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Protocol for Isolation of Cardiac Interstitial Cells from Adult Murine Hearts for Unbiased Single Cell Profiling.

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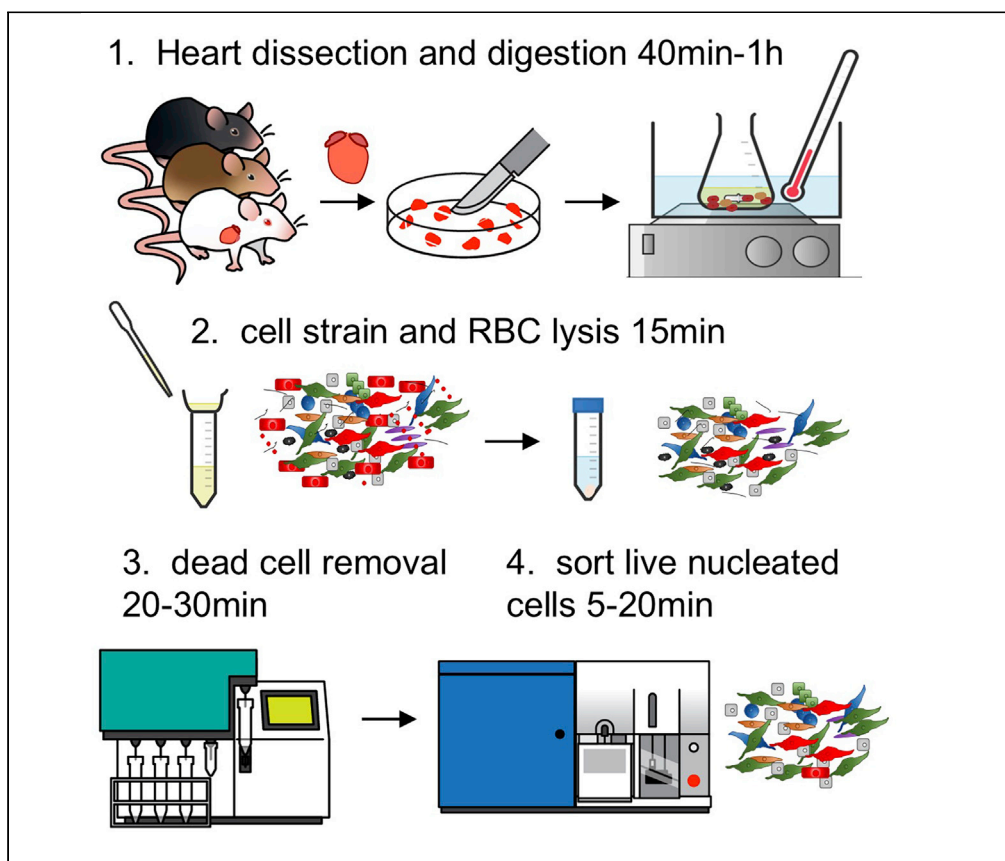
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Protocol

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Interstitial cells have a crucial role in cardiac fibrosis and repair of the mammalian heart. Single-cell profiling using droplet-based technology has revolutionized the investigation of cell states and identities. Here, we present a protocol for the efficient isolation of high-quality live nucleated non-cardiomyocytes from adult murine heart, for unbiased single-cell RNA sequencing using 10× Chromium technology. This protocol has been applied to homeostatic and injured hearts from different mouse strains.

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HIGHLIGHTS

The protocol is optimized to isolate interstitial cells from adult murine hearts

Dead cell removal and live nucleated selection maximize cells capture for scRNA-seq

The protocol has been used for different mice strains, pre and post ischemic injury

The combination of DRAQ5 and PI is compatible with green reporter mice

Forte et al., STAR Protocols 1, 100077

September 18, 2020 © 2020

The Author(s).

[https://doi.org/10.1016/](https://doi.org/10.1016/j.xpro.2020.100077)

[j.xpro.2020.100077](https://doi.org/10.1016/j.xpro.2020.100077)



Protocol

Protocol for Isolation of Cardiac Interstitial Cells from Adult Murine Hearts for Unbiased Single Cell Profiling

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<https://doi.org/10.1016/j.xpro.2020.100077>

SUMMARY

Interstitial cells have a crucial role in cardiac fibrosis and repair of the mammalian heart. Single-cell profiling using droplet-based technology has revolutionized the investigation of cell states and identities. Here, we present a protocol for the efficient isolation of high-quality live nucleated non-cardiomyocytes from adult murine heart, for unbiased single-cell RNA sequencing using 10× Chromium technology. This protocol has been applied to homeostatic and injured hearts from different mouse strains.

For complete details on the use and execution of this protocol, please refer to Forte et al. (2020).

BEFORE YOU BEGIN

⌚ Timing: 0.5–2 h

Preparation the Day before the Experiment

1. Prepare all buffers and solutions (see table below).

Note: Solutions, except for the Enzymatic solution, can be re-used for up to a month.

