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Isolation of Macrophage Subsets and Stromal Cells from Human and Mouse Myocardial Specimens

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March 19th, 2019

Dear Dr. Upponi,

Please find enclosed our manuscript entitled, “Isolation of Macrophage Subsets and Stromal Cells from Human and Mouse Myocardial Specimens,” which we are pleased to submit *JoVE*.

Immune cells are increasingly recognized to impart important effects on the heart under homeostasis and in the context of various cardiac diseases including myocardial infarction and heart failure. Among such immune populations, monocytes and macrophages have been implicated in coronary development, cardiac conduction, heart regeneration, and adverse left ventricular remodeling. Given these findings, there has been intense interest in this area and an expansion of laboratories studying cardiac immunology. At present, techniques to analyze and isolate cardiac immune and stromal cells vary considerably between laboratories.

In this manuscript, we present a universal protocol to isolate immune and stromal populations from the mouse and human myocardium. This technique is suitable for a variety of downstream applications including flow cytometry analysis and sorting, *ex vivo* activity assays, and transcriptomic profiling. We further discuss key protocol details and potential pttfalls. We believe that this manuscript will prove informative and useful to both established and new investigators interested in studying cardiac immune cell diversity and function.

This manuscript has not been submitted elsewhere, and all of the authors have seen and approved of this manuscript in its final form. We look forward to your comments on this manuscript.

Sincerely,

Kory J. Lavine MD, PhD
Assistant Professor
Department of Medicine, Cardiovascular Division
Washington University School of Medicine

TITLE:

Isolation of Macrophage Subsets and Stromal Cells from Human and Mouse Myocardial Specimens

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KEYWORDS:

Macrophages, human heart, flow cytometry, heart failure, stromal cells, mouse heart

SUMMARY:

Presented here is a protocol to isolate various subsets of macrophages and other non-immune cells from human and mouse myocardium by preparing a single cell suspension through enzymatic digestion. Gating schemes for flow cytometry based identification and characterization of isolated macrophages are also presented.

ABSTRACT:

Macrophages represent the most heterogeneous and abundant immune cell populations in the heart and are central in driving inflammation and reparative responses after cardiac injury. How various subsets of macrophages orchestrate the immune responses after cardiac injury is an active area of research. Presented here is a simple protocol that our lab performs routinely, for the extraction of macrophages from mouse and human myocardium specimens obtained from healthy and diseased individuals. Briefly, this protocol involves enzymatic digestion of cardiac tissue to generate a single cell suspension, followed by antibody staining, and flow cytometry. This technique is suitable for functional assays performed on sorted cells as well as bulk and single cell RNA sequencing. A major advantage of this protocol is its simplicity, minimal day to day variation and wide applicability allowing investigation of macrophage heterogeneity across various mouse models and human disease entities.

INTRODUCTION:

Macrophages represent the most abundant immune cell type in the heart, and they play significant roles in generating robust inflammatory and reparative responses following cardiac

injury[1-4]. Previously, our group identified two major subsets of macrophages in the murine heart derived from distinct developmental origins[5,6]. Broadly, distinct populations of tissue resident cardiac macrophage subsets can be identified based on the cell surface expression of CCR2 (C-C motif chemokine receptor 2). CCR2- macrophages (cell surface expression: CCR2-MHCII^{low} and CCR2-MHCII^{high}) are of embryonic origin (primitive and erythromyeloid lineages), able to self-renew, and represent a dominant population under homeostatic conditions. Resident CCR2+ macrophages are of definitive hematopoietic origin, are maintained through recruitment from circulating monocytes, and represent a minor population under homeostatic conditions. Functionally, CCR2- macrophages generate minimal inflammation and are critical for coronary development neonatal heart regeneration[5,7]. In contrast, CCR2+ macrophages initiate robust inflammatory responses following cardiac insults and contribute to collateral cardiomyocyte injury, adverse remodeling of the left ventricle, and heart failure progression[8,9].

Recently, we have shown that the human myocardium also contains two distinct subsets of macrophages identified similarly as either CCR2- or CCR2+[8]. Gene expression and functional analyses revealed that human CCR2- and CCR2+ macrophages represent functionally divergent subsets and are functionally analogous to CCR2- and CCR2+ macrophages found in the mouse heart. Human CCR2- macrophages express robust levels of growth factors, including IGF1, PDGF, Cyr61, and HB-EGF. CCR2+ macrophages are enriched in chemokines and cytokines that promote inflammation, such as IL-1b, IL-6, CCL-2, CCL-7, and TNF-a. Stimulated CCR2+ macrophages secrete markedly higher levels of the inflammatory cytokine interleukin-1β (IL-1β) in culture. How these subsets differentially contribute to tissue repair and left ventricular (LV) remodeling in the context of cardiac injury remains an area of active research.

Flow-cytometry based analysis of macrophage heterogeneity in the mouse and human heart requires digesting the cardiac tissue and generating a single cell suspension followed by flow cytometric analysis or cell sorting for further downstream processes such as bulk RNA sequencing/single cell RNA sequencing or culturing the cells for functional assays. The original protocol for making a single cell suspension from murine hearts were first reported by Nahrendorf group in Nahrendorf et al. 2007[10]. Our lab has adapted and modified the protocol to extract macrophages from the human myocardium. Using the same protocol but with slight modification in staining and gating scheme, CD45- stromal cells from the human myocardium can also be harvested. Presented here, in text and video, is a protocol that is performed routinely for the extraction of macrophages or stromal from the human myocardium.

