Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

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

We thank the editor for careful review of our manuscript. The resubmitted text has been proofread thoroughly by all authors.

2. 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. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

The protocol has been edited accordingly.

3. 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. See examples below.

The protocol has been edited accordingly.

4. 1.2: Please specify the amount/mass/concentration of protease and phosphatase inhibitors added in this step.

The text has been edited to clarify a final concentration of 1X, and the table of materials has been edited to reflect the 100x stock solutions of protease and phosphatase inhibitors.

5. 1.7: Please specify the pore size of the cell strainer.

The pore size of the cell strainer has been clarified.

6. 1.9: Please split into two steps.

This step has been split into a subsequent step.

7. 2.2: Please list an approximate volume of solutions to prepare.

Recommended volumes of iodixanol solutions have been added to the protocol.

8. 4.1: Please describe how protein quantification is done.

The required sample or standard volume for protein quantification using the recommended EZQ kit has been added to the protocol. The kit should be performed according to the manufacturer instructions, as specified in the text.

9. As data from mass spectrometry are shown, please describe how to perform mass spectrometry in the Protocol.

The mass spectrometry protocol section has been separated into clearer steps for the reader to follow. LC-MS/MS parameters followed in this study have been described in great detail in our previous publication, which is now cited in the text.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

The protocol has been simplified as much as possible, per the editor’s comments.

11. Please include single-line spaces between all paragraphs, headings, steps, etc.

The text has been edited accordingly.

12. 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.

Steps to be included in the video have now been highlighted.

13. 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 see above.

14. 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.

Please see above.

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

Figures have been uploaded as .tiff files.

16. Figure 1: Please change “268,000 g” to “268,000 x g” and include a space between numbers and their temperature units (i.e., 4 °C).

The figure has been edited as requested.

17. Figure 2D: Please explain what the different images represent (indicate the tissues).

The figure legend has been clarified.

18. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Items have been sorted as requested.

19. References: Please do not abbreviate journal titles.

The JOVE endnote style file has been used to create references for this manuscript.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:

Manuscript Summary:

In this manuscript, Hurwitz and colleagues reported the application of a method, previously published by the same authors (Hurwitz et al 2018), to isolate and purify EVs from tumor specimens in addition to brain tissue. The paper is well written and interesting however some issues need to be resolved before consideration for publication

Major Concerns:

1) In order to ensure the reproducibility and consistency of this method the authors should perform replicates, also in different tumors rather than representative findings displaying results. In fact is not clear how many replicates and which tumors were used.

We thank the reviewer for careful review of our manuscript. Replicate experiments of similar lung tumor xenografts were used in the application of this method, and are stated in lines 110-116. The method has been expanded in detail from our previous publication, as requested by the JOVE editors, and representative data reflects both brain and tumor-derived EVs to demonstrate the broader applicability of the technique. As per the Journal of Visualized Experiments author instructions, figures display representative findings of the methodology application.

2)Lines 99-100:"EVs could harbor great diagnostic value, particularly in specimens where histologic evaluation is limited". This is an important issue to translate EV information into the clinics. Since EV cargo should reflect tumor characteristics, the authors have verified the presence of tumor markers in EV samples?

The reviewer clearly poses an important question with regards to the biological significance and utility of tissue-derived EVs for cancer diagnostics and phenotyping. However, the direct comparison of EV protein to tumor protein is beyond the scope of this study. In addition, while immunohistochemical analysis of tissue specimens can provide clues toward histologic origin and variable broad tumor classifications, universal and reliable tumor markers have not yet been validated for lung tumors used in this study. As pointed out, future studies will be important to compare the protein expression in tumor-derived EVs to the original tumor sample. In this study, we simply provide a method to reliably harvest EVs from tissue, which may be used by researchers to answer these important questions in subsequent studies. This limitation is now stated in lines 322-324 in the discussion.

3) Is the focus and novelty of this paper is the application of isolation method to tumor tissues? If yes why the author shown results from brain samples?

The focus of this paper is an expanded detail of the method used in a study previously published by our laboratory (Hurwitz SN et al., J Neuro Methods, 2018), as invited by the JOVE journal editors. As our previous work aimed to optimize this method for the enrichment of EVs from brain tissue, we believe the inclusion of this data provides a necessary comparison for the novel application of the technique to whole tumor tissue.

4) to better read the content of figure Figure 3 it could be better present the results as a table.

While we agree that a table may equally demonstrate our findings in Figure 3, we believe the figure currently provides a simple and easy way to understand the numerous EV proteins enriched in our samples, and politely wish to maintain the structure of the figure.

Minor Concerns:

1) In Figure 2D add the number for the scale bars.

The scale bars for Figure 2D are currently present in the figure legend.

Reviewer #2:

The manuscript titled "Extraction of extracellular vesicles (EVs) from whole tissue" by Hurwitz and colleagues expand on a previous study in the Journal of Neurosciences Methods (Hurwitz, 2018) now describing their protocol for extracting small EVs from solid tumors. The authors indicate that "this method offers a reproducible technique to extract EVs from solid tissue for further downstream analyses." The suggested significant of this approach is that extracting EVs from ex vivo tissue specimens will provide a more direct insight of the physiological and pathological roles of these particles. The authors clearly stated the potential applications of their protocol and why this is important in the field. Overall, the protocol is straightforward; however, a number of important details are lacking.

