Identification of Nodal Tissue in the Living Heart Using Rapid Scanning Fiber-Optics Confocal Microscopy and Extracellular Fluorophores

Chao Huang, BS; Aditya K. Kaza, MD; Robert W. Hitchcock, PhD; Frank B. Sachse, PhD

Background—Risks associated with pediatric reconstructive heart surgery include injury of the sinoatrial node (SAN) and atrioventricular node (AVN), requiring cardiac rhythm management using implantable pacemakers. These injuries are the result of difficulties in identifying nodal tissues intraoperatively. Here we describe an approach based on confocal microscopy and extracellular fluorophores to quantify tissue microstructure and identify nodal tissue.

Methods and Results—Using conventional 3-dimensional confocal microscopy we investigated the microstructural arrangement of SAN, AVN, and atrial working myocardium (AWM) in fixed rat heart. AWM exhibited a regular striated arrangement of the extracellular space. In contrast, SAN and AVN had an irregular, reticulated arrangement. AWM, SAN, and AVN tissues were beneath a thin surface layer of tissue that did not obstruct confocal microscopic imaging. Subsequently, we imaged tissues in living rat hearts with real-time fiber-optics confocal microscopy. Fiber-optics confocal microscopy images resembled images acquired with conventional confocal microscopy. We investigated spatial regularity of tissue microstructure from Fourier analysis and second-order image moments. Fourier analysis of fiber-optics confocal microscopy images showed that the spatial regularity of AWM was greater than that of nodal tissues (37.5±5.0% versus 24.3±3.9% for SAN and 23.8±3.7% for AVN; P<0.05). Similar differences of spatial regularities were revealed from second-order image moments (50.0±7.3% for AWM versus 29.3±6.7% for SAN and 27.3±5.5% for AVN; P<0.05).

Conclusions—The study demonstrates feasibility of identifying nodal tissue in living heart using extracellular fluorophores and fiber-optics confocal microscopy. Application of the approach in pediatric reconstructive heart surgery may reduce risks of injuring nodal tissues. (Circ Cardiovasc Imaging. 2013;6:739-746.)

Key Words: atrioventricular node ■ confocal imaging ■ congenital cardiac defect ■ sinoatrial node ■ 2-dimensional

Of all birth defects, congenital heart defects are the most frequent cause of death among infants in the US. The state-of-the-art approach to repair congenital heart defects is open heart reconstructive surgery in the neonatal period. Complications that can occur as a result of these complex surgeries are sinus node dysfunction or atrioventricular block arising from trauma to the sinoatrial node (SAN) and atrioventricular node (AVN), respectively. These complications are associated with significant morbidity and mortality. Patients with persistent complications require short-term or chronic cardiac rhythm management using implantable pacemakers. The need for permanent pacemakers after repair for congenital cardiac defects ranges from 1% to 3% for ventricular septal defect to as high as 27% to 45% for congenitally corrected transposition of the great arteries.

The SAN, AVN, and other tissues of the conduction pathways are not visible using surgical loupes or microscopes. These instruments are based on reflection of light from the tissue surface and do not provide visual information from within the tissue, where nodal tissue resides. The localization of SAN and AVN tissue during surgery is based on their relative proximity to anatomic landmarks. The SAN is usually located proximal to the crista terminalis in the right atrium. The AVN is adjacent to the atrioventricular septum of the heart. Commonly, the AVN is identified from the triangle of Koch, which is defined as the space between the septal leaflet of the tricuspid valve, coronary sinus, and the tendon of Todaro. In hearts with congenital defects this localization is made more difficult by individual variations in the spatial relationship of landmarks to nodal structures. The aforementioned postoperative complications can arise as a result of this imprecise methodology for localization of nodal tissue. Thus, imaging
approaches for real-time visualization of nodal tissue in the intraoperative setting could reduce the incidence of nodal dysfunction and conduction block.

In this study, we establish a novel intraoperative imaging modality to discriminate cardiac tissue types using methodology based on fluorescent labeling, confocal microscopy, and image analysis. To achieve this aim, we investigated the microstructure of atrial working myocardium (AWM), the SAN, and the AVN in a rodent model. Using fluorescent markers for nodal cells and extracellular space and 3-dimensional (3D) conventional confocal microscopy, we created reconstructions at submicrometer resolution from fixed tissue. From these reconstructions we developed methods for characterization of tissue microstructure based on texture analysis. Subsequently, we evaluated our approach for tissue discrimination in the isolated perfused heart using a recently introduced fiber-optics confocal microscopy (FCM) system, a fluorescent marker of the extracellular space, and specialized imaging microscopes. Using the acquired image data, we characterized the tissue microstructure with previously developed methods for texture analysis. We performed statistical analyses of conventional confocal microscopy and FCM image data to investigate the proposed approach for discrimination of cardiac tissue types. Furthermore, we explored fetal and infant tissue microstructure in human using conventional confocal microscopy to provide further insights into clinical translation of the developed approach.

