High-Resolution Optical Mapping of the Right Bundle Branch in Connexin40 Knockout Mice Reveals Slow Conduction in the Specialized Conduction System

Houman S. Tamaddon, Dhananjay Vaidya, Alexander M. Simon, David L. Paul, José Jalife, Gregory E. Morley

Abstract—Connexin40 (Cx40) is a major gap junction protein that is expressed in the His-Purkinje system and thought to be a critical determinant of cell-to-cell communication and conduction of electrical impulses. Video maps of the ventricular epicardium and the proximal segment of the right bundle branch (RBB) were obtained using a high-speed CCD camera while simultaneously recording volume-conducted ECGs. In Cx40−/− mice, the PR interval was prolonged (47.4±1.4 in wild-type [WT] [n=6] and 57.5±2.8 in Cx40−/− [n=6]; P<0.01). WT ventricular epicardial activation was characterized by focused breakthroughs that originated first on the right ventricle (RV) and then the left ventricle (LV). In Cx40−/− hearts, the RV breakthrough occurred after the LV breakthrough. Additionally, Cx40−/− mice showed RV breakthrough times that were significantly delayed with respect to QRS complex onset (3.7±0.7 ms in WT [n=6] and 6.5±0.7 ms in Cx40−/− [n=6]; P<0.01), whereas LV breakthrough times did not change. Conduction velocity measurements from optical mapping of the RBB revealed slow conduction in Cx40−/− mice (74.5±3 cm/s in WT [n=7] and 43.7±6 cm/s in Cx40−/− [n=7]; P<0.01). In addition, simultaneous ECG records demonstrated significant delays in Cx40−/− RBB activation time with respect to P time (P-RBB time; 41.6±1.9 ms in WT [n=7] and 55.1±1.3 ms in [n=7]; P<0.01). These data represent the first direct demonstration of conduction defects in the specialized conduction system of Cx40−/− mice and provide new insight into the role of gap junctions in cardiac impulse propagation. (Circ Res. 2000;87:929-936.)

Key Words: optical mapping ■ specialized conduction system ■ knockout mice ■ connexin40

Intercellular coupling via gap junction channels is an important determinant of impulse propagation in the heart.1 These channels provide a low resistance pathway that is essential for the coordinated spread of electrical activation, which subsequently triggers the contraction of the heart. Recent studies have indicated that alterations in the expression pattern of cardiac connexin proteins may lead to abnormal electrical coupling and arrhythmias.2-4 Therefore, understanding the role of intercellular communication in impulse propagation is essential.

Three connexins, Cx40, Cx43, and Cx45, are thought to be involved in impulse propagation in the myocardium. Detailed immunolocalization studies have shown that each of these proteins has a unique pattern of expression in the adult heart;5-8 however, the functional role of connexin proteins in impulse propagation remains poorly understood. The presence and expression levels of these connexins vary considerably in cardiac tissues with different conduction properties.6,8,9 Cx45 is expressed in the atrioventricular node and proximal portion of the ventricular conduction system.7 Cx43 is expressed in both the atrial and ventricular myocardium.7,10,11 Immunohistochemistry studies in the mouse heart have indicated that Cx40 is expressed mainly in the atrial myocardium and His-Purkinje system.7,12,13 Previous studies have shown that deletion of Cx40 did not affect the expression of other cardiac connexins or the gross structure of the heart.4,5 On the basis of these studies, it is expected that the targeted deletion of Cx40 will result in conduction deficits in both the atria and specialized conduction system.

