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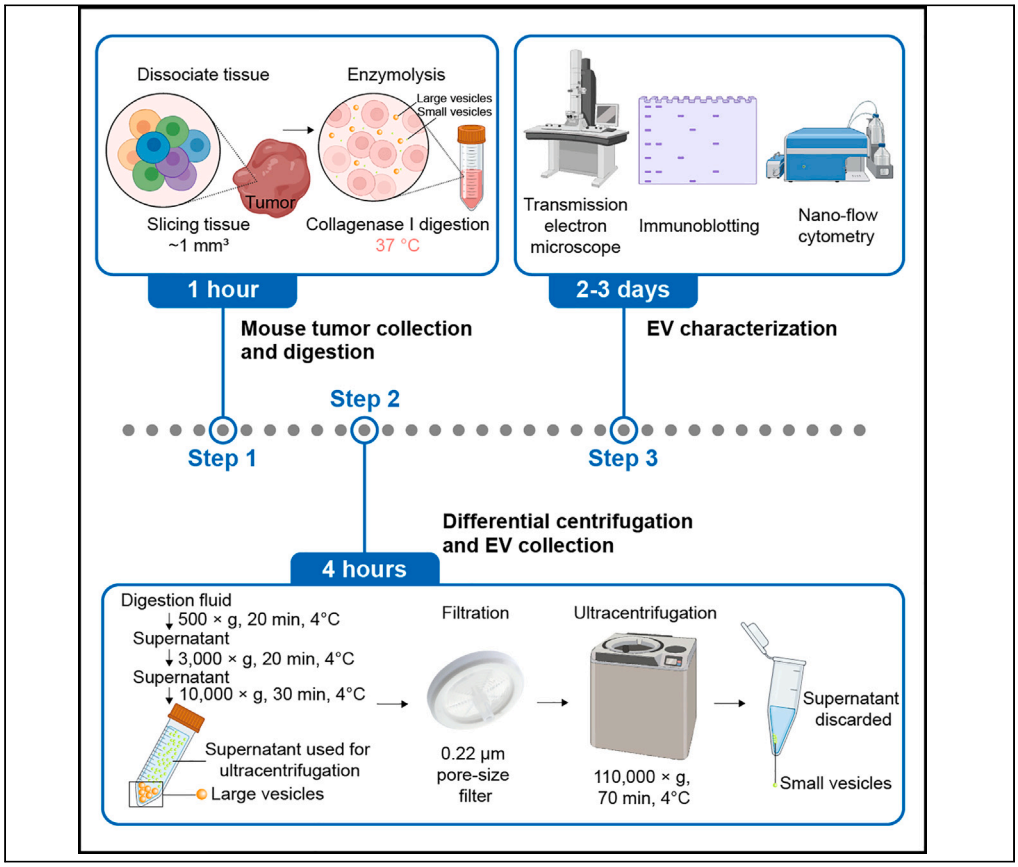
Protocol

Isolation and characterization of small extracellular vesicles from murine primary mammary tumor



Tumor-derived small extracellular vesicles (TEVs) play a pivotal role in cancer progression by transferring functional biomolecules between the parental and recipient cells. Here, we present a protocol to isolate TEVs directly from murine primary mammary tumor using differential centrifugation. We describe steps for tissue dissociation, enzymatic digestion, and centrifugation. We then detail procedures for characterization of TEVs through transmission electron microscopy, immunoblotting, and nano-flow cytometry. This protocol can be used to extract EVs from other solid tumor types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Sheng Hu, Xiaohui Zhang, Wei Yan

weiyan@whu.edu.cn

Highlights

Tumor-derived small extracellular vesicle (EV) isolation by differential centrifugation

Steps for tissue dissociation, enzymatic digestion, and centrifugation

Tips for acquisition of maximum yield of high-purity small EVs

EV characterization by transmission electron microscope and nano-flow cytometry

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Isolation and characterization of small extracellular vesicles from murine primary mammary tumor

Sheng Hu,[1,2] Xiaohui Zhang,[1,2] and Wei Yan[1,3,*]

[1]Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, TaiKang Center for Life and Medical Sciences, Wuhan University, Wuhan 430072, China

[2]Technical contact

[3]Lead contact

*Correspondence: weiyan@whu.edu.cn
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SUMMARY

Tumor-derived small extracellular vesicles (TEVs) play a pivotal role in cancer progression by transferring functional biomolecules between the parental and recipient cells. Here, we present a protocol to isolate TEVs directly from murine primary mammary tumor using differential centrifugation. We describe steps for tissue dissociation, enzymatic digestion, and centrifugation. We then detail procedures for characterization of TEVs through transmission electron microscopy, immunoblotting, and nano-flow cytometry. This protocol can be used to extract EVs from other solid tumor types.