Methods

Rodent Heart Preparations

All procedures were approved by the University of Utah Institutional Animal Care and Use Committee and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Sprague–Dawley male rats of 300 g body weight were anesthetized with pentobarbital (40 mg/kg) and anticoagulated with heparin (500 IU/kg). Following intraperitoneal administration, hearts were rapidly excised and Langendorff-perfused with cold zero calcium Tyrode solution (in mmol/L: 92 NaCl, 11 dextrose, 4.4 KCl, 5 MgCl₂, 24 HEPES, 20 taunine, 5 creatine, 5 C₃H₇NaO₃, 1 NaH₂PO₄, 12.5 NaOH; pH 7.2; ≈10°C) at a flow rate of 10 to 15 mL/min.

For preparations of living hearts, these were continuously Langendorff-perfused with cold zero calcium Tyrode solution. Before imaging, 5 μL of fluorescent dye was applied to the epicardial or endocardial surface of the heart to label the extracellular space within regions of interest. The fluorescent dye was Alexa Fluor 488 conjugated to dextran with a molecular weight of 3 or 10 kDA (Invitrogen, Carlsbad, CA) dissolved in phosphate buffered saline (PBS) solution to a final concentration of 125 μg/mL.

For fixed tissue preparations, perfusion with Tyrode solution ceased after 5 minutes. Afterward, hearts were either perfused for 5 minutes with a zero calcium Tyrode solution containing wheat germ agglutinin (WGA) conjugated to CF488A (29022-1; Biotium, Hayward, CA; 1:100), followed by a 10-minute perfusion with Tyrode solution containing 4% paraformaldehyde (pH 7.2), or the hearts were immediately perfused with fixative. The hearts were then immersed in PBS containing 4% paraformaldehyde for 24 hours at 4°C. Tissue from the AVM, SAN, and AVN was dissected from the fixed hearts (Figure 1; Figures I and IIA in the online-only Data Supplement).

Immunofluorescent Labeling of Fixed Tissue

Preparations of fixed tissue were washed 3× in PBS and then permeabilized and blocked with PBS solution containing 0.5% Triton X-100 and 4% normal goat serum (16210-064; Gibco, Grand Island, NY) for 1 hour. After washing with PBS, preparations were bathed in Image-iT FX Signal Enhancer (I36933; Invitrogen) for 1 hour. Next, preparations were washed in PBS and incubated overnight with primary antibodies (antihyperpolarization-activated cyclic nucleotide-gated potassium channel 4 [HCN4, APC-052, Alomone Labs, Jerusalem, Israel; 1:800]; antisarcomeric α-actinin [ab9465, Abcam, Cambridge, MA; 1:400]; 4′,6-diamidino-2-phenylindole [DAPI, D1306, Invitrogen; 1:400]) in PBS incubation solution containing 0.5% Triton X-100 and 4% normal goat serum. The following day, preparations were washed 3× in PBS and incubated overnight with secondary goat antirabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen; 1:400) and goat antimouse IgG conjugated to Alexa Fluor 633 (Invitrogen; 1:200) in PBS incubation solution. The next day, preparations were washed 3× in PBS and stored in PBS solution until imaging. All labeling was performed on a laboratory platform rocker (S-2035-D; Labnet International Inc, Woodbridge, NJ) at room temperature.

Imaging of Fixed Tissue

Fixed atrial tissue preparations were imaged using a confocal microscope (Zeiss LSM 5 Duo; Zeiss, Jena, Germany) equipped with a 40x oil immersion lens with a numeric aperture of 1.3. Tissue samples were placed on a glass slide and a coverslip was placed on top of the tissue (Figure IIB in the online-only Data Supplement). The coverslip was gently pressed on the tissue, bringing its surface close to the glass slide. Fluorophores within the labeled preparations were individually excited for each image slice, alternating between 364, 488, 543, and 633 nm laser lines. The emitted light was collected through a longpass filter at 385 nm, bandpass filter for 505 to 555 nm, longpass filter at 560 nm, and longpass at 650 nm. This multi-track imaging protocol reduced cross-talk of the applied fluorophores. 3D image stacks were acquired at a spatial resolution of 0.2×0.2×0.2 μm in x-, y- and z-direction, respectively, a field of view of (x) 204.8 μm by (y) 204.8 μm, and depth (z) up to 50 μm. Details on the processing and visualization of images are provided in online-only Data Supplement.