Cardiac tissue specimens are obtained from adult patients with dilated cardiomyopathy (DCM: idiopathic or familial) or ischemic cardiomyopathy (ICM) undergoing left ventricular assist device (LVAD) implantation or cardiac transplantation. Explanted hearts or LVAD cores are intravascularly perfused with cold saline prior to starting the digestion procedure. It is important to note that “quality” of tissue specimen determined in terms of degree of scarring or adipose tissue infiltration can greatly affect the yield of macrophages. Heart specimens with large areas of scarring will have much lower cell yield and can pose serious technical limitation when desired downstream analysis methods require in vitro cell culturing.

PROTOCOL:

The protocol presented has been approved by the Washington University in St. Louis Institutional Review Board (#201305086). All subjects provide informed consent before sample collection and the experiments are performed in accordance with the approved study protocol. The presented protocol is performed with the approval of the Institutional Animal Care and Use Committee at Washington University School of Medicine and follows the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals.

1. Preparation of human cardiac tissue specimens

1.1. Flush explanted hearts by cannulating the left and right coronary artery ostia and perfuse with 200 mL of cold saline.

1.2. Flush LVAD apical cored tissues by cannulating an epicardial vessel and perfuse with 50 mL of cold saline.

1.3. Dissect tissue specimen from the apical or lateral wall of the left ventricle in cold saline or HBSS using a sterile scissor.

1.4. Carefully dissect out epicardial fat and chordae tendineae from the specimen using fine scissors.

1.5. Dissect tissue chunks into pieces weighing approximately 200 mg with help of a sterile blade or scissors.

2. Preparation of mouse heart

2.1. Euthanize the mouse by CO_2 asphyxiation or cervical dislocation.

2.2. Open the chest cavity with the help of sharp scissors. Use blunt hemostats to lift the heart upwards. Perfuse the heart with cold PBS using a 25 G needle attached to a 5 mL syringe. Perfuse until heart appears blanched in color.

2.3. Remove the heart and place in a sterile Petri dish on ice.

3. Preparation of single cell suspension

3.1. Place human cardiac tissue chunks (~200 mg) or murine heart in a sterile Petri dish. Finely mince the tissue using a sterile blade or scissors.

3.2. Set up the digestions:

3.2.1. Use a final digestion volume of 3 mL per human cardiac tissue chunk (200 mg) or per one murine heart. Final enzyme concentrations are as follows: Collagenase1 (450 U/mL), DNase1 (60 U/mL), Hyaluronidase (60 U/mL).

3.2.2. For each digestion reaction add DMEM and all enzymes to a 15 mL conical tube. With the help of clean forceps, place the finely minced tissue into each reaction tube. Mix well by gentle vortexing.

3.3. Digest for 1 h at 37 °C in a shaking incubator set to a low to medium agitation speed.

3.4. After 1 h of digestion, take the tubes out from the incubator and place on ice. Set up 50 mL conical tubes with a 40 µm cell strainer on top. Wet the filters with 2 mL of enzyme deactivating (ED) buffer.

3.5. Deactivate the digestion enzymes by adding 8 mL of ED buffer to each digestion tube. Then pour the resulting 13 mL total mixture through the 40 µm cell strainer into the 50 mL conical tubes. Transfer the samples back in fresh 15 mL conical tube. This enables optimal cell pelleting and minimizes cell loss during centrifugation.

3.6. Spin the samples at 400 x *g* for 6 min (Centrifuge set at 4 °C). Discard the supernatant leaving 0.5 mL of media. Resuspend the cell pellet by gentle pipetting and add 1 mL of ACK lysis buffer. Gently swirl the tube and incubate at room temperature for 5 min to perform red blood cell (RBC) lysis.

3.7. After 5 min in ACK buffer, add 9 mL of DMEM to the sample. Put the lid back on to the tubes and gently invert the tubes to mix, and filter through a 40 µm cell strainer. Collect the filtrate in 15 mL conical tubes.

3.8. Centrifuge the tubes at 400 x *g* for 6 min and discard the supernatant.

3.9. Add 1 mL of FACS buffer and resuspend the pellet. Then transfer in the cells in FACS buffer to a 1.5 mL microcentrifuge tube. Centrifuge again at 400 x *g* for 5 min. Discard the supernatant and resuspend the pellet in 100 µL of FACS Buffer. A single cell suspension is now ready for antibody staining.

4. Antibody staining

4.1. A typical human antibody panel consists of the following antibodies: CD45-PercpCy5.5, CD14-PE, CD64-FITC, HLA-DR-APC/Cy7, CCR2-APC. Please refer to **Table 1**. Add all antibodies to the heart samples at 1:50 dilution and incubate for ~30–40 min at 4 °C in the dark. Proceed to step 4.4 below.

4.2. For stromal cells (endothelial cells, fibroblast, smooth muscle cells), add DRAQ5 (1 µM final concentration) and CD45-percpCy5.5. Please refer to **Table 1**. Incubate for 30 min at 4 °C in the dark. Proceed to step 4.4 below.

4.3. For murine heart macrophages add the following antibodies: CD45-PercpCy5.5, CD64-APC, MHCII-APC/Cy7, CCR2-BV421, and Ly6G-FITC. Please refer to **Table 2**. Add all antibodies to the heart samples at 1:100 dilution and incubate for ~30-40 min at 4 °C in the dark. Proceed to step 4.4 below.

