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TITLE:

Isolation and Culture of Resident Cardiac Macrophages from the Murine Sinoatrial and Atrioventricular Node

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KEYWORDS:

cardiac conduction system, sinoatrial node, atrioventricular node, resident cardiac macrophages, flow cytometry, magnetic activated cell sorting, MACS, fluorescence-activated cell sorting, microdissection, primary cell culture

SUMMARY:

The protocol presented here provides a step-by-step approach for the isolation of cardiac resident macrophages from the sinoatrial node (SAN) and atrioventricular node (AVN) region of mouse hearts.

ABSTRACT:

Resident cardiac macrophages have been demonstrated to facilitate the electrical conduction in the heart. The physiologic heart rhythm is initiated by electrical impulses generated in sinoatrial node (SAN) and then conducted to ventricles via atrioventricular node (AVN). To further study the role of resident macrophages in cardiac conduction system, a proper isolation of resident macrophages from SAN and AVN is necessary, but it remains challenging. Here, we provide a protocol for the reliable microdissection of the SAN and AVN in murine hearts followed by the isolation and culture of resident macrophages.

Both, SAN which is located at the junction of the crista terminalis with the superior vena cava, and AVN which is located at the apex of the triangle of Koch, are identified and microdissected. Correct location is confirmed by histologic analysis of the tissue performed with Masson's trichrome stain and by anti-HCN4.

Microdissected tissues are then enzymatically digested to obtain single cell suspensions followed by the incubation with a specific panel of antibodies directed against cell-type specific surface markers. This allows to identify, count, or isolate different cell populations by fluorescent activated cell sorting. To differentiate cardiac resident macrophages from other immune cells in the myocardium, especially recruited monocyte-derived macrophages, a delicate devised gating strategy is needed. First, lymphoid lineage cells are detected and excluded from further analysis. Then, myeloid cells are identified with resident macrophages being determined by high expression of both CD45 and CD11b, and low expression of Ly6C. With cell sorting, isolated cardiac macrophages can then be cultivated in vitro over several days for further investigation. We, therefore, describe a protocol to isolate cardiac resident macrophages located within the cardiac conduction system. We discuss pitfalls in microdissecting and digesting SAN and AVN, and provide a gating strategy to reliably identify, count and sort cardiac macrophages by fluorescence-activated cell sorting.

INTRODUCTION:

The sinoatrial node (SAN) physiologically initiates the electrical impulse and is, therefore, the primary pacemaker of the heart. The atrioventricular node (AVN) conducts the electrical impulse from the atria to the ventricles and also acts as a subsidiary pacemaker[1]. In general, generation and conduction of electrical impulses is a complex process that can be modulated by various factors[2], including resident macrophage in SAN/AVN regions. A recent study by Hulsmans et al. demonstrates a specific population of cardiac resident macrophages which are enriched in the AVN and function as key players in keeping a steady heartbeat[3]. They found that macrophages are electrically coupled to the cardiomyocytes and could change the electrical properties of coupled cardiomyocytes. The authors also note that such conducting cells interleaving with macrophages are also present in other components of the cardiac conduction system, such as the SAN.

Currently, it is not fully known if the phenotype of resident cardiac macrophages differs between the cardiac regions. However, it has been shown that the tissue microenvironment can affect transcription and proliferative renewal of tissue macrophages[4]. Furthermore, since the cardiomyocyte phenotype has been demonstrated to be different between regions, the functional effects of macrophages on cardiomyocytes may also be region-specific, even if the macrophage phenotype itself may be the same. Therefore, further studies on specific cardiac regions are needed.

Recent studies have demonstrated that, at steady state, the tissue resident macrophages are established prenatally, arising independently of definitive hematopoiesis, and persist into adulthood[5]. However, after macrophage depletion or during cardiac inflammation, Ly6c^{hi}

monocytes contribute to replenish cardiac macrophage population[6]. Studies involving genetic lineage tracing, parabiosis, fate mapping, and cell tracking showed the coexistence of a variety of tissue resident macrophages populations in organs and tissues, and, also, different cellular behavior of macrophage subsets that are potentially associated with their ontogeny[7-9].

Characterization of resident cardiac macrophages has benefited from the use of magnetic activated cell sorting (MACS) and fluorescent activated cell sorting. These methods are particularly useful for isolating specific cell populations from multiple tissue fractions by labeling them with their cell surface markers. This not only leads to a higher purity of the isolated immune cell type, but also allows for phenotypic analysis. Here, we present a protocol including magnetic beads-coated cells followed with fluorescent activated cell sorting for the enrichment of cardiac resident macrophages specifically isolated from the SAN and AVN region.

To explore the characteristics of cardiac resident macrophages in conduction system and their function for cardiac conduction and arrhythmogenesis, precise localization and dissection of SAN and AVN are critical. For microdissection of SAN and AVN, anatomical landmarks are used for the region identification[10]. In brief, SAN is located at the junction of the superior vena cava and right atrium. AVN is located within the triangle of Koch, which is anteriorly bordered by the septal leaflet of the tricuspid valve, and posteriorly by the tendon of Todaro[11]. We also provide an accurate microdissection procedure of SAN and AVN in mice which is confirmed by histology and immunofluorescence staining.

Isolated resident macrophages could be used for further experiments such as RNA sequencing or could be recovered and cultivated for more than two weeks allowing various in vitro experiments. Therefore, our protocol describes a highly valuable procedure for the immuno-rhythmologist. **Table 1** shows the composition of all the solutions needed, **Figure 1** shows the microdissection landmarks for SAN and AVN. **Figure 2** is schematic illustration of SAN and AVN localization. **Figure 3** shows the histological staining of SAN and AVN (Masson's trichrome and immunofluorescence staining). **Figure 4** shows a step-by-step gating strategy to isolate cardiac resident macrophages by fluorescence-activated cell sorting.

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. C57BL6/J mice were commercially obtained.

1. Preparations

1.1 Prepare Cell sorting buffer (**Table 1**) and store at 4 °C.

NOTE: During the whole experimental procedure, the cell sorting buffer should always be on ice.

1.2 Prepare Digestion buffer **(Table 1)** shortly before the digestion as the activity of

collagenase could only be detected for few hours at room temperature.

1.3 Refer to the previously published protocol for the preparation of dissection dish[10]. In brief, add 30 mL of agarose gel (3%-4%) into a 100 mm diameter Petri dish and cool down at room temperature.

2. Animal sacrifice and heart excision

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

2.2 After the injection of fentanyl for analgesia, open the rib cage and perfuse the heart by injecting 5-10 mL of ice-cold 1x PBS directly into the left ventricle (LV). Extract the mice heart and put it on the dissection dish. Experimental details have been described in detail previously[10].

3. Microdissection of SAN and AVN

3.1 After isolating the heart, perform the following microdissection procedures in the dissection dish with ice-cold 1x PBS under the dissecting microscope.

