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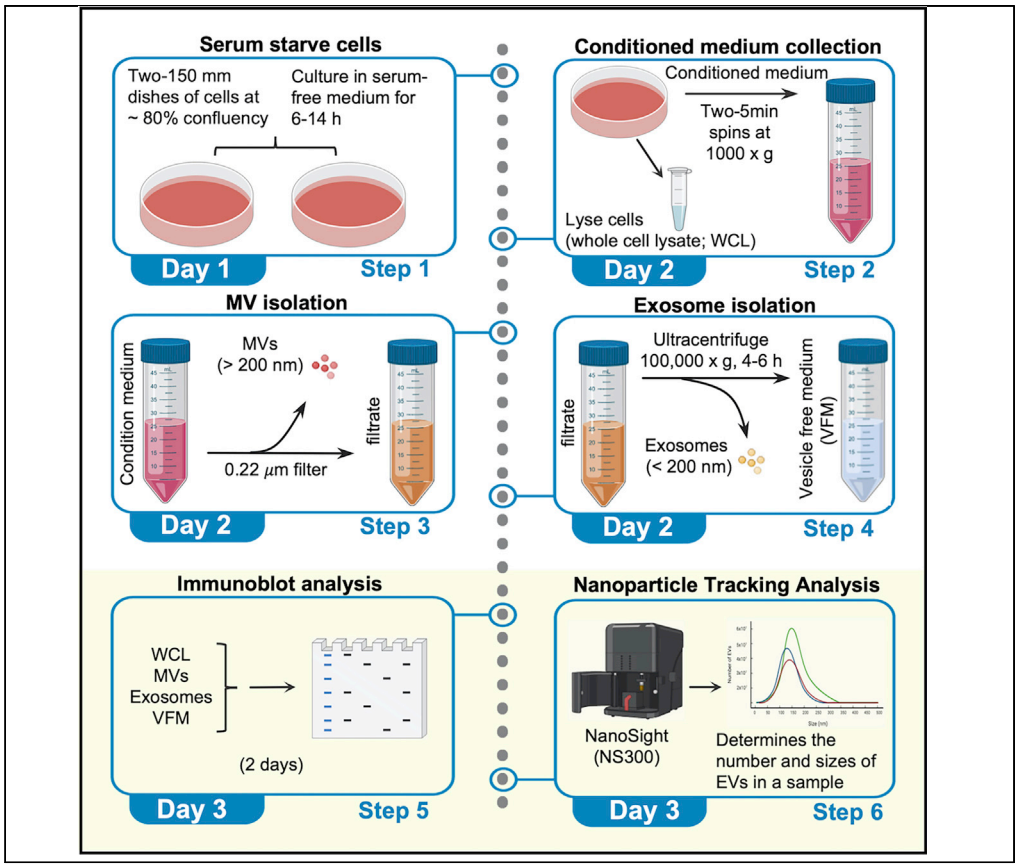
Isolation and characterization of extracellular vesicles produced by cell lines



Cells produce two broad classes of extracellular vesicles (EVs), exosomes and microvesicles (MVs). Exosomes are 30–150 nm vesicles derived from multivesicular bodies, while MVs are 200–1,000 nm vesicles that pinch off from plasma membranes. Reliable isolation of EVs is crucial to understand their biochemical and functional properties. Here, we describe a protocol to isolate and characterize EVs from conditioned medium from mammalian cell lines. This protocol has been optimized for adherent cells but can also be adapted for suspension cells.

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HIGHLIGHTS

Cells produce two major classes of extracellular vesicles, exosomes and microvesicles

Extracellular vesicles function as mediators of intercellular communication

The contents of EVs promote phenotypic changes and can be used as diagnostic markers

Here, we provide an optimized protocol for isolating exosomes and microvesicles

Wang et al., STAR Protocols 2, 100295
March 19, 2021
https://doi.org/10.1016/j.xpro.2021.100295

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Isolation and characterization of extracellular vesicles produced by cell lines

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https://doi.org/10.1016/j.xpro.2021.100295

SUMMARY

Cells produce two broad classes of extracellular vesicles (EVs), exosomes and microvesicles (MVs). Exosomes are 30–150 nm vesicles derived from multivesicular bodies, while MVs are 200–1,000 nm vesicles that pinch off from plasma membranes. Reliable isolation of EVs is crucial to understand their biochemical and functional properties. Here, we describe a protocol to isolate and characterize EVs from conditioned medium from mammalian cell lines. This protocol has been optimized for adherent cells but can also be adapted for suspension cells. For complete details on the use and execution of this protocol, please refer to Latifkar et al. (2019).

BEFORE YOU BEGIN

Preparation of cells

Timing: 1 h for plating cells; 72 h of culturing cells prior to EV isolation

Note: The EV isolation protocol described here has been optimized for adherent cells, and MDA-MB-231 breast cancer cells will be used as an example. However, the procedure can also be adapted for cells that grow in suspension (Zhang et al., 2019).

1. Plate cells in two 150-mm tissue culture dishes, such that they will be ~80% confluent in 72 h. For MDA-MB-231 cells, 2.0 × 10^6 cells are plated in each of the dishes containing growth media (RPMI-1640 medium supplemented with 10% fetal bovine serum; FBS).

Note: Expression plasmids, siRNAs and shRNAs can be introduced into the cells via transfection or viral infection 24 h after plating the cells (Latifkar et al., 2019; Antonyak et al., 2011; Desroches et al., 2016).

