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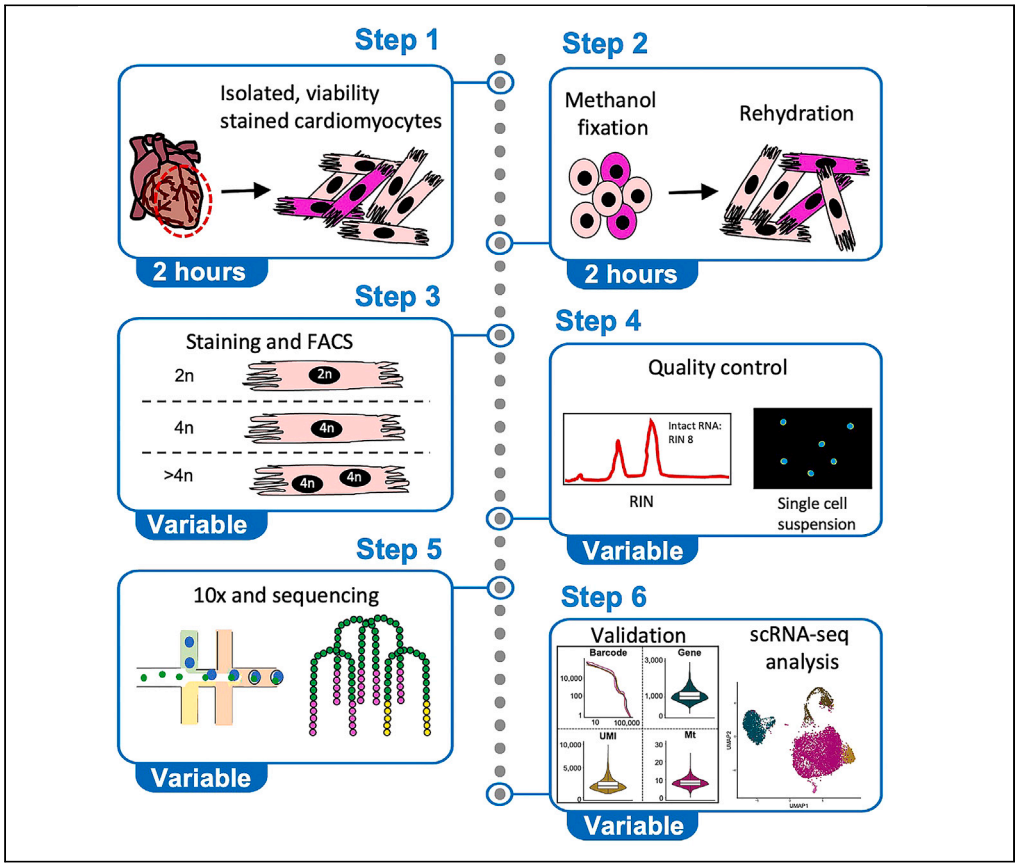
Protocol to achieve high-resolution single-cell transcriptomics of cardiomyocytes in multiple species



Single-cell RNA sequencing (scRNA-seq) remains state-of-the-art for transcriptomic cell-mapping. Here, we provide a protocol to generate high-resolution scRNA-seq of rare cardiomyocyte populations (e.g., regenerating/dividing, etc.) from mouse and zebrafish hearts as well as induced pluripotent stem cells, collected in time to achieve detailed transcriptomic insight. We describe the serial steps of viability staining, methanol fixation, storage, and cell sorting to preserve RNA integrity suited for scRNA-seq as well as the quality assessment of the data as shown by examples.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Guidance on cell viability, fixation, storage, and sorting for scRNA-seq of rare cells

Step-by-step scRNA-seq protocol of mouse, zebrafish, and iPS cell-derived cardiomyocytes

Quality assessment of scRNA-seq data verifies data integrity

Ellman et al., STAR Protocols 5, 103194
September 20, 2024
https://doi.org/10.1016/j.xpro.2024.103194

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Protocol to achieve high-resolution single-cell transcriptomics of cardiomyocytes in multiple species

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https://doi.org/10.1016/j.xpro.2024.103194

SUMMARY

Single-cell RNA sequencing (scRNA-seq) remains state-of-the-art for transcriptomic cell-mapping. Here, we provide a protocol to generate high-resolution scRNA-seq of rare cardiomyocyte populations (e.g., regenerating/dividing, etc.) from mouse and zebrafish hearts as well as induced pluripotent stem cells, collected in time to achieve detailed transcriptomic insight. We describe the serial steps of viability staining, methanol fixation, storage, and cell sorting to preserve RNA integrity suited for scRNA-seq as well as the quality assessment of the data as shown by examples.

For complete details on the use and execution of this protocol, please refer to Bak et al.[1]

BEFORE YOU BEGIN

Single cell RNA sequencing (scRNA-seq) is widely used for extensive gene expression analysis at the single cell level, thereby providing unique transcriptional profiles of individual subpopulations in heterogeneous tissues that cannot be achieved through bulk RNA-seq.[2] The technique has continuously improved since it was first introduced in 2009 and several platforms for scRNA-seq have emerged enabling transcriptional profiling of thousands of cells in a high-throughput, cost-efficient manner.[3,4]

Up until now, most scRNA-seq studies on cardiac cells have been performed on a heterogeneous population of cells and the cardiomyocytes (CMs) have been identified bioinformatically after sequencing based on their transcriptomic profile using well established CM markers.[5–8] In these scRNA-seq studies, CMs are analyzed together with non-myocyte populations, e.g., fibroblasts, endothelial cells, and immune cells.[5–8] Isolating CMs or even specific subpopulations of CMs prior to cDNA library generation enhances the potential sequencing saturation of each cell, and the number of CMs analyzed, thus allowing substantial increase in transcriptional resolution of CMs. The protocol presented below is based on a protocol that includes methanol fixation followed by rehydration of cells and that has previously proven compatible with scRNA-seq of neurogenic cells.[9] We have further developed this protocol enabling exclusion of cells dead prior to fixation, enrichment of the cell type of interest and ploidy stratification by fluorescence activated cell sorting (FACS) using intracellular staining.

It is also apparent that none of the scRNA-seq studies enable distinctive analysis of CMs with different ploidy, a key parameter in terminal CM differentiation where CMs around birth become

