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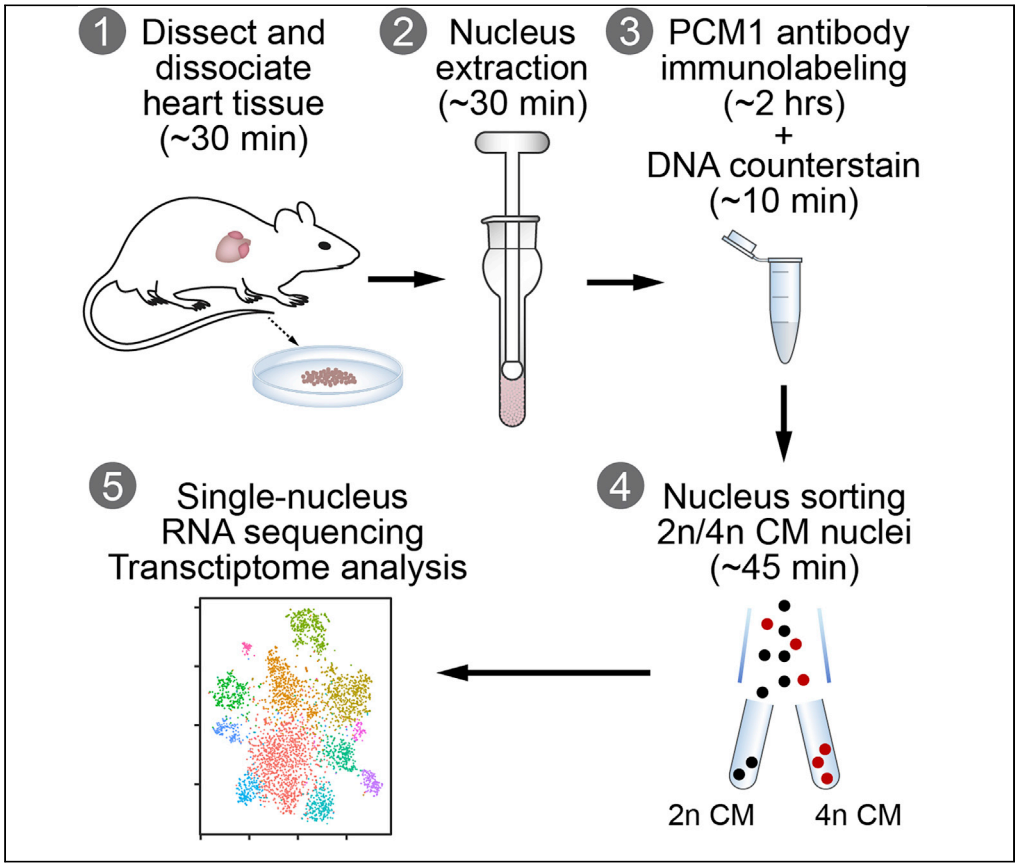
Protocol

Protocol for Single-Nucleus Transcriptomics of Diploid and Tetraploid Cardiomyocytes in Murine Hearts



Murine cardiomyocytes undergo proliferation, multinucleation, and polyploidization during the first 3 weeks of postnatal life, resulting in a mixture of diploid and tetraploid cardiomyocytes in the heart. Understanding the molecular differences between diploid and tetraploid cardiomyocytes from these processes has been limited due to lack of unique markers and their heterogenous origins. Here, we apply single-nucleus RNA-sequencing to fluorescence-activated cell sorting-selected diploid and tetraploid cardiomyocytes to characterize their heterogeneity and molecular distinctions.

Miao Cui, Eric N. Olson

eric.olson@utsouthwestern.edu

HIGHLIGHTS

PCM1 immunoselection isolates cardiomyocyte (CM) nuclei from murine hearts

Protocol profiles transcriptome of 2n and 4n cardiomyocytes at single-nucleus resolution

Integrating samples at various conditions identifies differential CM populations

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Protocol for Single-Nucleus Transcriptomics of Diploid and Tetraploid Cardiomyocytes in Murine Hearts

Miao Cui[1,2] and Eric N. Olson[1,3,*]

[1]Department of Molecular Biology, the Hamon Center for Regenerative Science and Medicine, and Sen. Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9148, USA

[2]Technical Contact

[3]Lead Contact

*Correspondence: eric.olson@utsouthwestern.edu
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SUMMARY

Murine cardiomyocytes undergo proliferation, multinucleation, and polyploidization during the first 3 weeks of postnatal life, resulting in a mixture of diploid and tetraploid cardiomyocytes in the heart. Understanding the molecular differences between diploid and tetraploid cardiomyocytes from these processes has been limited due to lack of unique markers and their heterogenous origins. Here, we apply single-nucleus RNA-sequencing to fluorescence-activated cell sorting-selected diploid and tetraploid cardiomyocytes to characterize their heterogeneity and molecular distinctions.

