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Whole-Mount Immunofluorescence Staining, Confocal Imaging and 3D Reconstruction of the Sinoatrial and Atrioventricular Node in the Mouse

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TITLE:

Whole-Mount Immunofluorescence Staining, Confocal Imaging and 3D Reconstruction of the Sinoatrial and Atrioventricular Node in the Mouse

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KEYWORDS:

Sinoatrial node, Atrioventricular node, Cardiology, electrophysiology, cardiac conduction system, Microdissection, Immunofluorescence, Confocal microscopy

SUMMARY:

We provide a step-by-step protocol for whole-mount immunofluorescence staining of the sinoatrial node (SAN) and atrioventricular node (AVN) in murine hearts.

ABSTRACT:

The electrical signal physiologically generated by pacemaker cells in the sinoatrial node (SAN) is conducted through the conduction system, which includes the atrioventricular node (AVN), to allow excitation and contraction of the whole heart. Any dysfunction of either SAN or AVN results in arrhythmias, indicating their fundamental role in electrophysiology and arrhythmogenesis. Mouse models are widely used in arrhythmia research, but the specific investigation of SAN and AVN remains challenging.

The SAN is located at the junction of the crista terminalis with the superior vena cava and AVN is located at the apex of the triangle of Koch, formed by the orifice of the coronary sinus, the

tricuspid annulus, and the tendon of Todaro. However, due to the small size, visualization by conventional histology remains challenging and it does not allow the study of SAN and AVN within their 3D environment.

Here we describe a whole-mount immunofluorescence approach that allows the local visualization of labelled mouse SAN and AVN. Whole-mount immunofluorescence staining is intended for smaller sections of tissue without the need for manual sectioning. To this purpose, the mouse heart is dissected, with unwanted tissue removed, followed by fixation, permeabilization and blocking. Cells of the conduction system within SAN and AVN are then stained with an anti-HCN4 antibody. Confocal laser scanning microscopy and image processing allow differentiation between nodal cells and working cardiomyocytes, and to clearly localize SAN and AVN. Furthermore, additional antibodies can be combined to label other cell types as well, such as nerve fibers.

Compared to conventional immunohistology, whole-mount immunofluorescence staining preserves the anatomical integrity of the cardiac conduction system, thus allowing the investigation of AVN; especially so into their anatomy and interactions with the surrounding working myocardium and non-myocyte cells.

INTRODUCTION:

Arrhythmias are common diseases affecting millions of people, and are the cause of significant morbidity and mortality worldwide. Despite enormous advances in treatment and prevention, such as the development of cardiac pacemakers, treatment of arrhythmias remains challenging, primarily due to the very limited knowledge regarding underlying disease mechanisms[1-3]. A better understanding of both the normal electrophysiology and the pathophysiology of arrhythmias may help to develop novel, innovative and causal treatment strategies in the future. Additionally, to comprehensively study arrhythmogenesis, it is important to localize and visualize the specific cardiac conduction system in animal models such as the mouse, as mice are widely used in electrophysiology research.

The major parts of the cardiac conduction system are the sinoatrial node (SAN), where the electrical impulse is generated in specialized pacemaker cells, and the atrioventricular node (AVN), which is the only electrical connection between the atria and the ventricles[4]. Whenever the electrophysiological properties of SAN and AVN are altered, arrhythmias such as sick sinus syndrome or atrioventricular block can occur which may lead to hemodynamic deterioration, syncope and even death, and thus underline the essential role of both SAN and AVN in electrophysiology and arrhythmogenesis[5].

Comprehensive studies on SAN or AVN require a precise localization and visualization of both structures, ideally within their physiologic environment. However, due to their small size and location within the working myocardium, without establishing a clear macroscopically visible structure, studying the anatomy and electrophysiology of SAN and AVN is challenging. Anatomical landmarks can be used to roughly identify the region that contains SAN and AVN[6-8]. In brief, SAN is located in the inter-caval region of the right atrium adjacent to the muscular crista

terminalis (CT), AVN is located within the triangle of Koch established by the tricuspid valve, the ostium of the coronary sinus and the tendon of Todaro. Thus far, these anatomical landmarks were mainly used to localize, remove and then study SAN and AVN as individual structures (e.g., by conventional histology). To better understand the complex electrophysiology of SAN and AVN (e.g., regulatory effects of adjacent cells of the working myocardium), however, studying the conduction systems within the physiologic 3D environment is necessary.

Whole-mount immunofluorescence staining is a method that is used to study anatomical structures *in situ* while preserving the integrity of the surrounding tissue[9]. Taking advantage of confocal microscopy and image analysis software, SAN and AVN can be visualized with fluorescently labeled antibodies targeting ion channels specifically expressed in these regions.

This following protocol explains the necessary steps to perform a well-established whole-mount staining method for SAN and AVN microscope localization and visualization. Specifically, this protocol describes how (1) to localize SAN and AVN by anatomical landmarks to prepare these samples for staining and microscopy analysis (2) to perform whole-mount immunofluorescence staining of the reference markers HCN4 and Cx43 (3) to prepare SAN and AVN samples for confocal microscopy (4) to perform confocal imaging of SAN and AVN. We also describe how this protocol can be modified to include additional staining of surrounding working myocardium or non-myocyte cells such as autonomous nerve fibers which allows a thorough investigation of the cardiac conduction system within the heart.

PROTOCOL:

Animal care and all experimental procedures were conducted in accordance with the guidelines of the Animal Care and Ethics committee of the University of Munich, and all the procedures undertaken on mice were approved by the Government of Bavaria, Munich, Germany (ROB-55.2-2532.Vet_02-16-106, ROB-55.2-2532.Vet_02-19-86). C57BL6/J mice were purchased from Jackson Laboratory.

NOTE: **Figure 1** shows the instruments needed for the experiment. **Figure 2** shows an illustration of the gross cardiac anatomy. **Figure 3** shows the location of SAN and AVN in an adult mouse heart. **Figure 4** shows the prepared sample loaded on the confocal microscopy.

1. Preparations

1.1. Prepare a 4% formaldehyde solution (4% PFA) by diluting 10 mL of 16% formaldehyde solution in 30 mL of PBS.

1.2. Prepare a 15% sucrose solution and 30% sucrose solution by dissolving 15 g and 30 g of sucrose powder into 100 mL PBS, respectively. After the sucrose powder is fully dissolved, filter the solution using 0.2 µm syringe filter before storing at 4 °C.

1.3. Prepare a 3%-4% agarose gel, add 3 g or 4 g agarose powder and 100 mL of 1x TAE (diluted

from 50x TAE stock solution) into a beaker. Place the beaker on a magnetic stirrer and boil it until the agarose is completely dissolved.

1.4. Prepare the dissection dish by gently pouring 30 mL of agarose gel (3%-4%) in a 100 mm diameter Petri dish. Leave the Petri dish on the bench at room temperature to cool down and harden.

1.5. Prepare blocking solution and washing solution according to the recipes provided in the **Table 1**. Prepare all of the solutions prepared at the day of use. Long term storage is not recommended.

1.6. Prepare the antibody solutions by diluting them in cold (4 °C) 1x PBS, protect the dilution from light (e.g., by wrapping aluminum foil around) and keep dilutions on ice until used. Dilute the antibodies shortly before incubation. Avoid leaving the diluted antibodies at room temperature.

NOTE: The appropriate antibody dilution should be tested with comparable tissue samples by performing a conventional immunofluorescent staining. Here, we tested the antibodies by staining frozen sections cut at a thickness of 10 µm.

2. Organ harvest and tissue preparation

2.1. Anesthetize the mouse by placing it into an incubation chamber connected with an isoflurane vaporizer and flush with 5% isoflurane/95% oxygen.

NOTE: Full anesthesia is confirmed by the loss of the postural reaction and righting reflex by gently rolling the chamber until the mouse is placed on its back.

2.2. Put the mouse in a supine position on the surgical table. Place the mouse nose into an anesthesia mask connected to a modified Bain circuit with its inner tube connected to the isoflurane vaporizer. To maintain anesthesia, use 1-2% isoflurane in oxygen at a flow rate of 1 L/min. Scavenge away excess anesthetic vapor from the mouse via the outer tube of the mask and draw through a canister of activated charcoal, which absorbs the excess anesthetic gas.

2.3. When full anesthesia is achieved, inject fentanyl for analgesia (0.1 mg/25 g body weight i.p.).

2.4. When the toe-pinch reflex is undetectable, make a clear cut from the jugulum to the symphysis using iris scissors to remove fur and skin. Make another cut from left to right underneath the ribs using iris scissors to carefully open the abdomen.

2.5. Life the xiphoid a little bit using curved forceps to allow cutting the diaphragm from left to right without injuring any organs. Cut the rib cage in a medial axillary line on both sides using iris scissors to flip it cranially and to allow access to the heart.

2.6. Cut the inferior vena cava and descending thoracic aorta at the level of the diaphragm using iris scissors. Puncture the heart with a 27 G needle in the area of the apex and then carefully push the needle into the left ventricle (LV). Gently inject 5-10 mL of ice-cold PBS into the LV to perfuse the heart.

NOTE: The color of the heart should turn from red to gray indicating successful perfusion with PBS.

2.7. Carefully lift the apex of the heart using a tweezer allowing to cut the large vessels and to remove the heart.

2.8. Excise the heart by cutting the large arteries and veins as far away from the heart as possible to avoid any damage to the superior vena cava (SVC). Conserve the SVC since it will serve as important landmark during later processing.

2.9. After heart removal, turn off the isoflurane vaporizer.

2.10. Put the heart into a dissection dish filled with ice cold PBS under the dissecting microscope. After determination of the left/right and front/back of the heart, turn the heart around with the front of the heart at the bottom of the dish (to expose the large vessels that are located posterior).

2.11. Immobilize the heart by putting little pins through the apex and the left atrial appendage (LAA) into the agarose at the bottom of the dissection dish (**Figure 2A**). Using fine tweezers and scissors, carefully remove non-cardiac tissue around the SVC and inferior vena cava (IVC) (e.g., lungs, fat, pericardium) to expose the inter-caval region (**Figure 3A**).

2.12. Remove the majority of the ventricles by cutting parallel the groove between the ventricles and the atria with the micro scissor. Preserve a small part of the ventricular tissue for the later loading on the plastic ring.

2.13. For fixation and dehydration, put the sample (containing the atria, SVC and IVC) in 4% PFA overnight at 4 °C.

2.14. The next day, transfer the heart to 15% sucrose solution for 24 hours at 4 °C.

2.15. The next day, transfer the heart to 30% sucrose solution for 24 hours at 4 °C.

NOTE: For the fixation and dehydration in step 2.13, 2.14 and 2.15, the samples are left at 4°C without further stirring or rocking.

3. Whole-mount immunofluorescence staining

3.1. Wash the heart in 1% Triton X-100 diluted in PBS and block and permeabilize in blocking solution (**Table 1**) overnight at 4 °C.

3.2. Place the heart in a 1.5 mL tube and incubate with rabbit anti-mouse connexin-43 (dilution of 1:200) and rat anti-mouse HCN4 (dilution of 1:200) antibodies diluted with blocking solution for 7 days at 4 °C.

NOTE: The optimal concentration of primary antibodies should be tested before using the datasheets as orientation. Other antigens of interest could also be stained in this step, as long as the host species of the primary antigen is different from the other ones. A higher concentration of Triton X-100 may help obtain more efficient antibody staining as demonstrated before[10], but concentration might be determined individually.

3.3. After 7 days, remove the solution containing primary antibodies using a pipette and wash the heart with 1%Triton X-100 solution 3 times (each time for 1 h at room temperature on the orbital shaker).

