AUTHOR RESPONSES:

We would like to thank the editor and reviewers for their insightful comments. We will address each of these comments point by point below. Thank you again for your time and consideration.

Editor:

1) Please copyedit the manuscript for numerous grammatical errors. In particular, the protocol is full of comma splices. Please use conjugates like "and" appropriately. See 1.2.3.15 as an example, but please correct this throughout.
The proper conjugates are now present throughout the manuscript. The manuscript has been edited for further grammatical errors.

2) "Forceps" is plural. See 1.2.3.13 note, 1.2.4.2, etc. This also applies to scissors, which should not be preceded by "a" – see 1.2.3.15.
The "a" preceding forceps was deleted.

3) 2.5 – "pellet cells 751 x g"
The sentence was edited and now reads "pellet cells at 751 x g".

4) 2.7 – Please use imperative tense.
The sentence was re-written using imperative tense.

5) Discussion – "that can hamper with"
The following sentence was corrected and "prevent" is used instead of hamper.

6) Please ensure that the references appear in the following format: [Last name, F.I., Las tName, F.I., Last Name, F.I. Article Title. Source. Volume (Issue), First Page – Last Page, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.
a. Please abbreviate all journal titles.
b. Please include volume, issue numbers, and DOIs for all references.

The listed references now meet the proper format.

7) Please include y axis tick labels on Figure 3 A (center, right).
This issue have been addressed and corrected.

8) Please use larger fonts for Figure 1, Figure 3, Figure 4, x and y axis tick labels.
A larger font size was used for all axes tick labels.

9) Please add more details to steps 1.2.2.1 and 1.2.2.2 describing how to eliminate air bubbles.
The method used to eliminate air bubbles was described.

10) Please specify the instrument used to make the incision in steps 1.2.3.3, 1.3.3.13.
The instrument used in these steps was specified.

11) Please correct step numbers referenced in 1.2.4.4. (18 and 19), and 2.4.4. (Step 4.4)

The referenced steps (mentioned above) were corrected.

12) Please describe centrifuge speeds as "x g" instead of the machine-dependent "rpm", in step 1.3.4.3.
All centrifuge speeds are now described as x g.

13. Formatting:
-1.3.3.16 and 1.3.3.17 should be substeps of 1.3.3.15.
-1.3.3.19-1.3.3.30 should be substeps of 1.3.3.18.
-2.4.4 – There is no step 4.4. Please correct the step number.
-2.5 – Paraformaldehyde is toxic. Please provide a caution statement as a note.
-First sentence of results section – Please fix the text formatting.

All the above formatting errors mentioned have been corrected.

14) Length exceeds 2.75 pg of highlighted material and should be reduced accordingly (1 pg minimum). We cannot film the procedures for all three tissues so it is recommended to only focus on one.

The highlighted material has been reduced to 2pgs and focuses solely on the liver dissociation protocol.

15) Visualization: Protocol is discontinuous. For instance, the aorta dissection should not be highlighted as insufficient detail will be filmed. Please choose which sections are most important to be visualized and highlight a continuous section of protocol. Only steps that are not essential to the section being filmed should be unhighlighted. We will not be able to produce a video from isolated steps in the sections.

Highlighted material now focuses on the liver dissociation protocol (the most challenging tissue) and provides a continuous script.

16) Additional detail is required:
-1.1.3.1, 1.3.3.2 – Is the mouse placed ventral side up or down? One assumes down, but the subsequent incisions made suggest that it is in fact up.
-1.2.3.2 – What orientation is the mouse placed in?

Additional details regarding the orientation have been provided.

17) Results:
-Please discuss the results shown in Figure 3 and Figure 4 in the Results section. What is the interpretation of the data?
Each figure and its sub content have been thoroughly discussed within the result section.

-Please define all statistical tests and p values and also define the error bars (SD, SEM, etc.) in the figure legends of Figures 2 & 3.
All statistical tests, p values and error bars are now properly defined.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

(A) Title: The title is to the point, describes the intent of the work and offers a practical example of the work.

The reviewer expressed no concerns, section remains unchanged.

