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A protocol for rapid and parallel isolation of myocytes and non-myocytes from multiple mouse hearts.

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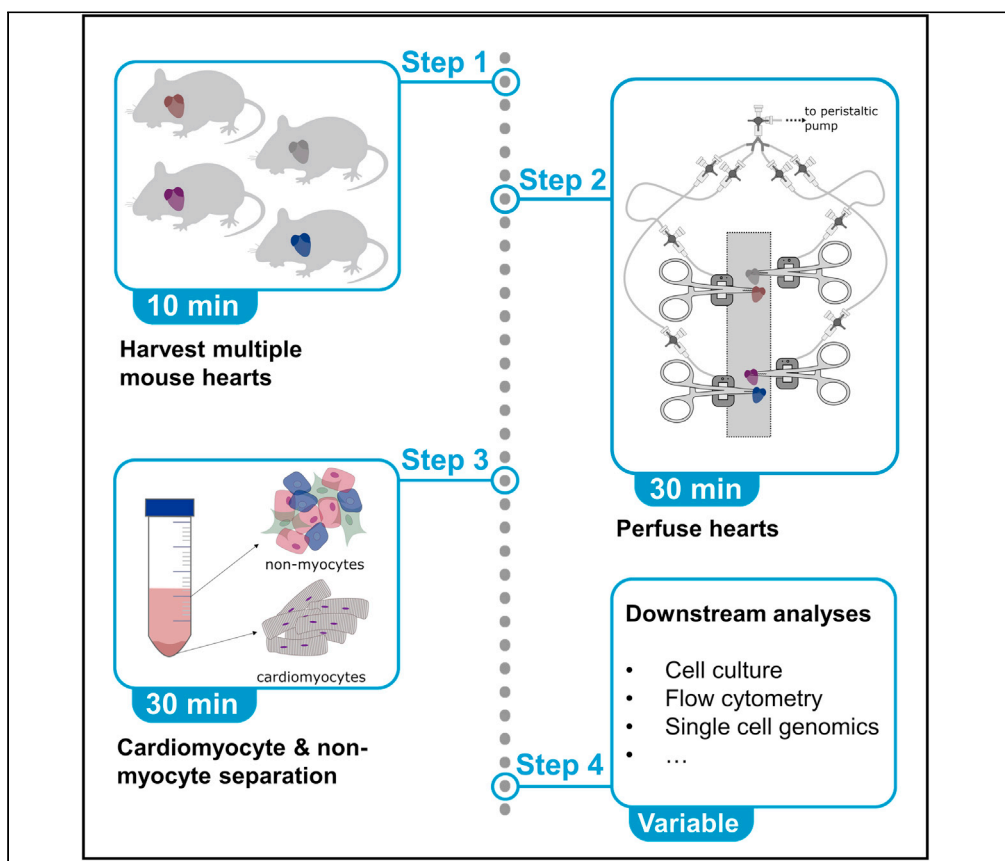
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Protocol

A protocol for rapid and parallel isolation of myocytes and non-myocytes from multiple mouse hearts



This protocol features parallel isolation of myocytes and non-myocytes from murine hearts. It was designed with considerations for (1) time required to extract cardiac cells, (2) cell viability, and (3) protocol scalability. Here, a peristaltic pump and 3D-printed elements are combined to perfuse the heart with enzymes to dissociate cells. Myocytes and non-myocytes extracted using this protocol are separated by centrifugation and/or fluorescence-activated cell sorting for use in downstream applications including single-cell omics or other bio-molecular analyses.

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Highlights
Protocol for extracting myocytes and non-myocytes from multiple mouse hearts

Protocol is simple and scalable for processing one or many hearts

Significant time saving for rapid isolation of viable and high-quality cardiac cells

Isolated cells are suitable for cell culture, single-cell omics, and various assays

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Protocol

A protocol for rapid and parallel isolation of myocytes and non-myocytes from multiple mouse hearts

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SUMMARY

This protocol features parallel isolation of myocytes and non-myocytes from murine hearts. It was designed with considerations for (1) time required to extract cardiac cells, (2) cell viability, and (3) protocol scalability. Here, a peristaltic pump and 3D-printed elements are combined to perfuse the heart with enzymes to dissociate cells. Myocytes and non-myocytes extracted using this protocol are separated by centrifugation and/or fluorescence-activated cell sorting for use in downstream applications including single-cell omics or other bio-molecular analyses.

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

BEFORE YOU BEGIN

The protocol below describes the methodology for parallel isolation of both cardiomyocytes and non-myocytes from four adult mouse hearts and innovates upon the approach by Ackers-Johnson et al. (2016). We have routinely applied this protocol on mice aged 8–20 weeks and anticipate it can be readily used to process hearts from younger or older mice if the hearts can be isolated and clamped as described below. Further, while the protocol describes the processing of four hearts, the approach can be scaled to isolate cells from fewer or more hearts (e.g., 2–16 hearts). Note, the parallel processing of a larger number of hearts may require more technicians to aid in heart dissection and monitoring of the cell dissociation system.

Preparation prior to day of experiment

⌚ Timing: 2–4 h

1. Prepare all stocks, buffers and solutions listed under *Prepare prior to the day of the experiment* in the **Buffers and stocks required** table (below).



