Nuclei Preparation from Frozen Tissue for 10X Multiome using Dounce Homogenization, Iodixanol Gradient Centrifugation, and FANS

Reagents List:

Reagent	Concentration	Vendor	Catalog Number
Sucrose	-	Sigma	S1888-500G
KCl	1M	Invitrogen	AM9640G
$MgCl_2$	1M	Invitrogen	AM9530G
Tris-HCl, pH 7.5	1M	Invitrogen	15567-027
Tris-HCl, pH 8.0	1M	Invitrogen	15568-025
DTT (DL-Dithiothreitol)	-	Sigma	D9779-10G
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets	-	Sigma	5056489001
Recombinant RNasin (Ribonuclease Inhibitor), 10000 U	-	Promega	N2515
Molecular biology water	-	Corning	46-000-CV
IGEPAL CA-630	-	Sigma	I8896-50ML
Tween-20	10%	BioRad	1662404
NaCl	5M	Invitrogen	AM9760G
OptiPrep Density Gradient Medium (Iodixanol)	-	Sigma	D1556-250ML

Fatty acid-free BSA	-	Lampire Biological Laboratories	7500804
7-AAD	-	Invitrogen	A1310
PBS	-	Corning	21-040-CV
Trypan Blue	0.4%	Invitrogen	T10282

Equipment:

- Sony Cell Sorter (SH800)
- Eppendorf tabletop swing-bucket centrifuge (Eppendorf, 5920R)

Consumables

- Wheaton Dounce Tissue Grinder, 1 mL (DWK Life Sciences, 357538)
- Sony Sorting Chip-100 µm for SH800 and MA900 (Sony, LEC3210)
- Thermo Scientific™ NERL™ Diluent 2 Hematology Reagent for Flow Cytometry (Fisher Scientific, 23-029-361)
- 30 µm CellTrics (Fisher Scientific, NC9682496)
- 1.5 mL Lo Bind Centrifuge tubes (Eppendorf, 022431021)
- 5 mL Eppendorf DNA LoBind tubes (Eppendorf, 0030108310)
- Thermo Scientific™ SoftFit-L™ Filtered Pipette Tips in Hinged Racks, 200 µL (Fisher Scientific, 21-402-561)
- Thermo Scientific™ SoftFit-L™ Filtered Pipette Tips in Hinged Racks, 20 µL (Fisher Scientific, 21-402-550)
- xTIP4™ Racked Pipette Tips, Rainin® LTS® Pipette Compatible, Biotix, 1000 µL (Fisher Scientific, 76266-146)
- Olympus Plastics 0.2 mL 8-Strip PCR Tubes, Flex Free Individual Attached Flat Caps (Genesee Scientific, 27-125U)
- Serological Pipets, 10 mL, Sterile, Individually Wrapped (Genesee Scientific, 12-104)

Reagent preparation:

1. Prepare stock Diluent Buffer (1 mL) and 50% iodixanol (6 mL) at room temperature, if needed.

Diluent Buffer			
Reagent	**Stock Concentration**	**Final Concentration**	**1 mL**
Tris-HCl, pH 8	1 M	120 mM	120 µL
KCl	2 M	150 mM	75 µL
$MgCl_2$	1 M	30 mM	30 µL

Molecular biology water	-	-	775 µL

50% Iodixanol			
Reagent	**Stock Concentration**	**Final Concentration**	**6 mL**
OptiPrep Density Gradient Medium	60%	50%	5 mL
Diluent Buffer			1 mL

2. Prepare all other buffers fresh on ice.

NIM			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**
Sucrose in water	1M	0.25M	1 mL
KCl	2M	25 mM	50 µl
$MgCl_2$	1M	5 mM	20 µl
Tris-HCl, pH 7.5	1M	10 mM	40 µl
Molecular biology water	-	-	4 mL
TOTAL	-	-	5.114 mL

NIM-DP			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**
NIM buffer	1X	1X	4 mL
DTT in water	200 mM	1 mM	20 µl
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	160 µl
Recombinant RNasin	40 U/µL	1 U/ µl	100 µl
TOTAL	-	-	4.28 mL

NIM-DP-L			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**

NIM-DP	-	-	1.1 mL
IGEPAL CA-630	10%	0.1%	11 µl

20% Iodixanol			
Reagent	**Stock Concentration**	**Final Concentration**	**2 mL**
OptiPrep Density Gradient Medium	50%	20%	800 µL
NIM	-	-	1.2 mL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 µL
DTT in water	200 mM	1mM	10 µL
Recombinant RNasin	40 U/µL	1 U/µL	50 µL

25% Iodixanol			
Reagent	**Stock Concentration**	**Final Concentration**	**1 mL**
OptiPrep Density Gradient Medium	50%	25%	500 µL
NIM	-	-	500 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	40 µL
DTT in water	200 mM	1 mM	5 µL
Recombinant RNasin	40 U/µL	1 U/µL	25 µL

