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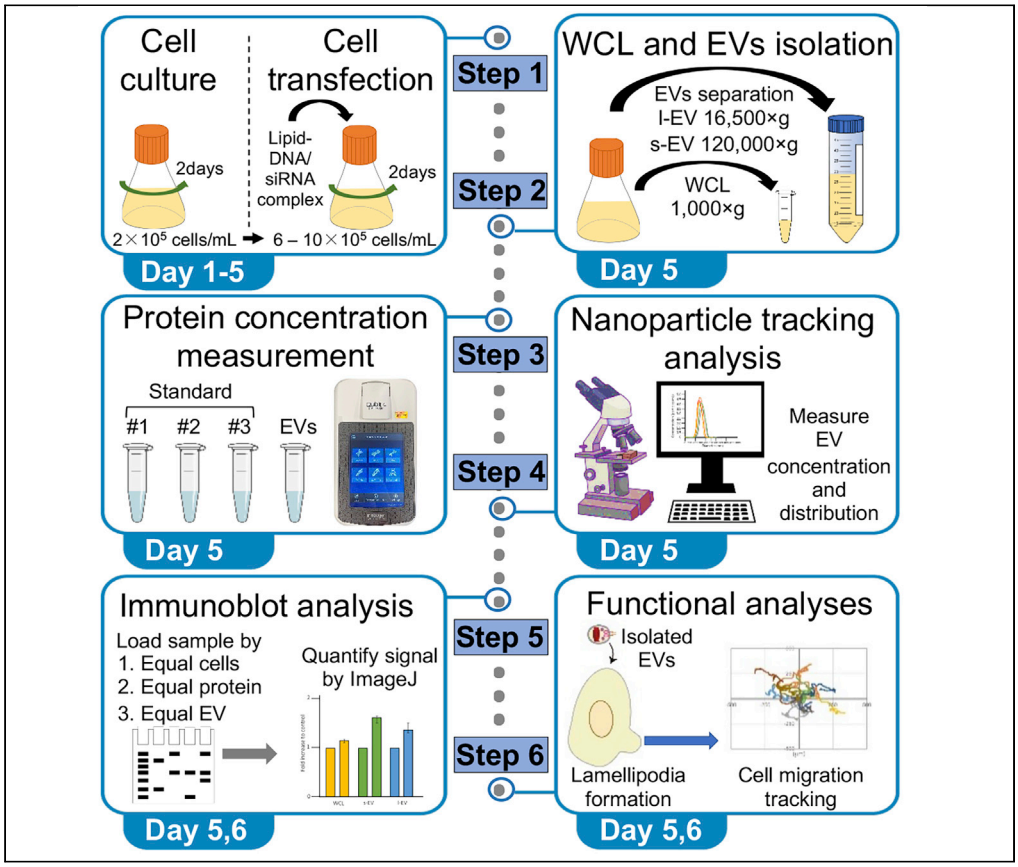
Ultracentrifugal separation, characterization, and functional study of extracellular vesicles derived from serum-free cell culture



Extracellular vesicles (EVs) play important roles in extracellular trafficking and signaling. Here, we separate EVs by differential centrifugation. EVs separated by this approach are called large EVs and small EVs, reflecting particle size, which sediment based on different ultracentrifugation forces. The resulting EVs can be quantified and analyzed using nanoparticle tracking analysis, immunoblotting, and functional assays. This protocol was applied to a suspension cell line with high transfection efficiency adapted to a high-density, serum-free culture.

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Highlights

Extracellular vesicle (EV) separation by differential centrifugation

EV collection from serum-free suspension culture

Determination of EV yield and measurement of EV size and protein amount

Analysis of EV protein content by immunoblotting and EV-induced cell migration

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Ultracentrifugal separation, characterization, and functional study of extracellular vesicles derived from serum-free cell culture

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SUMMARY

Extracellular vesicles (EVs) play important roles in extracellular trafficking and signaling. Here, we separate EVs by differential centrifugation. EVs separated by this approach are called large EVs (l-EVs) and small EVs (s-EVs), reflecting particle size, which sediment based on different ultracentrifugation forces. The resulting EVs can be quantified and analyzed using nanoparticle tracking analysis, immunoblotting, and functional assays. This protocol was applied to a suspension cell line with high transfection efficiency adapted to a high-density, serum-free culture.

For complete details on the use and execution of this protocol, please refer to Nishimura et al. (2021).

BEFORE YOU BEGIN

EVs are vesicles naturally secreted from cells (Thery et al., 2018). EVs can be generally separated into two groups, which are s-EVs (50–150 nm in diameter) and l-EVs (100–1000 nm in diameter) (van Niel et al., 2018), by different ultracentrifugation forces in the range of 100,000–200,000 × *g* for s-EVs and 10,000–20,000 × *g* for l-EVs, as the sedimentation efficiency of EVs is proportional to their sizes (Jeppesen et al., 2019). The protocol described below aims to separate the EVs secreted by FreeStyle™ 293-F cells into FreeStyle 293 Expression Medium after transfection (Nishimura et al., 2021). This cell line is adapted to suspension culture in FreeStyle 293 Expression Medium, which does not contain any EVs because it is free of materials and proteins of animal origin and is chemically defined. Hence, the step for preparing EV-free medium can be omitted. This protocol can also be applied to separate EVs from any culture medium of suspension or adherent cells, however, modification of culture condition and depletion of EVs from the serum-containing medium are required.

