Journal of Visualized Experiments

Preparation of adipose progenitor cells from mouse epididymal adipose tissues

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61694R1
Full Title:	Preparation of adipose progenitor cells from mouse epididymal adipose tissues
Corresponding Author:	Jason Doles Mayo Clinic Minnesota Rochester, MN UNITED STATES
Corresponding Author's Institution:	Mayo Clinic Minnesota
Corresponding Author E-Mail:	doles.jason@mayo.edu
Order of Authors:	Dong Seong Cho
	Jason Doles
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.	Rochester, Minnesota USA

MAYO CLINIC

Jason D. Doles, Ph.D.
Assistant Professor/Senior Associate Consultant
Department of Biochemistry & Molecular Biology
Mayo Clinic College of Medicine

200 First Street SW, Rochester, MN 55905 USA
Telephone: (507) 284-9372
Fax: (507) 284-3383
E-mail: doles.jason@mayo.edu

Date: July 27, 2020

Re: Manuscript resubmission

Dear Dr. Troyer and *JOVE* Editorial Staff:

We are pleased to submit our revised invited protocol manuscript titled “Preparation of adipose progenitor cells from mouse epididymal adipose tissues” for consideration for publication in the *Journal of Visualized Experiments*. In addition to this cover letter, please find our revised manuscript text, figures, and materials table. Thank you in advance for your attention to this submission and should you require any further information, please do not hesitate to contact me. I am reached best by email at: doles.jason@mayo.edu.

Sincerely,

Jason D. Doles, Ph.D.
Assistant Professor
Department of Biochemistry and Molecular Biology
Mayo Clinic

TITLE:

Preparation of Adipose Progenitor Cells from Mouse Epididymal Adipose Tissues

AUTHORS AND AFFILIATIONS:

Dong Seong Cho[1], Jason D Doles[1*]

[1]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, USA

Email Address of Corresponding Author:
Jason D Doles (doles.jason@mayo.edu)

Email Address of Co-Author:
Dong Seong Cho (cho.dong@mayo.edu)

KEYWORDS:

adipose tissue, adipose progenitor cell, adipose tissue-derived stem cell, preadipocyte, single cell isolation, flow cytometry, epididymal fat pad

SUMMARY:

We present a simple method to isolate highly viable adipose progenitor cells from mouse epididymal fat pads using fluorescence activated cell sorting.

ABSTRACT:

Obesity and metabolic disorders such as diabetes, heart disease, and cancer, are all associated with dramatic adipose tissue remodeling. Tissue-resident adipose progenitor cells (APCs) play a key role in adipose tissue homeostasis and can contribute to the tissue pathology. The growing use of single cell analysis technologies – including single-cell RNA-sequencing and single-cell proteomics – is transforming the stem/progenitor cell field by permitting unprecedented resolution of individual cell expression changes within the context of population- or tissue-wide changes. In this article, we provide detailed protocols to dissect mouse epididymal adipose tissue, isolate single adipose tissue-derived cells, and perform fluorescence activated cell sorting (FACS) to enrich for viable $Sca1^+/CD31^-/CD45^-/Ter119^-$ APCs. These protocols will allow investigators to prepare high quality APCs suitable for downstream analyses such as single cell RNA sequencing.

INTRODUCTION:

Adipose tissue plays a key role in energy metabolism. Excess energy is stored in the form of lipids, and adipose tissue is capable of significant expansion or retraction depending on nutritional status and energetic demand. Expansion of adipose tissue can result from an increase in adipocyte size (hypertrophy) and/or from an increase in adipocyte number (hyperplasia); the latter process tightly regulated by proliferation and differentiation of adipose progenitor cells[1,2]. During obesity, adipose tissue excessively expands, and tissue dysfunction – including hypoxia, inflammation, and insulin resistance – often develops[3,4]. These complications are risk factors to many chronic diseases including hypertension, diabetes, cardiovascular

