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Nuclei isolation from fresh frozen tissues for 10X Single Cell Multiome ATAC + Gene Expression Sequencing and other technologies

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dGTEx Single Cell Proto... Multiome

dGTEx Protocols
Broad Institute of MIT and Harvard

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We use this protocol and it's working

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Abstract

This protocol is designed for isolating nuclei from fresh frozen tissue samples ranging from 6 mg to 30 mg. It has been primarily used to generate 10X Single Cell Multiome ATAC + Gene Expression sequencing data across 21 different tissue types (see the guidelines and warnings section for details). For some tissues, debris removal is performed using a continuous density gradient—following the Daughter of Frankenstein protocol—instead of FACS sorting.
The protocol consistently delivers strong quality control (QC) metrics, including RNA metrics such as genes per cell and UMIs per cell, and ATAC metrics like TSS enrichment ratio and high-quality fragment fractions. Beyond 10X 3' v3.1 single-cell RNA sequencing, nuclei isolated using this method have also been successfully applied to other single-cell platforms, such as Fluent BioSciences PIPseq and Scale Biosciences RNA sequencing. This protocol is compatible with a wide range of downstream technologies.

Guidelines

21 Fresh frozen tissues optimized by this protocol

	Tissues	Density Gradient	Protocol Deviation
1	Adipose - Subcutaneous	No	
2	Adrenal Gland	No	
3	Artery - Aorta	Yes	2% BSA was used in the PBS + BSA buffer
4	Colon - Sigmoid	No	
5	Colon - Transverse	No	
6	Esophagus - Gastroesophageal Junction	No	
7	Esophagus - Mucosa	No	
8	Esophagus - Muscularis	No	
9	Heart - Atrial Appendage	No	
10	Heart - Left Ventricle	No	
11	Liver	No	
12	Lung	Yes	
13	Muscle - Skeletal	No	
14	Nerve - Tibial	No	
15	Ovary	No	
16	Pancreas	No	RNAse Inhibitor quantity was doubled
17	Prostate	No	
18	Skin - Not Sun Exposed (Suprapubic)	No	
19	Skin - Sun Exposed (Lower leg)	No	
20	Small Intestine - Terminal Ileum	No	
21	Stomach	No	

List of fresh frozen tissues for which the 10X Multiome data was generated using this protocol. Density gradient centrifugation is only necessary for certain tissues. Protocol deviation for aorta and pancreas are also listed in the table.

Note: Before applying this protocol to a new tissue type, optimize it using the provided buffers (without RNase inhibitors). After completing **Section I**, assess debris by staining the nuclei with **Propidium Iodide**. If significant debris is observed, proceed to the **density gradient centrifugation** step; if not, proceed directly to **Section III**.

Materials

Reagents:

- Nuclei Buffer 20X **10x Genomics** **Catalog #**2000153/2000207
- Digitonin (5%) **Thermo Fisher** **Catalog #**BN2006
- Trizma Hydrochloride Solution pH 7.4 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T2194
- Sodium Chloride Solution 5 M **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**59222C
- Magnesium Chloride Solution 1 M **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M1028
- Nonidet P40 Substitute **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #** 74385
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**3335402001
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D0632
- MACS BSA Stock Solution **Miltenyi Biotec** **Catalog #** 130-091-376
- RNase-Free Disposable Pellet Pestles, With tube **Thermo Fisher** **Catalog #**12141368
- MACS SmartStrainers (70 µm) **Miltenyi Biotec** **Catalog #**130-098-462
- 10% Tween 20 **Bio-Rad Laboratories** **Catalog #**1662404
- Sterile single-pack CellTrics™ filters 30 uM **Sysmex** **Catalog #**04-004-2326
- RNaseZap® **Thermo Scientific** **Catalog #**AM9780
- Noyes Spring Scissors - Tungsten Carbide **Fine Science Tools** **Catalog #**15514-12
- Bel-Art® Disposable Pestles **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**BAF199230001-100EA
- Filter, CellTrics ,5um,30um Diam,St(50/BX) **Sysmex**
- Optiprep (Iodixanol) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D1556-250ML
- INCYTO C-Chip™ Disposable Hemacytometers **VWR International (Avantor)** **Catalog #**82030-468

NP-40 nuclei Lysis buffer: (1.2 mL/sample)- Prepare fresh and keep on ice

	A	B	C	D	E
		Stock	**Final**	**For 2 mL (uL)**	**For 4 mL (uL)**
	Tris-HCl (pH 7.4)	1 M	10 mM	20	40
	NaCl	5 M	10 mM	4	8
	MgCl2	1 M	3 mM	6	12
	Nonidet P40**	10%	0.10%	20	40
	DTT	1000 mM	1 mM	2	4
	RNase inhibitor 40 U/µl	40 U/µl	1 U/µl	50	100
	Nuclease-free Water			1898	3796