2. Autoclave dissection tools (2 set of dissecting forceps and scissors) and 1.5 mL Eppendorf tubes in a glass beaker.

Preparation on the Day of the Experiment

⌚ Timing: 3 h

3. Cool down the centrifuges at 4°C. Turn on the water-bath to 37°C for tissue digestion. Prepare two ice buckets (one for samples, one for the AutoMACS running buffer).
4. Turn on the AutoMACS and select the “Clean” program from the main screen, in order to have the instrument ready for cell separation.
5. Prepare the dissection stage:
 - a. Place a Styrofoam lid covered with bench paper in a glass tray at a 45-degree angle.
 - b. Prepare labelled petri dish or 15 mL tubes for each of the hearts to be collected.
 - c. Have 2 set of dissecting forceps and scissors clean and sterilized.
6. In a laminal flow cabinet/tissue culture hood place:



- a. the enzymatic solution at 20°C–22°C.
- b. the red blood cell (RBC) lysis buffer at 20°C–22°C.
- c. the containers used for tissue digestion.
- d. the collection tubes, one for each sample: 50 mL collection tubes each with a 40µm cell strainer on top, pre-wet with 1 mL of fetal bovine serum (FBS).
- e. bacteriological petri dish and scalpel/blades for dissection.
- f. a dish or Falcon tube containing ethanol 70% and one with sterile PBS to clean the dissection tools.

△ **CRITICAL:** keep samples and wash buffer on ice at all time; work under sterile conditions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Phosphate Buffered saline (PBS) 1×. pH 7.4	Thermo Fisher Scientific	Cat# 10010023
Hanks' Balanced Salt Solution (HBSS) 10×	Thermo Fisher Scientific	Cat# 14185052
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat# A2153
Premium Grade Fetal Bovine Serum (FBS)	VWR- Seradigm	Cat# 89510-194
DRAQ5	eBioscience	Cat# 65-0880-92
Propidium Iodine	Sigma-Aldrich	Cat# P4864
Red Blood Cell (RBC) Lysis Buffer	Sigma-Aldrich	Cat# R7757
Collagenase type II	Worthington Biochemical Corporation	Cat# LS004177
Trypan Blue Stain (0.4%)	Thermo Fisher Scientific	Cat# T10282
Ethanol 70% -140 Proof	Koptec - DeconLabs	Cat# V1401
Ethanol 100% - 200 Proof	Koptec - DeconLabs	Cat# V1016
EDTA disodium salt	Fisher Scientific	Cat# BP120500
Heparin sodium salt	Sigma-Aldrich	Cat# H3393
Critical Commercial Assays		
Dead Cell Removal MicroBeads	Miltenyi Biotec	Cat# 130-090-101
Chromium Single Cell 3' Library & Gel Bead Kit v2	10x Genomics	Cat# PN-120237
Chromium Single Cell A Chip Kit	10x Genomics	Cat# PN-120236
Chromium i7 Multiplex Kit	10x Genomics	Cat# PN-120262
Single Cell 3' Reagent Kits v2 Document	10x Genomics	Cat# CG00052
Experimental Models: Organisms/Strains		
Mouse: B6. Cg-Gt(ROSA)26Sor ^{tm6(CAG-ZsGreen1)Hze/J}	The Jackson Laboratory	JAX: 007906
Mouse: Tg(Wt1-cre)#Jbeb	(del Monte et al., 2011 , Wessels et al., 2012)	MGI: 5308608
Mouse: Tg(Col1a1-EGFP)#Dab	(Yata et al., 2003)	MGI: 4458034
Mouse: C57BL/6J	The Jackson Laboratory	JAX: 000664
Mouse: 129S1/SvImJ	The Jackson Laboratory	JAX: 002448
Other		
Refrigerated benchtop centrifuge for 5, 15, 50 mL tubes	N/A	N/A
Refrigerated benchtop microcentrifuge for 1.5–2 mL tubes	N/A	N/A
IKA magnetic stirrer	IKA	Cat# 0003690601

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
3 Eisco™ glass pneumatic trough (for water bath)	Fisher Scientific	Cat# S89305
Autoclaved Erlenmeyer flask (50 mL)	N/A	N/A
Disposable transfer pipette	N/A	N/A
200 µL wide bore, low retention pipette tips with filter	Rainin	Cat# 30389188
DNA LoBind 1.5 mL tubes	Eppendorf	Cat# 022431005
Sterile bacteriological petri dish	N/A	N/A
Scalpel or blades	N/A	N/A
2 curved serrated Graefe forceps	Fine Science Tools	Cat# 11051-10
2 curved Walton scissors	Fine Science Tools	Cat# 14077-09
AutoMACS separator	Miltenyi Biotec	Cat# 140-00-531.03
AutoMACS column	Miltenyi Biotec	Cat# 130-021-101
FACS Aria II (5 lasers, 130 µm nozzle)	BD Biosciences	RRID:SCR_018091
Chromium controller	10x Genomics	N/A
Hemocytometer	N/A	N/A
Autoclave	N/A	N/A
0.2 µm syringe filters	FisherScientific	Cat# NC9103939
Cell Strainers 40 µm	Celltreat	Cat# 229481
50 mL syringes	N/A	N/A
Magnetic bars	N/A	N/A