⚠ CRITICAL: To ensure that sufficient amounts of exosomes and/or MVs are collected for subsequent analyses, it is recommended that at least two-150 mm dishes of nearly confluent (~80%) cells be used as a starting point. This amount can be scaled up or down to meet experimental needs.

Preparation of reagents

Timing: 1–2 h, reagents can be prepared prior to the day of the procedure

2. Filter sterile phosphate buffered saline (PBS) twice with a 0.22 μm filter unit, 200 mL, store at 4°C.
3. Lysis buffer, 10 mL, store at 4°C.
4. Wash ultracentrifuge tubes and their lids thoroughly with sterile deionized and distilled water. Fill the ultracentrifuge tubes with 70% ethanol. Place their lids on, and occasionally invert them. Approximately 15 min later, move the tubes to a tissue culture hood (sterile environment) and remove the 70% ethanol. Allow the tubes and lids to dry completely.
5. Pre-cool a Type 45 Ti fixed-angle rotor at 4°C.

Note: If the isolated EVs are going to be used for RNA analyses, nuclease-free water can be used for preparing reagents and washing and sterilizing the ultracentrifuge tubes/lids.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-IkBα antibody	Cell Signaling Technology	4812S; RRID: AB_10694416
Anti-Flotillin-2 antibody	Cell Signaling Technology	3436S; RRID: AB_2106572
Anti-HSP90 antibody	Cell Signaling Technology	4877S; RRID: AB_2233307
Anti-CD63 antibody	Abcam	ab59479; RRID: AB_940915
Anti-Annexin I antibody	Santa Cruz Biotechnology	SC-12740; RRID: AB_2057007
Anti-Rabbit IgG-HRP Conjugate antibody	Cell Signaling Technology	7074S; RRID: AB_2099233
Anti-Mouse IgG-HRP Conjugate antibody	Cell Signaling Technology	7076S; RRID: AB_330924
Chemicals, peptides, and recombinant proteins		
Leupeptin	Sigma	L9783
Aprotinin	Sigma	10197777001
Dithiothreitol (DTT)	Sigma	10197777001
Fetal bovine serum	Gibco	10437028
RPMI-1640	Gibco	11875-093
Experimental models: cell lines		
Human: MDA-MB-231 cells	ATCC	ATCC®HTB-26™
Other		
0.22 μm Steriflip filter	Millipore	SEM1M179M6
Amicon Ultra-15 centrifugal filter units, 10 kDa	Millipore	UFC901024
Trypsin	Corning	MT25052CI
Polycarbonate bottle assembly (70 mL)	Beckman Coulter	355622

MATERIALS AND EQUIPMENT

The ultracentrifugation steps described in this protocol use the Beckman Coulter Ultracentrifuge Optima XE (Beckman Coulter, Inc., USA).

Lysis buffer	
Reagent	Final concentration
Tris-HCl (1 M, pH 7.4)	25 mM
NaCl (5 M)	100 mM
Triton X-100	1%
EDTA (0.5 M)	1 mM
DTT	1 mM
$NaVO_4$ (200 mM)	1 mM
β-Glycerol phosphate (1 M)	1 mM
Aprotinin	1 ug/mL
Leupeptin	1 ug/mL

10× Tris-buffered saline and tween 20 (TBST)	
Reagent	Amount
Tris-HCl	24.23 g
NaCl	80.06 g
dH_2O	Bring volume up to 1 L
Tween 20	5 mL
pH 7.6 (with HCl or NaOH)	N/A
Total	**1 L**

5× protein sample buffer	
Reagent	Amount
Tris-HCl (1 M, pH 6.8)	3.75 mL
20% SDS	3 mL
Bromophenol blue	9 mg
DTT	1.16 gm
Glycerol	4.5 mL
dH_2O	Bring volume up to 15 mL
Total	**15 mL**

STEP-BY-STEP METHOD DETAILS

Note: This protocol is modified from (Latifkar et al., 2019).

Serum starve cells: day 1

Timing: 10 min per experimental condition to exchange the growth medium with serum-free medium (i.e., serum starve two 150-mm tissue culture dishes of cells); 6–14 h of serum starvation to generate the conditioned medium

Culturing cells in medium devoid of serum is critical for the isolation of EVs produced by a given cell type. FBS and calf serum (CS) contain large amounts of vesicles that, if not removed from the culturing medium, will be present together with the EVs isolated from cells.

⚠ CRITICAL: Caution: All steps described in this section must be carried out in a biosafety cabinet under sterile conditions.

1. Aspirate the serum-containing (i.e., growth) medium from two nearly confluent 150 mm plates of cells and rinse them with 15 mL of sterile, 37°C PBS per dish.
   a. Aspirate the PBS.
   b. Repeat the rinse step two additional times.

   Note: Serum-free medium can also be used for the wash steps.

2. Rinse each plate of cells with 10 mL of serum-free medium that had been warmed to 37°C. For MDA-MB-231 cells this is RPMI-1640 medium.
   a. Aspirate the medium from the cells.

   Note: All rinsing steps should be performed gently, as to not cause cell detachment.

3. Add 20–22 mL of serum-free RPMI-1640 medium that was warmed to 37°C into each 150 mm dish of cells and culture them in a cell incubator (37°C, 5% CO_2) for a minimum of 6 h to as long as 14 h.

We suggest a minimum of 6 h of serum starvation be used, so that the cells release enough EVs for isolation purposes. Longer duration of serum starvation can result in better EV yields.