PBS + 2% BSA + 1U/µl RNase I: (3.5 mL/sample)-Prepare fresh and keep on ice

	A	B	C	D	E
		Stock	**Final**	**For 4 mL (uL)**	**For 8 mL (uL)**
	PBS	1 M	10 mM	2700	5400
	BSA	10%	1%	800	1600

	A	B	C	D	E
	RNase inhibitor 40 U/µl	40 U/µl	1 U/µl	100	200

Wash Buffer: (1 mL/sample)-Prepare fresh and keep on ice

	A	B	C	D	E
		Stock	Final	For 1 mL (uL)	For 2 mL (uL)
	Tris-HCl (pH 7.4)	1 M	10 mM	10	20
	NaCl	5 M	10 mM	2	4
	MgCl2	1 M	3 mM	3	6
	BSA	10%	1%	100	200
	Tween-20	10%	0.10%	10	20
	DTT	1000 mM	1 mM	2	1
	RNase inhibitor	40 U/µl	1 U/µl	25	50
	Nuclease-free Water			849	1698

1X Lysis Buffer: (250uL/sample) – Make at least 500 uL -Prepare fresh and keep on ice

	A	B	C	D	E
		Stock	**Final**	**For 2 mL (uL)**	**For 0.5 (uL)**
	Tris-HCl (pH 7.4)	1 M	10 mM	20	5
	NaCl	5 M	10 mM	4	1
	MgCl2	1 M	3 mM	6	1.5
	Tween-20	10%	0.10%	20	5
	Nonidet P40**	10%	0.10%	20	5
	Digitonin*	5%	0.01	4	1
	BSA	10%	1%	200	50
	DTT	1000 mM	1 mM	2	0.5
	RNase inhibitor 40 U/µl	40 U/µl	1 U/µl	50	12.5

	A	B	C	D	E
	Nuclease-free Water			1674	418.5

Lysis Dilution buffer: (100 uL/sample) Make at least 500 uL-Prepare fresh and keep on ice

	A	B	C	D	E
		Stock	**Final**	**For 2 mL (uL)**	**For 1 mL (uL)**
	Tris-HCl (pH 7.4)	1 M	10 mM	20	10
	NaCl	5 M	10 mM	4	2
	MgCl2	1 M	3 mM	6	3
	BSA	10%	1%	200	100
	DTT	1000 mM	1 mM	2	2
	RNase inhibitor 40 U/µl	40 U/µl	1 U/µl	50	25
	Nuclease-free Water			1718	858

0.1X Lysis buffer: (100uL/sample)-Keep on ice

	A	B	C	D	E
		Stock	**Final**	**For 2 mL (uL)**	**For 0.5 mL (uL)**
	1X Lysis Buffer	1X	0.1X	200	50
	Lysis Dilution Buffer			1800	450

Diluted Nuclei Buffer

	A	B	C
		For 0.5 mL (uL)	**For 0.25 mL (uL)**
	20 X nuclei buffer	25	12.5
	DTT	0.5	0.25
	RNAseI	12.5	6.25
	diH2O	462.5	231.25

BUFFERS TO MAKE IN ADVANCE FOR DENSITY GRADIENT (Filter Sterilize GD and GH)

	A	B	C
	Stock Solutions	grams	mL diH2O
	500 mM Tricine	4.48	50
	1M KCl	3.725	50
	1 M MgCl2 X 6 H2O	10.15	50

For 2 Samples: (Add RNAse Inhibitors at 1U/uL)

		G60	GD	GW	GH
	GW	3 mL	0.6 mL	-	-
	G30	-	-	3 mL	2 mL

Safety warnings

Materials Guidelines

- Use **low-bind plastics** (e.g., 1.5 mL tubes, P200, and P1000 tips) as recommended by **10x Genomics**.
- Utilize a **swinging-bucket centrifuge** for optimal nuclei yield.

Additional Recommendations

- Maintain an **RNase-free environment** throughout the procedure.
- Keep **nuclei on ice at all times** to preserve integrity.
- **Minimize protocol duration** to improve data quality.

Before start

Previous Day Preparation

- Clean forceps and scissors with **RNaseZap**.
- Wrap the cleaned instruments in aluminum foil and store them at **-20°C overnight** in preparation for the experiment.

