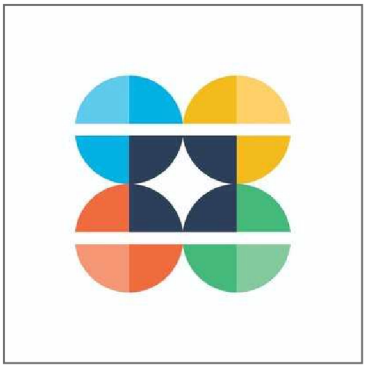

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Protocol status: Working
We use this protocol and it's working

Nuclei Preparation from Frozen Tissue for 10X Multiome using Dounce Homogenization, Iodixanol Gradient Centrifugation, and FANS (v1.2, Jan 2024)

ereisner[1]

[1]Center for Epigenomics

Cellular Senescence Network (SenNet) Method Development Community

SenNet TMC-University of California San Diego

1 more workspace

ereisner
Center for Epigenomics, University of California, San Diego

ABSTRACT

This protocol describes isolation of nuclei from frozen tissue using dounce homogenization, iodixanol gradient centrifugation, and FANS. Nuclei are permeabilized, washed, and counted; single-nucleus suspensions of sufficient concentration and nuclei quality may then be processed using the Chromium Next GEM Single Cell Multiome ATAC + Gene Expression (CG000338, Rev F) protocol from 10X Genomics.

ATTACHMENTS

Nuclei Preparation from Frozen Tissue for 10X Multiome using Dounce Homogenization, Iodixanol Gradient Centrifugation, and FANS, v1.2, Jan 2024.pdf

Created: Feb 24, 2024

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PROTOCOL integer ID: 95698

Keywords: Multiome, Dounce homogenization, Gradient Centrifugation, FANS, Nuclei Isolation

MATERIALS

Reagents List:

	A	B	C	D
	Reagent	**Concentration**	**Vendor**	**Catalog Number**
	Sucrose	-	Sigma	S1888-500G
	KCl	1M	Invitrogen	AM9640G
	MgCl2	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.

	A	B	C	D
	A	B	C	D
	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
	MgCl2	1 M	30 mM	30 µL
	Molecular biology water	-	-	775 µL

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

2 On ice Prepare all other buffers fresh on ice.

	A	B	C	D
	NIM			
	Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**
	Sucrose in water	1M	0.25M	1 mL

A	B	C	D
KCl	2M	25 mM	50 µl
MgCl2	1M	5 mM	20 µl
Tris-HCl, pH 7.5	1M	10 mM	40 µl
Molecular biology water	-	-	4 mL
TOTAL	-	-	5.114 mL

A	B	C	D
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

A	B	C	D
NIM-DP-L			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**
NIM-DP	-	-	1.1 mL
IGEPAL CA-630	10%	0.1%	11 µl

A	B	C	D
20% Iodixanol			

A	B	C	D
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	1 mM	10 µL
Recombinant RNasin	40 U/µL	1 U/µL	50 µL

A	B	C	D
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

A	B	C	D
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

A	B	C	D
7-AAD (in DMSO)	1 mM	2 µM	4 µL
Recombinant RNasin	40 U/µL	1 U/µL	50 µL
PBS	-	-	1666 µL
TOTAL	-	-	2000 µL

A	B	C	D
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

A	B	C	D
Nuclear Permeabilization Buffer (NPB)			
Reagent	**Stock Concentration**	**Final Concentration**	**For 4 samples**
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

	A	B	C	D
	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	10 mM	20 µL
	DTT	200 mM	1 mM	10 µL
	MgCl2	1M	3 mM	6 µL
	NaCl	5M	10 mM	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

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

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

5 Add 1 mL of NIM-DP-L buffer to each dounce homogenizer.

5.1 ****If tissue mass is very small (< 50 mg), instead add 600 µL of NIM-DP-L.**

6 Transfer each sample to a dounce homogenizer.

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

8 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.

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

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

11 Centrifuge for 10 mins at 1000 rcf, 4°C, and 3/3 acceleration/deceleration.

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

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

13 Centrifuge for 10 mins at 1000 rcf, 4°C, and 3/3 acceleration/deceleration.

14 Discard the supernatant and resuspend the pelleted nuclei in 2 mL of 20% iodixanol.

14.1 ****If tissue mass is very small (< 50 mg), instead resuspend in 400 uL of 50% iodixanol (for a final iodixanol concentration of 20%).**

15 **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.****

16 Centrifuge for 30 mins at 4000 rcf, 4C, and **3/1 acceleration/deceleration**.

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

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

19 Sort 120,000-130,000 nuclei into a 1.5 mL Eppendorf Lobind tube containing 90 µL of collection buffer using a Sony SH800 Cell Sorter.

20 Centrifuge the sorted nuclei for 5 mins at 500 rcf, 4°C, and 3/3 acceleration/deceleration.

21 Discard the supernatant.

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

23 Add 900 µL of wash buffer.

24 Centrifuge for 5 mins at 500 rcf, 4°C, and 3/3 acceleration/deceleration.

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

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

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

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

29 Capture images from the microscope field at 10X and 20X magnification. Assess nuclei quality.

30 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.