⚠ CRITICAL: The length of serum starvation used to generate the conditioned medium can vary depending on how sensitive the cells being used are to dying when deprived of nutrients (i.e., serum starved). It is important to make certain that the serum-starved cells are still viable when collecting the conditioned medium (see below), as fragments of dead cells can contaminate the MV and exosome preparations.

Collecting conditioned medium and cell lysis: day 2

Timing: 1–1.5 h

The conditioned medium is collected from cells and placed in 50 mL conical tubes. The samples are subjected to low speed centrifugation to remove intact cells and cell debris from the conditioned medium. At this point, the partially clarified medium contains soluble factors (i.e., growth factors), MVs, and exosomes.

Note: The transfer of medium between tubes (i.e., conical and ultracentrifuge tubes) must be carried out in a sterile tissue culture hood. All centrifugation steps should be performed at 4°C. The reagents and collected samples need to be maintained on ice.

4. The conditioned medium from two-150 mm dishes of serum-starved cells is collected and placed in sterile 50 mL conical tubes on ice.
5. Treat the cells with trypsin to detach them from the plate. The cells are subsequently counted and lysed.
   a. Trypsin treat the cells by adding 5 mL of trypsin to each 150 mm plate of cells. Once the cells have completely detached from the plate, add 5 mL of growth medium (serum-containing medium) to inactivate the trypsin. The cells are pipetted up and down repeatedly until a single-cell suspension is achieved. The cells are then transferred to a 15 mL conical tube.
   b. Determine the number of cells present in the solution using an automated cell counter or hemocytometer.
   c. Centrifuge the cells at 500 × *g* at 4°C for 5 min to pellet the cells.
   d. Rinse the cell pellet with 5 mL of ice cold PBS.
   e. Centrifuge the cells at 500 × *g* at 4°C for 5 min to pellet the cells and remove the PBS.
   f. Lyse the cells using 2.0 mL of ice cold lysis buffer and pipet the cells up and down several times. As our lysis buffer contains 1% Triton X-100, it effectively disrupts cell and EV membranes. Thus, there is no need for sonication. Transfer the lysate to a 1.5 mL microcentrifuge tube.
   g. Incubate the samples on ice for 15 min.
   h. Centrifuge the samples at 16,000 × *g* for 10 min and place the clarified cell lysate in a new 1.5 mL microcentrifuge tube on ice for use later.

Note: This is considered to be the whole cell lysate (WCL).

Note: Determining the number of cells from which the conditioned medium was derived is important because it allows for the determination of the numbers of EVs that are produced per cell when performing Nanoparticle Tracking Analysis (NTA) (see below).

6. The conditioned medium in 50 mL conical tubes is centrifuged at 1,000 × *g* for 5 min at 4°C to remove intact cells and debris.
7. The supernatant is carefully removed using a 25 mL serological pipet and transferred to a new 50 mL conical tube.

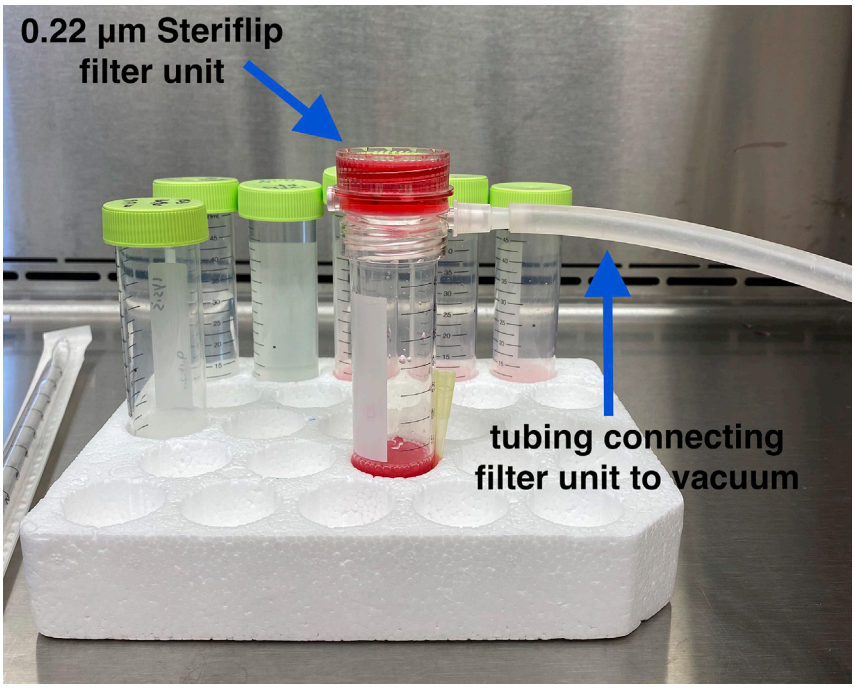

Figure 1. Filtration system setup for MV isolation

The 0.22 µm Steriflip filter unit is connected to the vacuum system. The partially clarified conditioned medium is slowly added to the filter unit. The MVs are retained by the filter, while the filtrate (i.e., the flow-through) contains soluble factors and exosomes.

8. Steps 6 and 7 are repeated.

⚠ CRITICAL: When removing the supernatant from the centrifuged conditioned medium, it is important to leave a small volume (i.e., ~1 mL) behind, to avoid disrupting the pellet.

Note: This is referred to as the partially clarified medium. A small aliquot (1.5 mL) of the medium can be added to a 2 mL microcentrifuge tube. Store the sample at 4°C for NTA (optional; see below).