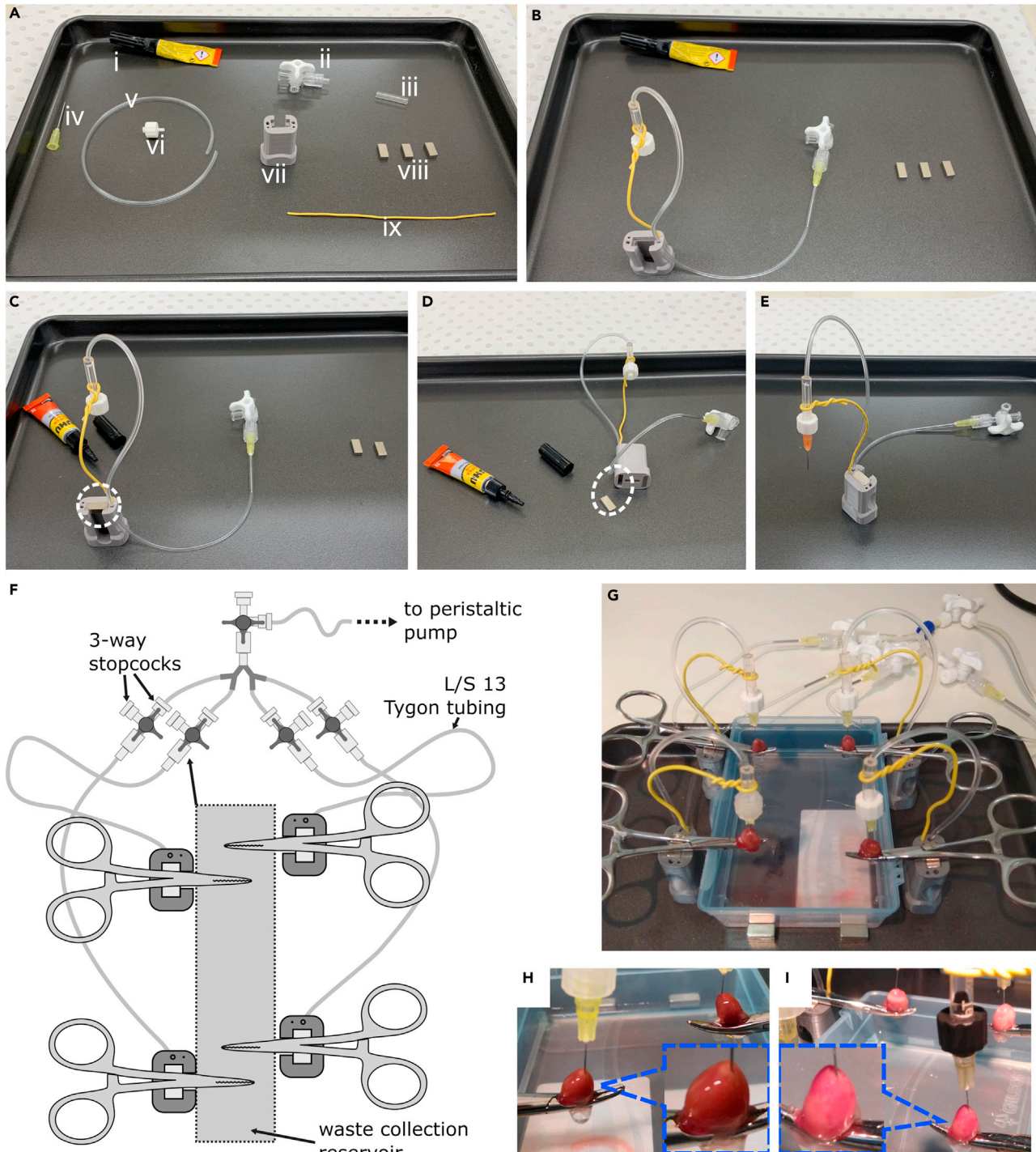


Figure 1. Assembly of 3D-printed perfusion platform

(A) Collection of all required elements. Image shows: (i) superglue; (ii) 3-way stop-cock; (iii) LS-13 nylon tubing; (iv) 23½ gauge needle with beveled tip removed and needle tip filed; (v) Tygon tubing; (vi) male luer to hose barb adapter fitting; (vii) 3D-printed perfusion platform; (viii) neodymium magnets; (ix) solid-core 22AWG electrical wire. Steel baking tray is also shown (but not labeled).

(B) Assembly of components before insertion of magnets. Note: LS-13 tubing is used as an intermediate tubing to couple the adapter fitting (component vi) and the narrow Tygon tubing.

(C) Mounting of magnet to the top of the platform. Note: a small amount of superglue is applied to the plastic seat which will house the magnet.

(D) Insertion of magnets to the bottom of the platform. Note: a small amount of superglue is applied to the holes which house the magnets.

Figure 1. Continued

- (E) Fully assembled platform with 30G needle (orange) attached.
- (F) Setup of fluidics system for processing of four hearts.
- (G) Example image of perfusion system processing four hearts.
- (H) Example image of a heart being processed that is not completely digested.
- (I) Example image of a heart being processed, that is completely digested and ready to be removed from platforms.

Note: In this protocol we demonstrate simultaneous perfusion and digestion of four hearts. As aforementioned, the setup can be easily modified to process different numbers of hearts. However, an even number of hearts is recommended to maintain equal fluidic pressure.

2. If required, autoclave dissection tools.
3. If required, print magnetic hemostat holders (Figure 1A, vii) and assemble perfusion platform (see Figures 1A–1G). The STL file required for printing the magnetic hemostat holder is downloadable in the Pinto Laboratory Github repository (https://github.com/pinto-lab/Farrugia-et-al_2021-magnetic-hemostat-holder).
4. Cut 177 μm nylon mesh to appropriately cover 50 mL tube openings.

Optional: Mesh with different pore size could be used (for example 250 μm). 177 μm pore diameter mesh used here readily permits passage of cardiomyocytes and other cells while excluding undigested tissue.

Preparation on day of experiment

⌚ Timing: 1–2 h

5. Optional for cardiomyocyte cell culture: In sterile conditions, add laminin to 15 $\mu\text{g}/\text{mL}$ to the required volume of PBS without Ca^{2+} Mg^{2+} . Coat 60 mm tissue culture dish with 2 mL of 15 $\mu\text{g}/\text{mL}$ laminin in sterile PBS without Ca^{2+} Mg^{2+} . Incubate at 37°C, 5% CO_2 for at least 1 h prior to plating cardiomyocytes.

Note: For plating, one heart will require two 60 mm tissue culture dishes. Alternatively, one 100 mm tissue culture dish can be used per heart. If so, use 3 mL of 15 $\mu\text{g}/\text{mL}$ laminin in sterile PBS without Ca^{2+} Mg^{2+} for coating. The laminin coated tissue culture dishes should be prepared on the day of the experiment and allowed to incubate with laminin at least 1 hour prior to use to allow adhesion of laminin. The laminin solution can be left in the dish for several hours during the day however not overnight. Once used, laminin coated tissue culture dishes should be discarded.

6. Place on ice 10 mL syringes loaded with 7 mL of ice-cold *EDTA Buffer* with 25G hypodermic needles attached.
7. Prepare heparin solution to a final concentration of 1 U/ μL in PBS without Ca^{2+} Mg^{2+} . Administer 100 μL of heparin solution to each mouse by intraperitoneal injection. Allow 15–30 min for adequate absorption of heparin.
8. While waiting for heparin to take effect (step 7):
 - a. Pre-chill the swing-bucket centrifuge and required accessories (buckets and tube holders) to 4°C. Alternatively, if the primary objective is to isolate cardiomyocytes for tissue culture, leave a swing-bucket centrifuge at room temperature (RT).
 - b. Pre-heat the water bath to 37°C and place the bottle containing *EDTA buffer* in the water bath (after loading 10 mL syringes with 7 mL cold *EDTA buffer* in step 6 above).
 - c. Setup fluidic system as shown in Figures 1F–1G.
 - d. Prime tubing and needles for perfusion with *EDTA buffer* by running solution through the tubing to expel any trapped air.

Note: While priming, ensure there are no leaks from fixtures and fluidics. Attend to any leaks as this will affect the efficiency of perfusion of the hearts, cell isolation and viability. It is prudent to have spare fluidic fixture components available in case replacement of a broken element is required. For further considerations regarding fluidic setup preparation see [problem 2](#) in the [troubleshooting](#) section for more details.

