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## Single nuclei isolation from frozen human adipose tissue for 10x Genomics multiome sequencing

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Human Cell Atlas Method ...

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## Abstract

Here we present a modified version of a 10x Genomics demonstrated protocol that we adapted for the isolation of nuclei from human adipose tissue. Single-nuclei are isolated from 1-1.2g frozen surgery-derived biopsies of human omental or subcutaneous abdominal adipose tissue and stained with 7-AAD for FACS sorting. The sorted nuclei are permeabilized and final resuspension takes place in Nuclei Buffer supplied in the 10x Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Reagent bundle. This protocol does not describe library construction or sequencing.

## Guidelines

Work within a BSL-2 biosafety cabinet.

Keep frozen tissue in the frozen state while grinding in the mortar with a pestle.

Keep sample on ice after transfer to the conical glass grinding tube.

Use only swinging bucket rotors for centrifugation to maximize nuclei recovery.

## Materials

### Equipment

#### Thermo-Flask Benchtop Liquid Nitrogen Container 2122<sup>NAME</sup>

Thermo Scientific BRAND

11-670-25C SKU

1L capacity SPECIFICATIONS

### Equipment

#### Tissue Grinder with PTFE Pestle and Glass Tube<sup>NAME</sup>

Kimble Kontes Duall BRAND

885480-0023 SKU

15mL SPECIFICATIONS

## Equipment

## Porcelain Mortar 60319

NAME

Coorstek

BRAND

Z247480-1EA

SKU

300mL

SPECIFICATIONS

## Equipment

## Porcelain Pestle 60320

NAME

Coorstek

BRAND

Z247529-1EA

SKU

 Tris-HCl (ph7.4) Merck MilliporeSigma (Sigma-Aldrich) Catalog #T2194-100ML NaCl Promega Catalog #V4221 MgCl<sub>2</sub> Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1028-100mL Nonidet P40 Substitute G-Biosciences Catalog #DG001 14-Dithiothreitol (DTT) Merck MilliporeSigma (Sigma-Aldrich) Catalog #43816-10mL Nuclease-free water Invitrogen Catalog #10977 Protector RNase inhibitor Merck MilliporeSigma (Sigma-Aldrich) Catalog #3335402001 Dulbeccos Phosphate-Buffered Saline no calcium no magnesium (DPBS/-) Gibco - Thermo Fischer Catalog #14190-144 Bovine Serum Albumin (BSA) Merck MilliporeSigma (Sigma-Aldrich) Catalog #A7030-100G Tween-20 Bio-Rad Laboratories Catalog #1610781 Digitonin Invitrogen - Thermo Fisher Catalog #BN20061 7-ADD Ready Made Solution Merck MilliporeSigma (Sigma-Aldrich) Catalog #SML1633-1ML

## CONSUMABLES:

100um Cell Strainer (Biologix, Ref. No. 15-1100)  
40um Cell Strainer (Falcon, Ref. No. 352340)  
2mL polystyrene pipet (Fisherbrand, Ref. No.13-678-11C)  
Transfer pipet (Samco Scientific, Ref. No. 202-20S)  
50mL conical polypropylene centrifuge tube (ThermoScientific, Ref. No. 339652)  
15mL conical polypropylene centrifuge tube (Fisherbrand, Ref. No. 05-539-12)  
5mL polystyrene, round-bottom (FACS) tube (Corning Falcon, Ref. No. 352054)

## Safety warnings

- ! This protocol uses liquid nitrogen for the first grinding step.

## Before start

Read through the entire protocol before beginning. This protocol has been developed using a two-step homogenization process utilizing a porcelain mortar with a porcelain pestle, and a conical glass grinder tube with a PTFE pestle. Substitutions for this equipment will require optimization.

During the homogenization process, lipids will aggregate and form solid lumps while on ice. They will also smear along the side of the conical glass grinder tube. Since they can interfere with downstream processing and clog the strainers, we transfer only the liquid portion of the homogenate to the 100um strainer using a 2mL pipet with a large bore.

We strongly suggest testing the protocol using buffers prepared without RNase inhibitor to observe the number of nuclei resulting from the homogenization process, and the expected **volume** of nuclei sample coming off the FACS sorter, before proceeding with any experimental samples.