3.4. After washing, incubate the heart = with Alexa Fluor 488 goat anti-rat IgG (dilution of 1:200) and Alexa Fluor 647 goat anti-rabbit IgG (dilution of 1:200) for 7 days at 4 °C.

3.5. After 7 days, remove all the solution containing the secondary antibodies using a pipette. Then wash the heart using washing solution (**Table 1**) 3 times (each time for 1 h at room temperature on the orbital shaker).

3.6. To stain nuclei, incubate the heart in DAPI solution (10 µg/mL) overnight at 4 °C.

3.7. On the next day, wash the heart with washing solution 3 times (each time for 1 h at room temperature on the orbital shaker).

NOTE: For the blocking, permeabilization, antibodies incubation and DAPI staining in step 3.1, 3.2, 3.4 and 3.6, leave the samples at steady state in the 4 °C, no stirring is required. The stained tissue could be preserved fully covered with washing solution at 4 °C and protected from light for a few days until imaging at the confocal microscope.

4. Confocal microscopy

4.1. Prepare plastic rings and fill with plasticine (**Figure 1**). In the center of the plasticine a little groove is formed in the center for loading the heart. Make a shallow groove, as this would be easier to acquire a flat imaging plane, and to adjust the space and avoid air bubbles between the sample and the coverslips in step 4.4.

4.2. Use the same heart sample for the imaging of both SAN and AVN sequentially. For SAN

imaging, directly load the heart whereas for AVN imaging, perform microdissection before.

4.2.1. **SAN imaging**

4.2.1.1. Identify the SAN by anatomical landmarks: it is located on the dorsal side of the heart within the inter-caval region (the region between the superior and inferior vena cava). The crista terminalis (CT) is the muscle streak between the SAN and RAA.

4.2.1.2. Place the heart into the plasticine groove with the back of the heart facing up. Add PBS onto the heart to displace all air within the cavity until the heart is fully covered with PBS (usually a few drops of PBS are sufficient).

4.2.1.3. Under the dissection microscope, gently press the RAA, LAA and remaining parts of the left ventricle into the plasticine to fix the heart. Make sure that the whole inter-caval region and the crista terminalis could be clearly seen (not covered by plasticine). The area that will be imaged is shown in **Figure 3A**.

4.2.2. **AVN imaging**

4.2.2.1. After finish the imaging of SAN, recollect the same sample and subsequently use for the AVN imaging. For AVN microdissection, place the heart = on a dissection dish under the dissection microscope.

4.2.2.2. Orient the heart with the right side facing up (including the remaining parts of the right ventricle). Put pins through the remaining part of the LV free wall (which is now at the bottom) to immobilize the tissue (**Figure 2B**).

4.2.2.3. Cut the remaining RV free wall upwards through the tricuspid valve and superior vena cava. Then flip the RV and RA away to expose interventricular and interatrial septum. (**Figure 3B**)

NOTE: Pay attention not to damage the coronary sinus (CS), as it is an important anatomical landmark to find the triangle of Koch which then allows to localize the AVN. The CS runs transversely in the left atrioventricular groove on the posterior side of the heart, and the CS orifice opening is located between IVC and the tricuspid valve at the inferior part of the interatrial septum (**Figure 3A and B**).

4.2.2.4. Identify the triangle of Koch that can be found on the endocardial surface of the right atrium, bordered anteriorly by the hinge-line of the septal leaflet of the tricuspid valve (TV), and posteriorly by the tendon of Todaro. The base is formed by the orifice of the coronary sinus (**Figure 3B**). Since this is the target region for AVN imaging, it needs to be clearly visible.

4.2.2.5. Transfer the heart into the plasticine groove within the plastic ring with the triangle of Koch clearly being exposed (i.e. not covered by plasticine). Gently press the tissue

around the Koch triangle into the plasticine to fix the sample. Add PBS onto the heart to displace all air within the cavity until the heart is fully covered with PBS (usually a few drops of PBS are sufficient).

4.3. Apply silicone to the edges of the plastic rings to allow covering the hearts loaded within the plasticine-filled plastic rings with cover slips.

4.4. Gently press the back side of the plasticine to squeeze out parts of the PBS and to attach the heart to the coverslip while avoiding any air bubbles within the imaging area.

NOTE: Make sure that the regions of interest are not folded and covered during pressing the back side of the plasticine. A flat imaging plane without sample overcompression is necessary for conserving the anatomy and for proper confocal imaging of the samples. For SAN, it is important to make sure the inter-caval region is clearly exposed. For AVN, the triangle of Koch should be fully exposed.

4.5. Put the whole-mount staining samples up-side down on the platform of the confocal microscope. The exposed SAN/AVN attached to the cover slip is now on the bottom of the microscope platform (**Figure 4**).

NOTE: To take images, we use the Carl Zeiss LSM800 with Airyscan Unit and the software ZEN 2.3 SP1 black.

4.6. Choose plate "BP420-480 + LP605" for the excitation of Alexa Fluor 647 conjugated anti-HCN4. Vary the master gain from 650-750.

4.7. Take an overview image of the whole SAN and AVN region by using **Tile Scan** function. Then select the HCN4-positive region by clicking and adding a square on the overview image around the area of interest that will be scanned.

4.8. Check the parameters, including plates and master gain for the remaining channels (Alexa Fluor 488 and DAPI) of the confocal microscope and set as described in step 4.6.

4.9. Slowly adjust the focus from top to bottom of the sample to preview the whole sample and to set the **First** and **Last** for the Z-stack range. Set an optimal interval for the Z-stack based on the thickness of the optical sample. We use an 20x objective, and 0.8-1 µm as the interval for Z-stack.

4.10. After all the parameters are properly set, scan the whole area of the SAN and AVN.

4.11. Perform 3D reconstruction of the images using software (e.g., Imaris version 8.4.2).

4.11.1. Select **Images Processing | Baseline Subtraction** to remove background staining.

4.11.2. Select surface creation onto setting for selected channel and region of interest for processing.

REPRESENTATIVE RESULTS:

By using the protocol outlined above, confocal microscopy imaging of both SAN and AVN can be reliably performed. Specific staining of the conduction system using fluorescent antibodies targeting HCN4 and staining of the working myocardium using fluorescent antibodies targeting Cx43 allows the clear identification of SAN (**Figure 5, Video 1**) and AVN (**Figure 6, Video 2**) within the intact anatomy.

Figure 1: Instruments for dissection and plastic ring holder. A. Plastic ring filled with plasticine (red arrow shows the groove formed in the center for loading the heart). **B**. Dissection Petri dish with agarose gel. **C**. Spring scissors. **D**. Iris scissors. **E**. Curved forceps. **F**. Fine forceps.

Figure 2: Illustration of the gross cardiac anatomy. A. Schematic illustration of the SAN microdissection. Pins are applied through the apex and the left atrial appendage (LAA). SAN is indicated by red dashed circle. **B.** Schematic illustration of the AVN microdissection. Pins are put through the remaining part of the LV free wall (which is now at the bottom). AVN is indicated by a red dashed circle. SVC, Superior vena cava; IVC, inferior vena cava; CS, coronary sinus; LAA, left atrial appendage; RAA, right atrial appendage; PA, pulmonary artery; PV, pulmonary vein; LV, left ventricle; IVS, interventricular septum; IAS, interatrial septum; OF, oval fossa.

Figure 3: Location of SAN and AVN in an adult mouse heart. A. Location of the sinoatrial node (SAN) in an adult mouse heart. View from the back of the heart. Location of the SAN is indicated by red dashed line within the inter-caval region (black dashed lines). SVC, Superior vena cava; IVC, inferior vena cava; CS, coronary sinus; LAA, left atrial appendage; RAA, right atrial appendage; PV, pulmonary vein; CT, Crista terminalis; LV, left ventricle; RV, right ventricle. **B.** Location of the atrioventricular node (AVN) in an adult mouse heart. View from the right. The AVN (red dashed circle) is located at the apex of the triangle of Koch (white dashed triangle) near the bottom of the membranous septum. The triangle of Koch is formed by the tendon of Todaro (TT, green dashed line), tricuspid valve (TV, blue dashed line) and the orifice of the coronary sinus (CS, yellow dashed line). SVC, superior vena cava; IVC, inferior vena cava; IVS, interventricular septum; OF, oval fossa.

Figure 4: Prepared sample loaded on the confocal microscopy. A. The prepared sample (red arrow) for confocal microscopy mounted into the plastic ring with plasticine (black arrow). View from the bottom. **B.** Plastic ring with mounted sample loaded up-side-down on the platform of the confocal microscope (inverse microscope).

Figure 5. Reconstruction of confocal microscopy imaging of SAN in a C57BL6/J mouse stained with anti-HCN4 (red) and Cx43 (white) antibodies. A. The dashed area delineates the SAN, which is oriented along the inter-caval region between the superior and inferior vena cava. **B.** Magnification of the inlayfrom panel A. **C.** 3D reconstruction of panel B. RAA, right atrial appendage; RA, right atrium; SAN, sinoatrial node.

Figure 6. Reconstruction of confocal microscopy imaging of AVN in a C57BL6/J mouse stained with anti-HCN4 (red) and Cx43 (white) antibodies. A. Dashed area indicates the AVN. **B.** Magnification of the inlay from panel A. **C.** 3D reconstruction of panel B. IVS, interventricular septum; IAS, interatrial septum; AVN, atrioventricular node.

Figure 7. **Negative control image. A.** negative control whole-mount staining for SAN. The dashed circle showed the SAN region confirmed by the anatomy landmarks. **B.** negative control staining for SAN only with second antibodies. **C.** DAPI staining for SAN. **D.** negative control whole-mount staining for AVN. The dashed circle showed the AVN region confirmed by the anatomy landmarks. **E.** negative control staining for AVN only with second antibodies. **F.** DAPI staining for AVN. RAA, right atrial appendage; RA, right atrium; SVC, superior vena cava; IVS, interventricular septum; IAS, interatrial septum;

Video 1: 3D Reconstruction of the SAN

Video 2: 3D Reconstruction of the AVN

DISCUSSION:

Cardiac anatomy has traditionally been studied using thin histological sections[11]. However, these methods do not preserve the three-dimensional structure of the conduction system and thus, only provides 2D information. The whole-mount immunofluorescence staining protocol described here allows to overcome these limitations and can be routinely used for SAN and AVN imaging.

In comparison to standard methods such as conventional immunohistochemistry that require paraffin-embedding, sectioning and antigen retrieval, the whole-mount methodology is advantageous for addressing the exact 3D localization and morphology of SAN and AVN, and to examine the relationship with the surrounding tissue since the tissue morphology is considerably preserved with only minimum tissue dehydration and physical rupture. Also, the immunoreactivity (i.e., the binding of antibodies to tissue antigens) is also highly maintained.

We established a practical sample procession method for confocal microscopy by using a plastic ring mounted holder filled with plasticine. Plasticine is ideal since it can be individually adjusted to the size of the tissue, it is hard enough to reliably hold the tissue without excessive pressure of the tissue. Most importantly, it is not fluorescent, avoiding auto-fluorescent background and therefore not interfering with the fluorescent signal of the antibodies used.

We used the confocal laser-scanning microscope (LSM) with the Airyscan detector, which can offer a distinct advantage in obtaining images with superior quality. Using a widefield imaging microscope can only offer tissue-level information but lacks cellular resolution. On the other hand, the widefield microscope carries a risk of high background[12]. By using an Airyscan detector for confocal LSM, improved resolution and signal-to-noise ratio (SNR) can be acquired, compared to the traditional one pinhole-and-detector confocal imaging system[13].