Fourier Analysis of Tissue Microstructure

The spatial distributions of the extracellular space in images from AVM and nodal tissue were characterized from their Fourier spectra. A discrete 2-dimensional (2D) Fourier transform was applied on xy images after multiplying them with a Gaussian window function. Intensities in circular sectors with an angle of 10° at spatial frequencies from 0.07 to 0.2 μm⁻¹ were integrated from the image spectra. A measure of spatial regularity was determined from a ratio of summed intensities within 15° of the peak of the angular spectra to the overall sum of intensities in angular spectra.
Image Moment Analysis of Tissue Microstructure
Higher-order image moments were determined to characterize the spatial distributions of the extracellular space. Images were decomposed in subregions with a dimension of 64×64 pixels. A circular mask was applied to each subregion and the central second-order moment calculated. Local orientation and scaling of the moment were derived from singular value decompositions. A histogram of local orientation angles was determined. A measure of spatial regularity was determined from the ratio of occurrence of an orientation angle within 15° of the peak of the occurrences to the overall occurrences.

FCM Imaging of Tissue in Living Hearts
2D image sequences of AWM, SAN, and AVN regions labeled with dextran-conjugated Alexa Fluor 488 were acquired using a fiber-optics confocal microscope (FCM1000; Leica Microsystems GmbH, Wetzlar, Germany) equipped with a custom fiber-optics microscope (UltraMiniO; Mauna Kea Technologies, Paris, France). A schematic diagram of the FCM imaging setup is shown in Figure III in the online-only Data Supplement. Excitation of the fluorophore was at a wavelength of 488 nm and emitted light was collected at wavelengths 505 to 700 nm. 2D image sequences were acquired with a lateral resolution of 1.8×1.8 μm in x- and y-direction, an optical sectioning (z) of 10 μm, a field of view (xy) of 169 by 120 μm, depth (z) ≤50 μm, and frame rate of 12 Hz. The micropipe tip diameter was 2.6 mm, which simplified maneuvering of the micropipe. It was attached to a manual micromanipulator (M3301L; World Precision Instruments, Inc, Sarasota, FL) allowing for fine-precision 3D movement of the microprobe in proximity to the heart surface.

Processing and Visualization of FCM Images
The brightness and contrast of images acquired with the FCM1000 were auto-adjusted using the imadjust function in MATLAB (The Mathworks Inc, Natick, MA).

Statistical Analysis
Statistical data are presented as means±SD. Statistical significance was assessed by 1-way ANOVA followed by post hoc Tukey–Kramer (P<0.05). A summary intensity (Iq) was determined from the averaged angle spectra that resulted from the image moment and Fourier analyses for conventional confocal microscopic and FCM images of AWM, SAN, and AVN tissue samples. Iq was defined as the sum of occurrences of local orientation angles along grouped orientation angles between −15° and +15° from the Fourier analyses. From the image moment analyses, I0 was determined from the sum of occurrences of local orientation angles along grouped orientation angles between −15° and +15°. A comparative intensity based on SD of intensities or occurrence of local orientation along grouped orientation angles between −89° and +90° was also determined (Iq). Differences in Iq of conventional confocal microscopic and FCM images of AWM, SAN, and AVN tissue samples were assessed. A similar analysis was done to determine significance based on Iq.

Human Heart Preparations, Immunofluorescent Labeling, and Imaging
Studies were designated as nonhuman subjects research and granted Institutional Review Board exemption by the University of Utah. We obtained deidentified, formalin-fixed sections from AWM, SAN, and AVN regions of 2 autopsy hearts. The autopsy hearts were from a 4-month-old infant and a 38-week-old fetus. Both did not have congenital heart disease. Preparations of fixed sections were washed 3× in PBS and incubated overnight with WGA conjugated to CF488A (Biotium; 1:25). The following day, preparations were washed 3× and stored in PBS solution until imaging. We applied conventional confocal microscopy and texture analyses as described above.

Results

Imaging of Cardiac Tissue Using Conventional Confocal Microscopy
We hypothesized that fluorescent labeling of extracellular space and imaging with conventional confocal microscopy allows for identification of nodal tissue. We tested this hypothesis on tissue preparations from adult Sprague–Dawley rat hearts. Locations of SAN and AVN in this species are grossly similar as in other mammals including human, rabbit, and pig.

