Journal of Visualized Experiments

Isolation of Nuclei from Adipose Tissue for Single-Cell Genomic Applications

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61230R1
Full Title:	Isolation of Nuclei from Adipose Tissue for Single-Cell Genomic Applications
Section/Category:	JoVE Genetics
Keywords:	Adipocyte; Thermogenesis; Diabetes; Genomics; biochemistry; Metabolism
Corresponding Author:	Kosaku Shinoda Albert Einstein Medical Center Bronx, New York UNITED STATES
Corresponding Author's Institution:	Albert Einstein Medical Center
Corresponding Author E-Mail:	kosaku.shinoda@einsteinmed.org
Order of Authors:	Gabrielle J. Benitez
	Kosaku Shinoda
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bronx, New York, the Unites States

TITLE:

Isolation of Adipose Tissue Nuclei for Single-Cell Genomic Applications

AUTHORS AND AFFILIATIONS:

Gabrielle J. Benitez[1] and Kosaku Shinoda[1–3]

[1]Departments of Medicine, Albert Einstein College of Medicine, Bronx, New York, NY, USA
[2]Departments of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York, NY, USA
[3]Fleischer Institute of Diabetes and Metabolism, Bronx, New York, NY, USA

Corresponding Author:

Kosaku Shionda (kosaku.shinoda@einsteinmed.org)

Email Address of Co-author:

Gabrielle J. Benitez (gabrielle.benitez@einstein.yu.edu)

KEYWORDS:

adipocyte, thermogenesis, diabetes, genomics, biochemistry, metabolism

SUMMARY:

This publication describes a protocol for the isolation of nuclei from mature adipocytes, purification by fluorescence-activated sorting, and single-cell level transcriptomics.

ABSTRACT:

Brown and beige fat are specialized adipose tissues that dissipate energy for thermogenesis by UCP1 (Uncoupling Protein-1)-dependent and independent pathways. Until recently, thermogenic adipocytes were considered a homogeneous population. However, recent studies have indicated that there are multiple subtypes or subpopulations that are distinct in developmental origin, substrate use, and transcriptome. Despite advances in single-cell genomics, unbiased decomposition of adipose tissues into cellular subtypes has been challenging because of the fragile nature of lipid-filled adipocytes. The protocol presented was developed to circumvent these obstacles by effective isolation of single nuclei from adipose tissue for downstream applications, including RNA sequencing. Cellular heterogeneity can then be analyzed by RNA sequencing and bioinformatic analyses.

INTRODUCTION:

Studies have shown that brown adipose tissue (BAT) has a remarkable capacity to dissipate energy. Two types of thermogenic adipocytes with distinct developmental features exist in both rodents and humans: beige adipocytes and classical brown adipocytes. While classical brown adipocytes are located mostly in interscapular BAT depots, beige adipocytes sporadically emerge in white adipose tissue (WAT) in response to certain physiological cues, such as chronic cold exposure, a process referred to as “browning” or “beiging”. Through the use of advanced imaging, it is now clear that adult humans have substantial depots of $UCP1^{+}$ BAT, especially in the

supraclavicular region[1–4]. The amount of adult human BAT inversely correlates with adiposity and can be increased by external cues, such as chronic cold exposure[5,6] or β3-adrenergic receptor agonist[7]. BAT-mediated energy expenditure may offer a viable approach to combat obesity.

Until recently, thermogenic adipocytes have been considered a homogeneous population. However, studies have revealed the existence of multiple subtypes or subpopulations that are distinct in developmental origin, substrate usage, and transcriptome[8–10]. For instance, a type of beige adipocyte that preferentially uses glucose for thermogenesis, the g-beige adipocyte, was recently described[10]. The incomplete understanding of cell types in brown and beige adipose tissue and the lack of specific markers constitute a critical barrier to studying their biological functions.

Traditional methods for isolating subpopulations of cells are based on expression of only a few known marker genes. Recent advances in single-cell genomics enables the use of global gene expression data of single cells to provide an unbiased estimate of the number of subpopulations in a tissue. The ultimate goal of this protocol is to determine all adipose tissue subtypes under various thermogenic stimuli at a single-cell resolution. In contrast to other tissues and cell types, determining cellular subtypes of adipose tissue is challenging due to the fragility of lipid-filled adipocytes. This paper introduces a robust protocol to isolate single nuclei from adipose tissue for downstream application to snRNA sequencing. Importantly, recent literature comparing well-matched single-nuclei RNA sequencing (snRNA-seq) and single-cell RNA sequencing (scRNA-seq) datasets revealed that snRNA-seq is comparable to scRNA-seq in cell type detection, and superior in cellular coverage for a complex tissue like the brain[11]. This protocol combines a density gradient centrifugation method optimized for adipose tissues by Rosen et al.[12] with a nuclei "cleanup" step with a MoFlo XDP High Speed Sorter. As seen in the representative results, an analysis of 7,500 single nuclei from mouse interscapular brown adipose tissue identified multiple cell types within seemingly homogeneous brown adipocytes. Overall, this simple and robust protocol can be applied to study tissue-level organization of adipocytes and adipose-resident cells, identification of subtype-specific marker genes, and development phenotyping of adipose-selective knockout/transgenic mice.