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

BEFORE YOU BEGIN

Prepare the below media and precool on ice for at least 30 min prior to beginning each respective section of this protocol. Refer to the Key Resources Table for a complete list of materials.

1. Lysis Buffer

Make 5 mL Per Sample

Reagent	Final Concentration	Volume (μL)
Sucrose (2.5 M)	320 mM	680
Tris-HCl (1 M, pH=8)	10 mM	50
$CaCl_2$ (1 M)	5 mM	25
Magnesium Acetate (1 M)	5 mM	25
EDTA (0.5 M)	2 mM	20
EGTA (0.2 M)	0.5 mM	12.5
DTT (1 M)	1 mM	5
Halt Protease Inhibitors Cocktail (100×)	1×	50
RNase OUT Recombinant Ribonuclease Inhibitor (40 U/μL)	200 U/mL	25
Nuclease-free H_2O	N/A	4,107.5
Total	N/A	5,000

2. Sucrose Buffer

Make 10 mL Per Sample

Reagent	Final Concentration	Volume (μL)
Sucrose (2.5 M)	1 M	4,000
Tris-HCl (1 M, pH=8)	10 mM	100
Magnesium Acetate (1 M)	5 mM	50
DTT (1 M)	1 mM	10
Halt Protease Inhibitors Cocktail (100×)	1×	100
RNase OUT Recombinant Ribonuclease Inhibitor (40 U/μL)	200 U/mL	50
Nuclease-free H_2O	n/a	5,690
Total	n/a	10,000

3. Nuclei Storage Buffer

Make 10 mL Per Sample

Reagent	Final Concentration	Volume (μL)
Sucrose (2.5 M)	440 mM	1,750
Tris-HCl (1 M, pH=7.2)	10 mM	100
KCl (1 M)	70 mM	700
$MgCl_2$ (1 M)	5 mM	100
Spermidine (2 M)	1.5 mM	7.5
Halt Protease Inhibitors Cocktail (100×)	1×	100
RNase OUT Recombinant Ribonuclease Inhibitor (40 U/μL)	200 U/mL	50
Nuclease-free H_2O	N/A	7,192.5
Total	N/A	10,000

4. 2% BSA/PBS

Make 5 mL Per Sample

Reagent	Final Concentration	Volume (μL)
BSA (10%)	2%	1,000
PBS (1×)	10 mM	3,975
RNase OUT Recombinant Ribonuclease Inhibitor (40 U/μL)	200 U/mL	25
Total	N/A	5,000

5. 1× Red Blood Cell Lysis Buffer: Make 2 mL per sample by diluting the 10× stock in ddH2O and store at 20–22°C.
6. 1× Phosphate-Buffer Saline (PBS): Dilute 10× PBS with ddH_2O or directly purchase 1× PBS from manufacturer.

Alternatives: If omitting DDT, Protease inhibitor, and Ribonuclease Inhibitor, Lysis Buffer, Sucrose Buffer and Nucleus Storage Buffer can be premade in a larger volume and stored at 4°C.

Before each experiment, aliquot appropriate volume of each buffer and add DDT, Protease inhibitor, and Ribonuclease Inhibitor right before use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-PCM1 antibody produced in rabbit	Sigma-Aldrich	Cat#HPA023374; RRID:AB_1855073
Goat anti-Rabbit IgG secondary antibody, Alexa Fluor 647	Thermo Fisher Scientific	Cat#A-21245
Chemicals, Peptides, and Recombinant Proteins		
Fetal Bovine Serum	Gemini Bio Products	Cat#100-106
Halt Protease Inhibitors Cocktail (100×)	Thermo Fisher Scientific	Cat#78430
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen	Cat#10777019
Hoechst 33342	Invitrogen	Cat#H3570
Tris-HCl (1 M, pH=8)	Thermo Fisher Scientific	Cat#15568025
Tris-HCl (1 M, pH=7.2)	Thermo Fisher Scientific	Cat#50-843-264
Sucrose	Sigma-Aldrich	Cat#S0389
DTT	Sigma-Aldrich	Cat#3483-12-3
Red Blood Cell Lysis Solution (10×)	Promega	Cat#Z3141
Spermidine trihydrochloride	Sigma	S2501
Phosphate Buffered saline (1×)	Thermo Fisher Scientific	Cat#10010023
Experimental Models: Organisms/Strains		
Mouse: ICR/CD-1	Charles River Laboratories	ICR/CD-1
Software and Algorithms		
Cell Ranger v2.1	10X Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger
Seurat v3.0	Stuart et al., 2019	https://satijalab.org/seurat/
Scrublet	Wolock et al., 2019	https://github.com/AllonKleinLab/scrublet
Other		
Conical tube 15 mL	Corning	Cat#352096
40 μm Cell strainer	Corning	Cat#352340
70 μm Cell strainer	Corning	Cat#352350
Vertical rotator	N/A	N/A
Refrigerated centrifuge	N/A	N/A
Vacuum Drive Disposable Filtration System (0.22 μm)	Millipore	Cat#S2GPU05RE
Premium Microscope Slide	Fisher Scientific	Cat#22-178-277
VWR micro cover glass	VWR	Cat#48366-227

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eric. N Olson (eric.olson@utsouthwestern.edu).