For complete details on the use and execution of this protocol, please refer to Li, Mei-Xin et al. (2023).[1]

BEFORE YOU BEGIN

The impact of TEVs on other organs has been continuously reported.[2–4] The effect is mainly due to the bioactive cargoes by encapsulation including lipids, proteins, nucleic acids and metabolites.[5–7] Several studies reveal the function of TEVs including tumorigenesis, formation of the metastatic niche and affecting the immune balance.[8–13] Isolation of TEVs is crucial for studying the physiological functions and diagnostic and therapeutic applications.

> ***Note:*** The EV isolation protocol described here is optimized for breast cancer (BC) cell xenografted tumor, and mouse triple-negative BC (4T1) xenografted tumor has been applied as an experimental model to display. However, the procedure can also be adapted for other types of cancer cell-xenografted tumor models.

Institutional permissions

Before starting, please make sure to conform to regulatory standards and acquire the required permissions for working with murine cell lines. All mouse experiments in this protocol were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the College of Life Sciences, Wuhan University (WDSKY0202008).

Preparation of xenograft tumor

Timing: 20–30 days

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1. Inject 2×10^5 of 4T1 cells (resuspended in 50 μL DMEM supplemented with 1% penicillin/streptomycin) into the fourth mammary fat pat of 6 weeks-old female BALB/c mice to induce the xenograft tumor. Maintain the mice in a specific pathogen-free (SPF) environment under a 12 h light/12 h dark cycle and with standard feed and water supply.
2. Measure the tumor volume and body weight every other day. Once the tumor volume reaches 500 mm^3 (calculated by length × $width^2/2$), transfer mice to the procedure room for further euthanasia and tissue harvesting.

Preparation of reagents, tools and equipment

Timing: 1–2 h

3. General laboratory preparation.
   a. Prepare enzymatic digestion mixture: prewarm the working digestion solution to 22°C–26°C before use.
   b. Prepare 200 mL of sterile phosphate-buffered saline (PBS) by filtering through a 0.22 μm filter unit, store at 4°C before use.
   c. Prepare sterile surgical scissors and forceps by autoclaving.
   d. Prepare centrifuge tubes (1.5 mL and 5 mL), cell culture dishes (100 mm × 20 mm), plastic syringes and ultracentrifuge tubes (Hitachi, S308892A).
   e. Turn on the ultraviolet lamps of the biosafety cabinet for pre-sterilization of about 30 min.
   f. Pre-cool the centrifuges.
   g. Pre-cool a P50A3 rotor at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-CD63 antibody; 1:1,000	Proteintech	25682-1-AP; RRID: AB_2783831
Rabbit polyclonal anti-Alix antibody; 1:1,000	Proteintech	12422-1-AP; RRID: AB_2162467
Rabbit polyclonal anti-CD9 antibody; 1:1,000	Proteintech	20597-1-AP; RRID: AB_2878706
Rabbit polyclonal anti-GOLGA2/GM130 antibody; 1:1,000	Proteintech	11308-1-AP; RRID: AB_2115327
Rabbit polyclonal anti-syntenin-1 antibody; 1:1,000	Proteintech	22399-1-AP; RRID: AB_2879100
Rabbit polyclonal anti-cytokeratin 18 antibody; 1:1,000	Affinity Biosciences	AF0191; RRID: AB_2833384
Rabbit polyclonal anti-Ki-67 antibody; 1:1,000	Affinity Biosciences	AF0198; RRID: AB_2834152
Rabbit monoclonal anti-FABP4 antibody; 1:1,000	ZenBio	R381753
Goat anti-rabbit IgG (H + L) secondary antibody, HRP; 1:10,000	Thermo Fisher Scientific	31460; RRID: AB_228341
Chemicals, peptides, and recombinant proteins		
DMEM, high glucose	Gibco	11965118
PBS, pH 7.4	Gibco	10010049
HBSS, calcium, magnesium, no phenol red	Gibco	14025092
DNase I solution (2500 U/mL)	Thermo Fisher Scientific	90083
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140163
NanoFCM cleaning solution, 50×	NanoFCM	17159
NanoFCM quality control nanospheres (QC beads), 250 ± 5 nm, 2E+10 particles/mL	NanoFCM	QS2503
Silica nanospheres cocktail (size beads), 68–155 nm, 100×	NanoFCM	S16M-Exo
Trypsin-EDTA (0.25%), phenol red	Gibco	25200056
Collagenase, type I	BasalMedia	S361RV
Collagenase, type IV	BasalMedia	S364RV
SDS-PAGE sample loading buffer, 5×	Biosharp	BL502B
10 × Tris-glycine-SDS buffer	Monad Biotech	CR05301S

