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Human Sinoatrial Node Structure: 3D Microanatomy of Sinoatrial Conduction Pathways

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Abstract

Introduction—Despite a century of extensive study on the human sinoatrial node (SAN), the structure-to-function features of specialized SAN conduction pathways (SACP) are still unknown and debated. We report a new method for direct analysis of the SAN microstructure in optically-mapped human hearts with and without clinical history of SAN dysfunction.

Methods—Two explanted donor human hearts were coronary-perfused and optically-mapped. Structural analyses of histological sections parallel to epicardium (~13-21µm intervals) were integrated with optical maps to create 3D computational reconstructions of the SAN complex. High-resolution fiber fields were obtained using 3D Eigen-analysis of the structure tensor, and used to analyze SACP microstructure with a fiber-tracking approach.

Results—Optical mapping revealed normal SAN activation of the atria through a lateral SACP proximal to the crista terminalis in Heart #1 but persistent SAN exit block in diseased Heart #2. 3D structural analysis displayed a functionally-observed SAN border composed of fibrosis, fat, and/or discontinuous fibers between SAN and atria, which was only crossed by several branching myofiber tracts in SACP regions. Computational 3D fiber-tracking revealed that myofiber tracts of

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Conflicts of interest

There are no conflicts of interest.

SACPs created continuous connections between SAN #1 and atria, but in SAN #2, SACP region myofiber tracts were discontinuous due to fibrosis and fat.

Conclusions—We developed a new integrative functional, structural and computational approach that allowed for the resolution of the specialized 3D microstructure of human SACPs for the first time. Application of this integrated approach will shed new light on the role of the specialized SAN microanatomy in maintaining sinus rhythm.

Keywords

human sinoatrial node; sinoatrial conduction pathway; fibrosis; optical mapping; 3D reconstruction; sinus node dysfunction

1. Introduction

The sinoatrial node (SAN) is the primary pacemaker of the heart and responsible for initiating and regulating cardiac rhythm¹⁻⁹. SAN automaticity and conduction depends on the unique heterogeneous distribution of intracellular ion channels, Ca^{2+} handling proteins and autonomic receptors within the SAN¹⁰⁻¹² as well as the unique structure of the SAN complex¹³. The specialized microanatomy allows the small SAN to pace the large atria efficiently by maintaining a balanced source-sink relationship¹⁴. Multiple factors affecting SAN structure could lead to sinus node dysfunction (SND)¹⁵, when the SAN inadequately paces the atria, which may result in a number of cardiac diseases such as heart failure, atrial fibrillation, malignant ventricular arrhythmias, and eventually cardiac arrest¹⁶⁻²¹. SND is the predominant prognosis for electric pacemaker implantation, which is currently the only available treatment²¹⁻²⁴. Despite over a century of research on the SAN, limited knowledge of the relationship between the human SAN microarchitecture and SAN function remains a critical barrier to properly understanding SND mechanisms and developing new alternatives to implantable pacemaker therapy.

Since the discovery of the SAN by Keith and Flack in 1907, multiple studies have investigated the SAN structure and its role in the formation and regulation of sinus rhythm in human and different animal hearts^{1, 3, 5, 6, 8, 9, 25-28}. Located at the junction of the superior vena cava (SVC) and the right atrium, the human SAN structure consists of a compact mass of specialized cardiomyocytes enmeshed in a dense matrix of collagen, fibroblasts and fatty tissue¹⁵. In general, the macrostructural features of the SAN, such as SAN size, the relationship between increased collagen tissue percent with age^{29, 30}, the defined SAN artery, and the banana-shaped 3D structure of the SAN, are generally accepted and agreed upon^{3, 27-29, 31, 32} (**Figure 1**). However, due to the complexities of this 3D structure, several microstructural features remain disputed and/or undefined.

Among the debates over human SAN microstructure are the contradictory hypotheses of how the SAN is electrically connected to the atria. One hypothesis is that the SAN is electrically insulated from the surrounding atria by a structural border of fibrosis, fat layers and myocyte discontinuity, and that functional and structural connection between the SAN and atria is limited to discrete SAN conduction pathways (SACPs)^{3, 5, 8, 9, 33-35}. An alternative hypothesis is that SAN and atrial cells are extensively connected by diffuse inter-

digitations of the SAN border with the atrial myocardium, and that no discrete pathways exist^{7, 27, 28, 36, 37}. These discrepancies may be explained by methodological limitations of previous SAN studies, such as restricting analyses of SAN structure to 2D instead of utilizing a 3D computational model, insufficient spatial resolution of 3D structural studies, and/or conducting structural studies without functional mapping of SAN conduction.

The importance of the functional-structural SAN to atria connection lies in its fundamental role in the mechanism of atrial activation from SAN pacemaker activity¹³⁻¹⁵ and the maintenance of normal sinus rhythm in the human heart. New methodologies need to be developed to resolve the discrepancy of the SAN-atrial connections. In the present study, we developed an integrated approach including high-resolution optical mapping, serial histological sectioning and computational 3D fiber tracking to provide for the first time a detailed description of the functionally-identified SAN structure and a 3D reconstruction of the specialized SACP microstructure in human hearts with and without SND.