Precaution: for the ATAC-Seq portion of the 10x Genomics multiome analysis process, you will need to use the proprietary 20x Nuclei Buffer supplied in the "Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Reagent Bundle."

## Buffer Preparation

- 1 Prepare all the buffers listed in the tables below. Any buffers that contain RNase Inhibitor must be prepared the day of the nuclei isolation. Buffers without RNase Inhibitor may be prepared a day ahead.

### NP40 Lysis Buffer: (A)

A	B	C	D	E	F
Ingredient	Source	Ref. No.	Stock Conc.	Final Conc.	Volume for 10mL
Tris-HCl (pH 7.4)	Sigma	T2194-100ML	1 M	10 mM	100uL
NaCl	Promega	V4221	5 M	10 mM	20uL
MgCl <sub>2</sub>	Sigma	M1028-100mL	1 M	3mM	30uL
Nonidet P40 Substitute	G-Biosciences	DG001	10%	0.1%	100uL
DTT	Sigma	43816-10mL	1000 mM	1 mM	10uL
Nuclease-free water	Invitrogen	10977			9490uL
RNase inhibitor	Sigma	3335402001	40 U/uL	1U/uL	250uL

### DPBS/1% BSA/1U/uL RNase Inhibitor Buffer:

A	B	C	D	E	F
Ingredient	Source	Ref. No.	Stock Conc.	Final Conc.	Volume for 3mL
DPBS/-	Gibco	14190-144	1X		2625uL
BSA*	Sigma	A7030-100G	10%	1%	300uL
RNase inhibitor	Sigma	3335402001	40 U/uL	1U/uL	75uL

\* Bovine Serum Albumin powder dissolved in DPBS/- and filter sterilized.

### Lysis Buffer (to prepare 0.1 X Lysis Buffer): (B)

A	B	C	D	E	F
Ingredient	Source	Ref. No.	Stock Conc.	Final Conc.	Volume for 0.5mL
Tris-HCl (pH 7.4)	Sigma	T2194-100ML	1 M	10 mM	5uL

A	B	C	D	E	F
NaCl	Promega	V4221	5 M	10 mM	1uL
MgCl <sub>2</sub>	Sigma	M1028-100mL	1 M	3mM	1.5uL
Tween-20	Bio-Rad	1610781	10%	0.1%	5uL
Nonidet P40 Substitute	G-Biosciences	DG001	10%	0.1%	5uL
Digitonin	Invitrogen	BN20061	5%	0.01%	1uL
BSA	Sigma	A7030-100G	10%	1%	50uL
DTT	Sigma	43816-10mL	1000 mM	1 mM	0.5uL
Nuclease-free water	Invitrogen	10977			418.5uL
RNase inhibitor	Sigma	3335402001	40 U/uL	1U/uL	12.5uL

#### Lysis Dilution Buffer (to prepare 0.1 X Lysis Buffer): (C)

A	B	C	D	E	F
Ingredient	Source	Ref. No.	Stock Conc.	Final Conc.	Volume for 1mL
Tris-HCl (pH 7.4)	Sigma	T2194-100ML	1 M	10 mM	10uL
NaCl	Promega	V4221	5 M	10 mM	2uL
MgCl <sub>2</sub>	Sigma	M1028-100mL	1 M	3mM	3uL
BSA	Sigma	A7030-100G	10%	1%	100uL
DTT	Sigma	43816-10mL	1000 mM	1 mM	1uL
Nuclease-free water	Invitrogen	10977			859uL
RNase inhibitor	Sigma	3335402001	40 U/uL	1U/uL	25uL

#### 0.1 X Lysis Buffer:

A	B	C	D	E
Ingredient	Source	Stock Conc.	Final Conc.	Volume for 1mL
1 X Lysis Buffer (B)	Prepared in lab	1 X	0.1 X	100uL
Lysis Dilution Buffer (C)	Prepared in lab			900uL

