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Nuclei Isolation Protocol Multiome 10X Genomics

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We use this protocol and it's working

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Abstract

Nuclei were isolated from frozen rat brain tissue for Chromium Next GEM Single Cell Multiome ATAC + Gene Expression assay (10 X Genomics). The protocol allowed us to obtain good quality individual nuclei with intact nuclei membrane. The isolated nuclei were assessed for transposition reaction followed by GEM (Gel beads-in-emulsion) generation and barcoding in order to simultaneously profile epigenetic landscape and gene expression in the same nuclei. The protocol is optimized to prevent wetting failure due to nuclei clogging during GEM generation step and to reduce detection of ambient RNA.

Guidelines

Procedural guidelines

- Use RNaseZap™ RNase Decontamination Solution (Cat. no. AM9786) to remove RNase contamination of working station and non-disposable items such as centrifuges and pipettes used during nuclei isolation.
- Perform all steps on ice if possible unless otherwise noted.
- Use disposable, RNase-free pipettes tips, and tubes. If possible, use broad tips as per the need to prevent nuclei damage during pipetting.
- High quality nuclei with well-preserved round morphology are optimal for single-cell multiome library preparation.

Materials

- HB Buffer with IGEPAL
- ATAC-RSB Buffer (without IGEPAL)
- Sucrose
- PBS containing 1% BSA
- ATAC permeabilization buffer (IGEPAL + Digitonin)
- Sterile water
- 70% Isopropyl alcohol
- Dounce Tissue Grinder Set
- 70 um Flomi cell strainers
- Eppendorf LoBind tubes (2 mL and 1.5 mL)
- Swinging-bucket centrifuge
- Pipettes and tips
- Ice

Safety warnings

Perform all steps on ice or at 4 ºC to preserve cell integrity.

- Perform all steps on ice or at 4 ºC to preserve cell integrity.
- Nuclei sticking together could be a sign of overlysis. Thus optimize the protocol before proceeding to GEM (Gel beads-in-emulsion) generation steps.

Ethics statement

Procedures using animals were approved by our Institutional Animal Care and Use Committee (IACUC)

Before start

Preparation: Maintain Cold Conditions: Perform all steps on ice or at 4 ºC to preserve cell integrity.

Procedures of nuclei isolation from brain tissues for 10 X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression assay

1 Sample **Preparation**: Maintain Cold Conditions: Perform all steps on ice or at 4ºC to preserve cell integrity

2 **Homogenization:** 5m

2.1 Use 450 µL of cold Homogenization buffer (HB buffer) with IGEPAL for each sample. Homogenize the tissue using a 2ml Dounce homogenizer with pestle "A" for 10-12 strokes followed by pestle "B" for 10-12 strokes, ensuring to push down and twist. 1m

3 **Incubation and Filteration:** 5m 30s

3.1 Incubate the homogenate for 2 minutes on ice. Gently mix by pipetting up and down, repeating this process two more times during the incubation period. 2m

3.2 Filter the homogenate using a 70 µm Flowmi cell strainer. 2m

3.3 Collect the filtered suspension into a pre-chilled 2 mL LoBind tube. 30s

3.4 Add 550 µL of HB buffer (without IGEPAL) and mix well before centrifugation. 30s

4 **Centrifugation:** 5m

4.1 Centrifuge at 500 RCF for 5 minutes at 4ºC to pellet nuclei. Carefully remove the supernatant, leaving behind approximately 50 µL if the pellet is not clearly visible. Keep the supernatant on ice for later steps, if quality check for nuclei loss is needed.

5 **Sucrose Cushion** 35m

5.1 Resuspend the nuclei in 1 mL of ATAC-RSB buffer (without IGEPAL).

5.2 Carefully underlay with 400 µL of ATAC-RSB buffer containing 1.5 M Sucrose.

5.3 Look for the separation of layers and then centrifuge at 5500Xg for 30 minutes at 4ºC.

6 **Nuclei permeabilization** 20m

6.1 Remove most of the supernatant, being careful not to disturb the pellet. Leave behind 50-200 µLif necessary.

6.2 Resuspend the nuclei in 450 µL of ATAC permeabilization buffer and incubate for 1 minute on ice.

6.3 Filter the suspension using a 40 µm Flowmi cell strainer into a new 1.5 mL LoBind Eppendorf tube.

6.4 Add 550 µL of PBS containing 1% BSA, centrifuge at 500 RCF for 5 minutes at 4ºC, discard the supernatant, and resuspend the nuclei in 300 µL of the same PBS solution + 1%BSA.