2. Materials and methods

2.1 Optical mapping of coronary-perfused human atrial preparations

Explanted human hearts were obtained from Lifeline of Ohio in accordance with The Ohio State University Institutional Review Board. Patient-specific data can be found in **Table 1**. Explanted human hearts were cardioplegically-arrested and cooled to 4°C in the operating room following cross-clamping of the aorta. Hearts were stored in cold cardioplegic solution (4°C) during transport, dissection and cannulation. Human atrial preparations were isolated as previously described^{9, 38}, coronary-perfused and superfused with 36.5±0.5°C oxygenated Tyrode's solution under constantly maintained pH (7.35±0.05) and pressure (55±5mm Hg)^{9, 39}. Thus, stable heart rhythm, atrial conduction and repolarization were maintained in the entire preparation for 4-8 hours^{9, 38}. The atrial preparations were immobilized with 10µM blebbistatin and stained with voltage sensitive, near-infrared dye di-4-ANBDQBS⁹. All mapped atrial preparations excluded regions of poor coronary perfusion/ischemia.

Atrial preparations were mapped and optical action potentials (OAPs) were recorded using a high-resolution (optical field-of-view 3.3×3.3cm², 330µm resolution) CMOS camera (MiCAM Ultima-L, SciMedia Ltd, CA), which was focused on the epicardium (Epi) (**Figure 2**). OAPs from the SAN and atria were analyzed using a custom Matlab computer program as previously described⁸. As previously described⁹, OAP morphology and reconstruction of activation patterns allowed for the identification of the leading pacemaker, or area of earliest SAN depolarization, as well as areas of earliest atrial activation, or breakthrough sites where SAN activation exited the SAN and activated atrial myocardium through SACP. To determine SAN activation during pacing, SAN action potentials were extracted from total optical signals⁴⁰. The preparations were instrumented with customized 2 bipolar pacing electrodes placed on the right atrial epicardial surface. Electrical activity was continuously recorded from a 2mm bipolar sensing catheter (7Fr, 8mm tip, Biosense Webster, CA) placed on the right atrial epicardial surface, and a far-field pseudo atrial ECG was recorded by two Ag-AgCl plaque electrodes (9-mm diameter).

2.2 Tissue dissection and staining

Two mapped heart preparations were chosen for detailed 3D reconstruction to represent SAN structure in non-SND (SAN #1) and SND (SAN #2) conditions, as SAN #2 had clinically-diagnosed SAN dysfunction as well as an implantable pacemaker and defibrillator. SAN activation maps were projected on the Epi surface of preparations to guide SAN histological dissection (**Figure 2**). SAN pacemaker complex and surrounding atrial myocardium, including crista terminalis (CT), right atrial free wall (RAFW), SVC and interatrial septum (IAS), were formalin-fixed, paraffin-embedded and serial sectioned from Epi to endocardium (Endo) (**Figure 4**). Histological sections were 5µm thick, but to adjust for shrinking due to dehydration and compression of the tissue during processing, these sections more closely represented 7.5µm thick tissue, and this size was used for subsequent analyses. Sections at average intervals of 21µm and 13µm for SAN #1 and SAN #2, respectively, were stained with Masson's trichrome (Sigma Aldrich) and sister sections were immuno-labeled with Connexin43 (Cx43, Sigma Aldrich) and Vimentin (Abcam). Additional perpendicular-cut histological sections of tissue above or below the parallel sectioned SAN region was used to determine whether the complete pacemaker tissue was included in the parallel sections. In Heart #2, the SAN tail continued for ~2mm past the sectioned tissue, but this piece of the SAN was omitted from the SAN reconstruction and size measurements. Histology sections were imaged with a 20x digital slide scanner (0.5×0.5µm² X-Y resolution, Aperio ScanScope XT, Leica). High-resolution images of immuno-labeled slides were captured by an Olympus FV1000 Filter confocal (**Figure 7D**), and whole slide images were imaged by a Typhoon 9410 imager (GE Healthcare) (**Figure 4B**). In each 2D histology section, myocardial tissue was delineated from fat, blood vessels and fibrosis for subsequent 3D analysis (**Figure 5**). Furthermore, histology and immunostaining images were used to identify and delineate the SAN pacemaker tissue from the surrounding right atria based on positive (atrial myocardium) and negative (SAN) Cx43 expression, distinct cell morphology, cell diameter and percent tissue fibrosis as previously described by our group and other human SAN studies (**Figures 7-9**)^{3, 7, 9, 28, 31, 40, 41}. SACP structure was identified as tracts of myofibers with transitional cells in the SAN border that merge with atrial myobundles and had distinctly different fiber orientations compared to the main SAN and atrial fiber orientations.

2.3 3D computer reconstruction of SAN complex

High-resolution histology images of human right atria and the SAN pacemaker complex were sequentially stacked and artificial deformation across the **z**-axis was minimized using a novel 3D image alignment approach by applying a global elastic constraint via an ImageJ Plugin (TrakEM2) (**Figure 4D**)⁴². The package automatically aligns a series of registered RGB images by employing a suite of tools including an affine transformation and least squares (linear) to find effective feature correspondences among the neighboring original images⁴² in order to accurately align and combine 2D images for subsequent 3D reconstruction and analysis (**Figure 4E**). Then a semi-manual segmentation was performed on the 2D stacks of Masson's trichrome images with 0.5×0.5µm² in-plane and 21µm (Heart #1) or 13µm (Heart #2) across-plane resolution to separate the SAN from neighboring atrial tissue based on functional and structural data (**Figure 4A-C**). The resulting 3D surface was