3.2 Use the cardiac anatomical landmarks, i.e., aorta, pulmonary artery, coronary sinus, left/right ventricle, etc. to determine the left/right (left: LV; right: RV) and anterior/posterior (anterior: aorta; posterior: coronary sinus) of the heart. After the orientation is determined, turn around the heart with the front of it at the bottom of the dish (to expose the large veins that are located posterior).

3.3 Microdissection of SAN

NOTE: Microdissection of the SAN have been previously described[10]. The process is described in brief below.

3.1. Expose the inter-caval region by pining the right atrial appendages (RAA) and the tissue adjacent to superior vena cava (SVC) and inferior vena cava (IVC) on the microsection dish using insect pins.

3.2. Cut the heart along the interatrial septum parallel to the crista terminalis (CT) to separate the inter-caval region and to obtain the SAN sample (**Figure 1A, Figure 2A**). Put the sample in an empty 1.5 mL microcentrifuge tube on ice.

3.4 Microdissection of AVN

3.4.1 After collection of the SAN sample ensure that the RAA and parts of the right atrium (RA) have already been cut away leaving only the interatrial septum (IAS) and, interventricular septum (IVS).

3.4.2 Pin the remaining parts of the heart through the tissue adjacent to the IAS and IVS using insect pins to make the right atrial side of IAS facing up.

3.4.3 Look at the right atrium on the endocardial surface for the triangle of Koch. It will be bordered anteriorly by the hinge-line of the septal leaflet of the tricuspid valve (TV), and posteriorly by the tendon of Todaro. The orifice of the coronary sinus is observed at the base. (**Figure 1B, Figure 2B**).

3.4.4 Cut the triangle of Koch, which contains the AVN, and directly put it in an empty 1.5 mL microcentrifuge tube on ice.

4. Digestion

4.1 Prepare the Digestion buffer **(Table 1)** shortly before use.

4.2 Mince the SAN and AVN tissue well with scalpels.

NOTE: Mincing the tissue well will increase the digestion efficiency and help to get good cell suspension for sorting. As the SAN and AVN samples are quite small, mincing the tissue directly inside the 1.5 mL microcentrifuge tube is recommended to reduce the loss of sample.

4.3 Add 500 µL of digestion buffer per sample and wash down all minced tissue from the wall of the 1.5 mL microcentrifuge tube. Gentle pipetting helps digesting the sample.

4.4 Homogenize the tube on a vortex machine (settings: 37 °C, 750 rpm for 1 h).

4.5 After digestion, transfer the tissue suspension to a fresh 15 mL centrifuge tube by passing through a 40 µm cell strainer. Rinse the cell strainer with an additional 5 mL of cell sorting buffer to stop the digestion.

4.6 Centrifuge the 15 mL tube at 350 x *g* for 7 min at 4 °C. Then remove the supernatant completely using the pipette. Resuspend the cell pellet with 90 µL cell sorting buffer.

NOTE: Before magnetic separation, pipette the cell suspension gently a few times or pass through a 30 µm cell strainer to remove cell clumps if necessary, to obtain a single cell suspension for optimal performance of magnetic enrichment of interesting cell populations.

5. Magnetic enrichment of CD45 and sample staining

NOTE: To isolate the cardiac macrophages with high sorting efficiency, exclusion of undesired cells including lymphocytes was performed with CD45 microbeads according to the manufacturer's protocol. Based on the sorting panel, cardiac resident macrophages were identified as $CD45^{high}CD11b^{high}CD64^{high}$ $Ly6C^{low/int}$ $F4/80^{high}$.

5.1 Add 10 µL of CD45 microbeads per 10^7 total cells to the cell suspension in the 15 mL centrifuge tube. Mix the samples well and incubate them for 15 min at 4 °C.

NOTE: The cell counting using a hemocytometer should be briefly done to make sure that each tube contains no more than 10^7 total cells. When working with higher cell numbers, the volume of magnetic beads needs to be scaled up.

5.2 Prepare the antibody mixture by diluting the following antibodies in cell sorting buffer (1:100 dilution for each antibody): CD45-PE, CD11b-APC-Cy7, CD64-APC, F4/80-PE-Cy7, Ly6C-FITC. DAPI will be added later to the staining for live/dead discrimination.

5.3 After 15 min of magnetic bead incubation, add 100 µL of antibody mixture directly into the cell suspension in the 15 mL tube (then all the antibodies' final concentration is 1:200) and incubate for 20 min at 4 °C.

5.4 After 20 min of antibody incubation, wash the cell suspension by adding 1-2 mL of cell sorting buffer per 10^7 cells and centrifuge at 350 x *g* for 10 min. Completely remove the supernatant by pipetting.

5.5 Resuspend up to 10^8 cells in 500 µL of cell sorting buffer.

NOTE: The maximum cell number for magnetic separation should be determined according to the manufacturer's protocol.

5.6 Prepare the magnetic separation set.

5.6.1 Attach the magnetic column to a suitable magnetic separator and place a collection tube under the magnetic column.

5.6.2 Prepare the magnetic columns by rinsing with cell sorting buffer: add 500 µL of cell sorting buffer at the top of the column and let the buffer pass through.

5.7 Apply the cell suspension immediately onto the column while the cell sorting buffer is passing through.

NOTE: Avoid the formation of air bubbles in the column. As per the manufacture's protocol, although the column filling time might change from storage conditions, it has no influence on the quality of the separation.

5.8 Wash the column with 3 x 500 µL cell sorting buffer. The flow-through in step 5.7 and step 5.8 contain unlabeled cells, which can be discarded if no further experiment is needed.

NOTE: Add the cell sorting buffer immediately when the column reservoir is nearly empty.

5.9 Remove the column from the magnetic separator and place it on a new collection tube.

5.10 Add 1 mL of cell sorting buffer onto the column. Immediately flush the column by firmly applying the plunger supplied with the column. The flow-through contains the magnetically labeled cells.

5.11 Add DAPI solution into all the collected magnetically labeled cell suspensions shortly before running them on the cell sorter. Adjust the final concentration of DAPI to 0.3-0.5 µg/mL.

5.12 Perform FACS analysis.

6. Samples for compensation

6.1 Prepare 6 brown 1.5 mL microcentrifuge tubes labeled as "PE", "APC-Cy7", "APC", "PE-Cy7", "FITC", and "DAPI" respectively to protect antibodies from light. Prepare one more 1.5 mL microcentrifuge tube labeled as "unstained".

NOTE: This could be done at the same time as incubating the cell suspension with the microbeads and antibodies. The unstained sample could be the cardiomyocyte tissue collected randomly from the spared heart tissue and also treated according to step 4.

6.2 Dilute each single fluorescence-conjugated antibody with cell sorting buffer into 1:50 in the 1.5 mL brown microcentrifuge tubes that are marked accordingly.