Cardiac tissue preparations were dissected from SAN and AVN regions using anatomic landmarks as described above (Figure 1; Figure I in the online-only Data Supplement). Briefly, SAN tissue was dissected from the junction of the superior vena cava and right atrium, and AVN tissue was dissected from the region of the membranous septum, which is bordered by the septal leaflet of the tricuspid valve, coronary sinus, and the tendon of Todaro. Tissue dissections were labeled with DAPI to mark nuclei and WGA conjugated to a fluorophore to label constituents of the extracellular space. WGA binds to glycoconjugates of the extracellular space, including those associated with collagen strands and the glycocalyx on the surface and periphery of cell membranes.11,12 Additionally, tissues were labeled with antibodies for both HCN4 and sarcomeric α-actinin. HCN4 labeling has been previously established as a positive marker of nodal cells.13–16 Sarcomeric α-actinin served to identify cardiomyocytes. Conventional confocal microscopic imaging of the tissue preparations yielded 3D distributions of the 4 labels at submicrometer resolution. Image stacks from a preparation of AWM are presented in Figure 2A through 2H. A hallmark of this tissue is the dense and aligned arrangement of myocytes, which is reflected in the striated distribution of WGA (Figure 2B). Fluorescence associated with anti-HCN4 was absent (Figure 2C). The myocytes labeled positive for α-actinin (Figure 2D). The myocytes were covered by an epicardial layer with a thickness of 3.6 μm. Figure 2I through 2P shows image stacks from SAN tissue. The stacks comprised 3 distinct layers (ie, epicardium, nodal cells, and subnodal layer of AWM myocytes). In this stack, the epicardial and nodal layer had a thickness of 2.4 and 11.2 μm, respectively. The microstructural arrangement in the nodal layer was in various aspects different from the arrangement in the subnodal layer of AWM myocytes in this stack (Figure 2N versus Figure 2M). It was also very different from the arrangement of myocytes in the stack from AWM tissue (Figure 2E). SAN cells formed a highly irregular reticulum and had notably smaller diameters than myocytes of the AWM. However, SAN cells also exhibited similar features of striated myocytes (ie, the regular transversal striations of the α-actinin signal with a longitudinal spacing of ≈2 μm). The extracellular space in the SAN layer was more prominent than in AWM and comprised oval-shaped clearings with variable sizes. Figure IV in the online-only Data Supplement illustrates image stacks from the compact region of the AVN, which presented a microstructural arrangement similar to SAN tissue. Here, the epicardial layer had a thickness of 0.8 μm. The orientation of AVN cells was diverse, and they formed a reticulum with ample extracellular space. Figure V in the online-only Data Supplement presents unprocessed image stacks corresponding to Figure 2 and Figure IV in the online-only Data Supplement.
Statistical analyses were performed on acquired images from a group of animals (Table). Image analysis from the AWM and SAN tissue showed that the epicardial layer had a thickness of 7.5±3.5 and 5.5±2.9 µm, respectively. In the compact AVN the thickness of the endocardial layer was 3.2±2.4 µm. In SAN and AVN tissue, the thickness of the nodal layer was 10.3±3.2 and 17.2±8.7 µm, respectively. Analysis of HCN4 signal intensities showed that these were significantly (P<0.005) higher in the nodal layer of the SAN and AVN preparations than in the AWM (Figure VI in the online-only Data Supplement). Also, intensities in the nodal layers were significantly higher than in their respective subnodal layers. We did not find significant differences between HCN4 intensities in the AWM and in subnodal SAN and AVN layers.

Quantitative Characterization of Tissue Microstructure

We evaluated the ability of 2 methods of 2D texture analysis to discriminate between AWM and nodal tissue using WGA images. The first method measured the texture orientation from Fourier transformed images. The second method characterized texture orientation from second-order image moments of image regions. Figure 3A through 3C presents exemplary images and results from these analyses applied to the WGA images presented in Figures 2B and 2J and Figure IVB in the online-only Data Supplement. These images were overlaid with glyphs characterizing local orientation and anisotropy ratio of second-order image moments. It is apparent that the microstructural arrangement of AWM (Figure 3A) differs from that of nodal tissue (Figure 3B and 3C).
In this example, both methods of texture analysis indicated a larger degree of orientation in AWM than in SAN and AVN tissue (Figure 3D and 3E).