The shapes and position of SAN and AVN are presented as 3D reconstruction and additional virtual section planes derived from 3D reconstruction can enhance the interpretation of histological sections, whereas conventional histology allows only a 2D assessment of the anatomy with the inherent risk of non-detection of small but relevant structures. Moreover, 3D reconstruction provides the opportunity to examine anatomical structures of interest transmurally and within the intact microenvironment, for example intrinsic autonomic nervous plex innervating the heart[14]. Whole mount in situ staining and 3D reconstruction allows investigation of the cellular environment as well as the specific cell types and their orientation. Furthermore, regional and cell-type specific protein expression can be visualized and may further support western blot and proteomics analyses[15].

SAN and AVN are specialized structures within the heart with a different expression pattern of ion channels and connexins that can be used for reliable identification[10,16]. Hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN) establish the diastolic depolarization in pacemaker cells and are therefore specifically expressed within the conduction system with HCN4 being the predominant isoform in the mouse[17]. Studies have shown that HCN4 is one of the detectable and stably expressed HCN isoforms throughout the SAN and AVN[11,18]. Connexins directly connect neighboring cells and allow the passage of ions from cell to cell; they are therefore essential for the conduction of the electrical impulse through the heart[19]. Connexin expression patterns vary between different regions in the heart with Cx43 being the most abundant isoform that has been found in almost all parts of the heart except for the SAN and AVN[20]. Combining microdissection to allow exposure of specific regions of interest with anti-HCN4 and anti-CX43 antibodies as well as in silico approaches for three-dimensional reconstruction of confocal images allow reliable identification of SAN and AVN and to comprehensive investigation of the SAN/AVN morphology as well as their interaction with the surrounding tissue.

The SAN and AVN might be easy to distinguish after staining with a specific antibody. To localize the SAN and AVN by their anatomical landmarks, however, some practice is needed before the correct microdissection can be performed.

Using the above protocol, a number of other antigens can also be applied. The protocol also works well on both perfusion-fixed and immersion-fixed tissue. However, the incubation time may be adjusted for optimal fixation since certain antibodies show reduced immunoreactivity when the antigen is over-fixed. As the whole-mount staining approach needs 7 days for primary and secondary antibody incubation, it is important to completely submerge the samples in adequate volumes of antibody-containing solutions. The dilution ratio of each antibody should be tested before the experiment. For optimal staining, any irrelevant tissue should be removed. For the protocol, we only preserved little parts of the ventricles for better orientation and remove all the surrounding fat tissue and pulmonary veins, as surrounding (irrelevant) tissue might bind antibodies as well, especially when the target antigens are globally expressed.

Whole-mount in situ staining, confocal imaging and 3D reconstruction is an invaluable and

powerful technique for researchers from various fields. However, the method also has some limitations. (i) It requires specialized equipment such as a confocal microscope that is not commonly available at every institution. (ii) As mentioned above, microdissection of specialized anatomic structures such as the SAN or AVN but also confocal imaging and post-imaging processing requires intensive practicing and experienced personnel. (iii) Success of the method highly relies on the quality of the antibodies used and may therefore be very challenging in some cases. Also (iv) 3D reconstruction can be difficult to achieve and may require intensive troubleshooting to optimize the procedure. For example, optimal intervals have to be set during image acquisition since sections spaced too far apart will leave gaps and might not allow an adequate 3D reconstruction of the anatomy. Sections that are too close may cause oversampling and bleaching due the excessive illumination and will take a long time to complete one image. Finally (v) the major limitation of confocal microscopy is the imaging depth which means that if sample thickness is beyond the maximum operating depth of the confocal microscope, the further fluorophore cannot be detected.

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

REFERENCES:

1 Clauss, S. et al. Animal models of arrhythmia: classic electrophysiology to genetically modified large animals. *Nature Reviews Cardiology.* **16** (8), 457-475 (2019).

2 Clauss, S. et al. Characterization of a porcine model of atrial arrhythmogenicity in the context of ischaemic heart failure. *PLoS One.* **15** (5), e0232374 (2020).

3 Schuttler, D. et al. Animal Models of Atrial Fibrillation. *Circulation Research.* **127** (1), 91-110 (2020).

4 van Weerd, J. H., Christoffels, V. M. The formation and function of the cardiac conduction system. *Development.* **143** (2), 197-210 (2016).

5 Vogler, J., Breithardt, G., Eckardt, L. Bradyarrhythmias and Conduction Blocks. *Revista Española de Cardiología (English Edition).* **65** (7), 656-667 (2012).

6 Wen, Y., Li, B. Morphology of mouse sinoatrial node and its expression of NF-160 and HCN4. *International Journal of Clinical and Experimental Medicine.* **8** (8), 13383-13387 (2015).

7 Verheijck, E. E. et al. Electrophysiological features of the mouse sinoatrial node in relation to connexin distribution. *Cardiovascular Research.* **52** (1), 40-50 (2001).

8 Glukhov, A. V., Fedorov, V. V., Anderson, M. E., Mohler, P. J., Efimov, I. R. Functional anatomy of the murine sinus node: high-resolution optical mapping of ankyrin-B heterozygous mice. *American Journal of Physiology Heart and Circulatory Physiology.* **299** (2), H482-491 (2010).

9 Sillitoe, R. V., Hawkes, R. Whole-mount immunohistochemistry: a high-throughput screen for patterning defects in the mouse cerebellum. *Journal of Histochemistry and Cytochemistry.* **50** (2), 235-244 (2002).
10 Hulsmans, M. et al. Macrophages Facilitate Electrical Conduction in the Heart. *Cell.* **169** (3), 510-522 e520 (2017).
11 Liu, J., Dobrzynski, H., Yanni, J., Boyett, M. R., Lei, M. Organisation of the mouse sinoatrial node: structure and expression of HCN channels. *Cardiovascular Research.* **73** (4), 729-738 (2007).
12 Shaw, P. J. in *Handbook Of Biological Confocal Microscopy* 10.1007/978-0-387-45524-2_23 (ed James B. Pawley) 453-467 (Springer US, 2006).
13 Huff, J. The Airyscan detector from ZEISS: confocal imaging with improved signal-to-noise ratio and super-resolution. *Nature Methods.* **12** (12), i-ii (2015).
14 Rysevaite, K. et al. Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations. *Heart Rhythm.* **8** (5), 731-738 (2011).
15 Acar, M. et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature.* **526** (7571), 126-130 (2015).
16 Brahmajothi, M. V., Morales, M. J., Campbell, D. L., Steenbergen, C., Strauss, H. C. Expression and distribution of voltage-gated ion channels in ferret sinoatrial node. *Physiological Genomics.* **42A** (2), 131-140 (2010).
17 Mesirca, P. et al. Cardiac arrhythmia induced by genetic silencing of 'funny' (f) channels is rescued by GIRK4 inactivation. *Nature Communication.* **5**, 4664 (2014).
18 Liang, X. et al. HCN4 dynamically marks the first heart field and conduction system precursors. *Circulation Research.* **113** (4), 399-407 (2013).
19 Verheule, S., Kaese, S. Connexin diversity in the heart: insights from transgenic mouse models. *Frontiers in Pharmacology.* **4**, 81 (2013).
20 van der Velden, H. Cardiac gap junctions and connexins: their role in atrial fibrillation and potential as therapeutic targets. *Cardiovascular Research.* **54** (2), 270-279 (2002).

figure 1

Figure 1.

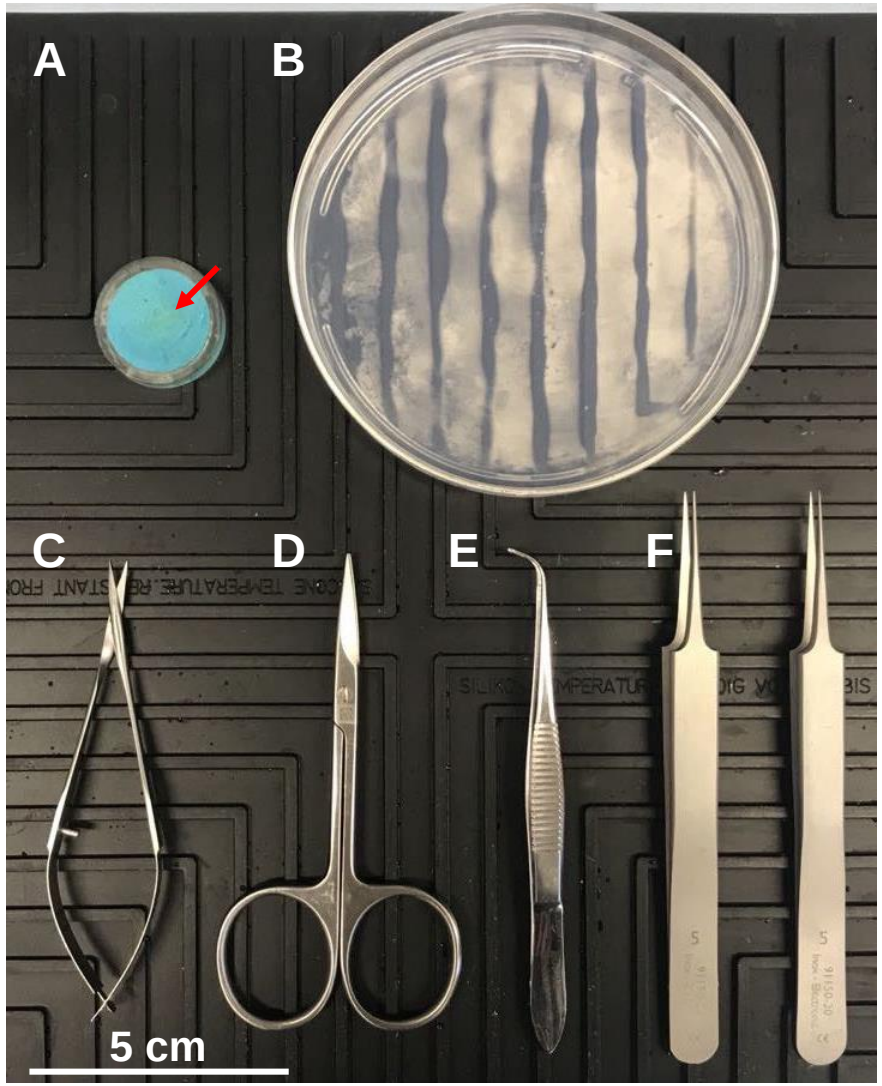


Figure 2.