#### Wash Buffer: (D)

A	B	C	D	E	F
Ingredient	Source	Ref. No.	Stock Conc.	Final Conc.	Volume for 2mL
Tris-HCl (pH 7.4)	Sigma	T2194-100ML	1 M	10 mM	20uL
NaCl	Promega	V4221	5 M	10 mM	4uL
MgCl <sub>2</sub>	Sigma	M1028-100mL	1 M	3mM	6uL
BSA	Sigma	A7030-100G	10%	1%	200uL
Tween-20	Bio-Rad	1610781	10%	0.1%	20uL
DTT	Sigma	43816-10mL	1000 mM	1 mM	2uL
Nuclease-free water	Invitrogen	10977			1698uL
RNase inhibitor	Sigma	3335402001	40 U/uL	1U/uL	50uL

#### 2X Diluted Nuclei Isolation Buffer:

A	B	C	D	E	F
Ingredient	Source	Ref. No.	Stock Conc.	Final Conc.	Volume for 0.2mL
Nuclei Buffer*	10X Genomics	2000153/2000207	20x	2x	20uL
DTT	Sigma	43816-10mL	1000 mM	2 mM	0.4uL**
RNase inhibitor	Sigma	3335402001	40 U/uL	2 U/uL	10uL
Nuclease-free water	Invitrogen	10977			169.6uL

\* Nuclei Buffer supplied in the 10x Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Reagent bundle.

\*\* Can dilute DTT 1:5 in water and use 2uL. Reduce water volume to 168uL.

## Nuclei Isolation

- 2 Transfer frozen adipose tissue to a 300mL porcelain mortar pre-chilled and filled with liquid nitrogen. Press/tap on the frozen tissue with a pre-chilled porcelain pestle to pulverize it. A little grinding may be necessary.
- 3 Transfer the pulverized tissue to an ice-cold 15mL conical glass grinder tube, (using a pre-chilled metal spatula), just after the liquid nitrogen disappears.