MV isolation: day 2

Timing: 1–2 h, depending on the number of samples

In this step of the procedure, filtration is performed to isolate the large EVs (i.e., the MVs) from the partially clarified medium.

Note: Each step should be carried out in a sterile tissue culture hood. All centrifugation steps should be performed at 4°C. The reagents and collected samples need to be maintained on ice.

9. Set up the 0.22 µm Steriflip filter unit as shown in Figure 1. Ensure that the unit is vertical.

Note: We use the Steriflip filtration system (Millipore) for MV isolation, but similar 0.22 µm filter systems can also be used.

10. Add the partially clarified conditioned medium to the filter, and slowly begin to apply the vacuum. Keep increasing the strength of the vacuum until a drip-rate of ~one drop/s is achieved.

⚠ CRITICAL: It is critical to control the strength of the vacuum that is applied to the filter unit, because too strong of a vacuum can cause the MVs to pass through the filter. The desired vacuum pressure applied should not result in a flow-rate that exceeds more than one drop per second throughout the entire filtration process. If it does, the tubing connecting the vacuum to the filtration unit should be immediately disconnected to stop the flow of the medium through the filter. Then, it can be reattached and the vacuum can be applied again.

11. Continue to add the partially clarified conditioned medium to the filter unit until all the medium has been filtered.

⚠ CRITICAL: Do not let the filter dry out. The MVs tend to adhere to the filter when it becomes dry, making it difficult to resuspend them.

12. Rinse the filter with 5 mL of PBS and apply the vacuum to remove any residual medium and exosomes from the filter.
13. Repeat step 12

14. When all the PBS had passed through the filter, immediately disconnect the vacuum tubing from the filter unit, and remove (unscrew) the filter from the tube containing the filtrate.
    a. Place the filtrate on ice.

Note: The filtrate has been depleted of MVs but contains exosomes and soluble factors.

    b. Remove 1.5 mL of the filtrate and put it in a new 2 mL microcentrifuge tube. Store at 4°C for NTA. (optional; see below)

15. Add 250 μL of ice cold lysis buffer to the top of the filter unit.
    a. Thoroughly rinse the entire surface of the filter with lysis buffer by pipetting the solution up and down at least 20 times (Figure 2).

⚠ CRITICAL: Be sure not to puncture the filter with the pipette tip, as this will result in the loss of sample. If yields of MV lysates are low, the amount of lysis buffer can be reduced.

    b. Transfer the lysate to a 1.5 mL microcentrifuge tube, and place it on ice.
    c. Incubate the samples on ice for 15 min.
    d. Centrifuge the samples at 16,000 × *g* at 4°C for 10 min and place the clarified MV lysate in a new 1.5 mL microcentrifuge tube on ice for later use.

Note: This sample is considered the MV lysate.

Alternatives: If the MVs are to be used for functional studies, instead of adding lysis buffer to the top of the filtration unit, the MVs can be resuspended in 500 μL of sterile ice cold PBS and transferred to a sterile 1.5 mL microcentrifuge tube.

Note: The MVs resuspended in PBS can be frozen at −80°C and used for functional studies at a later time. A small aliquot of this sample can also be subjected to NTA to quantify the MVs. (optional; see below)

Exosome isolation: day 2

Timing: 6–8 h

Figure 2. Rinsing the 0.22 μm filter unit to collect the MVs

The filter unit is rinsed thoroughly with 250 μL of lysis buffer or sterile PBS by pipetting up and down multiple times to collect the MVs retained on the filter following filtration.

Ultracentrifugation is used to isolate the exosomes from the filtrate.

Note: These steps should be carried out in a sterile tissue culture hood. All centrifugation steps should be performed at 4°C. The reagents and collected samples need to be maintained on ice.

16. The filtrate in the collection tube (see step 14) is transferred to polycarbonate ultracentrifuge tubes that had been cleaned and sterilized (see Preparation of reagents). The lids are attached to the tubes.
17. Weigh the ultracentrifuge tubes containing medium and normalize their weight using sterile PBS.

⚠ CRITICAL: Caution: Take care that the centrifuge tubes are properly balanced.

18. Place the ultracentrifuge tubes into a pre-cooled Type 45 Ti fixed-angle rotor.

Note: Prior to placing the ultracentrifuge tubes in the rotor, it is helpful to indicate (using a marker) the sides of the tubes that will face the outer edge of the rotor (Figure 3). This will be used to help locate where the exosome pellets will be following centrifugation. Make sure the rubber O-rings on the lid of the rotor are evenly coated with silicon vacuum grease. Place the lid on the rotor and tighten it firmly.

19. Spin samples at 100,000 × *g*, 4°C for 4 h.
20. Immediately remove the tubes from the rotor following centrifugation and carefully place them on ice.

Note: The exosome pellet will be located toward the bottom of the tube, below where the indication was made. It is often difficult to see at this point.

