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Nuclei Isolation from Human Subchondral Bone for 10x Multiome

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Abstract

This protocol describes isolation of nuclei from fresh-frozen human knee subchondral bone for use in Omics analyses, including RNA-sequencing of ATAC-sequencing. Tissue dissociation was the most critical step in optimization which was monitored by nuclei yield, integrity and purity. Methods for quality control are also described.

Nuclei Isolation from Human Subchondral Bone for 10x Multiome

Materials
5-ml centrifuge tubes (Eppendorf, catalog number: 0030108310)
50-ml conical centrifuge tubes (Falcon, catalog number: 352098)
Cell strainers, 40-µm (Fisher, catalog number: 22363547)
Cell strainers, 20-µm (pluriSelect, catalog number: 43-50020-03)
Cell strainers, 10-µm (pluriSelect, catalog number: 43-50010-03)
Connector Ring (pluriSelect, catalog number: 41-50000-03)
Mortar and Pestle
Liquid Nitrogen (LN)
Ladle for LN handling
Ultracentrifuge tubes (Beckman Coulter, catalog number: 344059)
RNase inhibitor (Sigma Aldrich, catalog number: 3335402001)
Recombinant Albumin solution (New England Biolabs, catalog number: B9200S)
PBS (Thermo Fisher Scientific, catalog number: 10010023)
DTT, Sigma-Aldrich, Catalog No. 646563
Tween 20 (Fisher, catalog number: BP337500)
Nonidet P40 Substitute (Sigma Aldrich, catalog number: 74385)
Acridine Orange/Propidium Iodide Stain (Logos Biosystems, catalog number: F23001)

Nuclei buffer (10× genomics)
Lysis Buffer (see Recipes)
Wash Buffer (see Recipes)
Diluent Buffer (see Recipes)
*Diluent buffer in Distilled Nuclease free water and sterile filtered before use (see Recipes)

Lysis Buffer	Stock	Final	20 ml
Tris-HCL (pH 7.4)	1 M	10 mM	200 ul
NaCl	5 M	10 mM	40 ul
MgCl2	1 M	3 mM	60 ul
Tween-20	10 %	0.01%	20 ul
Nonidet P40 Substitute	10 %	0.05%	100 ul
Recombinant Albumin	20 mg/ml	0.3 mg	300 ul
DTT	1 M	1 mM	20 ul
RNase inhibitor	40 U/uL	1 U/uL	500 ul
Nuclease-free water			18760 ul

Wash buffer	Stock	Final	20 ml
Tris-HCL (pH 7.4)	1 M	10 mM	200 ul
NaCl	5 M	10 mM	40 ul
MgCl2	1 M	3 mM	60 ul
Recombinant Albumin	20 mg/ml	0.3 mg/ml	300 ul
Tween-20	10%	0.10%	200 ul
DTT	1 M	1 mM	20 ul
RNase inhibitor	40 U/ul	1 U/ul	500 ul
Nuclease-free water			18680 ul

Diluted Nuclei Buffer	Stock	Final	1 ml (ul)
Nuclei Buffer (20X)	20X	1X	50 ul
DTT	1 M	1 mM	1 ul
RNase inhibitor	40 U/ul	1 U/ul	25 ul
Nuclease-free Water			924 ul

Nuclei Isolation Protocol Using the Singulator S200

We use fresh snapfrozen tissues stored in Liquid Nitrogen (LN).

1.1. Pre-chill mortar and pestle with LN. Place 200mg snapfrozen bone into a mortar, add LN and smash (do not grind) several times with a pestle until it is dissociated. Make sure that the tissue is in tiny fragments while trying not to grind it, as grinding creates more nuclei damage.

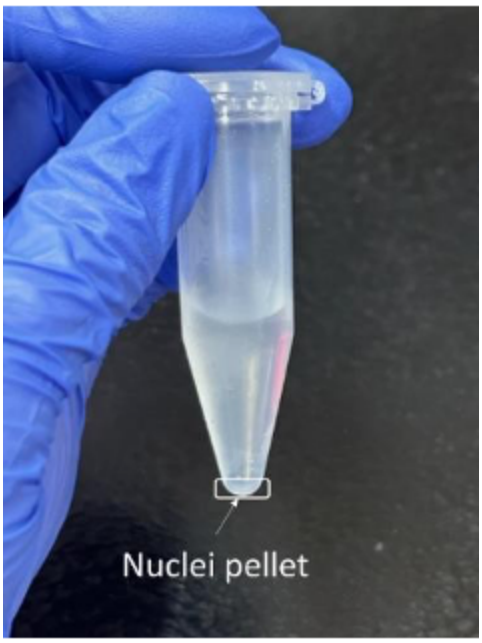
1.2. Transfer tissue fragments to a 50 ml tube with 2 ml lysis buffer and incubate for 20 min at 4°C.