(B) Abstract:

1. Short abstract could mention isolation of resident macrophages, not just characterization

The short abstract was re-written to address the reviewer's feedback.

2. Long abstract should still be to the point; the authors spend 4 sentences with background about obesity before mentioning that this publication is essentially about the techniques of isolating and characterizing macrophages. The abstract, at times, is the only thing that many readers will read. It needs to grab the attention of the reader and convey the purpose of the article.

The long abstract was re-written to address the reviewer's feedback.

(C) Introduction:

The introduction provides sufficient background to preface the purpose of the study. The authors include a motivation for the work.

The reviewer expressed no concerns, section remains unchanged.

(D) Results: This submission does not present novel results. The strength is the detailed protocol and as a way of demonstrating the quality of isolated macrophages, the authors cite the results of their previous publication. The figures included with this submission are DIRECT copies of the figures from the previous publication.

1. According to the Peer Review Specialist that was consulted, this is permissible, providing that, "they gained permission from the original journal. "

The permission for re-print was granted by the Journal of Immunology.

2. This reviewer recommends that the flow cytometry data figures (histograms) have better labels, including the specific fluorochromes used for the staining and detection. As a methods paper, details to this degree would be extremely useful in reproducing the protocol and results. Just because JI let you publish poorly labeled figures, the authors should be held to a higher standard for such a well written method based paper such as this.

The labels for all flow cytometry plots have been improved and included as required.

(E) Methods:

The methods described are well written, have adequate detail and are well organized.

The reviewer appreciates the attention to detail and enjoyed working through the protocol.

The reviewer expressed no concerns, section remains unchanged.

(F) Discussion: The Discussion is a very useful component of the overall submission. It includes not only discussion of results, but in depth troubleshooting and practical tip and advice. This discussion is very well done and useful.

The reviewer expressed no concerns, section remains unchanged.

1. Although the authors mention the use of viability dyes to enrich viable collections, there are no examples of this technique in the figures.
A plot demonstrating the use of the viability dye Texas Red live/dead stain has been included (Figure 1).

2. The authors mention that sorting can be used to collect cells for DNA and RNA analysis. The authors also mention the collection of cells in fetal bovine serum to enhance recovery (the reviewer assumes that this would be to collect live functional cells, not cells for DNA/RNA analysis). However, serum contains RNAses that would harm RNA downstream analysis. The authors should specify the collection medium for the specific downstream application

The reviewer makes an important point and consequently this was addressed. The collection medium needed for specific downstream application that minimizes RNA degradation is now explained.

References: Appropriate references cited.
The reviewer expressed no concerns, section remains unchanged.

Reviewer #2:
Manuscript Summary:
The manuscript by Allen et al. gives an excellent overview about current methods for cell isolation of adipose tissue, liver and the aorta. The step wise procedure is clear and the materials are listed properly.

(1) Especially, for researchers that are new in the field, this manuscript could be very useful. However, more established M2 markers, such as CD206 and CD301 should be discussed.

This is an excellent point and we have addressed more established M2 and M1 macrophage markers for flow cytometry and QRT PCR applications.

(2) Further, background staining of CD11c (clone N418) is usually very high, compared to the isotype control. Therefore, comparison of blanks, CD11c isotype and CD11c staining in lean and obese mice should be shown and the gating schemes should be recommended.

Although this is an excellent observation, the purpose of this publication is to focus on the isolation and characterization of macrophage populations from different tissues inflamed during diet induced metabolic disorders and consequently we wish to not focus on the disadvantages of the CD11c (clone N418) antibody. The high background of the CD11c antibody (clone N418) can be corrected for by using the proper controls to identify accurate gating boundaries. The use of proper controls (including SS, isotype controls and FMO) to define reliable gating boundaries is stressed in the manuscript. Additionally alternative M1 phenotype antibodies are available and can be used in substitute. Unfortunately, isotype controls could not be included for each experimental group (normal chow, HFD, HFHCD). This is because doing so will require additional time to obtain the data files from the other co-first author of the 2016 JI publication and this individual is no longer at our institution. Again, the aim of this publication is not to focus on the disadvantage of mouse CD11c antibodies since the selection for other M1 specific antibodies have grown over the years.