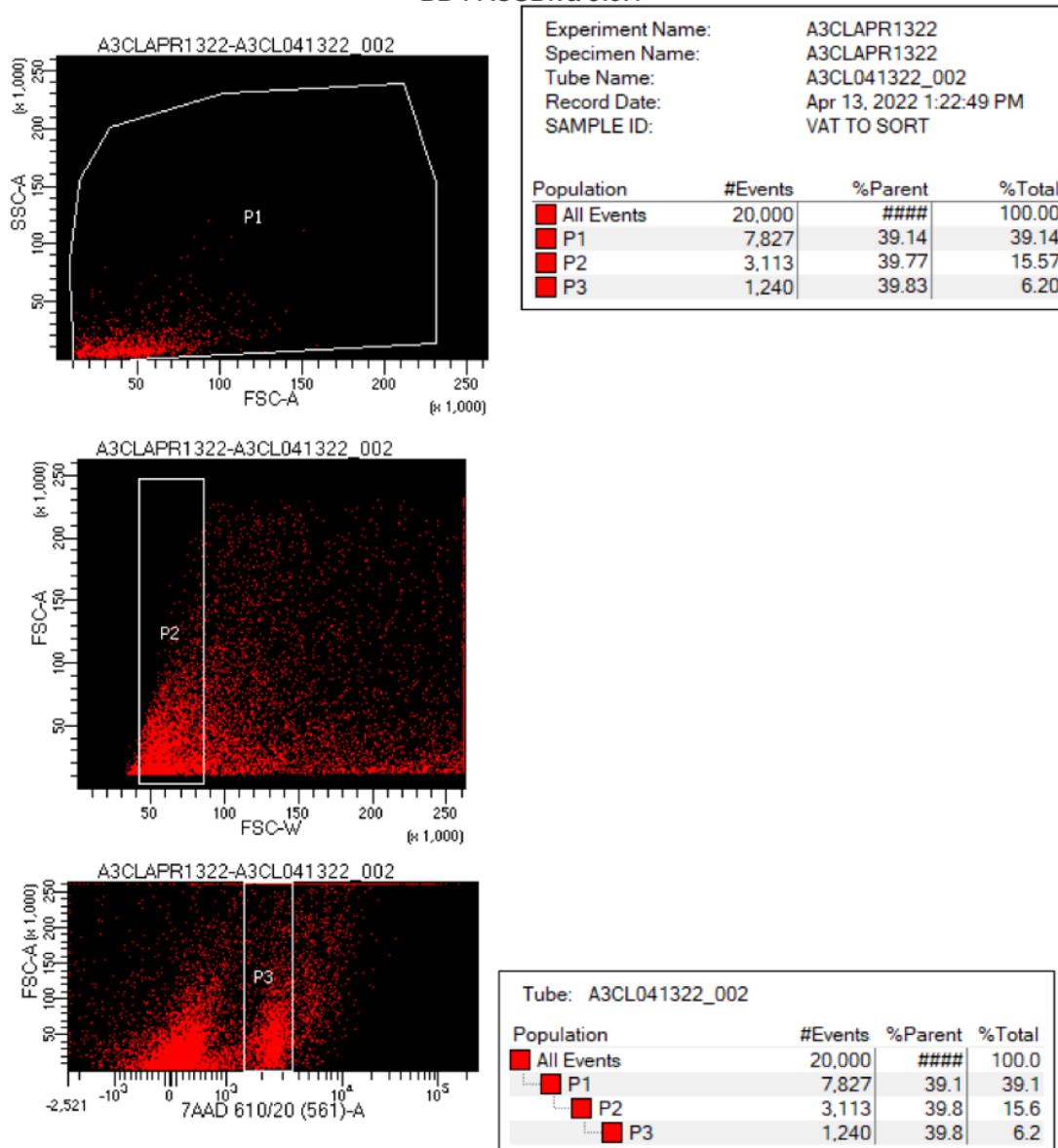
- 4 Immediately add 2mL of cold NP40 Lysis Buffer to the tissue.
- 5 Homogenize the tissue/cells in the grinder tube with a cold conical PTFE pestle. Use twisting strokes with the pestle. The number will be determined by the appearance of tissue, (e.g.,12 strokes).  
Tips:
  - keep on ice all the time, (the conical grinder tube should be immersed in ice)
  - use gentle strokes, (with pressure if necessary)
  - avoid foaming
  - add more NP40 Lysis Buffer, if necessary, (not more than 900uL)
  - use the number of strokes necessary to reach the bottom of the tube with the pestle
- 6 Using a 2mL pipet with a wide bore, transfer the liquid portion of the homogenate only to a 100um cell strainer (Biologix, Ref. No. 15-1100, or other) pre-wetted with 150uL of NP40 Lysis Buffer and placed on a 50mL conical polypropylene centrifuge tube.
- 7 Wash the contents of the conical glass grinder tube with 500uL of NP40 Lysis Buffer and transfer this to the strainer as well.
- 8 Wash the strainer with 500uL of NP40 Lysis Buffer.
- 9 Pass the filtered homogenate through a 40um cell strainer (Falcon, Ref. No. 352340, or other) pre-wetted with 150uL of NP40 Lysis Buffer into another 50mL conical polypropylene centrifuge tube. Wash the strainer with 500uL of NP40 Lysis Buffer.
- 10 Transfer the contents of the 50mL tube to a 15mL polypropylene centrifuge tube.
- 11 Centrifuge at 500 rcf for 5 min at 4°C in a swinging-bucket rotor.
- 12 Carefully remove any lipid layer with a plastic transfer pipet, then carefully remove the supernatent down to about 50uL using a 1mL pipet tip.
- 13 Add 1mL of DPBS/1% BSA/1U/uL RNase Inhibitor, but DO NOT mix.
- 14 Incubate on ice for 5 min.

- 15 Pipette mix to resuspend pellet (5x). Leave the sample in the 15mL centrifuge tube.
- 16 Centrifuge at 500 rcf for 5 min at 4°C.
- 17 Remove the supernatent.
- 18 Resuspend in 400uL of DPBS/1% BSA/1U/uL RNase Inhibitor and pipet to mix (5x). Keep on ice.
- 19 Repeat steps 2-18 for additional samples.