6.3 Add one drop of compensation beads solution and incubate for 20 min at 4 °C.

6.4 Add 2 mL of cell sorting buffer into each 1.5 mL brown microcentrifuge tube and centrifuge at 450 x *g* for 5 min. Completely discard the supernatant and resuspend the bead containing pellet with 300 µL cell sorting buffer and transfer them into cell sorter tubes that are also marked accordingly.

7. Running on the cell sorter and gating strategy

7.1 Apply the unstained sample and compensation tubes first and adjust the voltages of each channel to align both the positive and negative peak to the proper position of the axis. Save the compensation settings and apply it to the following samples.

7.2 Apply the samples on the cell sorter. Set the gating strategy as described in **Figure 4.** Cardiac resident macrophages are identified as $CD45^{high}CD11b^{high}CD64^{high}Ly6C^{low/int}$ $F4/80^{high}$. DAPI is used as a cell viability marker.

7.3 Check the flow cytometry charts to confirm that the cell population of interest is properly shown on the charts. If not, adjust the voltage of each channel to the center view of each chart.

7.4 If the voltage settings are satisfactory, start the sorting procedure. Collect the sorted cell population into culture medium composed of DMEM containing 10% fetal bovine serum, supplemented with 100 µg/mL streptomycin and 100 U/mL penicillin.

8. Resident macrophages culture

8.1 After gathering the sorted macrophages, transfer the cells immediately either to 35 mm tissue culture dishes or 24 well plate, or directly use them for subsequent experiments.

8.2 To culture sorted macrophages, incubate the cells at 37 °C, 5% CO_2 incubator.

8.3 Change the culture medium every 48-72 h. Floating dead cells can be easily removed by medium change. Use live macrophages attaching to the bottom of culture dish for subsequent experiment.

REPRESENTATIVE RESULTS:

We describe a practical procedure for the isolation of cardiac resident macrophages specifically from the SAN and AVN region. To confirm a correct dissection, Masson's Trichrome staining and immunofluorescent HCN4-staining is performed (**Figure 3**)[12]. With this protocol, we could collect approximately 60,000 macrophages from one whole heart. **Figure 4** shows the gating strategy for sorting cardiac macrophages. Live resident cardiac macrophages were identified as $CD45^+CD11b^+F4/80^+CD64^+Ly6C^-$. **Figure 5** shows freshly sorted cardiac macrophages which were identified by their surface antigens CD45, F4/80 and CD11b. Freshly sorted cells were observed under brightfield view of microscope (**Figure 5A**). The sorted cells were positive for CD45 ($CD45^+$) when observed under the fluorophore-PE channel (**Figure 5B**). The same view of the sorted cells when observed under fluorophore-APC-Cy7 channel showed $CD11b^+$ phenotype (**Figure 5C**). The same view of the sorted cells when observed under fluorophore-PE-Cy7 channel showed $F4/80^+$ phenotype (**Figure 5D**). **Figure 5E** is the merged image obtained with the fluorescent microscope for the sorted cells. These triple positive cells were identified as cardiac resident macrophages. **Figure 6** shows isolated cardiac macrophages cultured in medium up to 6 days. White arrows indicate macrophages, black arrows indicated floating round-shape dead cells.

FIGURE AND TABLE LEGENDS:

Figure 1: Anatomy of the SAN and AVN under the dissection microscope. (**A**) Anatomy of the SAN under the dissection microscope. The location of the SAN is indicated by red dashed line within the inter-caval region (black dashed lines). (**B**) Anatomy of the AVN under the dissection microscope. This figure has been modified from previously published article[10]. 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; IAS, interatrial septum; RA, right atrium; RAA, right atrial appendage; RV, right ventricle; CT, Crista terminalis; IVS,

interventricular septum; OF, oval fossa. PA, pulmonary artery; RV, right ventricle; LV, left ventricle.

Figure 2: Schematic illustration of SAN and AVN localization. (**A**) schematic illustration of SAN localization. SAN is indicated by a red dashed circle within the inter-caval region besides the CT (black dashed line). (**B**) schematic illustration of AVN localization. AVN is indicated by a red dashed circle inside the Koch triangle (grey dashed triangle) composed of TV and the orifice of CS. SVC, superior vena cava; IVC, inferior vena cava; IAS, interatrial septum; RA, right atrium; RAA, right atrial appendage; CT, Crista terminalis; IVS, interventricular septum; OF, oval fossa; IVS, interventricular septum.

Figure 3: Identification of the SAN and AVN with histological stain. Identification of the SAN (**A**,**B**) and AVN (**C,D**)**.** Immunofluorescent staining of HCN4 positive conduction system cells in SAN (**A**) and AVN (**C**) as well as Masson's trichrome staining of SAN (**B**) and AVN (**D**). Red arrows indicate sinus node artery (SNA, **B**), black arrow and black dashed line indicate compact AVN, blue arrow indicates the central fibrous body (CFB). CT, crista terminalis; CFB, central fibrous body; CN, compact AVN; RA, right atrium; RV, right ventricle; IAS, interatrial septum; IVS, interventricular septum; TV, tricuspid valve; MV, mitral valve.

Figure 4: Gating strategy for cell sorting of resident cardiac macrophages. Mononuclear cells are identified, doublets are excluded by FSC-W vs. FSC-A and dead cells are excluded by DAPI (**A-D**). Live cells are gated on $CD45^+$ leukocytes (**E**), and then gated on $CD11b^+$ myeloid cells (**F**). Cardiac macrophages were identified by the expression of both F4/80 and CD64 (**G**), and then finally stratified by Ly6C expression (**H**). Live resident cardiac macrophages are identified as $CD45^+CD11b^+F4/80^+CD64^+Ly6C^-$. SAN, sinoatrial node; AVN, atrioventricular node; CT, crista terminalis; CFB, central fibrous body; CN, compact AVN; RA, right atrium; RAA, right atrial appendage; RV, right ventricle; IAS, interatrial septum; IVS, interventricular septum; TV, tricuspid valve; MV, mitral valve.

Figure 5: Freshly sorted cardiac macrophages and immunofluorescent staining. (**A**) Freshly sorted cardiac macrophages. Immunofluorescent staining of specific surface antigens such as CD45 (**B**), CD11b (**C**), or F4/80 (**D**). According to the gating strategy, cardiac macrophages are identified as triple positive cells (**E**). Scale bar represents 50 µm.

Figure 6: Culture of sorted macrophages. Culture of sorted macrophages in culture medium for 48h (**A, B**), 96 h (**C,D**) and 6 days (**E,F**) respectively. Two individual culture dishes per time point are shown (dish 1: A, C, E; dish 2: B, D, F). White arrows indicate live macrophages with spindle-like shape and typical protrusions[3]. Black arrows indicate floating round-shape dead cells.

Table 1: Composition of solutions needed.

DISCUSSION:

In this manuscript, we describe a protocol for the enrichment of cardiac resident macrophages specifically from the SAN and AVN regions at high purity.