diseases, stroke, and cancer[5]. Hence, limiting uncontrolled adipose tissue expansion and mitigating adipose tissue pathologies are top biomedical research priorities. During adipose tissue expansion, resident adipose tissue-derived stem cells (ASCs) proliferate and differentiate sequentially into preadipocytes (committed progenitor cells) and then into mature adipocytes[6]. Recent single-cell RNA-sequencing (scRNA-seq) studies show that these adipose progenitor cell (APC) populations (ASCs and preadipocytes) exhibit substantial molecular and functional heterogeneity[7-12]. For example, ASCs display a reduced adipogenic differentiation capacity, while also exhibiting higher proliferation and expansion capabilities, compared to preadipocytes[7]. Further molecular differences are reported within ASC and preadipocyte populations, although the functional relevance of these differences remains unclear[7]. Together, these data highlight the complexity of the adipose progenitor cell pool and underscore the need to develop and standardize tools to better understand and manipulate these critical cell populations.

This protocol details the isolation of high viability $Sca1^+$ adipose progenitor cell populations from mouse epididymal fat pads that are suitable for sensitive downstream analyses, including single-cell studies (scRNA-sequencing) and cell culture. Isolation and dissociation of epididymal fat pads was performed as previously described[7,13] with slight modifications that improve the viability of isolated APCs. In brief, dissociated cells from epididymal fat pads are stained with antibodies against Sca1, a marker for both ASCs and preadipocytes[6,7], and other lineage (Lin) markers: Ter119 (erythroid cells), CD31 (endothelial cells), and CD45 (leukocytes). Viable $Sca1^+/Ter119^-/CD31^-/CD45^-/DAPI^-$ cells are then sorted by fluorescence activated cell sorting (FACS). Importantly, this protocol was validated by successful isolation and analysis of viable $Sca1^+/Lin^-$ adipose progenitor cells reported in a recent single cell RNA sequencing study that identified functionally heterogeneous subpopulations within ASCs and preadipocytes[7].

PROTOCOL:

All animal experimental procedures were performed under approval by the Mayo Clinic Institutional Animal Care and Use Committee.

1. Solution preparation

1.1. Prepare collagenase 2% (w/v) solution by dissolving collagenase II 2 g in 100 mL Hanks' balanced salt solution (HBSS). Aliquot 200 µL each for each use.

1.2. Prepare neutralization medium by mixing 84 mL F-12 medium, 15 mL horse serum, and 1 mL penicillin/streptomycin.

1.3. Prepare flow cytometry buffer by dissolving 500 mg of bovine serum albumin (BSA) and 400 µL of 0.5 M EDTA in 100 mL of Dulbecco's phosphate-buffered saline (DPBS).

2. Dissection of epididymal fat pad and tissue dissociation

2.1. Humanely euthanize (isoflurane/cervical dislocation) one male mouse. In this protocol, a

four-month-old FVB mouse was used.

2.2. Apply/spray 70% ethanol on the abdomen and expose the lower abdominal cavity using clean scissors and forceps.

2.3. Locate epididymal fat pad (proximal/attached to testes). Hold the junction between testes and epididymal fat pad with blunt forceps and pull gently to liberate the epididymal fat pad.

2.4. Remove testes by cutting using scissors and incubate epididymal fat pad in 5 mL of HBSS supplemented with 3% BSA in a 50 mL conical tube at room temperature for 15 min.

2.5. Centrifuge at 150 *x g* for 7 min at 4 °C.

2.6. Take floating epididymal fat pad from the 50 mL conical tube and finely mince the tissue using clean scissors.

2.7. Add 200 µL collagenase 2% solution into 3.8 mL of HBSS in a 13 mL culture tube. Add the minced tissue and incubate in a rotating incubator at 5 rpm for 1 h at 37 °C. Instead of a rotating incubator, cell culture incubator at 37 °C can be alternatively used with occasional agitations.

2.8. Transfer entire contents into a 50 mL conical tube and add 10 mL of neutralization medium. Mix gently by inverting the tube 2-3 times.

2.9. Prepare another 50 mL conical tube fitted with a 70 µm cell strainer. Filter the digested tissue into the new tube using the cell strainer.

2.10. Transfer the flow-through to a 15 mL conical tube and centrifuge at 350 *x g* for 10 min at 4 °C.

2.11. Carefully remove the supernatant and resuspend cell pellets with 5 mL of DPBS. Centrifuge at 350 *x g* for 10 min at 4 °C.