Materials Availability

This study did not generate new unique reagents

Data and Code Availability

This study did not generate any unique datasets or code

MATERIALS AND EQUIPMENT

1. 2.5 M Sucrose: Dissolve 855.75 g of Sucrose in 1 L ddH_2O, pass through 0.22 μm filtration system, and store at 4°C for up to 1 year.

   Note: It is necessary to filter the Sucrose buffer to prevent bacterial growth. Sucrose can crystalize at 4°C. Make a new batch of Sucrose Buffer when that occurs.

2. 1 M DTT: Dissolve 1.55 g of DTT in 10 mL ddH_2O. Make 500 μL of aliquots and store at −20°C.
3. 1 M $CaCl_2$: Dissolve 55.49 g of $CaCl_2$ in 500 mL ddH_2O, filter, and store 20°C–22°C.
4. 1 M Magnesium Acetate: Dissolve 71.2 g of Magnesium Acetate in 500 mL ddH_2O, filter, and store at 20°C–22°C.
5. 0.5 M EDTA: Dissolve 29.2 g of EDTA in 200 mL ddH_2O, filter, and store at 20°C–22°C.
6. 0.2 M EGTA: Dissolve 15.2 g of EGTA in 200 mL ddH_2O, filter, and store at 20°C–22°C.
7. 1 M KCl: Dissolve 14.9 g of KCl in 200 mL ddH_2O, filter, and store at 20°C–22°C.
8. 1 M $MgCl_2$: Dissolve 19 g of $MgCl_2$ in 200 mL ddH_2O, filter, and store at 20°C–22°C.
9. 10% BSA: Dissolve 10 g of bovine serum albumin in 100 mL ddH_2O, filter, and store at 4°C.
10. 2 M spermidine: Dissolve 1 g of spermidine in 2 mL ddH_2O. Make 50 μL of aliquots and store at −20°C.
11. 40 mL dounce homogenizer with type A pestle.
12. Refrigerated centrifuge with swing buckets (e.g., AccuSpin 3R from Fisher Scientific).
13. Fluorescence-activated cell cytometer (e.g. BD FACSARIA).
14. Vertical rotator.

STEP-BY-STEP METHOD DETAILS

Nuclei Extraction from Cardiac Tissues

Timing: 1–2 h

This step isolates nuclei from freshly dissected mouse heart tissue.

1. Transfer 50-100 mg freshly dissected mouse heart tissue into a 10 cm dish on ice and mince into small pieces (around 1 mm^3 cube) with razor blade washed in ethanol.

   Note: If using neonatal hearts, multiple heart samples can be pooled to get enough starting material.

   Note: The minced tissue should be easily resuspended in PBS without seeing large connected tissue pieces (Figure 1A)

2. Resuspend the minced tissue in 5 mL PBS and transfer into a 15 mL tube. Centrifuge the tube at 500 × *g* for 2 min at 20°C–22°C. Discard the supernatant.
3. Resuspend the tissue pellet in 2 mL 1× Red Blood Cell Lysis Buffer. Vortex the tube for 20 s and incubate at 20°C–22°C for 2 min. Add 2 mL PBS to stop the lysis and centrifuge the sample at 500 × *g* for 5 min at 4°C.

