Nuclei preparation from frozen tissue for Chromium Single Cell Multiome ATAC + Gene Expression (10x Genomics)

A. Reagents and consumables:

Prepare buffers fresh and leave on ice.

NIM (Can be stored at 4C)			
Reagent	**Stock c**	**Final c**	**for 5 mL**
Sucrose (S1888, Sigma)	1M	0.25M	1.25 mL
KCl (AM9640G, Invitrogen)	2M	25 mM	62.5 µl
$MgCl_2$ (194698, Mp Biomedicals Inc)	1M	5 mM	25 µl
Tris-HCl, pH 7.5 (15567027, Thermos Fischer Scientific)	1M	10 mM	50 µl
Molecular biology water (46000-CM, Corning)	-	-	3.613 ml

NIM-DP (Make fresh)			
Reagent	**Stock c**	**Final c**	**For 2 mL**
NIM buffer	1X	1X	1.9 ml
DTT (D9779, Sigma)	200 mM	1 mM	10 µl
Pierce Protease Inhibitor	50X	1X	40 µl
Recombinant RNAsin (Promega, PAN2515)	40 U/µl	1.0 U/µl	50 µl

NIM-DP-L			
Reagent	**Stock c**	**Final c**	**for 1 mL**
NIM-DP	1X	1X	990 µl
Triton X-100 (Sigma, T8787-100ML)	10% (in water)	0.1%	10 µl

Sort buffer (SB) (Can be stored at 4C without RNasin or protease inh)			
Reagent	**Stock c**	**Final c**	**5 samples** (500uL each)
EDTA (Invitrogen, 15575020)	500 mM	1 mM	5 µl
Recombinant RNAsin (Promega, PAN2515)	40 U/ul	1 U/ul	62.5 µl
Pierce Protease Inhibitor	50X	1X	50 µl
Fatty acid free BSA in PBS	10%	1%	250 uL
PBS	-	-	2.133 mL

Collection buffer (CB) (Can be stored at 4C without RNasin or protease inh)			
Reagent	**Stock c**	**Final c**	**5 samples** (87.5 uL each)
Recombinant RNAsin (Promega, PAN2515)	40 U/µl	5 U/µl	54.7 µl
Pierce Protease Inhibitor	50X	1X	8.75 µl
Fatty acid free BSA in PBS	10%	5%	218.75 µl
PBS	-	-	155.3 µl

5X OMNI (Permeabilization buffer) (Make fresh)			
Reagent	**Stock c**	**Final c**	**for 200 µL**
Tris-HCl (pH 7.4) (15567027, Thermos Fischer Scientific)	1M	50 mM	10 µL
NaCl (Fischer, S271-3)	5M	50 mM	2 µl
$MgCl_2$ (194698, Mp Biomedicals Inc)	1M	15 mM	3 µl
Tween-20 (Sigma, P7949-100ML)	10%	0.05%	1 µl
IGEPAL (Sigma, I8896)	10%	0.05%	1 µl
Digitonin (Promega, G9441)	2%	0.005%	0.5 µl
Fatty acid free BSA in PBS	10%	5%	100 µl
DTT (D9779, Sigma)	200mM	5mM	5 µl
Recombinant RNAsin (Promega, PAN2515)	40 U/µl	1U/µl	5 µl
Pierce Protease Inhibitor	50X	5X	20 µl
Molecular biology water (46000-CM, Corning)	-	-	52.5 µl

Wash Buffer (Make fresh)			
Reagent	**Stock c**	**Final c**	**For 2 mL**
Tris-HCl (pH 7.4) (15567027, Thermo Fischer Scientific)	1 M	10 mM	20 µL
NaCl (Fischer, S271-3)	5 M	10 mM	4 µl
$MgCl_2$ (194698, Mp Biomedicals Inc)	1 M	3 mM	6 µl
Tween-20 (Sigma, P7949-100ML)	10%	0.1%	20 µl
Fatty acid free BSA in PBS	10%	1%	200 µl
DTT (D9779, Sigma)	200 mM	1 mM	10 µl
Recombinant RNAsin (Promega, PAN2515)	40 U/µl	1 U/µl	50 µl
Pierce Protease Inhibitor	50X	1X	40 µl
Molecular biology water (46000-CM, Corning)	-	-	1.65 ml