2.12. Carefully remove the supernatant and add 50 µL of flow cytometry buffer. Mix well by pipetting gently and keep cells on ice. Due to the volume of the cell pellets and small amount of residual supernatant, total volume of cells in the flow cytometry buffer will be greater than 50 µL (approximately 100 µL).

3. Antibody labeling and fluorescence activated cell sorting (FACS)

3.1. After mixing the cells well with gentle pipetting, transfer 54 µL of the cell suspension into a 1.7 mL microcentrifuge tube. Add 6 µL of FcR blocking reagent and mix well by pipetting gently. Incubate at 4 °C for 10 min. Save any leftover cells after taking 54 µL and keep them in

the ice to use as an unstained control in step 3.5 and step 3.6.

3.2. During the incubation in step 3.1, prepare an antibody cocktail by combining 11 µL each of anti-Sca1-APC, anti-Ter119-FITC, anti-CD31-FITC, and anti-CD45-FITC antibodies in a microcentrifuge tube. Mix well by gentle pipetting and keep on ice protected from light.

3.3. After incubation with FcR blocking reagent for 10 min, add 40 µL of the antibody cocktail and mix well by gentle pipetting. Incubate at 4 °C for 10 min.

3.4. Add 500 µL of flow cytometry buffer supplemented with 1 µg/mL of DAPI into the stained cells. Mix well by gentle pipetting and filter cells using a 5 mL test tube with cell strainer snap cap. Keep cells on ice.

3.5. Add 500 µL of flow cytometry buffer to remaining cells in step 3.1 and mix well by gentle pipetting. Filter cells using a 5 mL test tube with cell strainer snap cap. Keep these cells on ice and use as an unstained control in the following step.

3.6. Take the stained cells and unstained control to FACS analysis or sorting instrument.

3.6.1. Identify and isolate the $APC^+/FITC^-/DAPI^-$ population with gating strategies shown in **Figure 1**. These cells represent viable $Sca1^+/Ter119^-/CD31^-/CD45^-$ cells. Debris and cell aggregates are depleted using forward (FSC) and side scatter (SSC) plots. Then, $DAPI^-$ population is gated, followed by gating of $APC^+/FITC^-$ population. Unstained controls should be used to aid in setting gating parameters.

3.6.2. Collect the cells into 500 µL of flow cytometry buffer in a 1.5 mL microcentrifuge tube. Moving forward, all procedures should be performed under sterile conditions to minimize a contamination. Approximately 50,000 – 100,000 cells are collected from one mouse.

3.7. Count cells using a hemocytometer to evaluate cell viability. Since cell aggregates can interfere with further downstream analyses, the presence of cell aggregates is also evaluated to ensure a minimal presence (<5% of viable cell number) of these aggregates.

REPRESENTATIVE RESULTS:

Four-month-old male FVB mice were used in this experiment. After exclusion of debris and doublets using FSC/SSC plots, viable cells ($DAPI^-$ population) were gated, followed by the selection of $APC^+/FITC^-$ population (**Figure 1**). DAPI, APC, and FITC gates were drawn based on the unstained control. Gating strategies are shown in **Figure 1**.

After 1 h of sorting, the quality of isolation was quantitatively evaluated by flow cytometry analysis (**Figures 2,3**). The cells were stained with propidium iodide solution (1:100) for viability staining. Using the same gating for sorting, the cells maintained high viability and purity: 92.6 ± 2.2% single cells (third panel in **Figure 2**), 86.4 ± 3.0% viability (PI^- cells), and 86.0 ± 2.8% $APC^+/FITC^-$ cells (n= 4, average ± standard deviation). These percentages were defined as

percentages of each gated population (single cells, PI^- cells, and $PI^-/APC^+/FITC^-$ population, respectively, in **Figure 2**) relative to total cell number.

FIGURE LEGENDS:

Figure 1: Isolation of viable $Sca1^+$ adipose progenitor single cells by FACS. Gating strategies are shown in both stained cells and in an unstained control.