further incorporated and smoothed through commercial software Amira (FEI, Oregon, USA) (**Figure 4E**). High-resolution fiber fields were obtained using Eigen-analysis of the 3D structure tensor. The 4D myofiber field was further computed using a state-of-art fiber tracking approach by seeding uniformly throughout a region of interest via a custom-developed Matlab package (MathWorks Inc., Natick, Massachusetts, USA) and visualized by commercial software Amira^{43, 44}. The fiber tracking approach utilized a linear line-propagation algorithm i.e., a single fiber line propagated by connecting pixels from a seed point by following the local vector orientation until it reaches beyond tissue region or predefined angle change⁴⁴. This approach was utilized to visualize not only SAN and atrial myofibers (**Figure 5B**), but also fibrosis (**Figure 5C**) and fat (**Figure 5D**). Defined SACP regions were further segmented by tracking individual myofiber tracts appearing to make continuous physical connections between the SAN and atria in 2D histology sections (**Figure 9**). 3D reconstruction and visualization of the SACP were based on individually segmented structure masks of each SACP and a 3D region growing approach⁴³. Discontinuous myofiber tracts in SACP were defined as having $>50\mu\text{m}$ 3D gaps between myofibers. All image processing and structure visualization was run on an IBM3850 (32 dual thread Intel chips, 256 GB shared memory, Linux operating system) at Auckland Bioengineering Institute, University of Auckland.

2.4 Statistical Analysis

Data are presented as Mean $\pm$ SD. Analysis was done in IBM SPSS Statistics using repeat measurements ANOVA. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Functional identification of SAN pacemaker complex

Near infrared optical mapping revealed that during baseline conditions, SAN #1 had stable sinus rhythm (61 BPM) with the leading pacemaker in the central region of the SAN tail (**Figure 2A**). SAN activation preferentially traveled superiorly from the leading pacemaker at 11.8 ± 3.1 cm/s and slowed to 6.5 ± 1.6 cm/s before exiting SAN through lateral SACP to excite the atria in the superior CT (atrial breakthrough in **Figure 2A**). While additional septal and inferior lateral exit and entrance SACP were observed in functional studies of SAN #1, the lateral SACP and corresponding atrial breakthrough was the primary observation during normal sinus rhythm, which is consistent with previous clinical and experimental observations^{8, 9, 40, 45}. For this reason, subsequent detailed structural analyses were focused on this particular SACP.

In diseased SAN #2, slow intrinsic automaticity (~ 48 BPM) was unmasked during baseline conditions, but permanent SAN exit block prevented atrial activation without pacing (**Figure 2B** and **Figure 3**). Intrinsic SAN conduction was considerably slower than SAN #1, as activation traveled inferiorly at 3.6 ± 1.1 cm/s from the leading pacemaker in the SAN head, and conduction was greatly slowed at the SAN center (0.6 ± 0.3 cm/s), which resulted in 2:1 intranodal block between the SAN head/center and tail. **Figure 3** shows that during pacing (Beats #1-3) and atrial beats (Beat #4), the atria paced the SAN from a left superior pathway, an observation consistent with previous studies of the canine SAN, which is

functionally and structurally similar to the human SAN⁴⁶⁻⁴⁸. However, this entrance pathway never functioned as an exit pathway during our experiment. Moreover, the region of intranodal block was correlated with an underlying structure consisting of local fibrosis strands and a lateral bifurcation of the SAN artery (**Figure 6**). This observation of functional block due to strands of intranodal fibrosis has been shown in previous studies of canine models with SAN structural remodeling^{47, 48}.

3.2 Structural identification of SAN pacemaker complex

A 3D computer reconstruction of SAN #1 (**Figure 4**) was created from 300 total tissue sections representing 2.25mm tissue thickness, of which 106 were stained with Masson's trichrome, allowing an overall Epi-Endo resolution of 21µm. **Figure 5A** shows the 3D reconstruction of all SAN complex tissues while subsequent panels (**Figure 5B-D**) show individual reconstructions of only myofibers, fibrosis, and fat, representing our ability to isolate the different tissue compositions of the SAN and surrounding atria. A 3D computer reconstruction of SAN #2 was created from 90 Masson's trichrome stained sections from a total of 160 tissue sections, providing an overall Epi-Endo resolution of 13µm (**Table 2**). Each SAN was centered on the nodal artery, and SAN sizes were within the range of previous human SAN size investigations (**Table 2**). SAN myofibers in Heart #1 and Heart #2 were primarily oriented parallel with the CT (**Figure 5-6**). Average fibrosis density was $54.0 \pm 2.0\%$ and $50.1 \pm 2.8\%$ in SAN #1 and the dysfunctional SAN #2, respectively. Apart from SACP, a structural border of fibrosis, fatty tissue, and/or discontinuous fibers was observed in both SAN. The septal side of the SAN border was comprised of 1-2mm of fatty tissue, while the lateral boundary proximal to the CT was not as defined (**Figure 4C** and **Figure 5D**). In this lateral region, the boundary demarcating the SAN from the atria was less distinct and the separation decreased to less than 100µm in multiple areas. However, high-resolution images as well as a 3D fiber tracking approach showed that other than SACP, no physical myocyte-to-myocyte connection between SAN and atria existed (**Figures 6** and **8**). Importantly, the functional and structural borders of both SAN#1 and SAN#2 are highly correlated (**Figures 4** and **6**).

3.3 Microstructure of SACP

In SAN #1, integration of SAN function and structure allowed for the identification of the SACP region corresponding to the lateral SACP from functional experiments (**Figure 5A**). Out of the 106 histology slides, 20 appeared to contain continuous SACP myofibers crossing the SAN border, representing 280µm thickness in total. Within this region, myofibers with similar orientation coalescing into several tracts (2-3mm long) were observed to make continuous physical connections between the SAN and atria (**Figure 9**). Analysis of these slides showed a smooth transition along these tracts of increasing cell diameter from SAN to atria in selected SACP regions, but an abrupt increase in non-SACP regions (**Figures 7-8**). Furthermore, Cx43 immunolabeling showed a progressive transition from Cx43 negative SAN pacemaker cells to intermediate expression in SACP region and distinct Cx43 gap junctions in the atria (**Figures 7B** and **7D**).

