Single Cell and Single Nuclei Analysis Human Heart Tissue

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Human Cell Atlas Method Development Community

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ABSTRACT

This protocol provides a walk through for complex processing of fresh human cardiac tissue followed by tissue dissociation for single cell RNA sequencing and nuclei isolation for single nuclei RNA sequencing.

The protocol can be used for various regions of the heart muscle, including the ventricles, atria and septum. Each section looks slightly different and will contain different amount of tissue, meaning that the number of applications and the amount of stored tissue will vary.

The protocol is also applicable to animal cardiac tissue with minor modifications adjusting for size and amount of the tissue.

PROTOCOL STATUS

Working

We use this protocol to process fresh cardiac tissues in our group and it is working

MATERIALS TEXT

Equipment:

Centrifuge for Falcon tubes and eppendorf tubes
Ultracentrifuge for Falcon tubes
Water bath
Light microscope
Countess II automatic cell counter

Tools:

Dissection tools (forceps, small sharp scissors, scalpel)
Round cell culture petri dish, 6cm diameter
Eppendorf tubes, 1.5ml and 2ml
Falcon tubes, 15ml and 50ml,
High speed falcon tubes, 50 ml
Serological pipettes, 5ml and 10ml
Cell strainers, Falcon, 40µm, 70µm and 100µm
Syringe, 1ml
96-well plate

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Hemocytometer
Dounce homogenizer, 7ml
Counting slides for Countess II

Chemicals and Solutions:

Nuclei isolation buffer 1 (**NIM1**) Make in advance, store up to 6 months at 4°C.		
Component	Volume [µl]	Final Concentration [mM]
1.5M Sucrose	2 500	250
2M KCl	187.5	25
1M MgCl2	75	5
1M Tris HCL, pH 8	150	10
NFW	12 087.5	-
Total	15 000	-
Nuclei isolation buffer 2 (**NIM2**) Prepare fresh, keep on ice.		
Component	Volume [µl]	Final Concentration
NIM1	4 895	-
1mM DTT	5	1µM
50x Protease Inhibitor	100	1x
Total	5 000	-
Homogenization Buffer (**HB**) Prepare fresh, keep on ice.		
Component	Volume [µl]	Final Concentration
NIM2	**4 850**	-
40U/µl RNaseIn	50	0.4U/µl
20U/µl SuperaseIn	50	0.2U/µl
10% (v/v) Triton X-100	50	0.10%
Total	5 000	-
Storage Buffer (**SB**) Prepare fresh, keep on ice.		
Component	Amount	Final Concentration
PBS (--)	1 492.5 µl	-
BSA	60 mg	4%
40U/µl Protector RNaseIn	7.5 µl	0.2U/µl
Total	1 500 µl	

Buffers for Nuclei Isolation

	Volume [µl]	Final Concentration
Liberase TL (5 mg/ml)	**150**	**0.5 mg/ml**
DNase I (2 U/µl)	**30**	**0.04 U/µl**
Hepes (1 M)	**15**	**1 mM**
DMEM	**1 305**	**-**
Total	**1 500**	

Additional Chemicals:
Isopentane
Liquid Nitorgen
OCT
Formalin cup (4% paraformaldehyde)

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Paraffin
DPBS, Calcium and Magnesium free (Gibco, 14190136)
Bovine Serum Albumin (Thermo Fisher Scientific, AM2616)
10X PBS
FBS
Percoll
Trypan Blue
NucBlue
Chromium kit (10X Genomics)

BEFORE STARTING

Prepare all the buffers and necessary consumables.
Prepare the dissection tools (scalpel, forceps, scissors) and cool them on ice.
Prepare a container with liquid nitrogen.
Prepare a container filled with isopentane and cool down to -80C using dry ice.
Cool down the centrifuges to 4C.
Switch on a water bath and warm up to 37C.

Pre-processing

1 Transfer the tissue into a petri dish with 1ml of the dPBS. Carefully clean off the fat without damaging the pericardium layer.

2 Determine the orientation of the tissue for sectioning.
In the ventricular tissue, the outer layer, pericardium, is very stiff, light-pink to white with patches of fat. The middle layer, myocardium, and the inner layer, endocardium, are dark red and softer.
The atrial tissue is generally more stiff and much thinner with fat deposited on the outside.