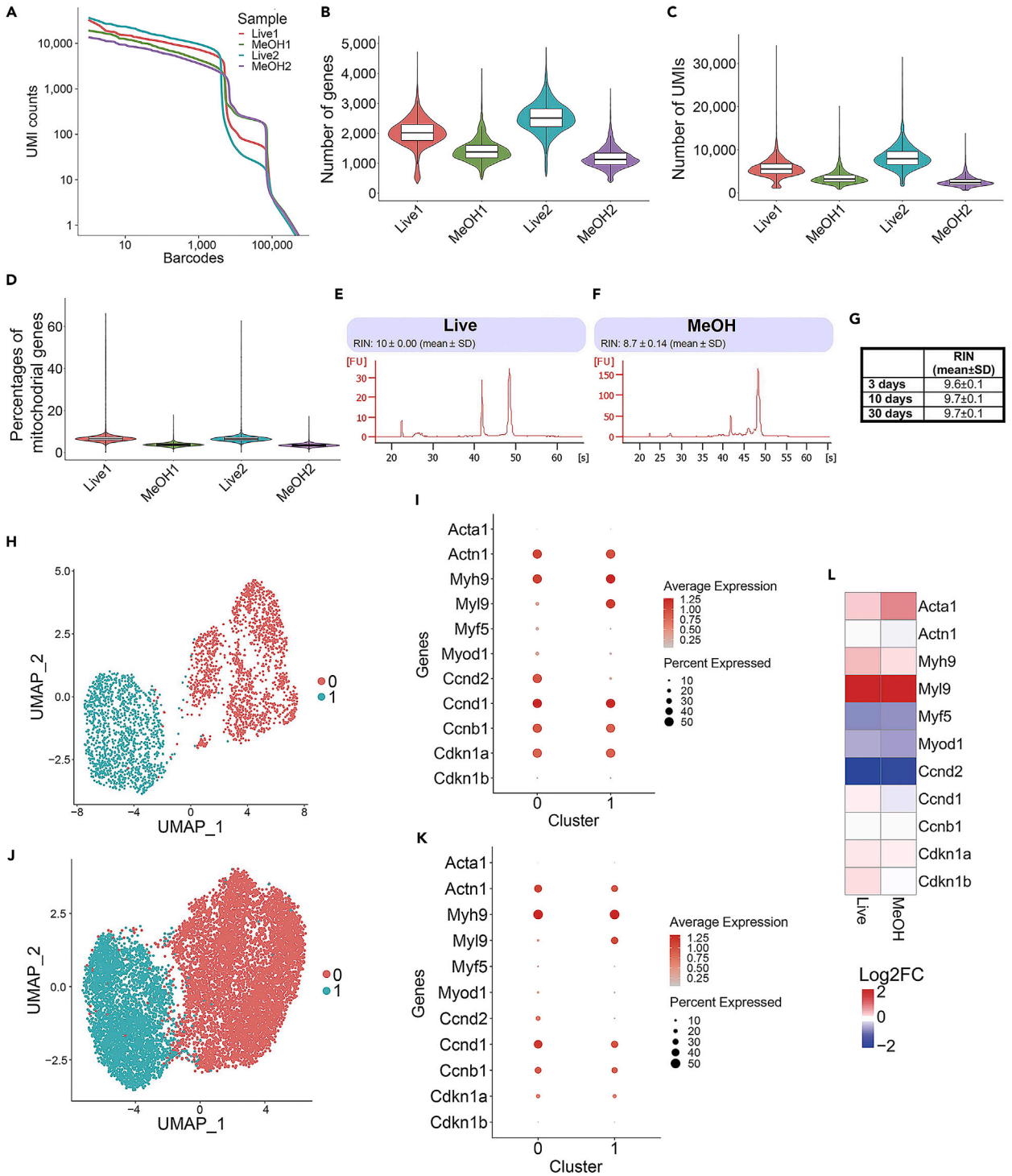


Figure 1. Methanol fixation does not alter gene expression of C2C12 cells

(A–D) Quality analyses of corresponding Live and methanol (MeOH) fixed C2C12 cells originating from two different culture flasks. (A) Barcode rank plot (knee-plot) showing log scaled barcodes ranked by number of Unique molecular identifier (UMI) vs. log scaled UMI counts, (B) Violin plot showing "Median Genes per Cell", (C) "Median UMI Counts per Cell", and (D) percentage of mitochondrial transcripts.

(E and F) Electropherograms showing the RNA integrity number (RIN) of Live and MeOH fixed C2C12 cells, respectively.

(G) Table showing RIN of MeOH fixed C2C12 cells stored in rehydration buffer at −80°C for 3-, 10-, or 30 days.

Figure 1. *Continued*

(H–K) Uniform manifold approximation and projection (UMAP) embedding and dotplot of select myoblast marker genes for Live (H–I) and MeOH fixed (J–K) C2C12 cells. UMAP representation was embedded based on highly variable genes and colored by Louvain algorithm clusters, at resolution = 0.084. Dotplots show average expression of genes indicated by color of dots and dot-size representing the percentage of total cells expressing the individual gene.

(L) Heatmap of average Log2-Fold-Change (Log2FC) for select myoblast genes between 0 and 1 Louvain algorithm clusters in Live and MeOH fixed C2C12 cells. Refer also to Table S1.

polyploid and lose their ability to divide.[10,11] During development the mammalian heart forms through CM proliferation,[12] however around birth the CMs enter cell cycle arrest where some CMs exit the cell cycle and become quiescent in G0,[13] while other CMs undergoes a final round of mitosis, but fails to complete karyokinesis and/or cytokinesis. This G2/M challenge results in polyploid and binucleated CMs and is considered one of the major hindrances for heart regeneration through CM proliferation.[14] Hence, we developed our novel approach to be able to investigate what drives CMs to exit the cell cycle in a terminally differentiated state by unveiling transcription factors regulating cell cycle associated genes that are differentially expressed in tetraploid CMs before and after polyploidization/binucleation and cell cycle withdrawal in the developing mammalian heart.

By the current approach, we have established a method for enzymatic dissociation, methanol fixation, rehydration, and intracellular fluorescent immunocytochemical staining, allowing identification of the cell type of interest, but also ploidy stratification by FACS prior to scRNA-seq and with a strict focus on preserving the RNA integrity. With this protocol, a defined population of cells, and herein specifically CMs, can be isolated prior to generation of the single cell cDNA libraries, increasing the amount of information from the cell type of interest by eliminating dropouts due to dead cells and unnecessary sequencing data of irrelevant cell types for a certain specific question. In particular, small cell populations such as proliferating CMs may be analyzed in detail with this protocol. Also, fixation of cells, enables pooling of samples collected over time, since cells can be stored at −80°C for at least a month before scRNA-seq as discussed above. This method additionally allows the transportation of samples to other facilities, if an in-house solution for library preparation and sequencing is unavailable, solving several logistical restrictions related to the use of fresh cells.

Initially, test you protocol in a simple setup

It is of utmost importance to test the entire protocol in a simple setup like a cell line. In line, the impact of the present protocol on the RNA quality and -integrity was validated in the myogenic cell line, C2C12. Cells originating from the same culture flask, were either fixed with methanol followed by rehydration or stored on ice. The cells were then used for single cell cDNA library generation using the 10× Genomics Chromium system, with subsequent sequencing on a NextSeq system. Computational analyses of sequencing data using the 10× Genomics Cell Ranger software package at default settings were performed on 4,134–6,960 cells/sample with a sequencing depth of 12,436–16,324 and 9,074–12,343 "Mean Reads per Cell" in Live and methanol (MeOH) fixed cells, respectively. This showed a high-quality sequencing of the samples with a percentage of "Valid Barcodes" of at least 94% in all samples and "Q30 Bases in RNA Read" values between 79.2% and 80.3% which is within the expected range following the Single Cell Gene Expression recommendations from 10× Genomics. The percentage of "Reads Mapped Confidently to the Transcriptome" were 60.5%–62.7% in Live cells and 56.9%–57.9% in MeOH fixed cells, substantially exceeding the recommended minimum threshold by 10× Genomics recommendations and reports,[15] indicating high quality of sequencing data. To investigate the quality of individual cells in the data, a Barcode rank plot (knee plot) was visualized (Figure 1A). The Barcode rank plot was used as a quality measure for the encapsulation efficacy of the methanol fixation protocol. Both Live and MeOH fixed data showed the expected characteristic sharp descent followed by plateaued "knee", indicating optimal separation of intact cells and empty droplets. Live samples had a generally higher "Mean Reads per Cell" count. Decreases in "Median Number of Genes per Cell" (2,018–2,508 in Live cells and 1,126–1,375 in MeOH fixed cells) and "Median Number of UMIs per Cell" (5,515–7,944 in Live cells and

2,407–3,233 in MeOH fixed cells) were observed after MeOH fixation as compared to Live cells. This is in line with previous observations[16,17] (Figures 1B and 1C; Table S1), but downstream analysis indicated that this reduction did not compromise cellular resolution. Also as reported by others,[16] "Fraction Reads in Cells" was significantly reduced after MeOH fixation (56.5%–57.8%) as compared to Live cells (84.9–92.0%) (Table S1), but again did not affect the relative analysis between fixed samples and thus the biological output. Importantly, the mean percentage of mitochondrial genes was lower in MeOH fixed cells (3.5%–3.7%) as compared to Live cells (6.8%–8.0%) suggesting that methanol fixation does not impair cell quality (Figure 1D). In line, the RNA integrity number (RIN) was determined using the Agilent RNA 6000 Nano kit and Agilent 2100 Bioanalyzer (Agilent Technologies). The RIN of Live cells were 10 corresponding to intact RNA. In MeOH fixed cells, the RIN was 8.7 $\pm$ 0.14 (Figures 1E and 1F), indicating only a mild degree of RNA degradation, which could explain the drop in "Fraction Reads in Cells" after methanol fixation. Still, this RIN value is considered within the acceptable range defining a high quality sample.[18]

