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TITLE:

Extraction of Extracellular Vesicles from Whole Tissue

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SUMMARY:

Here, we provide a detailed protocol to isolate small extracellular vesicles (EVs) from whole tissues, including brain and tumor specimens. This method offers a reproducible technique to extract EVs from solid tissue for further downstream analyses.

ABSTRACT:

Circulating and interstitial small membrane-bound extracellular vesicles (EVs) represent promising targets for the development of novel diagnostic or prognostic biomarker assays, and likely serve as important players in the progression of a vast spectrum of diseases. Current research is focused on the characterization of vesicles secreted from multiple cell and tissue types in order to better understand the role of EVs in the pathogenesis of conditions including neurodegeneration, inflammation, and cancer. However, globally consistent and reproducible techniques to isolate and purify vesicles remain in progress. Moreover, methods for extraction of EVs from solid tissue *ex vivo* are scarcely described. Here, we provide a detailed protocol for extracting small EVs of interest from whole fresh or frozen tissues, including brain and tumor specimens, for further characterization. We demonstrate the adaptability of this method for multiple downstream analyses, including electron microscopy and immunophenotypic characterization of vesicles, as well as quantitative mass spectrometry of EV proteins.

INTRODUCTION:

Small extracellular vesicles (EVs) include endosomal-derived exosomes and small membrane-shed microvesicles that are of broad biomedical interest. Small EVs are composed of a heterogeneous population of 50-250 nm membrane-bound vesicles containing biologically active proteins, lipids, and nucleic acids that are collectively believed to play important roles in a multitude of disease processes. Advancing research has particularly implicated these vesicles in

neurodegenerative and prion disorders, infectious processes, autoimmune or inflammatory conditions, and tumor growth and metastasis[1-13]. Rapidly growing biomedical research into the significance of EVs in disease pathogenesis has generated parallel interest in developing reproducible and rigorous methods for the isolation and purification of these vesicles.

A historical and current challenge in EV characterization has been the inability to fully separate small EV subpopulations. This challenge is largely due to our limited understanding of the differing molecular mechanisms governing the biogenesis of distinct vesicles. Overlapping size, density, and biologic cargo between subpopulations further convolutes these distinctions. Part of this challenge has also been the use of widely differing enrichment techniques, providing inconsistency in downstream analysis of isolated vesicles across laboratories, and undermining the global effort to illuminate categorical EV populations.

It is worth noting that the majority of EV characterization has been performed from *in vitro* collection of cell culture supernatant, with more recent *in vivo* studies describing techniques to isolate vesicles from animal or human body fluids, including plasma, urine, and saliva. While EVs are present in large quantities in circulation, it also recognized that these vesicles play important roles in cell-to-cell communication events and are present in the interstitium of cellular tissues. In the context of cancer, interstitial EVs may be particularly important in modulating the tumor microenvironment for cancer cell seeding and metastatic growth[14,15]. Consequently, there is value in the development and optimization of techniques to extract vesicles from solid tissue specimens. These methods will provide a means to directly study organ- or tumor- derived EVs harvested from clinical specimens, including small biopsies and partial or full organ resections.

In this study, and in a previous report published by our laboratory[16], we aim to address several major current concerns in EV enrichment methodology: 1) to describe a reproducible technique to isolate and purify EVs to the highest standards currently accepted in the field; 2) to attempt to isolate small EV subpopulations highly enriched in endosomal-derived exosomes; and 3) to provide a protocol for the extraction of these vesicles from solid tissue specimens for the purpose of further characterization.

Recently, Kowal and colleagues described a relatively small-scale iodixanol density gradient to separate and purify EV subpopulations with greater efficacy than comparable sucrose density gradients[17]. In the cited study, dendritic cell-derived EVs captured in a relatively light density fraction, consistent with a density of 1.1 g/mL, were highly enriched in endosomal proteins believed to be most consistent with a high proportion of exosomes present in this fraction. According to the authors, these “bona fide” exosomal proteins included tumor susceptibility gene 101 (TSG101), syntenin-1, CD81, ADAM10, EHD4, and several annexin proteins[17]. We later adapted this technique to succeed a method of tissue dissociation described by Perez-Gonzalez et al[18] and a subsequent differential centrifugation protocol to isolate whole brain-derived EVs[16]. We also demonstrated the utility of this method in characterizing EV proteomes by combining a sequential protocol for downstream quantitative and comparative mass spectrometry of vesicular protein, previously described by our laboratory[19]. This work paralleled that from the Hill laboratory, in which EVs were enriched from the frontal cortex of brains[20].