Figure 2: Representative flow cytometry analysis to assess post-isolation cell viability. Viability staining was performed using propidium iodide.

Figure 3: Quantification of percentage of cells 1 h post-isolation in flow cytometry analysis. Percentages of single cells, viable cells, and $APC^+/FITC^-$ cells were quantified to evaluate purity and viability after isolation of cells. Data shown represents the average ± standard deviation. n=4.

DISCUSSION:

Single cell RNA sequencing (scRNA-seq) is rapidly gaining traction as a powerful tool to simultaneously study diverse cell populations at the single cell level. Due to high costs associated with sample preparation and high throughput sequencing, it is imperative to optimize cellular inputs (high viability and purity) to increase the likelihood of experimental success. Some cell preparation protocols rely on removal of dead cells and debris using low-spin washes and column-based separation without FACS sorting[14]. Many of these methods, however, significantly reduce the number of viable recovered cells, and in many cases, dead cells are not completely removed[14]. This protocol outlines a simple method to isolate highly viable $Sca1^+$ adipose progenitor cells containing minimal cellular debris, cell doublets, and dead cells. Although cells isolated using this protocol exhibited high viability, we still recommend minimizing time between cell isolation and further downstream analysis to maintain high viability. In addition, successful antibody labeling is critical to isolate $Sca1^+$ adipose progenitor cells with high purity. Hence, determination of optimal concentration of antibodies is strongly recommended for each antibody, and dilution of the antibodies in step 3.2 can be adjusted accordingly.

This flow cytometry-based protocol is amenable to customization depending on individual interest in specific adipose progenitor cell sub-populations. Here, alternative antibody combinations can be used to identify and isolate the population(s) of interest. Indeed, due to extensive APC marker diversity, multiple labs studying APCs using scRNA-seq report isolating cells using other surface markers. For example, $PDGFRA^+/CD44^+$ cells, $CD31^-/CD45^-/Ter119^-/CD29^+/CD34^+/Sca1^+$ cells, and $Pdgfrb^+$ cells have been isolated by FACS for downstream APC scRNA-seq analyses[8,9,11]. $CD142^+$ subpopulations have also been characterized and are shown to exhibit limited adipogenic differentiation capacity and an ability to suppress adipogenesis[10,11]. In addition to Sca1, combinations of antibodies against CD55, CD81, and CD9 were recently used to prospectively isolate ASC and preadipocyte subpopulations (using this protocol) and study subpopulation molecular and functional heterogeneity[7]. Moreover, since adipose

progenitor cells from other fat pads including inguinal fat pads and brown adipose tissues have been isolated using collagenase dissociation like the present protocol[15,16,17], the present protocol may be applicable to isolation of adipose progenitor cells from other adipose tissues.

One caveat of the current approach is that it is tailored towards the isolation of immature APCs. Although the present protocol yields high viability APCs, the efficacy and efficiency of isolation of other adipose tissue resident cell types (i.e., immune cells, fibroblasts, endothelial cells, etc.) is unclear. Importantly, this protocol is not suitable for the isolation of mature adipocytes. Adipocytes have been extremely difficult to isolate using FACS due to their large cell size and fragility. Recently, isolation of mature adipocytes by FACS was reported using a large nozzle size (150 μm) and low sheath pressure (6 psi) with a specific FSC/SSC gating strategy[18]. Future studies could perhaps merge this adipocyte isolation protocol with the protocol described here to establish a more comprehensive pipeline to isolate and study diverse cell types within adipose tissue.

ACKNOWLEDGMENTS:
We acknowledge the Mayo Clinic Microscopy Cell Analysis Core Flow Cytometry Facility for assistance with FACS sorting.

DISCLOSURES:
The authors have nothing to disclose.