Macrophages are divided into subpopulations based on their anatomical location and functional phenotype. They can also switch from one functional phenotype to another in response to variable microenvironmental signals[13]. Compared to other organs such as bone marrow and liver, cardiac tissue contains a lower percentage of immune cells and lower absolute numbers of each cellular subpopulation[14]. Therefore, cell sorting, enrichment and purification methods are necessary tools to obtain sufficient amounts of the cell population of interest. Fluorescence-activated cell sorting, and MACS allow to obtain pure, sorted cell populations as it permits simultaneous measurement of various properties of the cells.

Different flow cytometry panels have been described for the identification of subpopulations of cardiac macrophages[3,6,15]. The function of macrophages in steady state and disease not only depend on their developmental origin but also on the tissue environment. In general, the adult heart contains two major subsets of $Ly6C^{low}/CCR2^{-}$ resident macrophages that express different levels of MHC-II and which can maintain themselves via local proliferation at steady state whereas during disease classical $Ly6C^{high}$ monocytes are recruited to sites of inflammation, where they differentiate into macrophages[16]. During development, different subpopulations of macrophages occupy different cardiac locations associated with distinct functions[17]. We aimed to study the resident macrophages specifically from the cardiac conduction system, especially the resident macrophages from the SAN and AVN regions. According to Hulsmans et al. cardiac resident macrophages are identified as $CD45^{high}$ $CD11b^{high}CD64^{high}F4/80^{high}Ly6C^{low/int}$.

The harvest of cardiac resident macrophages from one adult mouse for cell sorting requires approximately 3 hours. It is important to arrange the experimental procedures logically and to allow incubation in parallel to save time and to minimize handling of the possibly fragile macrophages in the suspension. As the sorting procedure could exert pressure on the sorted cells, we recommended to reduce the sorting time by using magnetic beads which could increase the sorting efficiency tremendously and also allows to obtain higher purity of resident macrophages.

The application of this protocol includes but is not limited to purification of macrophages and/or any other non-cardiomyocyte cell type from the cardiac tissue and any other mice strains. The sorted macrophages could be used for subsequent experiments, for example cell motility assays, gene or protein expression studies, etc. Single-cell RNA sequencing is also possible by collecting cells one-by-one directly from the collecting tube of the cell sorter.

However, flow cytometry-based cell sorting has its limitations. A precisely designed antibody panel is important and must consider the expression of antigens on the cell population of interest and the fluorophore conjugated to the antibodies. Cells function and viability might be altered by the binding antibodies, which might affect the outcomes of subsequent experiments. In addition, the complex cell sorting instruments are expensive, sophisticated, and also prone to problems with fluidics system blockages and laser calibration. Hence maintenance by highly trained specialist and properly operation by an experienced professional technician are required. Even though cell sorting could provide a pure cell population of interest, the overall efficiency is still relatively low.

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DISCLOSURES:

No potential conflict of interest relevant to this article was reported.

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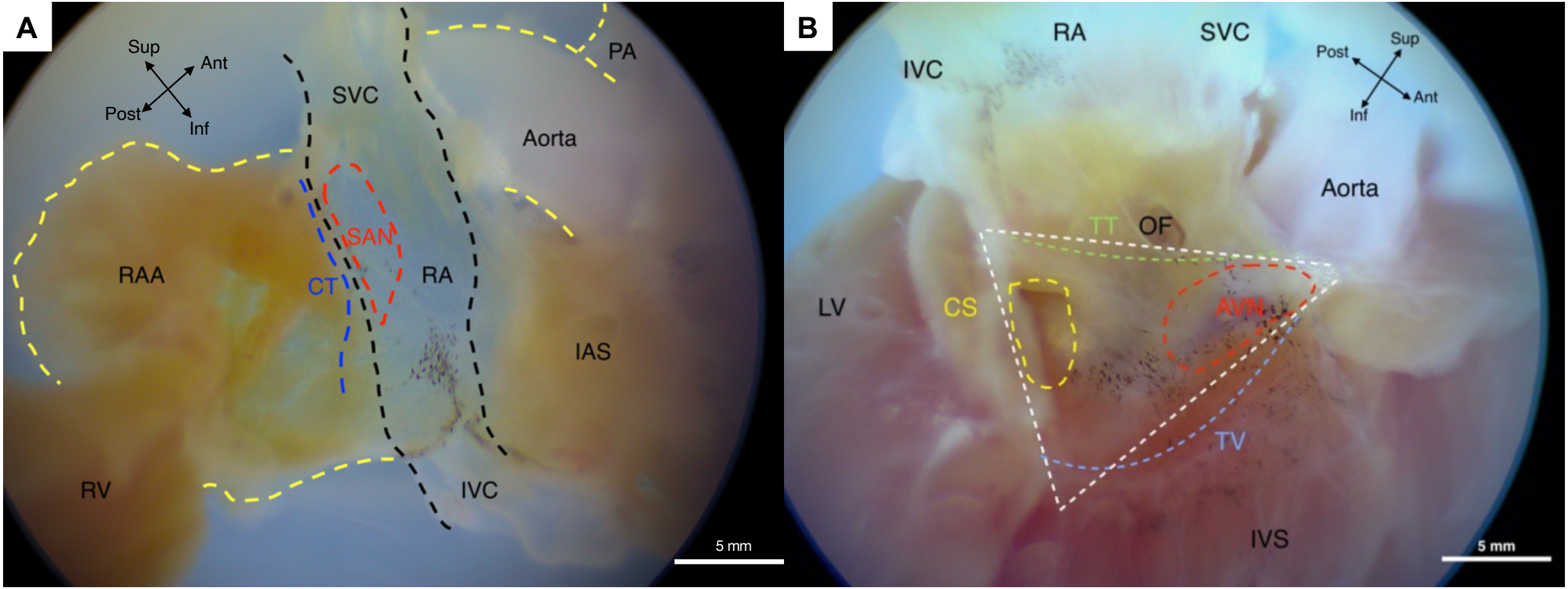
Figure 1.



