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



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Protocol status: Working

We use this protocol and it's working

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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 f...

648KB

Materials

Reagents List:

A	B	C	D
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

	A	B	C	D
	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
	MgCl ₂	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

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🧊 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
	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

	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



A	B	C	D
Recombinant RNasin	40 U/ μ L	1 U/ μ l	100 μ l
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 μ l

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



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

A	B	C	D
Sort Buffer (SB)			
Reagent	Stock Concentration	Final Concentration	For 4 samples
Fatty acid-free BSA in PBS	10%	1%	200 μ L
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 μ L
7-AAD (in DMSO)	1 mM	2 μ M	4 μ L
Recombinant RNasin	40 U/ μ L	1 U/ μ L	50 μ L
PBS	-	-	1666 μ L
TOTAL	-	-	2000 μ L



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

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 μ L
DTT	200 mM	1 mM	5 μ L
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	40 μ L
Recombinant RNasin	40 U/ μ L	1 U/ μ L	25 μ L
PBS			928 μ L



A	B	C	D
Wash Buffer (WB)			
Reagent	Stock Concentration	Final Concentration	Volume per Sample
Fatty acid-free BSA in PBS	10%	1%	200 μ L
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 μ L
Tris-HCl, pH 7.5	1M	10 mM	20 μ L
DTT	200 mM	1 mM	10 μ L
MgCl ₂	1M	3 mM	6 μ L
NaCl	5M	10 mM	4 μ L
Tween-20	10%	0.01%	2 μ L
Recombinant RNasin	40 U/ μ L	1 U/ μ L	50 μ L
Molecular biology water	-	-	1628 μ L

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 μ L 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 μ m 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 μ L of 50% iodixanol (for a final iodixanol concentration of 20%).**
- 15 **Slowly** pipette the suspension dropwise onto a 500 μ L 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 ($< 20 \mu\text{L}$) to avoid disturbing the pellet.
- 18 Resuspend the pellet in $500 \mu\text{L}$ 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 $90 \mu\text{L}$ 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 $100 \mu\text{L}$ of NPB. Incubate on ice for 1 minute.
- 23 Add $900 \mu\text{L}$ of wash buffer.
- 24 Centrifuge for 5 mins at 500 rcf, 4°C , and 3/3 acceleration/deceleration.
- 25 Carefully remove the supernatant, leaving $10\text{-}15 \mu\text{L}$ to avoid disturbing the pellet.
- 26 Gently resuspend in $12 \mu\text{L}$ of 1X Nuclei Buffer (prepared from 10X Genomics Multiome protocol).
- 27 Stain an aliquot of nuclei with 0.4% Trypan Blue. Load $10 \mu\text{L}$ 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.