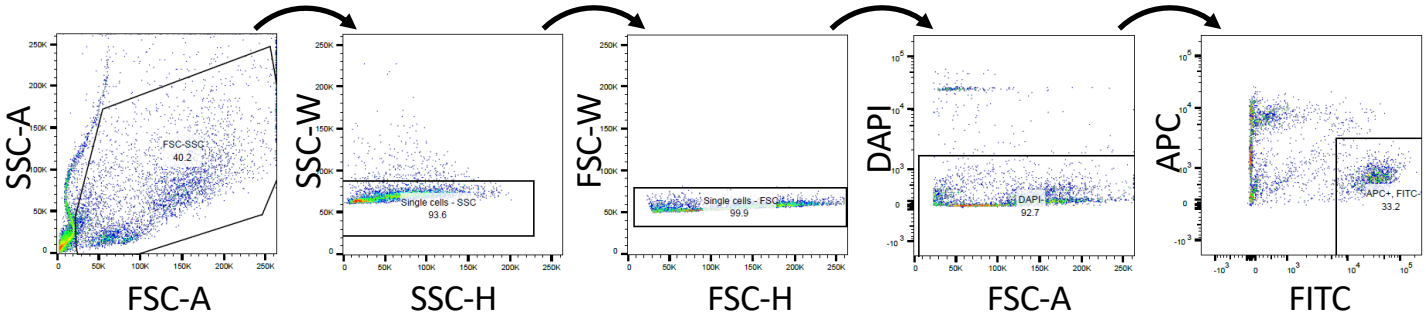
REFERENCES:
1. Krotkiewski, M., Bjorntorp, P., Sjostrom, L., Smith, U. Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. *The Journal of Clinical Investigation.* **72**, 1150-1162 (1983).
2. Salans, L. B., Horton, E. S., Sims, E. A. Experimental obesity in man: cellular character of the adipose tissue. *The Journal of Clinical Investigation.* **50**, 1005-1011 (1971).
3. Trayhurn, P. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiological Reviews.* **93**, 1-21 (2013).
4. Halberg, N. et al. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Molecular and Cellular Biology.* **29**, 4467-4483 (2009).
5. Upadhyay, J., Farr, O., Perakakis, N., Ghaly, W., Mantzoros, C. Obesity as a Disease. *Medical Clinics of North America.* **102**, 13-33 (2018).
6. Cawthorn, W. P., Scheller, E. L., MacDougald, O. A. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. *Journal of Lipid Research.* **53**, 227-246 (2012).
7. Cho, D. S., Lee, B., Doles, J. D. Refining the adipose progenitor cell landscape in healthy and obese visceral adipose tissue using single-cell gene expression profiling. *Life Science Alliance.* **2**, e201900561 (2019).
8. Burl, R. B. et al. Deconstructing Adipogenesis Induced by beta3-Adrenergic Receptor Activation with Single-Cell Expression Profiling. *Cell Metabolism.* **28**, 300-309 (2018)
9. Hepler, C. et al. Identification of functionally distinct fibro-inflammatory and adipogenic stromal subpopulations in visceral adipose tissue of adult mice. *eLife.* **7**, e39636 (2018).

10. Merrick, D. et al. Identification of a mesenchymal progenitor cell hierarchy in adipose tissue. *Science.* **364**, eaav2501 (2019).
11. Schwalie, P. C. et al. A stromal cell population that inhibits adipogenesis in mammalian fat depots. *Nature.* **559**, 103-108 (2018).
12. Raajendiran, A. et al. Identification of Metabolically Distinct Adipocyte Progenitor Cells in Human Adipose Tissues. *Cell Reports.* **27**, 1528-1540 (2019).
13. De Matteis, R. et al. In vivo physiological transdifferentiation of adult adipose cells. *Stem Cells.* **27**, 2761-2768 (2009).
14. Hanamsagar, R. et al. An optimized workflow for single-cell transcriptomics and repertoire profiling of purified lymphocytes from clinical samples. *Scientific Reports.* **10**, 2219 (2020).
15. Marinovic, M. P. et al. Crotamine induces browning of adipose tissue and increases energy expenditure in mice. *Scientific Reports.* **8**, 5057 (2018).
16. Takahashi, H. et al. Biological and clinical availability of adipose-derived stem cells for pelvic dead space repair. *Stem Cells Translational Medicine.* **1**, 803-810 (2012).
17. Cowan, C. M. et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nature Biotechnology.* **22** (5), 560-567 (2004).
18. Hagberg, C. E. et al. Flow Cytometry of Mouse and Human Adipocytes for the Analysis of Browning and Cellular Heterogeneity. *Cell Reports.* **24**, 2746-2756 (2018).