The gross structure of the adult mouse specialized conduction system has not been studied in any great detail.14,15 Some histological studies have demonstrated broad similarities between the structure of the specialized conduction system of the mouse and that of larger mammals.14,16 In the mouse, the atrioventricular (AV) bundle has been shown to give rise to a compact right bundle branch (RBB), whereas many left bundle branch (LBB) fibers originate progressively over a wide range. Studies of the canine intraventricular conduction system have shown that the fibers of the RBB and LBB insert into the interventricular septum and the ventricular free...
was clearly prolonged, indicating atrioventricular defects. 16,18,19

Septal LBB fibers have previously been shown to initiate the first ventricular depolarization (Q wave) in humans and larger mammals, whereas septal RBB insertions contribute to later phases of ventricular activation. 16,17 The objective of this study was to characterize the electrophysiological consequences of the null mutation of Cx40 (Cx40–/–) on patterns of ventricular activation as well as conduction in the RBB. To achieve these objectives, we have developed an imaging system that is capable of obtaining high-resolution optical maps of electrical excitation in the ventricles and from the proximal segment of the RBB. Our results indicate that deletion of Cx40 does not result in impaired conduction velocity (CV) within the ventricular myocardium. However, in all Cx40–/– mice, epicardial breakthrough activation patterns during sinus rhythm and atrial pacing suggested slowed conduction in the RBB, which was confirmed by direct measurements of CV in the RBB. These data provide a detailed mechanism for the alterations recorded in surface ECGs from Cx40 deficient mice3–5 and provide important insight into basic mechanisms underlying impulse propagation in the heart.

Materials and Methods

All mapping studies were performed using Langendorff perfused mouse hearts in the absence of any motion reduction techniques on an upright microscope equipped with a cooled CCD camera (Dalsa Inc, CA-D1 128T). The right atrial appendage was paced to study activation patterns in intact left ventricle (LV) and right ventricle (RV) and on the exposed RBB. To confirm the location of the RBB, high-resolution images of acetylcholinesterase (AChE) staining were correlated with the optical mapping results. Ventricular conduction velocities (CVmax and CVmin) were studied by pacing the RV free wall. Ventricular conduction velocities were measured as described previously.21,22 CV in the RBB was determined by linear regression of activation time as a function of distance using the method of least squares.

Results

Conscious 6-Lead and Langendorff ECG

Figure 1 shows examples of 6-lead ECG recordings obtained from a conscious wild-type (WT) and a Cx40–/– mouse. Although the heart rates of the 2 animals were similar (WT, 730 bpm; Cx40–/–, 725 bpm), the PR interval was clearly prolonged, indicating atrioventricular defects.

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<th>ECG Parameters in Conscious Cx40 Knockout Mice</th>
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Data are mean±SEM, expressed in ms.

Optical Mapping of Purkinje-Activated Ventricular Myocardium

To determine the mechanisms underlying the surface ECG alterations, ventricular epicardial activation patterns were recorded from the anterior surface of both WT and Cx40–/– mice. Figure 2 shows color activation maps obtained during sinus rhythm and right atrial pacing at a cycle of 120 ms. In the left column, the WT activation pattern is characterized by 2 focused breakthroughs that originate on the free walls of the right and left ventricles. The breakthroughs form wavefronts that fully activate the field of view within 3 ms. In all WT mice during sinus rhythm, the RV breakthrough either preceded or occurred simultaneously with the LV breakthrough. These breakthrough sites represent 3D-wave propagation.
from Purkinje-muscle junctions (PMJ) originating from the RBB and LBB. Similar activation patterns were observed in all WT hearts.

In the right column, the activation patterns recorded from a Cx40\textsuperscript{−/−} mouse appear grossly different. Firstly, the focused LV breakthrough site seen in WT mice was replaced by a more diffuse and patchy activation pattern. In the example shown, the arrows indicate the direction of propagation from multiple breakthrough sites covering a large region of the LV. Secondly, the location of the first breakthrough site on both the LV and RV in Cx40\textsuperscript{−/−} mice was more variable than in WT mice. In this example, the first RV breakthrough in the Cx40\textsuperscript{−/−} heart occurred in the center of the RV free wall, whereas in other cases, the location of the first RV breakthrough site occurred elsewhere on the RV. Finally, in all Cx40\textsuperscript{−/−} mice the breakthrough activation sequence was reversed (ie, the RV breakthrough occurred after the first LV breakthrough). Note that in the example shown, the RV breakthrough during atrial pacing is delayed and is less apparent than during sinus rhythm.