MATERIALS AND EQUIPMENT

Solutions

Name	Reagents	Volume (mL)
Cleaning solution (for AutoMACS)	70% ethanol	600 mL
Rinsing solution (for AutoMACS)	Autoclaved PBS w/o Ca ²⁺ /Mg ²⁺ pH 7.4	600 mL
Running buffer (for AutoMACS)	0.5% BSA in PBS w/o Ca ²⁺ /Mg ²⁺ pH 7.4	600 mL
Enzymatic solution	268 IU/mL of Collagenase type II in PBS w/o Ca ²⁺ /Mg ²⁺	15 mL per sample
Binding buffer (for dead cell removal)	diluted 1:20 from stock provided with the kit in autoclaved ddH ₂ O	6 mL per sample
Wash buffer (for cells)	2% FBS in PBS w/o Ca ²⁺ /Mg ²⁺	50 mL
FACS buffer	2% FBS in PBS with 2 mM EDTA	50 mL
Collection buffer	0.04% BSA in PBS w/o Ca ²⁺ /Mg ²⁺	50 mL

Note: The FBS is heat inactivated for 30 min at 56°C. Heat inactivated serum can be aliquoted in 50 mL tubes and stored at –20°C. Before use, thaw in a water bath at 37°C. Remaining thawed serum can be kept at 4°C for four weeks. To avoid extensive periods of refrigeration, refreeze once in smaller aliquots. Avoid more than two freeze-thaw cycles to limit protein degradation.

△ **CRITICAL:** BSA precipitates rapidly and irreversibly when heated at 50°C or above. Do not autoclave but use sterile PBS for the running buffer, and syringe filter for the collection buffer (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/a4919pis.pdf)

△ **CRITICAL:** Lyophilized proteins, like collagenase, tend to absorb humidity (<http://www.worthington-biochem.com/tissuedissociation/working.html>). For accurate measurements, leave the Collagenase in a desiccator or at 20°C–22°C for at least 30 min before weighing.

△ **CRITICAL:** The dead cell removal beads are coated with molecules (i.e. Annexin V) which bind to the phospholipid phosphatidylserine, normally located in the inner leaflet of the plasma membrane and exposed on the surface of dying cells and during platelet activation (Nagata et al., 2016, van Genderen et al., 2008). This binding requires Ca⁺², therefore EDTA is not added to the AutoMACS running buffer for this application (for other applications, running buffer contains 2 mM EDTA).

Alternatives: The protocol has been applied to 8–12 week-old mice from several different strains and genetic backgrounds.

Alternatives: For tissue digestion, this protocol utilizes an autoclaved Erlenmeyer flask (50 mL) with a magnetic bar, placed in glass container with water, on a magnetic stirrer with controlled temperature. Alternatively, the digestion can be performed in single-use plastic histology containers placed in a shaking water bath, manually pipetting the suspension with a transfer pipette every 10 min. The gentleMACS Octo Dissociator (Miltenyi Biotec) provides a more automated alternative but requires optimization to minimize cell death.

Alternatives: For dead cell removal, this protocol uses an AutoMACS with magnetic columns reusable for 100 times or 1 month, using the DepleteS program. Alternatively, single-use magnetic columns are available (LS columns, Miltenyi Biotec Cat# 130-042-401) and can be used on a MACS multistand (Miltenyi Biotec Cat# 130-042-303) with a magnetic separator like QuadroMACS separator (Miltenyi Biotec Cat# 130-090-976). In this case, prepare 12 mL of binding buffer per sample (4 × 3 mL washes are required for each column); the elution time may be longer than with the AutoMACS, depending on the cell density.

STEP-BY-STEP METHOD DETAILS

Cell Isolation

⌚ Timing: 3 h

Adult murine cardiac ventricular tissue is dissociated to isolate single interstitial cells (Figures 1 and 2).

Note: This protocol is adapted from a previous protocol (Chong et al., 2011).

1. After euthanizing the mouse according to the protocol approved by your own Institution, place it on the dissection stage in supine position and fix the paws in place with four needles/pins.
 - a. Spray with 70% ethanol, cut the skin below the thoracic cavity, pull it up and place it under the two top needles/pins.
 - b. Use a different set of tools for the internal organs (to avoid mycoplasma contamination deriving from the fur/skin).
 - c. Open the rib cage: hold the lower edge of the sternum and pull it slightly up, cut upward along the right side of the sternum, down on diaphragm from the right side to the left side of the mouse and finally up on the left side to expose the full cavity.

Note: the heart will be pointing to the left so avoid starting the cut from the left side. This is particularly important when dissecting mice previously subjected to myocardial infarction (MI), with possible adhesion to the rib cage on the left side, and having a good view of the heart from the right side is pivotal for correct dissection without damage.

