JoVE58649R1

We thank the Editors and reviewers for their thoughtful review and comments. A detailed annotated response to the comments follows:

**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: Noted

2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

Response: We have done this

3. Figure 3: Please include a space between numbers and their corresponding units (i.e., 50 x g, 10 min, etc.).

Response: These changes have been made

4. Figure 5: Please change 10e12 to 1012, and change ml to mL.

Response: We have revised Figure 5 accordingly

5. Please provide an email address for each author.

Response: Email addresses have been included

6. Short Abstract: Please revise it to be in complete sentences.

Response: We have made changes as appropriate

7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

Response: We have rephrased the introduction

8. Please spell out each abbreviation the first time it is used.

Response: We have made changes as appropriate

9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Response: We have made changes as appropriate

10. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Response: We have made these changes

11. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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 and Reagents. For example: Styrofoam, Nalgene, etc.

Response: We have made changes as appropriate

12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have made changes as appropriate

13. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

Response: We have made changes as appropriate

14. Please add more details 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. 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.

Response: We have made changes as appropriate

15. 1.5: What type of water is used here, running water or deionized water?

Response: Running water was used here.

16. 2.1: Please specify the age, gender and type of mouse used.

Response: The range of age, gender and type of mouse from which isolations have been performed is now listed in the representative results section.

17. 2.5: Please specify all surgical instruments used in the protocol.

Response: We have specified as appropriate

18. 3.3: Please specify the low flow rate used here.

Response: We have mentioned the flow rate used.

19. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Response: Revised

20. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: Done

21. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We have made changes as appropriate.

22. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

Response: Noted

23. Representative Results: Please move details about the protocol to the Protocol section, and move the discussion about the protocol to the Discussion. As Figure 5 shows the size and concentration of liver tissue EVs, please briefly mention the methods to measure the size and concentration of liver tissue EVs. For figures showing the experimental set-up, please reference them in the Protocol.

Response: We have made these changes as appropriate. Figure 1 is referred to in the Protocol session.

24. Discussion: Please also discuss any limitations of the technique.

Response: We have mentioned limitations within the discussion.

25. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Response: We have made changes as appropriate

26. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Response: We have made changes as appropriate

27. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Response: We have made changes as appropriate

28. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: We have made changes as appropriate

**Reviewers' comments:**  
  
**Reviewer #1:**  
  
1. The characterization of vesicles is inadequate to determine whether these are liver derived vesicles alone or whether there are other contributing particles such as lipoprotein particles and/or protein aggregates. To convincingly demonstrate that these are only liver derived vesicles, the authors would have to do either density gradient fractionation or column based separation and quantify vesicles versus protein aggregates versus lipoproteins. In addition, the characterization of vesicles in terms of nanoparticle tracking analysis alone is insufficient; they need to show via additional complementary methods including immunoblotting and electron microscopy with immune electron microscopy that they are isolating extracellular vesicles.

Response: We have included reference to this in the discussion section

2. Page 1, line 32. The method is really isolating vesicles ex vivo and not in vivo.

Response: We agree and have removed reference to in vivo.

3. Line 164. It should be clarified that this is the 10mL collagenase medium that was set aside in step 1.8.

Response: We have added this notation.

4. Line 139. The low flow rate for HBSS infusion should be included.

Response: We have included the flow rate.

5. Line 236. A range is provided 1.74 x 10e15 to 4 x 10e15 with a mean of 3.46 x 10e12 per mL. The mean does not fit within the range. Besides this calculation, number of vesicles obtain per liver or per gram of liver tissue should be also provided.

Response: Thank you for bringing this to our attention. The mean was 3.46 x 10e15 per mL. We have now corrected this.

6. While vesicles released during dissociation of the liver may be one way to collect liver derived vesicles, the authors do not demonstrate or compare how this method may compare with vesicles collected during perfusion of the liver by collecting perfusate. While this may not be important, as there is no established "gold standard," this should be included in the discussion as a possible alternative.

Response: We concur and have now included this within the discussion.

7. Page 1: Line 35: The first line of 'Introduction' is not grammatically correct. Please check.

Response: Thank you for bringing this to our attention. We have now corrected this.

8. Figure 5: Y axis is showing concentration 10e12 (particles/ml), but the average yield is 3.46X1015. In write up, there is also discrepancy between the ranges of EV concentration (1.74X1015 to 4.00X1015) and mean of 3.46X1012 particles per ml. Please recheck the values.

Response:   
  
Response: Thank you for bringing this to our attention. We have now corrected the typos and revised Figure 5.

**Reviewer #2:**  
  
For a balanced view, the potential drawbacks of the protocol shall be discussed. One common issue with the two-step perfusion of the liver is cell damage. The author shall add a step after the perfusion to check the cell viability. This can be easily done using Trypan blue. High viability is essential, as dying or dead cells will release intracellular components to the buffer affecting the purity of EVs.

Response: We thank the reviewer for the comments, and have included these considerations within the discussion.

In fact, though the author claims the method will produce EVs of high purity, they did not mention how the purity was checked.

Response: We have removed mention of purity of EVs.

During the process of perfusion, EVs can possibly be flushed out. How do the authors address this concern?

Response: We concur and have included these considerations within the discussion.

Another issue is the normalization of the yield of EVs. It appears that the yield of EVs with this method can only be normalized to the number of mouse used. Under pathological conditions, when the ratio between liver and body weight is significantly changed, this normalization approach can be problematic. I believe adding some discussions on these issues will make the manuscript more objective.

Response: We concur with the comments. While the yield can be normalized per mouse, we do not expect that the method will isolate 100% of the tissue EV. A loss of EV during ultracentrifugation can also be expected. Thus the comparison of yield is not likely to be useful. These issues are discussed in the representative results and discussion sections.

**Reviewer #3:**

Exosomes are secreted nanovesicles. Will liver perfusion and collagenase digestion cause the release of some kind of immature "exosomes"? Or will it contain other microvesicles - e.g. apoptotic bodies that are released from dead/dying cells resulted from liver perfusion?

Response: This protocol will isolate extracellular vesicles. These include exosomes, as well as microvesicles. Within the discussion, we have mentioned the use of cell viability as a quality measure to monitor for excessive cell death.

The authors didn't provide any western blots of EV markers. Thus, the quality of the liver tissue-derived EVs is not sure.

Response: We are not aware of any EV protein markers that provide information about quality of EVs. There are no universal markers of EVs.