**Synchronized Ventricular ECG Recordings**

To gain insight into the differences between RV and LV breakthrough activation patterns, RV and LV activation times were correlated with the Q time obtained from simultaneous volume-conducted ECG records. Figure 3 shows the relation between the Q time and the optically recorded RV and LV times while pacing the right atria at a basic cycle length (BCL) of 120 ms. Figures 3A and 3B show examples of these times and their relationship for a WT and Cx40\textsuperscript{−/−} mouse, respectively. Clearly, in the Cx40\textsuperscript{−/−} mouse, the RV activation time is delayed much more than the LV activation time relative to onset of the QRS complex. Figure 3C summarizes all right atrial stimulus (S) to Q time measurements obtained at the fastest BCL resulting in 1:1 AV capture (54.6±1.2 in WT [n=6] and 61.5±3.2 in Cx40\textsuperscript{−/−} [n=6]) and at BCL = 120 ms (47.4±1.4 in WT [n=6] and 57.5±2.8 in Cx40\textsuperscript{−/−} [n=6]; P<0.01 between genotypes; P<0.02 between cycle lengths). The increase in the S-Q interval is consistent with the ECG data obtained in conscious mice (see Table). Figure 3D shows a bar graph summarizing our measurements of Q to LV time (4.6±0.4 in WT [n=6] and 5.2±0.5 in Cx40\textsuperscript{−/−} [n=6]; NS) and Q to RV time (BCL=120: 3.7±0.7 in WT [n=5] and 6.5±0.7 in Cx40\textsuperscript{−/−} [n=6]; P<0.01) at a constant BCL of 120 ms. These data indicate that the RV but not LV time is significantly delayed with respect to the Q time in the Cx40\textsuperscript{−/−} mice. Figure 3E illustrates the interval changes between the 2 genotypes. Clearly, in Cx40\textsuperscript{−/−} mice, only the delayed LV breakthrough is fully accounted for by the late arrival of the Q wave.

**Ventricular Conduction Measurements**

The delayed arrival of the RV breakthrough is likely attributable to slowed conduction in the RBB; however, to exclude the possible contribution of conduction defects within the ventricular myocardium, epicardial conduction patterns and velocities were determined. Figure 4 shows activation maps obtained from the RV of a WT (panel A) and a Cx40\textsuperscript{−/−} (panel B) mouse while pacing the ventricles directly at a BCL of 120 ms. Pixels were systematically excluded near the pacing electrode (<1 mm) to remove any stimulus artifacts and at a distance (>3 mm) to exclude potential wavefront collisions and 3D-wave propagation. Clearly, the anisotropic conduction pattern in the Cx40\textsuperscript{−/−} was similar to that of WT. Figure 4C shows mean CV\textsubscript{max} and CV\textsubscript{min} for all WT and Cx40\textsuperscript{−/−} mice tested. No statistical differences were found between the 2 genotypes. Similar results were obtained at a BCL of 90 ms (data not shown). These data indicate that Cx40 does not significantly contribute to impulse propagation within the ventricular myocardium, which is in agreement with multielectrode\textsuperscript{20} and immunolocalization studies for Cx40.\textsuperscript{7}
optical maps of the proximal segment of the RBB were obtained. Figure 5A is an image of the right septal wall where the RBB has been stained with acetylthiocholine iodide, which precipitates in the presence of AChE activity. Figure 5B shows a color activation map of the RBB superimposed on the stained septal preparation. Optical signals recorded from the right septum are shown in the 2 insets. Voltage-dependent fluorescence recorded from pixels on the RBB is expected to originate from both the specialized conduction system and the underlying ventricular myocardium, giving rise to 2 temporally distinct action potential upstrokes. Therefore, during antegrade propagation, the first upstroke should result from a wave of activation through the RBB, whereas the second should result from the subsequent activation of the ventricular myocardium. Pixels not on the RBB are expected to show a single upstroke representing endocardial activation. The time sequence plots shown in the insets to the right and below panel B were obtained from pixels falling on the RBB and away from the RBB, respectively. Clearly, 2 action potential upstrokes are seen in all of the traces in the right inset, whereas only 1 action potential upstroke is seen in the bottom inset. In addition, these pixels showed activation that propagated from base to apex, consistent with antegrade propagation through the RBB. Figure 5C shows the pixels that were identified using a signal-to-noise ratio (SNR) criterion (>4) and the activation times from these pixels were used to measure RBB conduction velocity. Gaps in the activation sequence were attributable to uneven staining of the endocardium. The trace in Figure 5D shows a volume-conducted ECG obtained during the optical recording. The color bar above the trace demarcates the beginning and end of RBB activation. Thus, the RBB optical upstrokes preceded the QRS complex by ~3 ms and propagated distally toward the apex, which is again characteristic of RBB activation. These data represent the first optical recordings obtained from the specialized conduction system in the right ventricle.