In this study, we elaborate on this technique and extend the application of the protocol recently published from our laboratory to the isolation of EVs from solid lung tumors. To our knowledge, this is the first study to describe a protocol to enrich EVs from *ex vivo* tumor specimens. Given the widespread interest in EVs as novel diagnostic biomarkers and their role in tumorigenesis, this method will likely prove valuable to a growing number of scientific researchers. From a clinical perspective, interstitial EVs could harbor great diagnostic value, particularly in specimens where histologic evaluation is limited. Our hope is that the method outlined here will provide a foundation for a reproducible technique to harvest EVs from fresh or frozen animal or human surgical specimens, paving the way for future work to uncover the significant roles in disease pathogenesis these small vesicles may play.

PROTOCOL:

Whole brains were obtained with approval from the Institutional Animal Use and Care Committee (IACUC) of the Florida State University. A total of twelve mouse brains (3 brains from each age group: 2, 4, 6, and 8 months) from a C57BL/6 J background were used for EV extraction, as previously described[16]. Lung tumor specimens were generously donated by Dr. Mandip Sachdeva under approval of the Florida Agricultural and Mechanical University IACUC. Lung tumors were derived from the human adenocarcinoma cell line H1975 grown in immunodeficient Balb/c nu/nu nude mice. Data from two representative tumor replicates are highlighted in this study.

NOTE: A schematic overview of the vesicle isolation and purification method is provided in **Figure 1**.

1. Tissue Dissociation and Differential Centrifugation

1.1. Prepare 10 mL of dissociation buffer (10 mg of papain; 5.5 mM L-cysteine; 67 µM 2-mercaptoethanol; 1.1 mM EDTA) in Hibernate-E medium per approximately 0.4-1.0 g of tissue. Note that all solutions used for EV enrichment and purification should be diluted in ultrapure filtered water.

1.2. Add whole fresh or frozen tissue to dissociation buffer in a 50 mL conical tube, and incubate in a warm water bath at 37 °C for 20 min. Tissue may be cut into smaller fragments before incubation if needed.

1.3. Following the incubation, add protease and phosphatase inhibitors for a final 1x concentration to the dissociation buffer containing the tissue.

1.4. Pour the solution containing the tissue into a loose-fit Dounce homogenizer, and gently dissociate the tissue using approximately 30 slow strokes per sample. The number of strokes may be adjusted based on tissue consistency.

1.5. Transfer dissociated tissue in buffer to a 50 mL conical tube and centrifuge at 500 x *g* for

5 min at 4 °C to pellet the cells and remaining fibrous or cohesive tissue fragments.

1.6. Transfer the supernatant to a clean 50 mL conical tube and centrifuge at 2,000 x *g* for 10 min at 4 °C to pellet and discard large cellular debris.

1.7. Transfer the supernatant again to a clean 50 mL conical tube and centrifuge at 10,000 g for 40 min at 4 °C to pellet undesired larger vesicles or small apoptotic bodies.

NOTE: This pellet can be saved for additional study of larger vesicles if desired.

1.8. Decant the supernatant through a 0.45 µm filter into a clean 12 mL ultracentrifugation tube.

NOTE: Densely fibrotic tissue samples may not be easily filtered. These samples can be filtered through a 40 µm cell strainer, then passed through serially smaller needles (18 G, 20 G, 22 G) before filtering through the 0.45 µm filter.

1.9. Ultracentrifuge the sample at 100,000 x *g* for 2 h at 4 °C to pellet small EVs.

1.10. Decant the supernatant and leave the ultracentrifugation tubes inverted for 5-10 min, tapping frequently to remove residual liquid on the sides of tubes. Residual fluid can also be aspirated using an aspirating vacuum pipette.

1.11. Re-suspend EV pellet in 1.5 mL of 0.25 M sucrose buffer (10 mM Tris, pH 7.4). It is important to ensure full re-suspension of the pellet in this step. To do so, cover the tubes with parafilm before vortexing EVs into solution. Rock the ultracentrifuge tubes for 15-20 min at room temperature before a final vortex mix.

1.12. Briefly centrifuge the tubes at a speed no more than 1,000 x *g* to recover the liquid suspension at the bottom of the tube.

1.13. Proceed to the gradient purification steps below, or if needed, store EV suspension at 4 °C overnight.

2. Density Gradient Purification

2.1. Add 1.5 mL of 60% iodixanol (stock solution in water) to the 1.5 mL sucrose/Tris buffer containing EVs to create a final solution containing 30% iodixanol. Pipette up and down several times to mix solution thoroughly. Be cautious to avoid losing solution in the pipette tip, as the high iodixanol concentration is quite viscous.