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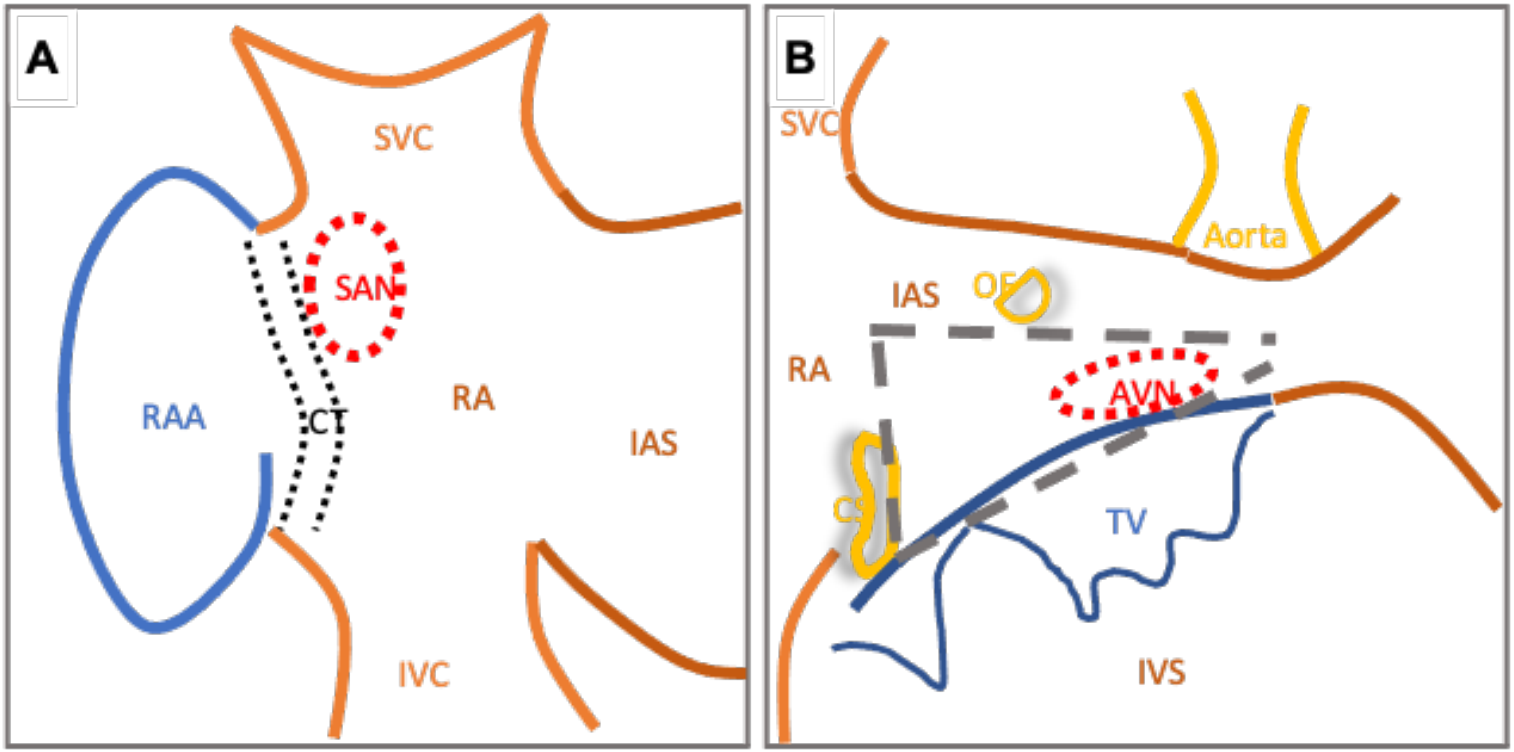

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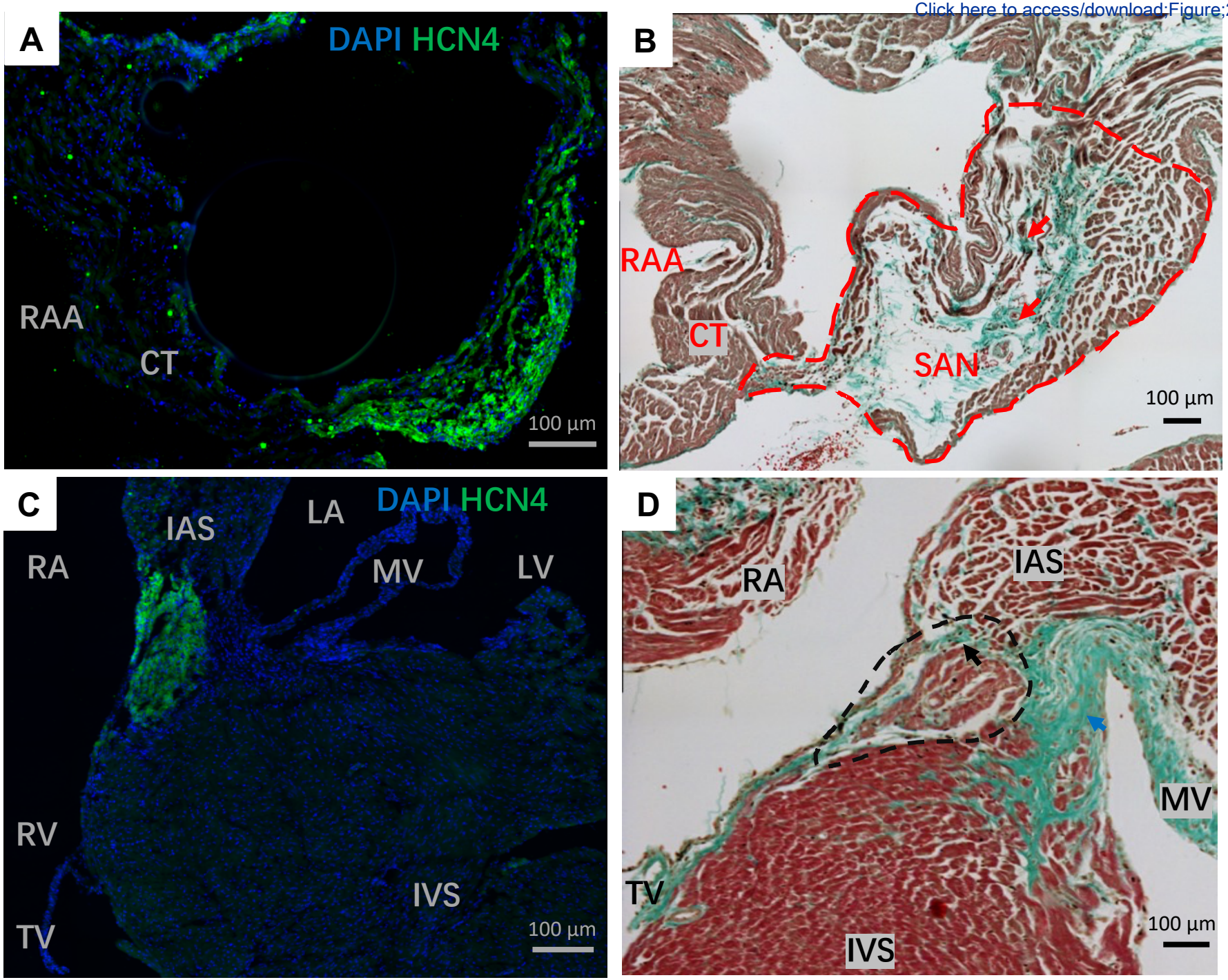

Figure 4.