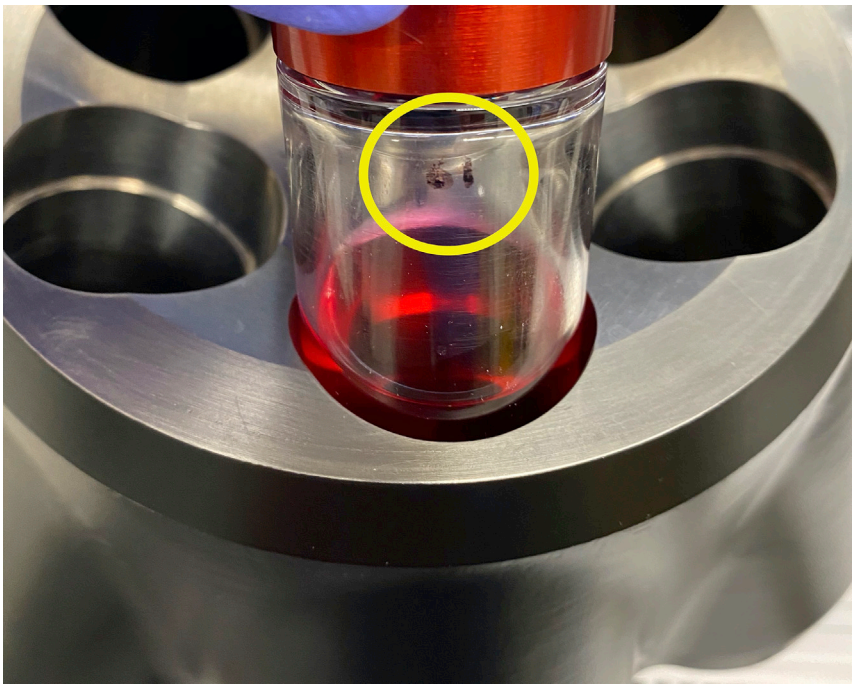

Figure 3. Placing the ultracentrifuge tubes containing the filtrate in the rotor
The part of the tube that is facing the outer edge of the rotor is indicated with a marker (circled above).

21. Carefully remove the lids from the ultracentrifuge tubes and transfer all the supernatant to a 50 mL conical tube using a 25 mL serological pipet. Store this tube on ice.

Note: The resulting supernatant has been depleted of MVs and exosomes, and is considered as the vesicle-free medium (VFM).

⚠ CRITICAL: Do not disturb the exosome pellets. Keep the pipet being used to remove the supernatant on the opposite side of the tube from where the exosome pellet is located.

22. Aspirate any remaining medium from the tube without disturbing the pellet.

Note: At this point, an off-white to transparent exosome pellet will be visible on the lower wall of the marked side of the ultracentrifuge tube.

23. Circle the pellet with a marker (Figure 4).
24. Wash the exosome pellet by resuspending it in 30 mL of ice cold sterile PBS. Weigh the ultracentrifuge tubes again and normalize their weight using sterile PBS. Centrifuge the samples at 100,000 × *g*, 4°C for 2 h.
25. Remove the PBS and lyse the pellet with 250 µL of ice cold lysis buffer.
    a. Resuspend the pellet by scrapping the pellet with a pipet tip and extensive pipetting.
    b. Transfer the sample to a new 1.5 mL microcentrifuge tube.
    c. Incubate the samples on ice for 15 min.
    d. Centrifuge the samples at 16,000 × *g* at 4°C for 10 min, and place the clarified lysate in a new 1.5 mL microcentrifuge tube on ice for later use.

Note: This is considered the exosome lysate.

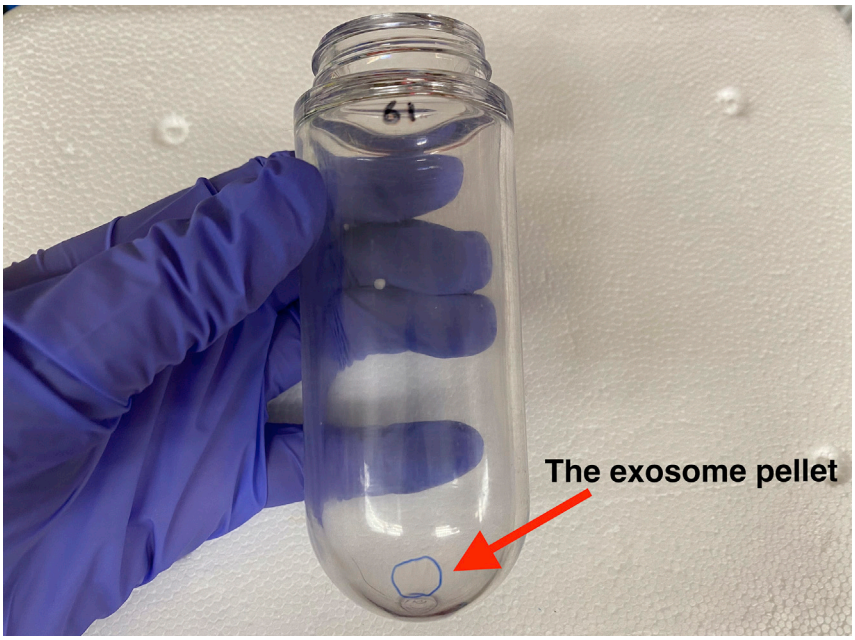

Figure 4. The location of the exosome pellet in the ultracentrifuge tube
Following centrifugation, the exosome pellet (see arrow above) will be located toward the bottom of the tube.

26. The WCL, MV and exosome lysates, and VFM can be stored at −80°C for future use.

Alternatives: If the exosomes are going to be used for functional studies, instead of adding lysis buffer to the exosome pellet, 500 μL of sterile PBS can be added. The exosome pellet is thoroughly resuspended and moved to a 1.5 mL microcentrifuge tube.

Note: The exosomes resuspended in PBS can be frozen at −80°C and used for functional studies at a later time. A small aliquot of this sample can also be subjected to NTA to quantify the exosomes (optional; see below).