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
30% Arc-Bis (29:1)	Biosharp	BL513B
Ammonium persulfate (AP)	Sigma-Aldrich	A3678
N, N, N′, N′-tetramethylethylenediamine (TEMED)	Sigma-Aldrich	411019
Skim milk powder	Biosharp	BS102-500g
20 × TBST buffer	Monad Biotech	CR10701S
NP-40 lysis buffer	Biosharp	BL653A
Protease inhibitor cocktail	Beyotime	P1010
Uranyl acetate	SPI-Chem	02624-AB
Critical commercial assays		
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Experimental models: Cell lines		
Mouse: 4T1	ATCC	CRL-2539
Experimental models: Organisms/strains		
Mouse: BALB/c; 6-week-old; female	Center for Disease Control (CDC; Hubei, China)	N/A
Software and algorithms		
Gatan DigitalMicrograph 3.5	Gatan	https://www.gatan.com/products/tem-analysis/gatan-microscopy-suite-software
NF Profession 2.0 software	NanoFCM	N/A
BioTek Gen5 Software	BioTek	https://www.agilent.com/en/product/microplate-instrumentation/microplate-instrumentation-control-analysis-software/imager-reader-control-analysis-software/biotek-gen5-software-for-detection-1623227
Other		
100 mm cell culture dishes	Labselect	12310
50 mL centrifuge tube	Labselect	CT-002-50A
1.5 mL microcentrifuge tube	Biosharp	BS-15-M
5 mL microcentrifuge tube	Biosharp	BS-50-M
PVDF membrane	Millipore	IPFL00010
Ultracentrifuge Himac CP80WX	Hitachi	N/A
P50A3 fixed-angle rotor	Hitachi	N/A
Millex-GP 0.22 μm filter unit (sterile)	Millipore	SLGP033RB
1.5 mL ultracentrifuge centrifuge tubes	Hitachi	S308892A
NanoFCM Flow NanoAnalyzer	NanoFCM	N/A
JEM-1400 Plus transmission electron microscope	JEOL	N/A
Chemiluminescent imaging device	Tanon	5200
Epoch microplate spectrophotometer	BioTek	Gen5
Copper grid with carbon-coated film	Daji Keyi Technology Co., Ltd.	D11023
Centrifuge	Eppendorf	5810 R
Microcentrifuge	Sigma	1-14K

MATERIALS AND EQUIPMENT

Step 1 of the protocol relies on the usage of collagenase to digest solid tumors into single-cell suspensions.

- Stocking digestion mixture: Dissolve the Collagenase I (100 mg) in 1 mL Hank's balanced salt solution (HBSS) to make the stocking solution.
- The stocking solution can be saved as aliquots at −20°C for several months and freeze-thaw cycles should be avoided.
- Working digestion solution: Add 200 μL of Collagenase I stocking solution (100 mg/mL) and 320 μL of DNase I (2500 U/mL) to 19.48 mL of HBSS. Filter the working solution with a 0.22 μm filter.

Working digestion solution contents		
Reagent	Final concentration	Amount
Collagenase I stocking solution	1 mg/mL	200 μL
DNase I	40 U/mL	320 μL
HBSS	N/A	19.48 mL
Stored at 4°C for 1–2 weeks.		

Step 2 of this protocol depends on suitable tubes for differential centrifugation.

- An Eppendorf centrifuge with an A-4-62 swinging bucket centrifuge rotor is used to process larger volumes of low speed in 5 mL tubes.
- A Sigma microcentrifuge is used for processing smaller volumes (100–2,000 μL) in 1.5 mL or 2 mL centrifuge tubes.
- The Himac Ultracentrifuge CP80WX (Hitachi, JAPAN) with P50A3 rotor is used for the ultracentrifugation steps.

Note: All the centrifuges should be maintained at 4°C and all the samples should be placed on ice.

Step 3 of this protocol requires the usage of the following specialized equipment.

- Transmission electron microscope (TEM) is used for EV morphology characterization via JEOL JEM-1400 Plus transmission electron microscope and images are analyzed by Gatan GMS 3 digital micrograph software.
- Immunoblotting is used to detect the biomarkers of EV.
- NanoFCM Flow NanoAnalyzer device is used for EV characterization such as particle number and size measurement. Obtained data was analyzed with the NanoFCM software NF Profession Version 2.0.

Alternatives: There are other methods available for basic EV characterization similar to nano-flow cytometry. For example, particle concentration and size distribution can also be detected by interferometric nanoparticle tracking analysis (iNTA)[14,15] or tunable resistive pulse sensing (TRPS)[16] measurements. For nano-flow cytometry, CytoFlex (Beckman Coulter) or Micro PLUS (Apogee) device can also be used for analysis of particle counts and size measurement. CytExpert 2.0 software and Apogee software-V255 software are respectively used to analyze the data.

STEP-BY-STEP METHOD DETAILS

Mouse tumor collection and digestion (step 1)

Timing: 1 h

This part of the protocol describes the collection and processing of the murine primary tumor. The purpose of this step is to dissociate solid tumor cells and fully release TEVs from the tissue into the solvent.

1. Euthanize the mouse by using inhaled CO_2 or cervical dislocation.

Note: By following applicable guidelines and laws, other euthanasia methods can be used to minimize animal pain.

2. Dissect the tumor tissue in the biosafety cabinet using sterilized surgical scissors and forceps. Once the tumor is removed from the mouse, immediately transfer it to a 50 mL conical tube containing 10 mL of ice-cold PBS and keep it on ice until the start of mincing.