9. Prepare:
 - a. Perfusion Buffer with enzymes and leave at RT.
 - b. Stop Buffer and keep on ice throughout protocol. Leave at RT if cardiomyocytes are to be cultured.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Collagenase Type II	Worthington Biochemical Corporation	LS004179
Collagenase Type IV	Worthington Biochemical Corporation	LS004188
Protease Type XIV	Sigma Aldrich	P5147
DPBS, no calcium, no magnesium	Gibco™	14190-250
UltraPure™ DNase/RNase-Free MQH2O	Invitrogen™	10977015
NaCl	Sigma-Aldrich	S7653
Glucose	Sigma-Aldrich	G8270
EDTA	Sigma-Aldrich	EDS
Taurine	Sigma-Aldrich	T0625
BDM	Sigma-Aldrich	B0753
(optional: Blebbistatin in lieu of BDM)	Sigma-Aldrich	B0560
NaH ₂ PO ₄	Sigma-Aldrich	71496
HEPES	Sigma-Aldrich	54457
KCl	Sigma-Aldrich	P9541
MgCl ₂	Sigma-Aldrich	208337
FBS	Gibco™	10099141
HBSS	Gibco™	14185052
CaCl ₂	Sigma-Aldrich	C5670
80% v/v Ethanol	Any	Any
Laminin Mouse Protein, Natural	Gibco™	23017015
MEM, Hanks' Balanced Salts	Gibco™	11575032
Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) (100X)	Gibco™	51300044
Insulin, Neutral, 1000units/10mL vial	Novo Nordisk	Actrapid
CD lipid concentrate	Thermo Fisher	11905031
Magnesium sulfate	Sigma-Aldrich	M7506
Sodium pyruvate	Sigma-Aldrich	P2256
Sodium bicarbonate	Sigma-Aldrich	S5761
L-Glutamic acid monosodium salt hydrate	Sigma-Aldrich	G5889
Sodium acetate	Sigma-Aldrich	S2889
Phenol red sodium salt	Sigma-Aldrich	P5530
Penicillin-Streptomycin (10,000 U/mL)	Gibco™	15140-122
Bovine serum albumin (BSA)	Sigma-Aldrich	A9418
Experimental models: Organisms/strains		
12- to 16-week-old male C57BL6	The Jackson Laboratory	000664
Other		
Allegra X-15R refrigerated swing bucket centrifuge (or equivalent)	Beckman Coulter	4882960
Water bath, 37°C	Any	Any

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
L/S Digital Pump System with Easy-Load II Pump Head (or equivalent)	Masterflex	HV-77921-70
L/S® Precision Pump Tubing, Tygon® E-LFL, L/S 13	Masterflex	HV-06440-13
25GX5/8 Needles (or equivalent)	Terumo	19005-TE
30GX1/2 Needles (or equivalent)	Terumo	19050-TE
10 mL Luer Lock Syringes (or equivalent)	Terumo	19046-TE
Graefe Forceps - Curved/Serrated, 0.8 × 0.7 mm, Stainless Steel	Fine Science Tools	11051-10
Walton Scissors - Curved/Sharp, 15 mm, 9.5 cm, Stainless Steel	Fine Science Tools	14077-09
Three-way stopcocks	Becton Dickinson	394600
Luer Assortment Kit	Cole-Parmer	45511-00
Neodymium magnet blocks (L: 12.5 mm, W: 6 mm, H: 3.5 mm, Grade 43-46 MGOe)	Alpha Magnetics Pty Ltd	N45
PETG 3D printing material	Prusa Research	PRM-PETG-URB-1000
3D printer	Prusa Research	Original PRUSA i3 MK3S
Curved hemostat	Any	Any
Solid-core 22AWG electrical wire	Adafruit Industries	289
Masterflex Tygon Tubing	Cole-Parmer	06419-03
Magnetic baking tray	Any	Any
Nylon mesh 177 μm (for cardiomyocytes) <i>Note: While we use nylon mesh with a pore size of 177 μm, we recommend mesh with pore sizes ranging from 170 μm to 250 μm for cardiomyocyte isolations.</i>	Fresh By Design	NM177
3 mL Pasteur pipettes, sterile	Any	Any
50 mL Tubes, conical base	Corning	352070
70 μm Nylon mesh (for non-myocytes)	Any	Any
15 mL Tubes, conical base	Falcon™	352096
60 mm Tissue culture dish	Falcon™	353002
14 mL Polypropylene Round Bottom Tube	Falcon™	352059
Sterile 70 mL specimen jars	SARSTEDT	75.9922.730
3D object file (STL) for hemostat holder	https://github.com/pinto-lab/	Farrugia-et-al_2021-magnetic-hemostat-holder

MATERIALS AND EQUIPMENT

Prepare prior to the day of the experiment

100 × collagenases II and IV

Reagent	Final concentration	Amount
Collagenase II	50 mg/mL	1 g
Collagenase IV	50 mg/mL	1 g
ddH ₂ O	n/a	20 mL
Total	n/a	20 mL

Reconstitute both collagenases in ddH₂O. Filter sterilize through 0.22 μm and store in 1 mL aliquots in -80°C. Stable for up to 6 months.

1000 × protease XIV

Reagent	Final concentration	Amount
Protease XIV	50 mg/mL	1 g
ddH ₂ O	n/a	20 mL
Total	n/a	20 mL

Reconstitute in ddH₂O. Filter sterilize through 0.22 μm and store in 200 μL aliquots in -80°C. Stable for up to 6 months.

EDTA buffer

Reagent	Final concentration	Amount
NaCl	130 mM	0.379 g
KCl	5 mM	19.6 mg
NaH ₂ PO ₄	0.5 mM	3 mg
HEPES	10 mM	119 mg
Glucose	10 mM	90.1 mg
BDM	10 mM	50.6 mg
(or blebbistatin)	5 mM	73.1 mg
Taurine	10 mM	62.6 mg
EDTA	5 mM	73.1 mg
ddH ₂ O	n/a	50 mL
Total	n/a	50 mL

Prepare 50 mL per heart. Prepare required volume of solution in ddH₂O with a final pH of 7.8. Filter sterilize through 0.22 μm and protect from light. Store at 4°C for up to 1 week.

Perfusion buffer

Reagent	Final concentration	Amount
NaCl	130 mM	0.456 g
KCl	5 mM	22.4 mg
NaH ₂ PO ₄	0.5 mM	3.6 mg
HEPES	10 mM	0.143 g
Glucose	10 mM	0.108 g
BDM	10 mM	60.7 mg
(or blebbistatin)	5 mM	87.7 mg
Taurine	10 mM	75.1 mg
MgCl ₂	1 mM	5.71 mg
ddH ₂ O	n/a	60 mL
Total	n/a	60 mL

Prepare 60 mL per heart. Prepare required volume of solution in MQH₂O with a final pH of 7.8. Filter sterilize through 0.22 μm and protect from light. Store at 4°C for up to 1 week.

Wash buffer

Reagent	Final concentration	Amount
CaCl ₂ (1M)	0.9 mM	90 μL
DPBS	n/a	9.91 mL
Total	n/a	10 mL

Prepare 10 mL per heart. Keep sterile and store at 4°C until experiment day. Can be made on or prior to experiment day.