Concentration of the VFM Day 3 (optional)

Timing: 3 h

The VFM contains soluble factors (i.e., growth factors) that have been secreted by cells. It can be concentrated using an Amicon centrifugal concentrator with a 10 kDa pore size to reduce the volume such that it can be analyzed by immunoblot analysis.

27. Transfer 15 mL of the VFM collected (see step 21) into the reservoir of a 15 mL 10 KDa Amicon centrifugal concentrator.

28. Centrifuge the sample at 2,700 rpm, 4°C for 25 min.

⚠ CRITICAL: Caution: To minimize protein degradation in the VFM, perform all centrifugation steps at 4°C. Also, make certain the tubes containing the VFM sample are stored on ice.

a. Continue adding the VFM to the same concentrator until all the medium has been concentrated to less than 250 μL.
b. Add 8 mL of sterile PBS to the concentrator, and centrifuge again.

c. Repeat step b.
d. Continue to centrifuge the sample until its volume is less than 250 μL.

29. Pipet the concentrated sample in the concentrator extensively (at least 10 times) to remove any proteins that might have adhered to the filter.
30. Transfer the solution to a 1.5 mL microcentrifuge tube.

Note: This is the concentrated VFM, which can be used immediately or stored at −80°C until a later time.

Characterization of EVs by immunoblot analysis: day 3 and day 4

Timing: 2 days for immunoblot analysis

In this section, the WCLs, the MV, and exosome lysates, as well as the VFM samples, will be subjected to immunoblot analysis. Various antibodies can be used as specific markers to distinguish each of these different fractions. The expression levels of various proteins of interest in EVs can also be assessed using this approach.

31. Determine the protein concentrations of the WCLs, the MV lysates, the exosome lysates, as well as the concentrated VFM using the Bradford assay and normalize their concentrations. Add 5× protein sample buffer to each sample and incubate them at 95°C for 5 min.

Note: The total amount of protein present in the EVs and VFM preparation can vary depending on the cell type from which the samples are collected. Assuming that the EVs isolated from two dishes of MDA-MB-231 cells are lysed in 250 μL of lysis buffer, we typically get ∼ 50–75 ng/μL of protein for MVs and ∼ 150–200 ng/μL of protein for exosomes.

Pause Point: Lysates prepared for immunoblot analysis can be stored at −20°C for several weeks.

32. Resolve the lysates by SDS-PAGE under reducing conditions to resolve proteins base on their size.
33. Transfer the gel to a PVDF membrane with a 0.45 μm pore size using a wet transfer system; 1.5 h.

Note: The PVDF membrane needs to be rinsed with methanol prior to transferring.

Alternatives: PVDF membranes with a smaller pore size (i.e., 0.22 μm) are also available, and can be used to more efficiently transfer smaller molecular weight proteins.

34. The membrane is blocked in 1× TBST containing 5% bovine serum albumin (BSA) and rocking for 1 h at 20°C–25°C.
35. The membrane is incubated with various primary antibodies diluted in 1× TBST at concentrations recommended by the manufacturer. The blot is then rocked at 4°C for 12 h.
    a. The CD63 antibody is used at a 1:2,000 dilution and is considered an exosome marker. This protein should only be detected in the WCLs and exosome fractions.
    b. The annexin antibody is used at a 1:1,000 dilution and is a MV marker. It should be detected only in the WCLs and MV fractions.
    c. The flotillin-2 and HSP90 antibodies are used at 1:1000 dilutions and are considered general EV markers (found in both MVs and exosomes). These proteins will be present in all lysates.
    d. The IκBα antibody is used at a 1: 1,000 dilution and is expressed by all cells, but is largely absent in EVs, as well as VFM samples. The lack of detectable levels of this protein in exosome, MV, and VFM preparations shows that they are devoid of cellular contaminants.

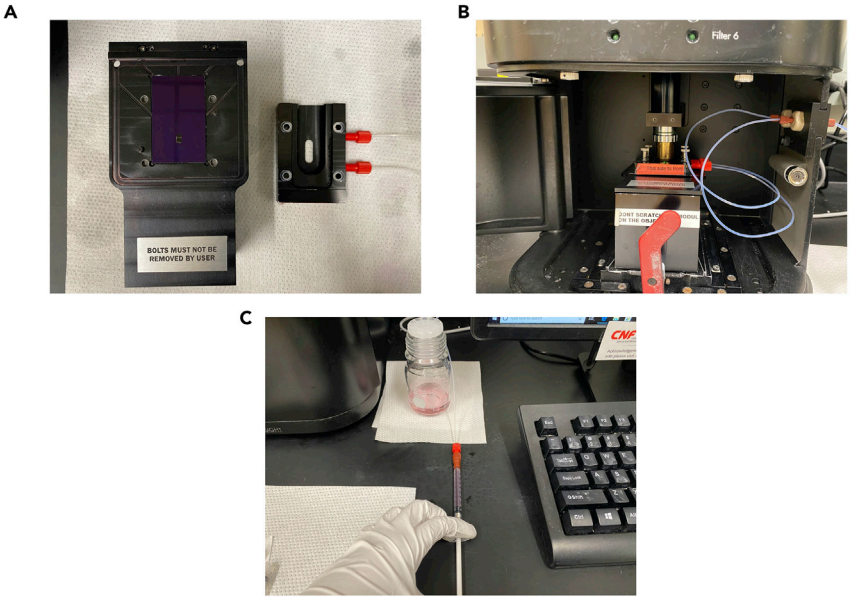

Figure 5. Setting up for nanoparticle tracking analysis (NTA)

(A) The laser module (left) and the flow cell top-plate (right).