2.2. Transfer the 30% iodixanol buffer solution containing EVs to the bottom of a 5.5 mL ultracentrifugation tube.

2.3. Prepare at least 1.5 mL of 20% and 10% iodixanol solutions per sample in ultrapure water from the 60% iodixanol stock solution.

2.4. Measure 1.3 mL of 20% iodixanol and carefully layer on top of the bottom gradient layer using a syringe and an 18 G needle. To avoid mixing the layers at the density interface, keep the bevel of the needle in contact with the inside of the ultracentrifugation tube just above the meniscus and add the solution dropwise.

2.5. Layer 1.2 mL of 10% iodixanol solution on top of the 20% iodixanol layer using the same technique as above.

2.6. Carefully balance and load the ultracentrifugation tubes into rotor buckets and centrifuge in a swing-bucket rotor at 268,000 x *g* for 50 min at 4 °C. Set the acceleration and deceleration speeds of the centrifuge to the minimum rates allowed to avoid disruption of the density layers.

2.7. Label ten 1.5 mL microcentrifuge tubes for each sample to correspond to fractions 1 through 10 of the density gradient. Fraction 1 is designated as the topmost fraction that will be first removed, while fraction 10 is the bottom fraction last removed.

2.8. Once the gradient ultracentrifugation has completed, gently remove the tubes from the rotor buckets and place in a stable holder. A visible band of vesicles will often be seen in the fraction of interest. Pipette ten serial fractions of 490 µL from the top of the gradient into the corresponding tubes. There will be a small amount of fluid remaining at the bottom of the tube that likely contains contaminating proteins. This sample can be discarded, or retained as fraction 11 if desired.

2.9. Measure the refractive indices of fractions using a refractometer. This can also be performed with a control gradient run in parallel if sample conservation is necessary. Pipette 20-30 µL of each fraction containing iodixanol onto the refractometer surface, and estimate the density by comparing the refractive indices to known iodixanol densities. Note that fractions can be stored overnight in the iodixanol-containing solution if needed before proceeding to the next ultracentrifugation wash step.

2.10. Transfer each fraction to a clean 12 mL ultracentrifugation tube. Add 5 mL of 1x phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to the tube and mix by pipetting slowly up and down.

NOTE: PBS added to the samples should be particle-free, ensured by filtering sterile PBS through a 0.22 µm filter and storing at room temperature to avoid salt precipitation.

2.11. Add an additional 6 mL of 1x PBS to the top and again mix carefully.

2.12. Ultracentrifuge the tubes at 100,000 x *g* for 2 h at 4 °C to re-pellet small vesicles. Decant the supernatant and tap the tubes dry before lysis of vesicles for protein analysis (Step 3) or re-

suspension of EVs for morphologic analysis (Step 5).

3. Lysis and Immunoblot Confirmation of EV Proteins

NOTE: If preservation of whole vesicles for morphologic characterization or biological activity is desired, researchers can proceed directly to Step 5. In initial experiments, or before mass spectrometry analysis, all fractions recovered should be analyzed by immunoblot to confirm fractions with EV proteins.

3.1. To lyse EVs for protein analysis, add 40 µL of strong lysis buffer (5% SDS, 10 mM EDTA, 120 mM Tris-HCl pH 6.8, 2.5% 2-mercaptoethanol, 8 M urea) with the addition of protease inhibitor to EV pellet in ultracentrifuge tube.

NOTE: If non-reducing conditions are desired for antibody detection of proteins, substitute ultrapure water for 2-mercaptoethanol in the strong lysis buffer.

3.2. Ensure complete re-suspension of pellet and lysis of EVs in buffer by placing parafilm over the ultracentrifugation tube, vortexing vigorously, then rocking for 20 min at room temperature before a final vortex.

3.3. Briefly centrifuge at 1,000 x *g* for 30-60 s to recover the entire sample volume. Transfer the sample to a new 1.5 mL microcentrifuge tube and store at -20 °C to -80 °C until further processing.

3.4. To prepare purified lysates for immunoblot analysis, add 5x Laemmli sample buffer (10% SDS, 250 mM Tris pH 6.8, 1 mg/mL bromophenol blue, 0.5 M DTT, 50% glycerol, 5% 2-mercaptoethanol) to the samples for a final concentration of 1x.

NOTE: If non-reducing conditions are desired, replace the DTT and 2-mercaptoethanol with ultrapure water.

