Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: Proof reading was performed.

2. Please provide an institutional email address for each author.

Response: Email addresses were provided.

Steven Negron: snegron@mmri.edu
Bing Xu: bing@mmri.edu
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3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: personal pronouns were removed.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Falcon, OptiPrep, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.
Response: Commercial language has been removed.

5. Line 73: Please elaborate on euthanasia. What % of CO2 was used? How long was the animal given CO2?
Response: Euthanize one adult mouse with CO_2 overdose. Briefly, 100% CO_2 was filled into the mouse cage a displacement rate of 10-30% cage volume per minute. 5 min later, death was confirmed by checking for the absence of visibly breathing.

6. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral, throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks
Response: The time units have been corrected.

7. Please convert stirring speeds/ centrifugation speeds to centrifugal force (x g) instead of revolutions per minute (rpm) throughout the protocol.
Response: The centrifuge speeds have been converted into centrifugal force.

8. Line 106-109: Please mention how long is the vortex performed.
Resposne: the duriation of vortex has been provided.

9. Line 125: Please include the details of the BCA assay. A citation will suffice.
Response: A reference was provided.

10. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
Response: Line space between protocol steps was provided. The protocol section for video was highlighted.

11. Please move the Figure Legends to the end of the Representative Results.

Response: Figure legends were moved to the end of the representative results.

12. Please include any limitations of the technique described in the protocol.
Response: One paragraph about BAs purity was added into the discussion section. Page 10, line 296 to 305.

13. Please do not use abbreviations for journal titles and book titles. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
Response: The reference format has been updated.

14. Figure 1: please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Examples: 5 h, 10 min.
Response: corrected.

15. Figure 2: Please include scale bars for all the images in the panel.
Response: scale bar provided.
16. Please sort the Table of Materials in alphabetical order.
Response: corrected.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this study, Lin and his colleagues developed a new method to isolate mouse BAs for gene and protein expression analysis. It is a topic of interest to researchers in metabolic areas. My detailed comments are as follows:

Major Concerns:
1. The rest of cells after removing the BAs layers should be validated by UCP1 IHC and staining of Lipophilic Dyes or tracing the UCP-1-Cre;tdTom BAs
Response: We thank the reviewer's suggestion. In this revised manuscript, we examined the cells exist in the solution below the BAs layer. The BAs were genetically labeled with tdTom, so that we can trace whether BAs were completely separated from the non-BA cells with our protocol. As shown in Figure 3 G, cells recovered from the clear 6% iodixanol solution were tdTom negative. Also, no obvious lipid droplets were visible in these cells. These data suggest that our new protocol can efficiently separate the BAs from the non-fat cells. On page 7, line 209 to line 217, we put a paragraph to describe these new results.

2. The thermogenic capacity of isolated BAs should be tested and compared between two methods.
Response: In this study, we developed a new protocol to isolate BAs for gene expression studies. The reviewer provided a good suggestion to test whether the BAs isolated with two different protocols have similar thermogenic capacity.
There are two methods to measure brown adipocytes thermogenic activity:
Seahorse XF Analyzer based respirometry analysis[1] and thermosensitive fluorescent dye based imaging analysis[2]. In these two methods, pre-adipocytes are first seeded on the plates and then differentiated into brown adipocytes. The primary BAs isolated with our current protocol are mature and lipid loaded cells. These low density BAs float in the cultue medium and cannot be seeded in culture plates/dishes, which makes these cells not suitable for either respirometry or imaging based thermogenic activity measurements. Therefore, due to the

current technical difficulties, we are unable to compare the thermogenic capacity of isolated BAs.

Minor Concerns:
It should be clear that this protocol is applicable to mouse brown adipocytes isolation, especially in the title and abstract.

Response:
We changed the title and revised the abstract to highlight that this protocol is for isolating mouse brown adipocytes.

Reviewer #2:
Manuscript Summary:
In this manuscript, Dr. Xu and colleagues describe a new method to isolate individual brown adipocytes from brown adipose depots. As the authors correctly indicated, the isolation of individual brown adipocytes is essential for the in-depth understanding of the functional variance and dynamics of this thermogenic cell type. The protocol in this manuscript is advantageous in that 1) it does not involve FACS sorting and 2) it is compatible with TRIZol-based protein purification from isolated brown adipocytes.

Major Concerns:
1. The purity is most important for an isolation protocol for individual cell types. The authors showed a DIC image of isolated BAs using the 6% iodixanol protocol (Fig. 3D) and qPCR and IB comparisons of BAs and non-BAs for some markers. However, these are not sufficient to quantify the purity of the isolation. I suggest that the authors isolate BAs from UCP1-TdTomato mice using the iodixanol protocol and quantify the purity of the isolated cells by FACS.
Response: We thank the reviewer for the suggestions of using FACS to analyse the BAs purity. Adipocytes are well known for their fragile nature. Large amount of debris or lipid particles are generated during the adipose tissue dissociation process, which makes the FACS analysis very challenging[3]. In our previous study, we labeled the brown adipocytes with tdTom, and isolated brown adipocytes from interscapular BAT. Consistent with published data[3], we found that most of the FACS events were tdTom negative[4]. Therefore, FACS will unlikely be a reliable method to quantify the purity of the purified BAs.