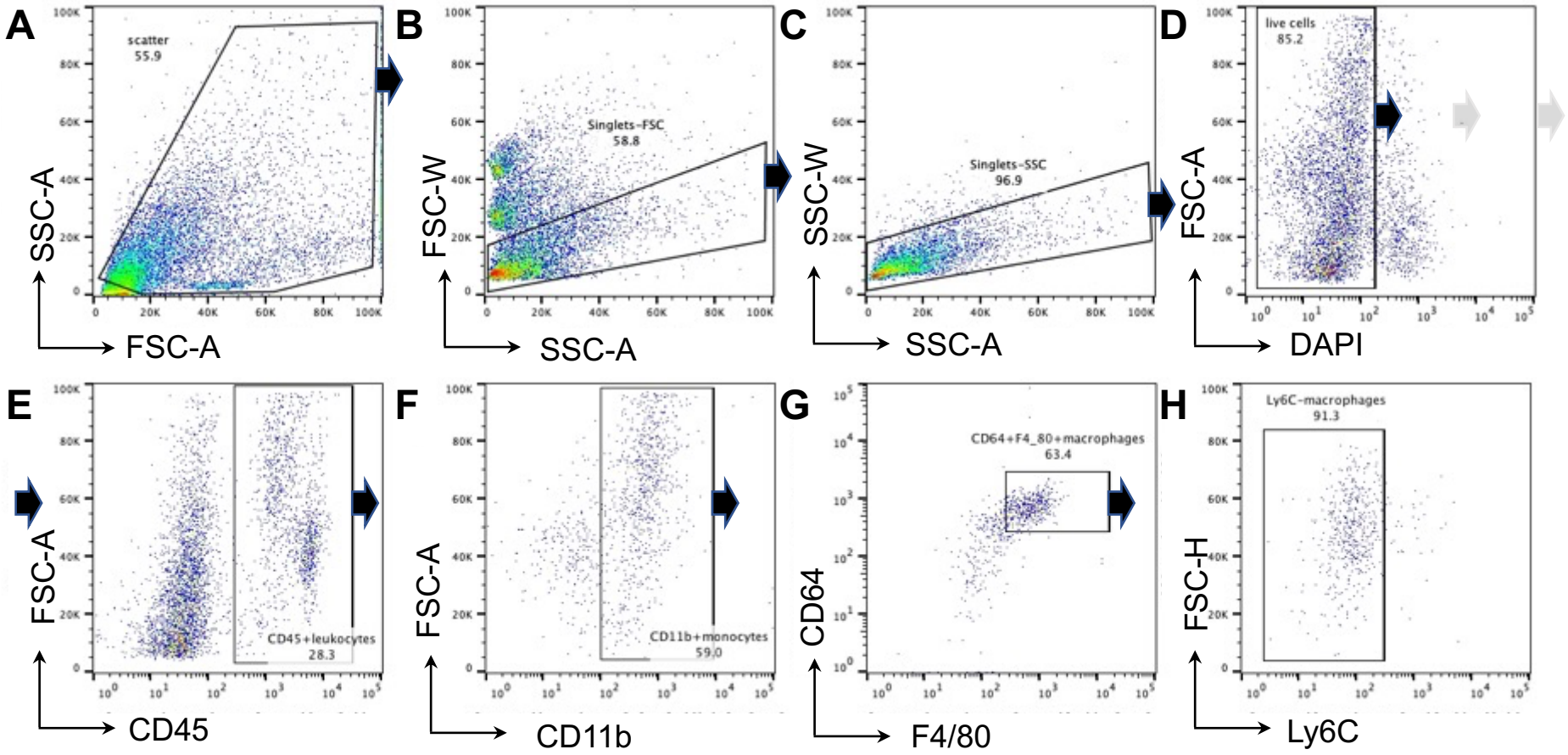

Figure 5.

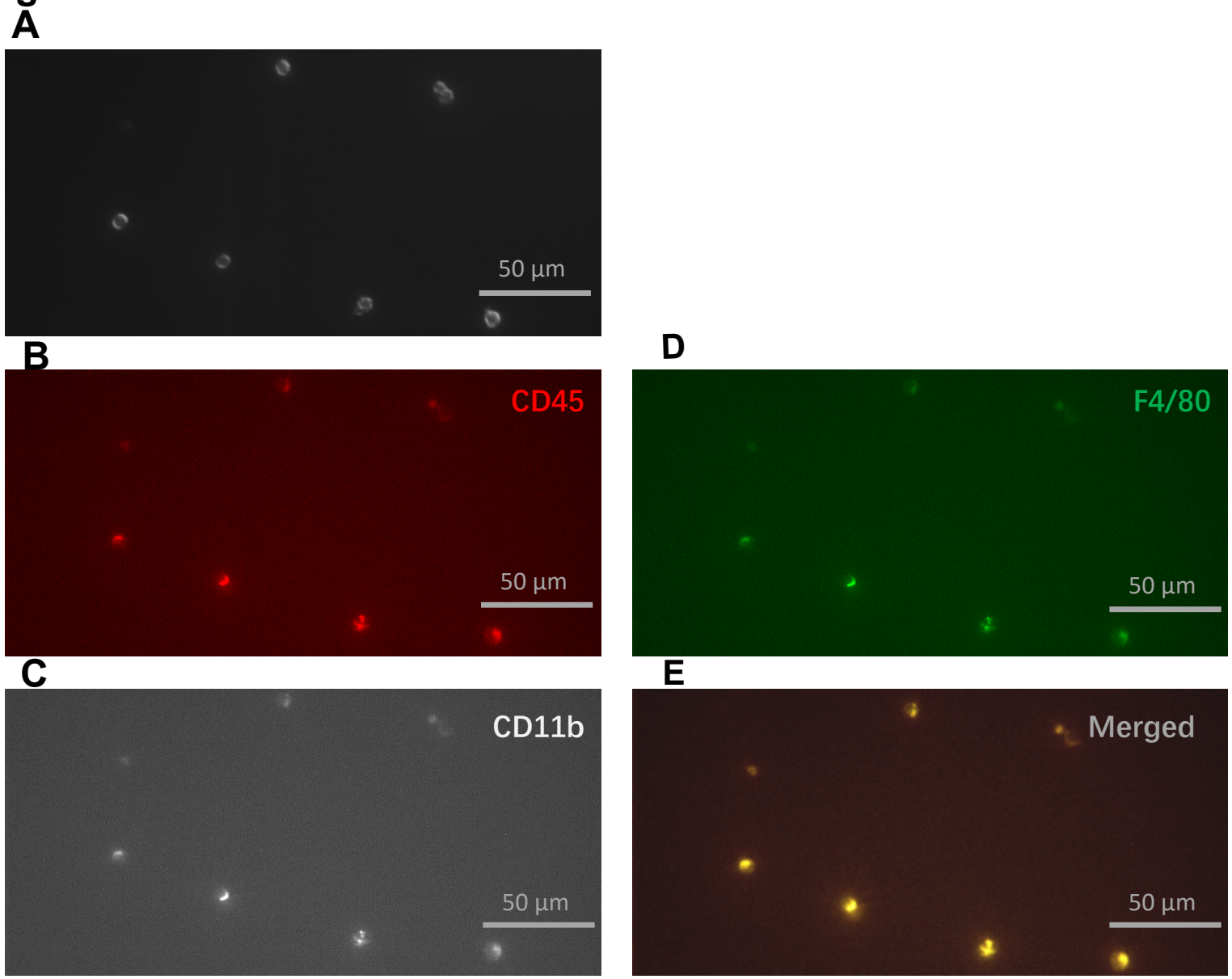

Figure 6.