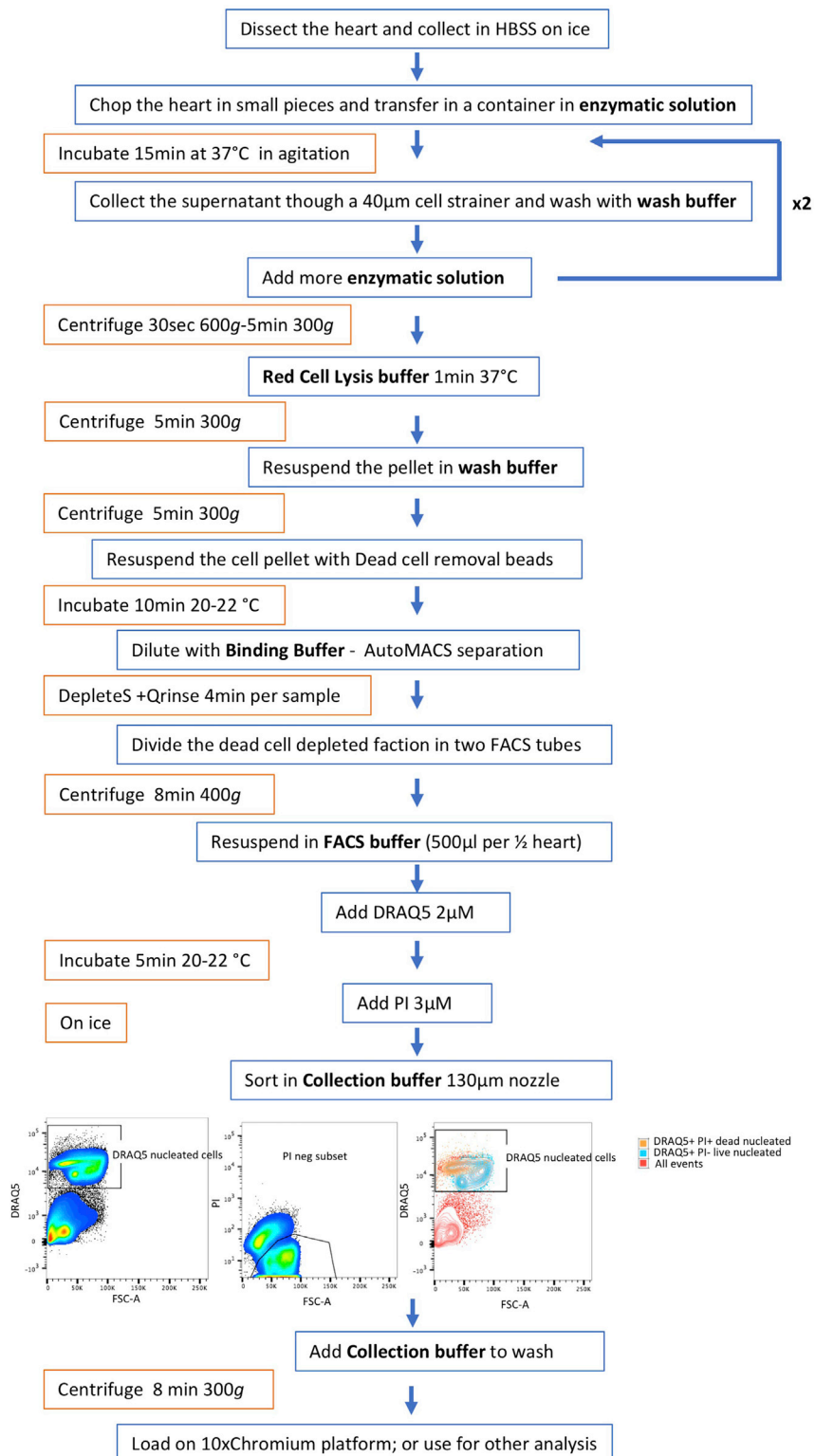


Figure 1. Workflow

Graphic summary of the main steps to obtain live nucleated single interstitial cells from adult murine hearts.



Figure 2. Images of Critical Steps

(A) Images of the set up before the experiment, which include preparing the buffers and turning on the AutoMACS; preparing the ice buckets, the collection tubes, the dissection stage; refrigerating the centrifuges at 4°C and warming up the water bath at 37°C.

(B) Images showing the dissection of the ventricles, enzymatic digestion in Erlenmeyer flask on a magnetic stirrer and collection of the cell suspension in a collection tube (CT) on ice.

(C) Images of the Red blood cell (RBC) lysis step and the pellet suspension in dead cell removal beads.

- d. Remove the heart (it is easier to run the tools along the abdominal aorta upward and cut the heart from the back), collect the heart still beating in a dish containing in HBSS, dissect out the atria and outflow tract, place on ice.

△ **CRITICAL:** If the cervical dislocation is not performed properly or the dissection takes too long and the heart is not pumping when placed in HBSS, there are high chances of getting solid blood clots in the ventricles. To reduce this risk, inject the mice intraperitoneally with Heparin, 5 min before culling, about 50 USP units per mouse. The recommended dose is 20–50 units per mL

of blood; an adult mouse has an average of 58.5 mL of blood/kg body weight; 1.46 mL in 25 g mouse (<https://www.nc3rs.org.uk/mouse-decision-tree-blood-sampling> from the National Center for Replacement Refinement & Reduction of Animals in Research).

Alternatives: To distinguish resident from circulating immune cells, labelled antibodies can be injected intravenously 1–3 min before culling the mice as in (Cochain et al., 2018).