In SAN #2, no functional SACP was identified, but a $\sim 5 \times 5 \text{ mm}^2$ area in the lateral boundary between the SAN and atria looking to contain a remnant of the SACP was analyzed (**Figure**

6B) with similar observations of myocardial cell diameter (**Figure 8**). Importantly, of the 34 histology sections containing this dysfunctional SACP region, no 2D slide was observed to contain a continuous myocyte-to-myocyte connection between the SAN and atria due to layers of fibrosis and fat (**Figure 9**). An unobstructed myofiber tract (blue) was observed in this region; however, it did not have direct connection to the CT atrial myocardium at the distal end in comparison to the SACP myofiber tracts in SAN#1.

3.4 3D reconstructions of SACP

Based on these 2D observations, the lateral SACP of SAN #1 and the similar region in SAN #2 were reconstructed to analyze the 3D aspect of the SACP. In SAN #1, a $2.7 \times 3.5 \times 0.28 \text{ mm}^3$ region containing transitional cells represented the SACP region (**Figure 10A**). Four discrete and continuous tracts of myofibers (**Figure 9A and Figure 10A**) formed an SACP that began in the SAN with 1.75mm width at the proximal end and fanned out to 2.2mm at the distal end in the atria. 3D fiber orientation analysis of myocytes with fiber tracking allowed for the visualization and confirmation of these continuous SACP myofiber tracts connecting the SAN to the atria. 3D structural analysis of SAN #2 provided a separate conclusion. In the selected $4.9 \times 5.4 \times 0.39 \text{ mm}^3$ SACP region, 3D fiber tracking analysis with the computational model did not reveal discrete continuous physical connections between the SAN and atria through these myofiber tracts due to fibrosis and fat infiltration (**Figure 10B**).

4. Discussion

4.1 3D histological reconstructions and structural models of the human SAN

Over a century ago, the anatomic structure of the SAN was discovered by Keith and Flack¹. Since this discovery, animal model and human studies have investigated how cardiac rhythm is initiated and regulated by the SAN^{1-7, 9}. One of the critical components of efficient SAN function as the leading pacemaker of the human heart is the specialized SAN structure which allows small SAN pacemaker clusters to pace the large atrial myocardium. Histological examinations of 2D human SAN sections have provided a greater knowledge regarding this specialized structure^{1, 3, 4, 9, 27-31, 49, 50}, but understanding the 3D microstructure of the pacemaker complex is necessary to properly describe SAN pacemaker and conduction functions. In the present methodological study, we developed an integrative approach that allowed for the resolution of the specialized 3D microstructure of human SAN responsible for conduction between SAN pacemaker clusters and surrounding the atrial myocardium – SAN conduction pathways (SACP). This integrated approach will allow for future study of the role of the specialized SAN microanatomy in maintaining sinus rhythm in health and disease.

Importantly, the present analysis of two human SAN structures is in agreement with the gross observations of human SAN size and shape by previous 3D histological SAN reconstruction studies (**Table 2**). In 1961, Thomas James provided the first estimation and figure of 3D human SAN size through the histologic study of 79 human hearts³. This model noted the close relationship of the SAN to the nodal artery, and included similar observations by Lev²⁹ such as the longitudinal orientation of SAN myocytes, the smaller

diameter of SAN cells compared to atrial cells, the higher amount of collagen in the SAN compared to the surrounding atria, and the increase of collagen content in the SAN with increasing age.

In 1967, Truex et al. provided the first 3D reconstruction of the human SAN by reconstructing 503, 10 μ m thick sections cut perpendicular to Epi³¹. Every 5th section of the 503 were stained for histology (50 μ m step between tissues), projected and traced onto colored dental wax plates 1mm in thickness. The model provided a SAN with a curved orientation located at the junction of the SVC and right atrium³¹.

Alings et al. (1995) later studied human SAN structure by sectioning the SAN perpendicular to Epi at 5 μ m and stained every 20th section for histological analyses (100 μ m step between tissue) of SAN size and collagen content³⁰. In 2005, Sanchez-Quintana et al. studied 41 human hearts by sectioning the SAN perpendicular to Epi at 10 μ m thickness, using every 20th section (200 μ m step between tissues) for histological analyses to examine the precise location of both the SAN and nodal artery in relation to the Epi and Endo. Later, Fedorov et al. (2010) provided a 3D human SAN model from optical mapping and histological study of 23 out of 300 5 μ m thick sections (400 μ m step between tissues). Importantly, this 3D SAN model was based on both functional and structural analysis of the same SAN, and was the first 3D SAN model to include functional SACPs⁹. In a separate study, Chandler et al. (2011) used histology staining from 58 sections taken at 500 μ m intervals perpendicular to Epi to provide a 3D model that included SAN myofiber orientation from diffusion tensor MRI (250 μ m $\times$ 250 μ m $\times$ 500 μ m resolution) as well as a description of a novel paranodal area²⁸. All of these 3D models provide SAN dimensions of varied but relatively similar size (**Table 2**); importantly, each acknowledges the crescent shape of the SAN, the tapering ends at the head and tail, and the common location of the SAN at the junction of the SVC and right atrium.