Being able to store the cells for prolonged time after fixation and rehydration without compromising the RNA integrity clearly benefits the logistics. It increases flexibility in timing and allows for pooling of samples for running in parallel. To investigate the effect of long-term storage methanol fixed, rehydrated cells were stored at −80°C in rehydration buffer (Phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and RNasin 1:80) for 3, 10, or 30 days. No difference in the RIN of cells stored for 3, 10, or 30 days was observed providing flexibility of the protocol (Figure 1G), and indeed we have obtained high quality scRNA-seq data with fixed cells stored for up to 3 years. The cellular heterogeneity of Live and MeOH fixed cells was determined through bioinformatic analysis and should expectably be equal, as the cells originated from the same cultures. To validate this, cells were dimensionally reduced using Uniform Manifold Approximation Projection (UMAP). A Gene Ontology analysis that directly compared differentially expressed genes between Live and MeOH fixed cells was computed. The highest induced genes (Log2FC 0.5–1.3, adjusted *p*-value < 0.001) in Live cells were associated with the terms *cytoplasmic translation*, *regulation of translation*, and *mRNA binding*, while the highest induced genes in MeOH (Log2FC 0.5–2.17, adjusted-P-value <0.001) were associated with *ATP hydrolysis activity* and *ATP-dependent protein folding chaperone* and *structural constituent of cytoskeleton*. No terms were associated with myocyte specific biological processes, indicating that MeOH fixation did not interfere with cellular phenotype. Unsupervised clustering grouped cells in two clusters in both Live and MeOH fixed samples (Figures 1H and 1J). To establish the consistency between the Live and MeOH fixed protocol selected known gene-markers of myogenic cells were visualized in dotplots split by the computed Louvain clusters (Figures 1I and 1K). The relative expressional differences between cluster 0 and 1 were highly comparable for all genes, indicating that methanol fixation does not alter the differential expression levels between cells. We note that the selected genes exhibited expression in a higher percentage of cells for Live samples and recommend being cautious in interpretations made in a combined Live and MeOH fixed settings, likely also applying to data integration and analysis of published datasets of live and fixed cells. Herein, we confirmed the expressional similarity between live and fixed cells by calculating the Log2 fold change of the myoblast markers in cluster 1 in comparison to cluster 0 and found that the relative fold change was highly similar between the Live and MeOH fixed protocol (Figure 1L).

Thus, by testing all reagents and procedures on a cell line, troubleshooting and adjustment are simplified and will benefit the later run of complex samples and designs.

Work in RNase free conditions

For all conditions, workspace, reagents, and utensils, use only RNase free conditions. Thus, make sure the workspace is RNase free to avoid RNA degradation. If possible, dedicate a workspace specifically for RNase free work. If the workspace is used for other work, clean thoroughly with RNaseZAP™ immediately before you start working RNase free. Cleaning with ethanol will not destroy the RNases.

Cleaning of instruments for dissection (two scissors and two tweezers)

1. Spray the instruments with RNaseZAP™ – let sit for a few minutes.
2. Wash off excess RNaseZAP™ in nuclease free water.
3. Let them dry.

Use only reagents free of RNase or tested for RNA degradation ability and use only RNase free utensils.

Institutional permissions

Animal experiments were approved by the Danish Council for Supervision with Experimental Animals (mouse experiments: #2016–15-0201–00,941 and #2022–15-0201–01119 and zebrafish experiments: #2016-15-0201-00874 and #2021-15-0201-01026).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Embryonic (E16.5) C57BL/6J mice	Bred at Biomedical Laboratory, University of Southern Denmark (Originating from Taconic Europe)	N/A
Neonatal (P1 and P5) C57BL/6J mice	Bred at Biomedical Laboratory, University of Southern Denmark (Originating from Taconic Europe)	N/A
C2C12	ATCC	Cat #CRL-1772
AB zebrafish	Kindly donated by Elke A. Ober, Faculty of Health and Medical Sciences, DanStem (Novo Nordisk Foundation Center for Stem Cell Biology)	N/A
WTC-mEGFP-MYL2-c127 hiPSC-CMs	Coriell Institute for Medical Research	Cat #AICS-0060-027
Chemicals, peptides, and recombinant proteins		
Nuclease-free water	Ambion	Cat #AM9932
Nuclease-free phosphate-buffered saline (PBS)	Ambion	Cat #AM9625
Bovine serum albumin (BSA)	Gemini Bio-Products	Cat #700-106P
Hank's buffered salt solution (HBSS)	Thermo Fisher Scientific	Cat #153963
RNaseZAP™	Sigma-Aldrich	Cat #R2020-250ML
RNasin® Plus RNase inhibitor 40 U/μL	Promega	Cat #N2615
Triton X-100	Sigma	Cat #T8787
Neonatal Heart Dissociation Kit, mouse and rat	Miltenyi	Cat #130-098-373
Methanol	Merck Millipore	Cat #1.006009.1000
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat #D2650
FACS flow	BD Biosciences	Cat #342003 or equivalent
FACS clean	BD Biosciences	Cat #340345 or equivalent
FACS rinse	BD Biosciences	Cat #340346 or equivalent
FACS Accudrop Beads	BD Biosciences	Cat #345249 or equivalent
Antibodies and dyes		
MYH1	DSBH	Cat #MF20-c
Donkey anti-mouse IgG Alexa Flour 488	Invitrogen	Cat #A-21202
Software and algorithms		
NucleoCounter v.1.3.0.0	ChemoMetec	Compatible with your NucleoCounter/cell counter
FACSDiva v.8.0.1–9.0	BD Biosciences	Compatible with your cell sorter
Other		
Fixable viability stain 570	BD Biosciences	Cat #564995
Hoechst 33342	Invitrogen	Cat #H3570
Micro tweezers	Lawton	Cat #09-3033-C or equivalent
Tweezers	Lawton	Cat #08-453-105 or equivalent
Micro scissors	Lawton	Cat #05-0064-C or equivalent

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Scissors	FST	Cat #14058-9 or equivalent
gentleMACS Octo Dissociator with heaters	Miltenyi Biotec	Cat #130-096-427
gentleMACS C tubes	Miltenyi Biotec	Cat #130-096-334
Incubator	Memmert	Cat #INB300 or equivalent
Microcentrifuge	Fisher Scientific	accuSpin Micro 17R or equivalent
Centrifuge for 15 and 50 mL tubes	Thermo Fisher Scientific	Cat #75007224 or equivalent
FACSAriaIII cell sorter	BD Biosciences	FACSAriaIII or equivalent
Sample injection tubing (12-inch lengths)	BD Biosciences	Cat#641059 or equivalent
Sample injection tubing (7-inch lengths)	BD Biosciences	Cat #641475 or equivalent
Pinch valve tubing	BD Biosciences	Cat #641900 or equivalent
Vortex mixer	Benchmark	Cat #BE-BV 1003-E or equivalent
Automated cell counter, NucleoCounter NC-200	ChemoMetec	Cat #900-0210 or equivalent
Laptop		Compatible with your NucleoCounter/cell counter
Agilent 2100 Bioanalyzer	Agilent Technologies	Agilent 2100 Bioanalyzer or equivalent
Agilent RNA 6000 Nano Kit	Agilent Technologies	Cat #G2939BA
Via1 Cassettes	ChemoMetec	Cat #941-0012 or what is compatible with your NucleoCounter/cell counter

⚠ CRITICAL: Methanol is a flammable and toxic substance. Avoid inhalation and contact with eyes and skin.

MATERIALS AND EQUIPMENT

All reagents needed for the isolation, fixation, rehydration, staining for FACS, and FACS are listed below.

RNase free HBSS/1% BSA*

Reagent	Final concentration	Amount
RNase free BSA	1%	500 mg
HBSS	N/A	To a final volume of 50 mL

Store at 4°C, up to 7 days.
*Amount needed is dependent on number of samples, 30.1 mL per litter of 10 pups.

1×PBS/1% BSA*

Reagent	Final concentration	Amount
RNase free BSA	1%	500 mg
Nuclease free 1× PBS (diluted from 10× PBS in nuclease free water)	N/A	To a final volume of 50 mL

Store at 4°C, up to 7 days, store at −20°C for several months.
*Amount needed is dependent on number of samples, 4,075 µL per sample is needed.

1× PBS/1% BSA with 0.1% Triton-X-100*

Reagent	Final concentration	Amount
Triton-X-100	0.1%	10 µL
1× PBS/1% BSA	N/A	9,990 µL

Store at 4°C, up to 7 days.
*Amount needed is dependent on the number of samples; 4,700 µL is needed per sample.