2. Move to the tissue culture cabinet, cut the myocardium in small pieces 1 mm circa using a blade or scalpel, on a bacteriological petri dish. Add 1 mL of enzymatic solution and mince again to obtain pieces of homogeneous size. Transfer in a container (autoclaved Erlenmeyer flask (50 mL) with a magnetic bar) with additional 7 mL of enzymatic solution using a wide bore 1 mL transfer pipette.

Alternatives: The tissue suspension can also be transferred using a 10 mL serological pipette; or 1 mL pipette tip, modified by cutting the narrow-edge of about 2 mm with a blade.

Note: aspirate some enzymatic solution before collecting the tissue fragments to avoid unwanted adhesion to the dry plastic surface of tip/pipette.

- a. Place the flask in the glass troughs filled with distilled water pre-warmed at 37°C on the magnetic stirrer.
- b. Leave in agitation 250 rpm at 37°C for the first 15 min digestion. After gently pipetting the suspension, collect the supernatant in the pre-assembled collection tube on ice (50 mL tube topped with a 40 µm cell strainer, pre-wet with 1 mL of FBS). Leave the remaining tissue fragments in the flask and add 7 mL of enzymatic solution for a second 15 min digestion at 37°C.
- c. Wash cell strainer with 3 mL of wash buffer (2% FCS in PBS)
- d. After the second digestion, collect the cell suspension through the strainer and wash with 3 mL of wash buffer.

Note: During the digestion pre-warm 1 mL aliquots of RBC lysis buffer at 37°C, in 1.5 mL tubes (one per each sample).

3. Spin down the cell suspension preferably in a refrigerated centrifuge, 30 s at 600 × g, 5 min at 300 × g.
4. Red blood cell removal: resuspend the cell pellets in 1 mL pre-warmed RBC lysis buffer and transfer in 1.5 mL Eppendorf tube. After 1 min, spin down in a refrigerated microcentrifuge at 300 × g for 5 min. Remove the supernatant, resuspend the pellet in 1 mL of wash buffer and spin down at 300 × g for 5 min.

Alternatives: to reduce one centrifugation step, the RBC lysis can be also done in the original 50 mL collection tube. After 1 min, dilute with 10 mL wash buffer and spin down at 300 × g for 5 min.

△ CRITICAL: It is important to perform RBC lysis before the dead cell removal to prevent unspecific depletion of viable immune cells. Immune cells can interact with activated platelets, which bind the magnetic microbeads in presence of Ca⁺². Therefore, complexes of immune cells and platelets could be retained in the magnetic columns and depleted.

5. Dead cell removal: remove the supernatant and resuspend the cell pellet in 200 µL dead cell removal beads. Incubate 10 min at 20°C–22°C in the dark. Place empty 50 mL tubes under each exit port of the AutoMACS; the live cells will be eluted through the negative selection