10 × ACM

Reagent	Final concentration	Amount
KCl	54 mM	0.403 g
MgSO ₄	35 mM	0.421 g
Na pyruvate	500 mM	5.502 g
NaHCO ₃	200 mM	1.680 g
Glucose	110 mM	1.982 g
HEPES	200 mM	4.766 g
Na glutamate	235 mM	3.974 g
Na acetate	48.7 mM	0.399 g
EDTA	1 mM	29.2 mg
Phenol red	2 mg/mL	0.2 g
ddH ₂ O		80 mL
Total	n/a	80 mL

Prepare in 80 mL MQH₂O (final volume 100 mL). Adjust pH to 7.25 with 1M NaOH, taking note of volume required. Add NaCl calculated by the following equation:

$$\text{NaCl to add (g)} = -0.0585 \times (\text{volume of NaOH added}) + 6.02$$

Make up to 100 mL with MQH₂O. Filter sterilize through 0.22 μm. Can be prepared 3–4 days in advance and stored at 4°C.

Prepare on day of the experiment, before you begin.

Perfusion buffer with enzymes

Reagent	Final concentration	Amount
100 × Collagenase II and IV	1 ×	400 μL
1000 × Protease XIV	1 ×	40 μL
Perfusion Buffer	n/a	40 mL
Total	n/a	40.440 mL

Prepare from cold reagents 40 mL per heart on day of experiment and allow to warm to room temperature.

Stop buffer

Reagent	Final concentration	Amount
Fetal calf serum	5%	1 mL
Perfusion buffer	n/a	19 mL
Total	n/a	20 mL

Prepare 20 mL per heart on day of experiment.

Note: Keep Stop buffer on ice for cells with downstream applications requiring maintaining a cold temperature. Keep Stop buffer at RT for myocytes destined for cell culture.

ACMBICT

Reagent	Final concentration	Amount
BDM	1.01 mg/mL	50.5 mg
Creatine	0.657 mg/m	32.85 mg
Taurine	3.75 mg/mL	187.5 mg
Insulin	0.1 IU/mL	5 IU
10 × ACM	1 × ACM	5 mL
ddH ₂ O	n/a	45 mL
Total	n/a	50 mL

Prepare 50 mL per heart. Keep at RT

ACMBICT/FCS

Reagent	Final concentration	Amount
Fetal calf serum	10%	3 mL
ACMBICT	n/a	27 mL
Total	n/a	30 mL

Prepare 30 mL per heart. Keep at RT

Modified MEM

Reagent	Final concentration	Amount
Fetal calf serum	10%	3 mL
BDM	1 mg/mL	30 mg
MEM	n/a	27 mL
Total	n/a	30 mL

Prepare 30 mL per heart. Keep at RT

Myocyte wash buffer 1

Reagent	Final concentration	Amount
ACMBICT/FCS	n/a	9 mL
Modified MEM	0.18 mM Ca ²⁺	1 mL
Total	n/a	10 mL

Prepare 10 mL per heart and store at RT until use.

Myocyte wash buffer 2

Reagent	Final concentration	Amount
ACMBICT/FCS	n/a	6 mL
Modified MEM	0.45 mM Ca ²⁺	2 mL
Total	n/a	8 mL

Prepare 8 mL per heart and store at RT until use.

Myocyte wash buffer 3

Reagent	Final concentration	Amount
ACMBICT/FCS	n/a	4 mL
Modified MEM	1.08 mM Ca ²⁺	6 mL
Total	n/a	10 mL

Prepare 10 mL per heart and store at RT until use.

Plating media

Reagent	Final concentration	Amount
FCS	10%	2 mL
BDM	1 mg/mL	20 mg
Penicillin/streptomycin	1 ×	200 μL
MEM	n/a	17.8 mL
Total	n/a	20 mL

Prepare 20 mL per heart. Incubate at 37°C, 5% CO₂ with loosened cap until use.

Maintenance media

Reagent	Final concentration	Amount
BSA	0.5%	100 mg
ITS-A	1 ×	200 μL
CD lipid concentrate	1 ×	200 μL
Penicillin/streptomycin	1 ×	200 μL
MEM	n/a	19.4 mL
Total	n/a	20 mL

Prepare 20 mL per heart and filter sterilize. Incubate at 37°C, 5% CO₂ with loosened cap until use.

STEP-BY-STEP METHOD DETAILS

Preparation of hearts for enzymatic digestion via perfusion

⌚ Timing: 0.5–1 h

If possible, we recommend three investigators participate in this initial step of the protocol—two dissecting tissue and one handling the perfusion platform. The number of investigators is dependent

on the competence and speed of technicians and the number of hearts being processed. For a detailed description of thoracotomy and externalization of the mouse heart, see [Pinto et al. \(2013\)](#).

1. Euthanize four mice by CO₂ asphyxiation and promptly pin in supine position—two per dissection board per investigator. Place freshly prepared *Perfusion buffer with enzymes* in 37°C water bath.

Note: Alternatively, euthanasia can be performed by administration of ethical and pharmaceutically-appropriate drugs. Euthanasia by cervical dislocation is NOT recommended as an intact circulatory system is desired.

△ **CRITICAL:** During dissection, outlined in steps (steps 3–8), take care not to pierce any organs, especially the heart.

2. Spray anterior thorax with 80% v/v ethanol solution to minimize fur dispersal.
3. Cut away a small 1–2 cm² area of skin above the intersection of the septum and diaphragm.
4. While holding the septum with forceps, slowly raise the ribcage and carefully make an incision in the abdominal cavity with surgical scissors, taking care to follow the lateral margins of the rib cage.

Note: Septum is held with forceps from steps 4–7.

5. While still holding the septum, if required, gently push away any exposed abdominal organs to reveal diaphragm. This can be achieved by closing the scissors (to minimize risk of damaging organs) and using them to push aside organs.
6. While exposing the inferior cavity of the diaphragm, cut the diaphragm along the margin of where the diaphragm and rib cage intersect.
7. With care and without damaging the lungs; cut the ribcage upwards towards the arms from the left- and right-hand side.
8. Pin the ribcage back adjacent to the left ear of the mouse and carefully remove the pericardium (if visible) from the heart gently.

Note: Perform steps 2–8 per mouse on dissection board first. Follow the next steps (9–14) in this section 1 mouse at a time per scientist. Steps 9–14 typically requires <5 mins.

9. Locate and cut the inferior vena cava and immediately perfuse the animal with 7 mL of ice-cold *EDTA buffer* in the pre-loaded syringes, through the right ventricle.
10. Place forceps below the heart and gently raise it, elevating it from other organs to expose the aorta.
11. Using a curved hemostat, clamp the aorta and secure firmly, taking care not to clamp the atria. For troubleshooting regarding atria clamping see [troubleshooting](#) section, [problem 3](#).
12. With the heart clamped, cut below the hemostat to isolate each mouse heart, and transfer the clamped heart to the perfusion platform.
13. Magnetically secure the hemostat in place, with the ventricles pointing upward and place a 30G needle in the apex of the left ventricle (as shown in [Figures 1G–1I](#)). If difficulties are experienced in maintaining needle position, refer to [problem 1](#) in troubleshooting.
14. Start perfusion using the peristaltic pump at a rate of 1 mL/min/heart with *EDTA buffer* at 37°C and proceed from step 9 with the next heart. Ensure a waste reservoir is placed beneath the perfusing hearts to collect perfusate.