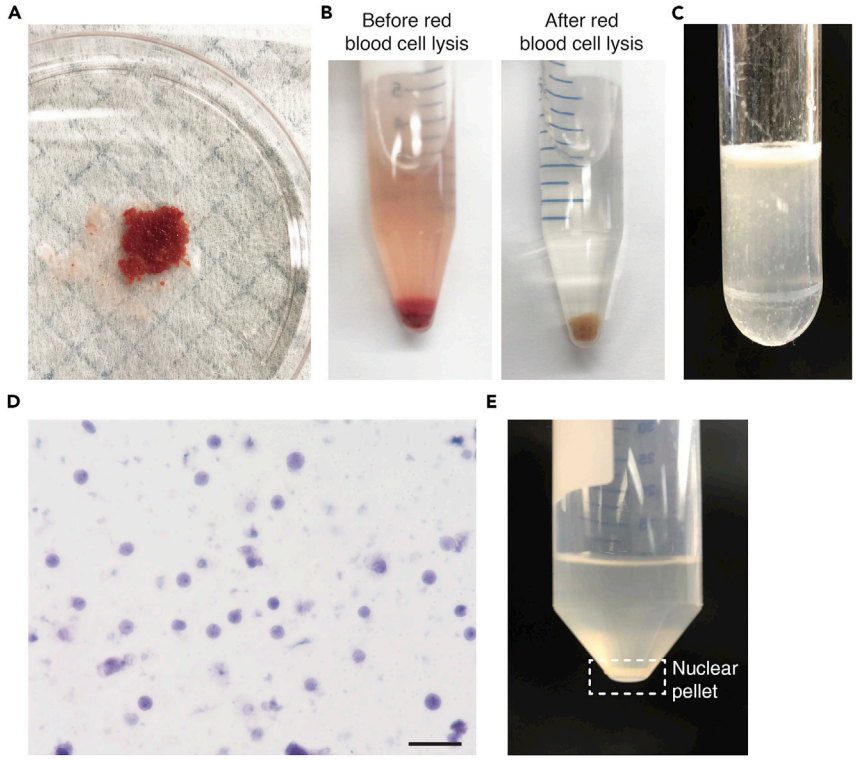

Figure 1. Nucleus Extraction from Cardiac Tissues

(A) Representative image showing heart tissues after being sufficiently minced.

(B) Images showing heart tissues before (left) and after (right) red blood cell lysis.

(C) Image of homogenized heart tissues during nucleus extraction.

(D) Brightfield images showing nuclear extract stained with Trypan blue. Scale bar, 20 μm.

(E) Image showing nuclear pellet after centrifugation.

⚠ CRITICAL: The heart is a highly vascularized organ; it is preferable to deplete red blood cells in the samples as the presence of red blood cells can significantly slow down the isolation of cardiomyocyte nucleus during flow cytometry sorting (step 14). (Figure 1B). It is critical to perform the red blood cell lysis step before proceeding to nucleus extraction, as the ammonium chloride in the lysis buffer will also lyse nuclei after they are extracted.

4. Wash the tissue pellet in 5 mL ice cold PBS. After centrifugation, resuspend the tissue in 5 mL Lysis Buffer and transfer to a 20 mL Dounce homogenizer placed on ice. Homogenize the tissue suspension with a type A pestle on ice for 15–20 strokes until no major tissue chunks are visible (Figure 1C).
5. Take 10 μL of the sample and check under microscope with Trypan blue to evaluate the nucleus extraction efficiency. Expect to see most nuclei separated and stained in blue (Figure 1D).
6. Once nucleus extraction is complete, filter the lysate first through a 70 μm strainer then pass over the flow-through onto a 40 μm cell strainer and centrifuge the flow-through at 1,000 × *g* for 5 min at 4°C to pellet nuclei.
7. Carefully aspirate the supernatant without disturbing the nuclear pellet (Figure 1E) and resuspend the pellet in 2 mL Sucrose Buffer. In a separate tube, aliquot 4 mL Sucrose Buffer and carefully transfer the nuclear suspension on top. Centrifuge the cushioned suspension at 1,000 × *g* for 10 min at 4°C to pellet nuclei.

Note: Centrifugation of the nuclear extract on sucrose cushion helps to clear out the tissue debris resulting from homogenization.

8. Wash the nuclear pellet with 1 mL Nuclei Storage Buffer and centrifuge at 1,000 × *g* for 5 min at 4°C. Resuspend the nuclei in 1 mL Nuclei Storage Buffer and keep on ice.

   Note: If cardiomyocyte enrichment is not required, you can resuspend the extracted nuclei in 200 μL of 2% BSA/PBS and proceed directly to step 16 for library preparation to profile total nuclei in the heart.

   Note: Nuclei Storage Buffer contains KCl and $MgCl_2$, which help the viability of the nuclei. When processing multiple samples, nuclei can be stored in Nuclei Storage Buffer on ice for up to 12 h before proceeding to the next steps.

Purify Diploid and Tetraploid Cardiomyocyte Nuclei by Cell Sorting

Timing: 2–3 h

This step enriches diploid and tetraploid cardiomyocyte nuclei by immunolabeling of the cardiomyocyte-specific nuclear membrane protein PCM1 and Hoechst staining for DNA content.

