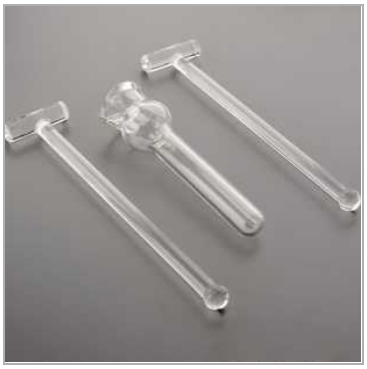

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Protocol status: Working
We use this protocol and it's working

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Nuclei isolation from frozen tissue

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Human Cell Atlas Method Development Community

Emily Stephenson

DISCLAIMER

Number of homogenisation strokes will be tissue specific as well as cell lysis, this may need to be optimised per tissue.

ABSTRACT

This protocol describes a method for the isolation of intact nuclei for further downstream methods such as single nuclei RNA-seq, ATAC-seq or 10x Multiome.

The protocol is adapted from Nadelmann et al doi: 10.1002/cpz1.132

MATERIALS

2M KCL
1M MgCL2
1M Tris buffer
Nuclease free water
1mM DTT
100x protease inhibitor
RNasin plus 40 U/ul
Superasin 20 U/ul
Triton X-100 10%
1x PBS
BSA powder
RNasin protector
Lymphoprep
10x PBS
Dounce homogeniser with pestle A and B
40uM cell strainer
Wide bore pipette tips
Trypan blue
Eppendorf tubes
C-chip haemocytometer

BEFORE START INSTRUCTIONS

Tissue can be collected before starting and kept on dry ice. Frozen tissue as small as a grain of rice can be used or OCT-embedded tissue sections.

Prepare reagents and buffers

1 Prepare nuclei isolation buffer 1 (NIM1) according to the table below. NIM1 can be made, filtered and stored at 4°C for 6 months. Record date on top of tube.

	Reagent	Volume/amount	Final conc. (mM)
	Sucrose 342.3 g/mol	4.279g	250
	2M KCL	625ul	25
	1M MgCl2	250ul	5
	1MM Tris buffer	500ul	10

	Reagent	Volume/amount	Final conc. (mM)
	Nuclease free water	48.625ml	-
	Total	50ml	

2 Prepare nuclei isolation buffer 2 (NIM2) according to the table below. NIM2 must be made on the day of experiment and kept on ice. Approx. 5mL per sample will be needed.

- Pre dilute 1M DTT in 4.995ml water
- For 100X protease inhibitor stock, dissolve 1 tablet in 500ul nuclease free water. Stock solution can be kept at 4°C for 2 weeks or 12 weeks at -20°C (record date on top of tube).

	Reagent	Volume	Final conc.
	NIM1	4946ul	1x
	1mM DTT	5ul	1uM
	100x protease inhibitor	50ul	1x
	Total	5mL	

3 Prepare homogenisation buffer (HB) according to the table below. HB must be made on the day of experiment and kept on ice. Do not vortex. Approx. 5mL per sample will be needed.

	Reagent	Volume	Final conc.
	NIM2	4850ul	1x
	RNasin plus 40 U/ul	50ul	0.4U.ul
	Superasin 20 U/ul	50ul	0.2U/ul
	Triton X-100 10%	50ul	0.1%
	Total	5ml	

4 Prepare wash buffer (WB) according to the table below. WB must be made on the day of experiment and kept on ice. Do not vortex. Make approx. 1ml per sample, more may be needed if performing extra washes or clean-up steps.

	Reagent	Volume	Final conc.

	Reagent	Volume	Final conc.
	1x PBS	975ul	1x
	BSA powder	0.05g	5%
	Rnasin protector	25ul	40U/ul
	Total	1ml	

5 Optional step: make up lymphoprep solution according to table below if samples have a lot of debris that need to be removed. Keep on ice.

	Reagent	Volume	Final conc.
	Lymphoprep	1350ul	90%
	10x PBS	150ul	1x
	Total	1500ul	

Nuclei isolation

6 Collect tissue or tissue sections on dry ice and transfer to the dounce homogeniser

7 Add 3mL homogenisation buffer to the tissue.

7.1 If using tissue embedded in OCT, leave for 5 mins on ice. Mix half way and use pestle A to push OCT to the bottom of the homogeniser. If OCT still remains, keep mixing until dissolved.

8 On ice, gently homogenise the tissue using pestle A. Up to 20 strokes may be needed until no

more resistance is felt.

9 Rinse pestle with 500mL homogenisation buffer.

10 On ice, gently homogenise the tissue using pestle B. Up to 20 strokes may be needed until no more resistance is felt.

11 Rinse pestle with 500mL homogenisation buffer.

12 Pour homogenate through a 40um cell strainer into a 50mL Falcon tube on ice. Rinse homogeniser with 500mL homogenisation buffer. Transfer any droplets underneath the filter with a pipette.

13 Centrifuge the tube at 500 x g for 6 mins at 4°C. Acc 0, dec 3.

14 Carefully remove the supernatant with 1mL pipette and transfer to another tube, do not throw away.

15 Add 500mL wash buffer and leave for 2 mins on ice.

16 Resuspend nuclei with a wide bore tip and transfer to 1.5mL eppendorf tube.

17 Look at nuclei underneath microscope using filtered trypan blue and C-Chip. If there appears to be a lot of debris, either pass nuclei through a 40mM FlowMi filter or perform a clean-up (steps at the end of protocol), if not proceed to count.

18 Nuclei will stain blue with trypan. Count nuclei in all four corners then use the following calculation: (count/4) x 2 (trypan dilution factor) x 10 (volume factor) = conc. (nuclei per mL)

Multiply by 500 to get total nuclei. Record this number as the “unclean count”.

19 Centrifuge the tube at 500 x g for 3 mins at 4°C.

20 If unclean count is more than 500k nuclei, remove as much supernatant as possible without disturbing pellet and wash again with 500ml of wash buffer. If count is less than 500k, remove supernatant and leave 10-50mL in tube depending on count (use tube of water as a guide). Do not throw away supernatant.

21 Resuspend pellet gently and count nuclei using filtered trypan blue and C-Chip. May need to do a 1 in 10 dilution using the supernatant as diluent. Count nuclei in all four corners then use the following calculation:
(count/4) x 2 (trypan dilution factor) x 10 (volume factor) x 10 (dilution factor) = conc. (nuclei per mL)

Multiply by volume to get total nuclei. Record this number as the “clean count”.

22 Proceed with next protocol such as 10x Genomics Multiome or scRNA-seq.

Optional clean-up to remove debris

23 Add 475ml wash buffer to 2 Eppendorf tubes (not LoBind) for each sample.

24 Add 22ul nuclei solution followed by 300ul 90% lymphoprep to each tube. Mix by inverting (do not pipette mix).

25 Centrifuge sample at 20,000 x g for 15mins at 4°C.

26 Remove top 500-800mL layer and add 500mL wash buffer. Be careful not to disrupt the nuclei ‘cloud’ which will hopefully be visible near the bottom of the tube, but may not be seen as a pellet. Mix by pipetting using a wide bore tip.

27 Proceed to step 19 in original protocol.