### Nuclei Sorting (protocol for each sample)

- 20 Transfer 25uL of unstained nuclei sample to a 5mL polystyrene, round-bottom (FACS) tube (Corning Falcon, Ref. No. 352008) containing 250uL of DPBS/1% BSA to reserve unstained. This only needs to be performed for one sample.
- 21 Transfer the rest of the nuclei prep, plus 100uL of DPBS/1% BSA/1U/uL RNase Inhibitor used to rinse the 15mL tube, to another 5mL FACS tube. Add 7AAD Ready-Made Solution (Sigma-Aldrich, Ref. No. SML1633) to this nuclei sample, (1uL 7AAD per 100uL sample). *7-AAD is excited with a 488 laser and emission is detected around 647nm.*
- 22 Incubate for at least 5 minutes on ice with light exclusion.
- 23 Fill a 5mL FACS collection tube with DPBS/5% BSA and incubate for 5 minutes to coat the collection tubes with protein.
- 24 Remove the DPBS/5% BSA from the collection tube and rinse with PBS/1% BSA.
- 25 Pipet enough DPBS/10% BSA and 40U/uL RNase Inhibitor into the collection tube to achieve a final concentration of 1% BSA and 1U/uL RNase Inhibitor after the addition of the sorted nuclei. *Volumes should be determined empirically through testing of FACS.*
- 26 Proceed to FACS machine for nuclei sorting. Use a 100um nozzle. See the gating strategy below.

### BD FACSDiva 9.0.1



This is the gating strategy we used with the BD FACSAria III cell sorter to avoid collecting doublets. Single nuclei were collected in the P3 gate. Previous testing showed that the more fluorescent nuclei were actually doublets or larger.

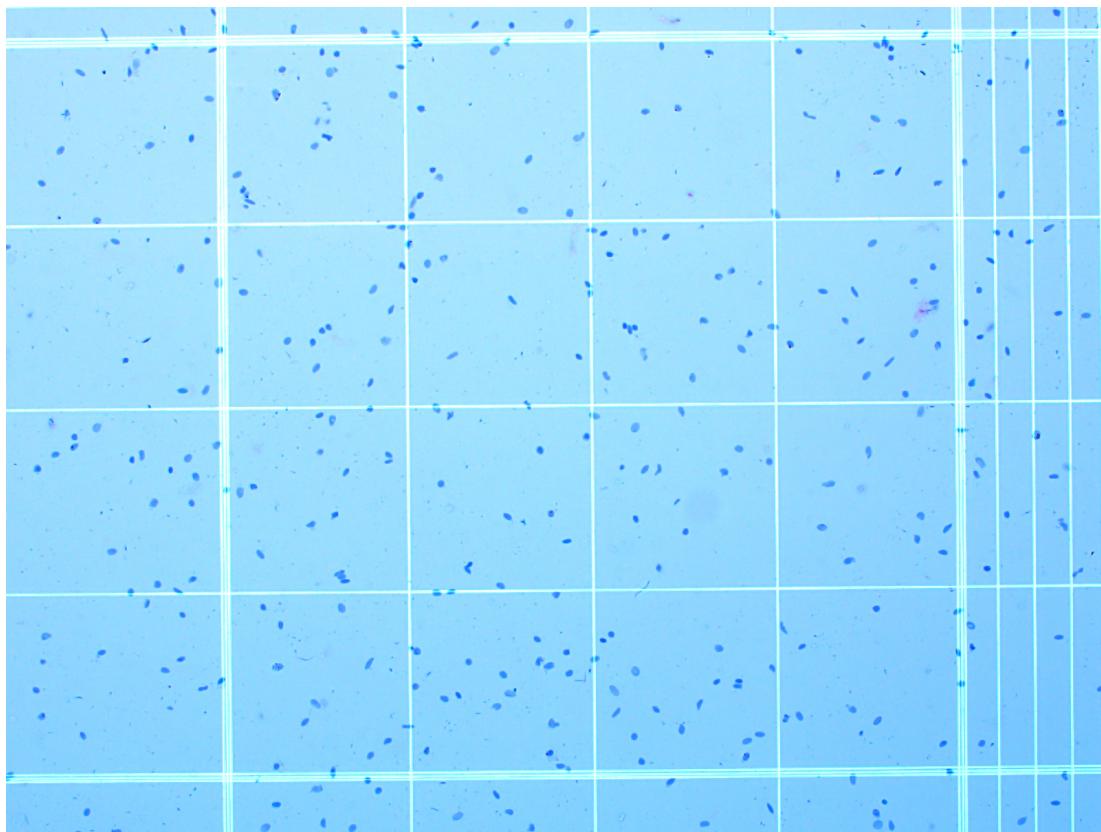
### Nuclei Permeabilization (protocol for each sample)

- 27 Transfer sorted nuclei to a 15mL polypropylene tube and centrifuge at 500 rcf for 5 min at 4°C.
- 28 Remove the supernatent without disrupting the nuclei pellet down to about 25uL. The pellet will not be visible.