9. Divide the nuclear suspension from step 8 into four tubes labeled as "unstained", "PCM1", "Hoechst", and "PCM1+Hoechst". In the "PCM1" and "PCM1+Hoechst" tubes, add anti-PCM1 antibody at a 1:200 dilution. Incubate the nuclei on a rotator for 45 min at 4°C. Leave the other two tubes on ice.
10. After the incubation, centrifuge the "PCM1" and "PCM1+Hoechst" tubes at 1,000 × *g* for 5 min at 4°C, wash twice with Nuclei Storage Buffer, and resuspend in 1 mL Nuclei Storage Buffer.
11. To all tubes, add secondary antibody conjugated with Alexa Fluor 647 at 1:500 dilution and incubate the nuclei on a rotator for 30 min at 4°C.
12. Wash the nuclei twice with 1 mL Nucleus Storage Buffer and resuspend in 500 μL 2% BSA/PBS. Only to "Hoechst" and "PCM1+Hoechst" tubes, add Hoechst at a 1:2,000 dilution and incubate for 10 min at 20°C–22°C.
13. Wash the nuclei twice with 1 mL 2% BSA/PBS and resuspend in 500 μL 2% BSA/PBS for FACS.
14. Sort the nuclei using a cell-sorter (e.g. BD FACSARIA). Gate single nuclei with the FSC-H versus FCS-W plot and the SSC-H versus SSC-W plot to avoid doublets. Use the "unstained" and "PCM1" samples to gate for PCM1+ nuclei. Use "unstained" and "Hoechst" samples to gate for diploid (2n) and tetraploid (4n) nuclei (Figure 2). From the "PCM1+Hoechst" sample, sort a minimum of 20,000 PCM1+ 2n and PCM1+ 4n nuclei into 1.5 mL tubes containing 500 μL of 2% BSA/PBS.

15. After sorting, pellet the PCM1+ nuclei at 1,500 × *g* for 10 min at 4°C , resuspend in 20 μL of 2% BSA/PBS, and immediately process to the next step.

    Note: Perform all washes at 1,000 × *g* for 5 min at 4°C.

    ⚠ CRITICAL: PCM1 signal intensity may vary between sample preparations, thus it is important to individually gate the samples using their own unstained and single-color controls.

Library Preparation for Single-Nucleus RNA-Sequencing

Timing: 2–3 days

Count number of nuclei and prepare single-nucleus RNA-sequencing libraries.

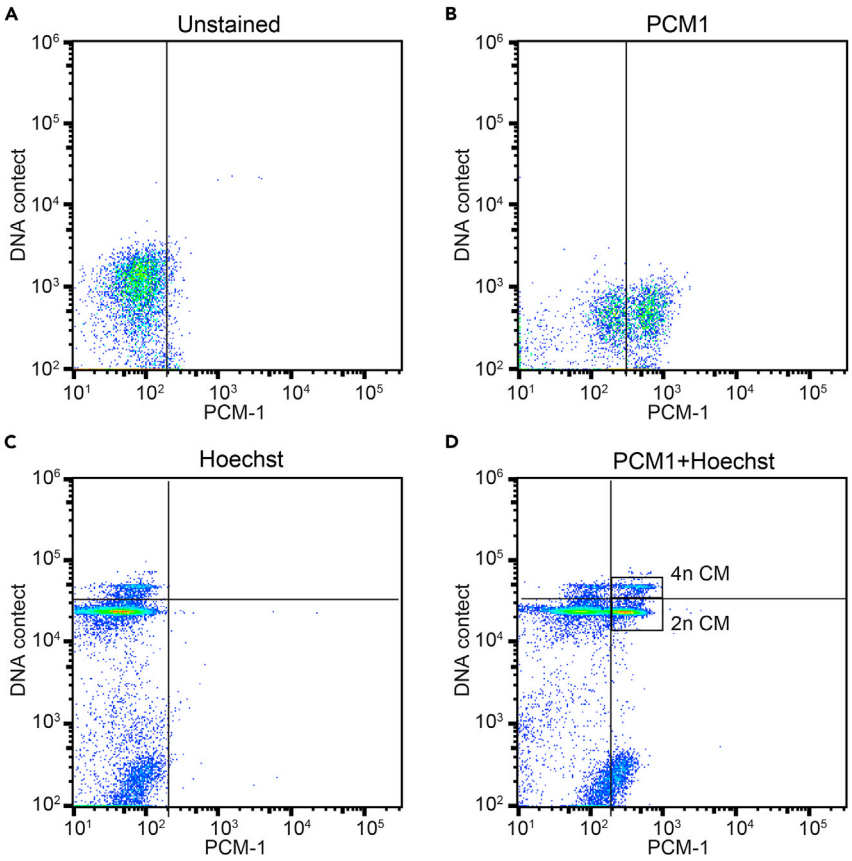

Figure 2. Gating strategy for Isolation of Diploid and Triploid Cardiomyocyte Nuclei

Representative flow cytometry plots for immunolabeling of pooled heart samples collected at P4 with antibody against PCM1 to label cardiomyocyte nuclei and Hoechst for DNA staining.

(A–D) Unstained sample (A) and samples only stained for PCM1 (B) or Hoechst (C) were used as negative controls for gating. 2n cardiomyocytes and 4n cardiomyocytes are identified in samples stained with both Hoechst and PCM1 antibody (D). The different fluorescent intensities (y-axis) of Hoechst staining indicate the difference in DNA ploidy (2n vs 4n).