PROTOCOL:

Animal care and experimentation were performed according to procedures approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine.

1. Preparation of tissue digestion and lysis buffers

1.1. Prepare tissue digestion buffer.

1.1.1. Prepare ~1 mL of digestion buffer for every gram of adipose tissue.

1.1.2. Weigh out 1.5 U/mL collagenase D and 2.4 U/mL dispase II and add phosphate buffered saline (PBS).

1.2. Prepare nuclei preparation buffer (NPB).

1.2.1. Prepare 10 mM HEPES, 1.5 mM magnesium chloride, 10 mM potassium chloride, 250 mM sucrose, and 0.1% NP-40 in nuclease-free water. Mix well. Sucrose may take more time to dissolve than the other components.

1.3. Prepare 0.5% bovine serum albumin (BSA) solution.

1.3.1. Prepare 0.5% BSA in PBS containing EDTA. It is recommended to use sterile-filtered cell culture grade PBS (see **Table of Materials**).

2. Enzymatic digestion of adipose tissue

2.1. Dissect mice to extract adipose tissue according to Aune et al.[13]. Collect isolated tissue in a dish containing PBS. Transfer the tissue to a paper towel to pat dry. Then place the tissue in a clean dish.

2.2. Add calcium chloride to the digestion buffer to a final concentration of 10 mM. Add a small volume of digestion buffer to the dish and thoroughly mince the adipose tissue. The volume of digestion buffer required will be based on the amount of tissue isolated. Usually ~1 mL or less is used.

2.3. Add about half of the amount of prepared digestion buffer (e.g., ~10 mL for five mice) to the minced tissue. Using a serological pipette, transfer the sample to a 50 mL conical centrifuge tube. Add any remaining amount of buffer to wash the dish and transfer to the 50 mL tube containing the sample. Pipette up and down several times. Then incubate with shaking at 200–210 rpm at 37 °C for 12–15 min.

3. Adipocyte isolation

3.1. After incubation, add 0.5% BSA at a 1:1 ratio to the sample. Mix well by pipetting up and down several times. Centrifuge the sample at 300 x *g* for 5 min at room temperature (RT) to spin down the stromal vascular fraction (SVF). The adipocyte fraction will remain in the top layer of the sample.

3.2. Place a 40 µm cell filter on top of a 50 mL conical centrifuge tube and filter the sample, collecting the top layer and leaving behind the SVF at the bottom of the tube. Transfer the top layer (adipocyte fraction) to a new tube by flipping the filter upside down over the tube and using 0.5% BSA to reverse wash the filter and collect the sample in the tube.

3.3. Bring the total volume of 0.5% BSA to 10–15 mL based on sample size and pipette up and down with a serological pipette or briefly shake up and down to mix the sample. Centrifuge the sample again at 300 x *g* for 5 min at RT.

4. Nuclei isolation

4.1. Use a wide-bore pipette tip and carefully transfer the top layer of the sample to a 100 µm cell filter placed on top of a new prechilled tube on ice, leaving behind any residual SVF.

4.2. Rinse/wash the filter with a sufficient amount of NPB and discard the filter. From this step forward have the sample on ice as often as possible and work quickly to avoid leaky and/or unhealthy nuclei. It is helpful to prechill microcentrifuge tubes to be used in future steps on ice.

4.3. Place the sample on ice for no more than 2 min with intermittent gentle inverting of the tube to mix. The incubation time on ice should be optimized for sample size and type of adipocytes. Centrifuge at 1,000 x *g* at 4 °C for 10 min.

4.4. Remove the supernatant and resuspend the pellet in 1 mL of NPB. Transfer the sample to a clean microcentrifuge tube. While transferring, avoid touching the walls of the tube, which contain debris and lipid. Add 0.6 U/µL RNase inhibitor to the sample and mix. Centrifuge again at 1,000 x *g* at 4 °C for 10 min.

NOTE: Centrifugation speed and time should be decreased at this step for smaller samples.

4.5. Remove the supernatant and resuspend in 1 mL of Nuclei Wash Buffer (2% BSA/PBS + 0.6 U/µL RNase inhibitor).

4.6. Double filter into a clean tube with stackable 30 µm filters. Wash filter with a small volume (~300 µL) of Nuclei Wash Buffer. Alternatively, for smaller samples, use a 30 µm filter that fits into a microcentrifuge tube and filter directly into it.