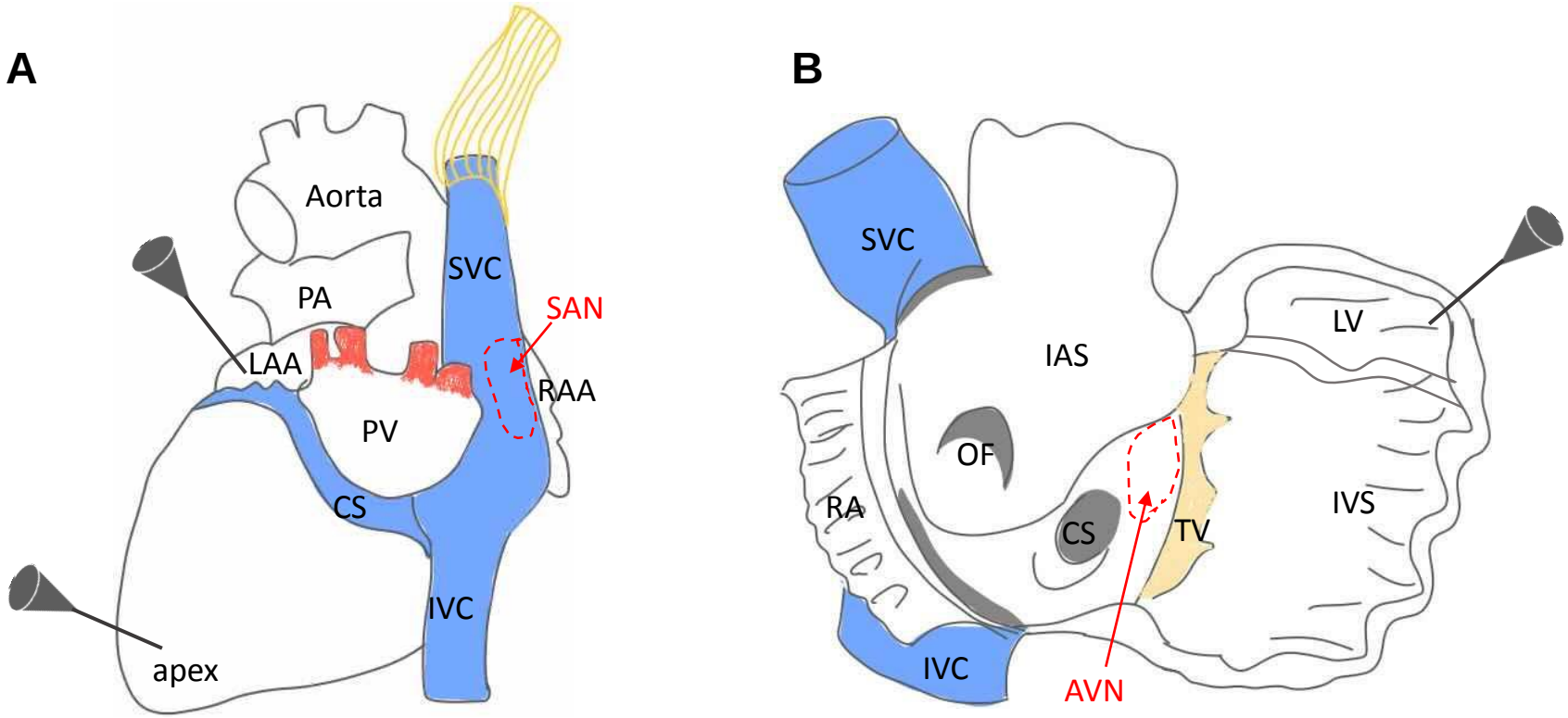
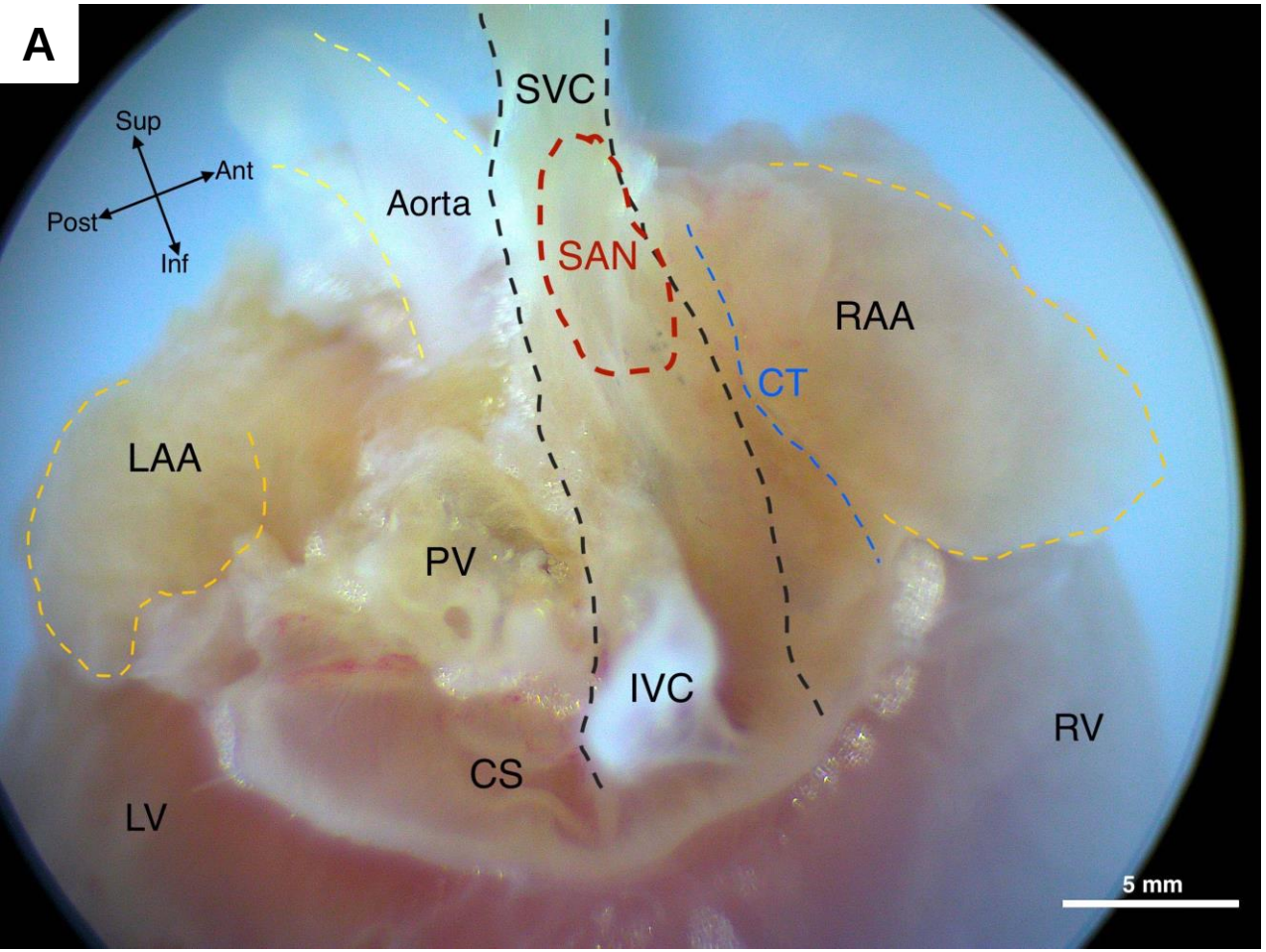
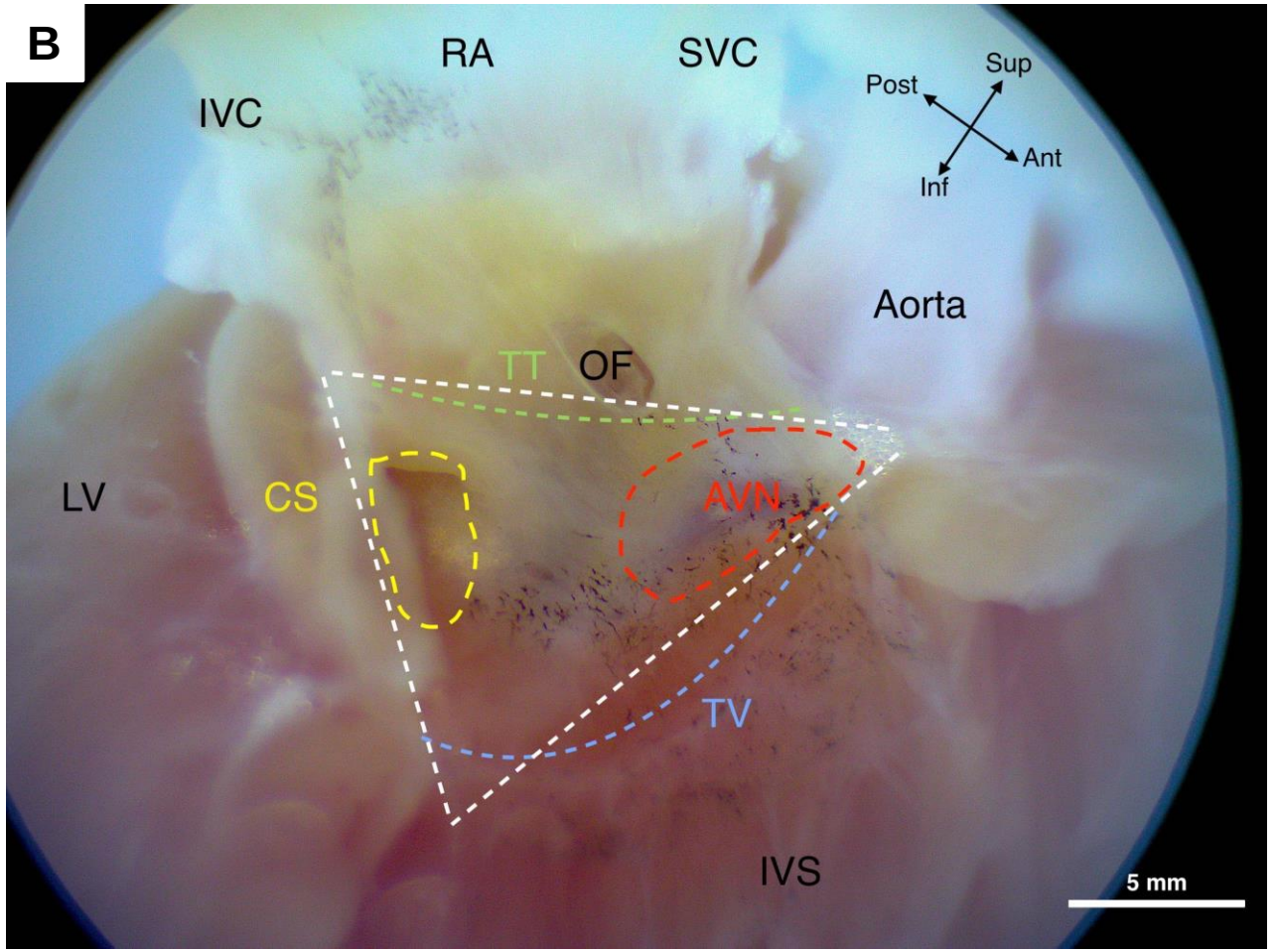


figure 3

Figure 3.





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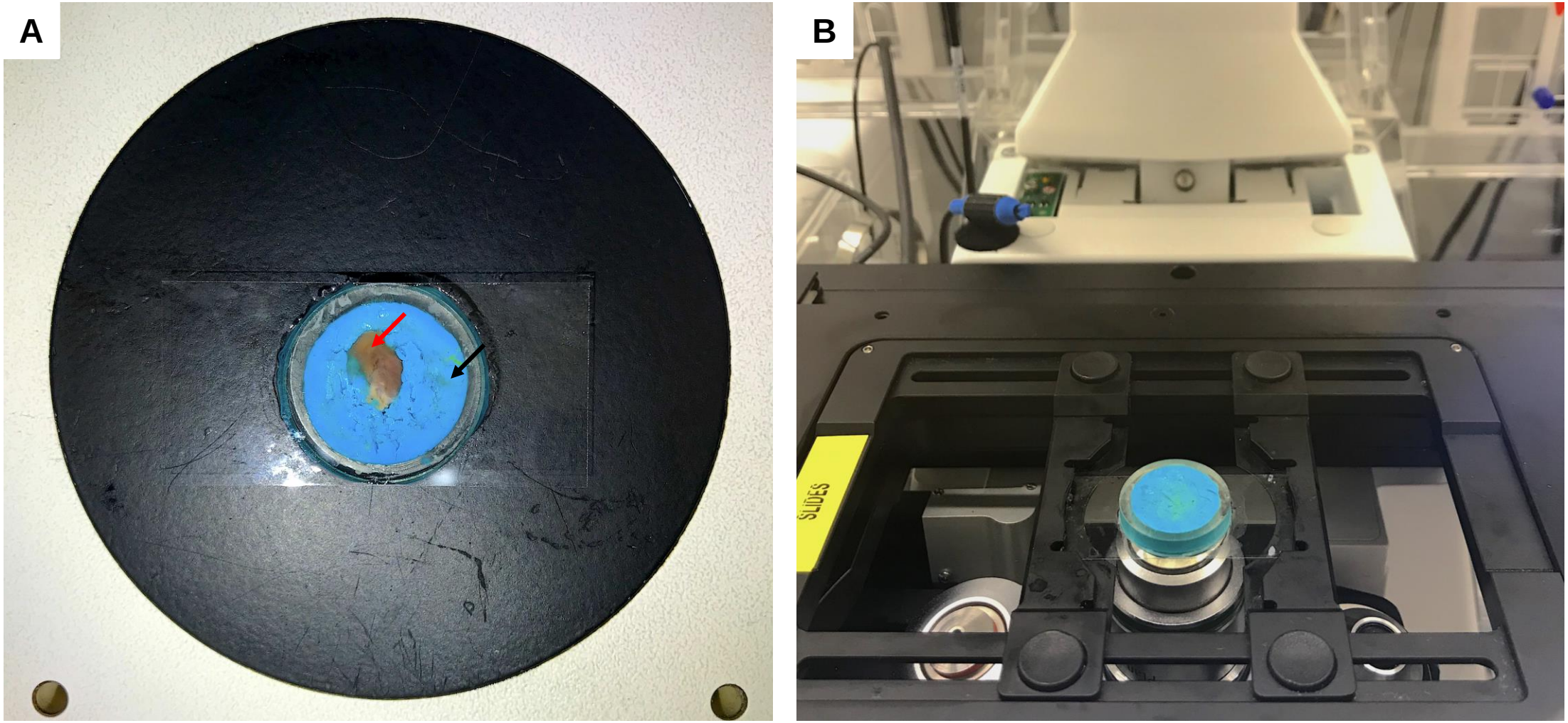