3.5. If whole tissue homogenate controls are desired, lyse the tissue in the urea-containing strong lysis buffer detailed above with protease inhibitor, then centrifuge at 10,000 x *g* for 10 min to discard the remaining extracellular matrix, whole cells, or intact cellular debris. Add 5x Laemmli sample buffer to the tissue homogenate for a final concentration of 1x.

3.6. Boil the sample buffer containing EV or tissue homogenate samples at 95 °C for 5-10 min, then load equal volume (from the equivalent of 0.1-0.3 g of starting tissue material; 1-2 µg of purified EV protein) of fractions 1-10 into a 10% SDS-PAGE gel. Load equal mass of tissue homogenate. Greater volume of samples may be needed for detection by less sensitive antibodies.

3.7. Proceed with gel electrophoresis and Western blot analysis as previously detailed[21] to confirm EV proteins in the purified lysates and compare relative EV abundance in fractions.

NOTE: Once EV protein has been reproducibly demonstrated in consistent fractions, the final ultracentrifugation wash described in Step 2.12 may be limited to gradient fractions of interest.

4. EV Protein Quantification

4.1. Use a detergent- and urea- compatible fluorescence-based assay if sensitive quantification of EV proteins is desired for further analysis (see **Table of Materials**). Note that some fluorescence-based assays are compatible with the bromophenol blue present in Laemmli sample buffer, facilitating direct lysis of the EV pellet (in Step 2.12) in this buffer if preferred.

4.2. If the aforementioned fluorescence-based protein quantification kit is used, spot 1 µL of sample or standard in at least duplicate on the provided filter paper, and continue protein quantification according to manufacturer's instructions.

5. Characterization of EV Morphology

5.1. To examine whole vesicles after the final centrifugation wash in Step 2.12, thoroughly re-suspend EV pellet in particle-free 1x PBS. Store EVs at 4 °C for no longer than one week until processing to avoid freeze-thaw cycles.

NOTE: It is beneficial to have previously confirmed fractions consistently containing enriched EV protein by immunoblot analysis to limit morphological analyses to fractions of interest.

5.2. Perform nanoparticle tracking analysis on whole EV samples to determine the concentration and size of vesicles in preparations, as previously detailed[22].

5.3. Characterize EV morphology and confirm size measurements by electron microscopy of vesicles, if desired. EM grids can be prepared from vesicle suspensions following Step 5.1[23], or alternatively can be processed as block preparations[24] from pellets following Step 2.12.

6. In-gel Purification and Trypsin Digestion for Mass Spectrometry Analysis

6.1. If characterization of EV proteomes by mass spectrometry is desired, load equal mass (at least 10 µg of protein) of fractions containing EVs into a pre-cast polyacrylamide gel to separate and purify proteins. Manufacturer supplied pre-cast gels are preferred to reduce the levels of potential contaminating proteins (e.g., keratin) being introduced into the samples.

6.2. Run the sample at least 0.5 inches into the polyacrylamide gel for protein purification, then fix and stain with Coomassie dye, as previously described in detail[25].

6.3. Cut the lanes into 1 mm^3 cubes for trypsin digestion, as previously detailed[19,26].

6.4. Load 5 µL of digested peptide sample into LC-MS/MS instrument for separation on the

analytical column and subsequent analysis by mass spectrometry according to parameters specified in detail[16].

REPRESENTATIVE RESULTS:

A schematic overview of the tissue dissociation, differential centrifugation, and gradient purification of vesicles is displayed in **Figure 1**. The morphologic and immunophenotypic confirmation of gradient-purified EVs is highlighted in **Figure 2.** A diagram of reproducible densities following ultracentrifugation of the 10-30% iodixanol gradient is shown, with two distinct vesicle populations migrating upward to fraction 2 (light EVs) and fraction 5 (dense EVs), dependent on tissue type. Representative immunoblots of gradient fractions exhibit the efficient separation and purification of small tumor-derived EVs in fraction 5. Of note, lung tumor specimens appeared enriched in dense EVs compared to light EVs (in fraction 2) previously harvested from whole brain tissue, again highlighted here. As a global tissue analysis of EVs emerge, it will be interesting to determine the distinct densities of EVs produced by unique tissues. Representative nanoparticle tracking analyses and electron microscopy of tissue-derived vesicles in the predominant vesicle-containing fraction demonstrate the enrichment and preservation of whole vesicles consistent with the known size and structure of small EVs. Finally, **Figure 3** highlights the utility of this method in facilitating downstream mass spectrometry characterization of gradient-purified vesicle proteins, demonstrating the detection of many proteins previously identified in small EVs. Many of these identified proteins are consistent with those enriched in small endosomal-derived EVs previously proposed by the Théry and Hill labs[17,20].