4.7. Confirm successful isolation of healthy nuclei.

4.7.1. Stain a small aliquot of the sample with trypan and use an automated cell counter to assess average dead size and percent dead cells. Average dead size for a sample containing mostly nuclei should range between 7–10 µm. Nuclei size is usually around 8 µm.

4.7.2. Stain a small aliquot of the sample with DAPI and examine under a microscope with a 20x objective or higher. The nuclear membrane should be intact and the nuclei should be round.

5. FACS cleanup and nuclei concentration step

5.1. Proceed immediately to the FACS step to clean up nuclei and remove any excess debris. The collection buffer should contain Nuclei Wash Buffer. During FACS the buffer is diluted. Preparing a higher concentration of BSA and RNase inhibitor to account for dilution during sorting is helpful. The FACS collection block should be on cooling mode.

5.2. After sorting the nuclei, centrifuge at 500 x *g* for 5 min at 4 °C to concentrate the sample. Reconstitute in an appropriate volume of Nuclei Wash Buffer to achieve a concentration of 500–1,500 nuclei/µL. Do not attempt to remove all the supernatant. Doing so will result in loss of nuclei.

5.3. Use a hemocytometer and a DAPI stained aliquot for a final count and to visualize the nuclei. Then proceed immediately with snRNA-seq according to the manufacturer's protocol.

REPRESENTATIVE RESULTS:

Unsorted adipocyte nuclei contain debris and doublets that create noise and high background in downstream single-cell RNA sequencing. The representative FACS gate strategy is shown in **Figure 1**. The nuclei were first selected based on forward scatter (FSC) and side scatter (SSC) (**A**), then, only singlets were selected based on the combination of width and heights of SSC (**B**). Finally, only DAPI-positive events were selected and sorted into collection buffer (**C**). This workflow provides highly-purified single nuclei with minimized nuclear aggregates and cellular debris (**Figure 2**).

To confirm the integrity of the sorted nuclei, a small aliquot of the sample was inspected by microscope after DAPI staining. The nuclear membrane should be intact and the nuclei should be round (**Figure 2B**). To confirm the success of the purification, it is also recommended to collect supernatant (i.e., collection buffer) after centrifugation and run real-time qRT-PCR of a marker gene. Use of a primer set (primer sequences in **Table 1**) designed to amplify nascent, intron-containing UCP1, confirmed that the supernatant of sorted nuclei did not contain a detectable level of nascent *Ucp1* mRNA (**Figure 3**). This step can be done for other marker genes highly abundant in brown, beige, or white adipocytes, such as *Cidea*, *Pgc1-a*, *Adipoq*, or *Fabp4*.

Nuclei isolated and confirmed through this protocol can be subjected to virtually any single-cell level gene expression platform. Use of the Chromium platform[14] (10x Genomics) determined a transcriptome of 7,500 nuclei from interscapular brown adipose tissue from 8-week-old male C57BL/6 mice (**Figure 4**). t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction and K-means clustering revealed seven cell types, including brown adipocytes, endothelial cells, mural cells marked by *Pdgfrb*, adipocyte progenitors, and immune cells. All clusters expressed *Fabp4*, a pan-adipocyte gene, to varying degrees (**Figure 4B**). A subset of clusters expressed a high level of *Ucp1* mRNA, whereas other clusters sporadically expressed *Ucp1* (**Figure 4A**,**C**). This is consistent with a previous study showing that interscapular brown adipose tissue contains at least two distinct populations with high and low *Ucp1* expression and thermogenic activity[8,9].

FIGURE AND TABLE LEGENDS:

Figure 1: Representative flow cytometry analysis of nuclei by MoFlo XDP High Speed Sorter. (**A**) Size and granularity. (**B**) Detection of nuclear aggregates and multiplets. (**C**) Final sorting gate selecting only DAPI-positive events.

Figure 2: Representative image of nuclei isolated from brown adipocytes. (**A**) Before and (**B**) after MoFLo XDP sorting. White arrows in **A** indicate nuclei without an intact membrane. Scale bar = 50 µm.

Figure 3: Representative qRT-PCR result for isolated nuclei and the supernatant.

Figure 4: Representative 10x chromium snRNA-seq results for brown adipocytes isolated from 8-week-old male C57BL/6 mice under RT. (**A**) t-SNE dimensionality reduction and K-Means clustering for 7,500 nuclei. *k* = 9. Canonical markers used for cluster annotations are shown in parentheses. (**B**) mRNA expression of *Fabp4*, an adipocyte-selective marker, in **A**. White = no expression, red = high expression. (**C**) mRNA expression of *Ucp1*, thermogenic marker in **A**. These data are from a single experiment. snRNA-seq data used for these figures was deposited in the Gene Expression Omnibus (GEO) under superseries accession number GSE144720.

Table 1: Primer sequences for real-time qPCR analysis.