Figure 6 shows a comparison between the activation patterns recorded from the RBB of a WT (panel A) and a Cx40–/– (panel B) mouse. These maps depict the similarities and differences of the RBB activation in the 2 mice. In both maps, activation begins near the RV base, distal to the septal leaflet of the tricuspid valve, crosses the septum along the main septal artery, and extends across the base of the anterior papillary muscle. In the Cx40–/– example shown, a smaller segment of the RBB was analyzed because of the early arrival of the underlying septal activation. Fusion of the RBB and
ventricular action potential upstrokes occurred at distal sites on the bundle, obscuring RBB upstrokes. This merging of the upstrokes may be attributable to either delayed RBB activation, resulting from slow conduction in the RBB, or earlier septal activation. These possibilities are studied below. Figure 6C displays the RBB conduction velocity in these 2 hearts. A >50% slowing of CV is apparent in the Cx40–/– heart. Summary of the RBB conduction velocity measurements is shown in Figure 6D. Clearly, the plots show a significant slowing of CV in the RBB of Cx40–/– mice. No cycle length dependence could be demonstrated at the cycle lengths tested. These data indicate that the slower CV contributed to the merging of RBB and ventricular action potentials upstrokes.

RBB-Synchronized ECG Recordings
To quantify the extent of RBB delay in relation to ventricular activation, RBB activation times were compared with Q time. Figures 7A and 7B illustrate synchronized volume-conducted ECG recordings from WT and Cx40+/– mice. Variable QRS morphologies were seen in the dissected RV preparations from both WT and Cx40+/–. Figure 7C summarizes S to RBB time and indicates a prolongation in Cx40+/– mice compared with WT mice (BCL=120 ms: 41.6±1.9 ms in WT [n=7] and 55.1±1.3 ms in Cx40+/– [n=7]; BCL=160 ms: 38.7±1.5 ms in WT [n=7] and 50.6±1.5 ms in Cx40+/– [n=7]; P<0.001 between genotypes; P<0.03 between cycle lengths). RBB to Q time measured in WT (BCL=120 ms: 4.30±0.8 ms; [n=7]) and Cx40+/– (BCL=120 ms: 1.2±0.5 ms; [n=7]) mice is shown in Figures 7D. Cx40+/– mice have a significantly shorter RBB to Q time (P<0.001). It is important to note that although the mean RBB to Q time was positive for Cx40+/– mice, 1 mouse showed RBB activation that occurred after the onset of the QRS complex. Figure 7E summarizes the interval changes seen between genotypes. Clearly, the delayed RBB activation that occurs in Cx40+/– mice is not fully accounted for by the late arrival of the Q wave (ie, the S-RBB increase is greater than the RBB-Q
Thus, the additional delay accounting for this difference must exist proximal to the optically mapped region of the RBB.