4.4. Wash the samples twice in FACS buffer. For each wash, add 1 mL of FACS buffer, gently vortex, and centrifuge at 400 x *g* for 5 min, resuspend in 350 µL of FACS buffer and add DAPI (1 µM, final concentration). Samples are now ready for FACS analysis/sorting.

REPRESENTATIVE RESULTS:

The protocol described allows isolation of macrophages from mouse and human myocardium. Using the same protocol, but with a different staining and gating strategy, stromal cells can also be harvested from the human myocardium. FACS results presented here were acquired either on BD LSRII or BD FACS ARIA III platform. Compensation controls were generated from single color control samples from stained splenocytes. **Figure 1** shows unprocessed and processed human LVAD core. **Figure 2** shows the gating scheme for the flow sorting of CCR2- and CCR2+ human macrophages. **Figure 3A** shows the gating scheme for CD45- stromal cells from human myocardium and **Figure 3B** shows images of Wright stained FACS sorted CD45+ and CD45- cells. **Figure 4** describes the gating scheme to sort macrophages from a mouse heart.

FIGURE AND TABLE LEGENDS:

Figure 1. The human LVAD tissue core before and after processing.

Figure 2. Flow cytometry gating scheme utilized to identify and characterize cardiac macrophage populations in dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) specimens.

Figure 3. Flow cytometry gating scheme to isolate CD45- and CD45+ stromal cells from human samples. (**A**) Flow cytometry gating scheme utilized to isolate CD45- stromal cells from human ischemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM) specimens. (**B**) Wright stained FACS sorted CD45- and CD45+ cells. Scale bars represent 100 µm.

Figure 4. Flow cytometry gating scheme to isolate various macrophage subsets from the mouse heart.

Table 1. Antibody panel for human myocardium specimen.

Table 2. Antibody panel for mouse heart specimen.

DISCUSSION:

The protocol allows for the extraction of various macrophage subsets from human myocardium. The protocol is simple and takes 3 to 4 hours to prepare single cell suspension ready for FACS analysis. Although the protocol is relatively simple to perform, there are certain technical aspects that need to be considered which will minimize variability. Firstly, working in timely fashion with human tissue is necessary for optimal cell viability. It is important to keep the tissue in cold saline/HBSS to minimize cell death. It is also necessary to remove epicardial fat and other connective tissue from the myocardial specimen. Consistent tissue mincing and digestion times will reduce sample to sample variation.

There is both intra-assay and inter-assay variability in tissue digestions and subsequent cell yields. This is one of the limitations in preparing a single cell suspension from tissues. The most important way to minimize this is by making sure enzymes are relatively new, properly aliquoted, and stored at -80 °C. Aliquots should be used one time only and should not be saved or frozen again. Enzymes used are sensitive to freeze thaw cycles. Another consideration is temperature of digestion and shaking speed. Using a thermostat-controlled shaker that evenly distributes heat helps to minimize digestion variability and improve cell viability.

It is important to mention that absolute yield of macrophages from human myocardium is generally not very high. Usually the cell yield varies from ~20,000 to 50,000 total macrophages per 1,200–1,500 mg of tissue. This becomes challenging when the desired downstream method of analysis involves cell culture assays. Phagocytosis, chemokine/cytokine production, cell stimulation, morphometry, and gene expression analyses (microarray, bulk RNA sequencing, and single cell RNA sequencing) can be easily performed. The quality of the tissue also determines the ease of digestion and subsequent cell yield. If the tissue is fibrous and scarred, digestion efficiency is suboptimal, and macrophage yield is likely to be very low.

Another aspect to consider is that myocardial tissue digestion leads to significant debris formation. This causes the pellet to appear loose. Thus, one must be careful to not discard the supernatant during wash steps by simple decanting. Using a suction/vacuum waste collection flask with a Pasteur pipette to collect the supernatant is advisable. Also, maintaining the centrifuge at 4 °C will help minimize the cell death and sample loss.

Although this protocol describes a way to extract macrophages from human myocardial tissue samples, successful isolation of macrophage subsets can also be achieved from mouse hearts without significant changes or modifications.

ACKNOWLEDGMENTS:

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DISCLOSURES:

Authors have nothing to disclose.