In the present study, high-resolution functional (330 $\times$ 330 μ m) and structural (0.5 $\times$ 0.5 μ m X-Y resolution, 13-21 μ m Epi to Endo step between tissue sections) mapping demonstrated the highly complex 3D microstructure of the functional and structural boundaries between the SAN and surrounding atrial myocardium in functional and diseased human SAN. Importantly, past histological examinations of serial sections of SAN tissue had large steps between subsequent slides that limited the studies' resolution and therefore, these studies may not have resolved and/or missed the SACP structure between slides^{9, 27, 28, 30, 31} (**Table 2**). Thus, this is the first integrative methodological study to analyze not only major human SAN components such as SAN fiber orientation, myofiber cellular size, fibrosis density and artery location as was done in previous classical studies (**Table 2**), but also functional and 3D structural analysis of the SAN and SACP microanatomy.

4.2 Functional-structural connection between the SAN and atria

Contradictory hypotheses remain regarding how the SAN is functionally and structurally connected to the atria. In his 1961 paper³, James first proposed that discrete Purkinje-like fiber tracts carry the SAN impulse to the atria. Although specific cell markers of Purkinje fibers were never found in the atria⁵¹, this idea of a discrete functional and structural connection between the otherwise functionally and structurally isolated SAN to the atria has

been proposed by multiple structural^{3, 8, 9, 31, 34, 52, 53} and functional^{4, 5, 8, 9, 34, 35} studies, including the present one. Previous animal⁸ and human⁹ studies have observed functional barriers of the SAN and proposed that a structural barrier separates the SAN and atria, other than at SACPs. However, the complete 3D structure of such a barrier was not examined or reconstructed. In both human hearts analyzed in the present study, a functionally-observed block zone correlated to a structural border that consisted of fibrosis, fatty tissue, and/or discontinuous myofibers in varying degrees depending on the border region (**Figure 4-6**).

Consequently, an alternative hypothesis presented by other studies suggests that the SAN and atria are connected extensively by diffuse inter-digitations along the SAN border with the atrial myocardium^{7, 27, 28, 54}. This hypothesis states that although the fibrous tissue provides a compact structure for the SAN, it does not surround and isolate the SAN from the atria, and that rather than discrete pathways between the two structures, extensions of nodal cells extensively merge the SAN periphery with the atria^{7, 27, 28, 54}. Direct evidence of the presence or absence of SACPs is critical for advancing our field in the understanding of SAN function in normal and disease conditions and will allow for the development of new diagnostic and treatment strategies.

The specific structural features of the human SAN are extremely important for its successful function as the leading pacemaker of the heart^{8, 13}. As normal SAN automaticity is dependent on a relatively depolarized state (about -60mV), the electrotonic influence from the surrounding atrial myocardium at a resting potential of about -85mV could hyperpolarize SAN pacemaker cells and inhibit automaticity^{6, 14, 55, 55}. However, this is prevented by a structural and functional insulation in the form of fibrosis and fat surrounding the SAN complex¹³. Other groups have proposed that the SAN collagenous sheath may also provide protection from overstretching caused by mechanical pressures of the atrial myocardium³⁰. Moreover, the specialized SAN structure and electrical coupling with the atria plays a crucial role in the synchronization of pacemaker clusters inside of the SAN (mutual entrainment), allowing for stability of SR, a relationship shown by earlier microelectrode and computer simulation studies conducted by the Jalife group⁵⁶.

Another important consideration in the normal function of the SAN is the source-sink relationship between the SAN leading pacemaker and atria. SAN pacemaker cell clusters are small and rely on a relatively weak action potential generated by the slow $I_{\text{Ca,L}}$ upstroke. Consequently, this means that the SAN is a relatively weak source of current, while the atrial cells, being much larger with a more negative resting potential, pose a very large sink of current before reaching threshold themselves. This apparent source-sink mismatch may be overcome by the specialized branching myofiber structure of SACPs, as was originally suggested for AV nodal conduction by the Rudy group⁵⁷. Previously, we hypothesized^{8, 9} that slow conduction through branching myofiber tracts of SACPs with weak Cx43 expression gives the SAN enough time to build up sufficient charge to excite the large atria in both canine and human SAN, as shown in **Figure 6A**. The Joyner et al. study¹⁴, one of the first computer simulations of SAN activation, revealed that weak electrical coupling between cells within the SAN border may be an essential feature of normal electrical communication between the SAN and the atrium. However, disease-induced structural and molecular remodeling may lead to disruption of the continuity of electrically coupled

myocytes, thereby disrupting SAN automaticity and slowing conduction, particularly in SACP, and ultimately leading to exit block and SND (**Figure 6B**).

4.3 Innovative methodology allows for analysis of SACP microstructure

The contradictory hypotheses of the SAN-atrial connection may stem from a lack of resolution of both functional and structural mapping of the SAN as well as human SAN studies that only analyzed SAN structure from 2D sections. Our results on the direct evidence of discrete SACP in the human SAN were made possible by the integration of multiple methodological and technologic advances listed below.

4.3.1 Integration of high-resolution functional and structural analysis—The integration of functional and structural analyses allows for direct functional evidence and support of structural observations. In the present study, functional mapping of human SAN with 330µm resolution located functional lines of block surrounding the SAN except for the SACP (**Figure 2**). As shown in previous optical mapping experiments of the canine SAN, without intramural optical mapping, the SAN activation pattern in the thick atrial wall could not be resolved^{8, 13, 40, 46}. Instead, only extensive areas of earliest atrial excitation (breakthroughs) across the CT would have been observed, as shown in multi-electrode mapping experimental³⁴ and clinical^{4, 58} studies. Only the integration of functional and structural mapping allows for SACP structures to be accurately defined and analyzed (**Figures 6-8**).