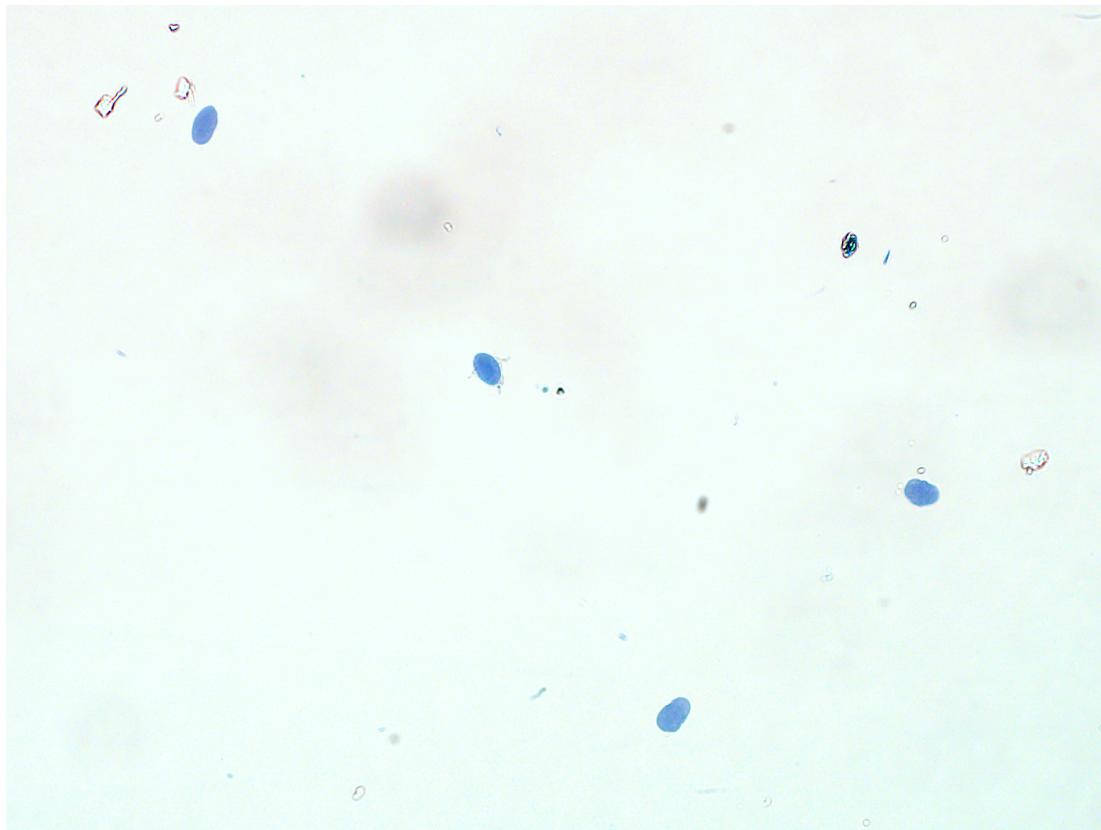
- 29 Resuspend the pellet in 100 $\mu$ L of 0.1X Lysis Buffer and pipet to mix (5x).
- 30 Incubate for 2 min on ice.
- 31 Add 1mL of Wash Buffer and pipette to mix (5x).
- 32 Centrifuge at 500 rcf for 5 min at 4°C.
- 33 Remove the supernatent without disrupting the nuclei pellet down to about 25 $\mu$ L.
- 34 Resuspend the pellet in an equal volume of 2X Diluted Nuclei Isolation Buffer. Pipet to mix (5x). The nuclei are now ready for counting and proceeding to the CG000338 Chromium Next GEM Multiome ATAC + GEX User Guide (Version F).