16. Transfer 10 μL of nuclear suspension to a microscope slide and mount with a glass cover slide. Inspect the integrity and purity of cardiomyocyte nuclei under fluorescent microscope (Figure 3). In a separate tube, mix 10 μL nuclear suspension with 10 μL Trypan blue. Take 10 μL of the mixture and count nuclei manually twice using hemocytometer.

17. Dilute the nuclei to 1,000 nuclei/μL with 2% BSA /PBS. Immediately load 6,000 nuclei to each reaction mix for single-cell partitioning and reverse transcription following the manufacturer's instructions from 10X Genomics.
18. Prepare single-nucleus RNA-sequencing libraries according to the manufacturer's instructions from 10X Genomics (v2 or v3 chemistry, https://www.salk.edu/wp-content/uploads/2017/11/10XUserGuide.pdf)
19. Perform sequencing using the 150-bp high output sequencing kit (Illumina), with the following pair-end sequencing settings: Read1, 26 cycles; i7 index, 8 cycles; Read2, 124 cycles.

Note: Library preparation is prone to batch-to-batch variation. Therefore, plan experiments ahead so that samples can be collected on the same day and processed together for library preparation.

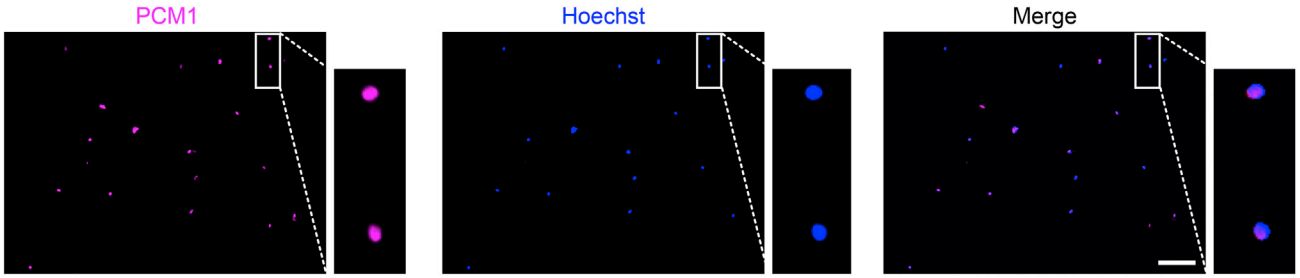

Figure 3. Immunofluorescent Images of Nuclei after Flow Cytometry Sorting

PCM1 positive nuclei are shown in violet. Hoechst staining is shown in blue. High magnification images of boxed areas are shown in panel on right. Scale bar, 50 μm.

EXPECTED OUTCOMES

Using the 10X Genomics Chromium kit, we usually obtain 2,000–5,000 nuclei that passed raw data filtering in each sample. A median of 700–1,500 genes per nucleus are detected when samples are sequenced at ~50,000 reads per nucleus with ~75% genome mapping rate. We noticed that the number of detected genes in tetraploid cardiomyocyte nuclei is significantly lower than that of diploid nuclei. The reduced transcriptome complexity in tetraploid nuclei likely reflects transcriptional repression during the G2/M cell-cycle phases (Prescott and Bender 1962, Parsons and Spencer 1997).

Although the PCM1 antibody selects cardiomyocyte nuclei, we often observe that about 20% of nuclei correspond to non-myocyte cell types in our final datasets. The presence of non-myocyte cells is likely due to the fact that PCM1 can also be localized to the nuclear membrane of terminally differentiated cells of other types, albeit it has been successfully used to purify cardiomyocyte nuclei in many independent studies (Alkass, Panula et al. 2015, Quaife-Ryan, Sim et al. 2017). Nevertheless, the non-myocyte cell types can be easily distinguished and removed *in silico* by the expression of cell type marker genes.

Mitochondrial transcripts are expected to be sequenced. This is due to co-isolation of mitochondria during the nucleus isolation process through their physical association with the endoplasmic reticulum. Our observations are consistent with reports from other groups where mitochondrial transcripts were detected with single-nucleus RNA-seq (Hu et al. 2018) as well as ATAC-seq (which also profiles nuclei) (Rickner et al. 2019). This is especially prevalent in cardiomyocytes, as they are among the cell types that have the most mitochondrial content with ~30% of the cellular volume being filled with mitochondria (Piquereau et al. 2013). Regardless, these mitochondrial transcripts can be removed before gene expression analyses, and thus will not affect the downstream analyses.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 2–3 days

Process raw sequencing data and perform data normalization and scaling for cell type identification and differential gene analysis. Standard data analysis and processing pipelines have been detailed (Lafzi, Moutinho et al. 2018, Kulkarni, Anderson et al. 2019). Here, we summarize the major steps and highlight the specific data filtering parameters we used in Cui et al. (2020).