**His-Bundle Branching and LBB Conduction Time**

To better understand the relation of the LBB conduction time and the Q time, calculations were made using RBB activation maps to estimate the activation time where branching of the common His bundle occurs (B time; see online Figure 2; available in an online data supplement at http://www.circresaha.org). Assuming that the branch point is ≈1.5 mm proximal to the optically mapped region of the RBB (see Lev et al.14 for histological description of branch point), the B time can be extrapolated from the RBB conduction velocity measurements and the B to Q time can be determined. Thus, the B to Q time delay provides an estimate of conduction time in the LBB fibers that give rise to the start of the QRS complex. From these estimates, B to Q time was not significantly different between the Cx40−/− and WT mice (BCL = 120 ms: 6.4 ± 0.8 in WT [n = 7] and 5 ± 0.5 in Cx40−/− [n = 7]; BCL = 160 ms: 6.9 ± 0.7 in WT [n = 7] and 5.5 ± 0.7 in Cx40−/− [n = 7]; NS). Therefore, no detectable conduction defect was found in the LBB using this model. This finding is in agreement with LV free-wall activation times, which also indicate nondetectable LBB conduction delays in Cx40−/− mice.

**Discussion**

Impulse propagation in cardiac tissue is the result of many parameters functioning in concert, such as cell excitability,24 intercellular coupling,25–28 and tissue geometry.29,30 Perturbation of any of these parameters during cardiac disease is associated with slowing of conduction and increased risk of cardiac arrhythmias. Previous reports characterizing the role of intercellular coupling have relied on pharmacological interventions to modulate gap junctional conductance25,26,31; hence, those studies have the limitation of nonspecific pharmacological effects. On the other hand, the development of genetic engineering technology has provided a new approach to investigate the role of specific proteins in cardiac disease in the absence of pharmacological interventions.32–34 In particular, targeted deletion of connexin proteins offers the potential to investigate the specific roles of these proteins in impulse propagation in the mammalian heart.4,5,35 Because the deletion of Cx40 is not associated with gross cardiac malformations within the myocardium or the His-bundle branches,5 the Cx40−/− mouse provides an elegant model to study the effects of cellular uncoupling on impulse propagation.

**ECG Intervals**

The ECG data obtained from conscious Cx40−/− mice confirm the PR and QRS prolongation that has been previously
reported in anesthetized mice.\textsuperscript{15} In this study, we focused on the role of Cx40 in the specialized conduction system and ventricular myocardium. However, Cx40 is also expressed in the atria. Other studies have reported in some Cx40\textsuperscript{−/−} mice slower atrial conduction velocities, prolonged P-wave durations, longer sinus node recovery times, and atrial arrhythmias.\textsuperscript{3,4} Together, these studies provide supporting evidence that Cx40 is an important determinant of impulse propagation in the atria as well as the specialized conduction system.

**Ventricular Activation in the Absence of Cx40**

The null mutation of Cx40 resulted in obvious differences in both RV and LV activation patterns and times. Unlike WT mice, the LV of Cx40\textsuperscript{−/−} mice showed multiple breakthrough sites on the anterior surface. In addition, the first breakthrough on the RV varied more in location than that of the LV and showed significant delays with respect to the first LV breakthrough. Because of the broader distribution of left compared with RBB fibers in mice,\textsuperscript{14} conduction failures resulting from cellular uncoupling in the absence of Cx40 is expected to manifest as islands of epicardial activations surrounded by areas of quiescence. This patchy failure of conduction may occur at branching Purkinje fiber sites or at PMJs, where source-sink mismatches exist because of a change in the geometry in the propagating pathway.\textsuperscript{29}

Effects on the safety factor for propagation attributable to a reduction in intercellular coupling and abrupt changes in tissue geometry have been studied in patterned cell cultures.\textsuperscript{27,29,36} In the whole heart, the best-known example of current-to-load mismatch is that of the PMJ.\textsuperscript{37,38} At PMJs, depolarizing current (source) originating from a small Purkinje fiber has to provide enough excitatory current for a large ventricular mass of tissue (sink). Studies conducted on this subject have shown that successful propagation is indeed challenged by this transition.\textsuperscript{36,38}