⚠ CRITICAL: Keep the sample in cold and wet conditions to avoid cell apoptosis and tissue degradation throughout the entire process. Move to the next step as soon as possible to collect high-quality EV samples. We recommend isolating the TEVs within 1 h from tissue collection.

3. Transfer the tumor tissue from the tube to a cell culture dish and gently cut the tissue into small pieces with the size of about 1 mm^3.

⚠ CRITICAL: The slicing process should be very gentle to avoid contamination of vesicles from broken cells. Do not let the tissue dry out, rinse it with cold PBS occasionally (see troubleshooting problem 1).

4. Transfer the tissue pieces to a sterile 5 mL tube and add the digestion mixture collagenase I (1 mg/mL) and DNase I (40 U/mL) to each tube according to 20× volume/mg tissue.

Note: Digestion solution should be prepared before use to avoid repeated freeze-thaw cycles.

5. Cap the tube and place it in the incubation shaker at 50 r/min and incubate at 37°C for 30 min.

Note: The enzyme concentration and the incubation time can be adjusted according to the stiffness of the tumor tissue to promote the complete dissociation of the tissue to get a high yield of EVs while decreasing the contamination derived within cells.

Differential centrifugation and EV collection (step 2)

Timing: 4 h

This part of the protocol describes the isolation of TEVs by employing differential centrifugation, which is relatively common and in wide use. The principle is to separate small EVs and impurity particles based on their different parameters such as size and density.

6. Cool down the centrifuge to 4°C prior to processing and concentrating the digestion supernatant.
7. Collect the supernatant (containing the secreted TEVs) by centrifugation (500 × *g*, 20 min, 4°C). At this point, the pellet can be discarded or alternatively culturing the primary cells isolated from tissue.
8. Carefully transfer the supernatant (containing the secreted TEVs) into a new 5 mL tube without touching the pellet.
9. Centrifuge the supernatant (3,000 × *g*, 20 min, 4°C) to further remove the fragments of tissue.
10. Carefully pipet the supernatant into a new 1.5 mL or 2 mL microcentrifuge tube without touching the pellet.
11. Centrifuge the supernatant (10,000 × *g*, 30 min, 4°C) to remove the larger microvesicles (MVs) and enrich small TEVs.

⚠ CRITICAL: Work as fast as possible to avoid the cell pellet from dissociating. A minimal amount of supernatant above the pellet can be left to avoid disturbing the supernatant.

12. Collect the supernatant with 1 mL of syringe and avoid suction of pellet.

13. Filter the collected supernatant through a 0.22 μm filter.

⚠ CRITICAL: It is critical to control the strength of squeezing the syringe, because too strong of the strength can cause the MVs to pass through the filter.

Note: The supernatant is now ready for concentration.

14. When the supernatant has passed through the filter, disconnect the syringe from the filter unit.
15. Pipet the filtered supernatant to a new/clean ultracentrifuge tube.

⚠ CRITICAL: Wash new ultracentrifugation tubes with water and rinse them extensively in ddH_2O before the initiation of TEVs isolation, since the factory coating on the inside of the tube will prevent the TEVs pellet from adhering to the tube. If necessary, the tubes should be washed extensively and sterilized if required after use.

16. Centrifuge the supernatant (110,000 × *g*, 70 min, 4°C) in a fixed-angle rotor of Hitachi ultracentrifuge (see materials and equipment) to collect small TEVs.

⚠ CRITICAL: It is extremely important to accurately balance the tubes in the ultracentrifuge. Unbalanced rotor may lead to spillage, contamination, run failure, or even damage to the rotor/ultracentrifuge.

Optional: If further purification of the TEV pellet is required, re-suspend the TEVs pellet in 2.5 mL of PBS and slowly overlay onto a 0.5 mL sucrose/deuterium oxide cushion layer by tilting the tube to 45°; then centrifuge at 110,000 × *g* for 70 min at 4°C.[17]

17. Pipet the supernatant (discard or save for later as a control) and resuspend the small TEVs pellet in 1 mL PBS to wash and collect by ultracentrifugation (110,000 × *g*, 70 min, 4°C).

⚠ CRITICAL: Pipetting the supernatant should be taken on the opposite side of the TEVs precipitation to avoid affecting the TEV pellets (Figures 1A and 1B).

18. Remove the supernatant and resuspend the collected TEVs pellet in 200 μL ice-cold sterile PBS.

⚠ CRITICAL: The PBS used for resuspension of the TEVs pellet should not be contaminated by microorganisms or small particles to avoid affecting subsequent identification (see troubleshooting problem 2).