- Demultiplex raw sequencing data using *cellranger mkfastq* function in the Cell Ranger Single-Cell Software Suite.
- Perform alignment, filtering, and barcode counting on the demultiplexed FASTQ files using *cellranger count* function in the Cell Ranger Single-Cell Software Suite. Align cDNA reads to mm10/GRCm38 pre-mRNA reference genome. Generate gene-barcode matrices with confidently mapped reads that have valid barcodes and unique molecular identifiers (UMI).

- Identify potential doublets using Scrublet and remove the doublets data entries from the gene-barcode matrices.
- Perform further data filtering using the Seurat R package. Remove cells in the 1% quantile or those that had fewer than 200 detected genes. Remove cells with more than 25% of the transcripts coming from mitochondrial genes.
- Perform data normalization and data scaling using Seurat to adjust for variations in the number of genes detected in each cell.
- Use the *IntegrateData* function implemented in Seurat V3 to aggregate datasets from multiple samples (e.g. diploid and tetraploid samples) for combined cluster calling and differential gene analysis (*FindMarkers* function) between cell clusters and datasets.

⚠ CRITICAL: Given that nuclear RNA has a high content of unspliced premature RNA molecules, it is important to use the pre-mRNA reference genome that contains both gene exons and introns for sequence alignment and read counting.

LIMITATIONS

It has been suggested that PCM1 localization and abundance in cardiomyocytes are dependent on the cell-cycle stage. Thus, a potential concern of the PCM1-based immunoselection of cardiomyocyte nuclei is that it may introduce biases to the populations of cardiomyocytes being captured. To address this, we compared the composition of cardiomyocytes in nuclear extracts of neonatal hearts at postnatal day (P) 4 before and after PCM1 FACS, hereafter referred to as "-PCM1" and "+PCM1", respectively. As expected, we identified more non-cardiomyocytes in the -PCM1 sample than in the +PCM1 sample (Figure 4). Most importantly, within the cardiomyocytes, we observed a similar composition of different populations between +PCM1 and -PCM1 samples (Figure 4E). In particular, CM2 and CM4, which are the two proliferative populations as shown by Mki67 expression (Figure 4C), are similarly abundant between the two samples, suggesting that PCM1 FACS does not introduce a significant bias in the composition of cardiomyocytes being profiled and is effective in isolating cycling cardiomyocytes in neonatal hearts.

Nucleus sequencing effectively solves the issue of the traditional single-cell RNA-sequencing where cardiomyocytes are generally too big to be captured. However, nuclear extraction disrupts the cellular integrity and thus does not distinguish nuclei from mononucleated cardiomyocytes from those of binucleated cardiomyocytes. To correlate the subtypes to nucleation, other supporting information such as visualization of the subsets via immunostaining or in-situ hybridization or direct comparison of isolated mononucleated and binucleated cardiomyocytes, as recently reported (Windmueller et al. 2020), are needed.

TROUBLESHOOTING

Problem 1

Low nucleus extraction efficiency or poor nucleus morphology after extraction.

Potential Solution

If less than 50% of nuclei are stained with Trypan blue in step 5, increase the stroke times until majority of nuclei are extracted and stained (Figure 1C). If poor nucleus morphology is seen after homogenization, such as nuclear membrane rupture, start over the experiment with less stoke times for homogenization.

Problem 2

After the reverse transcription, the cDNA quality is poor with averaged size smaller than 1,000 bp.

Potential Solution

The poor cDNA quality is due to RNA degradation during sample preparation. Make sure to add RNAase inhibitor to all the buffers and handle samples on ice all times.