**Imaging of Living Hearts Using FCM**

We hypothesized that imaging with FCM using a fluorescent marker of the extracellular space will allow discrimination of tissue types in living hearts. In these studies, we used dextran-conjugated fluorophores with a molecular weight of 10 kDa and a FCM microprobe with a working distance of 50 µm. Previous studies demonstrated that dextran conjugates of 3 to 10 kDa molecular weight penetrate endothelial endocardium, endothelial epicardium, and myocardial capillary endothelium in rat,17 and through the epicardium into ventricular myocardium in rabbit.18 However, dextran conjugates of this molecular weight do not penetrate intact cell membranes19 and thus label specifically the extracellular space. FCM image sequences were acquired at a rate of 12 images/s from the AWM and nodal regions following topical application of extracellular fluorophore (Figure IIIB in the online-only Data Supplement). The image sequences of nodal tissue were acquired from an intact living heart from similar regions that were excised for the aforementioned conventional confocal microscopy study (Figure 1; Figure I in the online-only Data Supplement). Examples of FCM image sequence are shown in Movie I in the online-only Data Supplement for AWM and Movie II in the online-only Data Supplement for SAN. In these images bright and dark regions correspond to fluorescence in the extracellular spaces and the absence of fluorescence in the intracellular spaces, respectively. The average SNR in 17 image sequences was 12.1±3.3 (see online-only Data Supplement for details). Images from the dextran-labeled AWM and nodal regions using FCM bear resemblance to WGA images using conventional confocal microscopy. FCM images of the AWM exhibited regular striations (Figure 3F) as found previously in WGA images using conventional confocal microscopy (Figure 2B). In contrast, images from nodal regions revealed irregular microstructural arrangements (Figure 3G and 3H) similar as in WGA images (Figure 2J; Figure IVB in the online-only Data Supplement).

**Table. Numbers of Images and Animals Used for Image Analysis**

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Imaging of Fixed Tissue</th>
<th>Imaging of Living Hearts</th>
<th>Depth and Thickness Profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWM</td>
<td>16/6</td>
<td>37/7</td>
<td>13/6</td>
</tr>
<tr>
<td>SAN</td>
<td>13/6</td>
<td>40/6</td>
<td>11/6</td>
</tr>
<tr>
<td>AVN</td>
<td>10/6</td>
<td>29/6</td>
<td>9/6</td>
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AVN indicates atrioventricular node; AWM, atrial working myocardium; and SAN, sinoatrial node.  

Figure 3. Quantitative characterization of microstructure in fixed and living tissue. Wheat germ agglutinin (WGA) images of fixed tissue preparations from atrial working myocardium (AWM; Figure 2B), sinoatrial node (SAN; Figure 2J), and atrioventricular node (AVN) regions (Figure IVB in the online-only Data Supplement) were analyzed using second-order moments. (A) AWM. (B) SAN. (C) AVN images overlaid with glyphs visualize orientation of the minor eigen-axis (color-coded, bar) and anisotropy ratio of eigen-values (shape-coded) of local image moments. Histogram of orientations based on (D) second-order moments and (E) Fourier transform. Kurtosis of histograms from second-order moment and Fourier analysis is a marker of myocyte alignment. Fiber-optics confocal microscopy images of living tissue from (F) AWM, (G) SAN, and (H) AVN regions labeled with a dextran–fluorophore conjugate. Living tissues exhibited similar microstructural features as in fixed tissues (A–C). (J) AWM, (K) SAN, and (L) AVN images overlaid with glyphs. Quantitative analysis of (I) second-order moments and (M) Fourier transforms suggest larger myocyte alignment in AWM regions versus SAN or AVN. Scale bar in A applies to B and C. Scale bar in F applies to G and H and J through L.
Characterization of FCM Images
We investigated if tissue types can be discriminated in images from FCM using our 2 methods of texture analysis. Examples of this evaluation are presented in Figure 3J through 3L. The FCM images shown in Figure 3F through 3H were overlaid with glyphs characterizing local orientation and anisotropy ratio of second-order image moments. Fourier and second-order image moment analysis of the image from the AWM demonstrated a prominent texture orientation (Figure 3I and 3M). Nodal tissue did not feature a similar degree of orientation and the intensities were approximately uniform over the range of angles.

Statistical Evaluation of Approach for Tissue Discrimination
Texture orientation from second-order image moments and Fourier transforms of sets of WGA (Figure 4A and 4E) and dextran-labeled images (Figure 4B and 4F) were obtained using conventional confocal microscopy and FCM, respectively. The resulting intensity and local orientation profiles from these sets of images were similar to those presented for exemplary images (Figure 3D and 3E versus Figure 4A and 4E and Figure 3I and 3M versus Figure 4B and Figure 4F). We used the sum of intensities and occurrence of local orientation for angles between −15° and +15° as a measure of spatial regularity. The summary intensity (I±15) was found to be significantly different in images of AWM and nodal tissue from both conventional confocal microscopy and FCM (Figure 4C and 4G). Similarly, the SD (Istd) of intensity profiles allowed discrimination of nodal and AWM tissue acquired with conventional confocal microscopy or FCM (Figure 4D and 4H).