Major Concerns:

1. The authors need to clarify the number of tissue samples used for their isolations and the source. The author state "Whole brains were obtained with approval from the Institutional Animal Use and Care Committee (IACUC) of the Florida State University. Tumor specimens were generously donated by Dr. Mandip Sachdeva under approval of the Florida Agricultural and Mechanical University IACUC". Based on the information provided, it is unclear the type of tissues used and number of samples evaluated. Therefore, they should add the tumor types, number of individual samples studied, and the animal species for which the tissues were derived.

We thank the reviewer for careful review of our manuscript. These details have now been expanded upon in lines 110-116, as requested.

2. Following the concerns above, the authors make a general statement, "we show that tumor specimens may harbor predominately dense small EVs compared to the lighter EVs isolated from brain tissue". Again, what tumor type and how many different tumors and tumor types were analyzed? Without an idea of the number of different samples and tumor types evaluated, this type of statement is unfounded.

The details of the tumor types used have been expanded upon in lines 112-116, and the limitations of this statement now discussed in lines 343-353.

3. Figure 2C. The authors show an example of NTA; however, the representation figure does not allow the reader to understand the overall number of particles isolated. They provide a plot of "percentile of total vesicles" relative to the vesicle size. First, this is an atypical way to plot this type of data and second it looks as though the vast majority of EVs are greater than 250 nm, or not sEVs. The authors should plot as number of particles versus size. Furthermore, it is also impossible to determine the number of vesicles isolated as a function of the starting tissue samples. It is essential that the authors provide this type of information.

The NTA plot has been revised to make the data interpretation easier for the reviewer and readers. It should be clear that the vast majority of EVs (92%) are smaller than 250 nm. The plot now includes concentration of vesicles harvested from the tissue.

4. Given one of the aims of the study was "to attempt to isolate small EV subpopulations highly enriched in endosomal-derived exosomes", it is surprising that the authors did not include CD9 analysis in their Western blots, given simultaneous expression of CD9, CD63, and CD81 is used to define exosomes. They suggest it is present in the summary of cherry-picked proteins in Figure 3, but was not validated by Western blot. Also, it was unclear why fewer proteins were evaluate in the brain versus tumor tissue-derived EVs (Figure 1B).

While CD9 is often present in small EVs, including exosomes, this tetraspanin is not always used universally to validate the presence of exosomes in samples. We highlight this protein found in our mass spectrometry analysis, but our laboratory does not routinely probe for the presence of this protein by immunoblot analysis. In our current proof of principle for this study, we have demonstrated the presence of multiple accepted EV protein biomarkers including two tetraspanin proteins. We have additionally confirmed the depletion of a non-EV protein calnexin in our preps. These efforts exceed the current expectations and guidelines set by the International Society of Extracellular Vesicles, and therefore we have not proceeded to validate all the EV proteins highlighted in Figure 3 by western blotting.

In Figure 2B, we aimed to more thoroughly characterize the tumor EVs isolated, as this was the novel application of our study. A more complete characterization of brain-derived EV isolates has been previously performed by our laboratory (J Neuro Methods, 2018). An abbreviated immunoblot analysis of the brain samples is simply shown here for visual comparison to the tumor-derived EV fractions.

5. Figure 3. What is the purpose of this figure? The authors do a very poor job of explaining the downstream proteomic analysis of "a representative tissue-derived EV sample". There is no information regarding what fraction(s) was analyzed by mass spectrometry, the amount of input protein, etc. Then the authors provide a summary of "highlighted vesicle proteins". Typically, MS yield over 1,000 proteins from isolated exosomes. How the proteomic data was analyzed is unclear and overall the description of this downstream analysis underwhelming.

A more clear methodology of the mass spectrometric protocol has been added to the manuscript and the associated figure legend, as requested. This protocol has been extensively described in detail by our laboratory in several publications that are now clearly cited in the method.

Minor Concerns:

1. The authors should include additional background regarding the heterogeneity of EV subpopulations (reference Zijlstra et al., 2018, Nature Cell Biology & Zhang et. al, 2018, Nature Cell Biology) and what EVs are enriched by their approach.

A summary of the findings of heterogeneous EV subpopulations identified in the study by Zhang et al. and commentary by Zijlstra et al. has been added to the discussion.

2. The authors should add a step for cutting the tissues into several pieces before adding it to the dissociation buffer.

We have added this optional step to the protocol, as requested.

3. In terms of using the Dounce homogenizer, the authors should indicate in their protocol that the number of strokes should be dependent on the sample if it is classified as a soft tissue or a hard tissue.

This is an interesting suggestion that we have discussed in the protocol.

4. The authors should state why they deviated from the protocol of Kowal et al., 2016 (350,000 g for 60 minutes) wherein they decreased ultracentrifugation speed and time (268,000 g for 50 mins) in the density gradient purification step.

Our protocol for the bottom-loaded iodixanol density gradient separation and purification of EVs has been adapted from the technique used by Kowal and colleagues. The ultracentrifugation speed was simply adjusted for use with our current MLS-50 rotor (max speed 268,000 g). The time of spin does not include deceleration time (without breaking mechanisms), which adds a substantial amount of time to the centrifugation (about 15-20 minutes). We have demonstrated similar densities of fractionation and efficiency in particle separation compared to the Kowal et al. study that we have previously published in two separate papers from our laboratory (Hurwitz et al., J Virology, 2017; Hurwitz et al., J Neuro Methods, 2018).