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Tissue dissociation and 10x Multiome for fetal heart tissue (Version 1) V.1

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We use this protocol and it's working

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Abstract

Congenital heart defects (CHD) arise in part due to inherited genetic variants that alter genes and noncoding regulatory elements in the human genome. These variants are thought to act during fetal development to influence the formation of different heart structures. However, identifying the genes, pathways, and cell types that mediate these effects has been challenging due to the immense diversity of cell types involved in heart development as well as the superimposed complexities of interpreting noncoding sequences. As such, understanding the molecular functions of both noncoding and coding variants remains paramount to our fundamental understanding of cardiac development and CHD. Here, we created a gene regulation map of the healthy human fetal heart across developmental time, and applied it to interpret the functions of variants associated with CHD and quantitative cardiac traits. We collected single-cell multiomic data from 734,000 single cells sampled from 41 fetal hearts spanning post-conception weeks 6 to 22, enabling the construction of gene regulation maps in 90 cardiac cell types and states, including rare populations of cardiac conduction cells. Through an unbiased analysis of all 90 cell types, we find that both rare coding variants associated with CHD and common noncoding variants associated with valve traits converge to affect valvular interstitial cells (VICs). VICs are enriched for high expression of known CHD genes previously identified through mapping of rare coding variants. Eight CHD genes, as well as other genes in similar molecular pathways, are linked to common noncoding variants associated with other valve diseases or traits via enhancers in VICs. In addition, certain common noncoding variants impact enhancers with activities highly specific to particular subanatomic structures in the heart, illuminating how such variants can impact specific aspects of heart structure and function. Together, these results implicate new enhancers, genes, and cell types in the genetic etiology of CHD, identify molecular convergence of common noncoding and rare coding variants on VICs, and suggest a more expansive view of the cell types instrumental in genetic risk for CHD, beyond the working cardiomyocyte. This regulatory map of the human fetal heart will provide a foundational resource for understanding cardiac development, interpreting genetic variants associated with heart disease, and discovering targets for cell-type specific therapies.

Materials

	Item	Catalog #	Vendor
	10% Tween-20	1662404	BioRad
	Eppendorf 1.5 ml tubes	22431021	Eppendorf
	BSA	A3311-10G	Millipore Sigma Aldrich
	1M Tris-HCl (pH 7.4)	T2194	Millipore Sigma Aldrich
	Protector RNase inhibitor	3335399001	Millipore Sigma Aldrich
	5M NaCl	59222C	Millipore Sigma Aldrich
	DTT (1000 mM)	646563	Millipore Sigma Aldrich
	Nonidet P40 Substitute (NP40)	74385	Millipore Sigma Aldrich
	RNase Inhibitor, 10000 units	3335402001	Millipore Sigma Aldrich
	PBS, pH 7.4	10010023	Thermo Fisher Scientific
	UltraPure Water	10977015	Thermo Fisher Scientific
	1M MgCl2	AM9530G	Thermo Fisher Scientific
	Digitonin	BN2006	Thermo Fisher Scientific
	HEPES	15630080	Thermo Fisher Scientific
	DMEM	10313021	Thermo Fisher Scientific
	ACK Lysing	A1049201	Thermo Fisher Scientific
	HBSS	14025092	Thermo Fisher Scientific
	B27 Serum	A1895601	Thermo Fisher Scientific
	DNAse I, 1mg/mL in water	LK 003172	Worthington

Introduction

1 This experimental protocol is designed to process and analyze fresh human fetal heart tissue using enzymatic dissociation techniques to explore gene expression and chromatin accessibility at the single-cell level. As the first iteration of our experimental pipeline when we began the project, this protocol was adapted from Miao et al. (2020) and 10X Genomics. The process begins with enzymatic dissociation of fresh heart tissue into single cells, followed by the isolation and purification of nuclei . These nuclei are then prepared for single-cell multiome library construction using the Chromium Single Cell Multiome ATAC + Gene Expression kit. By employing the single-cell multiome method, we can simultaneously analyze gene expression and regulatory elements, enabling us to map gene and enhancer activities across various cell types and developmental stages.