Indeed, in larger mammals, which show a larger spatial distribution of LBB fiber insertions, a patchy and diffuse epicardial activation pattern is seen in the LV but not the RV.\textsuperscript{17,39} In our experiments, large delays in the RV breakthrough times resulted in significant changes in the sequence of RV epicardial activation attributable to a sweeping wave of activation from the LV. Hence, we cannot exclude the possibility that patchy patterns of conduction block also exist in the RV of the mouse heart. On the other hand, from our data, these isolated, local propagation failures do not provide a satisfactory mechanism for the overall delayed activation of the RV in Cx40\textsuperscript{−/−} mice.

**Conduction Slowing in the RBB of Cx40\textsuperscript{−/−} Mice**

Using newly developed high-resolution imaging techniques, RBB activation was quantitatively measured to determine the source of RV activation delays. Qualitatively, the activation pattern of the RBB in the Cx40\textsuperscript{−/−} mice was similar to that seen in the WT. In both cases, activation began near the septal leaflet of the tricuspid valve and continued distally to the base of the anterior papillary muscle. However, quantitative measurements of RBB conduction velocities showed a significant slowing in Cx40\textsuperscript{−/−} mice. Therefore, the lack of Cx40 in the RBB resulted in significant conduction slowing, which additionally resulted in delayed RV free-wall activation.

During optical mapping, it was noticed that RV septal activation occurred before the complete activation of the RBB in the Cx40\textsuperscript{−/−} mice. Moreover, simultaneous ECGs indicated that the RBB in the Cx40\textsuperscript{−/−} mice activated later with respect to the start of the QRS complex. Because the LBB is presumed to activate the septum and cause the first deflection of the QRS complex, both the Q-time and RV septal activation represent surrogate markers of LBB activation.\textsuperscript{16,18} Thus, both the optical and ECG measurements suggest that the RBB is more affected than the LBB via these surrogate markers. With improved technology and strides in optical mapping techniques, future left septal mapping studies in Cx40\textsuperscript{−/−} mice may directly demonstrate the role of Cx40 in the LBB.

**Role of Connexin45**

The presence of Cx45 in the His-Purkinje system cannot be forgotten.\textsuperscript{7} In Cx40\textsuperscript{−/−} mice, the existence of successful conduction through the RBB and LBB provides reason to implicate Cx45 as the remaining intercellular coupler in these tissues. Moreover, the lack of a detectable delay in the LV conduction system in Cx40\textsuperscript{−/−} mice leads to speculation that Cx45 is maintaining near normal conduction through this safer pathway. It is also important to note that because the RBB is anatomically thinner and action potential durations are longer than those in the LBB,\textsuperscript{16,40,41} it is expected that a
lower safety factor for antegrade conduction exists through the right pathway.46,42

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References
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EXPANDED MATERIAL AND METHODS

Mice

Data were obtained from adult age-matched (ages 11-20 weeks) wildtype (WT; n=21) and Cx40-/- (n=21) mice. Separate groups of mice were studied for the conscious ECG, ventricular mapping and right bundle branch (RBB) mapping experiments. All animal care protocols conformed to institutional and NIH guidelines.