Note: All perfusate (here and in subsequent steps) is collected in a waste reservoir beneath the hearts. As the waste reservoir fills, use a Pasteur pipette to periodically empty the contents into a waste beaker.

15. During perfusion, ensure the surface of hearts remain moist by periodically basting the hearts using a Pasteur pipette with clean pre-warmed *EDTA buffer*. It is important to prevent tissue from drying out.
16. Once all hearts are being simultaneously perfused with *EDTA buffer*, increase perfusion flow rate to 6 mL/min (1.25 mL/min/heart).

TIP: When processing more than four hearts, multiple fluidic channels (Figure 1F) can be used to process hearts in batches of four. Alternatively, individual fluidic channels can be closed using the stopcocks to process fewer hearts.

Enzymatic digestion and cell isolation

⌚ **Timing:** 1–2 h

Observation: As *EDTA buffer* is perfusing through the heart, perfusate with blood will emerge from the heart. The perfusate will progressively clear as blood is removed from the tissue.

17. After blood is no longer dripping into the waste reservoir (~5 min after start of *EDTA buffer* perfusion), pause the peristaltic pump and switch buffers from *EDTA buffer* to *Perfusion buffer with enzymes* (which should be in the 37°C water bath – step 1) before resuming perfusion at 6 mL/min (1.25 mL/min/heart).
18. During digestion:
 - a. Baste hearts undergoing perfusion with the same pre-warmed *Perfusion buffer with enzymes* (at 37°C) until it appears slightly blanched, translucent, and dilated (~30 min).

Note: We have observed shorter perfusion times when agents such as sodium pentobarbitone are used for euthanasia. If uneven rates of digestion are observed, see [problem 4](#) in troubleshooting section. Further, as the hearts perfuse, the perfusate is accumulating in the waste reservoir – this is to be discarded.

- b. While waiting for hearts to digest, prepare the following per heart for the next steps and leave at RT:
 - i. A 30 mm petri dish with 1 mL of *Perfusion buffer with enzymes*.
 - ii. A 50 mL tube with 2 mL of *Perfusion buffer with enzymes*.
19. When digestion is complete (Figures 1H and 1I), hold the prepared 30 mm petri dish with buffer below the heart. Remove hypodermic needle from the heart. Indicating a well-digested heart, the tissue will appear soft. Ensuring the heart is held over the petri-dish (present at RT, prepared step – 18b.i), using the curved scissors, cut between the atria and the ventricles to collect the cardiac ventricles in the petri dish.
20. Repeat for remaining hearts.
21. At ambient temperature, gently pull apart the tissue within each petri dish using forceps to release the cells and break up the tissue so that it may be aspirated by a 3 mL Pasteur pipette. If heart appears incompletely digested, refer to the troubleshooting section, [problem 5](#).
22. Using a 3 mL sterile Pasteur pipette, transfer the suspension into the 50 mL tube with RT *Perfusion buffer* and triturate gently 10 times using the same pipette.
23. Place 50 mL tube in the 37°C water bath with caps loosely tightened for 5 min, and afterward pipette mix as aforementioned using a Pasteur pipette 15 times.

Note: If the objective of this protocol is to proceed with cell culture of cardiomyocytes, please follow alternative steps 31–37 “Alternative steps when isolating myocytes for cell culture” (instead of steps 24–30, which are primarily for isolation of non-myocytes). The alternative

steps prevent the exposure of cardiomyocytes to low temperatures and help preserve their physiological characteristics. The key differences of the alternative steps are the temperatures at which cells are maintained. If downstream processing of cardiomyocytes requires preservation of nucleic acids, protein and other elements that are labile at RT, then we recommend steps 24–30. We routinely use the steps at lower temperature for isolation of cardiomyocyte nuclei for RNA sequencing.

24. Place the 50 mL tube back in the 37°C water bath and incubate for another 5 min. During the incubation period, aliquot 5 mL of cold *Stop buffer* to a fresh 50 mL tube per heart and place on ice.
25. After incubation, gently pipette-mix the cardiac cell suspension using a Pasteur pipette for another 15 times.
26. Filter the cell suspension through 177 μm nylon mesh into the pre-prepared 50 mL tube containing *Stop buffer* and leave on ice, undisturbed, for 10 min.

Separation of myocytes from non-myocytes

⌚ Timing: 0.5–1 h

Note: In this final step of the protocol, non-myocytes are separated from myocytes and prepared for down-stream applications.

27. Centrifuge the cell suspensions at 50 \times g for 2 min at 4°C (with centrifuge breaks activated).
28. Prepare 15 mL tubes with 7 mL of cold *wash buffer* per heart and place on ice.
29. After centrifugation place tubes on ice. A loose pellet (primarily cardiomyocytes) and cloudy supernatant (primarily non-myocytes) will be present.
 - a. Supernatant – non-myocytes:
 - i. Transfer all but approximately 1 mL of supernatant to the 15 mL tubes (step 28) through 70 μm nylon mesh into the wash buffer and place on ice. Retain 50 mL tube for processing cardiomyocytes (step 29b, below).
 - ii. Top-up the volume of non-myocyte enriched samples to 15 mL with cold *wash buffer* and subsequently centrifuge at 200 \times g for 15 min at 4°C, with centrifuge breaks deactivated.
 - iii. Remove the supernatant and re-suspend the pellet (containing non-myocytes) in a desired buffer depending on subsequent application.
 - b. Pellet – Cardiomyocytes:
 - i. Add 5 mL of cold *Stop buffer* to the cardiomyocyte pellet in the 50 mL tube (after removing supernatant) and gently re-suspend the pellet.
 - ii. Spin down cardiomyocytes an additional time at 50 \times g for 2 min at 4°C (with centrifuge breaks activated).
 - iii. Aspirate supernatant and re-suspend pellet in desired buffer.

Note: Steps i and ii of step 29b may be repeated to decrease presence of non-myocytes

30. Use isolated myocytes or non-myocytes in desired application.

Alternative steps when isolating myocytes for cell culture

Follow steps 31–37 when preparing cardiomyocytes for cell culture to minimize stress cardiomyocytes may experience due to rapid changes in temperature and preserve their physiological characteristics. Note, the non-myocyte fraction is preserved either way.

31. Place the 50 mL tube back in the 37°C water bath and incubate for another 5 min. During the incubation period, aliquot 5 mL of RT *Stop buffer* to a fresh 50 mL tube per heart and leave at RT

32. After incubation, gently pipette-mix the cardiac cell suspension using a Pasteur pipette another 15 times.
33. Filter the cell suspension through 177 μm nylon mesh into the pre-prepared 50 mL tube containing *Stop buffer* and at RT, undisturbed, for 10 min.