Day of the Experiment PreparationRNase-Free Workspace

- Wipe down the working bench and all pipettes with **RNaseZap** to maintain an RNase-free environment.

Equipment Setup

- Set the **centrifuge to 4°C**.
- Preheat the **thermomixer to 65°C** to thaw **Digitonin**.

Required Plastics per Sample

- 70 µm strainers for 15 mL conical tubes (×1)
- 30 µm **CellTrics** strainers
- 15 mL conical tubes (×1)
- 1.5 mL **low-bind, RNase-free microfuge tubes** (×3)

Ice and Dry Ice Preparation

- Prepare an **ice bucket** for buffers and tissue chopping.
- Have **dry ice** ready to retrieve tissue from the **-80°C freezer**.
- Set up the **70 µm filter** on a **15 mL conical tube** and place it on ice, ready for use just before starting.

Section I :Tissue Homogenization

1 Do not thaw the tissue before lysis; keep it on dry ice at all times.

2 Weigh the tissue to confirm the desired input weight, ensuring it remains frozen throughout the process.

3 If tissue is not already in a 1.5 mL tube, transfer to a 1.5 mL tube.

4 After weighing the tissue, proceed to Section II.

Section II: Tissue Homogenization

31m

5 Add 400 µL of NP-40 lysis buffer to the tissue on wet ice.

6 Using forceps and scissors, chop the tissue 3 to 4 times in 400 µL NP-40 lysis buffer while keeping it on ice.

6.1 **Note**: Over-chopping can lead to debris.

7 After chopping, homogenize the sample using a pellet pestle in 400 µL of NP-40 lysis buffer on ice for 00:02:00 to maximum of 00:04:00 .

6m

7.1 Keep the tube on ice at all times, only lifting it briefly to check the progress of homogenization.

8 **Note:** Homogenizing in lysis buffer for longer than 5 minutes may negatively affect ATAC data quality.

9 Once homogenization is complete, add 800 µL of NP-40 lysis buffer to the tube.

10 Mix by pipetting up and down twice using a wide-bore pipette tip (a regular-bore tip may be used if the tissue disintegrates easily).

11 Pass the suspension through a 70 µm strainer into a 15 mL conical tube kept on wet ice.

11.1 Be sure to collect the flowthrough from the bottom of the filter, avoiding contact with the base of the strainer to prevent disturbing any retained debris.

12 Collect all the flowthrough and transfer it into a new 1.5 mL tube.

13 Centrifuge at 500 x g, 4°C, 00:10:00 10m

14 Carefully remove and discard the supernatant, leaving approximately 50 µL behind. Avoid disturbing the pellet.

15 Add 1 mL PBS + 1% BSA + 1U/µl RNase Inhibitor. DO NOT MIX.

16 Incubate for 00:05:00 on ice. 5m

17 Pipette mix to re-suspend the pellet.

18 Strain with 30 µm Celltrix strainer into a new 1.5 mL microfuge tube on ice.

19 Centrifuge at 500 x g, 4°C, 00:10:00 . 10m

19.1 If performing density gradient centrifugation, Make the GW AND G30 buffers in this step.

Note: If you're optimizing the protocol to determine whether density gradient centrifugation is necessary, examine the nuclei under a microscope after the next step, using propidium iodide staining.

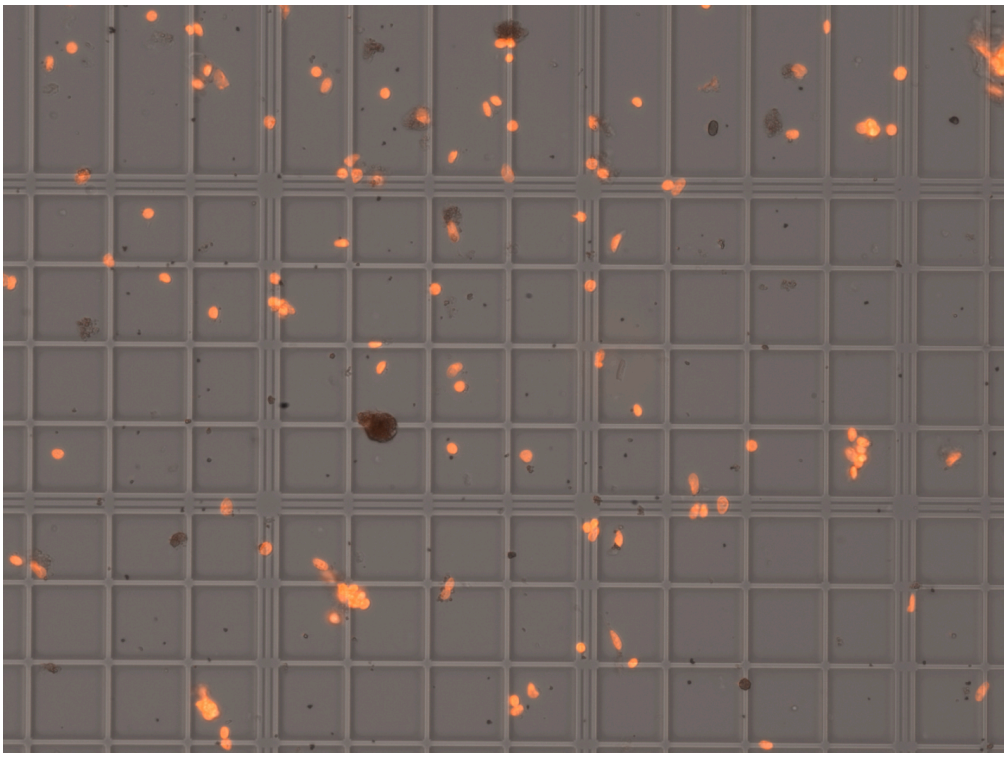
20 After centrifugation, carefully remove as much of the supernatant as possible without disturbing the pellet. If the pellet appears loose, leave approximately 50 µL behind.