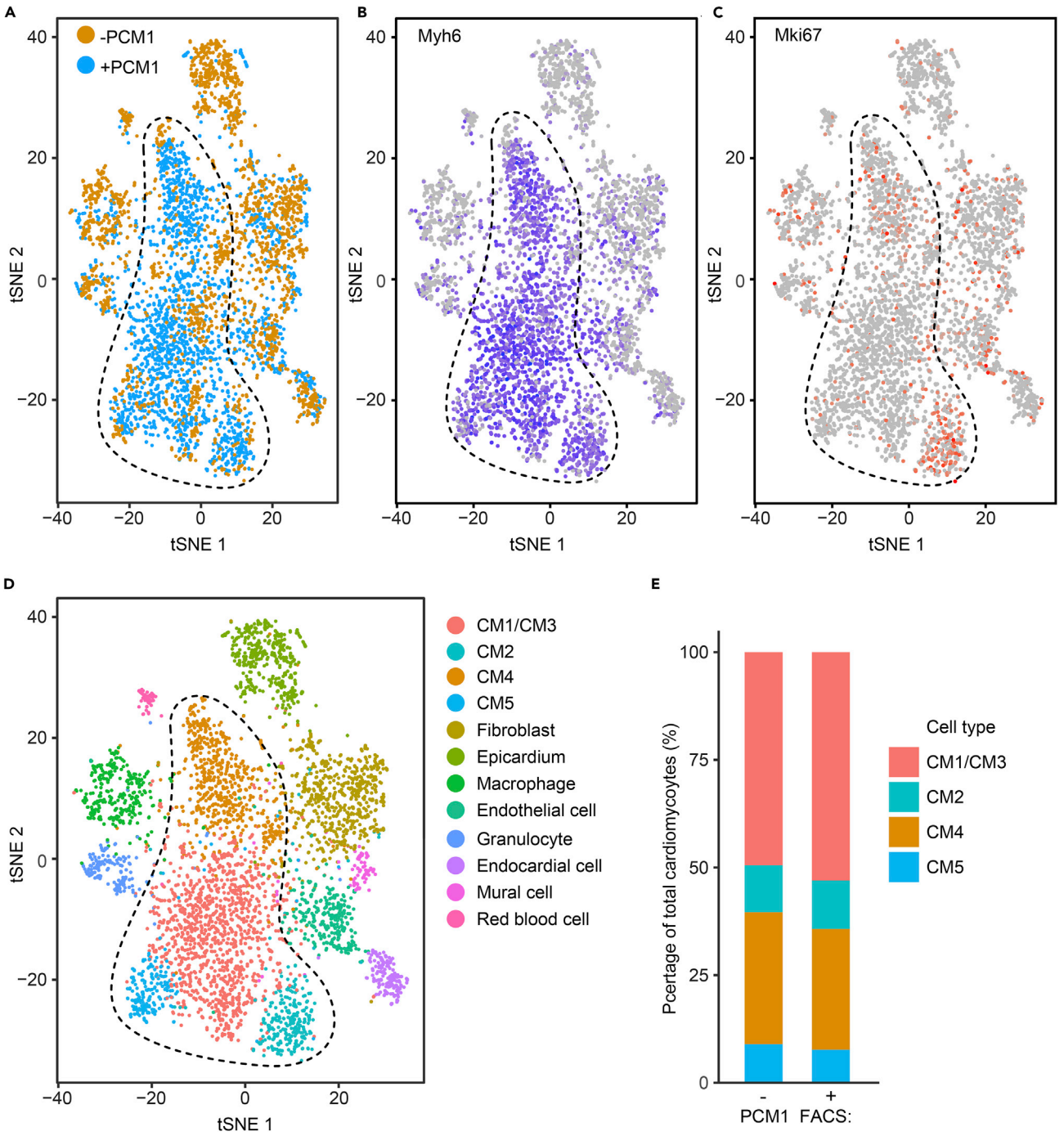

Figure 4. Comparison of Cardiac Nuclei (Marked by the Dashed Line) Purified with (+PCM1) and without (-PCM1) PCM1 Immunolabeling and Flow Cytometry Sorting

(A) Sample composition projected on TSNE plot. +PCM1, cardiac nuclei isolated with PCM1 FACS; -PCM1, total cardiac nuclei isolated without PCM1 FACS.

(B) Myh6 expression projected on TSNE plot showing cardiomyocyte populations.

(C) Mki67 expression projected on TSNE plot showing proliferating cells.

(D) Cell type identities projected on TSNE plot are color-coded. Cell types were identified based on de novo cluster calling and transferred cell labels in the +PCM1 dataset that were previously identified in Cui et al. (2020). Note that the previously identified CM1 and CM3 clusters are merged into one cluster due to their transcriptome similarity.

(E) Percentage of each cardiomyocyte cluster over total cardiomyocytes in -PCM1 and +PCM1 samples.

Problem 3

The number of nuclei being captured is much lower than the target number.

Potential Solution

The low capture rate is likely due to the presence of debris in the nuclear extract. Debris particles from fractured cell membranes can be encapsulated by oil droplets during the single-cell partitioning and will take up the barcoded beads. However, they yield very few sequenced RNA molecules and, thus, will be discarded during data filtering. Repeat the sucrose centrifugation step or filter the nucleus suspension with 40 μM FLOWMI cell strainers to help reduce the debris.

Problem 4

Non-myocyte cell types are captured at high proportions by PCM1 FACS.

Potential Solution

Make sure to use negative controls without PCM1 immunolabeling from the corresponding sample to gate $PCM1^{+}$ nuclei. Do not use unmatched samples, especially samples of other stages, for gating, as the PCM1 signal intensity may vary between sample preparation and differ by ages.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

M.C. conducted the experiments; M.C. conceptualized the study and analyzed the data; M.C. and E.N.O. wrote the paper; E.N.O. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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