figure 5

Figure 5.

Click here to access/download;Figure;2020-11-06 Figure 5 confocal microscopy of SAN.pptx

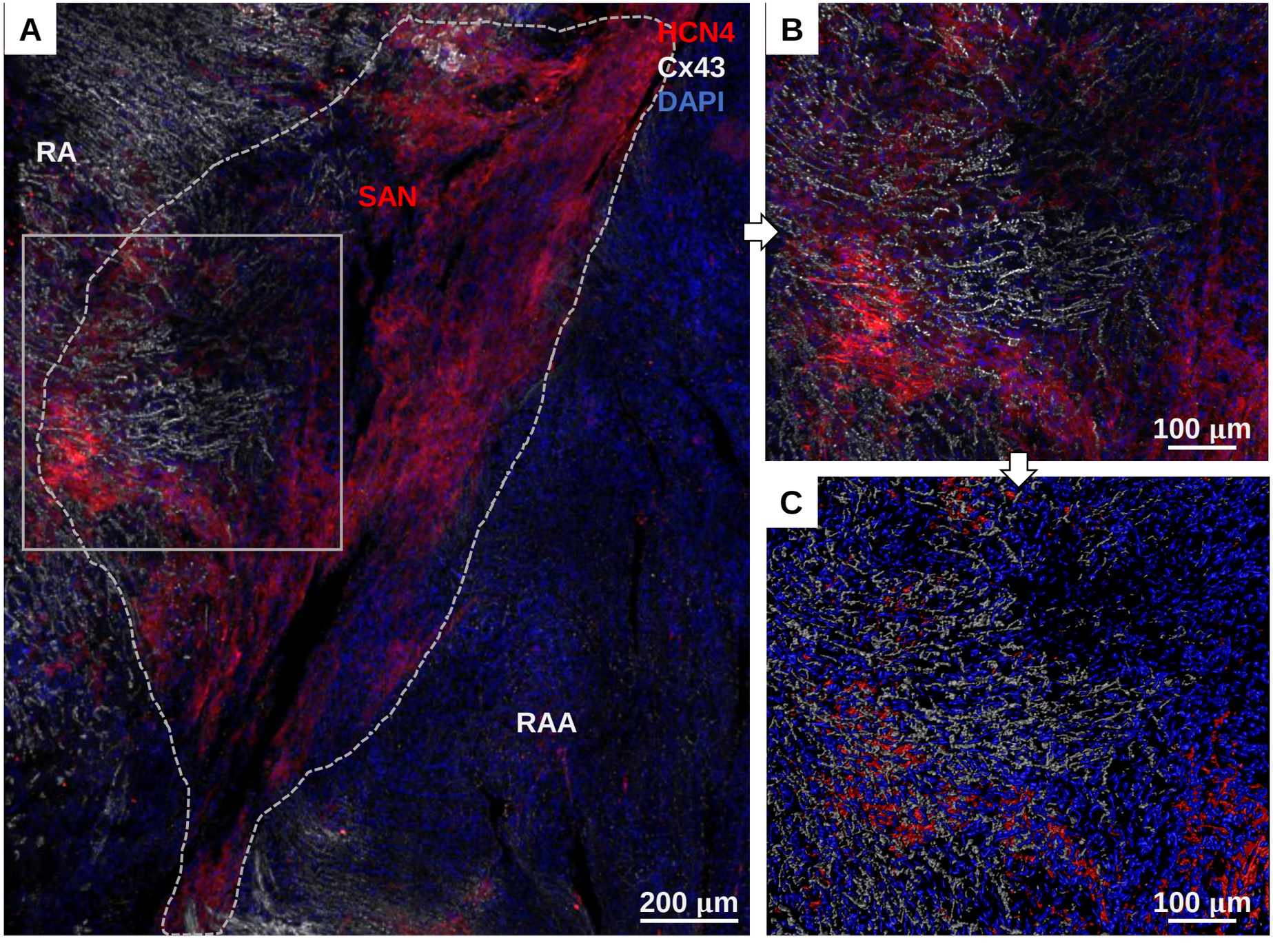


figure 6

Figure 6.

Click here to access/download;Figure;2020-11-06 Figure 6 confocal microscopy of AVN.pptx

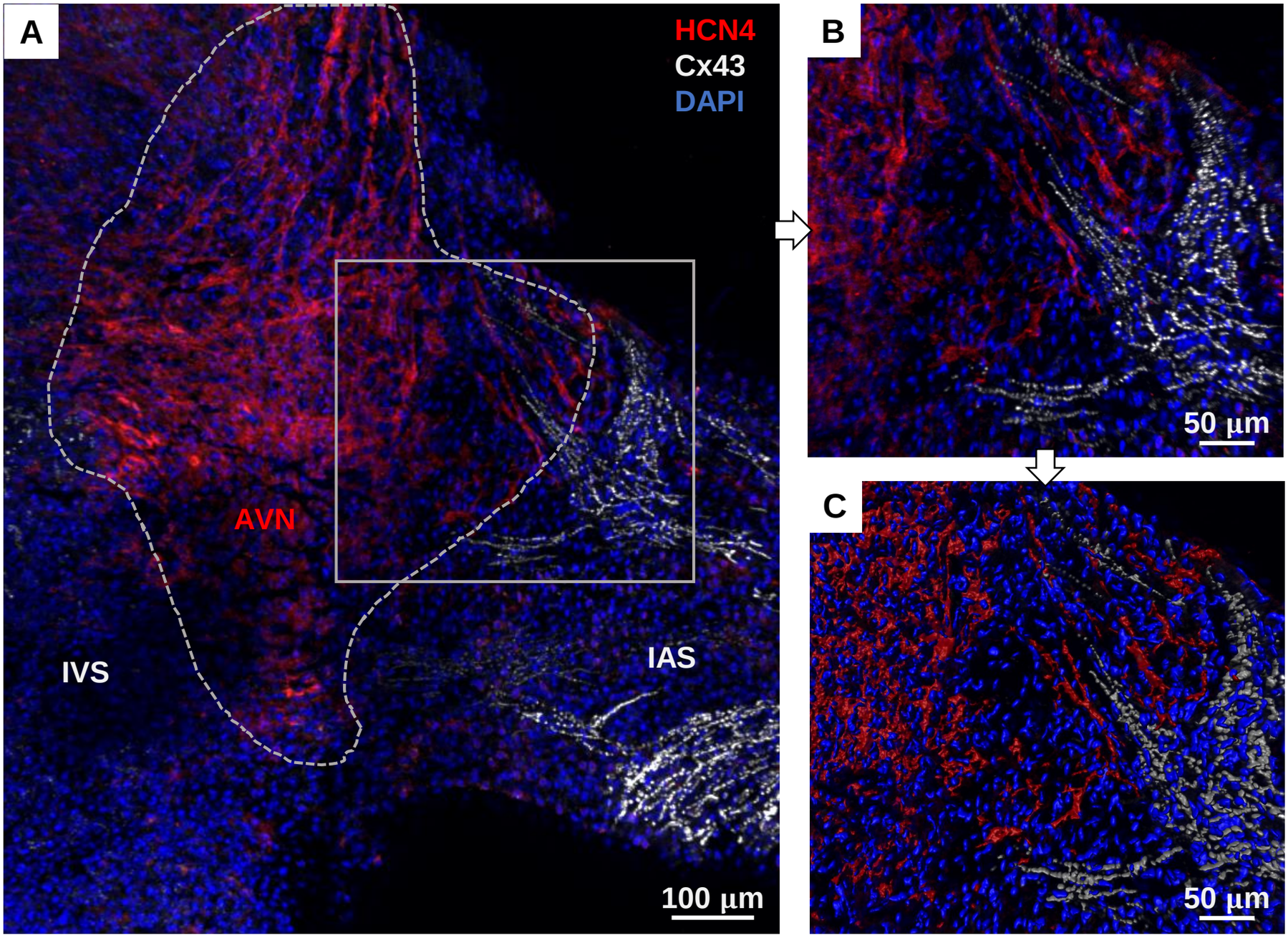
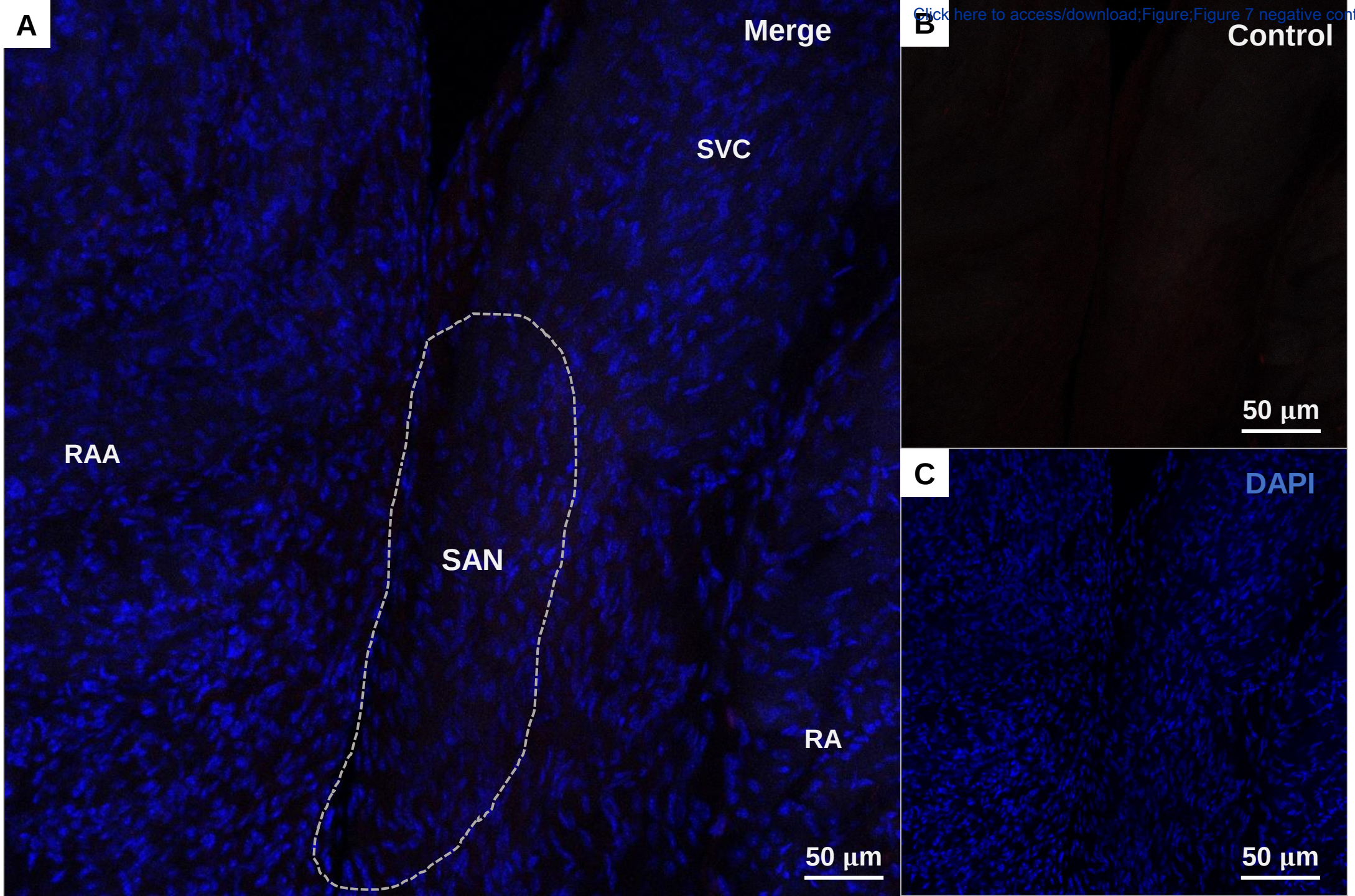
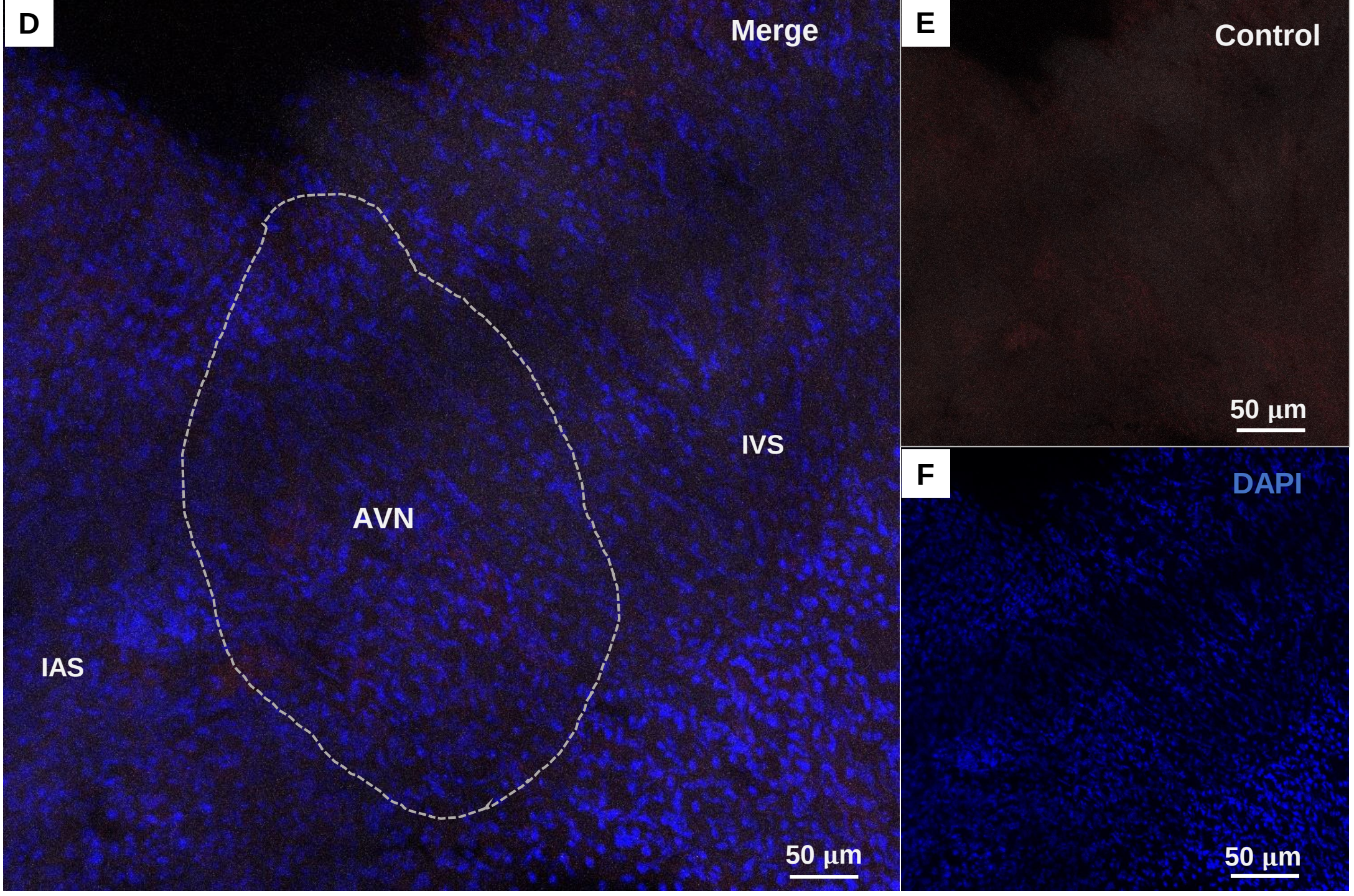


