle across portions of Heart #1. A. Locations of samples 1-5 and the normal zone (NZ) to infarct zone (IZ). B. Fiber orientations across samples 1-5. C. Fiber angles variation across the wall moving from the NZ to the IZ.

The segmented myocardium of Heart #1 was processed using topology preserving dilation (Section S2.3) and an image based network model of activation in the infarct border zone (Section S2.4) was constructed. The activation model was paced using identical protocols Heart #2 at a site on the endocardium and within the infarct. Activation time fields are shown in Online Figure XVII.

Online Figure XVII. Activation times in Heart #1 border zone model. A. Activation times on the 10th beat of a train of 300 ms BCL sub-endocardial stimuli (indicated by the red sphere). The left panel is a basal view and the right panel is an apical view. B. Activation times and block on the first beat for a stimulus located within the infarct (indicated by the red sphere).
These activation results in Heart #1 show an identical substrate of unidirectional block as observed in Heart #2. The unidirectional block occurs at a diffuse network of myocyte strands, similar to the unidirectional block through region 2 in Figure 5.

The topology of the imaged region of the Heart #1 does not exhibit multiple strands of surviving myocytes through the border zone. Consequently, some of the rate dependent features of Heart #2 are not seen in Heart #1. However, the range of APD shown in Online Figure XVIII is almost identical to that of Heart #2 for a sub-endocardial stimulus (see Online Figure X-A) and the conduction velocity range for Heart #1 of 0.4±0.2 m/s is also identical between the two models.

**Online Figure XVIII.** APD distributions for the 10th beat at 300 ms BCL pacing in Heart #1. The left panel is a basal view and the right panel is an apical view.

### S4. References