Note: If TEVs are used for micro library small RNA sequencing, all sampling steps must be followed strictly and rapidly, otherwise it is likely to lead to RNA degradation. The experimental instruments and containers need RNase-free treatment, and the reagents need to be prepared with RNase-free water. The samples should be stored at −80°C (<1 week) in each individual tube to avoid repeated freeze-thaw cycles. If the sample needs to be diluted for detection, the diluent can only be stored on ice or at 4°C, and cannot be frozen. The storage time should not exceed two days. If TEV samples are used for protein identification by mass spectrometry, wear disposable masks, hats, and gloves throughout the experiment to avoid protein contamination. Seal the centrifuge tube with a sealing film to avoid bubbles and prevent prolonged contact with air. The entire operation process should be sterile as much as possible.

19. Prepare 20 μL aliquots for TEM, immunoblotting, nano-flow cytometry and avoid freeze-thaw cycles.

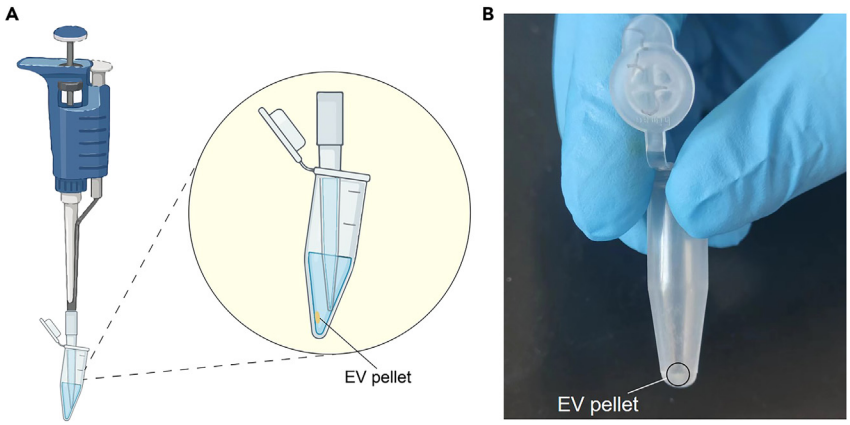

Figure 1. Careful handling the TEV pellets

(A) After ultracentrifugation, carefully pipet the supernatant to avoid contact with TEV pellets.

(B) TEV pellets adhere to the sidewall of the ultracentrifuge tube.

Pause point: If immunostaining is required for the resuspension, mix 20 μL of resuspension with 60 μL of NP-40 buffer (containing protease inhibitor cocktail) solution and suction several times with a pipette. The mixed solution is TEV lysate. Lysates prepared for immunoblotting analysis can be stored at −80°C for future use.

EV characterization (step 3)

Timing: 2–3 days

Timing: 2–4 h (for step 20)

Timing: 2 days (for step 21)

Timing: 2 h (for step 22)

Timing: 2 h (for step 23)

This section describes the identification of TEV immune characteristics and single-particle morphology, concentration and size.

20. Transmission electron microscope analysis:
    a. Pipet 10 μL of the TEVs sample onto a copper grid with carbon-coated film and incubate for 2 min.

Note: The TEVs sample should be gently dropped onto the copper grid, or the sample should be dropped onto a clean parafilm before the copper grid is inverted onto the droplet. If the copper grid is not effective in loading TEVs sample, the copper grid can be processed with glow discharge before use (see troubleshooting problem 3).

    b. Remove excess droplet by blotting.

Note: Pipetting should be performed from the edge of the copper grid to avoid the filter paper touching the copper grid with carbon-coated film.

c. Pipet 20 μL 2% uranyl acetate onto the parafilm, float the copper grid upside down on the uranyl acetate droplet, and negative stain for 30 s.

⚠ CRITICAL: The time of negative staining cannot be too long (see troubleshooting problem 4) and uranyl acetate needs to be carefully handled for safety due to its harmful property.

d. Remove the floating liquid using filter paper at the edge site.
e. Dry the copper grid at 22°C–26°C for a few minutes and store it in a dry container for TEM detection.

Note: If stored for a long time, the copper grid can be placed in a clean 0.2 mL tube to avoid contamination and damage.

21. Immunoblotting analysis:
    a. Determine the protein concentration of the TEVs using the BCA Protein Assay Kit and normalize the concentration. Add 5 × loading buffer to each sample and incubate them at 95°C for 2 min.
    b. 1 × running buffer is poured in the opening of the casting frame to fill the wells of the gel. Resolve the lysates by SDS-PAGE. The tank is then covered with a lid and the unit is connected to a power supply. The sample is allowed to run at 70 V for 30 min and 90 V for 1 h. SDS-PAGE is used to separate proteins by molecular weight.

SDS-PAGE gel contents

Reagent	Amount
4% Upper layer gel	
ddH_2O	1.5 mL
30% Arc-Bis	0.37 mL
Upper buffer	0.63 mL
10% Ammonium Persulfate (AP)	25 μL
N, N, N′, N′-Tetramethylethylenediamine (TEMED)	3 μL
Total	2.5 mL
10% Lower layer gel	
ddH_2O	3.11 mL
30% Arc-Bis	2.51 mL
Lower buffer	1.88 mL
10% Ammonium Persulfate (AP)	70 μL
N, N, N′, N′-Tetramethylethylenediamine (TEMED)	5 μL
Total	7.5 mL
Stored at 4°C for 1 week.	