1× PBS/1% BSA with 0.1% Triton-X-100 and RNasin® 1:80*

Reagent	Final concentration	Amount
RNasin	0.5 u/µL	42.5 µL
1× PBS/1% BSA with 0.1% Triton-X-100	N/A	3,357.5 µL

Make fresh, do not store.
*Amount needed is dependent on the number of samples, 3,700 µL per sample is required for all steps in the protocol. Extra is needed for running the FACS, 2,000 mL per run is normal.

1× PBS/1% BSA with 0.1% Triton-X-100 and RNasin® 1:40*		
Reagent	Final concentration	Amount
RNasin	1 u/μL	25.6 μL
1× PBS/1% BSA with 0.1% Triton-X-100	N/A	999.4 μL

Make fresh, do not store.
*Amount needed is dependent on the number of samples, 1,000 μL per sample is required for all steps in the protocol.

STEP-BY-STEP METHOD DETAILS

Isolation of mouse left ventricles

Timing: 45 min

Here we describe the steps for isolation of the left ventricle in mice. The protocol can be performed using mice at every developmental stage from E16.5 to P5. Exclusive steps specific for embryo and neonatal pups are described in separate steps.

The time used will depend on the number of pups. At present, indicated times refer to a litter of 6–9 pups.

1. Isolate the ventricles:
   For E16.5 mice, exclusively, all steps are carried out on ice.
   a. Sacrifice the pregnant female mouse by cervical dislocation, or according to institutional guidelines.
   b. Remove the embryos from the uterus and transfer them to a nuclease free 50 mL tube containing HBSS/1% BSA, leaving the embryos in their gestational sac.
   c. Transfer the embryos to a nuclease free 10 cm petri dish containing fresh HBSS/1% BSA.
   d. Remove the embryos from the sac.
   e. Transfer an embryo to another nuclease free 10 cm dish and isolate the heart under the microscope – The heart can be easily located since it is still beating (see also[19]).
   f. Transfer the heart to a nuclease free 6 cm dish containing a drop of 100 μL HBSS/1% BSA.
   g. Remove the vessel, atria and right ventricle.
   h. Repeat step e-g, for all individual embryos.
   i. Transfer the left ventricles to separate nuclease free 1.5 mL Eppendorf tubes containing 100 μL of RNase free PBS.
   For P1 and P5 mice, exclusively, all steps are carried out on ice.
   a. Sacrifice the mouse by decapitation with a pair of scissors or according to institutional guidelines.
   b. Make a cut of a few mm through the ribs along the sternum – make sure not to cut too long as this increases the risk of exposing the organs in the abdomen.
   c. Gently squeeze the mouse on the thorax and the beating heart will be visible.
   d. Take the heart out with a pair of tweezers and place it in a drop of RNase free PBS in a nuclease free 6 cm dish.
   e. Remove the vessels, atria, and right ventricle under a stereomicroscope.
   f. Transfer the left ventricles to separate nuclease free 1.5 mL Eppendorf tubes containing 100 μL of RNase free PBS.
2. Prepare the enzymes from the Miltenyi Neonatal Heart Dissociation Kit, mouse and rat, according to the manufacturer's recommendations.

Cell dissociation and counting

Timing: 1 h and 15 min

Here we describe the steps for cellular dissociation using the gentleMACS Octo dissociator, subsequent cell counting, and viability stain before methanol fixation.

3. Start by pre-cooling your centrifuge to 4°C and pre-heat your incubator to 37°C.
   a. With a pair of tweezers, gently move the left ventricles to the gentleMACS C tube pre-filled with the enzymes.
   b. Place the tube in an upside-down position at the gentleMACS Octo Dissociator and apply the heat block – Make sure no hearts are stuck to the side of the tube.
   c. Run program 37C_mr_NHDK_1 on the gentleMACS Octo Dissociator. This program takes approx. 1 h.
   d. Quickly spin (briefly at 300 × *g*) the dissociated cells to settle all cells from the lid of the gentleMACS C Tube.
   e. Transfer dissociated cells to a nuclease free 15 mL tube.
   f. Centrifuge the cells at 300 × *g* for 5 min at 4°C.
   g. Remove the supernatant without disturbing the cell pellet.
   h. Wash cells in 1 mL RNase free 1× PBS/1% BSA pre-heated to 37°C.
   i. Centrifuge the cells at 300 × *g* for 5 min at 4°C.
   j. Remove the supernatant without disturbing the cell pellet.
   k. Resuspend the cells in 1 mL RNase free PBS/1% BSA pre-heated to 37°C.
   l. Mix 25 μL of cell suspension with 75 μL RNase free PBS/1% BSA and determine the number of cells as well as the viability on the NucleoCounter (optimal range between 50,000 and 5,000,000 cells/mL).
   m. Add 1 μL of fixable viability stain 570 to the cells and incubate for 3 min at 37°C in an incubator.

Methanol fixation

Timing: 1 h

This step will accomplish the cellular fixation using methanol. Fixation with methanol has been found to preserve transcriptomes and RNA integrity, enabling construction of reliable cDNA libraries from single cells for next-generation sequencing.[9]

The time used will depend on the number of samples. At present, the indicated times refer to a total of 6–9 samples.

4. Start by pre-cooling your centrifuge to 4°C
   a. Centrifuge the cells at 300 × *g* for 5 min at 4°C.
   b. Remove the supernatant without disrupting the cell pellet.
   c. Add 1 mL of chilled RNase free PBS/1% BSA and gently pipette mix 10 times or until cells are completely resuspended.
   d. Centrifuge at 300 × *g* for 5 min at 4°C.
   e. Add 1 mL of chilled RNase free PBS and gently pipette mix 10 times or until cells are completely resuspended.

   Note: Until after removal of methanol, PBS without BSA should be used, since BSA is a protein that will precipitate in methanol.

   f. Centrifuge at 300 × *g* for 5 min at 4°C.
   g. Remove the supernatant without disrupting the cell pellet.
   h. Resuspend the cells in 100 μL RNase free PBS.
   i. Gently add 900 μL ice cold methanol to the cells and gently pipette mix 10 times or until cells are completely resuspended.

j. Fix the cells for 15 min on ice.
k. After fixation proceed immediately with the rehydration protocol.

⚠ CRITICAL: High quality RNA is crucial, when performing scRNA-seq. A challenging, yet essential part of cell preparation for isolation of an enriched CM population prior to scRNA-seq is fixation of cells while preserving the RNA integrity and accessibility. Conventional fixing reagents containing aldehydes modifies nucleic acids by cross linking of the RNA and requires reversal of cross linking prior to scRNA-seq.[20] Alcohols, however, act by dehydrating the cells leaving the RNA in a modified, collapsed state, which can be reverted by rehydration.[20]

Rehydration

Timing: 1 h

Here we describe the immediate rehydration of cells. Following this step, cells can be stored at −80°C for a prolonged period.

The time used will depend on number of samples. At present, the indicated times refers to 6–9 samples.

5. Start by pre-cooling your centrifuge to 4°C
   a. Centrifuge fixed cells at 3,000 × *g* for 10 min at 4°C.
   b. Make a master mix of RNase free PBS/1% BSA/RNasin® (1:80) (For rehydration you need 3 mL per sample).
   c. Remove the supernatant without disturbing the cell pellet.
   d. Add 1 mL of chilled RNase free PBS/1% BSA/RNasin® (1:80) and gently pipette mix 10 times or until cells are completely resuspended.
   e. Centrifuge at 3,000 × *g* for 10 min at 4°C.
   f. Remove the supernatant without disturbing the cell pellet.
   g. Repeat step d–f.
   h. Add 1 mL of RNase free PBS/1% BSA/RNasin® (1:80) to the cells and pipette mix 10 times or until cells are completely resuspended.
   i. Store the cells at −80°C or progress immediately with staining and FACS.

Staining for FACS

Timing: 2 h

Here we describe the staining that allows the isolation of cardiomyocytes. Fixation of cells enables immunocytochemical staining against intracellular markers, hereby allowing isolation of the cell type of interest by FACS. This is particularly important for identifying CMs where surface markers are generally lacking and where DNA for ploidy measures is inside the nucleus.

The time used will depend on number of samples, and here the indicated times refer to the use of 6–9 samples.

6. Start by pre-cooling your centrifuge to 4°C
   a. Thaw the cells on ice, if frozen.
   b. Pellet the cells at 3,000 × *g* for 10 min at 4°C.
   c. Remove the supernatant without disturbing the cell pellet.