4.3.2 3D Computational approach—The human SAN is 1-2mm thick and extends through hundreds of serial-sectioned histology slides. As such, analysis of a limited number of 2D slides is insufficient to provide accurate results of the SAN's intricate 3D structure. To successfully study the human SAN structure, it is necessary to utilize a 3D approach, which was accomplished in the current study by a computational model. The spatial resolution of our 3D computational model was optimized by limiting the step distance between 2D serial sections to 13-21µm. The high density of serial sections, and therefore spatial resolution, of our 3D computational model allowed for the proper identification and subsequent structural analysis of SACP #1, which measured 280µm thick at the SAN border. If sections at more than 300µm steps had been used for our model, SACP myofiber tracts could have been missed completely.

4.3.3 Parallel vs transverse SAN sectioning—In addition to including a dense number of 2D sections, spatial resolution of the 3D computational model of SAN structure was enhanced by sectioning the SAN parallel to Epi rather than perpendicular to Epi. Sectioning parallel to Epi optimizes fiber tracking analyses because SAN myofibers are primarily oriented parallel to the epicardium²⁸ (**Figure 5**). Furthermore, multi-branching myofiber tracts composing the lateral SACP are also primarily oriented parallel to Epi and occupy a roughly 3×3mm² area in this plane (XY axes of **Figure 10**), but are only 0.28-0.39mm deep in the transverse plane (Z axis of **Figure 10**). Sectioning parallel to the Epi allows for the greatest amount of SACP surface area to be included in the 3D fiber-tracking data analysis, which precisely traced individual SACP myofiber tracts as they curved in the X, Y and Z directions.

4.3.4 Novel fiber-tracking approach—The 3D structure-tensor approach⁵⁹ utilized in this study was imperative in distinguishing SACP structural differences that allowed for proper SAN conduction in SAN #1 but caused conduction abnormalities manifesting as exit block in SAN #2 (**Figure 2**). 2D histological analysis of SACP #1 and SACP #2 (**Figure 7-8**) showed no obvious differences as both had similar increases in cell diameter and myofiber density from SAN to SACP to atria, as well as similar individual SACP myofiber tracts and overall SACP size. However, 3D analysis with our novel fiber-tracking approach allowed for the resolution of continuous myofibers in functional SACP #1 but discontinuous myofibers in non-functional SACP #2 (**Figure 9-10**). From the results of the current human study as well as previous studies^{40, 60-66} linking structural remodeling to SND, we suggest that in dysfunctional SACP, structural remodeling caused by upregulated fibrosis and fat infiltration disrupts SACP myofiber tract continuity and may lead to exit block and SND.

4.4 Functional implications and future studies of SACP

Previous optical mapping studies of explanted human⁹ and canine^{8, 40, 46, 67} SAN revealed that the majority of atrial breakthroughs seen in earlier electrode mapping experiments^{4, 45} represent exit points of SAN electrical wave propagation through SACP. The presence of finite pathways conducting electrical impulses from the SAN to the atria shown here is consistent with clinical mapping studies that show earliest atrial activation sites (breakthroughs) that remain stable beat-to-beat under the same physiologic conditions^{4, 45}. We suggest that SACP microstructure plays a crucial role in human SAN conduction and potentially contributes to normal SAN pacemaking by providing beneficial source-sink relationships. These branching myofiber tracts with progressive increases in cell diameter and Cx43 expression may improve conduction safety by delivering electrical impulses from the SAN to several intramural layers of the atria (e.g. CT) simultaneously. This may explain previous observations of a large area of earliest atrial activation⁴⁵ that may accelerate global atrial activation and synchronize contraction. Conversely, the microanatomy of SACP tracts (transitional cell diameter and Cx43 expression) may allow for unidirectional conduction properties that protect the SAN from overdrive suppression during pacing and/or atrial fibrillation by creating SAN entrance block, as shown in canine studies^{39, 40, 67}. Computer simulations of the anatomically realistic human SAN structure are required to test the functional benefits and implications of these specialized branching myofiber tracts comprising SACP.

Future studies on the development of a biological pacemaker⁶⁸⁻⁷⁰ for SND treatment should consider not only the creation of active pacemaker cells but also the specialized microstructure (SACP vs. conduction border) that successfully delivers the impulse from these cells to the surrounding tissue. This study may provide the basis for future clinical studies of SAN arrhythmia and SND treatments where novel imaging techniques, such as contrast-enhanced MRI or microCT^{63, 71-74} are employed in conjunction with catheter electrode mapping to visualize SACP. The SACP may be a potential target for ablation of SAN arrhythmias or local gene/cell treatments aimed at restoring conduction impairments and normal SAN rhythm in diseased hearts.

4.5 Study limitations

Future studies are necessary to validate the current findings in a greater amount of hearts as well as in hearts with a wider variety of diseases. Moreover, additional studies are needed to determine the structural, molecular and functional differences between SACP within the same heart that govern which SACP is the preferential pathway. The present study reconstructed the lateral SACP of two human SANs due to the enormous effort required for 3D histological image alignment, reconstruction and analysis with the high 3D spatial resolution. Despite our efforts to minimize steps between subsequent tissue sections, not all tissue sections were utilized for the 3D reconstruction and we had gaps between 13-21µm. However, due to the relatively small size of these gaps, it is unlikely that any major structural features of SACP myofiber tracts were missed.