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic overview of vesicle isolation and purification from whole tissue. Following tissue dissociation, pre-clearing differential centrifugation steps, filtration, and ultracentrifugation, crude EV pellets can be resuspended on the bottom of an iodixanol density gradient for floatation separation.

Figure 2. Representative morphologic and immunophenotypic analysis of tissue-derived EVs. (A) Calculated densities of iodixanol gradient following ultracentrifugation, averaged over independent experiments, highlighting fractions containing light and dense EVs. (B) Representative immunoblots of tissue-derived vesicles demonstrating the isolation of predominately dense tumor EVs and light brain EVs. Vesicle isolates are depleted of non-EV protein calnexin. *H*, tissue homogenate. (C) Nanoparticle tracking analysis (NTA) of representative gradient-purified tissue EVs. The smallest detected particle in this sample was 78 nm, and approximately 92% of vesicles detected were 250 nm or smaller, consistent with a high enrichment of small EVs. (D) Representative electron microscopy images of brain tissue-derived EVs. Scale bars = 200 nm.

Figure 3. Summary of highlighted vesicle proteins detected by mass spectrometry of a representative tissue-derived EV sample. Following EV extraction and gradient purification, 10 µg of EV protein in enriched fraction was loaded into a polyacrylamide gel for electrophoresis and in-gel trypsin digestion. In this representative tissue-derived EV sample, a total of 918

proteins were identified by LC-MS/MS analysis. Peptides were identified, analyzed, and found to be enriched in exosomal, lysosomal, and plasma membrane proteins as previously described[16]. Here, we demonstrate the presence of small EV or exosomal proteins in our preparations that have been described by the Théry and Hill labs[17,20].

DISCUSSION:

Much scientific interest has been generated with regards to the roles small EVs play in the tumor microenvironment, as well as in organ development, maturation, and function. Overall, this study provides an optimized workflow for the extraction of intact EVs from whole brain or tumor specimens. While here we simply demonstrate the applicability of this technique to lung tumor-derived EVs, this method could easily be adapted for work on other solid tissues, providing opportunities for further *ex vivo* characterization of small secreted vesicles that are of broad biomedical interest. While beyond the scope of this study, future comparison of EV content to that of originating tissue expression will also be important to establish the utility of isolated vesicles as tissue and disease biomarkers.

In the study previously published by our laboratory, we clearly demonstrate the advantages of the floatation-based density gradient over sedimentation gradients for purification of enriched small vesicles. In essence, a buoyancy-based separation improves purification of vesicles by avoiding contaminant migration through the fractions of interest. This principle appears particularly important when starting from complex samples like whole tissue. Regardless, we stress the importance of the pre-analytical confirmation studies of isolated vesicles described, including examination of EV morphology through complementary techniques such as electron microscopy and nanoparticle tracking analysis, as well as biochemical analyses including immunoblotting. EV proteins in enriched fractions should be confirmed with at least three recognized EV markers, including one tetraspanin protein, as recommended by the *Minimal Information for Studies of EVs* (MISEV) report published in the Journal of Extracellular Vesicles[27]. A negative EV marker should be demonstrated to be absent or highly reduced in EV fractions compared to tissue homogenates. All steps stated above are crucial for quality assurance of small vesicle purification. In addition, early demonstration of laboratory- and user- specific consistency in EV-containing density fractions can save valuable time and cost associated with processing and analyzing all fractions following the density gradient ultracentrifugation step.

It is clear that vesicles from diverse tissues may float to slightly different density fractions, or may separate into predominately light or dense subpopulations. These differences have been previously seen in whole brain-derived EVs compared to EVs harvested from dendritic cells grown in culture[16,17], and are further demonstrated in this study. We show that lung tumor specimens may harbor predominately dense small EVs compared to the lighter EVs isolated from brain tissue. These differences highlighted by the method proposed in this study may promote valuable inquiries into the differential composition of EVs, including tumor EVs that may be packed with dense DNA strands[28-30]. Because of these differences in EV composition, we strongly underscore the importance of these initial technical experiments. Future application of this technique to the isolation of EVs from diverse tumor specimens will be important to determine if dense EVs are predominately secreted in the interstitium of other tumor types as well.