Figure 7.




D
Merge
AVN
IVS
IAS
50 µm
E
Control
50 µm
F
DAPI
50 µm

SAN 3D movie

Click here to access/download

Video or Animated Figure

3D reconstruction SAN HCN4 Cx43_Airyscan Processing_Stitch.avi

AVN 3D movie

Click here to access/download

Video or Animated Figure

3D reconstuction AVN Cx43 HCN4-Airyscan Processing_Stitch.avi

Table 1

Compound	Final concentration	g or mL/100 mL required
Fixing solution		
Formaldehyde (16%)	4%	25 mL
PBS (1x)		75 mL
1% Triton solution		
Triton X100	1%	1 mL
PBS		99 mL
Blocking solution		
Triton X-100	1%	1 mL
BSA	0.50%	0.5 g
Normal goat serum	20%	20 mL
PBS (1x)		89 mL
Washing solution		
Tween 20	0.10%	0.1 mL
BSA	0.50%	0.5 g
PBS (1x)		99.9 mL
TAE (50x)		
Tris-base	24.20%	24.2 g
100% acetic acid	5.71%	5.71 mL
0.5 M EDTA	0.05 M	10 mL
dH_2O		Add up to 100 mL

Description	Company	Cat.-No.	Comments
Anesthesia			
Isoflurane vaporizer system	Hugo Sachs Elektronik	34-0458, 34-1030, 73-4911, 34-0415, 73-4910	Includes an induction chamber, a gas evacuation unit and charcoal filters
Modified Bain circuit	Hugo Sachs Elektronik	73-4860	Includes an anesthesia mask for mice
Surgical Platform	Kent Scientific	SURGI-M	
In vivo **instrumentation**			
Fine forceps	Fine Science Tools	11295-51	
Iris scissors	Fine Science Tools	14084-08	
Spring scissors	Fine Science Tools	91500-09	
Tissue forceps	Fine Science Tools	11051-10	
Tissue pins	Fine Science Tools	26007-01	Could use 27G needles as a substitute
General lab instruments			
Orbital shaker	Sunlab	D-8040	
Magnetic stirrer	IKA	RH basic	
Pipette,volume 10 µL, 100 µL, 1000 µL	Eppendorf	Z683884-1EA	
Microscopes			
Dissection stereo- zoom microscope	VWR	10836-004	
Laser Scanning Confocal microscope	Zeiss	LSM 800	
Software			
Imaris 8.4.2	Oxford instruments		

ZEN 2.3 SP1 black	Zeiss		
General Lab Material			
0.2 µm syringe filter	Sartorius	17597	
100 mm petri dish	Falcon	351029	
27G needle	BD Microlance 3	300635	
50 ml Polypropylene conical Tube	Falcon	352070	
5ml Syringe	Braun	4606108V	
Cover slips	Thermo Scientific	7632160	
Eppendorf Tubes	Eppendorf	30121872	
Chemicals			
0.5 M EDTA	Sigma	20-158	Components of TEA
16% Formaldehyde Solution	Thermo Scientific	28908	use as a 4% solution
Acetic acid	Merck	100063	Components of TEA
Agarose	Biozym	850070	
Bovine Serum Albumin	Sigma	A2153-100G	
DPBS (1X) Dulbecco's Phosphate Buffered Saline	Gibco	14190-094	
Normal goat serum	Sigma	NS02L	
Sucrose	Sigma	S1888-1kg	
Tris-base	Roche	TRIS-RO	Components of TEA
Triton X-100	Sigma	T8787-250ml	Diluted to 1% in PBS
Tween 20	Sigma	P2287-500ml	
Drugs			
Fentanyl 0.5 mg/10 mL	Braun Melsungen		
Isoflurane 1 mL/mL	Cp-pharma	31303	
Oxygen 5 L	Linde	2020175	Includes a pressure regulator
Antibodies			
Goat anti-Rabbit IgG Alexa Fluor 488	Cell Signaling Technology	#4412	diluted to 1:200
Goat anti-Rat IgG Alexa Fluor 647	Invitrogen	#A-21247	diluted to 1:200
Hoechst 33342, Trihydrochloride, Trihydrate (DAPI)	Invitrogen	H3570	diluted to 1:1000

Rabbit Anti-Connexin-43	Sigma	C6219	diluted to 1:200
Rat anti-HCN4 (SHG 1E5)	Invitrogen	MA3-903	diluted to 1:200
Other			
Plastic ring			Self-designed and 3D printed
Plasticine	Cernit	49655005	
Silikonpasten, Baysilone	VWR	291-1220	
Animals			
Mouse, C57BL/6	The Jackson Laboratory		

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CAMPUS GROSSHADERN
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Nam Nguyen, Ph.D.

Manager of Review
JoVE

Ihr Zeichen: Unser Zeichen: Xia 06.11.2020

Dear Dr. Nguyen,

thank you very much for your kind and constructive comments and your invitation to re-submit our revised manuscript to JoVE. Please find enclosed a point-by-point response to the reviewers' comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank you for the comments. The manuscript has been proofread very carefully, and spelling or grammar issues are changed at tracking mode. Please check them in the revised manuscript enclosed.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.

Thank you for the comment. The protocol has been modified accordingly.

3. Please specify all surgical tools used to make the incisions.

We apologize for not mentioning every surgical tool at each step. We added this information as follows:

[…] 2.4 When the toe-pinch reflex is undetectable, a clear cut from the jugulum to the symphysis is made using iris scissors to remove fur and skin. Another cut from left to right underneath the ribs is made using iris scissors to carefully open the abdomen. The xiphoid is lifted a little bit using curved forceps to allow cutting the diaphragm from left to right without injuring any organs. The rib cage is then cut in medial axillary line on both sides using iris scissors to flip it cranially and to allow access to the heart.

2.5 Inferior vena cava and descending thoracic aorta are cut at the level of the diaphragm using iris scissors. […]

4. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

5. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We highlighted parts of the protocol as suggested.