Running buffer solution contents

Reagent	Final concentration
Tris	25 mM
Glycine	192 mM
SDS	0.1%
Stored at 4°C for 1 week.	

c. Transfer the gel to a PVDF membrane with a 0.45 μm pore size.

Note: The PVDF membrane should be rinsed with methanol before transferring.

d. Block the membrane in 1 × TBST containing 5% skim milk and rock for 2 h at 22°C–26°C.

e. Incubate the membrane with various primary antibodies diluted in TBST at concentrations recommended by the manufacturers.
f. Rock the antibody incubation box at 4°C for 12–16 h.
g. Wash the membrane using 1 × TBST by rocking for 10 min at 22°C–26°C and repeat for two times.
h. Incubate the membrane with HRP-conjugated secondary antibody diluted in 1 × TBST by rocking for 2 h at 22°C–26°C.
i. Wash the membrane using 1 × TBST by rocking for 10 min at 22°C–26°C and repeat for two times.
j. Incubate the membrane with the enhanced chemiluminescence (ECL) reagent and detect the images with a chemiluminescent imaging device (Tanon-5200).

22. Nano-flow cytometry:
    a. Prepare the cleaning solution: Dilute the NanoFCM Cleaning Solution with ultrapure water to 1 × working concentration.
    b. Prepare the PBS negative control for background correction.

    Note: Keep the samples on ice while performing the setup of the NanoFCM.

    c. Prepare sample and a series of gradient dilution samples for further assessment.
    d. Load the NanoFCM Quality Control Nanospheres (QC bead) for 1 min.

    Note: NanoFCM Quality Control Nanospheres (QC bead) solution is to establish a concentration standard and for initial system alignment.

    e. Boost the cleaning solution for 30 s, remove the microcentrifuge tube with the cleaning solution from the inlet and use deionized water to clean the sample line.
    f. Load the Silica Nanospheres Cocktail (size bead) solution and boost for 30 s.
    g. Repeat the washing steps (step 22e).
    h. Load the filtered sterile PBS and boost for 30 s.
    i. Repeat the washing steps (step 22e).
    j. Load the microcentrifuge tube with the sample and boost for 30 s.

    ⚠ CRITICAL: The concentration of the sample should be appropriate and not too high (see troubleshooting problem 5). The recommended number of EV particles is between 10^7–10^9 per mL.

    k. Repeat the washing steps (step 22e).
    l. Load the next sample and boost for 30 s.
    m. Load the NanoFCM Quality Control Nanospheres (QC bead) solution and boost for 1 min after the last sample.
    n. Perform the cleaning procedure and shut down the NanoFCM device.

23. Data analysis of nano-flow cytometry.
    Data analysis is analyzed by the NF Profession version 2.0 software.
    a. Load the NanoFCM data into the NF Profession software.
    b. Open the data of acquired QC beads.
    c. Set the side scatter (SSC) threshold automatically for the QC beads.
    d. Click on ''Count STD'' to set the standard.
    e. Generate a size calibration curve, load the Silica Nanospheres Cocktail (size beads) standard to fit the curve.
    f. Open the acquired PBS sample.
    g. Automatically set the threshold for PBS by clicking "AUTO".
    h. Click on "Count blank" to set the background and save to the settings.
    i. Open the data of acquired samples and subtract the blank background.

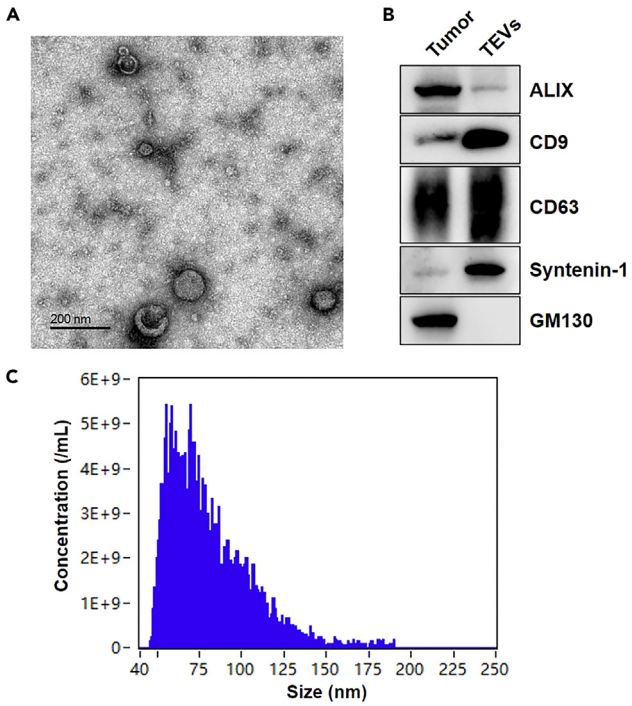

Figure 2. TEV characterization

(A) Representative transmission electron microscopy (TEM) image of TEVs. Scale bar, 200 nm.

