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Tissue dissociation and 10x Multiome for fetal heart tissue (Version 3) V.3

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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
	Dounce Tissue Grinder, 15mL	D9938-1SET	Millipore Sigma Aldrich
	Flowmi (40-um cell strainer)	H13680-0040	Fisher Scientific
	Flowmi (70-um cell strainer)	H13680-0070	Fisher Scientific
	100-um cell strainer	43-50100-51	Pluriselect
	60% Iodixanol	D1556-250ML	Millipore Sigma Aldrich
	Nonidet P40 Substitute (NP40)	74385	Millipore Sigma Aldrich
	PBS, pH 7.4	10010023	Thermo Fisher Scientific
	Protector RNase inhibitor	3335399001	Millipore Sigma Aldrich
	Ribolock RNase inhibitor	EO0382	Thermo Fisher Scientific
	Spermidine	S2501-1G	Millipore Sigma Aldrich
	Spermine	S3256-1G	Millipore Sigma Aldrich
	Sucrose	S7903-250G	Millipore Sigma Aldrich
	UltraPure Water	10977015	Thermo Fisher Scientific
	Complete Protease Inhibitors	11836170001	Millipore Sigma Aldrich
	DTT (1000 mM)	646563	Millipore Sigma Aldrich
	10% Tween-20	1662404	BioRad
	1M KCl	60142-100ML-F	Millipore Sigma Aldrich
	1M KOH	P5958-250G	Millipore Sigma Aldrich
	1M MgCl2	AM9530G	Thermo Fisher Scientific
	1M Tris-HCl (pH 7.4)	T2194	Millipore Sigma Aldrich
	5M NaCl	59222C	Millipore Sigma Aldrich
	BSA	A3311-10G	Millipore Sigma Aldrich
	Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units, 500mL	5660020	Thermo Fisher Scientific
	Eppendorf 1.5 ml tubes (DNA/RNA LoBind Tubes)	022431021	Eppendorf

Introduction

1 This experimental protocol is designed to process and analyze human (fetal and adult) and mouse heart tissues using single-cell multiome techniques to explore gene expression and chromatin accessibility at the single-cell level. The process begins with mechanical homogenization of flash-frozen heart tissue, followed by the isolation and purification of nuclei through density gradient centrifugation. These nuclei are then prepared for single-cell multiome library construction using the Chromium Single Cell Multiome ATAC + Gene Expression kit. By using 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 **Stable Solutions (prepare and store)**

Maintain at 4 °C . Filter using a 0.22 um PVDF filter system. This is stable at 4°C for at least **6 months**.

Sucrose (1M) Stock

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol.
	Sucrose (powder)	--	1000	--	102.69 g
	UltraPure Water	--	--	--	235.5 ml
				total volume	**300 ml**

1.034X Homogenization Buffer (Nuclei Lysis)

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)
	Sucrose	1 M	0.26	3.85	52
	KCl	2 M	0.03	66.67	3
	MgCl2	1 M	0.01	100	2
	Tricine-KOH pH 7.8	0.75 M	0.02	37.5	5
	UltraPure water	--	--	--	138
				total volume	**200 mL**

Diluent Buffer

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)
	KCl	2 M	0.15	13.33	7.5
	MgCl2	1 M	0.03	33.33	3
	Tricine-KOH pH 7.8	0.75 M	0.12	6.25	16
	UltraPure water	--	--	--	73.5
				total volume	**100 mL**

50% Iodixanol Solution

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)
	Diluent Buffer	--	1	--	8.3
	60% Iodixanol	60%	50%	1.2	41.7
				total volume	**50 mL**

ATAC-RSB Buffer

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)
	Tris-HCl ph 7.5	1 M	0.01	100	5
	NaCl	5 M	0.01	500	1
	MgCl2	1 M	0.003	333.33	1.5
	Water	--	--	--	492.5
				total volume	**500**

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.

1X Homogenization Buffer (Unstable Solution)

cOmplete Protease Inhibitors come as tablets. It is difficult to use less than ½ tablet.