While the complete purification of small EVs and isolation of distinct vesicle subpopulations remains a current limitation across laboratories, this method provides a basis for further studies to characterize small vesicles from a diversity of tissue or tumor types, and may therefore facilitate a greater understanding of EV diversity. Recent separation of EV particles by asymmetric-flow field-flow fractionation has illuminated the possibility of distinct cargo and functions of small (60-80 nm) versus larger (90-120 nm) exosomes, and further identified a subset of non-membrane bound <50 nm particles denominated as "exomeres"[31,32]. However, cargo packaged in these unique vesicle populations varied across cell lines, highlighting the substantial heterogeneity of secreted vesicles, and supporting the importance of comprehensive *in vitro* or *ex vivo* vesicle analysis. In this study, we most likely enrich for both classes of exosomes identified. A proportion of small microvesicles with similar densities to exosomes may be present in isolates as well. Although we cannot exclude the possibility of exomeres in our isolates, membrane-bound vesicles were certainly most abundant in the gradient fractions enriched in vesicle protein. In addition to small EVs, large oncosomes (> 1,000 nm) have been of recent interest to cancer biologists[33]. While the protocol described in this study filters out vesicles greater than 450 nm, exclusion of this step may facilitate examination of these larger EVs described. Future examination of densities associated with various vesicle subpopulations will be important to distinguish these entities in addition to their described sizes.

Finally, though we appreciate the limitations including cost and time associated with this proposed technique, we also acknowledge the need for rigorous foundational methods to characterize tissue EVs and to serve as an end-product comparison for constantly evolving and more sophisticated methods in the future. These advancing technologies will hopefully illuminate more rapid and clinically-compatible techniques to extract EVs from medical or surgical specimens.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