21 If your nuclei preparation contains a high amount of debris, perform density gradient centrifugation.

If proceeding with density gradient centrifugation, continue to **Section II**. Otherwise, skip ahead to **Section III, step 35**.

21.1 Only perform the steps in **Section III** if your downstream assay involves profiling chromatin accessibility using a single-cell ATAC assay. Otherwise, proceed with your chosen single-cell assay after completing **Section I** or **Section II**, depending on the quality of the nuclei.

22 **Nuclei images**
- Nuclei with debris



Nuclei from Lung before density gradient 20X EVOS M7000 Imaging System

Section II : Density Gradient Centrifugation

30m

23 Add 300 µL of G30 buffer to the pellet. Mix well but gently to re-suspend the pellet.

24 Carefully underlay 1 mL of the G30 buffer beneath the sample homogenate from the previous step. A clear separation between the homogenate and the G30 buffer should form—do not disturb this interface.

25 Centrifuge the nuclei at 8000 x g, 4°C, 00:20:00 . 20m

26 After centrifugation, the nuclei will form a pellet at the bottom, while contaminating membranes will float at the top.

27 After centrifugation, carefully remove the supernatant and add 1 mL of ice-cold PBS containing 1% BSA and 1 U/µL RNase inhibitor, **without re-suspending the nuclei.**

28 Centrifuge the nuclei at 500 x g, 4°C, 00:05:00 . 5m

29 Remove as much of the supernatant as possible without disturbing the pellet. If the pellet appears loose, leave approximately 50 µL behind. Then, re-suspend the pellet in 1 mL of PBS + 1% BSA + 1U/µl RNase inhibitor.

30 Centrifuge the nuclei at 500 x g, 00:05:00 5m

31 Remove the supernatant and re-suspend the pellet in 50 µL of ice-cold PBS + 2% BSA + 1U/µl RNase inhibitor.

31.1 If optimizing the nuclei isolation protocol, proceed with nuclei imaging and counting to evaluate yield and quality.

32 **Nuclei image after density gradient**

Lung nuclei after density gradient 20X EVOS M7000 Imaging System

Section III : Permeabilization

12m

33 Add 500 µL PBS + 1% BSA + 1U/µl RNase inhibitor to the pellet.

34 Centrifuge at 500 x g, 5°C, 00:05:00 5m

35 Remove the supernatant without disrupting the nuclei pellet.

36 Re-suspend the pellet in 100 µL 0.1X Lysis Buffer and pipette mix 5 times.

37 Incubate for 00:02:00 on ice. 2m

38 Add 1 mL Wash buffer and pipette mix 5 times.

39 Centrifuge at 500 x g, 00:05:00 . 5m

40 Carefully remove the supernatant without disturbing the nuclei pellet.

41 Re-suspend the pellet in nuclei suspension buffer appropriate for the chosen downstream single cell technology.

42 Count the nuclei after staining them with a dye such as propidium iodide.

43 Proceed with 10X Single Cell Multiome ATAC + Gene Expression Sequencing, or continue with your chosen assay.

Protocol references

1. Daughter of Frankenstein protocol for nuclei isolation from fresh and frozen tissues using OptiPrep® continuous gradient V.2 **dx.doi.org/10.17504/protocols.io.bs99nh96**
2. Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing **CG000375 • Rev C**