DISCUSSION:

A straightforward and robust method to isolate single nuclei and study adipose tissue heterogeneity is presented. Compared to whole tissue RNA sequencing, this workflow offers an unbiased view of cellular heterogeneity and population-specific markers. This is significant and innovative for the advancement of adipocyte biology, molecular metabolism, and obesity research.

This protocol is particularly optimized for downstream application of snRNA-seq. The "cleanup" step to achieve isolation of healthy nuclei with the MoFlo XDP High Speed Sorter completely removes debris and aggregates from the crude suspension while maintaining nuclear membrane integrity. Therefore, this increases capture efficiency in the droplet formation and recovers thousands of single-nuclei transcriptomes from just one run without loss of the number of detected genes. In addition to droplet-based single-cell platforms, which provide a higher number of surveyed single nuclei or cells, sorter-based microplate and Fluidigm C1 platforms[15] can also be used to obtain greater resolution and coverage of the transcriptome[16]. Because this workflow offers high-quality nuclei, assays for transposase-accessible chromatin using sequencing (i.e., ATAC-sequencing) can also be performed with minimum modifications to the existing scATAC-seq protocol[17].

One limitation of this protocol is the loss of information from the removal of nonnuclear compartments, such as the cytosol and cellular membrane. For instance, emerging multimodal single-cell analyses[18,19] that simultaneously measure cell membrane protein expression and the transcriptome cannot be applied to this protocol.

The future goal is to combine this protocol with nuclei multiplexing using barcoded antibodies[20]. Sorted nuclei from different adipose tissues under different experimental conditions will be barcoded and pooled using an antinuclear pore complex antibody and snRNA-seq will be performed. By sequencing these barcodes alongside the nuclear transcriptome, each nuclei can

be assigned to its original sample, cross-sample multiplets can be clearly identified, and droplet-based systems can be "super-loaded" for significant cost reduction per nuclei.

ACKNOWLEDGMENTS:

We would like to thank David Reynolds from the Albert Einstein Genomics core and Jinghang Zhang from the Flow Cytometry Core for technical support. We acknowledge support from the National Institutes of Health (NIH) (DK110426) and Pilot and Feasibility Grants from the Einstein-Mount Sinai Diabetes Research Center (DK020541), and New York Obesity Research Center (DK026687) (all to K.S.). We also would like to thank Albert Einstein Cancer Center (CA013330) for core support.

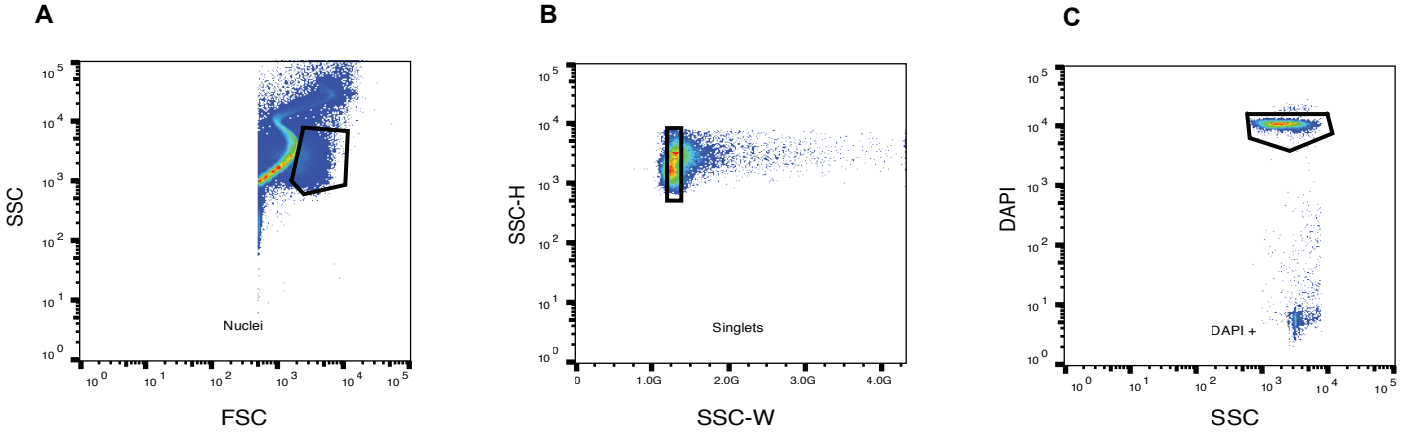
DISCLOSURES:

The authors declare that they have no competing financial interests.