Figure 1

Click here to access/download;Figure;Cho_figures_final_resub 1.pdf

Stained cells



Unstained control

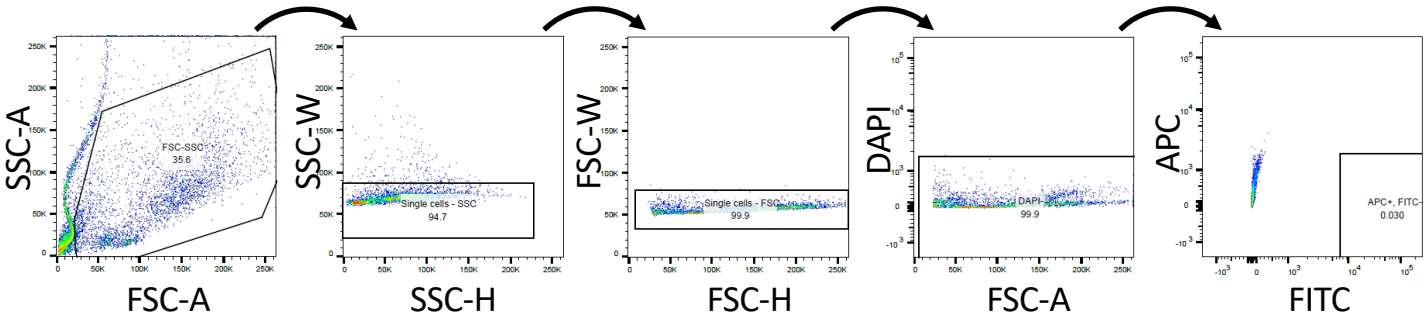


Figure 2

Click here to access/download;Figure;Cho_figures_final_resub 2.pdf

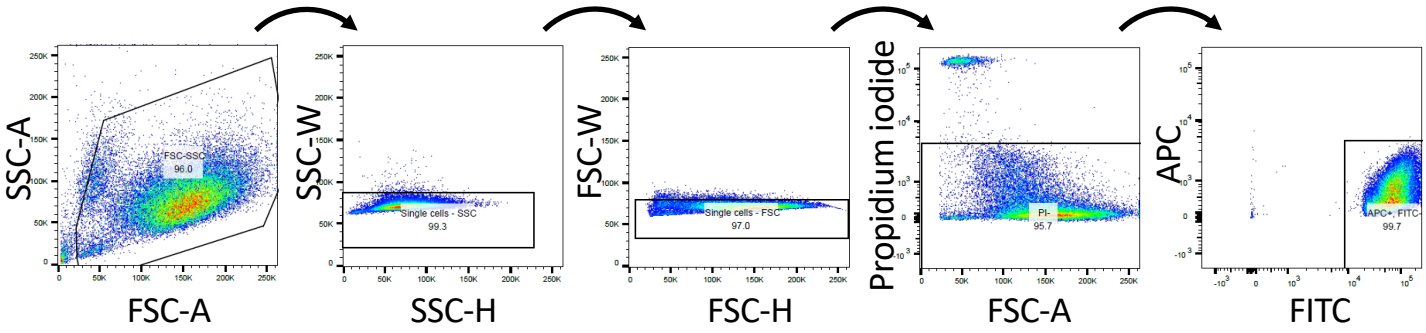


Figure 3

Click here to access/download;Figure;Cho_figures_final_resub 3.pdf

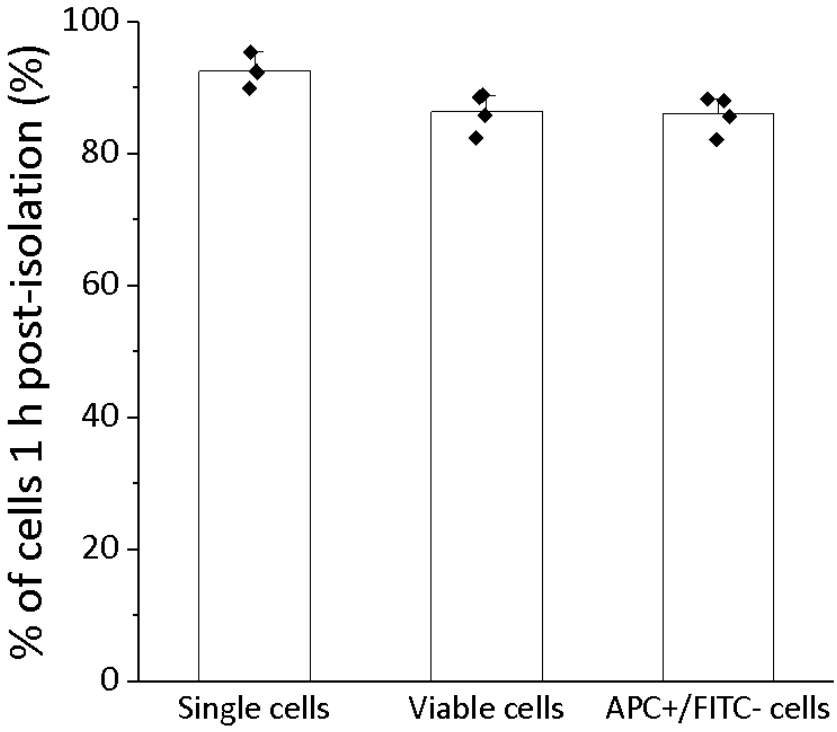


Table of Materials

Click here to access/download;Table of Materials;Cho_JoVE_Materials_resub.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.7 mL microcentrifuge tube	VWR	87003-294	
13 mL culture tube	Thermo Fisher Scientific	50-809-216	
15 mL conical tube	Greiner Bio-one	188 271	
5 mL test tube with cell strainer snap cap	Thermo Fisher Scientific	08-771-23	
50 mL conical tube	Greiner Bio-one	227 261	
70 µm cell strainer	Thermo Fisher Scientific	22-363-548	
Anti-CD31-FITC antibody	Miltenyi Biotec	130-102-519	
Anti-CD45-FITC antibody	Miltenyi Biotec	130-102-491	
Anti-Sca1-APC antibody	Miltenyi Biotec	130-102-833	
Anti-Ter119-FITC antibody	Miltenyi Biotec	130-112-908	
BSA	Gold Biotechnology	A-420-500	
Collagenase type II	Thermo Fisher Scientific	17101-015	
DAPI	Thermo Fisher	D1306	
Dulbecco's phosphate-buffered saline (DPBS)	Thermo Fisher Scientific	14190-144	
F-12 medium	Thermo Fisher Scientific	11765-054	
FcR blocking reagent	Miltenyi Biotec	130-092-575	
Hanks' balanced salt solution (HBSS)	Thermo Fisher Scientific	14025-092	
Horse serum	Thermo Fisher Scientific	16050-122	
Penicillin-streptomycin	Thermo Fisher Scientific	15140-122	
Propidium iodide solution	Miltenyi Biotec	130-093-233	

Dear Dr. Troyer and *JOVE* Editorial Staff:

Below please find our point-by-point response to reviewer/editorial comments.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 2.1: Mention animal strain and age.

We thank for your comments and we apologize for not mentioning details of animals used in the study. The details of used animals are now in the text.

2) 2.5, 2.10, 2.11, : Convert speed to x g.

We thank for your comments and we acknowledge that gravity is more appropriate than RPM for centrifugal force. We changed the unit into x g.

• **Protocol Numbering:**
1) Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary.

We apologize for not properly using the suggested numbering. We corrected this error.

2) All steps should be lined up at the left margin with no indentations.

We apologize for the incorrect formatting. We corrected the formatting accordingly.

3) Please add a one-line space after each protocol step.

We corrected the formatting accordingly.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow

from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We have highlighted the protocol section according to the suggested guidelines.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We appreciate these comments and we revised the discussion section appropriately.

• **Figures:** Remove the text “Cho_Figure #”

Figure text has been removed as requested.

• **References:** Please spell out journal names.

We apologize for this formatting error. We now corrected journal names in the References section.