Separation of myocytes from non-myocytes (for cell culture)

⌚ Timing: 0.5–1 h

Note: In this final step of the protocol, non-myocytes are separated from myocytes and prepared for down-stream applications.

34. Centrifuge the cell suspensions at $50\times g$ for 2 min at RT (with centrifuge breaks activated).
35. Prepare 15 mL tubes with 7 mL of cold *wash buffer* per heart and place on ice.
36. After centrifugation place tubes on ice. A loose pellet (primarily cardiomyocytes) and cloudy supernatant (primarily non-myocytes) will be present.
 - a. Supernatant – non-myocytes:
 - i. Transfer all but approximately 1 mL of supernatant to the 15 mL tubes (step 35) through 70 μm nylon mesh into the *wash buffer* and place on ice. Retain 50 mL tube for processing cardiomyocytes (step 36b, below).
 - ii. Top-up the volume of non-myocyte enriched samples to 15 mL with cold *wash buffer* and subsequently centrifuge at $200\times g$ for 15 min at 4°C , with centrifuge breaks deactivated.
 - iii. Remove the supernatant and re-suspend the pellet (containing non-myocytes) in a desired buffer depending on subsequent application.
 - b. Pellet – Cardiomyocytes:
 - i. Add 10 mL of RT *Stop buffer* to the cardiomyocyte pellet in the 50 mL tube (after removing supernatant) and gently re-suspend the pellet at RT
 - ii. Spin down cardiomyocytes an additional time at $20\times g$ for 3 min at RT (with centrifuge breaks activated).
 - iii. Aspirate supernatant and re-suspend in desired buffer.
37. Use isolated myocytes or non-myocytes in desired application.

Optional step: Preparation of myocytes for cell culture with Ca^{2+} reintroduction

If cardiomyocytes are to be used for applications such as cell culture, the following protocol is recommended to gradually restore physiological calcium levels prior to plating. The myocytes are sequentially incubated in *Myocyte Wash Buffers* containing increasing concentration of Ca^{2+} of 0.18, 0.45 and 1.08 mM (*Myocyte Wash Buffer 1, 2 and 3, respectively*) and are plated in *Plating Media* containing 1.26 mM Ca^{2+} .

Note: Cardiomyocytes are fragile. Avoid shocks/vibrations and pipette gently during cell resuspension steps. Further, handle tubes with care after centrifugation as the cardiomyocytes are loosely pelleted.

38. At step 36(b)iii, resuspend cardiomyocytes in 10 mL *Myocyte Wash Buffer 1*. Transfer cell suspension to a 70 mL specimen jar (to reduce cell clumping and to avoid ischemia) and incubate at RT for 10 min.
39. Post incubation, transfer cells to a 14 mL round-bottomed tube and centrifuge at $20\times g$ for 3 min at RT
40. Aspirate supernatant, leaving a small volume, $\sim(50\text{--}100\ \mu\text{L})$ so as not to disturb the pellet, and resuspend cardiomyocytes in 8 mL *Myocyte Wash Buffer 2*, transfer to a 70 mL specimen jar and incubate at RT for 10 min.
41. Transfer cells to a 14 mL round-bottomed tube and centrifuge at $20\times g$ for 3 min at RT

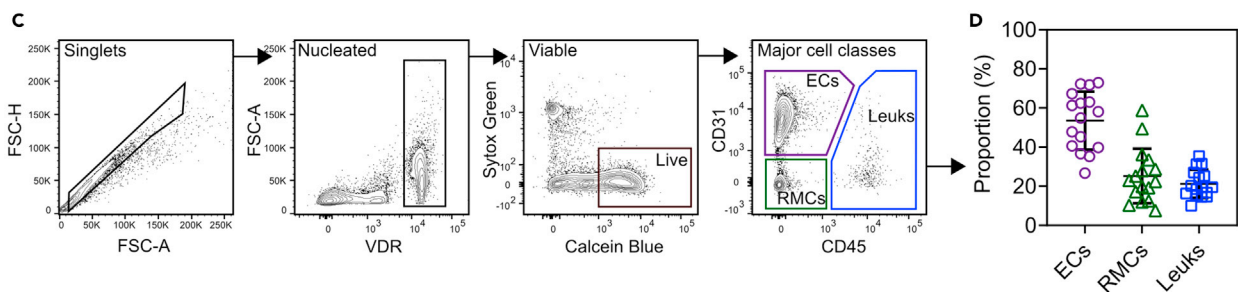
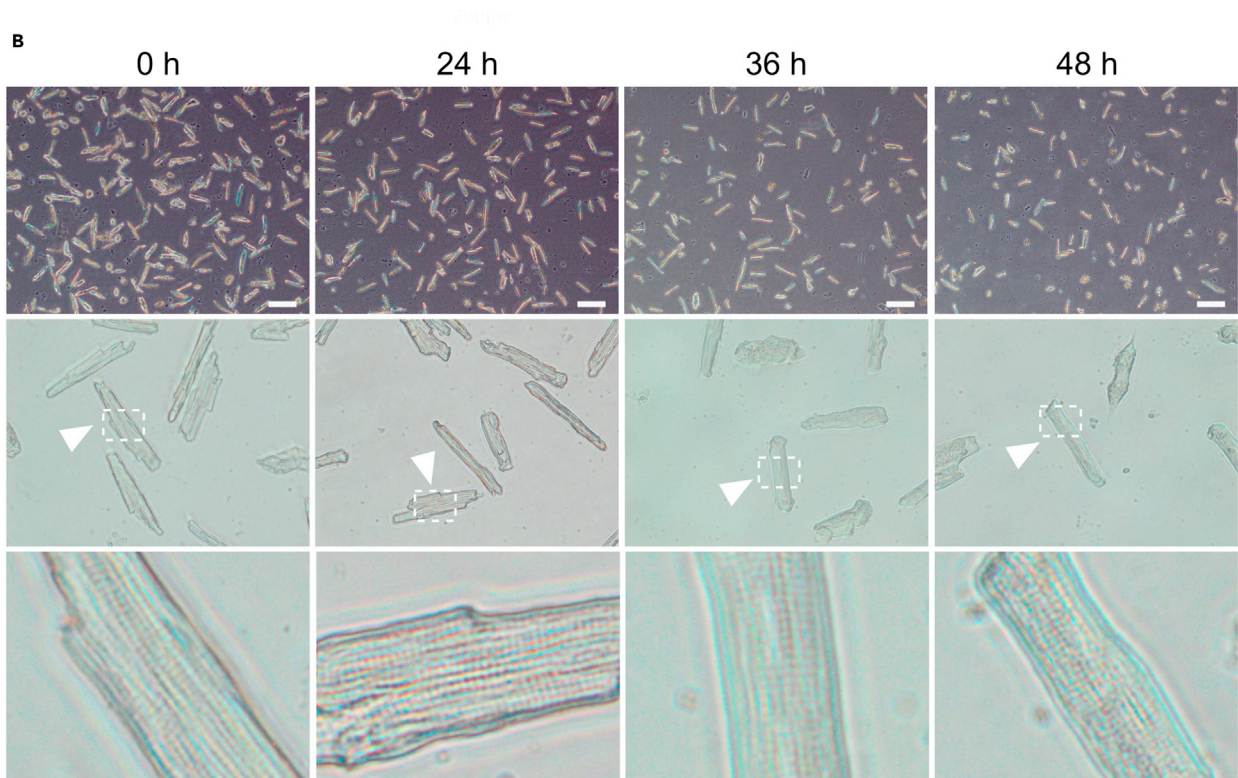
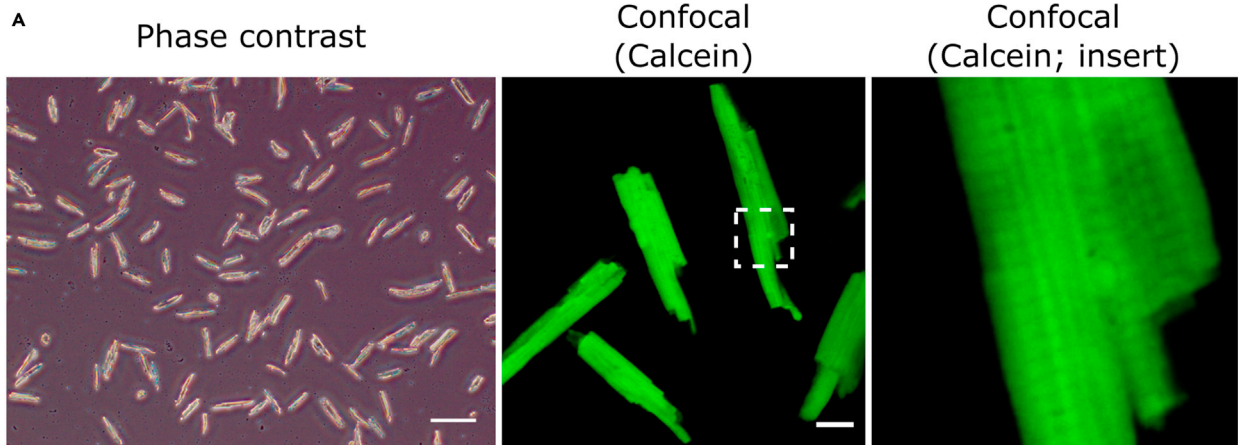