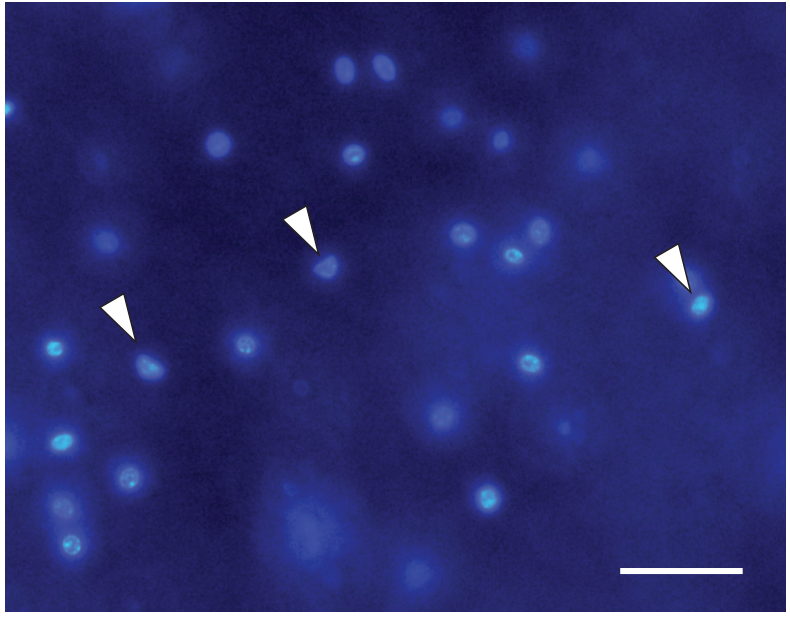
REFERENCES:

1. Cypess, A. M. et al. Identification and importance of brown adipose tissue in adult humans. *The New England Journal of Medicine*. **360** (15), 1509–1517 (2009).
2. van Marken Lichtenbelt, W. D. et al. Cold-activated brown adipose tissue in healthy men. The New England Journal of Medicine. **360** (15), 1500–1508 (2009).
3. Virtanen, K. A. et al. Functional brown adipose tissue in healthy adults. *The New England Journal of Medicine*. **360** (15), 1518–1525 (2009).
4. Nedergaard, J., Bengtsson, T., Cannon, B. Unexpected evidence for active brown adipose tissue in adult humans. *American Journal of Physiology—Endocrinology and Metabolism*. **293** (2), E444–E452 (2007).
5. Saito, M. et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes*. **58** (7), 1526–1531 (2009).
6. Yoneshiro, T. et al. Recruited brown adipose tissue as an antiobesity agent in humans. *The Journal of Clinical Investigation*. **123** (8), 3404–3408 (2013).
7. Cypess, A. M. et al. Activation of human brown adipose tissue by a β3-adrenergic receptor agonist. *Cell Metabolism*. **21** (1), 33–38 (2015).
8. Song, A. et al. Low- and high-thermogenic brown adipocyte subpopulations coexist in murine adipose tissue. The Journal of Clinical Investigation. **130** (1), 247–257 (2020).
9. Cinti, S. et al. CL316,243 and cold stress induce heterogeneous expression of UCP1 mRNA and protein in rodent brown adipocytes. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*. **50** (1), 21–31 (2002).
10. Chen, Y. et al. Thermal stress induces glycolytic beige fat formation via a myogenic state. *Nature*. **565** (7738), 180–185 (2019).
11. Bakken, T. E. et al. Single-nucleus and single-cell transcriptomes compared in matched cortical cell types. *PloS One*. **13** (12), e0209648 (2018).
12. Roh, H. C. et al. Simultaneous Transcriptional and Epigenomic Profiling from Specific Cell Types within Heterogeneous Tissues In Vivo. *Cell Reports*. **18** (4), 1048–1061 (2017).
13. Aune, U. L., Ruiz, L., Kajimura, S. Isolation and differentiation of stromal vascular cells to beige/brite cells. *Journal of Visualized Experiments*. (73), e50191 (2013).
14. Zheng, G. X. Y. et al. Massively parallel digital transcriptional profiling of single cells. *Nature*