Note: Since antibodies often are unavailable in a RNase free version, a higher concentration of the RNase inhibitor RNasin® (1:40 instead of 1:80) was added to the samples when they were exposed to an antibody during staining.

d. Make a master mix of PBS/1% BSA/0.1% TX100/RNasin® (1:80) and PBS/1% BSA/0.1% TX100/RNasin® (1:80) (for staining you need 700 μL (1:80) and 1000 μL (1:40) per sample).
e. Permeabilize the cells in 100 μL RNase free PBS/1% BSA/0.1% TX100/RNasin® (1:80) for 10 min on ice while shaking.
f. Pellet the cells at 300 × *g* for 3 min at 4°C.
g. Wash twice in 100 μL RNase free PBS/1% BSA/0.1% TX100/RNasin® (1:80).
h. Pellet the cells at 300 × *g* for 10 min at 4°C.
i. Remove the supernatant without disturbing the cell pellet.
j. Add the primary antibody MYH1 diluted 1:300 in 300 μL PBS/1% BSA/0.1% TX100/RNasin® (1:40) and resuspend the cells.
k. Incubate for 30 min on ice while shaking.
l. Pellet the cells at 300 × *g* for 3 min.
m. Remove the supernatant without disturbing the cell pellet.
n. Wash twice in 100 μL RNase free PBS/1% BSA/0.1% TX100/RNasin® (1:80).
o. Pellet the cells at 300 × *g* for 10 min at 4°C.
p. Remove the supernatant without disturbing the cell pellet.
q. Incubate for 30 min on ice while shaking resuspended in secondary antibody (Donkey anti-mouse IgG Alexa Fluor 488) diluted in 300 μL RNase free PBS/1% BSA/0.1% TX100/RNasin® (1:40).
r. Wash twice in 100 μL RNase free PBS/1% BSA/0.1% TX100/RNasin® (1:80).
s. Resuspend the cells in 100 μL RNase free PBS/1% BSA/RNasin® (1:40).

Note: For obtaining reliable scRNA-seq data it is important that cells are in a single cell suspension. To ensure this, cells were filtered through a cell strainer before accessing the cell sorter. In addition, cells were maintained in PBS containing 1% BSA to avoid clumping.

7. Filter cells in a FACS tube with a blue cell strainer cap (35 μm).
   a. Add 100 μL of RNase free PBS/1%BSA/RNasin (1:40) to the cell strainer.
   b. Filter the cell suspension through the cell strainer.
   c. Wash the cell strainer with another 200 μL of RNase free PBS/1% BSA/RNasin® (1:40).
8. Transfer cells to RNase free FACS tubes.
9. Add 2 μL of Hoechst (diluted according to the manufacture's recommendation) to the samples.
10. Sort the cells at a FACSAriaIII cell sorter (BD Biosciences).

FACS

Timing: depends on the number of cells and number of samples

This step provides a guide for FACS sorting, specifically using the FACSAriaIII cell sorter and FACSDiva software.

11. Make sure the nozzle 100 and the filter 1.5 is applied to the FACSAriaIII cell sorter.

Note: When doing FACS on CMs that are relatively large cells it is important to remember to choose a nozzle and filter applicable with the cell size.

12. A fluidic startup is performed using the FACSFlow buffer.
13. When the stream is stabilized, CST (Cytometer Setup and Tracking) is performed.

14. A new sample line (from sample to Flowcell, BD Tube Assembly) is mounted – remember to wear gloves, to not contaminate this with RNases, since this will go directly into the sample.
15. Drop delay is performed with dedicated BD FACS Accudrop Beads.
16. Make sure the FACSAriaIII cell sorter is properly cleaned before applying the cells.
    a. Run PBS/1% BSA/RNasin® (1:80) through the FACSAriaIII cell sorter.

Note: The protocol was performed with a strict focus on maintaining high quality RNA for reliable scRNA-seq data. All reagents and equipment were RNase free when possible and the RNase inhibitor, RNasin was added to the samples at all steps after fixation.
The FACSAriaIII cell sorter was properly cleaned with RNaseZap prior to cell sorting and the loop going directly into the sample was renewed prior to use. The quality of the RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies), which uses an algorithm referred to as the RNA integrity number (RIN) to provide a comparable measure of the RNA integrity. A RIN of 1 refers to totally degraded RNA and a RIN of 10 correlates to fully intact RNA.[21] To obtain reliable scRNA-seq data, the RNA from a small aliquot of the cells undergoing scRNA-seq is analyzed and the RIN should not be lower than 6.[18] Using the RIN for determining RNA integrity eliminates the subjective interpretation associated with determining RNA degradation based on agarose gel electrophoresis.[21]

17. First, take a small portion of one sample – this will be used for setting the gates without contaminating the remaining sample and for testing that the cells are sorted efficiently into four different tubes.
18. Set the gating hierarchically in the FACSDiva software (see also[1]).
    FSC/SSC → FSC/PE → Alexa-488/Hoechst – In the current set up – depends on your flow cytometry panel.
    a. FSC/SSC – Exclude cell debris.
    b. PE – Cells dead prior to fixation are stained more intensely with the fixable viability stain 570 as compared to viable cells. In this way, cells dead prior to fixation can be excluded by only gating the cells with low PE expression.
    c. Alexa-488 – Cells are stained with a primary antibody against MYH1 (MF20-c, DSHB) to distinguish CMs from the remaining heart tissue cells. The secondary antibody is Alexa flour-488 conjugated, making CMs positive to Alexa-488.
    d. Hoechst 33342 – Cells are stained with Hoechst 33342, which stains the DNA of the cells. The ploidy of the cells can be determined from the amount of DNA in each cell.

Note: Exclusion of dead cells prior to cDNA library generation for scRNA-seq is crucial for obtaining reliable transcriptomic profiling data.
This step, however, is often neglected in scRNA-seq studies.[22]This step is of particular importance since dead cells alter the transcriptomic profile and can be difficult to exclude bioinformatically.[22,23] In general a threshold of 5% mtRNA is used for computational exclusion of low quality cells in mice.[24] For CMs, however, the naturally high number of mitochondria present exceeds this threshold of mitochondrial transcripts substantially,[25] making it difficult to use mtRNA for computational exclusion of low-quality cells. Also, excluding CMs based on a high percentage of mitochondrial transcripts can create a bias and exclude specific subpopulations of CMs.[26] Thus, the use of a fixable viability stain is thus vital to our approach to minimize the presence of dead cells.

19. Collect four samples of cells; 2n, 4n, polyploid and non-myocytes
20. Re-run the samples to check the purity – at this point you might experience fading of the fluorescence dye, making the cells not fit accurately into the pre-set gates.
21. Progress to the actual samples – back flush + cleaning with RNase free PBS/1% BSA/RNasin® (1:80).

22. Place up to four RNase free FACS tubes pre-filled with 100 μL PBS/1% BSA/RNasin®1:40 in the sort collection chamber – before placing the tubes quickly vortex to coat the sides of the tubes with the buffer.
23. Record the first cells for quantification.
24. Immediately after FACS, centrifuge the cells at 400 × *g* for 5 min at 4°C to exchange the FACS flow solution with an RNase free buffer.
25. Remove the supernatant without disturbing the pellet.
26. Resuspend the cells in PBS/1% BSA/RNasin® 1:40 and transfer the cells to an RNase free 1.5 mL Eppendorf tube.
27. Store the cells at −80°C until scRNA-seq – Before progressing with the scRNA-seq, RIN can be verified.