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Figure 1

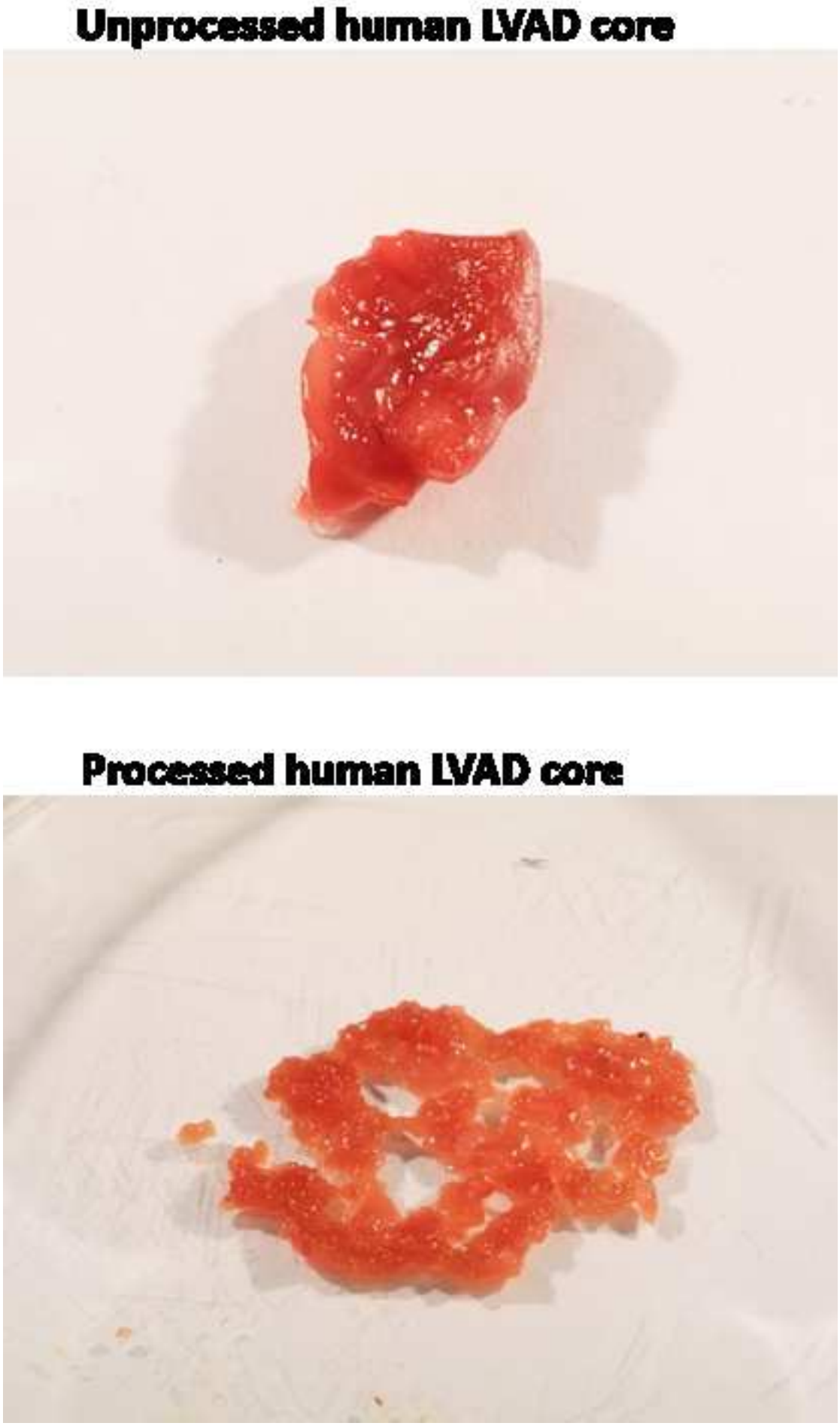

Figure 2