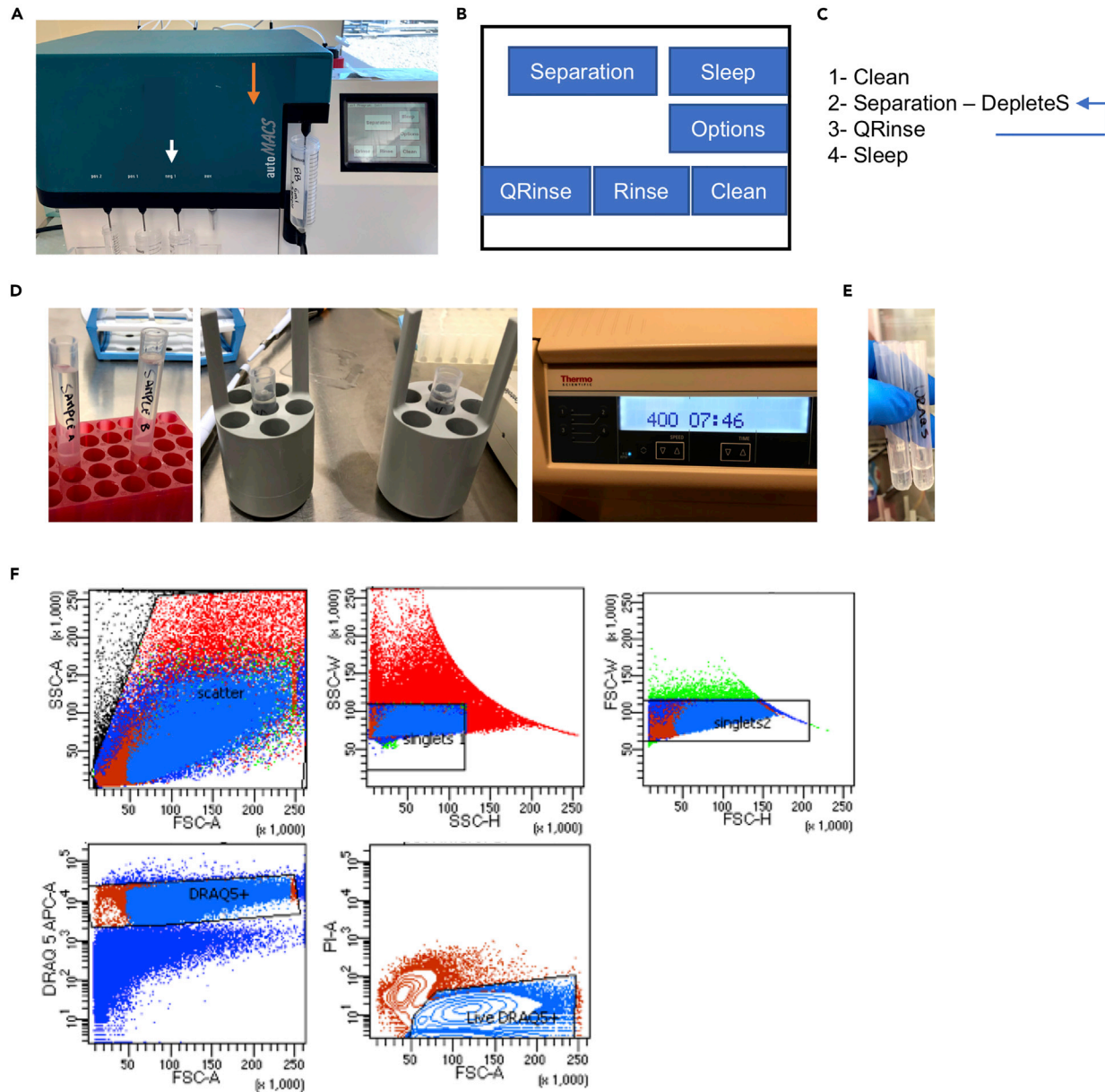


Figure 3. Images of Critical Steps

- (A) Image of the AutoMACS. The orange arrow indicates the intake port and the white arrow indicates the output or negative selection port (“neg”).
- (B) Schematic representation of the autoMACS main screen.
- (C) Workflow indicating the steps to perform on the AutoMACS per each sample.
- (D) The eluted cell suspension is divided in two 5 mL FACS tube and centrifuged.
- (E) Each pellet is resuspended in in 500 μ L of FACS buffer with DRAQ5. PI is added after 5 min, before sorting.
- (F) Full gating strategy for the selection of single live (PI⁻) nucleated (DRAQ5⁺) cells.

port (“neg”) (Figure 3A). Transfer the beads-cell suspension in a 15 mL tube with 6 mL of binding buffer, and place it under the uptake port. Using the touchscreen, select the “Separation” menu and the depletion program “DepleteS” (Figures 3B and 3C). After completion start the “QRinse” cleaning program before performing the separation on the next sample. Estimated time of DepleteS and QRinse is about 5 min per sample, and the live cells will be eluted in about 8 mL of running buffer.