In the current study, we aimed to develop a strait forward method to isolate brown adiopocytes from interscapular BAT for gene expression study. The BAs were enriched based on their relatively low density. Because white adipocytes (WAs) also have low density, the purity of isolated BAs can potentially be affected by WAs but not other cell types. The interscapular BAT is sometimes attached by white adipose tissue, such as in old or high fat diet treated mice. Although we described that the white adipose tissue need to be removed during the BAT preparation step, the white adipose tissue might not be completely removed. Therefore, if very high purity is essential, the isolated BAs need to be sorted by FACS. We put a new paragraph in the discussion section (Page 10, lines 296 to 305) to adress this purity issue.

2. The current iodixanol protocol is based on cell density. It is well known that lipolysis during brown adipose activation is accompanied with reduced triglyceride storage in BAs, which presumably increases the density of brown adipocytes (under certain conditions). It would be

essential to confirm that the 6% iodixanol protocol non-selectively enriches all differentiated brown adipocytes (UCP1+) rather than only those lipids-laden brown adipocytes. As such, the cells underneath the upper BA layer should be examined carefully (e.g., after dilution of iodixanol and centrifugation) for UCP1+ cells.

Response: The reviewer raised an excellent question about the BAs density dynamics. As shown in our previous study[4], BAs at different developmental stages contain different amount of lipids. At postnatal day 1, BAs contain very limited amount of lipid droplets. Using this protocol, we isolated BAs from different age mice: postnatal day 1 (P1), 8 and 30. BAs from the BAT of P8 and P30 but not from P1 mice can be isolated with the current protocol. Additionally, BAs from P1 can be enriched by low speed centrifuge.

In this study, we isolated BAs from adult mice (6-8 weeks old) breed in a thermoneutral condition. In this revised manuscript, we examined the cells exist in the solution below the BAs layer. The BAs were genetically labeled with tdTom, so that we can trace whether BAs were completely separated from the non-BA cells with our protocol. As shown in Figure 3 G, cells recovered from the clear 6% iodixanol solution were tdTom negative. Also, no obvious lipid droplets were visible in these cells. On page 7, line 209 to line 217, we put a paragraph to describe these new results. These data suggest that our new protocol can efficiently separate the BAs from the non-fat cells.

Minor Concerns:

Step 2 "let the flask containing BAT and digestion buffer sit on ice for 1 hour". The purpose and importance of this 1-hour incubation at 4'C is not clear. The authors could provide more information on this.

Response: Cold treatment usually slows down enzymatic activity and gene expression[5]. We put the tissue on ice for 1 hour to reduce the BAs cell activity and to let the digestion enzymes efficiently perfuse into the brown adipose tissue. One page 10, we put a paragraph to explain the rationals (lines 292-295).

Step 3 "the temperature of the incubator at 35°C". Is 35°C a critical temperature for the digestion? Is 5% CO2 recommended? After all, 37°C incubators are commonly found in biomedical laboratories for mammalian and bacteria cell culture.

Response: For the enzyme digestion process, 37°C is a theoretically best digestion setting. However, in our hands, the incubator sometimes is overheated and the temperature can reach up to 38°C. At 35°C, the digestion works as well as at 37°C, and we do not need to worry about the overheating.

This digestion does not need to be done in a 5% CO2 incubator.

References

1. Mahdaviani, K., Benador, I. & Shirihai, O. Assessment of Brown Adipocyte Thermogenic Function by High-throughput Respirometry. *Bio Protoc* **5**, (2015).

2. Kriszt, R. et al. Optical visualisation of thermogenesis in stimulated single-cell brown adipocytes. *Scientific Report.* **7**, 1383 (2017).

3. Hagberg, C. E. et al. Flow Cytometry of Mouse and Human Adipocytes for the Analysis

of Browning and Cellular Heterogeneity. *Cell Report*. **24**, 2746-2756.e5 (2018).

4. Negron, S. G., Ercan-Sencicek, A. G., Freed, J., Walters, M. & Lin, Z. Both proliferation and lipogenesis of brown adipocytes contribute to postnatal brown adipose tissue growth in mice. *Scientific Report*. **10**, 20335 (2020).

5. Sonna, L. A., Fujita, J., Gaffin, S. L. & Lilly, C. M. Invited review: effects of heat and cold stress on mammalian gene expression. *Journal of applied physiology* **92**, 1725-1742 (2002).