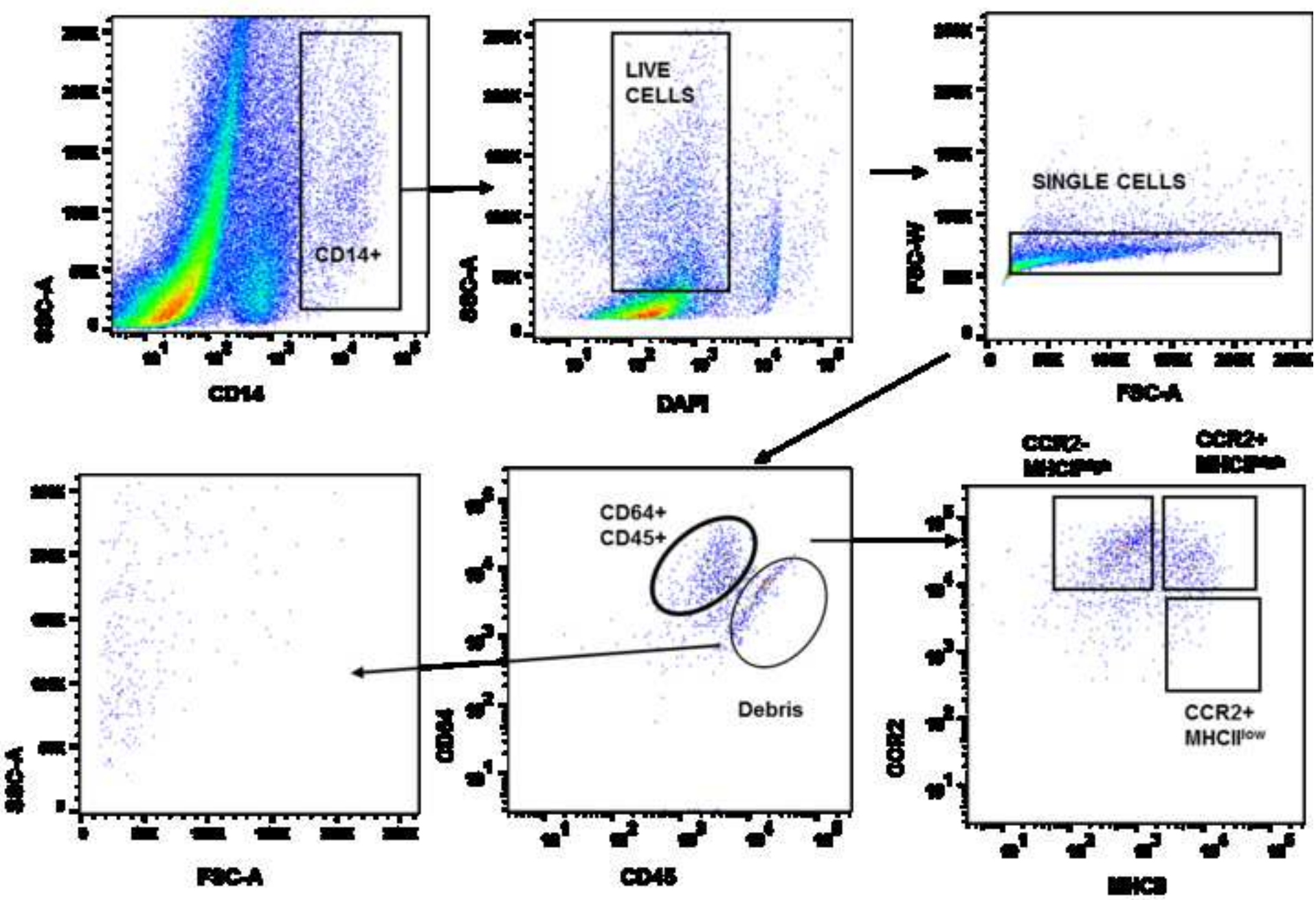

Figure 3

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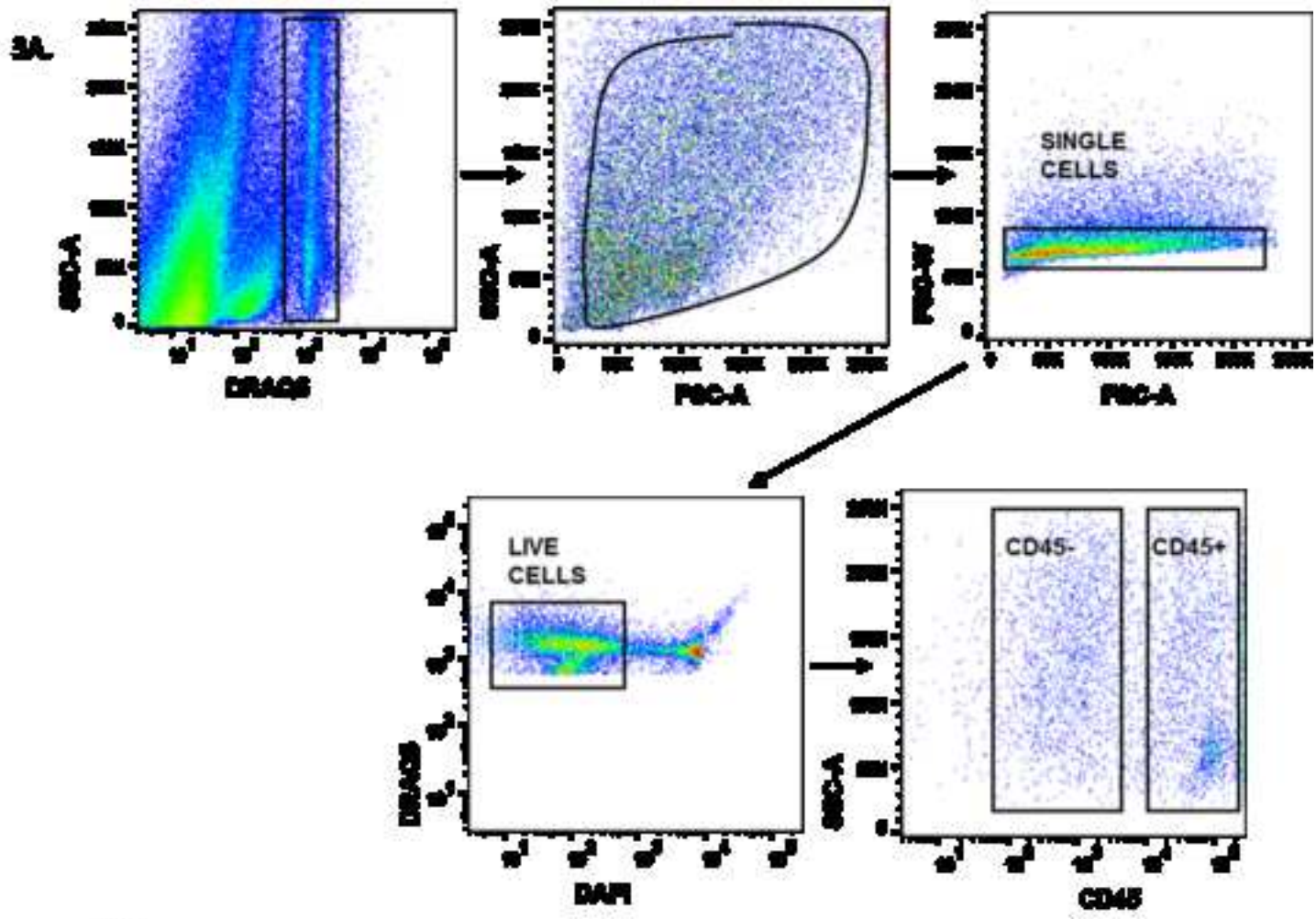