## Expected Results

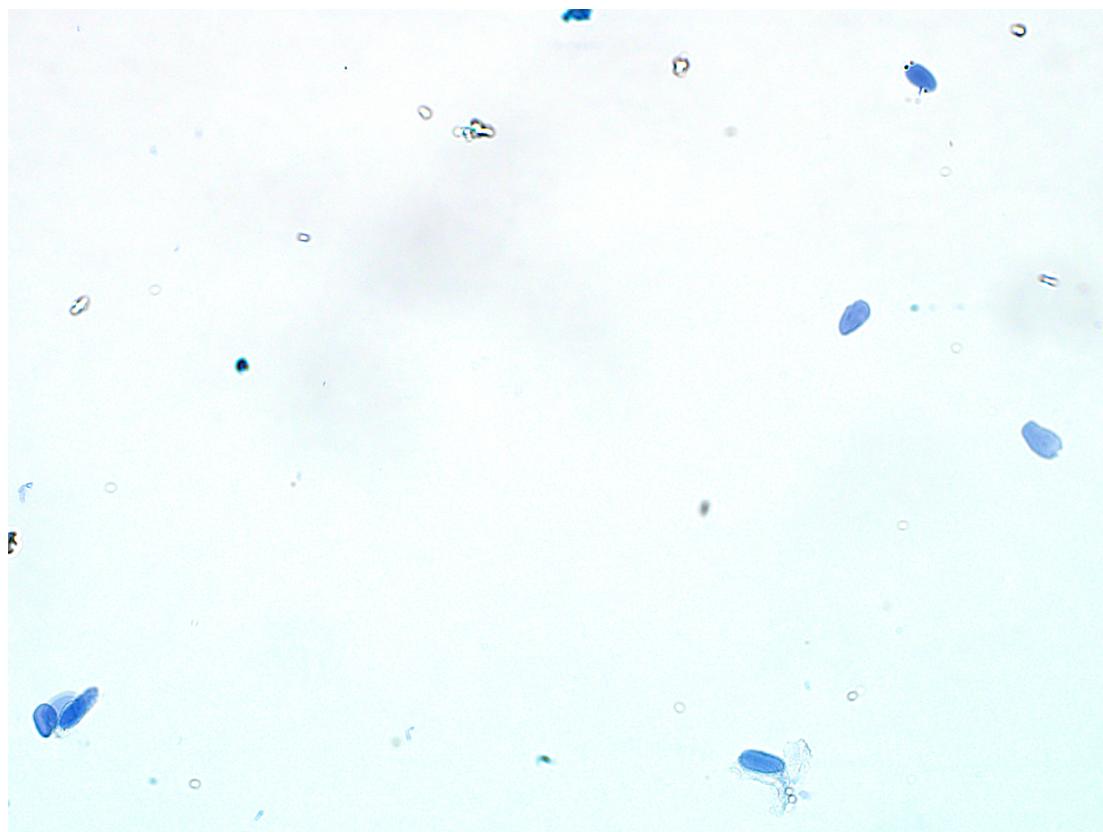
- 35 After sorting and permeabilizing the nuclei, they should appear to be mostly single and intact. We obtained about  $1.4 \times 10^5$  nuclei/gram of omental adipose tissue, and  $7.4 \times 10^4$  nuclei/gram of subcutaneous abdominal adipose tissue.



This photo shows sorted and permeabilized nuclei stained with Trypan Blue at 10x magnification on an Improved Neubauer hemacytometer.



This photo shows sorted and permeabilized nuclei stained with Trypan Blue at 40x magnification.



This photo shows sorted and permeabilized nuclei stained with Trypan Blue at 40x magnification.

## Protocol references

1. CG000375\_Demonstrated Protocol Nuclei Isolation Complex Sample ATAC GEX Sequencing RevB\_10x Genomics
2. C. Streider-Barboza., et.al., "Single-nuclei Transcriptome of Human AT Reveals Metabolically Distinct Depot-Specific Adipose Progenitor Subpopulations," 2022, BioRxiv, doi: <https://doi.org/10.1101/2022.06.29.496888>
3. David W. Burden, "Guide to the Disruption of Biological Samples - 2012," version 1.1, Random Primers, Issue No. 12, Jan. 2012, pp.1-25 (updated June 4, 2012).