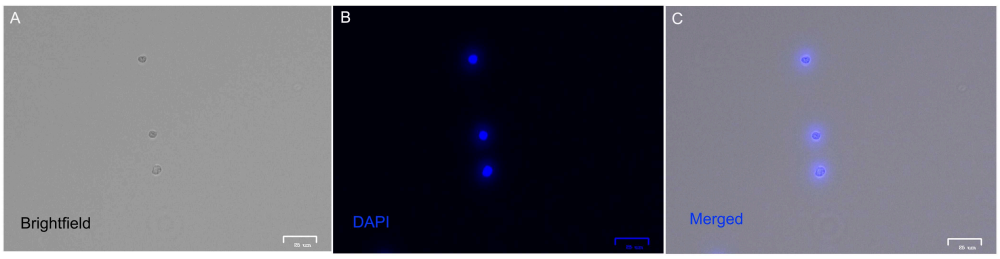
6.5 After centrifugation, remove the supernatant, leaving 10-20 µL for resuspension with 200 µL of 1X Diluted Nuclei Buffer (PN-2000207, 10X Genomics Kit).

6.6 Centrifuge at 350 RCF for 5 minutes at 4ºC.

6.7 After a final centrifugation, remove the supernatant, leaving 5-6 µL for resuspension.

7 **Nuclei Counting:** 30m
Prepare Hoechst Solution: Mix 1 µL of Hoechst stock solution (1 mg/mL in PBS) with 99 uL of PBS.

7.1 Counting: Prepare a dilution with 0.5 µL of the resuspended nuclei, 1 µL PBS + Hoechst and 8.5 µL of PBS. Place 10 µL on a counting chamber and count under a microscope, covering nine fields to calculate average and standard deviation. Take images to assess the nuclei status and to keep record.



Evaluating the quality of nuclei for single-cell multiome analysis: Nuclei with intact round morphology are regarded as high quality and are optimal for single-cell multiome library preparation. A. Brighfield image of brain nuclei, B. DAPI staining of brain nuclei, C. Merged image of brightfield and DAPI channel.

7.2 Calculation: Use the provided formula to determine the total number of nuclei. Total nuclei = (Average nuclei count in fields)* (final resuspension volume of nuclei * 10). 12,000-14,000 nuclei are loaded during GEM generation step in order to obtain a target of 6,000 nuclei for further analysis.

Stock Buffers and Solutions:

30m

8

Preparation: All stock solutions should be filtered using a 0.22 µm PVDF filter system. Stock solutions are stable at 4°C for at least 6 months.

	A	B	C	D	E
	1.034x Homogenization Buffer Stable Solution	For 200 ml stock solution			
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (µl)*
	1 M	Sucrose	0.26 M	3.87	51706.50
	2 M	KCl	0.03 M	77.36	2585.33
	1 M	MgCl2	0.01 M	193.40	1034.13
	0.75 M	Tricine-KOH pH 7.8	0.02 M	36.26	5515.36

	A	B	C	D	E
	-	Water	-	-	139158.69
	Total Volume (µL)				200000.00

	A	B	C	D	E
	Stable ATAC-RSB Buffer	For 500 mL stock solution			
	Stock	*Name*	Final Conc.	*Fold Dilution (x)*	*Total Vol. (µl)*
	1 M	Tris-HCl pH 7.5	0.01 M	100.00	5000.00
	5 M	NaCl	0.01 M	500.00	1000.00
	1 M	MgCl2	0.003 M	333.33	1500.00
	-	Water	-	-	492500.00
		Total Vol. (µL)	500000.00		

	A	B	C	D	E	F
	HB unstable buffer (without IGEPAL) [Prepare 2.2 mL per 1-2 sample (500 µLw/o IGEPAL +500 µL with IGEPAL 10% extra]					

	A	B	C	D	E	F
	Reagents	Stock conc.	Final conc	1-2X	1-2X (10% extra)	4X
	HB stable solution	1.0341X	1	1954	2149.4	4298.8
	DTT	1 M	1 mM	2	2.2	4.4
	Spermidine	0.5 M	0.5 mM	2	2.2	4.4
	Spermine	0.15 M	0.15 mM	2	2.2	4.4
	Pierce Proteinase Inhibitor	50X	1X	40	44	88
	Total			2000 µL	2200 µL	4000 µL

Note: Add RNase protector (40 U/ µL) at the last step. 12.5 µL per mL of buffer.