Electrocardiographic recordings

Six-lead body surface ECGs were recorded from conscious WT and Cx40-/- mice. Animals were placed in a restraint device and metal plate electrodes covered with electrolyte gel made contact with the sole of the animal’s feet. All surface leads were calculated from the recorded signals. In addition, volume-conducted ECG’s were recorded during Langendorff perfusion from Ag-AgCl electrodes (0.5mm × 1mm). In the study of ventricular conduction and breakthrough patterns, one electrode faced the right ventricular free wall and the other faced the left ventricular free wall. In the study of RBB activation, one electrode faced the posterior interventricular groove, while the other faced the anterior interventricular groove. Signals were amplified and low-pass filtered with a differential amplifier (CyberAmp 380, Axon Instruments), digitized (Digidata 1200) at 5 kHz and stored for offline analysis. Interval measurements were performed on signal-averaged ECG recordings. Signal averaging (10-15 beats) provided average interval duration measurements and improved the signal-to-noise for each mouse ECG trace. ECG intervals were defined as described previously.2
Optical Mapping System

Mice were heparinized (heparin sodium [0.5U/g]), anesthetized by brief CO₂ inhalation and sacrificed by cervical dislocation. Hearts were surgically removed via a thoracotomy and placed in a custom-built perfusion / superfusion apparatus. While the heart was fully immersed in Tyrode’s solution (containing in mmol/L: NaCl, 130; NaHCO₃, 24; NaH₂PO₄, 1.2; MgCl₂, 1; glucose, 5.6; KCl, 4; CaCl₂, 1.8 saturated with 95% O₂:5% CO₂ gas mixture), the aorta was cannulated using a 22g stainless steel cannula and perfused with warm (37-38 °C) Tyrode’s solution at a constant pressure of 68-74 mm Hg (∼1-2 ml/min). Figure 1 shows a schematic of the optical mapping system and the locations on the heart that were mapped. The perfusion/superfusion apparatus was composed of an outer water-jacketed dish (Radnoti Inc.) and inner custom-built perfusion chamber (panel A). The custom-built perfusion chamber allowed for horizontal positioning of the heart. All hearts were allowed to equilibrate for 15 minutes following perfusion.

High-resolution optical mapping studies were performed on an upright Olympus microscope (BX50WI) with a reflected light fluorescence attachment (BX-FLA) equipped with a cooled (−10 to 0 °C) CCD camera (Dalsa, Inc). Excitation light from a 100-W mercury arc lamp (Olympus, Inc) entered a filter cube that reflected green excitation light (480-550; dichroic mirror 570 nm) to the heart and passed the emitted fluorescent light (>590 nm) to the CCD camera. An electronic shutter (Uniblitz, Inc.) placed in the excitation light path was used to limit exposure time (50-60 msec) during systole. This technique allowed for multiple recordings with minimal photobleaching and
phototoxicity. Images were acquired at 912 frames/s with 12-bit resolution from a 64 × 64-pixel array, which provided a spatial resolution of 40 µm (4x objective, N.A. 0.28) during RBB experiments and 82 µm (2.5x objective, N.A. 0.08) during ventricular experiments (see below). All mapping studies were performed in the absence of any motion reduction techniques.

**Ventricular Activation Study**

After equilibration, the heart was rotated such that the anterior right ventricle (RV) and left ventricle (LV) apices of the heart faced the microscope objective (see figure 1, panel B). Hearts were stained with voltage sensitive dye (Di-4-ANEPPS, Molecular Probes) by injecting a 0.3 ml bolus containing 125 nmol/L into a 10 ml compliance chamber within the perfusion line. Imaging was begun after 8-10 minutes when fluorescent intensity reached a steady state. A unipolar electrode was placed on the right atrial appendage to pace the atria such that impulses propagated through the AV node and His-Purkinje system allowing for study of ventricular activation. To study the epicardial ventricular conduction velocities, a unipolar Ag-AgCl (0.5mm) pacing electrode was placed on the free wall of the right ventricle (RV). All stimuli were delivered at a constant cycle length (indicated below) using 2 ms rectangular pulses equivalent to 1.5x diastolic threshold.

**Right Bundle Branch Mapping Experiments**

After equilibration, hearts were rotated such that the RV free wall faced the microscope objective. As illustrated in panel C of figure 1, two incisions were made on the RV free wall (panel C) to expose the RV septal surface, taking care not to lacerate the right
coronary artery. The RV septum was stained by superfusing the heart for 10 minutes with voltage sensitive dye (Di-4-ANEPPS, 52 μmol/L). The anterior papillary muscle and the septal artery were brought into the field of view to study RBB activation during AV conduction. The right atrial appendage was paced at a constant cycle length as described above.