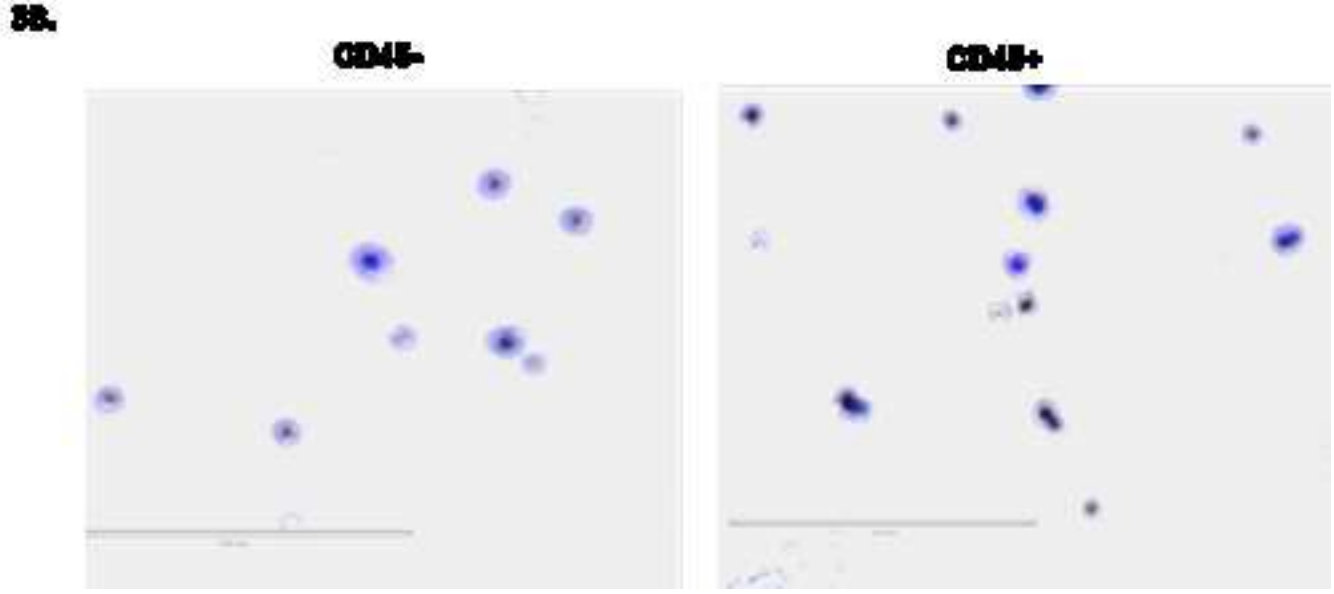

Figure 4

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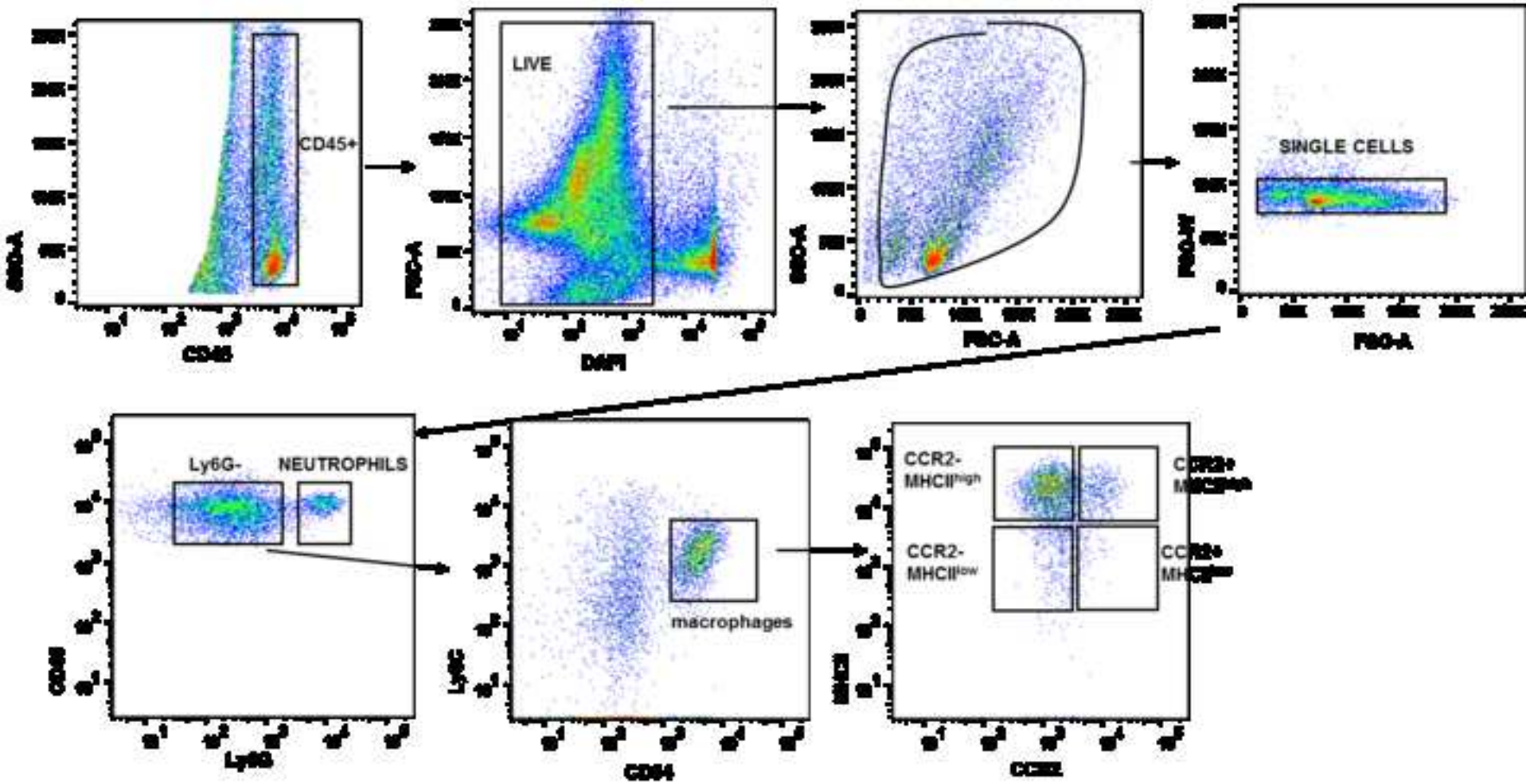