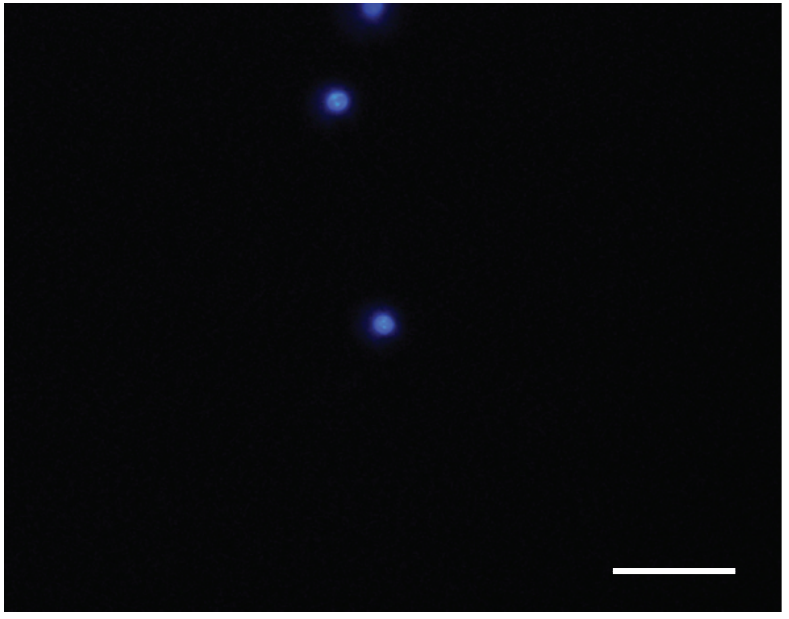
Communications. **8**, 14049 (2017).
15. Pollen, A. A. et al. Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. *Nature Biotechnology*. **32** (10), 1053–1058 (2014).
16. Hayashi, T. et al. Single-cell full-length total RNA sequencing uncovers dynamics of recursive splicing and enhancer RNAs. *Nature Communications*. **9** (1), 619 (2018).
17. Satpathy, A. T. et al. Massively parallel single-cell chromatin landscapes of human immune cell development and intratumoral T cell exhaustion. *Nature Biotechnology*. **37** (8), 925–936 (2019).
18. Stoeckius, M. et al. Simultaneous epitope and transcriptome measurement in single cells. *Nature Methods*. **14** (9), 865–868 (2017).
19. Peterson, V. M. et al. Multiplexed quantification of proteins and transcripts in single cells. *Nature Biotechnology*. **35** (10), 936–939 (2017).
20. Gaublomme, J. T. et al. Nuclei multiplexing with barcoded antibodies for single-nucleus genomics. *Nature Communications*. **10** (1), 2907 (2019).