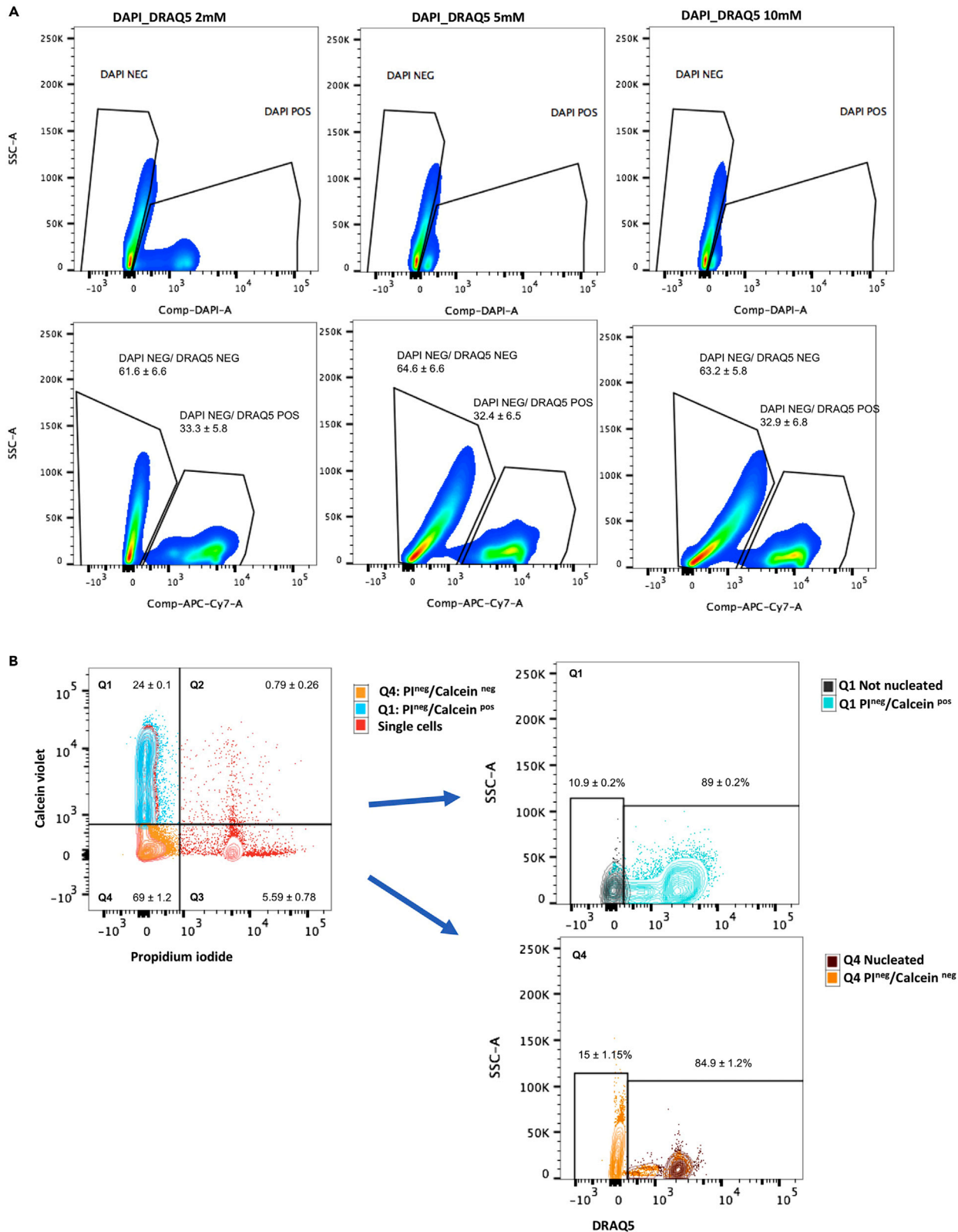


Figure 4. Additional Combination of Dyes Tested for the Selection of Live Nucleated Cells

(A) FACS plots showing cells labeled with DAPI (180nM) and 3 serial dilutions of DRAQ5 (0.1, 1, 10 μ M).

(B) FACS plots showing the gating strategy for cells co-labeled with PI, CalceinV450, and DRAQ5. Cells in Q1: Calcein +PI- (live metabolically active cells) and Q4: Calcein-PI- were further subclustered based on the DRAQ5 expression (right panels, top and bottom, respectively). Percentages of populations are reported on each plot and presented as average \pm SEM of 3 replicates.

6. Divide the cell suspension in two flow cytometry round bottom 5 mL tubes. Spin 8 min at $400 \times g$ (the pellet should not be too compact to avoid doublets and breaking cells) (Figures 3D and 3E).
7. Resuspend cell pellets in FACS buffer (500 μ L/tube). Add DRAQ5 at the final concentration of 2 μ M and after 5 min at 20°C–22°C, add Propidium iodide at the final concentration of 3 μ M and transfer the cells on ice. Proceed to the flow cytometer. In the 5 min incubation time, prepare the collection tubes: 1.5 mL tubes with 50–100 μ L collection buffer, and vortex and/or place on a tube roller shaker to equilibrate the tube walls with the buffer. The complete gating strategy can be found in Figure 3F.

Note: The combination of DRAQ5 and PI is compatible with cells expressing green fluorescent reporter proteins as ZsGreen1 and eGFP, when using a 5-laser flow cytometer. Other combinations of dyes were tested as shown in Figure 4. We tested DRAQ5 5, 10, 20 μ M in combination with DAPI 180 nM (stock 1 mg/mL, diluted to 1 μ g/mL used 1:20). Both dyes intercalate DNA: DRAQ5 permeates all cells, whereas DAPI permeates only dead cells. The percentage of DAPI^{neg} DRAQ5^{pos} cells was very similar in the three conditions (Figure 4A) but by increasing the concentration of DRAQ5, the intensity of DAPI staining would decrease, making the separation DAPI^{neg}/DAPI^{pos} less clear. Therefore, we used the lowest concentration of DRAQ5 (2mM) and replaced DAPI with PI which intercalates both DNA and RNA.

Note: We also tested Calcein Violet 450 AM, a dye which labels metabolically active live cells. Once inside the cell this membrane-permeable compound is converted by intracellular esterases in a membrane-impermeable violet fluorescent dye, which can be retained only by live cells with intact membrane. We tested 3 concentrations (0.1, 1, 10 μ M) with incubation of 20 min or 1h at 20°C–22°C, and found that 10 μ M to be the optimal concentration, labelling about 30% of total events both a 20 min and 1h. When combining PI 3 μ M, DRAQ5 2 μ M and Calcein 10 μ M, we observed a good overlap between DRAQ5 and Calcein but about 10% of the PI^{neg}Calcein^{pos} cells were not nucleated (Figure 2B). Given also the longer incubation time (20 min 20°C–22°C followed by an extra wash) we decided not to use Calcein in the final experiments.

8. Use a flow cytometer temperature at 4°C, preferably with a 130 μ m nozzle. Collect 24,000–32,000 events, keeping the flow rate between 900–1,100 events/s and the sorting efficiency over 90%. In these conditions, about 2 min per samples should be sufficient to collect the above-mentioned number of events. See Troubleshooting.

Note: We used a 130 μ m nozzle as the pressure on cells is 12 psi, which is half of the pressure exerted by a 100 μ m nozzle.

Note: We observed that with our preparation the cell counter Countess® II Automated Cell Counter underestimated the real number of cells, possibly due to the wide range of cell sizes. Additionally, time is an important factor, so rather than sorting the whole sample, counting and risking cell death post-selection, we sorted just the minimum number of events required for loading the right number of cells in the 10 \times Chromium chip. We aimed to load 12,000 cells per lane, which with the capture efficiency of 50% (Zheng et al., 2017) would result in about 6,000 cells lysed and sequenced, and a low percentage of multiplets (4.6% multiplet rate). Based on previous tests and calculations we estimated an error of 20% between the number of events sorted (when sorting with a digital efficiency over 90% on our instrument, a customized FACSAria II- BD Biosciences), and the number of cells counted with a hemocytometer.