Note: For description of the protocols for scRNA-seq, we refer to our own published papers,[1,27] as well as.[28]

EXPECTED OUTCOMES

In our primary publication using the current approach, we obtained a highly enriched population of CMs from embryonic and neonatal mouse hearts bioinformatically assigned to a CM phenotype based on known CM markers by using the protocol described herein. Ploidy stratification allowed identification of genes that were differentially expressed in tetraploid CMs before (E16.5) and after (P5) the time of CM polyploidization/binucleation. With a specific interest in differentially expressed cell cycle associated genes, we were able to bioinformatically predict a list of transcription factors (TFs) regulating cell cycling activity of this subpopulation of CMs. This was only accomplished by the increased scRNA-seq resolution achieved by the protocol provided herein. By adeno-associated virus (AAV) transmission, the effect of ectopic expression of 11 of these TFs on cell cycle progression was determined in primary cell cultures of murine neonatal (P0) cardiac cells. We found that six out of the 11 TFs induced an increase in cell cycle progression as determined by EdU incorporation, thereby validating our current approach.[1]

Validation and quality control in embryonic and neonatal mouse-derived cardiac cells

Following the protocol left heart ventricles of embryonic and neonatal mice were prepared for scRNA-seq, using ploidy stratification specifically for CMs into diploid and tetraploid CM fractions (Figure 2A). Sorted cells were examined by fluorescence microscopy to confirm that the cells were indeed in a single cell suspension (Figure 2B). The RIN after fixation and rehydration of cells ranged from 7.1 to 8.7 in the nine samples (three biological replicas for each time point) with no clear pattern in RIN dependent on developmental stage (Figure 2C). Thus, the RIN exceeds the threshold of 6 recommended for obtaining reliable scRNA-seq data.[18] The three biological replicas performed for each time point were pooled into two samples containing diploid or tetraploid cells (six samples in total). cDNA library generation was performed using the 10× Genomics Chromium system (Chromium Single Cell 3′ Reagent Kits v2) and libraries were sequenced using the Illumina NextSeq platform. Initial computational analysis was performed using the 10× Genomics Cell Ranger software at default settings. Computational analysis was performed on sequencing data of 5,193 ± 1,309 cells per sample (31,156 cells in total). Visualizing each cell in a barcode rank plot showed expected characteristics for 5/6 samples (Figure 2D). For sample 16.5-4n we observed a weaker descent at the valid cell/empty cell cutoff, however Cell Ranger estimated the cutoff at a comparable number of cells (6,560 cells). Assessing single cell metrics, found samples were appropriate for further computational analysis (Figures 2E and 2F; Table S2). Mature CMs have a high percentage of mitochondrial transcripts as compared to other cell types due to their high energy demand.[25,26] This likely explains the increase in the percentage of mitochondrial transcripts as the CMs develop from E16.5 to the more mature P1 and P5 CMs (Figure 2G). Other quality control parameters remained constant, and thereby do not imply a decrease in cell quality (Table S2). As previously shown,[1] dimensional reduction of the data characterizes that samples sharing developmental stage are clustered

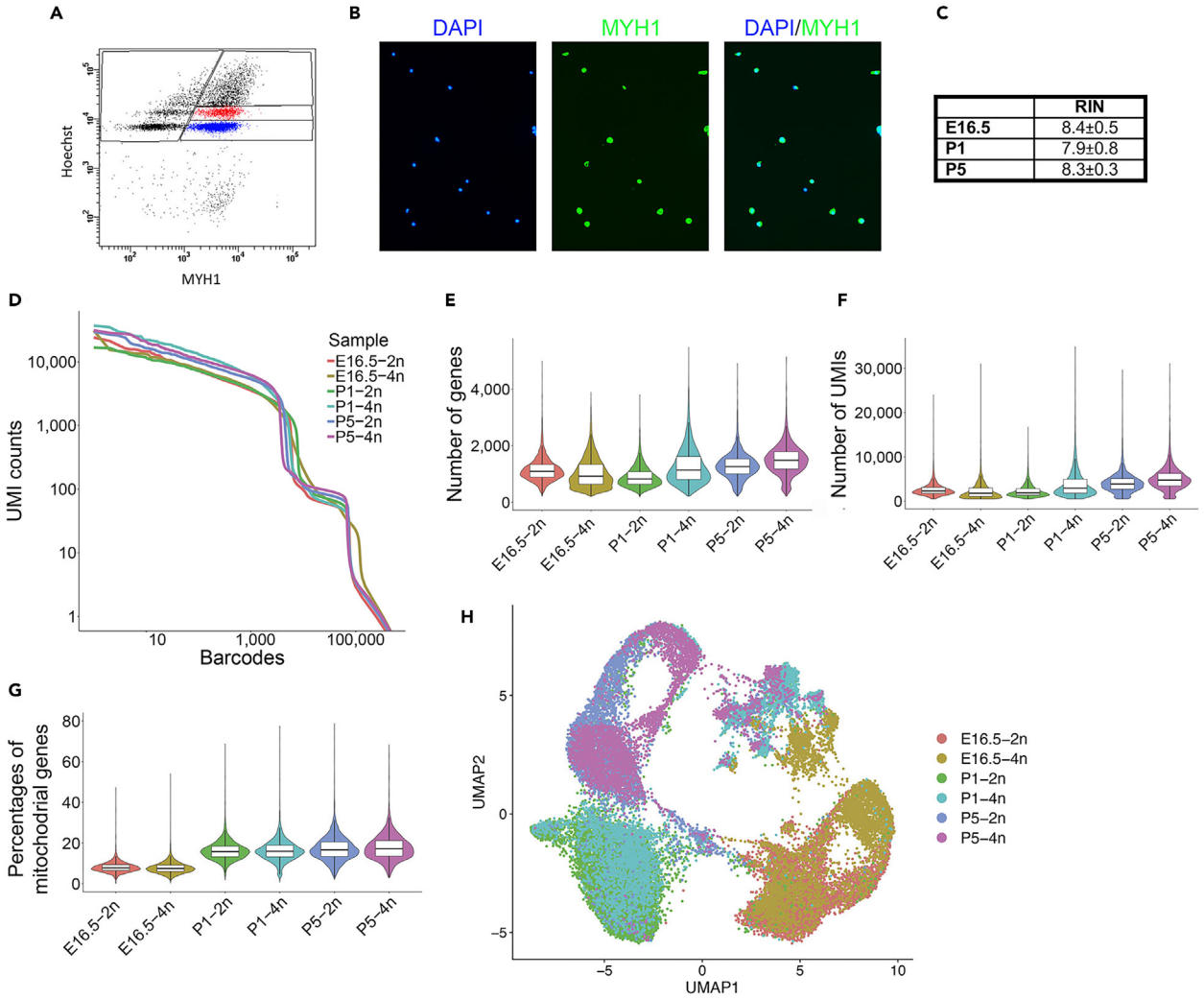


Figure 2. Embryonic and neonatal mouse cardiac cells can be fixed and enriched for cardiomyocytes (CMs) prior to scRNA-seq

(A) FACS plot showing CM identification and ploidy stratification based on MYH1 and Hoechst staining of live-gated cells.

(B) Immunofluorescence imaging confirming that sorted cells are in a single cell suspension (green: MYH1; blue: DAPI).

(C) Table of RNA integrity number (RIN) of cardiac cells after fixation at E16.5, P1, and P5. (D–G), Quality analyses of E16.5-2n, E16.5-4n, P1-2n, P1-4n, P5-2n, P5-4n samples.

(D) Barcode rank plot (knee-plot) for barcodes ranked by Unique molecular identifier (UMI) count, log transformed against total number of UMIs per cell,