**Analysis**

Ten to fifteen beats were averaged to obtain a representative activation sequence in all optical mapping records. Pixels with voltage-dependent signals were identified by a signal-to-noise ratio (SNR, rms) exclusion procedure. For ventricular mapping studies, a digital low pass filter (6th order Butterworth; cutoff of 200 Hz) was applied. Since mechanical contraction begins during repolarization, it is possible to identify motion-free action potential upstrokes by finding \( \frac{dF}{dt_{\text{max}}} \) on the optical action potential. Activation time was defined as the instant at which the fluorescent signal reached 50% of the AP amplitude since this measure has been shown to be more robust against noise present in signals.\(^3\) Ventricular conduction velocities (CV\(_{\text{max}}\) and CV\(_{\text{min}}\)) were measured as described previously.\(^4,5\) CV in the RBB was determined by linear regression of activation time as a function of distance using the method of least squares. The inverse slope of this line was taken as the RBB conduction velocity.

The following intervals were measured from synchronized volume conducted ECGs and optical recordings: stimulus to RBB activation (S-RBB), stimulus to left ventricular breakthrough (S-LV), and stimulus to right ventricular breakthrough (S-RV). In addition,
stimulus to ventricular activation (S-Q) was measured from the volume conducted ECG recording (Figure 2). In all experiments, stimulus time was defined as the beginning of the rectangular stimulus artifact obtained from volume-conducted ECG records, and ventricular activation (Q time) was defined as the first measurable deflection (above noise) of the QRS complex. LV and RV activation times were defined as the earliest optical breakthroughs identified on the LV and RV, respectively. Breakthrough sites were identified as islands of activation that propagated radially. RBB activation was defined as the time at which the first pixel on the RBB activated in the field of view (see Figure 6 below), which remained anatomically similar throughout all optical recordings.

**Histochemistry**

In a subset of WT RBB mapping experiments (n=3), the location of the RBB was confirmed by staining the right septal wall for AChE activity. Immediately following mapping studies, hearts were fixed in 4% formaldehyde for 15min. The right septal wall was then stained for 24 hrs using the staining solutions and protocol described by El-Badawi. High-resolution images of the staining pattern were obtained and correlated with the optical mapping results.

**Statistics**

Data are presented as means ± standard error of mean (SEM). When appropriate, statistical analyses was performed using ANOVA and differences were considered significant when p < 0.05.
FIGURE LEGENDS

**Figure 1. Experimental Setup.** Panel A depicts a schematic of the optical components of the upright microscope mounted with a high-speed CCD camera. The microscope was mounted on a x-y stage allowing for micrometer alignment of the optical mapping system without movement of the isolated perfused heart. Volume conducted ECGs were recorded simultaneously with optical mapping from the Ag-AgCl posts in the inner dish. Panels B and C display the position of the murine heart in the ventricular and RBB optical mapping experiments, respectively.

**Figure 2. Anatomical Schematic of AV Conduction and Timeline, Marking Interval Measurements.** Stimulus time (S, open circle) is shown to the left of the schematic where a unipolar electrode was placed on the right atrial appendage. The activation wavefront spreads anatomically through the AV node (yellow circle), the common Bundle of His, divides at a branch point (B), and continues distally through the bundle branches. In the LV, activation of the septum occurs via septal LBB Purkinje fibers (Q Time, green oval) whereas activation of the anterior LV occurs via free wall Purkinje fibers (LV Time, blue oval). In the RV, RBB time (orange oval) occurs at a proximal segment of the RBB and anterior RV activation occurs distally throughout the free wall (RV Time, red oval). All measured intervals are shown.
REFERENCE LIST


FIGURE 2 supplement