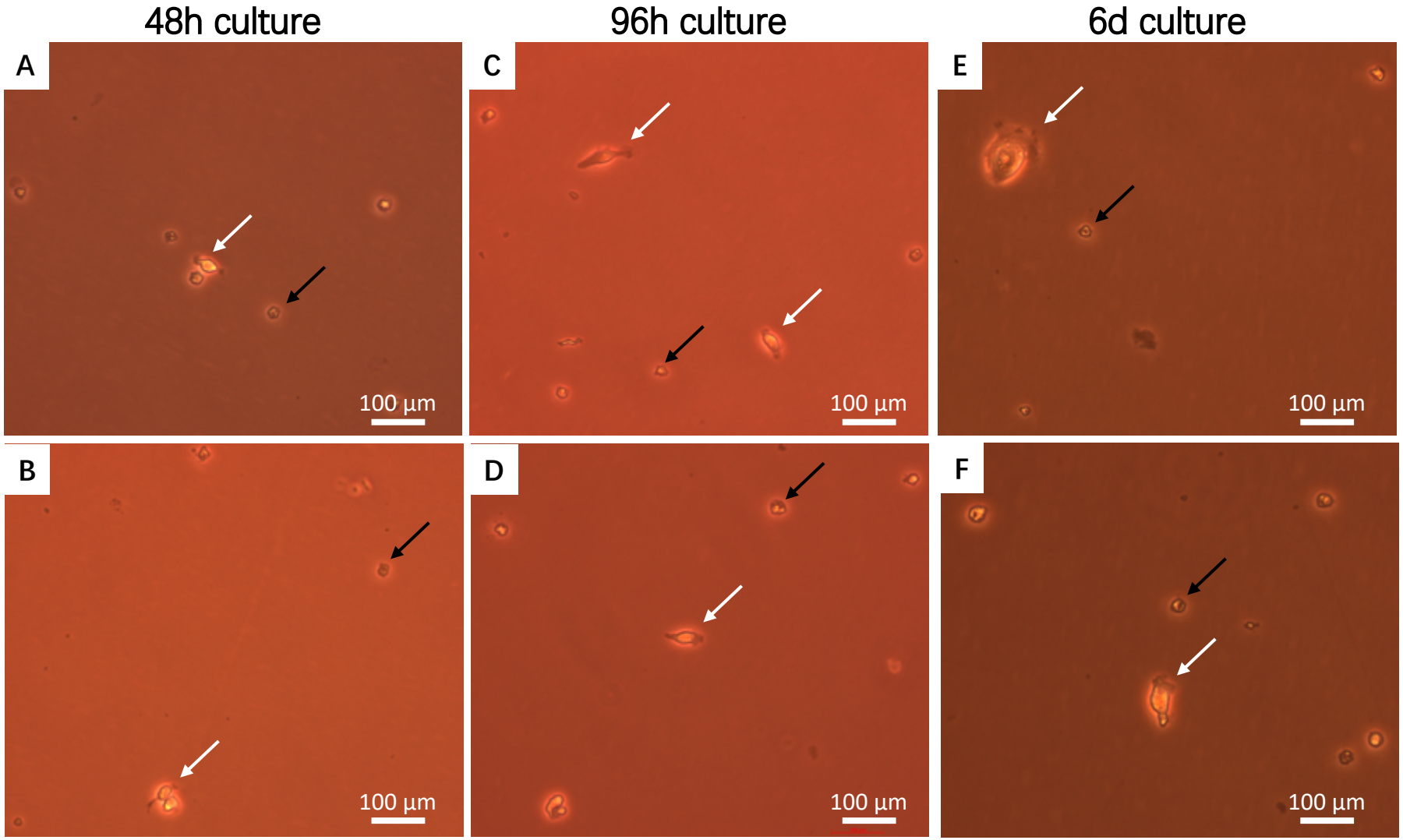

Table 1. Solutions.

Compound	Final concentration
FACS buffer	
BSA	0.50%
EDTA	2 mM
PBS (1X)	
Digestion buffer	
Collagenase I	450 U/mL
Collagenase XI	125 U/mL
DNase I	60 U/mL
Hyaluronidase	60 U/mL
HEPES buffer	20 µl per 1 ml
PBS (1X)	Add up to 1 ml for 2 samples
Culture medium	
DMEM	
Penicillin/streptomycin	1%
FBS	20%
TAE (50x)	
Tris-base	24.20%
100 % acetic acid	5.71%
0.5 M EDTA	0.05 M
dH_2O	

g or ml required
500ml
79 ml
1 ml
20 ml
24.2 g
5.71 ml
10 ml
Add up to 100ml

Table Materials

Description	Company
Anesthesia	
Isoflurane vaporizer system	Hugo Sachs Elektronik
Modified Bain circuit	Hugo Sachs Elektronik
Surgical Platform	Kent scientific
***In vivo* instrumentation**	
Fine forceps	Fine Science Tools
Iris scissors	Fine Science Tools
Spring scissors	Fine Science Tools
Tissue forceps	Fine Science Tools
Tissue pins	Fine Science Tools
General lab instruments	
Orbital shaker	Sunlab
Pipette,volume 10ul, 100ul, 1000ul	Eppendorf
Magnetic stirrer	IKA
Microscopes	
Dissection stereo- zoom microscope	vwr
Leica microscope	Leica microsystems
Flow cytometry machine	
Beckman Coulter	Beckman coulter
Software	
FlowJo v10	FlowJo
Name of Material	**Company**
General Lab Material	
0.2 µm syringe filter	sartorius
100 mm petri dish	Falcon
27G needle	BD Microlance 3
50 ml Polypropylene conical Tube	FALCON
Cover slips	Thermo scientific
Eppendorf Tubes	Eppendorf
5ml Syringe	Braun
Chemicals	
0.5 M EDTA	Sigma
Acetic acid	Merck
Agarose	Biozym
Bovine Serum Albumin	Sigma
Collagenase I	Worthington Biochemical
Collagenase XI	Sigma
DNase I	Sigma