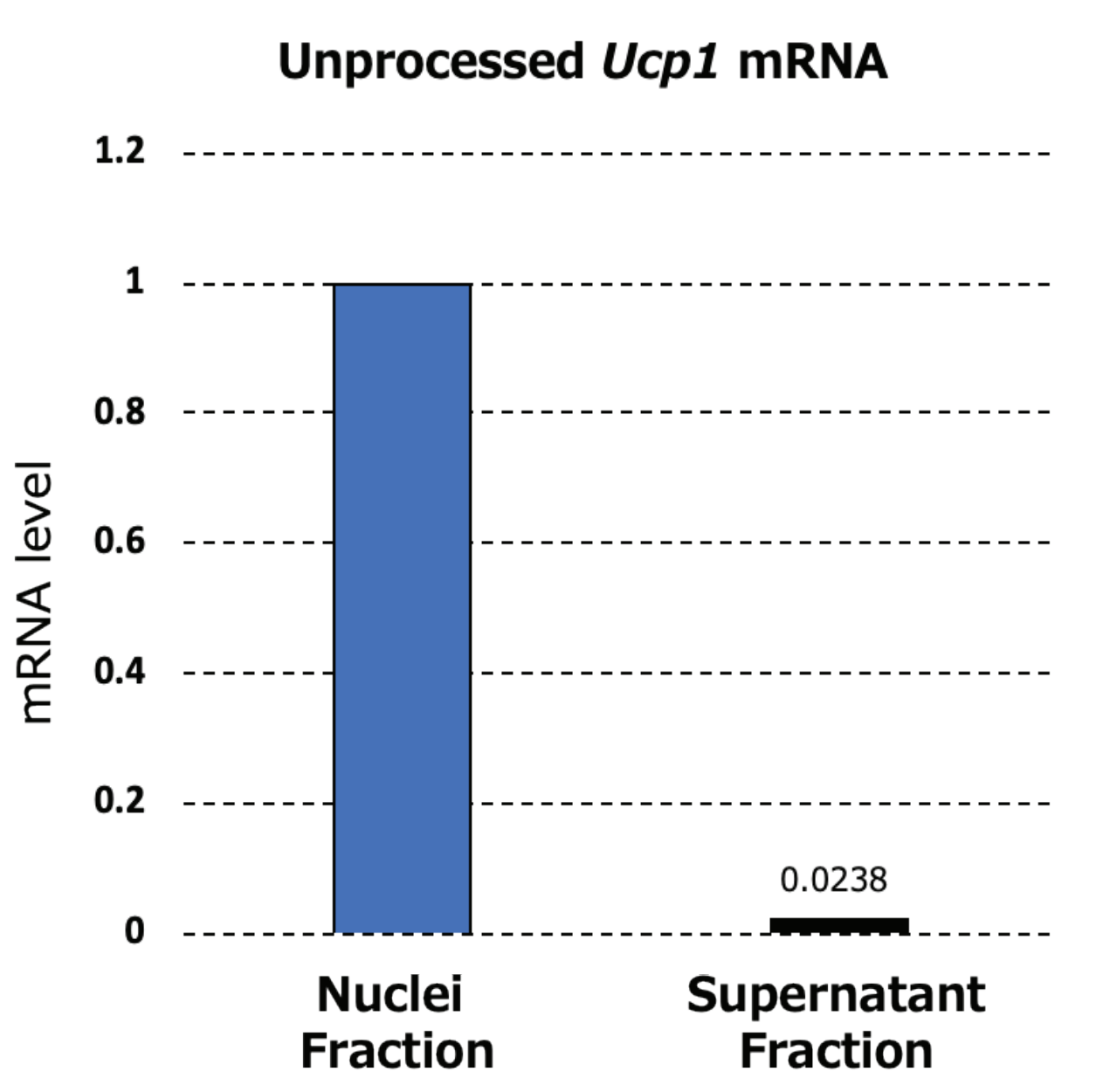

A
SSC
FSC
Nuclei
B
SSC-H
SSC-W
Singlets
C
DAPI
SSC
DAPI +

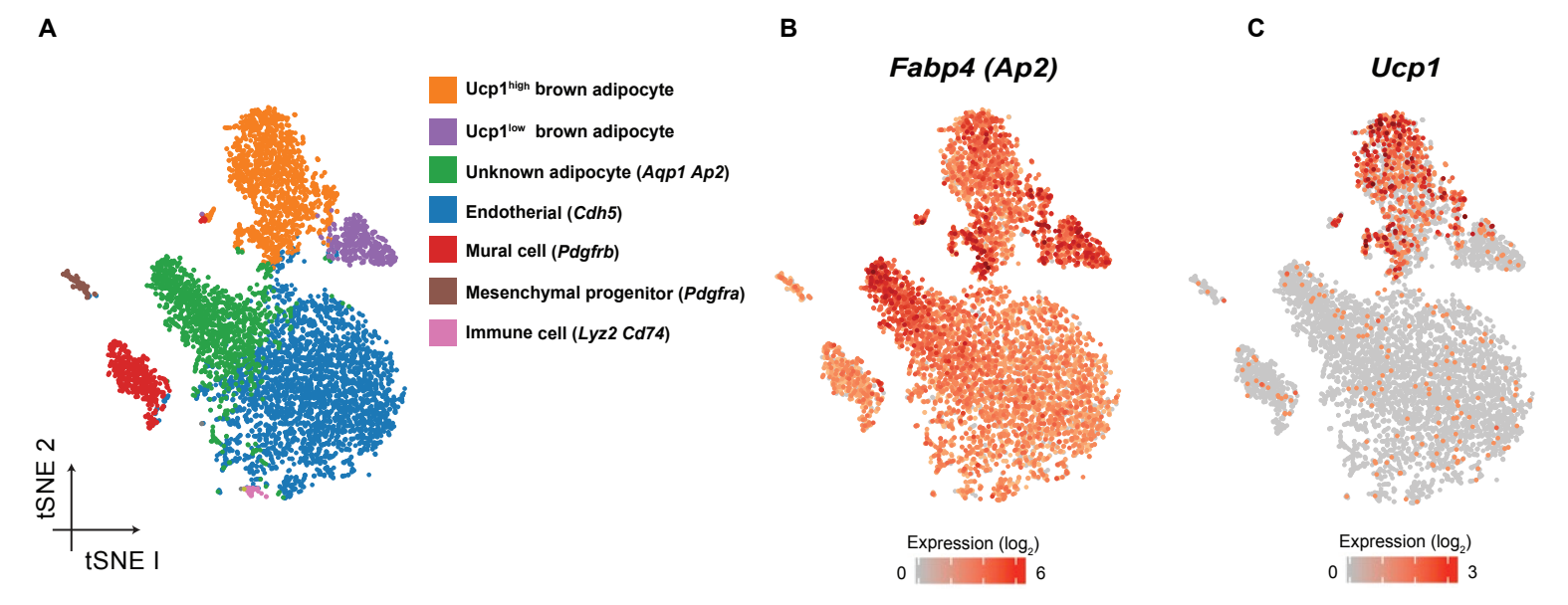
A



B




Unprocessed *Ucp1* mRNA
1.2
1
0.8
0.6
0.4
0.2
0
mRNA level
0.0238
Nuclei Fraction
Supernatant Fraction


A
Ucp1high brown adipocyte
Ucp1low brown adipocyte
Unknown adipocyte (Aqp1 Ap2)
Endotherial (Cdh5)
Mural cell (Pdgfrb)
Mesenchymal progenitor (Pdgfra)
Immune cell (Lyz2 Cd74)
tSNE 2
tSNE I
B
Fabp4 (Ap2)
Expression (log2)
0
6
C
Ucp1
Expression (log2)
0
3