(B) Image showing how to connect the inlet and outlet tubing with the flow cell.

(C) The beam bath is rinsed with water/PBS/medium using a 1 mL syringe.

36. Wash the membrane using 1× TBST with rocking for 5 min at 20°C–25°C.
    a. Repeat two additional times.
37. The membrane is incubated with HRP-conjugated secondary antibody diluted in 1× TBST for 1 h at 20°C–25°C.
38. The membrane is washed using 1× TBST and rocking for 10 min at 20°C–25°C.
    a. Repeat two additional times.
39. For protein detection, the membrane is incubated with the enhanced chemiluminescence (ECL) reagent for 1 min and exposed to autoradiography film in a dark room, or imaged with chemiluminescent imaging device.

Characterization of EVs by nanoparticle tracking analysis: day 3

Timing: 30 min per sample

The amount and size of MVs and exosomes present in the partially conditioned medium, or in the isolated MVs or exosomes, can be determined using nanoparticle tracking analysis (NTA). This approach allows for the capturing movies of EVs as points of diffracted light moving under Brownian motion.

Note: The NanoSight NS300 (Malvern, Malvern, UK) is used to perform NTA in this protocol. Green laser with a wavelength of 532 nm is used. Detailed procedures can be found in NS300 Operating Manual from Malvern.

40. Mount the top-plate of the flow cell top-plate on the laser module and connect the inlet and outlet tubing with the flow cell (Figure 5).
41. Rinse the beam path of the device with ~900 μL ultrapure water using a 1 mL syringe.
42. Rinse the beam path again with ~900 μL of either serum-free RPMI-1640 medium or PBS using a 1 mL syringe (Figure 5).

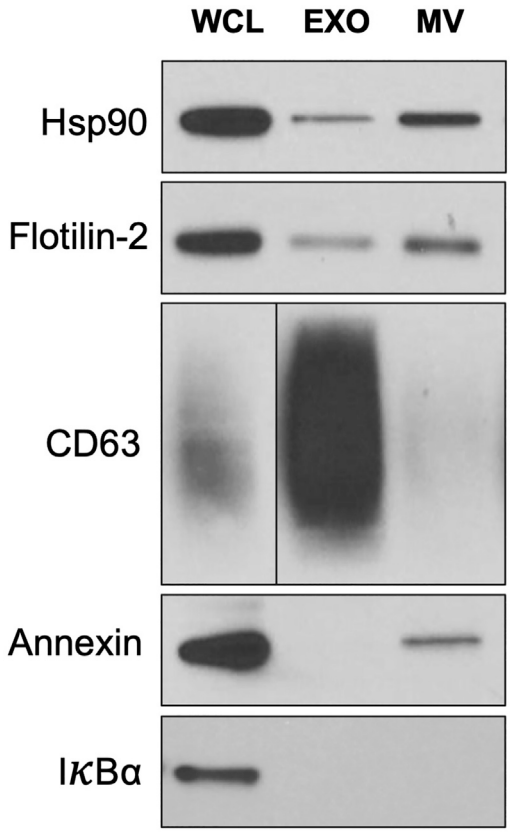

Figure 6. Immunoblot analysis of the whole cell lysates, and the exosome and MV lysates collected from MDA-MB-231 cells

Lysates of MDA-MB-231 cells (WCL), as well of the exosomes and MVs these cells generate, were incubated with antibodies to detect the expression levels of the general EV markers Hsp90 and Flotillin-2, the exosome marker CD63, the MV marker Annexin, and the WCL marker IκBα.

Note: It is important to rinse the device of impurities before experimental samples are analyzed.

⚠ CRITICAL: It is important to analyze EV samples within 48 h of their collection to minimize vesicle aggregation and lysis.

43. Use a syringe to draw 1.5 mL of a given sample (i.e., partially clarified conditioned medium, MV preparations, or exosome preparations), and remove any air bubbles from the syringe by holding it upright and tapping it with your finger to move the bubbles to the top of the syringe. Then apply gentle pressure to the plunger to push the air bubbles out of the syringe.

Alternatives: The NanoSight syringe pump can be used for sample loading.

44. Slowly inject the sample into the beam path until many particles are visible on the screen.
45. Focus the NS300 camera to obtain a clear image of the particles on the screen.
46. Capture a 60 s digital movie of the EVs under Brownian motion.
47. Slowly inject more (~100 μL) sample into the beam path, record another 60 s digital video.
48. Repeat step 47 three more times until a total of five movies have been captured. The numbers and sizes of EVs present in each sample will be determined by the NTA software.