3 Proceed to the sectioning (Step 4 and 5).
Afterwards mince the rest of the tissue into small cubes, approximatelly 2 mm large to minimize spatial bias.
Separate about 200mg for Tissue Dissociation (Step 6).
Split the rest of the tissue into approximatelly 200mg portions, place into cryotubes and flash-freeze by placing into liquid nitrogen. Store the portions in -80C until further processing.
Select one of those portions for Nuclei Isolation (Step 16).

Sectioning for Spatial Transcriptomics

4 Choose an area with clean cross section throught all the layers of the tissue. The final section should have dimensions of max 7mm x 7mm and depth of 2-3mm.
Place the section into a mold (pericardium facing up) and lower into the pre-cooled isopentane.
Once frozen, cover the tissue in OCT and leave on dry ice for OCT to freeze.
Store the filled mold at -80°C until sectioning.

Sectioning for Immunohistochemistry

5 Choose an area with clean cross section throught all the layers of the tissue. The final section should have dimensions of max 7mm x 7mm and depth of 2-3mm.
Place the section into a formalin cup for 16-24h for fixation. After that gradually dehydrate the section into ethanol and store frozen at -20C.
Embed the tissue into parafin and store until sectioning.

Tissue Dissociation

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6 Transfer the 200mg of the minced tissue into 1.5ml epi filled with the digestion buffer using scalpel. Minimize the amount of transferred dPBS. Seal the epi with parafilm.

7 Place the epi into a waterbath and digest for 15 minutes at 37°C with agitation. Every 5 minutes remove from the waterbath and gently shake by hand.

8 After the digestion is finished, place the epi on ice and triturate using a 5ml pipette. Pipette up and down 5-10 times.

9 Filter the solution through 100µm cell strainer. Using a 1ml plunger gently rub the tissue through the strainer. Wash the strainer with 8.5ml of DMEM to final volume of 10ml cell suspension.

10 If the sample will be applied to Chromium controller, filter the solution using 70µm strainer. Wash the strainer with another 5ml of DMEM.

11 Centrifuge at 300 x g, 4°C for 5 min.

12 Discard the supernatant and wash the pellet with 10ml of dPBS + 2%FBS. 1. Add 2.86ml of Percoll solution to create 27% Percoll-cell solution. Transfer the solution to a high speed Falcon tube and prepare the balance tube.

13 Centrifuge at 15 000 x g for 20min at 4°C, with break option set to 4 (or slowed down).

14 Collect the cell-cloud using a 1ml pipette. Wash the cells in dPBS + 0.04% BSA. Resuspend in 100ul of dPBS + 0.04% BSA.

15 Count the cells and asses the cell morphology with microscopy. Asses the cell viability using Trypan Blue. Adjust the concentration and proceed with the 10X protocol.

Nuclei Isolation

16 Pick up the minced frozen tissue from the storage and keep on dry ice until processing.

17 Transfer the tissue pieces into a precooled Dounce homogenizer (7ml) and add 3 ml of HB. Homogenize with 8 strokes of the loose pestle and 8 strokes of the tight pestle. Keep on ice to avoid heating. If homogenization seems incomplete, add strokes (dependent on the amount of tissue).

18 Filter the homogenized tissue through 40µl cell strainer into a 50ml tube. Wash the homogenizer with 2x 1ml of the rest of the HB.

19 Centrifuge at 500xg for 5 min at 4 degrees to get nuclei pellet. Remove the supernatant entirely.

20 Re-suspend the nuclei pellet in 1ml of SB. Stain the suspension with 2 drops of NucBlue. Wait for 10 min for staining to take place.

21 Proceed to FACS sorting into an eppi with 200µl of SB. Set the gates to select for DAPI stained nuclei.

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Exclude particles smaller than 1µm to remove small debris and damamged nuclei. Exclude doublets and unregular-shaped debris.

22 Centrifuge the sorted nuclei at 500xg for 5 min at 4 degrees to get a pellet. Remove the supernatant entirely. Re-suspend in 100µl of SB.

23 Count the nuclei 4x using Countess and Trypan Blue. Adjust the concentration and proceed to loading to the 10X chip.

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