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Tissue dissociation and 10x Multiome for fetal heart tissue (Version 2) V.2

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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
	UltraPure Water	10977015	Thermo Fisher Scientific
	DTT (1000 mM)	646563	Millipore Sigma Aldrich
	Spermidine	S2501-1G	Millipore Sigma Aldrich
	Spermine	S3256-1G	Millipore Sigma Aldrich
	Nonidet P40 Substitute (NP40)	74385	Millipore Sigma Aldrich
	1M Tris-HCl (pH 7.4)	T2194	Millipore Sigma Aldrich
	5M NaCl	59222C	Millipore Sigma Aldrich
	1M MgCl2	AM9530G	Thermo Fisher Scientific
	BSA	A3311-10G	Millipore Sigma Aldrich
	Protector RNase inhibitor	3335399001	Millipore Sigma Aldrich
	10% Tween-20	1662404	BioRad
	Digitonin	BN2006	Thermo Fisher Scientific
	PBS, pH 7.4	10010023	Thermo Fisher Scientific
	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
	Eppendorf 1.5 ml tubes (DNA/RNA LoBind Tubes)	22431021	Eppendorf

Introduction

1 This experimental protocol is designed to process and analyze human fetal 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, which involves 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 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.

Protocol version 2 was the first protocol developed specifically for isolating nuclei from flash-frozen tissue samples. While it retains the same buffer compositions as protocol version 1, the tissue dissociation step (douncing) was adapted from the method described by Corces et al. (Science, 2018). This version provides a fast and efficient way to dissociate tissue into nuclei, making it particularly suitable for early-stage samples (<9 PCW). If nuclei retention is a concern, especially when working with small or limited tissue samples, protocol version 2 is recommended as an initial approach before considering the more complex steps in protocol version 3.

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.

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

0.5x Homogenization Buffer (for nuclei lysis step)

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	Nuclear-Free Water	--	--	--	14.707	14,707
	DTT	1M	1 mM	1000	0.17	170
	Spermidine	500 mM	0.5 mM	1000	0.17	170
	Spermine	150 mM	0.15 mM	1000	0.17	170

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	NP40	10%	0.10%	100	0.085	85
	Tris-HCl (pH 7.4)	1M	10 mM	100	0.17	170
	NaCl	5M	10 mM	500	0.034	34
	MgCl2	1M	3 mM	333.3	0.051	51
	BSA	10%	1%	10	1.7	1,700
	RNase Inhibitor	40 U/uL	1 U/uL	40	0.100	100
	Tween-20	10%	0.10%	100	0.085	85
	Digitonin	5%	0.01%	500	0.017	17
				Total Volume	**17.5 mL**	**17,459 uL**

Nuclei Wash Buffer

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (mL)	Total Vol. (uL)
	PBS (1X)	100%	--	--	19.92	19,920
	MgCl2	1M	3 mM	333.3	0.060	60
	BSA	10%	0.01%	1000	0.020	20
				Total Volume	**20 mL**	**20,000 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	1M	1mM	1000	0.8
	Nuclei Buffer (20X)	20X	1X	20	40
	Protector RNase Inhibitor	40 U/uL	1 U/uL	40	20

	Reagent	Stock Conc.	Final Conc.	Fold Dilution	Total Vol. (uL)
	Nuclease-free water	--	--	--	740
				Total Volume	800uL

Experimental Preparation

4 Prepare fresh solutions the same day as the experiment.

5 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.

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

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

Experiment

5m

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

9 Add 4 mL 0.5x 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.

10 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.

11 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

12 **Tissue-to-Nuclei Homogenization**

When working with heart tissue (human fetal), 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.

12.1 Consider using this ratio range for each pestle:

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

12.2 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 Incubate sample(s) for 5 minutes on ice.

14 Transfer samples to individual 15ml conicals. Keep the samples separate if they are not being mixed right away.

15 Centrifuge samples: 20 x g, 4°C, 00:01:00 1m

16 Collect the supernatant (contains the nuclei) into new 15mL conicals.

17 Add enough Nuclei Wash Buffer to top off each conical to 8 mL total.

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

19 Transfer filtered samples to individual 15ml conicals.

20 Centrifuge samples: 400 x g, 4°C, 00:05:00 5m

21 Remove the supernatant from every tube.

22 Wash the nuclei in 4 mL of Nuclei Wash Buffer.

23 Centrifuge samples: 400 x g, 4°C, 00:05:00 5m

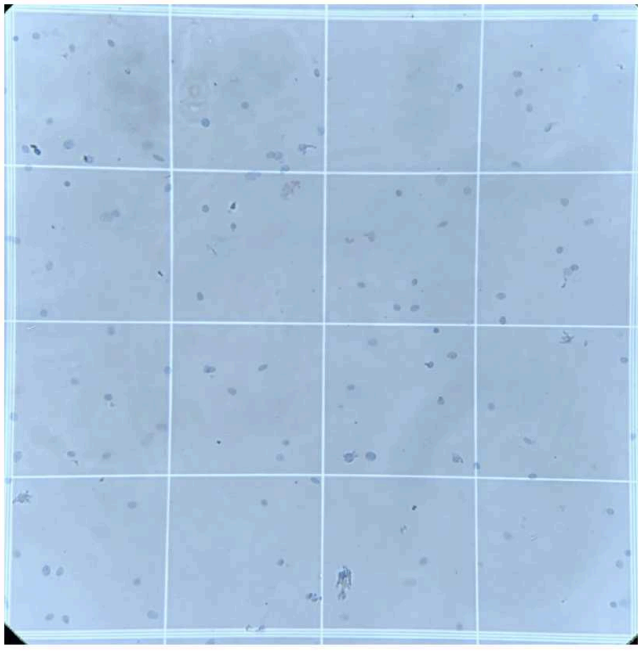
24 Remove the supernatant from every tube.

25 Add 700 µL of Nuclei Wash Buffer to each sample.

26 Gently pipette each sample using a 1000uL tip and filter them using a 70um Flowmi strainer (Fisher Scientific, #H13680-0070) to a new 1.5ml low-binding tube. Filter bit by bit, not the entire sample all at once (~400-500uL at a time).

27 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.

28 Your nuclei should not have clumps. You should see round, intact nuclei like the example below:



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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29

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