• **Table of Materials:** Please sort in alphabetical order.

The materials are now all sorted in alphabetical order.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this protocol, Cho and Doles present a method for isolating Sca1+ adipocyte progenitor cells that can be used for demanding downstream applications such as single cell-RNA sequencing.

General Comments:

1. The choice of Sca1 as a marker to broadly identify adipocyte progenitor cells (APCs) is appropriate as Sca1 is a common marker of mesenchymal stem cells and us a surface marker for the various currently identified subpopulation of APCs.

2. This protocol is well-described and follows standard steps for obtaining adipose tissue (here, epididymal fat).

3. The rationale for using flow cytometric sorting to obtain the viable cells is well-supported.

4. The authors note that the protocol is specific for SCA1+ APCs only.

We greatly appreciate these positive comments.

Major Concerns:

1. It would be helpful if the authors indicate whether this approach is also applicable to other fat pads, particularly inguinal and brown fat since those depots are often targeted for analysis.

We appreciate these comments, and we acknowledge that discussing other adipose tissues will be helpful for readers in this field. Other adipose tissues, such as inguinal fat pad and brown adipose tissue, have also been similarly dissociated with collagenase to isolate adipose progenitor cells, so the present protocol can likely be applied to other adipose tissues. The text has been revised to discuss this issue.

2. Section 3, step 6: This is the first mention of "PI" in the protocol. The addition of propidium iodide to determine cell viability should be specifically included in an earlier step. Alternatively, if DAPI alone is being used to eliminate nonviable cells, please delete mention of propidium iodide throughout the text (in this step, under Representative Results, Figure 2 and Fig. 2 legend, Name of Material/Equipment).

We appreciate your comments, and we apologize for making any confusion by not clearly describing the procedures. “PI” in step 3.6 was typo, and it is now corrected to “DAPI”, as DAPI was used to deplete nonviable cells in FACS. However, PI was also used in our protocol to further evaluate cell viability after cell sorting (Figure 2). Because DAPI was already used for cell sorting to isolate viable cells during cell sorting, PI was alternatively used for following assessment of viability.

3. Figure 3. How is percent single cells determined? This is important to clarify for the reader/viewer since the highlighted downstream application is single-cell sequencing.

We thank for your comments, and we apologize for not clearly explaining it. The percentage of each population was defined as percentage of each gated population in Figure 2 out of total cells. Especially, the single cell gated population is shown in the third panel in Figure 2. The text is now revised accordingly.

4. For the uninitiated in flow cytometry, it will be helpful to explain how aggregated cells are identified, when present.

We appreciate this comment, and it is now revised in the text in step 3.7.

Minor Concerns:
Section 2, step 7. In the absence of a rotating incubator at 37C, what might the reader substitute?

In the absence of a rotating incubator, we note that a regular incubator can be used with occasional agitation. This alternative is noted in the revised text.

Reviewer #2:
Manuscript Summary:
The author provides straightforward protocol to isolate adipose progenitor cells as defined by Sca1+/CD31-/CD45-/Ter119-. As adipocyte progenitors have a critical role in adipose tissue remodeling and are key to the health of adipose tissue, this work is of general interest. A strength of this protocol is that it can be modified to the interest of labs using alternative surface markers to isolate the APCs.

Major Concerns:
I don't have major concerns

Minor Concerns:
1. Typically, when adipose tissue progenitor cells are isolated, there is functional characterization of the cells. For example, they are transplanted into mice to become adipose tissue. Can the author refer to their previous work or other work to give context as to why they selected these markers and the functional characterization of sorted cells?

We greatly appreciate these comments. The protocol can be further applied to sort heterogeneous subpopulations within adipose progenitor cells. We note that we performed such analyses in a previously published study (now mentioned/cited in the Introduction section).

2. Please provide sorting scheme (even if simple) of the FACS experiment

We appreciate this comment and we now provide details of the FACS experiment in step 3.6.

3. Can the authors provide an estimate of the number of APCs. isolated from fat pads from 4 mice?

We thank for this comment. Typically 50,000 – 100,000 cells can be sorted, and this is described in step 3.6.