5. Conclusion

We developed a new integrated approach that allowed us to provide the first 3D structural delineation of human SACP connecting the human SAN to the atria. We suggest that SACP are a necessary structural component of proper SAN function and a major contributor for source-sink relationship. Structural remodeling of SACP due to aging and different cardiac diseases may lead to discontinuous myofiber tracts in SACP regions and ultimately exit block and SND. Future application of this approach will show if structurally impaired SACP may represent new targets for SND treatments.

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Abbreviations

BPM	beats per minute
CT	crista terminalis
Cx43	connexin43
Endo	endocardium
Epi	epicardium
IAS	interatrial septum
MRI	magnetic resonance imaging
OAP	optical action potential
RAA	right atrial appendages
RAFW	right atrial free wall

SACP	sinoatrial conduction pathway
SACT	sinoatrial conduction time
SAN	sinoatrial node
SND	sinus node dysfunction
SVC	superior vena cava

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A. James, 1961 **B. Truex et al., 1967** **C. Fedorov et al., 2010** **D. Chandler et al., 2011** **Figure 1. 3D reconstructions of the human SAN**

A. The 1961 illustration of the SAN proximal to the superior vena cava (SVC) with proposed Purkinje fibers connecting it to the surrounding atria was the first study to propose the size and shape of the 3D human SAN. *Adapted from James, 1961, Anat Rec.* **B.** A 1967 wax model of the human SAN reconstructed from serial histological sections was the first 3D human SAN reconstruction. *Adapted from Truex, et al. 1967, Anat Rec.* **C.** A 2010 3D model was the first computational model of the SAN created from histological and functional studies and included proposed sinoatrial conduction pathways (SACPs). *Adapted from Fedorov, et al. 2010, JACC.* **D.** A 2011 histologically validated 3D computational model of the SAN included for the first time SAN fiber orientation and a paranodal region. *Adapted from Chandler, et al. 2011, Anat Rec.* BB-Bachmann's bundle; CT- crista terminalis; Endo- endocardium; Epi- epicardium; IAS- interatrial septum; IVC- inferior vena cava; SACP- sinoatrial conduction pathway; SVC- superior vena cava.

Figure 2. Optical mapping of the two SAN complexes

A. Optical mapping during baseline conditions of the non-diseased SAN #1 shows optical action potentials (OAPs) from the leading pacemaker at point 1 and the point of earliest atrial activation at point 2. White arrow indicates the path of conduction from the SAN to the atria and star indicates atrial breakthrough point. **B.** Optical mapping of dysfunctional SAN #2 shows permanent SAN exit block during baseline conditions. OAPs from the head (1) and tail (2) illustrate that conduction from the leading pacemaker in the head of the SAN only reached the tail of the SAN every other beat due to intranodal block, seen by beat 1 and beat 2. White arrow indicates intranodal conduction. Abbreviations as in **Figure 1**; Atrial EG- atrial electrogram; OAP- optical action potential; RAA- right atrial appendage; RAFAW- right atrial free wall; SACT- sinoatrial conduction time; SCL- sinus cycle length.

SAN #2 Entrance Conduction and Exit Block

Figure 3. Diseased SAN #2 conduction during atrial pacing and intrinsic SAN rhythm

Schematic of SAN #2 conduction during atrial pacing and post-pacing SAN pacemakers recovery during adenosine 10 μ M perfusion. Colors of asterisks in the schematic correspond to the SAN OAPs extracted from that location. The atrial paced (Beats #1-3) or ectopic beat (Beat #4) entered the SAN head from a left superior entrance pathway. 2:1 intranodal block (gray bar) between the SAN head and tail was observed. During post pacing recovery, this intranodal block also decoupled SAN compartments, allowing for spontaneous depolarization from two leading pacemaker sites in the SAN head and tail (Beats #5 and #6). Despite this intrinsic SAN rhythm, persistent SAN exit block was observed. Abbreviations as in **Figure 2**.

Figure 4. 3D reconstruction of Human SAN #1

A. Optical mapping was used to locate the functional borders of the SAN. Green star indicates atrial breakthrough point. **B.** Immunostaining for Cx43 was used to define SAN borders based on Cx43 negativity in the SAN and high Cx43 expression in atrial myocardium. **C.** Masson's trichrome staining was used to further characterize the SAN as a region of compact fibrosis (blue). **D.** 106 serial histology sections with identified SAN tissue, representing the transmurial thickness of the SAN, were stacked within the computer program. **E.** Computational 3D human SAN from the histological slides visualizes nodal (blue) and arterial (red) tissue. Abbreviations as in **Figure 2**.

Figure 5. 3D myofiber, fibrotic tissue and fat composition in and around human SAN #1
A. 3D computational analysis by a fiber tracking approach with seeding in different textures displays microstructure of all tissue types including myofibers, fibrosis, and fat in the SAN complex (red) and surrounding atrial tissue (green). **B.** Computational model showing only the myofibers of the SAN complex and surrounding atrial tissue. **C.** Computational model showing only the fibrotic fibers of the SAN complex and surrounding atrial tissue. **D.** Computational model showing only the fat tissue of the SAN complex and surrounding atrial tissue. Abbreviations as in **Figure 2**.