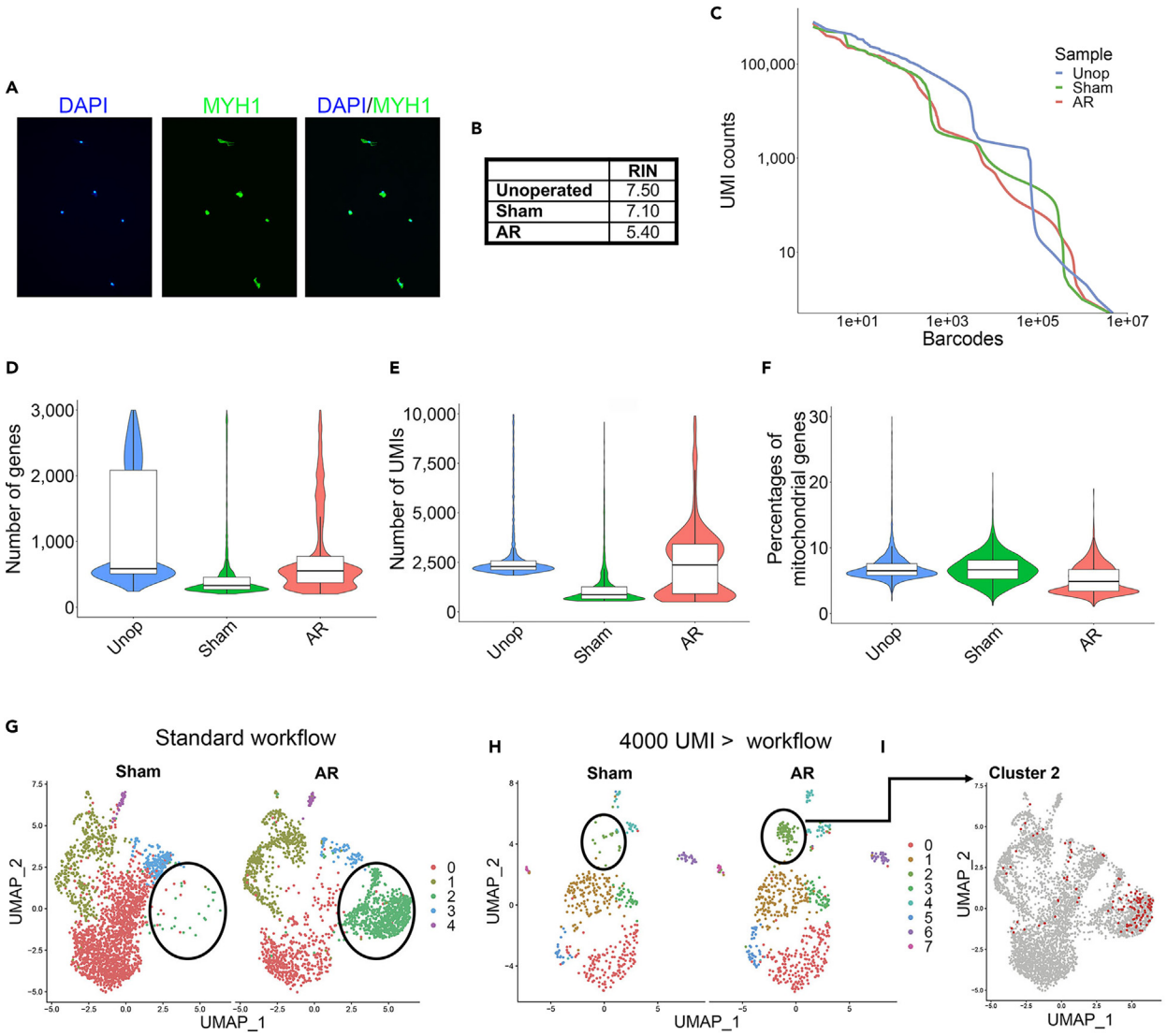
(E) Violin plot showing number of genes per cell, (F) Number of UMI counts per cell, and (G) Percentage of mitochondrial transcripts.

(H) Uniform manifold approximation and projection (UMAP) based on highly variable genes bioinformatically confirming our CM sorting approach. Cells are colored by sample. Refer also to Table S2.

together at consistent communities of cells (Figure 2H), indicating appropriate applicability of the protocol for investigations of cells in the mouse heart.

Validation and quality control in adult zebrafish cardiac cells and human iPS cell-derived CMs

In contrast to mammals, the zebrafish myocardium regenerates upon damage, making the zebrafish an interesting model for studying CM renewal.[29] However, the transcriptomic alterations leading to regeneration upon zebrafish myocardial damage have only been addressed at a single cell level in a few studies.[7,30,31] The present protocol was applied to adult zebrafish hearts without modifications. Apex resection (AR) was performed in parallel to Sham and unoperated controls. Cells were stained with fixable viability stain, MeOH fixed, and rehydrated before staining with the CM marker MYH1 and Hoechst to visualize the ploidy specifically of CMs and cells were sorted by FACS. The sorted



	RIN
Unoperated	7.50
Sham	7.10
AR	5.40

Figure 3. Validation of the protocol in adult zebrafish cardiac cells

(A) Immunofluorescence imaging confirming that sorted cells are in a single cell suspension (green: MYH1, blue: DAPI).

(B) Table showing RNase integrity number (RIN) values of zebrafish cardiac cells for unoperated, Sham and apex resected (AR) samples.

(C–F) Quality analyses of unoperated, Sham, and AR samples. (C) Barcode rank plot (knee-plot) derived from each detected droplet. Barcodes were sorted based on the number of Unique molecular identifiers (UMIs) and subsequent log-scaled, against number of total UMIs per droplet – log-scaled. (D–F) Violin plots showing (D) Number of genes per cell, (E) Number of UMI counts per cell and (F) Percentage of mitochondrial transcripts per cell.

(G–I) Stringent bioinformatic quality control confirming the continuity of Louvain clusters after removal of cells with low UMI count in Sham and AR samples. (G) Uniform manifold approximation and projection (UMAP) represented by highly variable genes in cells with > 500 UMIs (standard workflow). Colors indicate Louvain algorithm clusters at a resolution = 0.15. (H) UMAP based on highly variable genes in cells with > 4000 UMIs. Colors indicate Louvain algorithm clusters at a resolution = 0.30. (I) UMAP of standard workflow highlighted by cells present in Louvain cluster two of stringent quality control dataset. Refer also to Table S3.

fractions of cells were indeed in a single cell suspension as validated by fluorescence microscopy (Figure 3A). The RIN of the zebrafish cardiac cell RNA showed RNA quality compatible with scRNA-seq for the unoperated and Sham samples, whereas the RIN for AR group was slightly lower than the recommended value (Figure 3B). The barcode rank plot showed non-conforming characteristics in 2/3 zebrafish samples as both Sham and AR lacked a subset of expected characteristics (Figure 3C). However, we noted that cells with more than 4,000 UMI counts represented the expected characteristics of single cells encapsulated in individual droplets. Single-cell metrics for zebrafish

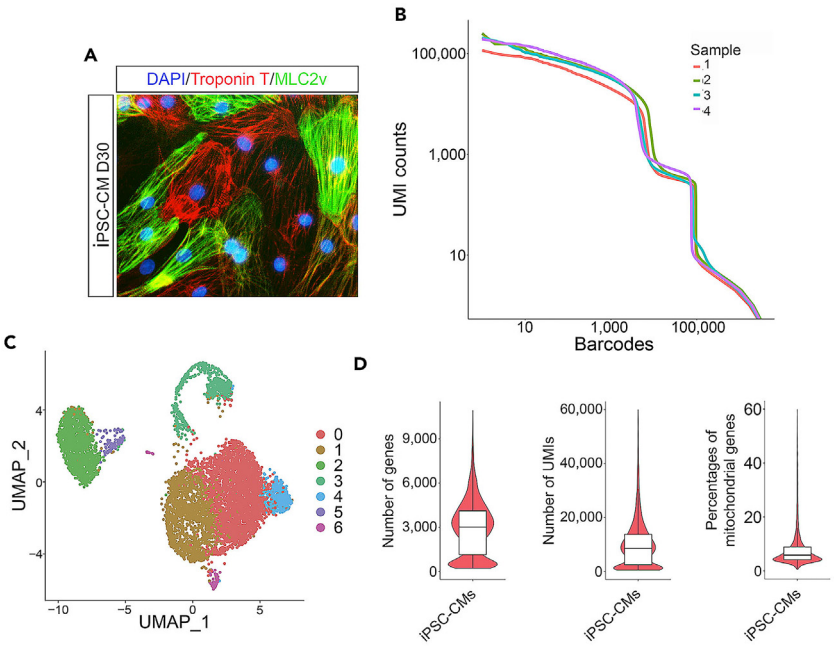


Figure 4. Validation of the protocol in day 30 human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)

(A) Immunofluorescence imaging showing the presence of cardiac contractile fibers in day 30 (D30) matured hiPSC-CMs (Blue: DAPI, red: Troponin T, green: Myosin light chain 2, MLC2v). (B–D), Quality analyses of hiPSC-CM samples.

(B) Barcode rank plot derived from each detected droplet. Barcodes were sorted based on the number of Unique molecular identifiers (UMIs) and subsequent log scaled against number of total UMIs per droplet - log scaled.

(C) Uniform manifold approximation and projection (UMAP) representation of hiPSC-CMs. Colors indicate Louvain algorithm clusters at resolution = 0.3.

(D) Violin plots quantifying quality metrics for hiPSC-CMs including, number of genes per cell, number of UMI counts per cell, and percentage of mitochondrial transcripts per cell. Refer also to Table S4.

samples is summarized in Figure 3D–F and Table S3. The cells exhibited a lower "Fraction of Reads per Cell" than standard at 51.1 ± 18.3%, which could indicate a higher amount of ambient RNA than recommended.