Figure 2. Expected outcomes

(A) An example image of healthy cardiomyocytes isolated using the perfusion system. Left panel, phase contrast images showing rod shaped cardiomyocytes. Image taken after Ca²⁺ reintroduction steps, plating onto a laminin coated dish, and media change. Scale bar indicates 200 μm. Center panel, confocal micrograph of adherent cardiomyocytes stained with Calcein Green. Scale bar indicates 20 μm. Right panel, zoomed image (insert from center panel) shows striations and sarcomeric organization within cardiomyocytes.

(B) Phase contrast images of adherent cardiomyocytes, captured at varying intervals from plating (0 h) to 2 days (48 h) in culture. Insert images (bottom row; arrows in middle row indicate insert positions) show sarcomeric organization of cultured cardiomyocytes. Scale bars indicate 200 μm.

(C) Flow cytometric analysis of non-myocytes isolated using the perfusion system. Gating strategy shows the determination of nucleated and viable single-cells and major non-myocyte cell classes (ECs, endothelial cells; RMCs, resident mesenchymal cells; Leuks, leukocytes).

(D) Quantification of the proportion of major non-myocyte cell classes determined using flow cytometry (n= 16). Error bars indicate standard deviation from the mean.

42. Aspirate supernatant, leaving a small volume (~50–100 μL) so as not to disturb the pellet, and resuspend cardiomyocytes in 10 mL *Myocyte Wash Buffer 3*, transfer to a 70 mL specimen jar and incubate at RT for 10 min.
43. Transfer cells to a 14 mL round-bottomed tube and centrifuge at 20×g for 3 min at RT
44. Aspirate supernatant and gently resuspend cardiomyocytes in desired volume of *Plating media* (e.g., 5 mL for a 60 mm tissue culture dish).
45. Immediately aspirate laminin from cell culture plates, wash with sterile PBS without Ca²⁺ Mg²⁺ and gently add cardiomyocytes in *Plating Media* to dish.
46. Incubate plates at 37°C, 5% CO₂ for 1 h to allow cells to adhere.
47. After 1 h, replace *Plating Media* with *Maintenance Media* and incubate cells at 37°C, 5% CO₂ for an additional 1.5–2 h prior to performing any treatments (e.g., stimulation with agonists for signaling studies).

EXPECTED OUTCOMES

Using this protocol, both myocytes and non-myocytes can be successfully isolated from multiple hearts in parallel. Isolated cardiomyocytes generally exhibit typical morphology with preserved sarcomeric organization (Figure 2A) and a yield of ~90,000 adherent cardiomyocytes can be attained per heart (Table 1). While we have not completed physiological assessment of isolated cardiomyocytes, we have cultured these cells up to 48 h with best morphology observed at 24 h or less (Figure 2B). However, this protocol innovates upon other published work (Ackers-Johnson et al., 2016) which comprehensively characterizes the physiological characteristics of cardiomyocytes isolated using buffers and general approach described here.

Using flow cytometry, we determine approximately 2 million non-myocytes can be extracted per heart (Table 1). It should be noted here; we use stringent criteria to calculate the yield of non-myocytes. This includes singlet-gating and viability dyes to exclude cell doublets and nucleated dead cells. We also used Vybrant™ DyeCycle™ and Calcein Blue dyes to ensure we only count nucleated and metabolically active cells, respectively (Pinto et al., 2016). Moreover, all three major cell classes of non-myocytes can be effectively isolated (Figures 2C and 2D). The major non-myocyte cell types are endothelial cells (ECs; both lymphatic and vascular), leukocytes (Leuks; granulocytes, myeloid and non-myeloid cells) and resident mesenchymal cells (RMCs; fibroblasts and mural cells). However, we find the proportion of endothelial cells are generally lower than in alternative protocols (Pinto et al., 2016; Squiers et al., 2020) where more mechanically rigorous methods are applied to disaggregate the dense vasculature of the heart (Table 2). However, as endothelial cells are

Table 1. Cardiomyocyte and non-myocyte yields per heart

Cell type	Cell count
Non-myocytes/heart determined by flow cytometry ^a	2,000,000
Cardiomyocytes (adherent cells in culture) per heart	90,000

All counts are equated to the nearest 10,000.

^aAverage count of 16 hearts of 14 week old animals; this number excludes cells that are not captured by the gates shown in Figure 2C.