Hyaluronidase	Sigma
HEPES buffer	Sigma
Bovine Serum Albumin	Sigma
DPBS (1X) Dulbecco's Phosphate Buffered Saline	Gibco
Fetal bovine serum	Sigma
Penicillin – Streptomycin	Sigma
DMEM	Gibco
Drugs	
Fentanyl 0.5 mg/10 ml	Braun Melsungen
Isoflurane 1 ml/ml	Cp-pharma
Oxygen 5L	Linde
Antibodies	
Anti-mouse Ly6C FITC (clone HK1.4)	BioLegend
Anti-mouse F4/80 PE/Cy7(clone BM8)	BioLegend
Anti-mouse CD64 APC (clone X54-5/7.1)	BioLegend
Anti-mouse CD11b APC/Cy7(clone M1/70)	BioLegend
Anti-mouse CD45 PE (clone 30-F11)	BioLegend
Hoechst 33342, Trihydrochloride, Trihydrate (DAPI)	Invitrogen
Animals	
Mouse, C57BL/6	Charles River Laboratories

Cat.-No.	Comments
34-0458, 34-1030, 73-4911, 34-0415, 73-4910	Includes an induction chamber, a gas evacuation unit and charcoal filters
73-4860	Includes an anesthesia mask for mice
SURGI-M	
11295-51	
14084-08	
91500-09	
11051-10	
26007-01	Could use 27G needles as a substitute
D-8040	
Z683884-1EA	
RH basic	
10836-004	
Leica DM6	
MoFlo Astrios	

Cat.-No.	Comments
17597	
351029	
300635	
352070	
7632160	
30121872	
4606108V	
20-158	
100063	Component of TEA
850070	
A2153-100G	
LS004196	
C7657	
D4527	

H3506	
H4034	
A2153-100G	
14190-094	
F2442-500ml	
P4083	
41966029	
31303	
2020175	Includes a pressure regulator
Cat# 128006	diluted to 1:100
Cat# 123114	diluted to 1:100
Cat# 139306	diluted to 1:100
Cat# 101226	diluted to 1:100
Cat# 103106	diluted to 1:100
H3570	diluted to 1:1000

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Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Ihr Zeichen: Unser Zeichen: 27.01.2021

Dear Dr. Bajaj,

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 your comments.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the revised version for revision.
Thank you very much. We keep the format without any change.

2. Please address all the comments marked in the manuscript.
We revised the manuscript according to all the comments. All changes can be followed in tracking mode.

Introduction part lines 104-105 are replaced by:
[…]

For microdissection of SAN and AVN, anatomical landmarks are used for the region identification.

[…]

Step 4.2 Note is replaced by:
[…]

Note: Mincing the tissue well will increase the digestion efficiency and help to get good cell suspension for sorting.

[…]

Step 4.6 Note is replaced by:

[…]

Note: Before magnetic separation, pipette the cell suspension gently a few times or pass through a 30 µm cell strainer to remove cell clumps if necessary, to obtain a single cell suspension for optimal performance of magnetic enrichment of interesting cell populations.

[…]

Step 5.7 Note is replaced by:
[…]

Note: Avoid formation of air bubbles in the column. Based on introduction of the manufacture's protocol, although the column filling time might change from storage conditions, it has no influence on the quality of the separation.

[…]

Step 5.8 Note is replaced by:
[…]

Note: Add the cell sorting buffer immediately when the column reservoir is nearly empty.

[…]

For the explanation of usage of magnetic beads CD45, note is added for procedure 5 as followed:
[…]

5. Magnetic enrichment of CD45 (Manufacture's Protocol) and sample staining

Note: *To isolate the cardiac macrophages with high sorting efficiency, exclusion of undesired cells including lymphocytes was performed with CD45 microbeads according to the manufacturer's protocol. Based on our sorting panel, we identify cardiac resident macrophages as* $CD45^{high}CD11b^{high}CD64^{high}$ $Ly6C^{low/int}$ $F4/80^{high}$.

[…]

REPRESENTATIVE RESULTS description for Figure 5 was modified as followed:

Figure 5

[…]

A*. Freshly sorted cells observed under brightfield view of microscope.* ***B****. Same view of the sorted cells observed under the fluorescent microscope with channel for fluorophore-PE showing the sorted cells are* $CD45^{+}$. ***C****. Same view of the sorted cells observed under the fluorescent microscope with channel for fluorophore-APC-Cy7 showing the sorted cells are* $CD11b^{+}$. ***D****. Same view of the sorted cells observed under the fluorescent microscope with channel for fluorophore-PE-Cy7 showing the sorted cells are* $F4/80^{+}$. ***E****. Merged figures of B, C and D with the fluorescent microscope for the sorted cells.*

[…]

Titles for each figure are added as followed:
[…]

FIGURE AND TABLE LEGENDS:

Figure 1. Anatomy of the SAN and AVN under the dissection microscope.
Figure 2. Schematic illustration of SAN and AVN localization.
Figure 3. Identification of the SAN and AVN with histological stain.
Figure 4. Gating strategy for cell sorting of resident cardiac macrophages.
Figure 5. Freshly sorted cardiac macrophages.
Figure 6. Culture of sorted macrophages.

Table 1. Material and Reagents.
Table 2. Composition of solutions needed.
[…]

3. The first half of the protocol is already published in our journal by your group. Please cite your previous publication instead. Please see the attached iThenticate report.

Thank you very much for this comment. Parts that are similar with previous published article are deleted and reference was added. Changes are as followed:

[…]

1.3 Procedures of preparation of dissection dish have been described previously[10]. In brief, add 30ml agarose gel (3%-4%) into a 100mm diameter petri dish and cool down at room temperature.

2. Animal sacrifice and Heart excise

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

2.2 Experimental details have been described previously[10]. In brief, after the injection of fentanyl for analgesia, open the rib cage and perfuse the heart by injecting 5-10ml ice-cold 1x PBS directly into the LV. Extract the mice heart and put it on the dissection dish.

3. Microdissection of SAN and AVN

3.1 After isolating the heart, operate the following microdissection procedures in the dissection dish with ice-cold 1x PBS under the dissecting microscope.

3.2 Use the cardiac anatomical landmarks, i.e., aorta, pulmonary artery, coronary sinus, left/right ventricle, etc. to determine the left/right (left: LV; right: RV) and anterior/posterior (anterior: aorta; posterior: coronary sinus) of the heart. After the orientation is determined, turn around the heart with the front of it at the bottom of the dish (to expose the large veins that are located posterior).

3.3 Microdissection of SAN

Microdissect the SAN microdissection procedures as described previously10. In briefly, expose the inter-caval region by pining the RAA and the tissue adjacent to SVC and IVC on the microsection dish using insect pins. Cut the heart along the interatrial septum parallel to the CT to separate the inter-caval region and to obtain the SAN sample (Figure 1A, 2A). Put the sample in an empty 1.5 mL microcentrifuge tube on ice.

[…]

REFERENCES:
[…]
10 Xia, R. et al. Whole-Mount Immunofluorescence Staining, Confocal Imaging and 3D Reconstruction of the Sinoatrial and Atrioventricular Node in the Mouse. Journal of Visualized Experiments. 10.3791/62058 (166), (2020).
[…]

4. Once done please ensure that the highlighted section is no more than 3 pages including headings and spacings.
We highlighted parts of the protocol as suggested.