Sort Buffer (SB)			
Reagent	**Stock Concentration**	**Final Concentration**	**For 4 samples**
Fatty acid-free BSA in PBS	10%	1%	200 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 µL
7-AAD (10% in DMSO)	1 mM	2 µM	4 µL
Recombinant RNasin	40 U/µL	1 U/µL	50 µL

PBS	-	-	1666 µL
TOTAL	-	-	2000 µL

Collection Buffer (CB)			
Reagent	**Stock Concentration**	**Final Concentration**	**For 4 samples**
Fatty acid-free BSA in PBS	10%	5%	200 µL
Recombinant RNasin	40 U/µL	5 U/µL	50 µL
PBS	-	-	150 µL
TOTAL	-	-	400 µL

Nuclear Permeabilization Buffer (NPB)			
Reagent	**Stock Concentration**	**Final Concentration**	**1 mL**
Fatty acid-free BSA in PBS	-	5%	50 mg
IGEPAL-CA630	10%	0.2%	2 µL
DTT	200 mM	1 mM	5 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	40 µL
Recombinant RNasin	40 U/µL	1 U/µL	25 µL
PBS			928 µL

Wash Buffer (WB)			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**
Fatty acid-free BSA in PBS	10%	1%	200 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 µL
Tris-HCl, pH 7.5	1M	10mM	20 µL
DTT	200 mM	1 mM	10 µL
$MgCl_2$	1M	3mM	6 µL
NaCl	5M	10mM	4 µL
Tween-20	10%	0.01%	2 µL

Recombinant RNasin	40 U/µL	1 U/µL	50 µL
Molecular biology water	-	-	1628 µL

Nuclei Preparation

1. Pre-chill a large, swing-bucket tabletop centrifuge to 4°C.

2. Retrieve a 1 mL dounce homogenizer with 2 pestles (“Loose” and “Tight”) for each sample. Place on ice and allow to chill.

3. Add 1 mL of NIM-DP-L buffer to each dounce homogenizer.
   ****If tissue mass is very small (< 50 mg), instead add 600 µL of NIM-DP-L.**

4. Transfer each sample to a dounce homogenizer.

5. Using the loose pestle, gently homogenize the sample until most of the tissue has broken into small pieces (usually 5-10 strokes).

6. Switch to the tight pestle and homogenize until the solution is uniform with no obvious particles (usually 15-25 strokes). Be gentle and avoid introducing bubbles.

7. Transfer the full volume of homogenized sample to a 30 µm filter in a 1.5 mL Eppendorf Lobind tube.

8. Rinse each dounce with 1 mL of NIM-DP buffer and transfer the rinse to the filter.

9. Centrifuge for 10 mins at 1000 rcf and 4°C.

   ****If tissue mass is very small (< 50 mg), skip steps 10-11****

10. Discard the supernatant and resuspend the pellet in 1 mL of NIM-DP.

11. Centrifuge for 10 mins at 1000 rcf and 4°C.

12. Discard the supernatant and resuspend the pelleted nuclei in 2 mL of 20% iodixanol.
    ****If tissue mass is very small (< 50 mg), instead resuspend in 400 uL of 50% iodixanol (for a final iodixanol concentration of 20%).**

13. Slowly pipette the suspension dropwise onto a 500 µL cushion of 25% iodixanol in a 5 mL Eppendorf Lobind tube. ****Do not mix this solution once transferred.****

14. Centrifuge for 30 mins at 4000 rcf and 4°C.

15. Discard the supernatant, leaving a small volume (< 20 µL) to avoid disturbing the pellet.

16. Resuspend the pellet in 500 µL of sort buffer and incubate on ice for 10 mins, protected from light.

17. Sort 120,000-130,000 nuclei into a 1.5 mL Eppendorf Lobind tube containing 90 µL of collection buffer.

18. Centrifuge the sorted nuclei for 5 mins at 500 rcf and 4°C.

19. Discard the supernatant.

20. Resuspend the pellet in 100 µL of NPB. Incubate on ice for 1 minute.

21. Add 900 µL of wash buffer.

22. Centrifuge for 5 mins at 500 rcf and 4°C.

23. Carefully remove the supernatant, leaving 10-15 µL to avoid disturbing the pellet.

24. Gently resuspend in 12 µL of 1X Nuclei Buffer (prepared from 10X Genomics protocol).

25. Stain an aliquot of nuclei with 0.4% Trypan Blue. Load 10 µL into one chamber of a hemocytometer.

26. Count nuclei in four quadrants. Average the count and determine the nuclei concentration (nuclei/µL).

27. Capture images from the microscope field at 10X and 20X magnification.

28. Follow the 10X Genomics protocol **"Chromium Next GEM Single Cell Multiome ATAC + Gene Expression" (CG000338, Rev F)** for the remainder of the experiment. Input 18,000 nuclei for each tagmentation reaction for a targeted recovery of ~10,000 nuclei.