To evaluate whether the immediate lower quality of the scRNA-seq data for Sham and AR samples harnessed the overall outcome, we compared the standard computational procedure with a more stringent approach and assessed the cluster composition. The standard data comprised 4,539 cells (AR: 2,048 cells; Sham: 2,491 cells) (Figure 3G), while the stringent approach, which filtered out cells with less than 4,000 UMIs, retained 1,125 cells (AR: 678 cells; Sham: 447 cells; 24.8% cell preservation) (Figures 3H and 3I), that could confidently be predicted as intact cells. In both procedures, the cells were grouped into 5 clusters, and a specific sample-related cluster (cluster 2) could be consistently identified in the stringent approach. The assessment indicated that the potential ambient RNA content in the data did not interfere with the resulting biological interpretations.

Alternative to studying CM division in mouse and zebrafish CMs, human iPS cell-derived CMs (hiPSC-CMs) (Figure 4A) remains a valid model and also provides a promising source of cells for CM replacement after myocardial damage[32] (reviewed in[33]). The current protocol for MeOH fixation followed by rehydration was applied to hiPSC-CMs. Since hiPSC-CMs cultures in our hands constitute a high concentration of viable CMs, the FACS approach was not applied, but may easily be included if required for a given study. The RIN of the hiPSC-CMs were 9.3 ± 0.64 (mean ± SD). The data quality of hiPSC-CMs was further validated through computational analysis. The barcode rank plot showed expected characteristics, as described above, for all samples (Figure 4B). 99.67%

of the cells were CMs (8,546 cells) as validated by expression of Troponin T. UMAP reduction showed hiPSC-CMs being distributed in seven clusters indicating recovery of a heterogeneous pool of hiPSC-CMs and underscored the utility of the approach developed also for hiPSC-CMs (Figure 4C). The quality metrics assessed for hiPSC-CMs indicates that data had optimal quality for valid downstream analysis (summarized in Figure 4D; Table S4).

This protocol may not only be applicable for cardiac cells. With minor modifications at relevant steps, it may also be applied to a variety of other cell types originating from different tissues and species for preparation of a single cell suspension for scRNA-seq.[27] The protocol may have limited use for primary cells from tissues with a naturally high level of proteases and RNases in which RNA degradation is difficult to avoid after fixation. This generally includes cells of the pancreas, gall bladder, skin, lymphatic-, and immune tissues.[9] In addition, this protocol makes it possible to investigate ploidy stratification which is highly relevant in e.g., the heart, liver, and bone marrow. In the heart, ploidy stratification prior to scRNA-seq may be important as polyploid CMs are thought to be inert to division,[14,34] whereas the diploid CM pool is suggested to have proliferative capacity.[35] Thus, our protocol implies several advantages not only when aiming for detailed scRNA-seq of CMs, but also other cells.[27]

LIMITATIONS

Since the 10× Genomics Chromium platform for cDNA library generation is restricted by a cell size limit of <30 μm, one limitation of the protocol is that it is not compatible with the larger adult CMs.[15] However, although we can only speculate, we do consider it likely that our protocol can be used in combination with other platforms for scRNA-seq cDNA library generation, e.g., the iCELL8 platform that has previously been used for transcriptomic profiling of large mature rod-shaped adult mouse CMs.[36] Moreover, since our protocol was applied for single whole cells, further investigation is needed to determine if the current protocol is applicable for experiments requiring prior nuclei isolation, including single-nucleus RNA sequencing and single-cell ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing). Of particular importance, we should emphasize that upon fixation and rehydration of cells, a reduction in the number of UMIs per cell compared to fresh cells (despite comparable sequencing depths across samples) was observed. While this reduction did not interfere with the current protocol validation, it may constrain the resulting transcriptomic resolution from the protocol in experimental setups with low initial mRNA concentration. One solution to that could be to recover the number of UMIs per cell by sequencing at a higher depth, yet this remains to be assessed.

TROUBLESHOOTING

Problem 1

Poor RIN is obtained (related to all steps).

Potential solution

At all steps, make sure you are working in an RNase free environment, using RNase free reagents, and adding RNasin® to the samples where indicated. Also, make sure all tubes, pipette tips etc. are RNase free and rinse frequently with RNaseZAP® Test the reagents once more as recommended.

Check all reagents for RNA degradation.

- Dilute the reagent of interest to the concentration applied to the protocol in RNase free PBS ± RNasin® and add 2.5 μg of RNA (irrelevant RNA with known quality).
- Incubate for 30 min at 37°C (to increase potential RNase activity).
- Measure the RIN at the Bioanalyzer.

Note that some reagents cannot be measured at the Bioanalyzer.

Before using the protocol on the desired primary cells, test on a standard cell line, lowering the number of animals needed during standardization.

Problem 2

Low number of CMs after FACS (related to all steps, and in particular step 7 and 11).

Potential solution

CMs are sensitive, hence, to avoid that they die during the process it is important to work as fast and efficient as possible and keep them on ice. Check viability of the CMs at all stages until fixation to encounter the problem. In addition, it is important to use the correct filters and nozzle size allowing the passage of the CMs as well as to check specificity of antibodies for CMs and sorting purity.

Problem 3

High content of non-CMs (related to step 6–23).

Potential solution

Make sure that you have validated the specificity of your antibody on your CMs and check your gating strategy, making sure the your gates doesn't include non-CMs. Check by purity sorting of the obtained CMs.

Problem 4

Not single cell solution (related to step 5–11).

Potential solution

Check the chosen filters, the size should match the size of the CMs in question. Remember to maintain CMs in PBS containing 1% BSA to avoid re-clumping after filtering.

Problem 5

Validation shows poor quality of scRNA-seq data (e.g., knee-plot shows non-conforming characteristics).

There are a number of indicators that predicts poor data quality, including step 3: low cell viability and step 6: cells are not in a single-cell suspension. These indicators can affect the overall outcome of the scRNA-seq library preparation.

Potential solution

Validate the quality of data by assessing number of reads per cell, number of genes per cell, and percent mitochondrial reads per cell. Furthermore, plot the number of UMIs per barcode, ranked (knee-plot) to validate the conformity of the cells. We recommend that you initially test the protocol in a pilot experiment, before doing your actual experiment, as described in the before you begin section.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ditte Caroline Andersen (dandersen@health.sdu.dk).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Ditte Gry Ellman (dellman@health.sdu.dk).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

ScRNA-seq data that support the findings of this study (only for mice) have been deposited to Gene Expression Omnibus (GEO: GSE162959). All other data supporting the findings of this study are available from the corresponding author on reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103194.

ACKNOWLEDGMENTS

The work was supported by research grants from the Novo Nordisk Foundation (#NNF17OC0028764), the Danish Council for Independent Research (Sapere Aude; # 8045-00019B), the Lundbeck Foundation (#R48-A4785 and #R313-2019-573), the Novo Nordisk Foundation Section for Basic Stem Cell Biology (National Collaboration), Danish Cardiovascular Academy (#PD2Y-2021004-DCA-67242), and Innovation Foundation Denmark (#2052-00021B) and research money from the Department of Clinical Biochemistry/Odense University Hospital.

We would like to thank Charlotte Nielsen and Tonja L. Jørgensen (Andersen group, Odense University Hospital) for excellent technical assistance on this study, Wolfgang Hofmeister (Andersen group, Odense University Hospital; Clinical Institute, University of Southern Denmark, Odense; and Faculty of Health and Medical Sciences, DanStem, Novo Nordisk Foundation Center for Stem Cell Biology, Copenhagen) for performing the zebrafish surgeries, Lars Vitved (Department of Cancer and Inflammation Research, University of Southern Denmark, Odense) for assisting with the FACS, Christina D. Fenger (Amplexa Genetics, Odense) for cDNA library preparation and assistance of sequencing, and Mark Burton (Department of Clinical Genetics, Odense University Hospital) for assisting with guidance and analysis of raw sequencing data.

AUTHOR CONTRIBUTIONS

D.G.E., F.A.B., S.T.B., S.B.M., and E.B.H.: collection of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. C.H.J.: conception and design and final approval of the manuscript. D.C.A.: conception and design, collection of data, data analysis and interpretation, manuscript writing, final approval of the manuscript, and financial support.

DECLARATION OF INTERESTS

E.B.H. and F.A.B. possess concurrent employment at the University of Southern Denmark and Amplexa Genetics A/S. Amplexa Genetics A/S maintains an interest in gene expression analysis.

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