Imaging and Characterization of Human Tissue Microstructure
We investigated fetal and infant tissue microstructure in human. We observed similar microstructural features in human AWM tissue as described above for our rodent model (Figure 5A and 5B versus Figure 2B). The thickness of the epicardial layer in the fetal and infant AWM tissues was 11.2 and 14.6 μm, respectively. Features of AVN regions were similar in rodent and human (Figure 5C versus Figure IVB in the online-only Data Supplement). The thickness of the endocardial layer in the fetal AVN tissue was 3.6 μm. In addition, texture analysis based on second-order image moments and Fourier transforms indicated a larger degree of orientation in AWM than in AVN tissue (Figure 5D and 5E; Figure VII in the online-only Data Supplement).

Discussion
The microstructure of the SAN and AVN has been used for identification since their discovery by Keith and Flack in 190720 and Tawara in 1906,21 respectively. These studies were based on thin sections of nodal tissues, which revealed the characteristic microstructure using light microscopy. Here, we demonstrate that nodal tissue types can be identified in living hearts based on confocal microscopy and fluorescent labeling of the extracellular space. Our approach demonstrated that confocal microscopy can acquire image data at the appropriate specific depth within the intact heart. We found that AWM, SAN, and AVN tissue is beneath a thin surface layer of tissue that does not obstruct confocal microscopic imaging in the rodent model (Movies III-VIII in the online-only Data Supplement). In our exemplary study on human tissue we found AWM and AVN tissue at similar small depths. We also introduced methods for texture analysis, which provided us with a quantitative tool for automated tissue identification. We suggest that the presented approach has the potential to identify nodal tissue in the in situ heart and complement other methods for tissue identification in a clinical setting. An obvious clinical application is related to pediatric reconstructive heart surgery. The described approach allows surgeons to map out the location of nodal tissue and thus avoid tissue damage, such as what might occur by suture injury. Suture injury is a common clinical cause for SAN and AVN dysfunction.22,23

Figure 4. Statistical characterization of images from atrial working myocardium (AWM), sinoatrial node (SAN), and atrioventricular node (AVN) regions. Histograms of orientations calculated from (A) second-order moments and (B) Fourier transforms of images from fixed, wheat germ agglutinin–labeled tissue acquired with conventional confocal microscopy (CCM). Histograms of orientations from (E) second-order moments and (F) Fourier transforms of FCM images from tissue labeled with a dextran–fluorophore conjugate. I±15 of AWM and nodal tissues were significantly different using the (C) second-order image moment and (G) Fourier analysers. D and H, Istd showed similar significant differences. *P<0.05, compared with nodal tissue (1-way ANOVA with post hoc Tukey–Kramer).
instances where repair must be made relatively close to nodal regions, surgeons could place sutures or incision lines with a higher level of confidence using the FCM. We suggest that our approach for tissue identification can help to reduce the incidence of complications caused by injury of nodal tissues.

FCM imaging complements other emerging imaging technologies suggested for in vivo tissue imaging, such as micro-optical coherence tomography, conventional fluorescence imaging with miniaturized microscopes, and fluorescence spectroscopy. All these technologies have the potential to provide clinicians with real-time visual information beyond reflected visible light. In contrast to the other emerging imaging technologies, FCM systems are already available commercially and Food and Drug Administration approved for applications in the gastrointestinal and pulmonary tracts. However, the application of FCM in other clinical fields is hampered, in part, because of the lack of reliable fluorescent labeling strategies. Commonly, fluorescent labels consist of fluorophores conjugated to a ligand. The ligand typically targets a specific site of interest. Here, we investigated dextran conjugated to Alexa Fluor 488 to target the extracellular space. We demonstrated that the dextran conjugate was able to label the extracellular space in the living perfused heart with a high SNR. The labeling allowed for visualization of tissue microstructure and subsequent image processing and quantitative analysis.

A limitation of the presented approach for clinical translation is the potential toxicity of fluorophores. The toxicity of many fluorophores has been recently reviewed, and several fluorophores are routinely used for human applications. The quantity of dextran–Alexa conjugate used in this study was ≤50 pg per imaging trial. While no toxicity-related information is available for this conjugate and Alexa Fluor 488, other hydrophilic conjugates of dextran and Food and Drug Administration–approved fluorophores are available. A promising fluorophore for labeling of the extracellular space is Fluorescite®, which is an Food and Drug Administration class IIa drug and already approved for use in ophthalmic angiography. A study exploring the use of intravenously injected Fluorescite® in FCM imaging of the gastrointestinal tract in vivo produced a SNR from acquired images that was comparable to the value obtained in our living heart studies.