With a 25% loss after centrifugation, we estimated that 32,000 events would give us 12,000 cells to load. In preparations from hearts in later phases of remodeling (d14-d28 post-MI) we observed higher efficiency of recovery (possibly due to the prevalence of larger-sized cells), therefore 24,000 events were used.

9. Transfer the collection tubes with sorted cells on ice. Add 800 μL of collection buffer per tube.
10. Centrifuge at 300g for 8 min in a refrigerated swing bucket microcentrifuge.

Alternatives: When using a centrifuge with fixed rotor, we found that spinning for 4 min, then turning the tubes and spinning for additional 4 min, helps to collect the cells at the bottom of the tube, and reduce loss due to cells sticking to the wall of the tube. We recommend using Low Retention Tube.

11. Aspirate the supernatant leaving ~ 40 μL of collection buffer
12. Proceed following the 10 \times Chromium protocol. In (Forte et al., 2020) we used the version 2 chemistry, according to the protocol described here (https://assets.ctfassets.net/an68im79xiti/16hQRgC8GoEgMOagGGCucm/a773eeb791949f8f9f6d6472771a7455/CG000075_Chromium_Single_Cell_3_V2_Ref_Cards_Rev_C.pdf)

△ CRITICAL: Always use fresh 80% Ethanol for all the purification steps.

Note: Agilent Bioanalyzer High Sensitivity Chips were used for the quality control of QC the cDNA and Library samples.

EXPECTED OUTCOMES

This protocol has been optimized for the isolation of single live nucleated cells, to reduce capture of membrane fragments, debris, and dying cells in downstream single cell analysis. From an adult 8–12-week-old mouse this protocol yields about 30% live nucleated cells (percentage of total events) and recovers $0.8\text{--}1.6 \times 10^6$ interstitial cells, with less than 10% Trypan blue positive cells (by manual counting with a hemocytometer).

Sorting 24,000–32,000 events for 10 \times Chromium, we captured an average of 5,210 cells per lane (number of cells estimated with CellRanger 2.0 analysis; 7,384 with CellRanger 3.0). We obtained an average of 217 ± 91.2 ng of cDNA per sample and 0.025 ng of cDNA per estimated cell, in samples from homeostatic hearts at 14–28 days post-MI (late remodeling). These values are nearly doubled in samples from 1, 3, 5, and 7 days post MI (inflammatory proliferative phase; 0.04–0.06 ng of cDNA per estimate cells).

LIMITATIONS

The current protocol is highly selective for viable and transcriptionally active cells due to the two steps to remove dead cells/debris (magnetic beads removal and sorting). While this reduces the chances of co-isolating dead cells or debris, cell types that are more susceptible to dissociation-induced cell death may be underrepresented. While precise information on the absolute cell numbers is not feasible, relative changes in cell composition can be compared among samples processed in the same way. Compared to previous methods (Pinto et al., 2016), we observed a prevalence of mesenchymal/stromal cells in homeostatic conditions and reduced relative percentage of endothelial cells, hence we did not require an artificial down-sampling of endothelial cells for downstream scRNA-seq analysis (Skelly et al., 2018). The unbiased sampling of endothelial cells allowed the capture of lymphatic endothelial cells and different subpopulations in the main endothelial cell cluster.

In the future, new transcriptomic approaches that do not require tissue dissociation will provide more precise information on the localization and contribution of different interstitial cell populations to the cardiac tissue (Asp et al., 2020).

TROUBLESHOOTING

Problem

Prolonged sorting time may be due to excessive cell death during preparation resulting in a significantly lower percentage of nucleated live cells.

Potential Solution

Chopping the heart too finely in step 2 can significantly increase the number of dead cells.

Check also the water bath temperature and the concentration of the enzymatic solution.

Removing the supernatant carefully by aspiration, instead of tilting the tubes, can help reducing loss of cells in all the washing steps.

A fast spin (600 × *g*) for 30 s before centrifugation can concentrate the cells pelleted at the bottom of the tube rather than on the side walls.

Be sure to pipette the cell gently at every step, and pre-coat the collection tubes with collection buffer.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elvira Forte (elvira.forte@jax.org).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

This work was supported by the JAX Director's Innovation Fund, the British Heart Foundation, and the Leducq Foundation Transatlantic Network of Excellence in Cardiac Research.

We thank Dr. Dhanushi Abeygunawardena and Dr. Vaibhao Janbandhu for helping with the optimization of the original cell isolation protocol. We acknowledge the use of JAX Flow Cytometry, Microscopy, and Single Cell Sequencing Cores. JAX Cores are supported by The Jackson Laboratory Cancer Center grant (P30 CA034196).

AUTHOR CONTRIBUTIONS

Methodology, E.F.; Investigation, E.F., S.D.; Writing E.F., N.R.; Visualization, E.F.; Funding Acquisition, N.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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