Table 1

Antigen	Fluorophore	Clone	Manufacturer
CD45	PercpCy5.5	2D1	Biolegend
CD64	FITC	10.1	Biolegend
CD14	PE	M5E2	Biolegend
CCR2	APC or BV421	K036C2	Biolegend
MHCII	APC/Cy7	L243	Biolegend
DRAQ5			Thermo Fisher
DAPI			Thermo Fisher

Table 2

Antigen	Fluorophore	Clone	Manufacturer
CD45	PercpCy5.5	30-F11	Biolegend
CD64	APC	X54-5/7.1	Biolegend
Ly6G	PE/Cy7	1A8	Biolegend
Ly6C	FITC	HK1.4	Biolegend
CCR2	BV421	SA203G11	Biolegend
MHCII	APC/Cy7	M5/114.15.2	Biolegend
DRAQ5			Thermo Fisher
DAPI			Thermo Fisher

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
15 mL Conocal Tubes	Thermo Fisher	14-959-53A
40 µm Cell Strainers	Thermo Fisher	50-828-736
50 mL Conical Tubes	Thermo Fisher	352098
ACK Lysis Buffer	Gibco	A10492-01
Bovine Serum Albumin	Sigma	A2058
Collagenase 1	Sigma	C0130-1G
DAPI	Thermo Fisher	D1306
DMEM 1x	Gibco	11965-084
DNAse 1	Sigma	D4527-20KU
DRAQ5	Thermo Fisher	62251
EDTA 0.5M pH 8	Corning	46-034-CI
Enzyme Deactivating Buffer		
FACS Buffer		
Fetal Bovine Serum	Gibco	A3840201
Forceps	VWR	82027-406
HBSS 1x	Gibco	14175-079
Hemostats	VWR	63042-052
Hyaluronidase type 1-s	Sigma	H3506-500MG
PBS 1x	Gibco	14190-136
Petridishes	Thermo Fisher	172931
Razor Blade	VWR	55411-050
Scissors	VWR	82027-578

Comments/Description

490 mL HBSS, 10 mL FBS, 1 g BSA
976 mL PBS, 20 mL FBS, 4mL EDTA (0.5M)

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Comments to Editor

We sincerely appreciate the careful review of our manuscript entitled "Isolation of Macrophage Subsets and Stromal Cells from Human and Mouse Myocardial Specimens (reference JoVE60015)". After considering each of the points raised by the editors and the reviewers, we have revised this manuscript in accordance. For your reference, the original comments have been copied and are shown in italics. The specific responses to the editorial suggestions are given below. We believe that the manuscript has improved significantly based on your suggestions and hope that you will find the revised manuscript acceptable for publication.

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

Authors Response: We have proof-read the manuscript to the best of our abilities and corrected grammatical and spelling errors.

2. *Authors and affiliations: Please provide an email address for each author.*

Authors Response: Email address for each author has now been included.

3*. Please define all abbreviations before use.*

Authors Response: All abbreviations have been defined when they appear first in the text.

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

Authors Response: SI abbreviations have been used for all units. Micro symbol has been corrected.

5. Please include a space between all numerical values and their corresponding units: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

Authors Response: Space between all numerical values and their corresponding units has been added.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Authors Response: Ethics statement has been provided before the numbered protocol text.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Thermo Fisher, VWR, Fisher Scientific, Gibco, Sigma, Corning, etc.

Authors Response: All commercial products have been listed as instructed without the commercial language.

8. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Authors Response: Ethics statement has now been provided before the numbered protocol text between lines 102 to 113.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Authors Response: Numbering of the protocol follows JoVE guidelines. Bullets, dashes or indentations were not used.

10. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Authors Response: The text has been revised to remove personal pronouns

11. Lines 94-124: The Protocol should contain only action items that direct the reader to do something. Please move the solutions, materials and equipment information to the Table of Materials.

Authors Response: Solutions, materials and equipment information has been removed.

12. Please specify all surgical tools used throughout the protocol.

Authors Response: All the tools have been specified in the text and in the materials list.

13. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Authors Response: Text has now been simplified and subsections have been added in the protocol.

14. Please reference Table 1 and Table 2 in the manuscript.

Authors Response: Tables 1 and 2 have been referenced in the manuscript.

15. Figure 3: Please combine panels A and B into a single image file. Please include a title and define scale bars in the figure legend.

Authors Response: Panels A and B have been now compiled as single image file. Title has been added and scale bar has been defined.

16. Please remove the titles and figure legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

Authors Response: Titles and figure legends from the uploaded figures have been removed.

17. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Authors Response: Figures have been uploaded individually as .png files

18. Please upload each Table individually to your Editorial Manager account as an .xlsx file.

Authors Response: Tables have now been uploaded in the account as .xlsx file.

19. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Authors Response: Items have been sorted in alphabetical order.

20. In-text citations: Please remove the parentheses enclosing the reference numbers. The corresponding number from the reference list must appear superscripted without a space after the word/group of words it applies to but before any punctuation.

Authors Response: References now appear as superscripted as required.

21. References: Please do not abbreviate journal titles.

Authors Response: We have corrected the journal titles.