Reagent and Buffer Preparation

2 **Same Day Solutions (prepare fresh)**

Maintain at 4 °C . These are solutions that are made fresh the day of the experiment. These solutions do not need to be filtered prior to use.

Note: Read the protocol first to understand the volume needed per sample. Adjust the table values based on these final volumes.

Tissue Digestion Buffer

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	DMEM	--	--	--	3.045	3,045
	Liberase	5 mg/ml	0.5 mg/ml	10	0.350	350
	DNase I	1 mg/ml	20 mg/ml	50	0.070	70
	HEPES	1M	10 mM	100	0.035	35
				Total Volume	**3.5 mL**	**3,500 uL**

Stop Digestion Buffer

Freeze and store aliquots of 0.3 mL B27 at -20 °C

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol (uL)
	RPMI	100%	98%	1.02	14.7	14,700

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol (uL)
	B27	100%	2%	50	0.300	300
				Total Volume	**15 mL**	**15,000 uL**

General Buffer

Maintain at 4 °C

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	PBS	--	--	--	9.96	9,960
	0.04% BSA	10%	0.04%	250	0.04	40
				Total Volume	**10 mL**	**10,000 uL**

Nuclei Wash Buffer

Prepare fresh and maintain at 4 °C

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	Nuclease-free water	--	--	--	2.63	2,629
	Tris-HCl (pH 7.4)	1M	10 mM	100	0.031	31
	Protector RNase Inhibitor	40 U/uL	1 U/uL	40	0.078	77.5
	NaCl	5M	10 mM	500	0.006	6.2
	MgCl2	1M	3 mM	333.3	0.012	12
	Tween-20	10%	0.1%	100	0.031	31
	BSA	10%	1%	10	0.310	310
	DTT	1M	1 mM	1000	0.003	3.1

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
				Total Volume	**3.1 mL**	3,100 uL

Lysis Dilution Buffer

Prepare fresh and maintain at 4 °C . This is used to create 0.5X lysis buffer.

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	Nuclease-free water	--	--	--	0.172	171.80
	Tris-HCl (pH 7.4)	1M	10 mM	100	0.002	2
	Protector RNase Inhibitor	40 U/uL	1 U/uL	40	0.005	5
	NaCl	5M	10 mM	500	0.0004	0.40
	MgCl2	1M	3 mM	333.3	0.0006	0.60
	BSA	10%	1%	10	0.020	20
	DTT	1M	1 mM	1000	0.0002	0.20
				Total Volume	**0.200 mL**	200 uL

1X Lysis Buffer

Prepare fresh and maintain at 4 °C . This is used to create 0.5X lysis buffer.

For the digitonin, incubate at 65 °C to dissolve precipitate before use.

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	Nuclease-free water	--	--	--	0.167	167.40
	Tris-HCl (pH 7.4)	1M	10 mM	100	0.002	2

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	Protector RNase Inhibitor	40 U/uL	1 U/uL	40	0.005	5
	NaCl	5M	10 mM	500	0.0004	0.40
	MgCl2	1M	3 mM	333.3	0.0006	0.60
	BSA	10%	1%	10	0.02	20
	DTT	1M	1 mM	1000	0.0002	0.20
	Tween-20	10%	0.10%	100	**0.002**	2
	Digitonin	5%	0.01%	500	0.0004	0.40
	NP-40	10%	0.10%	100	0.002	2
				Total Volume	**0.200 mL**	**200 uL**

0.5X Lysis Buffer

Prepare fresh and maintain at 4 °C . This is used to lyse the cells into nuclei.

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (uL)
	1X Lysis Buffer	1X	0.5X	2	100
	Lysis Dilution Buffer	--	--	--	100
				Total Volume	**200 uL**

Experimental Preparation

3 Prepare fresh solutions the same day as the experiment.

4 Clean materials with RNase Away and 70% ethanol, and use Eppendorf Lobind tubes. Maintain samples and solutions ice-cold during the whole experiment.