1X Nuclei Buffer (Make fresh)			
Reagent	**Stock c**	**Final c**	**100 uL**
20X Nuclei Buffer (10X Genomics kit)	20X	1X	5 µl
DTT (D9779, Sigma)	200 mM	1 mM	0.5 µl
Recombinant RNAsin (Promega, PAN2515)	40 U/µl	1 U/µl	2.5 µl
Molecular biology water (46000-CM, Corning)	-	-	92 µl

- 7-AAD (Invitrogen, A1310)
- Sony Cell Sorter 100 µM Chip (Sony, LE-B3001)
- Sony Cell Sorter (Sony, SH800S)
- Eppendorf centrifuge (Eppendorf, 5920 R)
- 30 µm CellTrics (Sysmex, 04-0042-2316)
- 1 ml Dounce Tissue Grinder (Wheaton, 357538)
- 1.5 ml LoBind tubes (Eppendorf, 22431021)

B. Nuclei preparation

1. Prechill any tubes, buffers or tools. Set the centrifuge to 4˚C.
2. For each sample to be homogenized, prepare a Dounce Tissue Grinder with 2 pestles (“Loose” and “Tight”). Remove from 70% ethanol storage, rinse with MilliQ water three times. Place mortars (buckets) in ice. Dry pestles with kimwipe and place on clean parafilm on top of ice to chill.
3. Remove excessive water collected at the bottom of mortars with p1000.
4. Add 990 µL of NIM-DP and 10 µL of Triton X-100 to each bucket to make 1mL of NIM-DP-L. Pipette mix.
5. Tip tissue into mortar or resuspend tissue in 1 ml of NIM-DP-L buffer and transfer to a Dounce homogenizer.
6. Be gentle and avoid introducing bubbles. Homogenize the sample by using the Loose pestle (usually 5-10 strokes) followed by the tight pestle (usually 15-25). Switch pestles when most of the tissue has been broken up into small pieces and use the tight pestle to homogenize until the solution is uniform (no obvious particles).
7. Filter using a 30 µm CellTrics filter into LoBind tube.
8. Centrifuge nuclei (1000 rcf, 10 min, 4˚C).
9. Discard the supernatant and gently resuspend pellet in 1 mL of NIM-DP. Centrifuge nuclei (1000 rcf, 10 min, 4˚C).
10. Add 7-AAD to Sort Buffer (1:1000) to a final concentration of 2 µM.

11. Discard supernatant from pelleted nuclei. Gently resuspend pellet in 400 µL of sort buffer + 7-AAD by mixing with a p1000 pipette ~5 times or until no clumps are visible.
12. Sort 120,000 nuclei into a LoBind tube containing 87.5 µL of collection buffer.
13. Measure the total volume of the sorted nuclei in the collection buffer via reverse pipetting.
14. Add the appropriate amount of 5X permeabilization buffer for a final concentration of 1X, and gently pipette mix with p1000 5 times.
15. Incubate on ice for 1 minute, then centrifuge (500 rcf, 5 min, 4˚C).
16. Slowly remove the supernatant until 20-30 µL remains in the tube (Do not disturb pellet).
17. Slowly add 650 uL of Wash Buffer along wall of tube (try not to disturb pellet) and immediately centrifuge (500 rcf, 5 min, 4˚C).
18. Prepare tubes for counting nuclei on hemocytometer by adding 7 µL of 1X Nuclei Buffer to each tube for a 1:16 dilution.
19. Very carefully remove the supernatant, switching to a p20 pipette once the supernatant volume is <20 µL. Leave ~1 µL to avoid disturbing the pellet.
20. Add 7 µL of 1X Nuclei Buffer and very gently resuspend pellet 4 times. Immediately take 1 µL and add to tube for counting containing 7 µL of 1X Nuclei Buffer.
21. Count nuclei by stain with 8 µL Trypan Blue (Invitrogen, T10282), count on a hemocytometer and record images from the microscope field. For calculating nuclei concentration of original stock multiply count average x 10 x 16 (dilution factor).
22. Refer to Chromium Single Cell Multiome ATAC + Gene Expression protocol (10x Genomics) Step 1. Load 18 - 20K nuclei per tagmentation.