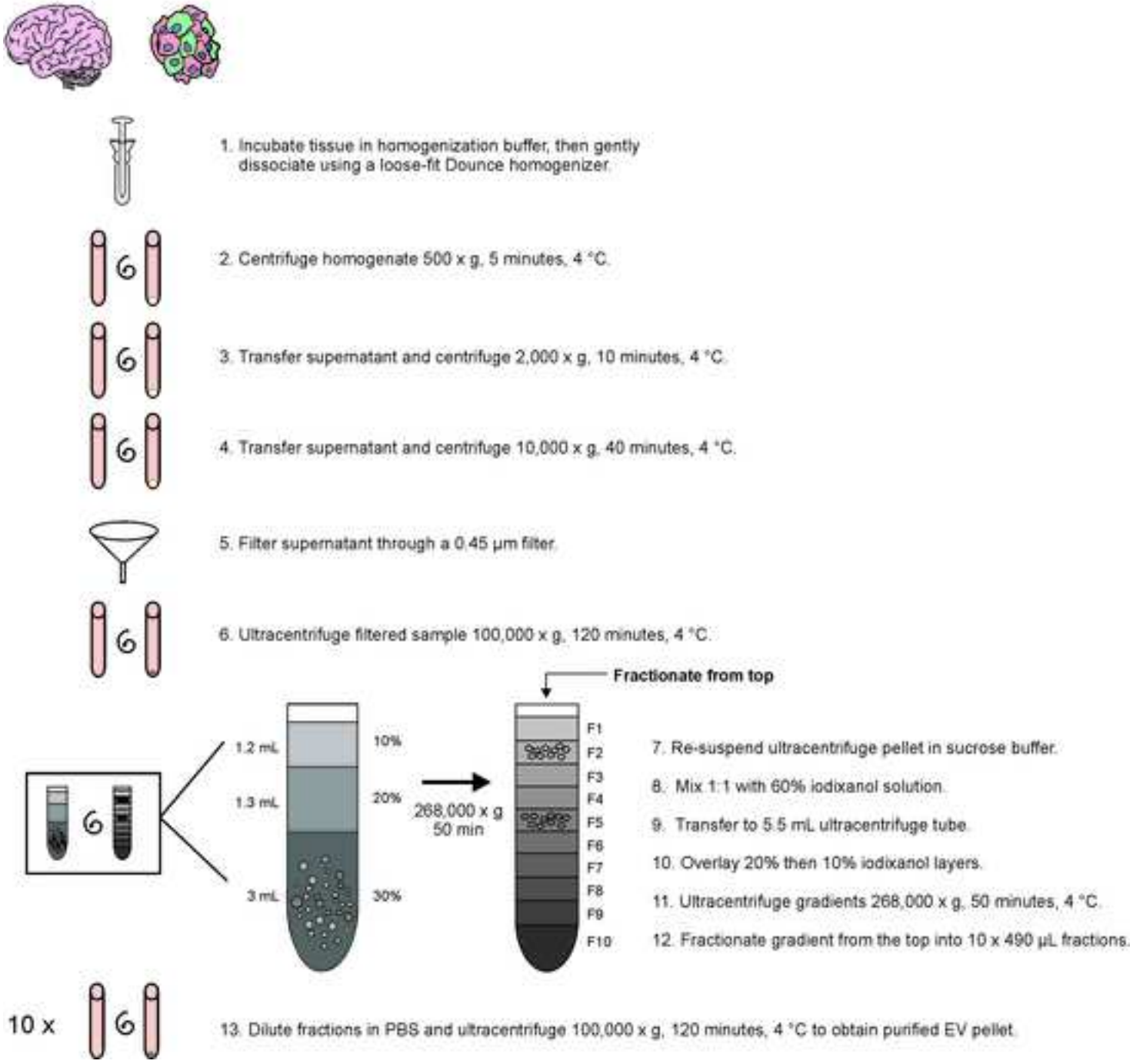


Figure 2

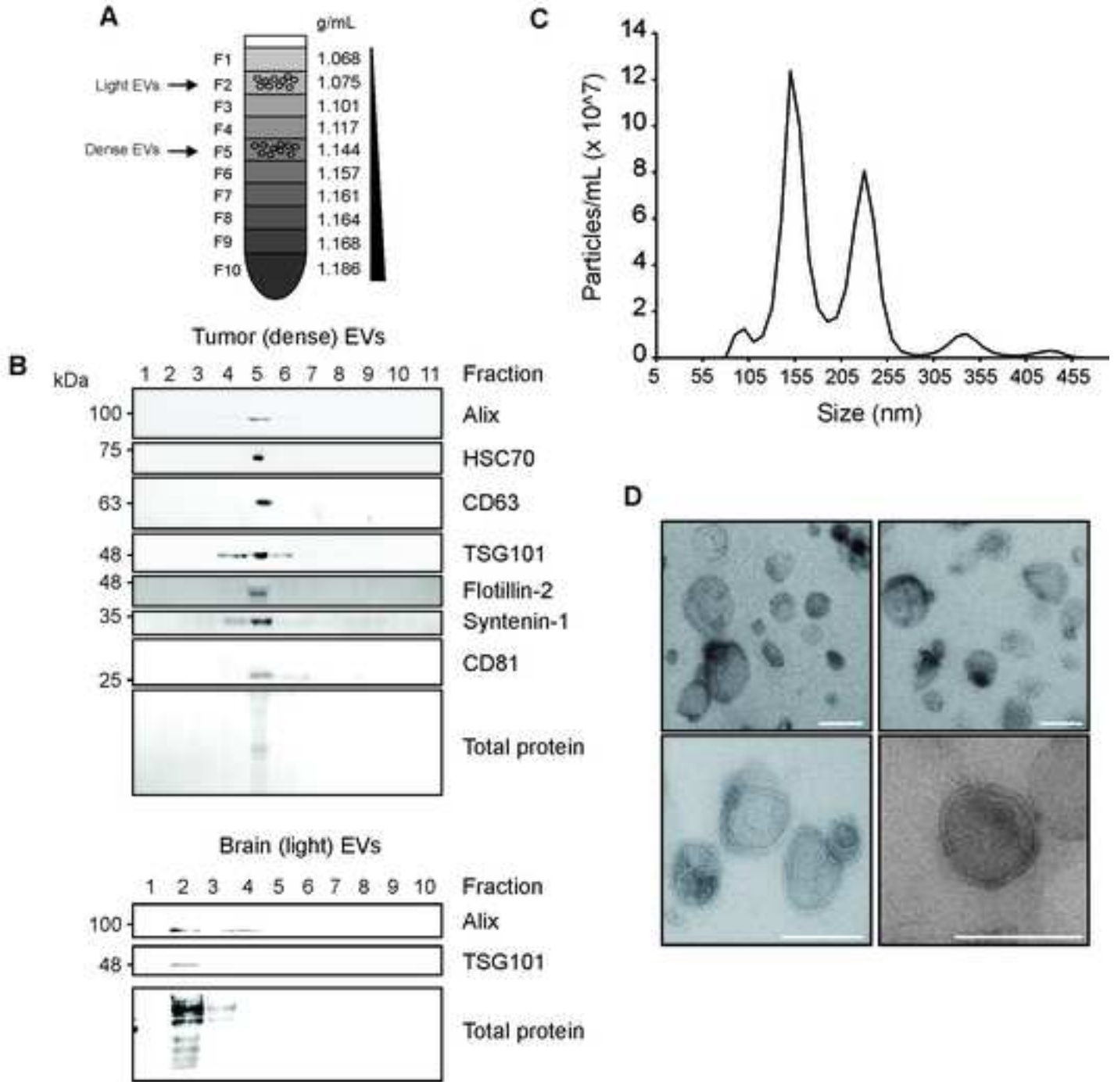


Figure 3

Tetraspanins

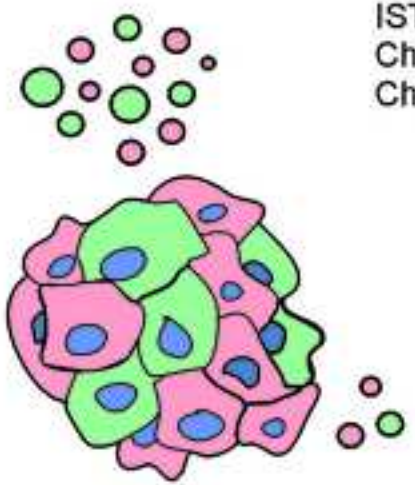
CD81
CD63
CD9
Tspan2
Tspan7

Annexins

Annexin A2
Annexin A3
Annexin A4
Annexin A5
Annexin A6
Annexin A7
Annexin A11

ESCRT proteins

Alix (PDCD6IP)
IST1 homolog
Charged multivesicular body protein 4B (CHMP4B)
Charged multivesicular body protein 1A (CHMP1A)



Integrins

Integrin alpha-V
Integrin beta-1

Rab proteins

Hras
Mras
Rab1a
Rab1b
Rab2a
Rab3a
Rab3c
Rab5a
Rab5b
Rab5c
Rab6b
Rab7a
Rab8b
Rab10
Rab11b
Rab14
Rab21
Rab35

Vesicle-associated proteins

Flotillin-1
Flotillin-2
Syntenin-1
Syntaxin-12
Sortilin-1
Vesicle trafficking protein Sec22b
Vesicle-associated membrane protein 1 (VAMP1)
Vesicle-associated membrane protein 2 (VAMP2)
Vesicle-fusing ATPase (NSF)
Vacuolar protein sorting-associated protein (VPS35)
Transferrin receptor
Clathrin
ADAM10

Name of Material/ Equipment	Company	Catalog Number
0.45 µm filter	VWR	28145-505
12 mL ultracentrifuge tubes	Beckman Coulter	331372
5.5 mL ultracentrifuge tubes	Beckman Coulter	344057
anti-Alix antibody	Santa Cruz	sc-7129
anti-Calnexin antibody	Santa Cruz	sc-11397
anti-CD63 antibody	Abcam	ab59479
anti-CD81 antibody	Santa Cruz	sc-9158
anti-Flotillin 2 antibody	Santa Cruz	sc-25507
anti-HSC70 antibody	Santa Cruz	sc-7298
anti-Syntenin-1 antibody	Santa Cruz	sc-100336