We developed our approach based on a microstructural characterization of cardiac tissue using conventional 3D confocal microscopy. The thicknesses of endocardial and epicardial layers determined from WGA images indicated that these layers do not constitute obstacles to confocal imaging of underlying tissue layers, for instance by depth-dependent attenuation of the excitation and emitted light. We used labeling for HCN4 to confirm the presence of nodal tissue and measured the thickness of nodal layers. The measured thickness of the nodal layers is sufficiently large versus the thickness of optical sections acquired with FCM (≈10 μm). We suggest that this type of microstructural analysis can provide important input for the development of imaging microprobes that are specialized for imaging applications. This analysis can assist in the development of microprobes tailored to age groups, disease states, and anatomic regions for FCM imaging of cardiac and other tissues.

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Disclosures
None.

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Supplemental Methods

Processing and Visualization of Images from Fixed Tissue. The three-dimensional image stacks were corrected for background signals and depth-dependent attenuation.\(^1\)\(^\text{,}^2\) We applied the Richardson-Lucy algorithm based on measured point spread functions (PSFs) to deconvolve the image stacks. 3D volume visualizations of exemplary image stacks of AWM, SAN, and AVN were produced using VolView (Kitware, Inc. Clifton Park, New York). The depth of the epicardial tissue layer was detected from the WGA associated signal. WGA signal intensity from XY images was averaged along the z-direction for samples of AWM, SAN and AVN tissue. The maximal increase and decrease of averaged WGA signal served as a marker of the start and end of an epicardial layer. The depth of nodal tissue was detected from the HCN4 associated signal using a similar approach. The maximal increase and decrease of averaged HCN4 signal served as a marker of the start and end of a nodal tissue layer.

Signal-To-Noise Ratio (SNR). The SNR was calculated from image sequences of SAN regions labeled with dextran conjugated Alexa Fluor 488 and acquired with a fiber-optics confocal microscope. SNR is defined as the ratio between the mean of the signal and the standard deviation of the signal. In our case, regions of interest (ROI) of approximately 700 pixels were selected that represented an area of high signal intensity and an area of low signal intensity for each image sequence. These high and low intensity areas corresponded to regions with both signal and noise-only regions, respectively. An average SNR was determined based on background corrected image sequences. Image sequences with an SNR below two deviations of the average SNR were excluded from Fourier and image moment analyses.

Quantitative Analysis of HCN4 Signal Intensity. Signal and background intensities were measured from acquired images of fluorescent dye solutions excited with the 543 nm laser line. We sampled those intensities in a range of laser powers, gains, and pixel dwell times. A three-dimensional calibration curve was calculated based on the measured mean intensities for the imaged solution with respect to the sampled laser powers, gains, and pixel dwell times. The AWM, nodal and subnodal layers were determined based on WGA and HCN4 depth profiles generated from image stacks of rat AWM (\(n = 7/4\)), SAN (\(n = 5/4\)), and AVN (\(n = 6/3\)). Sample size \(n\) is denoted as \(n = l/A\) with \(l = \text{images}\) and \(A = \text{animals}\). The HCN4 mean intensities from these layers were corrected based on the established calibration curve in a similar manner as used to correct depth dependent attenuation.