A. Functional SAN #1 and SACP Located Functionally and Histologically

B. Dysfunctional SAN #2 and SACP Region Located Histologically

Figure 6. Functional and structural identification of the SACP regions

A. Human SAN #1, from left to right: 3D reconstruction of SAN (blue) and main SAN arteries (red). Optical mapping (**Figure 2A**) revealed conduction within the SAN complex traveled from the leading pacemaker to the lateral border of the SAN. White arrows indicate the path of conduction and the conduction velocity (cm/s) at that point is labeled. White oval represents leading pacemaker site. Green star indicates atrial breakthrough point. Histology staining of the entire SAN complex 2.3mm from Epi. A magnified section of the histology slide shows the boxed off SACP region with myofibers branching off from the compact nodal tissue. **B.** Human SAN #2, from left to right: 3D reconstruction of SAN (blue) and main SAN arteries (red). Optical mapping (**Figure 2B**) revealed conduction began in the head of the SAN and traveled inferiorly to the tail but failed to exit the SAN and excite the atria in Beat 1. In Beat 2, conduction beginning in the SAN head failed to excite the SAN tail due to intranodal block. White arrows indicate the path of conduction and the conduction velocity at that point is labeled. Histology staining of the SAN complex 1.68mm from Epi; perpendicular-cut section of SAN tail shows ~2mm inferior continuation of SAN. A magnified section of the histology slide shows a section boxed off of the lateral SAN where myofibers appear to transition into the nodal tissue; however, the region is extensively infiltrated by fibrotic tissue. Region of intranodal conduction block coincided with strands of intranodal fibrosis and the SAN artery. Abbreviations as in **Figure 2**.

Figure 7. Microanatomy of the functional SACP tracts in SAN #1

A. Masson's Trichrome histological staining with lateral SACP outlined. **B.** A sister section to the one shown in panel **A** is immunostained for Cx43 (green) and Vimentin (red) and compiled from a mosaic of high-resolution confocal images. **C.** High resolution of histological regions shows the myofiber morphology of SAN, proximal SACP, and atrial tissue. **D.** High resolution of immunostained regions shows the Cx43 expression and fibrotic content of the SAN, proximal SACP, and atrial tissue. Abbreviations as in **Figure 2**. Dist-distal, Prox-proximal.

Figure 8. Cell diameter across the SAN borders and SACP

A. Histology staining of human SAN #1 and surrounding atrial tissue. The SACP and the SAN border superior to the SACP are boxed and subdivided into SAN (Region 1), proximal transition (Region 2), distal transition (Region 3), and atria (Region 4) for analysis. **B.** Histology staining of human SAN #2 and surrounding atrial tissue. Regions across the SAN border are selected in accordance with panel **A**. **C.** Graph depicting average myocyte diameter (manually measured, $n=12$ for each region) in regions listed above for SACP tracts and non-SACP borders in human SAN #1 show cell diameter transitions smoothly from Region 1 to Region 4 only in the SACP. **D.** Graph depicting average myocyte diameter (manually measured, $n=12$ for each region) in regions listed above for SACP tracts and non-SACP borders in human SAN #2. Abbreviations as in **Figure 2**.

Figure 9. SACP myofiber tract segmentations

Multiple histology sections of human SAN #1 (**A**) and SAN #2 (**B**) showing individual SACP myofiber tract 2D segmentation. 2D histological outline of individual SACP myofiber tracts used for subsequent 3D computational analysis of the entire SACP region. SACP and their designated colors correspond to the 3D SACP myofiber tracts in **Figure 10**. The SAN-atrial border is represented by a black dashed line. Abbreviations as in **Figure 2**.

Figure 10. Computational 3D reconstructions of functional and dysfunctional SACP

A. Human SAN #1, from left to right: 3D reconstruction of serial histological sections found to contain SACP transitional tissue. Delineated SACP myofiber tracts colored for myofiber angle show a smooth transition from the vertical SAN fibers out towards the atria. A computer model of a functional SACP containing four continuous myofiber tracts bridging the gap between the SAN and atria. **B.** Human SAN #2, from left to right: 3D reconstruction of transmural histological sections found to contain SACP transitional tissue. Delineated SACP myofiber tracts colored for myofiber angle shows that myofiber tracts composing the SACP were discontinuous due to $>50\mu\text{m}$ gaps composed of fibrosis and fat. A computer model of a dysfunctional SACP containing four discontinuous myofiber tracts between the SAN and atria. Abbreviations as in **Figure 2**.

Table 1

Human heart information

Heart #	Heart ID	Sex	Age	Cardiac Disease (Cause of Death)	Implantable Devices
1	474083	F	41	HTN, Trivial MR (CVA, SAH)	None
2	618200	F	Age41 58	AF, MI, HTN, CAD, COPD (Cranial Trauma)	Pacemaker/ Defibrillator

Abbreviations: AF- atrial fibrillation; BPM- beats per minute; CAD- coronary artery disease; COPD- chronic obstructive pulmonary disease; CVA- cerebral vascular accident; HTN- hypertension; MI- myocardial infarction; MR- mitral valve regurgitation; SAH- subarachnoid hemorrhage.

Table 2

Studies of SAN structure dimensions

Study	Length (mm)	Width (mm)	Depth (mm)	Step Size (μm)	Sectioning
James, 1961 (n=79)	15	5	1.5	NA	Perpendicular to Epi
Truex et al., 1967 (n=5)	7.3	NA	1.6	50	Perpendicular to Epi
Alings et al., 1995 (n=32)	NA	4.8	1.2	100	Perpendicular to Epi
Sanchez-Quintana et al., 2005 (n=47)	13.5	5.3	1.5	200	Perpendicular to Epi
Chandler et al., 2011 (n=1)	29.5	6.4	1.8	500	Perpendicular to Epi
Fedorov et al., 2010 (n=4)	14.3	6.7	1	480	Perpendicular to Epi
Current Study: SAN #1 SAN #2	12.0	3.3	2.2	21	Parallel to Epi
	17.0	3.5	1.2	13	Parallel to Epi