anti-TSG101 antibody	Santa Cruz	sc-7964
Dounce homogenizer	DWK Life Sciences	885300-0015
EZQ protein quantification kit	ThermoFisher Scientific	R33200
FA-45-6-30 rotor	Eppendorf	5820715006
FEI CM120 Electron Microscope	TSS Microscopy	
goat anti-rabbit IgG (Fab fragment)	Genetex	27171
HALT phosphatase inhibitor (100x solution)	ThermoFisher Scientific	78420
HALT protease inhibitor (100x solution)	ThermoFisher Scientific	78438
Hibernate E medium	ThermoFisher Scientific	A1247601
MLS-50 swinging-bucket rotor	Beckman Coulter	367280
NanoSight LM10	Malvern	
Optima MAX-XP Benchtop Ultracentrifuge	Beckman Coulter	393315
Optima XE-100 ultracentrifuge	Beckman Coulter	A94516
Optiprep	Sigma	D1556
Q Exactive HF Mass Spectrometer	ThermoFisher Scientific	
rabbit anti-goat IgG	Genetex	26741
rabbit anti-mouse IgG	Genetex	26728
Refracto 30PX (refractometer)	Mettler Toledo	51324650
S-4-104 rotor	Eppendorf	5820759003
SW 41 Ti swinging-bucket Rotor	Beckman Coulter	333790
Tabletop 5804R centrifuge	Eppendorf	22623508

Comments/Description

Loose-fit pestle (clearance of 0.889–0.165 mm) used.

60% iodixanol in sterile water solution

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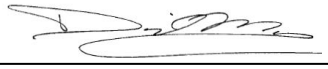
Signature:	
Date:	9/25/18

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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).