	A	B	C	D	E	F	G
	HB unstable buffer (with IGEPAL) (calculation is done for 10% extra reagent/sample)						
	Reagents	Stock conc.	Final conc	1X	2X	3X	4X
	HB unstable solution (w/o IGEPAL)	1.0341X	1	544.5 µL	1089	1633.5	2178
	IGEPAL	10%	0.1%	5.5 µL	11	16.5	22
	Total			550 µL	1100	1650	2200

Note: Add RNase protector (40 U/ µL) at the last step. 12.5 µL per ml of buffer.

	A	B	C	D	E	F	G
	Preparation of 1.5 M sucrose in ATAC RSB buffer (without IGEPAL): For 4 samples, prepare in total volume of 2 mL						
	Reagents	Stock conc.	Final conc	1X	2X	3X	4X
	ATAC RSB buffer (w/o IGEPAL)	1.0341X	1	500 µL	1000 µL	1500 µL	2000 µL
	Sucrose		1.5 M	0.256 gm	0.513 gm	0.768 gm	1.0269
	Total			500 µL	1000 µL	1500 µL	2000 µL

Note:
1 . Vortex for 2 min in order to dissolve sucrose in ATAC RSB buffer. After no crystals are visible, place the sucrose solution in ice.
2. Add RNase protector (40 U/ µL) at the last step. 12.5 µL per ml of sucrose solution.

	A	B	C	D
	ATAC permeabilization buffer			
		Stock Conc.	Final conc.	**For 4 samples**
	ATAC-RSB			2994 µL
	IGEPAL	10%	0.01%	2 µL

	A	B	C	D
	Tween	10%	0.01%	2 µL
	Digitonin	5%	0.001%	0.4 µL
	DTT	1000 mM	1 mM	2 µL
	TOTAL			2000 µL

Note: 1. Add RNase protector (40 U/ µL) at the last step. 12.5 µL per ml of buffer.

	A	B	C	D
	1X Diluted Nuclei Buffer			
	Diluted Nuclei Buffer: Maintain at 4 degree Celcius	Stock	Final	1 mL
	20X Nuclei Buffer (PN-2000207)	20X	1X	50 uL
	DTT	1,000 mM	1 mM	1 uL
	RNase Inhibitor (Confirm vendor-specific stock concentration)	40 U/ µL	1 U/ µL	25 µL
	Nuclease-free Water	-	-	924 µL

Reagent Order Lists

9 **Order List**

	A	B	C
	Item	**Supplier**	**Cat Number**

	A	B	C
	Eppendorf 2 mL Lo-Bind tubes	Sigma	Z666556-250EA
	Eppendorf 1.5 mL Lo-Bind tubes	Sigma	Z666548-250EA
	Nunc cryovials	Thermo	375418PK
	Iodixanol (comes at 60%)	Sigma	D1556-250ML
	Sucrose	Sigma	S7903-250G
	NP40	Roche (Sigma)	11332473001
	Tricine	Sigma	T0377-25G
	Potassium Hydroxide (KOH)	Sigma	P5958-250G
	Complete Protease Inhibitors	Roche	11697498001
	MgCl2	Ambion (Thermo)	AM9530G
	KCl	Ambion (Thermo)	AM9640G
	DTT	Thermo	R0861
	Spermidine	Sigma	S2501
	Spermine	Sigma	S3256-1G
	70 µm Flowmi cell strainers	Fisher	03-421-228
	70 µm bucket-style cell strainers	BD Falcon	352350

	A	B	C
	Tris-HCl pH 7.5	Invitrogen	15567-027
	NaCl	Ambion (Thermo)	AM9759
	Tween 20	Roche (Sigma)	11332465001
	H2O	Invitrogen	10977-015
	Dounce Tissue Grinder Set	Sigma	D8938-1SET
	INCYTO Disposable hemocytometers	Fisher	22-600-100
	BAM Banker	Wako Chemicals	302-14681
	RiboLock	Thermo	EO0384
	0.22 µm PVDF Filter Units (500 ml)	Millipore	SCGVU05RE
	0.22 µm PVDF Filter Units (50 ml)	Millipore	SE1M179M6
	RNase inhibitor	Roche	03335402001
	RNaseZap TM RNase Decontamination solution	Invitrogen	AM9786