6. Please combine Tables 1-3 into one single Table of Materials and Equipment.

Thank you for the comment. We have combined Tables 1-3 into one single Table "Materials and Equipment" and replaced the original tables with the new combined table.

7. Please specify any limitations of the protocol in the discussion.

Thank you for your comment that is very helpful for us. It shows that our manuscript and especially our discussion was not written well enough. Two paragraphs discussing potential limitations of the protocol were added as follows:

Discussion Section:

[…] The SAN and AVN might be easy to distinguish after staining with a specific antibody. To localize the SAN and AVN by their anatomical landmarks, however, might need some practice before the correct microdissection can be performed. […]

[…] Whole-mount in situ staining, confocal imaging and 3D reconstruction is an invaluable and powerful technique for researchers from various fields. However, the method also has some limitations: (i) it requires specialized equipment such as a confocal microscope that is not commonly available at every institution, (ii) as mentioned above, microdissection of specialized anatomic structures such as the SAN or AVN but also confocal imaging and post-imaging processing requires intensive practicing and experienced personnel, (iii) success of the method highly relies on the quality of the antibodies used and may therefore be very challenging in some cases. Also, (iv) 3D reconstruction can be difficult to achieve and may require intensive troubleshooting to optimize the procedure. For example, optimal intervals have to be set during image acquisition since sections spaced too far apart will leave gaps and might not allow an adequate 3D reconstruction of the anatomy. Sections that are too close may cause oversampling and bleaching due the excessive illumination and will take a long time to complete one image. Finally, (v) the major limitation of confocal microscopy is the imaging depth which means that if samples' thickness is beyond the maximum operating depth of the confocal microscope, the further fluorophore cannot be detected. […]

And two references were added:

14 Rysevaite, K. et al. Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations. Heart Rhythm. 8 (5), 731-738, (2011).

15 Acar, M. et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature. 526 (7571), 126-130, (2015).

8. Please spell out journal titles in the references.

All the journal titles were fully spelled.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, Xia and colleagues describe a protocol for dissection of mouse sinoatrial node and atrioventricular node tissue for whole-mount immunostaining. They detail a relatively simple and straightforward technique that should be easily adopted by any lab interested in conducting mouse studies of the cardiac conduction system.

Major Concerns:

None

Minor Concerns:

A few clarifications are needed:

1) For Notes #2: how long is a "longer time at room temperature"?

Thank you for the comment. We changed the manuscript as follows to avoid misunderstanding:

[…] NOTES: […] #2. The dilution of antibodies should be done shortly before incubation. Avoid to leave the diluted antibodies at room temperature. […]

2) Step 2.5: Please provide more details for the LV perfusion step. Was a closed circuit system used?

We thank the reviewer for pointing towards this issue. No, we performed the perfusion by simply injecting PBS directly into the LV using a syringe, and no closed circuit system was used. This method has also been described by some other researchers[1].

3) Step 2.8: Is washing of the heart sample required prior to putting on the dissection microscope stage?

Thank you for the comment. After harvesting the heart, it was put directly into cold PBS for the following microdissection. We changed this part of the manuscript:

[…] 2.8 The heart is put directly into a dissection dish filled with ice cold PBS under the dissecting microscope. […]

4) For each step requiring fixation or washing (e.g. 2.10, 2.11, 2.12, 3.1, 3.2), please state how the samples should be incubated? Are rocking or stirring required?

Thank you very much for the comment. For the fixation and antibody incubation, no rocking or stirring is required. The sample is directly put into the corresponding buffer at 4°C. We clarified our manuscript as follows:

[…] 2.12 […] NOTE: For the fixation and dehydration in step 2.10, 2.11 and 2.12, the samples are left at 4°C without further stirring or rocking. […]

*[…] 3.7 […] NOTES: **#1**. For the blocking, permeabilization, antibodies incubation and DAPI staining in step 3.1, 3.2, 3.4 and 3.6, leave the samples at steady state in the 4°C, no stirring is required. […]*

5) For the dissection of SAN and AVN starting from whole heart, it would be helpful to include a cartoon diagram of the heart to show which regions are pinned to the petri dish along with the dissection plane. The images from the dissection microscope are difficult to visually in 3D space.

Thank you for the comments. We added an illustration as Figure 2 to make our microdissection procedures easier to understand.

6) It was not clear from the protocol that the SAN and AVN images would be derived from the same piece of tissue. Please clarify this point earlier in the text.

Thank you very much for this comment. We used the same heart for the imaging of both SAN and AVN. In order to avoid the misunderstanding, we clarified our manuscript as follows:

[…] 4.2 Loading the heart. The same heart sample can be used for imaging of both SAN and AVN sequentially. For SAN imaging the heart can be directly loaded whereas for AVN imaging microdissection has to be performed before. […]

7) The authors state that "confocal laser scanning microscopy" was used to capture images, but no details are provided. I would suggest that at least some details regarding instrument specifications are added to the protocol, and a discussion of the various types of confocal imaging should be provided in the Discussion section. Also, are there special considerations during image acquisition to enable proper 3D reconstruction by the Imaris software package?

The reviewer's comments are highly appreciated. As the image analysis is beyond the scope of the protocol, we limited the length of this part in our manuscript. However, we believe that adding some more details will make the manuscript easier to understand. And indeed, this specific issue about confocal imaging and usefulness of 3D construction was not extensively discussed in our original manuscript. We improved our manuscript and discussed the confocal imaging by adding the following paragraph:

Protocol section:

[…] 4.6 For taking images, we use the Carl Zeiss LSM800 with Airyscan Unit and the software ZEN 2.3 SP1 black.

4.7 Plate "BP420-480 + LP605" was chosen for the excitation of Alexa Fluor 647 conjugated anti-HCN4. The master gain varied from 650-750.

4.8 An overview image of the whole SAN and AVN region is taken by using "Tile Scan" function. Then the HCN4-positive region is selected by clicking and adding a square on the overview image around the area of interest that will be scanned.

4.9 The parameters, including plates and master gain for the remaining channels (Alexa Fluor 488 and DAPI) of the confocal microscope are also checked and set as described in step 4.7.

4.10 The focus is slowly adjusted from top to bottom of the sample to preview the whole sample and to set the "First" and "Last" for the Z-stack range. An optimal interval for the Z-stack based on the thickness of the optical sample is set. We use an 20x objective, and 0.8-1 µm as the interval for Z-stack.

4.11 After all the parameters are properly set, the whole area of the SAN and AVN is scanned.

4.12 3D reconstruction of the images is done using the software Imaris version 8.4.2.

4.12.1 "Images processing-> baseline subtraction" is used to remove background staining.

4.12.2 Surface creation onto setting for selected channel and region of interest is selected for processing.…]

Discussion section:

[…] We used the confocal laser-scanning microscope (LSM) with the Airyscan detector, which can offer a distinct advantage in obtaining images with superior quality. Using a widefield imaging microscope can only offer tissue-level information but lacks cellular resolution. On the other hand, the widefield microscope carries a risk of high background.[12] By using Airyscan detector from ZEISS for confocal LSM, improved resolution and signal-to-noise ratio (SNR) can be acquired, compared to the traditional one pinhole-and-detector confocal imaging system.[13] […]

[…] 3D reconstruction can be difficult to achieve and may require intensive troubleshooting to optimize the procedure. For example, optimal intervals have to be set during image acquisition since sections spaced too far apart will leave gaps and might not allow an adequate 3D reconstruction of the anatomy. Sections that are too close may cause oversampling and bleaching due the excessive illumination and will take a long time to complete one image. Finally, (v) the major limitation of confocal microscopy is the imaging depth which means that if samples' thickness is beyond the maximum operating depth of the confocal microscope, the further fluorophore cannot be detected. […]

And two references were added:

12 Shaw, P. J. in Handbook Of Biological Confocal Microscopy 10.1007/978-0-387-45524-2_23 (ed James B. Pawley) 453-467 (Springer US, 2006).

13 Huff, J. The Airyscan detector from ZEISS: confocal imaging with improved signal-to-noise ratio and super-resolution. Nature Methods. 12 (12), i-ii, (2015).

Reviewer #2:

Manuscript Summary:

The authors present a detailed method for locating the sinoatrial node (SAN) and atrioventricular node (AVN) in intact cardiac tissue, thus allowing the study of these regions within their 3D anatomical structure. The authors compare this method to 2D imaging techniques, which require sectioning tissue in thin slices and eliminates both anatomical markers for locating the SAN or AVN, and the potential to study their relationship to any surrounding tissue. The authors detail how to harvest the tissue, identify the relevant structures, and stain and image the regions of interest.

Major Concerns:

Discussion on the relevance or usefulness of the 3D reconstructions is lacking, despite the 3D assessment of the SAN and AVN being critical components of the authors' justification for the whole-mount preparation. Panel C of Figures 4 and 5 are the most visually-striking in the protocol, and the discussion should include how those 3D reconstructions could be leveraged.

We thank the reviewer for pointing towards this important issue. To address this issue, we added a paragraph to the discussion:

Discussion Section:

[…]The shapes and position of SAN and AVN are presented as 3D reconstruction and additional virtual section planes derived from 3D reconstruction can enhance the interpretation of histological sections, whereas conventional histology allows only a 2D assessment of the anatomy with the inherent risk of non-detection of small but relevant structures. Moreover, 3D reconstruction provides the opportunity to examine anatomical structures of interest transmurally and within the intact microenvironment, for example intrinsic autonomic nervous plexi innervating the heart.[14] Whole-mount in situ staining and 3D reconstruction allows to investigate the cellular environment as well as the specific cell types and their orientation. Furthermore, regional and cell-type specific protein expression can be visualized and may further support western blot and proteomics analyses.[15] […]

[…]3D reconstruction can be difficult to achieve and may require intensive troubleshooting to optimize the procedure. For example, optimal intervals have to be set during image acquisition since sections spaced too far apart will leave gaps and might not allow an adequate 3D reconstruction of the anatomy. Sections that are too close may cause oversampling and bleaching due the excessive illumination and will take a long time to complete one image. Finally, (v) the major limitation of confocal microscopy is the imaging depth which means that if samples' thickness is beyond the maximum operating depth of the confocal microscope, the further fluorophore cannot be detected. […]

And two references were added:

14 Rysevaite, K. et al. Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations. Heart Rhythm. 8 (5), 731-738, (2011).

15 Acar, M. et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature. 526 (7571), 126-130, (2015).

This reviewer does not identify any instances of scientific inaccuracy, and commends the authors for assembling a rigorous and detailed protocol. The following comments focus on avoiding confusion in following the protocol.

While the authors generally demonstrate a strong command of the English language, a thorough proofread is necessary to avoid instances of confusing language. Examples of such include:

- Line 80: "is a method that allows to study"

Thank you for the considerable comment. The manuscript has modified as followes:

[…] Whole-mount immunofluorescence staining is a method used to study anatomical structures in situ while preserving the integrity of the surrounding tissue. […]

- Line 127-128: "The appropriate antibody dilution should be tested with comparable tissue samples performing a conventional immunofluorescent staining." (This reviewer assumes the authors mean, "by performing.")

The manuscript has modified accordingly.

Line 217: Please clarify the phrase "a few drops." Is the goal to displace all air within the cavity?

We thank the reviewer for pointing to that issue. The reviewer is correct, one goal is to remove all the air from the cavity, but another goal is to fully cover the sample. To avoid any misunderstanding changes were made in Step 4.2.1.2 and step 4.2.2.3 as follows:

[…] 4.2.1.2 […]PBS is added onto the heart to displace all air within the cavity until the heart is fully covered with PBS (usually a few drops of PBS are sufficient). […]

[…] 4.2.2.3 […] PBS is added onto the heart to displace all air within the cavity until the heart is fully covered with PBS (usually a few drops of PBS are sufficient). […]

Line 251: Please clarify how much the heart should be "squished," or such squishing avoided, when attaching the coverslip. Is a flat imaging plane necessary or vital for proper imaging, and in which direction should the user err (i.e. Too deep a cavity, or too shallow in 4.1 - line 208)?