(B) Immunoblotting analysis of samples from tumor tissue and TEVs. ALIX, CD9, CD63, and Syntenin-1 are serving as positive markers for EV characterization.

(C) Particle size distribution (nm) and concentration (/mL) of isolated TEVs by nano-flow cytometry.

j. Export the PDF report including the sample size distribution (nm) and the particles concentration (particles/mL).

EXPECTED OUTCOMES

Here, a detailed protocol for the isolation and characterization of the TEVs from mice xenograft tumors is provided according to the isolation of extracellular vesicles from tissue samples.[18,19] Transmission electron microscopy (TEM) analysis of the concentrated TEVs reveals the characteristic cup-shaped form of exosomes derived from the mice breast tumor (Figure 2A). Mince solid tumors into small pieces and add lysis buffer to grind in order to prepare for the whole tissue lysate. The immunoblotting of tissue and TEVs samples shows that the small EV positive markers accumulate in the TEVs while negative marker GM130 vanishes in the tissue derived EV sample (Figure 2B). The measured size distribution of the TEVs is shown in Figure 2C.

In addition, we employed similar method to extract and analyze other tissues derived EVs such as liver and adipose tissue. We systematically studied the effects of collagenase types and digestion time on the efficiency of tissue EV extraction. We used type I and type IV collagenase to digest liver and adipose tissue for 0.5 and 1 h respectively. Digestion of liver tissue with type IV collagenase for 0.5 h resulted in the highest acquisition of EVs, and prolonged digestion time reduced the amount of EV extraction (Figure 3A). For adipose tissue, the maximum efficiency of EV isolation was observed under the digestion of type I collagenase for 0.5 h. Moreover, extended digestion time significantly reduced the yield of EV extraction with type I collagenase rather than type IV collagenase (Figure 3B). Conclusively, TEM analysis of EVs further confirmed that either liver tissue digested by type IV collagenase for 0.5 h (Figure 3C), or adipose tissue digested by type I