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Volume (mL)	Volume (uL)
	1.034x HB Stable Soln	1.0341	1	1.03	30.3	30,292
	DTT	1 M	0.001	1000	0.035	35
	Spermidine	500 mM	0.5	1000	0.035	35
	Spermine	150 mM	0.15	1000	0.035	35
	NP40	10%	0.3%	33.33	1.05	1,050
	Ribolock RNase Inhibitor	40U/ul	60U/ml	667	0.052	52
	BSA	10%	1%	10	3.5	3,500
	cOmplete Protease Inhibitor	--	--	--	1 tablet	1 tablet
				total volume	**35 mL**	**35,000 uL**

30% Iodixanol Solution

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	1X Homogenization Buffer (unstable)	--	--	--	2.016	2,016
	50% Iodixanol	50%	30%	1.67	3.018	3,018
				total volume	**5.034 mL**	**5,034 uL**

40% Iodixanol Solution

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	1X Homogenization Buffer	--	--	**--**	1.008	1,008

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	(unstable)					
	50% Iodixanol	50%	40%	1.25	4.032	4,032
				total volume	**5.04 mL**	**5,040 uL**

ATAC-RSB-Tween Buffer

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	ATAC-RSB	--	--	--	3.46	3,460
	Tween-20	10%	0.1%	100	0.040	40
	BSA	10%	1%	10	0.400	400
	RNase Inhibitor	40 U/µL	1 U/µL	40	0.100	100
				Total Volume	**4 mL**	**4,000 uL**

3 **Diluted Nuclei Buffer**

Use this buffer to dilute nuclei for **10X Genomics Chromium** loading.

Note: Nuclei Buffer (20X) is from the 10X Genomics kit.

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (uL)
	DTT	1 M	1 mM	1000	0.4
	Nuclei Buffer (20X)	20X	1X	20	20
	Protector RNase Inhibitor	40 U/uL	1 U/uL	40	10
	Water	--	--	--	369.60
				Total Volume	**400 uL**

Experimental Preparation

4 Prepare stock solutions (stable) ahead of time. They can be stored at 4 °C for 6 months, except 50% iodixanol, which needs to be refreshed every month.

5 Prepare fresh solutions the same day as the experiment.

6 Clean materials with RNase Away and 70% ethanol, use filtered+sterile wide-bore tips, and use Eppendorf Lobind tubes. Maintain samples and solutions ice-cold during the whole experiment. When handling nuclei, use P1000 wide-bore tips. Use P200 wide-bore tips for smaller volumes.

7 Fill up a 2L beaker with 500mL sterile water, which will be used to soak dirty dounces and pestles.

8 Pre-chill swinging bucket centrifuges to 4 °C . Pre-chill all dounces, pestles, and tubes to 4 °C in a fridge.

Experiment: Adding the Tissue to the Dounce Homogenizer 5m

9 Remove samples from cold storage and keep on dry ice until use.

10 Add 4 mL 1x Homogenization Buffer to every dounce you plan to use. For this protocol, we use the 15mL capacity dounce, which you find under **Materials**. Make sure the lysis buffer is cold before adding the flash frozen tissue.

11 Shave and cut the tissue in a petri dish that is resting on top of an ice block that's in dry ice. Add around 20-100 mg of flash frozen tissue to its respective dounce. It is recommended to work with one dounce at a time.

12 Once the tissue is added to the dounce - Leave for 00:05:00 so the tissue can thaw slowly. A good sign to look for is if the tissue sunk to the bottom of the homogenizer. 5m

13 **Tissue-to-Nuclei Homogenization**

When working with heart tissue (human [adult, fetal] or mouse), you may need to adjust the douncing ratio (loose or tight pestle) based on the tissue type and amount.

Avoid grinding the tissue against the dounce. Instead, focus on gently pressing it with each pass. Keep the pestle below the liquid surface to minimize bubble formation. Begin with the loose pestle, as it is more gentle, and assess whether further use of the tight

pestle is necessary. If the loose pestle sufficiently breaks down the tissue, the tight pestle may not be required.

13.1 Consider using this ratio range for each pestle:

10-20 times with pestle A (loose) and repeat **10-20 times** with pestle B (tight).

13.2 Place pestles in a bucket of sterile water to soak until properly cleaned.
If you're running several conditions: clean the dounce with ethanol and then distilled water (i.e. UltraPure, Thermo Fisher Scientific #5660020), maintain extracted samples on-ice and repeat steps above until finished collecting all conditions.

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

13.4 Add 400 µL 1x Homogenization Buffer to each dounce homogenizer to rescue the rest of the nuclei, then add the volume to the same 100um filter.

13.5 Gently pipette each sample using a 1000uL tip and filter them using a 70um Flowmi strainer (Fisher Scientific, #H13680-0070) to a new 5ml low-binding tube. Filter bit by bit, not the entire sample all at once (~400-500uL at a time).