Table

Click here to access/download;Table;Benitez et al JoVE_Table 1.xlsx

Gene	Species	Forward primer
Ucp1 (Intron-containing)	mouse	GAT CTT CTC AGC CGG AGT TTC

Reverse primer
CCT TCC TAA TAG CAC CCA TTC C

Name of Material/Equipment	Company	Catalog Number	Comments/Description
autoMACS Rinsing Solution	Miltenyi Biotec	130-091-222	PBS with EDTA; sterile-filtered
BSA	Sigma	A1595	
CaCl2	Sigma	21115	
Cell filter 100 µm	Corning	431752	
Cell filter 40µm	Corning	431750	
CellTrics (30 µm)	Sysmex	04-004-2326	
Collagenase D	Roche	11088866001	
Countess II FL Automated Cell Counter	Invitrogen	AMQAF1000	
DAPI	Sigma	D9542	
Dispase II	Roche	4942078001	
HEPES	Sigma	H4034	
KCl	Fisher	P217-3	
MACS SmartStrainers (30 µm)	Miltenyi Biotec	130-098-458	Stackable filters
MgCl2	Sigma	M1028	
MoFloXDP Cell Sorter	Beckman Coulter	ML99030	
NP-40	Sigma	74385	
Protector RNase Inhibitor	Roche	3335402001	
Sucrose	Fisher	S5-3	

Dear Phillip,

Thank you for handling our manuscript (JoVE61230) on our isolation protocol for nuclei from adipocytes for single cell RNA-sequencing. As you can see below, all the editorial as well as referees' comments have been addressed. I believe that the manuscript is ready to move on to video production. Please feel free to contact me if you have any questions.

Sincerely,
Kosaku

Kosaku Shinoda, Ph.D.
Assistant Professor, Albert Einstein Medicine

Editorial comments:

E-1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We carefully proofread the manuscript for spelling or grammatical errors.

E-2. Please define all abbreviations before use, e.g., SVF, t-SNE.

We have defined all the abbreviations including SVF (Stromal Vascular Fraction) and t-SNE (t-Distributed Stochastic Neighbor Embedding).

E-3 JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We have removed all commercial language from main text.

E-4. Please provide at least 6 key words or phrases in a separate section in the manuscript.

We have provided 6 keywords on page 1.

E-5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Animal care and experimentation were performed according to procedures approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine. This statement is included in page 3 line 78.

E-6. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

E-6A. Please provide more detail or a reference about collecting adipose tissue.

The detailed protocol for collecting adipose tissue has been published in JoVE in 2013 (Aune et al.). We have included the citation.

E-6B. 'More digestion buffer' is vague; do you mean the entire volume prepared in 1.1? Also, how strong is the shaking?

The recommended amount of buffer is about half of the prepared digestion buffer (10ml is prepared for 5 mice). This information has been added in page 3 line 109.

E-6C. How exactly do you wash the sample? Is that step 3.3?

This information has been added in page 3 line 123.

E-7. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, or .pdf file (4 files in total).

This has been corrected.

E-8. Figures 3 and 4 do not appear to be cited correctly in the Results section.

This has been corrected.

E-9. Figure 4B, C: Please explain the red coloring, e.g., with a scale.

mRNA level is indicated as follows: white (no expression) and red (high expression). This is added to the legend. We also have included scale for Figure 4B.

E-10. Please do not abbreviate journal titles.

This has been corrected.

E-11 Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have included all the materials and equipment used, including sorter, in the Table of Materials.

Reviewers 1's comments:

R1-1 This is a very detailed, well-written protocol of isolating nuclei from adipose tissue. Isolating high quality, pure nuclei from adipose tissue has been a barrier for single cell sequencing analysis of adipocyte in vivo. The current manuscript provides a practical protocol to address this unmet need, and should generate strong interest to the adipocyte field.

We appreciate the reviewer's comment.

Reviewer 2's comments:

R2-1 - Well done manuscript, very useful protocol for isolation of Nuclei from adipose tissue.

We appreciate the reviewer's comment.

R2-2 - Even though floating adipocytes fraction by centrifuge, still many of the cell contaminated-endothelial, immuncells etc.Because recently other paper reported single cell analysis from adipose tissue (eLife 2019;8:e49501 doi: 10.7554/eLife.49501), I re-analyzed with RAW data and found much of the cell types-adipocytes, endothelialcells, immunecells ... like this. Figure4- I would request to add the anotation using canonical marker(endothelial, adipocytes, immunecells etc...) . Otherwise the authors can not say subpopulations of adipocytes exists.

We have added the annotation using canonical markers in Figure 4.

R2-3 - In this protocol, it is not clear to filter/how collect toplayer with 40um filter. Using cell filter 40um and then centrifuge to correct top layer? Please clarify this protocol.

This is now clarified in 3.2 (page 4 line 122).