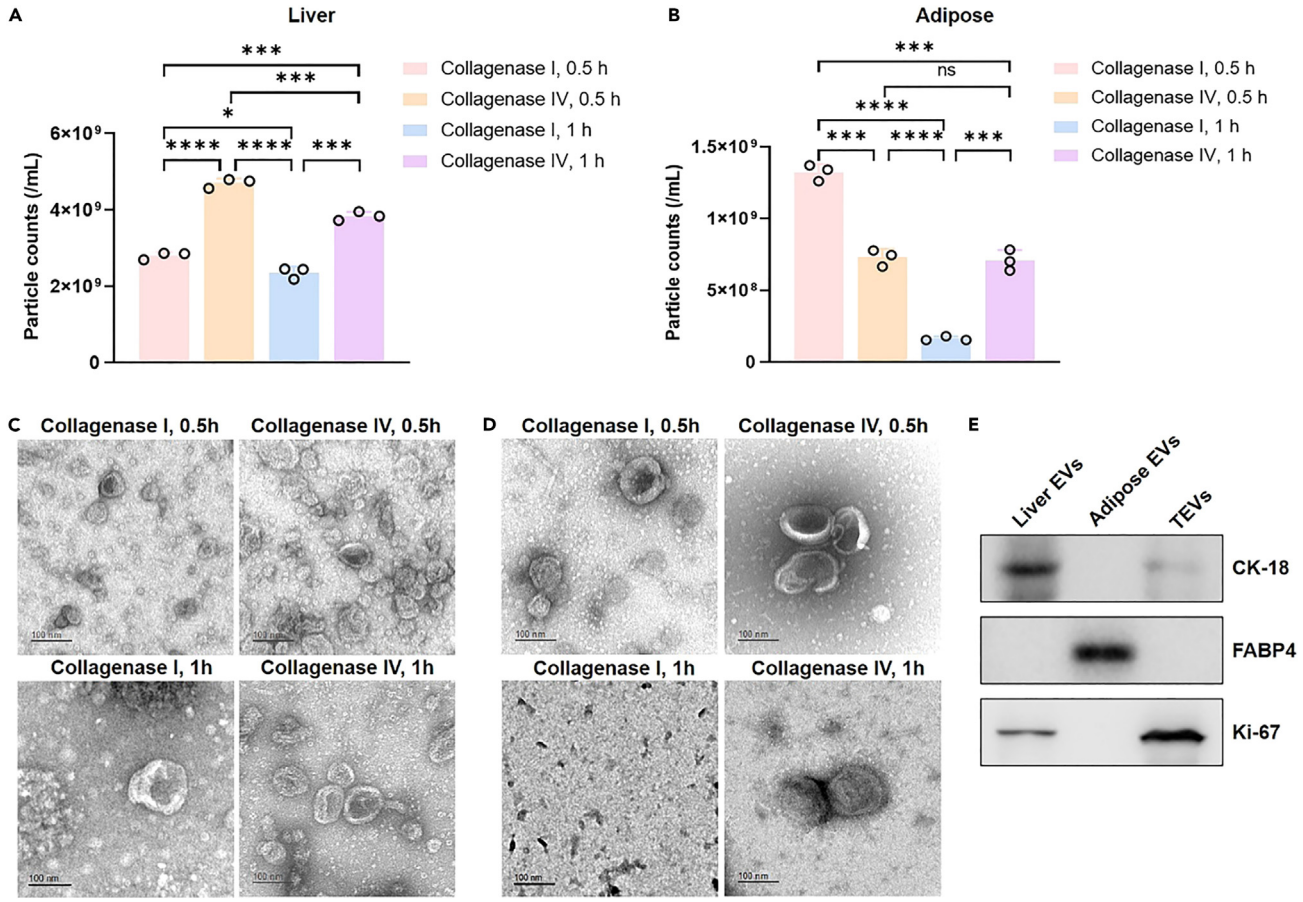

Figure 3. Characterization of EVs isolated from liver and adipose tissue and tissue immune markers for EVs

(A) Particle concentration (particles/mL) of isolated liver EVs acquired by nano-flow cytometry. Data are represented as mean ± SD of three independent biological replicates (n = 3). *P* value (*P*): $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$, unpaired two-tailed Student's t test.

(B) Particle concentration (particles/mL) of isolated adipose tissue EVs acquired by nano-flow cytometry. Data are represented as mean ± SD of three independent biological replicates (n = 3). *P* value (*P*): $^{***}P < 0.001$, $^{****}P < 0.0001$, unpaired two-tailed Student's t test.

(C) Transmission electron microscopy (TEM) images of EVs derived from liver. Scale bar, 100 nm.

(D) Transmission electron microscopy (TEM) images of EVs derived from adipose tissue. Scale bar, 100 nm.

(E) Immunoblotting analysis of samples from liver, adipose, and tumor tissue derived EVs.

collagenase for 0.5 h, exhibiting the best extraction efficiency (Figure 3D). Eventually, we tested the tissue immune markers FABP4, CK-18 and Ki-67 when characterizing the isolated EVs from adipose, liver and tissue tumor. The enrichment of tissue-specific biomarkers accurately represents the origin of isolated EVs (Figure 3E).

LIMITATIONS

Isolation of TEVs by differential centrifugation is an efficient and productive strategy. However, there are some limitations compared to other EV isolation techniques such as density gradient centrifugation, size-exclusion chromatography, immunoprecipitation and ultrafiltration. The differential centrifugation is relatively simple to operate, but it is not suitable for the study of trace and precious samples. For tissue samples, filtering with a filter unit is essential because the components of the digested tissue include complex vesicular disruptors such as protein polymers, cell fragments, and microbes. These substances of similar size to TEVs will seriously interfere with subsequent identification and biological function research of TEVs without filtering. Moreover, filtration also affects the concentration of TEVs, resulting in a decrease in its production.

TROUBLESHOOTING

Problem 1

After the digestion of tumor tissue, there are still a large number of small fragments in the digestive fluid (step 3).

Potential solution

The tumor tissue should be minced into small pieces (approximately 1 mm^3). If the tissue is completely cut, it can be considered to appropriately extend the collagenase digestion time.

Problem 2

The PBS used for resuspension of the TEVs pellet should not be contaminated by small particles (step 18).

Potential solution

The cleanliness PBS is crucial during the characterization process of small TEVs. The contamination of particles in PBS directly affects the transmission electron microscopy image background and incorrect particle concentration in the nano-flow cytometry analysis. We recommend using commercial PBS instead of self-made PBS.

Problem 3

The copper grid carries too many EVs clusters, or EVs cannot be loaded by the copper grid even with sufficient sample abundance (step 20a).

Potential solution

After gas glow discharge process, the surface of the copper grid with carbon-coated film is negatively charged and hydrophilic, making the aqueous solution easily dispersed.

Problem 4

The transmission electron microscopy results of TEVs have a deep background and unclear morphology (step 20c).

Potential solution

The negative staining time should not be too long, usually between 30 s and 1 min, and should not exceed 2 min.

Problem 5

When the TEVs concentration is too high, the nano-flow cytometer receives signals from multiple particles merging. This will cause individual particles no longer be captured as single particles resulting in erroneous data interpretation (step 22j).

Potential solution

Perform gradient dilution on the original sample such as 1:5, 1:50, and 1:200 until there are no significant fluctuations in the particle size range after dilution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wei Yan (weiyan@whu.edu.cn).

Technical contact

Additional requests regarding technical details should be directed to the technical contacts, Sheng Hu (husheng@whu.edu.cn) and Xiaohui Zhang (zhangyfai@whu.edu.cn).

Materials availability

This study did not require any new materials or reagents.

Data and code availability

This study did not generate any new database or code.

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AUTHOR CONTRIBUTIONS

W.Y. designed and supervised the study. S.H. and X.Z. performed the experiments. W.Y., S.H., and X.Z. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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