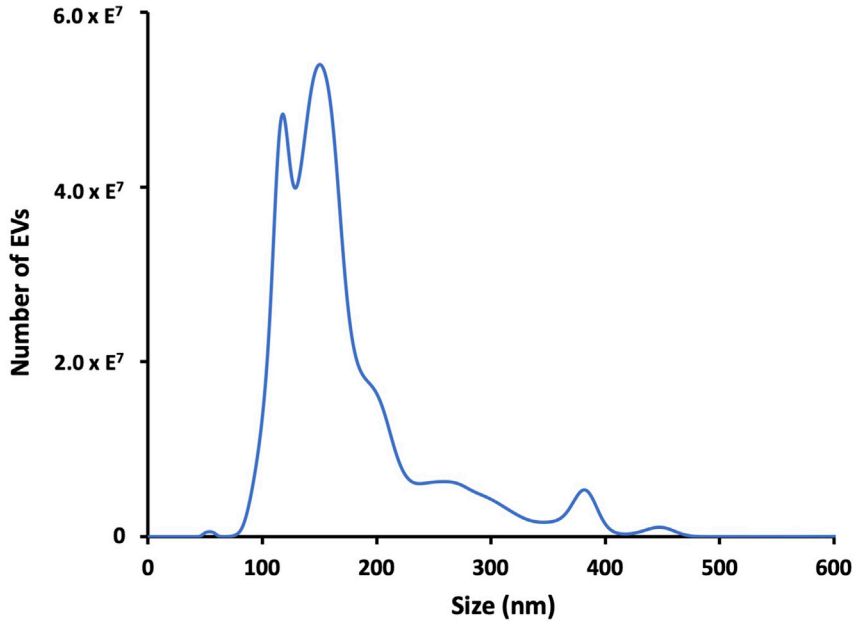

Figure 7. Nanoparticle tracking analysis (NTA) of the partially clarified medium collected from MDA-MB-231 cells
NTA was performed on the partially clarified conditioned medium collected from MDA-MB-231 cells, which contains exosomes (vesicles less than 200 nm) and MVs (vesicles larger than 200 nm).

Note: If multiple samples will be analyzed, repeat steps 41–48.

EXPECTED OUTCOMES

Here, a detailed protocol for the isolation and characterization of the two major classes of EVs (i.e., MVs and exosomes) produced by cells is provided. EVs isolated from two 150 mm dishes of MDA-MB-231 cells (i.e., ~4.0 × 10^7 cells) using this approach typically yields approximately 15–20 μg of MV lysate and 40–50 μg of exosome lysate, which is sufficient to run several western blots, perform proteomics, and carry-out biological assays. Figures 6 and 7 show examples of immunoblots performed on whole cell lysate (WCL), exosomes lysate (EXO), and MV lysate (MV) probed with various antibodies (Figure 6), as well as example of NTA performed on the partially clarified conditioned medium from MDA-MB-231 cells (Figure 7). The International Society for Extracellular Vesicles (ISEV) has recently updated their guidelines for characterizing the different classes of EVs, and it is an excellent source of additional information (Théry et al., 2018).

LIMITATIONS

There is a growing appreciation that there are likely to be multiple subclasses of MVs and exosomes that contain distinct cargo and have unique functions (Willms et al., 2016). Performing sucrose gradient centrifugation, or an iodixanol density gradient fractionation, on isolated MVs and exosomes may help to resolve the subclasses further. The MVs and exosomes isolated and resuspended in PBS can be loaded at the bottom a discontinuous sucrose (0.4–2.5 M) or iodixanol (12%–36%) gradient with decreasing concentration. The sample is subjected to ultracentrifugation (100,000 × *g*) for 15 h, which will produce 10–12 different fractions, that can be collected and subjected to immunoblot analysis using different markers of interest (Jeppesen et al., 2019; Willms et al., 2016).

A limitation of this protocol is that it has been optimized for adherent cells. To isolate MVs and exosomes from cells that grown in suspension, the conditioned medium needs to be initially cleared of

all cells and debris. This is accomplished using the two low speed spins (1,000 × *g*, 5 min). Extra centrifugation steps are also required to pellet the cells for serum starvation.

TROUBLESHOOTING

Problem 1

Low yields of MVs and exosomes.

Potential solution

The number of cells used for EV isolation can be scaled up. Although it is generally accepted that all cells generate EVs, the amounts of MVs and exosomes that different cell types produce can vary greatly.

Problem 2

MV yields are low.

Potential solution

If the amount of MVs isolated is lower than expected (but the exosome yields are sufficient), it is likely that their resuspension from the filter is incomplete (see step 15). Make sure to thoroughly rinse the filter with lysis buffer or PBS by pipetting the solution over the entire filter at least 20 times.

Problem 3

MV yields are low and MV markers are detected in the exosome preparations.

Potential solution

If MV yields are low and the exosome lysates contain MV markers (i.e., annexin), the strength of the vacuum applied to the 0.22 μm filter in step 10 of the protocol may be too strong. MVs (and exosomes) are lipid-enclosed packages and can pass through a small pore size if the vacuum pressure is strong.

Problem 4

The filter unit becomes clogged.

Potential solution

Cells and debris in the conditioned medium can cause the 0.22 μm filter unit to become clogged. Make sure that all of the cells and debris have been removed from the conditioned medium using two low speed (1,000 × *g*, 5 min) spins. Removing the supernatant immediately following each spin and ensuring any unnecessary movements are avoided will help reduce contaminants in the medium.

Problem 5

Cannot visualize the exosome pellet following ultracentrifugation of the conditioned medium.

Potential solution

Avoid unnecessary movements of the ultracentrifuge tubes following centrifugation. Use the mark made on the side of the tube to indicate the location of the exosome pellet, and aspirate the medium from the opposite side of the tube (as to not disrupt the pellet).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard A. Cerione (rac1@cornell.edu).

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

This study did not generate new databases or code.

ACKNOWLEDGMENTS

This research was supported by grants from the NIH (R35 GM122575, R01 CA101402, and U54 CA210184) to R.A.C. NTA was performed at Cornell NanoScale Facility and was supported by NSF grant NNCI-1542081. The graphical abstract was created with BioRender.com.

AUTHOR CONTRIBUTIONS

F.W. performed the experiments and wrote the manuscript. R.A.C. and M.A.A. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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