References

Supplemental Figure I. Preparation for localization and characterization of AVN tissue. (A) Fixed rat heart with right atrial lateral wall and right atrial appendage removed shown from cranial. Arrow indicates location of coronary sinus. (B) Tissue section from region marked in (A). (C) Conventional confocal microscopic image of tissue preparation in (B) labeled for anti-HCN4 (green) and anti-sarcomeric α-actinin (red). HCN4-positive regions were outlined region. Circle in (B,C) marks location of coronary sinus. AM, atrial muscle; AO, aorta; CN, compact node; INE, inferior nodal extension; LA, left atrium, RV, right ventricle; TV, tricuspid valve; VM, ventricular muscle.
Supplemental Figure II. Preparation and imaging of fixed cardiac tissue with conventional confocal microscopy. (A) Sketch of heart with SAN and AVN regions. Tissue preparations were excised from those regions after fixation of the heart. (B) Schematic of the imaging setup. Fluorescently labeled tissue was brought in close proximity to the glass slide for imaging.
Supplemental Figure III. Preparation and imaging of living cardiac tissue with FCM. (A) Setup for FCM imaging of living heart. The 3D manual micromanipulator is used to maneuver the imaging microprobe in close proximity to the epicardial or endocardial surface. (B) Illustration of imaging of 2-layered tissue. The fluorophore diffuses into the tissues after superficial application. The imaged region (*) is beneath a thin epicardial or endocardial tissue layer.
Supplemental Figure IV. Confocal microscopic images of AVN tissue. The tissue was labeled with (A) DAPI, (B) WGA, (C) anti-HCN4, and (D) anti-sarcomeric α-actinin. Irregular shaped, HCN-4 positive myocytes were located beneath the epicardial layer. An overlay of DAPI, anti-HCN4, anti-sarcomeric α-actinin images illustrates the irregular arrangement of myocytes located (E) at a depth of 16.2 µm and (F) beneath the epicardial layer. (G) Three-dimensional reconstruction of the region marked in (F) labeled with DAPI, WGA, and anti-sarcomeric α-actinin. (H) Three-dimensional reconstruction of the region marked in (F) after removal of the endocardial layer reveal the complex arrangement of AVN tissue. Scale bar in (A) applies to (B-F).
Supplemental Figure V. Unprocessed images acquired with conventional confocal microscopy. Cross-sections through unprocessed image stacks corresponding to those presented in (A-D) Figure 2, (E-H) Figure 3, and (I-L) Supplemental Figure 3.
Supplemental Figure VI. Quantitative analysis of HCN4 signal intensity in AWM (n = 7/4), SAN (n = 5/4), and AVN (n = 6/3) tissue. The HCN4 signal in the SAN region was approximately 2.5 times higher in the nodal layer as compared to its subnodal layer. We found that the HCN4 intensity in the nodal layer of the AVN region was 3.1 times higher than its subnodal layer. The HCN4 level in the nodal layers of the SAN and AVN regions were 2.8 and 3.1 times higher respectively as compared to the HCN4 level in the AWM region. *P < 0.005, compared with AWM. †P < 0.005, compared with SAN nodal layer. §P < 0.005, compared with AVN nodal layer. Sample size n is denoted as n = I/A where I = images and A = animals.
Supplemental Figure VII. Histogram of orientations based on (A) 2nd order image moments and (B) Fourier transforms of human AWM and AVN images shown in Figure 5.
**Legends for Movies**

**Movie I.** Real-time live imaging of rat AWM. The AWM tissue was labeled with dextran conjugated Alexa Fluor 488 and imaged using FCM. The dark regions shown are of muscle cells and the bright regions are of the fluorophore. The image sequence was taken at a rate of 20 images/s using a manual micromanipulator to maneuver the imaging microprobe in the region of interest.

**Movie II.** Real-time live imaging of rat SAN. The same imaging protocol used to acquire recordings in Movie I was followed.

**Movie III.** Three-dimensional reconstruction of AWM. The tissue was imaged using conventional confocal microscopy. The 3D section has a field of view of (x) 118.9 µm, (y) 118.9 µm, and depth (z) 26.8 µm. Signals associated with DAPI, WGA, and sarcomeric α-actinin labeling are shown in blue, yellow, and red respectively.

**Movie IV.** Three-dimensional reconstruction of AWM with epicardial layer removed. Signals associated with DAPI, WGA, and sarcomeric α-actinin labeling are shown in blue, yellow, and red respectively.

**Movie V.** Three-dimensional reconstruction of SAN. Reconstruction was performed from image stack acquired using conventional confocal microscopy with a field of view of (x) 118.9 µm, (y) 118.9 µm, and depth (z) 35.0 µm. Signals associated with DAPI, WGA, and sarcomeric α-actinin labeling are shown in blue, yellow, and red respectively.

**Movie VI.** Three-dimensional reconstruction of SAN with endocardial layer removed. Signals associated with DAPI, HCN4, and sarcomeric α-actinin labeling are shown in blue, green, and red respectively.

**Movie VII.** Three-dimensional reconstruction of AVN. Reconstruction was performed from image stack acquired using conventional confocal microscopy with a field of view of (x) 57.9 µm, (y) 57.9 µm, and depth (z) 28.4 µm. Signals associated with DAPI, WGA, and sarcomeric α-actinin labeling are shown in blue, yellow, and red respectively.

**Movie VIII.** Three-dimensional reconstruction of AVN with epicardial layer removed. Signals associated with DAPI, HCN4, and sarcomeric α-actinin labeling are shown in blue, green, and red respectively.