13.6 Centrifuge samples: 350 x g, 4°C, 00:05:00 5m

13.7 Remove all but 50 µL of supernatant (containing cytoplasmic RNAs). If the pellet is not clearly visible, then leave more supernatant in the tube. Gently resuspend the nuclei in a total volume of 800 µL 1x Homogenization Buffer . Note: You should add 750 µL if you have 50 µL of supernatant.

Experiment: Density Gradient 15m

14 **Tip**: To avoid mixing of layers, wipe the side of the pipette tip with a Kimwipe to remove excess Iodixanol solution from the external surfaces of the pipette tip. Keep the tube straight/upright/not tilted.

Add 800 µL 50% Iodixanol Solution to each sample (total volume will be 1600 µL). Gently pipette **5 times** to mix, ensuring thorough mixture. This is now a

25% nuclei mixture.

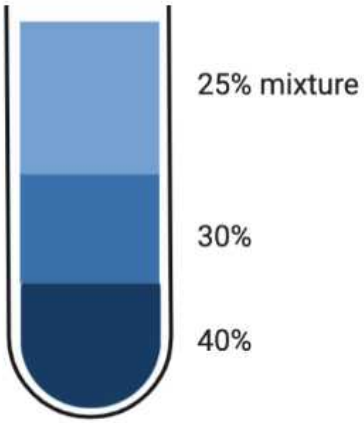
Calculation: (800ul)(50%) / (1,600ul) = 25%

15 Slowly layer 1000 µL 30% Iodixanol Solution **under** the 25% mixture. Do not eject into the 25% layer as you bring the tip out.

16 Slowly layer 1000 µL 40% Iodixanol Solution **under** the 30% mixture.

Important: During this step, you will need to gradually draw your pipette tip up to avoid overflowing the tube. However, the tip of your pipette must stay below the 30%-40% interface at all times. Do not eject into the layers as you bring the tip out.

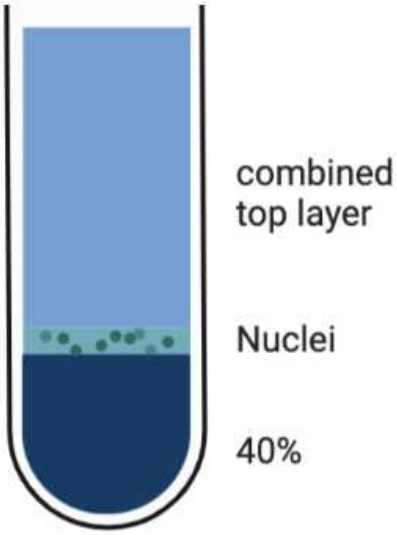
Your layers should look like this:



17 Handling the tubes gently - Place them in a pre-chilled **swinging bucket** centrifuge. Centrifuge samples: 3500 x g, 4°C, 00:15:00 with the brake off (deceleration 0, acceleration 5). 15m

This step should take around **30-40 minutes**.

Your layers should look like this:



18 Using a vacuum, aspirate the top layers down to within 200-300uL of the nuclei band at the middle interface (see previous drawing). Be careful not to disrupt the nuclei band.

19 Using a P200, collect the nuclei band and transfer to a fresh 1.5mL Eppendorf LoBind tube. Do not aspirate more than 200ul at this step as this can cause you to take too much of the 40% layer which sometimes contains debris.

20 Wash the nuclei in the 1.5mL Eppendorf LoBind tube in a total volume of
1 mL 1x Homogenization Buffer

Note: High concentrations of iodixanol can be too viscous for hemocytometers.

21 Centrifuge samples: 1200 x g, 4°C, 00:05:00 5m

It is highly recommended that you use a **swinging bucket** centrifuge.

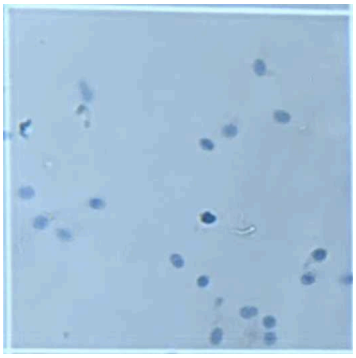
22 Remove the wash buffer and add 700 µL ATAC-RSB-Tween Buffer to every sample.

Note: You may need less volume depending on your tissue input (mg) and cell pellet.

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

24 Check nuclei using Trypan blue staining (1:1 dilution of 5ul sample to 5ul Trypan) and a manual hemocytometer. Do not use automated cell counters.

Your nuclei should look like this:



If you still have debris, then filter samples with the 40um Flowmi strainer (Fisher Scientific #H13680-0040).

The nuclei are now ready for downstream applications.

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25
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