Comments to Reviewer 1

Major Concerns:

How can these macrophages be classified as related to M1, M2 and M0? Some specific markers should be added. The method includes the digestion of cardiac tissue specimens with Collagenase type 1, Hyaluronidase and DNase I, but the details concerning the isolation by FACS are missing. It is not even mentioned the type of the FACS equipment. All the control isotypes are missing and thus, one cannot understand how the gates are set. How was the compensation done? Which was the gain used for each fluorochrome? Other FACS details must be shown. Mention for each step the percentage of the gated cells from the total population.

Fig. 2. Panel left - X axis : CD14 - how CD45 was identified? How the gate for Live cells was set (where are the controls? - dead cells and live cells). How the debris were identified in the second row of the fig 2 (left part). Note A-E the panels.

Authors Response: M1, M2 and M0 classification of macrophage activation is based on *in vitro* stimulation by selected ligands. However, *in vivo* macrophages do not exist as M1 or M2. Instead, they exist as a continuum of various polarization states and express several overlapping markers depending on the tissue context and disease process. Recent studies have highlighted the limitations of classifying macrophages M1 and M2 *in vivo.* Our lab identified two major subsets of cardiac macrophages in the murine heart derived from distinct developmental origins and are identified as either CCR2- or CCR2+ based on the expression of cell surface receptor CCR2 (C-C motif chemokine receptor 2) (PNAS 111, no. 45 (2014): 16029-16034, Immunity 40, no. 1 (2014): 91-104). Recently, we have shown that analogous to the murine heart, the human myocardium also contains two distinct subsets of CCR2- and CCR2+ macrophages. Microarray analysis reveals that human CCR2- and CCR2+ macrophages represent functionally divergent subsets (Nature medicine 24, no. 8 (2018): 1234). Recent studies highlight the importance of ontogeny in defining tissue macrophages that govern their divergent functions as well.

The type of FACS instrument used in results shown has now been added in the text in the representative results section. The gating strategy including the isotype controls, compensation controls and other details have already been published from our lab previously (Nature medicine 24, no. 8 (2018): 1234, PNAS 111, no. 45 (2014): 16029-16034, Immunity 40, no. 1 (2014): 91-104). Presented here are the representative results of macrophages/stromal cells harvested from human or murine myocardium.

We chose not to present the percentage of gated cells from total population in each step to avoid misleading the reader. Enzymatic digestion of tissues leads to generation of debris. Unfortunately, generation of debris is variable from day to day and depends largely on tissue quality. The first step in the gating scheme is the identification of either CD45+ (all leukocytes) or CD14+ (myeloid cells) from the debris field. The percentage of first gate thus varies from digestion to digestion. Our focus here is to provide a step by step gating strategy for users to follow. Adding percentages

at each step may lead to confusion as the absolute values will greatly depend on tissue quality, digestion conditions, and the instrument used. We have added the requested information regarding the instrument used for cell sorting. Voltage settings are instrument dependent.

Thank you for pointing out the major typing error in the Fig 2. Panel left should be CD14+ cells. Typing error in the figure has now been fixed. Live and dead gates were set based on single stains of cells with DAPI, and also based on FSC and SSC parameters to identify the debris in second row of the figure. FSS-A and SSC-A plot for debris has been included in the figure.

Minor Concerns:

All the materials needed should be in one section (at the beginning of the protocol, some of the Materials and reagents, but not antibodies are mentioned).

-Row 69: "left ventricular (LV)" instead of "LV"

-Remove Rows 104 -114 because the complete list of reagents (Table of materials) is at the end of manuscript. Anyway, in row 111 the company and catalog number is missing for Bovine serum albumin.

-Row 120: "left ventricular coronary assist device (LVAD)" instead of "LVAD"

-Row 138: "Petridish" instead of "pertidish"

-Row 158: "red blood cells (RBC)" instead of "RBC"

-Row 205: Delete "for"

-Legend of Figures 2 and 3: Dilated cardiomyopathy (DCM) instead of "DCM" and "ischemic cardiomyopathy (ICM)" instead of "ICM".

Authors Response: Thank you, all the above corrections have been included in the text. Thank you for pointing out the missing catalog number for Bovine serum albumin and has now been added.

Comments to Reviewer 2

Major Concerns:

The title indicates the protocol works for isolation of macrophages and stromal cells from human and mouse tissue although the text describes the isolation of macrophages from human tissue only.

There is no clear description for the different markers that distinguish human and murine macrophages/ stromal cells.

Authors Response: Preparation of the murine heart specimen section has now been added in the protocol (Lines 131 to 137). The markers used to identify human and mouse macrophages are quite similar with the following exceptions: CD14 marker has been used in gating strategy for human macrophages (Fig 2) while marker Ly6C has been used in identifying the murine monocytes. Similar subsets of macrophages (that are identified based on CCR2 receptor expression) exist within both human and murine myocardium and that is the focus of the protocol. We have defined the markers and gating strategy for the isolation of macrophage subsets and other stromal cells that can be used for various downstream assays. An exhaustive list of markers to differentiate various cell types between human and mouse is beyond the scope of protocol presented.

Minor Concerns:

Some of the FACS plots and axes are misleading and not all FACS gating schemes include live/dead staining.

Some of the catalog numbers for the reagents used are missing.

Authors Response: Thank you for pointing out, mislabeled axes in Fig 2 has been corrected. Live/dead staining has been added in Fig 4. Catalog numbers for DAPI, DRAQ5 and BSA have now been added.