1.3. After this incubation, add 2 ml wash buffer to the tube.

1.4. Pass the nuclei suspension sequentially through 40 µm, 20 µm, and 10µm strainers into a 50-ml conical centrifuge tube on ice. Do not apply negative pressure.

1.5. Transfer the flow-through to a 5 ml LoBind tube.

1.6. Centrifuge at 500 rcf for 5 min at 4°C.

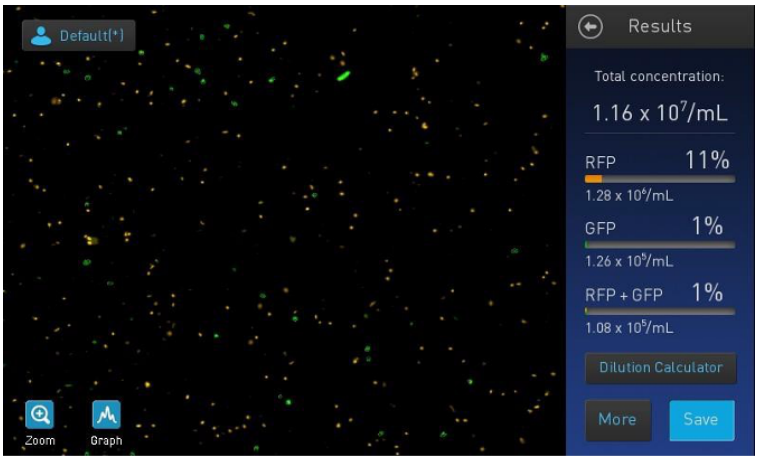


1.7. Remove supernatant carefully and resuspend in 400µL PBS + Recombinant Albumin 0.03 mg/ml + 1U/µl RNase Inhibitor.

1.8. To remove cellular debris, spin down at 4℃, 150 rfc for 10 min to pellet the nuclei and retain the small debris in the supernatant.

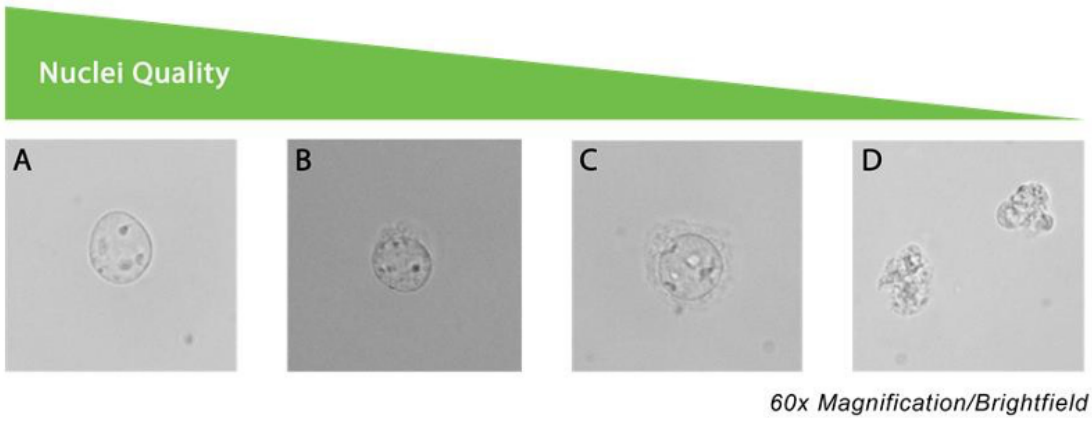
1.9. Remove supernatant and resuspend in 400µL PBS + Recombinant Albumin (0.03mg/ml) + 1U/µl RNase Inhibitor.

1.10. Count nuclei using a Countess II FL Automated Cell Counter (AOPI staining).



Example of AOPI counts: Nuclei stained in RFP (orange), live cells and debris stain in GFP (green).

1.11. Nuclei should be visualized under a microscope at 40x or 60x magnification (AOPI staining) to assess nuclear membrane integrity, according to the nuclear integrity quality check guidance by 10x (https://10xgenomics.com).



A: High-quality nuclei have well-resolved edges. Optimal quality for single-cell gene expression libraries.
B: Mostly intact nuclei with minor evidence of blebbing. Quality single-cell gene expression libraries can still be produced.
C: Nuclei with strong evidence of blebbing. Proceed at your own risk.
D: Nuclei are no longer intact. Do not proceed!

1.12. Centrifuge at 500 rcf for 5 min at 4°C and remove the supernatant without disrupting the nuclei pellet.

1.13. Based on the nuclei concentration estimated at step 1.9, resuspend nuclei pellet in chilled Diluted Nuclei Buffer to a target concentration of 4,000 – 5,000 nuclei/ul, for a target nuclei recovery of 20,000 nuclei.
* Put only 5 µL into the PCR tube

1.14. Proceed immediately to Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide (CG000338) and minimize the time between nuclei preparation and chip loading.