Table 2. Non-myocyte comparison yield per heart determined by flow cytometry

Non-myocyte cell type	This protocol	Alternate protocol (Pinto et al., 2016; Squiers et al., 202)	Proprietary Miltenyi Biotec Multi Tissue Dissociation Kit 2 and gentleMACS™ Octo Dissociator ^b
Endothelial cells, ECs ^a	1,200,000	4,100,000	200,000
Resident mesenchymal cells, RMCs ^a	500,000	1,200,000	390,000
Leukocytes, Leuks ^a	400,000	420,000	610,000

^aAll counts are averaged from 16 hearts from 14 week old animals and equated to the nearest 10,000

^bFollowing manufacturer's protocol.

abundant in the heart, this does not limit subsequent studies which require endothelial cells. Further, the parallel non-myocyte and cardiomyocyte isolation methodology described here performs better overall compared to widely used commercial protocols.

LIMITATIONS

Variability of results depending on ambient temperature: Temperature during perfusion (step 18.a) of the hearts with *Perfusion buffer with enzymes* is optimal at 37°C. Although the buffer was kept in a 37°C-set water bath, we recorded the hearts' surface temperature at ~18°C. Although colder than expected, we were still able to isolate cardiomyocytes and non-myocytes. In laboratories with warmer ambient temperatures, we have successfully isolated both myocyte and non-myocytes in 15 min of perfusion.

Inability to weigh ventricles before dissociation: Due to the necessity to keep atria and base of the ascending aorta intact to facilitate perfusion of buffers and enzymes through the coronary vasculature, the ventricles cannot be precisely weighed. This may be problematic for studies which aim to measure heart mass to determine hypertrophy.

Variability of results depending on the investigator: We have observed that some differences in cellular composition may arise from different scientists performing the protocols. If consistency is required, we recommend that the same investigators perform the same tasks of the protocol. Despite this limitation, in preparing this report, investigators from an independent laboratory were able to successfully follow the protocol described.

3D printed components: While we have provided CAD generated files for 3D printing perfusion platforms, we recognize that 3D printers may not be available to all researchers. In this event, we recommend you contact a local 3D printing service which are now widely available.

TROUBLESHOOTING

Problem 1

Inserting hypodermic needle into heart ventricle and maintaining needle position above the ventricle while perfusing the heart

It is sometimes difficult to align the hypodermic needle attached to the platform with a heart ventricle (clamped by a hemostat). It may be also challenging to maintain the needle position, while the heart is being perfused. This is primarily due to the electrical wire (attached to the tube holding the hypodermic needle) which may change position after needle insertion.

Potential solution

At the final stage of setting-up the perfusion platforms, pre-align the hypodermic needle to the approximate position a heart would be using the curved hemostat placed on the perfusion platform as a guide. After commencing the protocol and placing the clamped heart on the perfusion platform, the needle can be easily inserted and its position stably maintained. If the position or angle of the

heart requires to be altered, gently move the hemostat. Moving the hemostat allows two dimensions of adjustment to ensure the needle position is directly above the heart.

Problem 2

Temperature control

Keeping ambient air temperature consistent around the hearts being perfused may be challenging and potentially affect enzyme activity. This may be a significant issue in areas such as those adjacent to high traffic walkways, doors or ventilations ports.

Potential solution

Setup perfusion workspace in an area that is not likely to experience temperature or air movement fluctuations. Alternatively, consider placing an acrylic (Perspex) enclosure around the back and sides of the perfusion platform.

Problem 3

Accidental clamping of atria

When clamping the heart after cold *EDTA buffer* perfusion through the right ventricle, the atria may be clamped as well.

Potential solution

If atria are accidentally clamped with the hemostat, do not discard this heart. Continue with the protocol and avoid removing the hemostat at attempting to re-clamp the heart as this could potential damage the heart tissue further. We have found that this protocol can be forgiving without impacting cell yield or viability.

Problem 4

Uneven rate of digestion in multiple hearts

During digestion with *Perfusion buffer* some hearts may appear better digested than others. This could be due to multiple reasons including (1) experimental treatments to hearts may have been present – for example, hearts with significant presence of fibrosis may require longer perfusion times. (2) Issues relating to the perfusion platform.

Potential solution

If the perfusion discrepancies are associated with the platform follow the below steps to ensure that the fluidic system is set up and operating correctly: -

- i. Pause the pump.
- ii. Ensure that the aspirating tube is placed within the buffer being perfused
- iii. Ensure that there are no leaks from tubing or fixtures, and tighten or replace tubing and fixtures as necessary.
- iv. Ensure the 30G needle placed at the apex of the hearts is positioned well and expelling fluid in the left ventricular chamber
- v. Resume flow of buffer and perfusion.

Problem 5

Undigested heart after perfusion

A heart that is incompletely digested will be more rigid than the soft and delicate hearts that are completely digested.

Potential solution

While the ability to extract large quantities of cells may be limited if the heart is incompletely digested, proceed with the protocol as described above since cells can still be extracted from the heart. If the tissue is too intact to gently pull apart, use surgical scissors to cut the heart into 4-6 pieces before proceeding with the digestion in 50 mL tubes.

RESOURCE AVAILABILITY

Lead contact

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Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

A.R.P. conceived the project and designed and assembled 3D-printed components; G.E.F., K.L.W., A.M., and A.R.P. wrote the manuscript; G.E.F., M.A.M., K.L.W., A.M., C.D.C., C.K., T.L.G., J.R.M., and A.R.P. edited the manuscript; G.E.F., M.A.M., K.L.W., A.M., C.D.C., C.K., A.C.P., T.L.G., and A.R.P. tested and optimized the technology and/or performed experiments; J.R.M. and A.R.P. managed the team; J.R.M. and A.R.P. funded the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Ackers-Johnson, M., Li, P., Holmes, A., O'Brien, S., Pavlovic, D., and Foo, R. (2016). A simplified, langendorff-free method for concomitant isolation of viable cardiac myocytes and nonmyocytes from the adult mouse heart. *Circ. Res.* 119, 909–920.
- McLellan, M., Skelly, D., Dona, M., Squiers, G., Farrugia, G., Gaynor, T., Cohen, C., Pandey, R., Diep, H., Vinh, A., et al. (2020). High-resolution transcriptomic profiling of the heart during chronic stress reveals cellular drivers of cardiac fibrosis and hypertrophy. *Circulation* 142, 1448–1463.
- Pinto, A., Chandran, A., Rosenthal, N., and Godwin, J. (2013). Isolation and analysis of single cells from the mouse heart. *J. Immunol. Methods* 393, 74–80.
- Pinto, A., Ilinykh, A., Ivey, M., Kuwabara, J., D'Antoni, M., Debuque, R., Chandran, A., Wang, L., Arora, K., Rosenthal, N., and Tallquist, M. (2016). Revisiting Cardiac Cellular Composition. *Circ. Res.* 118, 400–409.
- Squiers, G., McLellan, M., Ilinykh, A., Branca, J., Rosenthal, N., and Pinto, A. (2020). Cardiac cellularity is dependent upon biological sex and is regulated by gonadal hormones. *Cardiovasc. Res.* 117, 2252–2262.