Experimental Preparation

5 Set a water bath / incubator / thermomixer to 37 °C . All of these options work but you will need to be to pipette mix your sample.

6 Pre-chill swinging bucket centrifuges to 4 °C .

Experiment (Tissue to Single Cells) 5m

7 Place heart tissue in a 150 cm culture dish. Using dissection scissors, separate tissue into sections (if applicable). Add dissected sections to 60 or 100 cm culture dishes to keep separate. Mince the tissue into small 1mm3 pieces in their respective plate.

8 Add each dissected tissue to a 15mL or 50mL tube containing 3mL of Tissue Digestion Buffer.

9 Incubate in a 37 °C incubator (or water bath / thermomixer) for 15 minutes and agitate with a pipette every ~2-3 minutes.
Important: We cut the tip off of the P1000 tip for easier pipetting.

10 Add 7 mL Stop Digestion Buffer to each tube.

11 Filter each sample through a 100um filter (PluriSelect #43-50100-51) into a 50mL conical.

12 Centrifuge samples: 300 x g, 4°C, 00:05:00 5m

13 Remove the supernatant from the tubes.

14 **RBC Lysis**

This next step is to remove the red blood cells (RBC) from the samples.

14.1 Resuspend cell pellets in 1 mL ACK Lysis Buffer to lyse red blood cells.

14.2 Incubate 5 min on ice.

14.3 Add 5 mL PBS + 0.04% BSA (General Buffer)

14.4 Centrifuge samples: 300 x g, 4°C, 00:05:00 5m

14.5 Remove most of the supernatant.

14.6 Resuspend cell pellet in 1 mL PBS + 0.04% BSA (General Buffer)

14.7 Remove most of the supernatant.

14.8 Resuspend cell pellet in 1 mL PBS + 0.04% BSA (General Buffer)
Gently pipette mix 5x.

14.9 Transfer to a 1.5mL Eppendorf LoBind tube. Rinse the 50-ml tube with 500 µL PBS + 0.04% BSA (General Buffer) . Add the 500uL to the 1.5mL tube.

14.10 Determine the cell concentration using the Countess.

Experiment (Single Cells to Nuclei)

15 Prepare 100,000-1,000,000 cells in a 1.5 ml microcentrifuge tube.

15.1 Centrifuge samples: 300 x g, 4°C, 00:05:00 5m

15.2 Remove all of the supernatant without disrupting the cell pellet.

15.3 Add 100 µL 0.5X Lysis Buffer . Pipette mix 10x.

15.4 Incubate for 3 mins on ice.

[Note: You can modify / optimize lysis by decreasing or adding time.]

15.5 Add 1 mL Nuclei Wash Buffer to the lysed cells. Pipette mix 5x.

15.6 Centrifuge samples: 500 x g, 4°C, 00:05:00 5m

15.7 Remove all of the supernatant without disrupting the cell pellet.

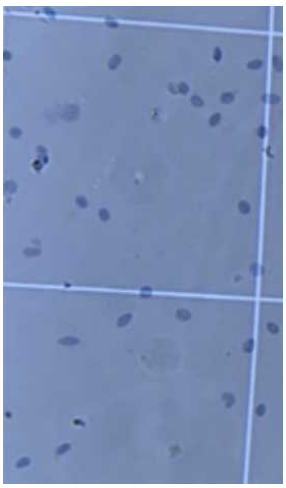
15.8 Repeat steps 15.5-15.7 two more times for a total of 3 washes.

15.9 Resuspend in 1 mL PBS + 0.04% BSA (General Buffer)

15.10 Filter each sample with a 40um Flowmi (Fisher Scientific, #H13680-0040) into new 1.5 mL Eppendorf Lobind tubes.

15.11 Determine the nuclei concentration and quality with a hemacytometer. Do not use an automated cell counter! Use a manual microscope.

Your nuclei should look like this:



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