Thank you for the comment. The heart should not be squished too much, as long as there are no air bubbles between the sample and the coverslip. Based on our experience, a flat imaging plane will

increase the efficiency of confocal imaging. In order to clarify the sample loading, we rephrase the steps as follows:

[…] 4.1 […] NOTE: We recommend to make a shallow groove, as this would be easier to acquire a flat imaging plane, and to adjust the space and avoid air bubbles between the sample and the coverslips in step 4.4. […]

[…] 4.4 […] NOTE: Make sure that the regions of interest are not folded and covered during pressing the back side of the plasticine. A flat imaging plane without sample overcompression is necessary for conserving the anatomy and for proper confocal imaging of the samples.. […]

Line 272: Figures for SAN and AVN are misidentified as Figs. 3 and 4, should be Figs. 4 and 5, respectively.

We apologize for the mistake and corrected figure labels.

Minor Concerns:

Figure 2: This reviewer appreciates the difficulty of identifying anatomical structures on a mouse heart under magnification, and notes the authors' excellent use of notation to identify relevant regions. Still, the images are somewhat disorienting. Perhaps a 3D cartoon model of the full heart in the relevant orientation for A/B would be helpful in better orienting the reader.

We agree with the reviewer that a 3D cartoon would be very helpful to help the reader understanding the anatomy of SAN and AVN. However, since we are not professional graphic designers we are not able to draw a 3D comic. Instead of a 3D comic we added a 2D illustration as new Figure 2 to our manuscript. We hope that this figure will also improve orientation. Furthermore, the video that will be linked to the article will show the whole process of the microdissection and will therefore allow the reader/viewer to easily understand where exactly SAN and AVN are located.

Line 136: Please clarify how achievement of full anesthesia is confirmed while in incubation chamber (Unlikely to be a concern for experienced mouse handlers, but should be noted nonetheless).

Thank you for pointing to this issue. We clarified this by adding a NOTE to this step:

[…] 2.2 […] NOTE: Full anesthesia is confirmed by the loss of the postural reaction and righting reflex by gently rolling the chamber until the mouse is placed on its back. […]

Line 204: (3. NOTE): Is there a maximum recommended time frame before sample/signal degradation, or is "a few days" simply the extent of the authors' experience storing the samples?

Thank you for the comment. The time frame we mentioned for the preservation of stained samples is derived from our experience working with sections preserved with mounting medium, which can help to prevent the degradation of the fluorophore. For those sections, they can be stored at 4°C for several months.

For whole-mount stained samples we unfortunately cannot provide an exact time frame until the samples are preserved. Since the whole-mount stained samples are kept in PBS at 4°C we can only recommend to scan the sample as soon as possible.

Line 263: It may be helpful for the authors to note the laser intensity and/or gain used to acquire the images, as a starting/reference point for the user.

Thank you for the comment. Required laser intensity depends on the concentration of antibodies used for staining. For our manuscript, we diluted the antibodies 1:200. If lower antibody concentration is used, a higher laser intensity for the imaging might be necessary. We added the parameters we used for the confocal imaging as follows:

[…] 4.7 Plate "BP420-480 + LP605" was chosen for the excitation of Alexa Fluor 647 conjugated anti-HCN4. The master gain varied from 650-750.

4.8 An overview image of the whole SAN and AVN region is taken by using "Tile Scan" function. Then the HCN4-positive region is selected by clicking and adding a square on the overview image to around the area of interest that will be scanned.

4.9 The parameters, including plates and master gain, for the remaining channels (Alexa Fluor 488 and DAPI) of the confocal microscope are also checked and set as in step 4.7. […]

Line 266: While step-by-step instructions of Imaris software are beyond the scope of this protocol, it would be helpful for the authors to include a useful resource for the software in a note following 4.9.

Thank you very much for the comment. Indeed, a detailed description how to use Imaris software is beyond the scope of this protocol. However, we would like to offer a few simple steps for using Imaris and added the following paragraphs:

[…] 4.12 3D reconstruction of the images is done using the software Imaris version 8.4.2.

4.12.1 "Images processing-> baseline subtraction" is used to remove background staining.

4.12.2 Surface creation onto setting for selected channel and region of interest is selected for processing. […]

Reviewer #3:

Manuscript Summary:

The authors describe a whole-mount procedure to immune-label the two key "upstream" components of the cardiac conduction system (CCS), the SA and AV nodes. The staining looks great, the technique is definitely one that people in the field will want to learn how to do, and overall I think that this is a great contribution to the JoVE stable of videos.

We thank the reviewer for this very kind comment.

Major Concerns:

1) The title of the paper refers only to the whole-mount staining, but clearly the microscopy and the subsequent 3D-reconstructions associated with it are mentioned at several points in the text:

a) In the abstract

b) In the introduction, where confocal microscopy is even specified as the fourth and final goal of the protocol

c) in the protocol

d) in the figures, results are shown

However, the description of both the confocal procedure and the 3D reconstruction is limited to 2 sentences? Points 4.8 and 4.9, respectively. I will leave it up to the JoVE editors how to deal with this, but clearly one would not be able to reproduce the entire protocol without having more details about both the confocal conditions as well as the parameters chosen for 3D reconstruction. In addition, the

authors should submit a movie that will allow the viewer to appreciate the 3rd dimensionality of their reconstruction, this is standard practice.

We thank the reviewer for pointing towards this important issue. To make the title more consistent with the abstract and the content of the manuscript, we replace our title with the following one:

TITLE:

Whole-Mount Immunofluorescence Staining, Confocal Imaging and 3D Reconstruction of the Sinoatrial and Atrioventricular Node in the Mouse

Furthermore, we added some more paragraphs about confocal microscopy to the protocol:

PROTOCOL:

[…] 4.6 For taking images, we use the Carl Zeiss LSM800 with Airyscan Unit and the software ZEN 2.3 SP1 black.

4.7 Plate "BP420-480 + LP605" was chosen for the excitation of Alexa Fluor 647 conjugated anti-HCN4. The master gain varied from 650-750.

4.8 An overview image of the whole SAN and AVN region is taken by using "Tile Scan" function. Then the HCN4-positive region is selected by clicking and adding a square on the overview image around the area of interest that will be scanned.

4.9 The parameters, including plates and master gain for the remaining channels (Alexa Fluor 488 and DAPI) of the confocal microscope are also checked and set as described in step 4.7.

4.10 The focus is slowly adjusted from top to bottom of the sample to preview the whole sample and to set the "First" and "Last" for the Z-stack range. An optimal interval for the Z-stack based on the thickness of the optical sample is set. We use an 20x objective, and 0.8-1 µm as the interval for Z-stack.

4.11 After all the parameters are properly set, the whole area of the SAN and AVN is scanned.

4.12 3D reconstruction of the images is done using the software Imaris version 8.4.2.

4.12.1 "Images processing-> baseline subtraction" is used to remove background staining.

4.12.2 Surface creation onto setting for selected channel and region of interest is selected for processing. […]

We also added two movies as suggested by the reviewer.

2) It would be useful for those in the audience who do have experience with antibody stainings to comment upon / explain the various concerns:

1) High triton concentration: 1% incubation with both primary and secondary antibodies is a very high concentration.

We thank the reviewer for the comment. Triton X-100 is the most popular detergent for improving the penetration of the antibody. According to the datasheet the recommended working concentration of Triton X-100 is 0.1–0.5% (v/v, in PBS) for permeabilization. For staining of frozen sections we also use 0.5%, but for whole-mount staining a higher concentration of 1% helps to obtain more efficient antibody staining as demonstrated before.[2] Therefore, we added a NOTE to our protocol:

PROTOCOL:

[…] 3.2*[…] NOTE: […] #3. Higher concentration of Triton X-100 may help obtain more efficient antibody staining as demonstrated before[10], but concentration might be determined individually. […]*

REFERENCES:

10 Hulsmans, M. et al. Macrophages Facilitate Electrical Conduction in the Heart. Cell. 169 (3), 510-522 e520, (2017).

2) Autofluorescence of myocardial tissue: Myocardial tissue can deliver a very high level of autofluorescence, but the images the authors present show very little autofluorescence.

The reviewer mentioned a valid point. We appreciate this comment. We did observe autofluorescence signals showing the morphology of myocardial tissue when we scanned the samples. For that reason, we use the "baseline subtraction" during the image analysis with the Imaris software to remove the background staining of the images in order to show the SAN and AVN regions more prominent.

We assume that the "very little autofluorescence" the reviewer mentioned refers to the clean background showed in **Figure 5.** This, however, is the plasticine used for covering the connecting tissue around AVN, which does not show any fluorescence.

3) Could points 1) and 2) be connected to each other?

According to our experience with various triton concentrations (0.5% for frozen sections, 1% for whole-mount staining) we could not see a clear correlation between triton concentration and the degree of autofluorescence. A literature research on this topic also did not show any report on that. So, although a potential relationship is very unlikely, it cannot be fully excluded at the moment.

4) Concentration of antibodies seems to be high for both primary and secondary antibodies, could the authors comment upon this?

According to the information on the datasheets of the antibodies, we tested the optimal concentration of each antibody with frozen sections. During these initial experiments it turned out that only with higher antibody concentration (higher than 1:400) appropriate images could be obtained. Therefore we decided to use higher concentrations of antibodies than recommended by the manufacturer.

5) Showing the results of a control staining without primary antibody?

Thank you for the comments. We added an additional Figure 7 showing a control staining without primary antibody

3) What about the entire downstream portion of the cardiac conduction system? The authors do not even mention the bundle of His, the Purkinje fibers, the moderator band, etc etc. I can imagine that readers of this article would be very interested, potentially, in seeing whether this whole mount method is effective for these downstream components of the CCS. At least at late embryonic stages, HCN4 should label the entire CCS.

The reviewer's comment is highly appreciated. Indeed, the bundle of His and Purkinje fibers are also important parts of the cardiac conduction system (CCS), but are not the focus of our group and therefore, we have not sufficient experience in microdissecting bundle branches for example. Therefore, we thought it might be more appropriate to focus on our research field and demonstrate the technique on SAN and AVN as an example. Although we did not try so far, we believe that whole-mount staining of lower CCS parts is possible as well but will need an optimized microdissection of the respective parts, as the major limitation of confocal is the imaging depth.

Minor Concerns:

1) Although the anatomical terminology used by the authors is going to be familiar to clinicians and those who study heart function, it is going to be lost on everyone else. From an American perspective, you won't even find the triangle of Koch or the tendon of Todaro in standard anatomy textbooks, that

is really a cardiologist's specialty term. But as far as I understand the protocol, the user will need to really understand the location of specialized structures in order to properly dissect and then mount the heart specimens for proper imaging. Not sure how specialized the lingo can get in a broad-interest journal like JoVE. The authors could point out these structures in their video of course.

We thank the reviewer for the comment. Indeed, we admit that this microdissection procedure might be difficult for researchers who do not specialize in cardiology or the anatomy of the cardiovascular system. Therefore, we hope that our protocol in combination with the video could help a little bit for clarifying the complex cardiac anatomy.

1 Pinto, A. R., Chandran, A., Rosenthal, N. A. & Godwin, J. W. Isolation and analysis of single cells from the mouse heart. *J Immunol Methods.* **393** (1-2), 74-80, (2013).

2 Hulsmans, M. *et al.* Macrophages Facilitate Electrical Conduction in the Heart. *Cell.* **169** (3), 510-522 e520, (2017).