General laboratory preparation

Timing: 30 min

1. Warm the cell culture medium at 37°C.
2. Place the rotors in centrifuges and cool them at 4°C.

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Note: Cool down the rotors in the centrifuges for minimum 20 min. However, we suggest users to always keep the rotors in 4°C fridge to reduce the waiting time.

3. Filtrate PBS through a 0.22 μm filter and keep it on ice.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-MIM	Cell Signaling	Cat# 93065; clone D2H4C; RRID: AB_2800199
Mouse anti-CD63	Santa Cruz	Cat# sc-5275; clone MX-49.129.5; RRID: AB_627877
Rabbit anti-Nectin-2	Abcam	Cat# ab135246
Mouse anti-CD81	Santa Cruz	Cat# sc-166029; RRID: AB_2275892
Rabbit anti-integrin α2	Abcam	Cat# ab133557; RRID: AB_2833020
Mouse anti-ALIX	Santa Cruz	Cat# sc-53540; RRID: AB_673819
Mouse anti-Tsg101	Santa Cruz	Cat# sc-7964; RRID: AB_671392
Mouse anti-IRS4	Santa Cruz	Cat# sc-393207
Rabbit anti-WAVE2	Cell Signaling	Cat# 3659; clone D2C8; RRID: AB_2216981
Mouse anti-Annexin A1	Santa Cruz	Cat# sc-12740; RRID: AB_2057007
Mouse anti-calnexin	Santa Cruz	Cat# sc-23954; RRID: AB_626783
Mouse anti-GM130	BD Biosciences	Cat# 610822; RRID: AB_398141
Mouse anti-Rac1	Millipore	Cat# 05-389; RRID: AB_309712
Rabbit anti-GFP	MBL Life Science	Cat# 598; RRID: AB_591819
Mouse anti-GAPDH	Santa Cruz	Cat# sc-166574; RRID: AB_2107296
Alexa Fluor 647-conjugated highly cross-adsorbed goat anti-rabbit IgG	Thermos Fisher Scientific	Cat# A21245; RRID: AB_2535813
Alexa Fluor 488-conjugated highly cross-adsorbed goat anti-rabbit IgG	Thermos Fisher Scientific	Cat# A11034; RRID: AB_2576217
Horseradish-peroxidase-conjugated anti-mouse IgG	Promega	Cat# W401B; RRID: AB_430834
Horseradish-peroxidase-conjugated anti-rabbit IgG	Promega	Cat# W402B; RRID: AB_430833
Alkali-phosphatase (AP)-conjugated anti-mouse IgG	Promega	Cat# S373B; RRID: AB_430871
Alkali-phosphatase (AP)-conjugated anti-rabbit IgG	Promega	Cat# S372B; RRID: AB_430872
Chemicals, peptides, and recombinant proteins		
5-Bromo-3-chloro-indolyl phosphate (BCIP)	Roche Diagnostics	Cat# 1585002
4-Nitro blue tetrazolium chloride (NBT)	Roche Diagnostics	Cat# 1585029
Enhance chemiluminescent substrate (ECL) Prime Western Blotting Detection Reagents	GE	Cat# RPN 2232
Rhodamine-labeled phalloidin	Thermo Fisher Scientific	Cat# R415
Polyethylenimine (PEI MAX)	Polysciences	Cat# 24765-1
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	Cat# 31985070
293fectin™ Transfection Reagent	Thermo Fisher Scientific	Cat# 12347019
FreeStyle 293 Expression Medium	Thermo Fisher Scientific	Cat# 12338026
Critical commercial assays		
Qubit Protein Assay Kit	Thermo Fisher	Cat# Q33211
Rac1/Cdc42 Activation Assay Kit	Merck	Cat# 17-10394
Experimental models: Cell lines		
FreeStyle 293-F cells	Thermo Fisher Scientific	Cat# R79007
Recombinant DNA		
pEGFP-N3	Clontech	Cat# 6084-1
pEGFP-N3-MIM I-BAR	Nishimura et al. (2021)	N/A
pEGFP-C1-Fascin	Nishimura et al. (2021)	N/A
pmVenus-C1-IRSp53 I-BAR (1-228)	Nishimura et al. (2021)	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
ImageJ	NIH, USA	https://imagej.nih.gov/ij/download.html
MTrackJ	NIH, USA	https://imagescience.org/meijering/software/mtrackj/
Manual Tracking	NIH, USA	https://imagej.net/plugins/track/track.html
NTA version 3.1	Malvern Panalytical	https://www.malvernpanalytical.com/en/learn/events-and-training/webinars/W150326NanoSightSoftwareRelease
Other		
Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized	Merck	Cat# SLGP033RB
MX-307 high speed refrigerated microcentrifuge	TOMY	Cat# MPN 280630012
Rotor Rack AR510-04	TOMY	Cat# MPN 100035
Rotor Rack AR015-24	TOMY	Cat# MPN 100031
Optima™ L-90K Ultracentrifuge	Beckman Coulter	N/A
Type 45 Ti Fixed-Angle Titanium Rotor	Beckman Coulter	Cat# 339160
Ultracentrifuge Beckman Coulter Optima™ TLX 120,000 RPM	Beckman Coulter	N/A
TLA-120.2 Fixed-Angle Rotor	Beckman Coulter	Cat# 357656
70 mL, Polycarbonate Bottle Assembly	Beckman Coulter	Cat# 355622
1 mL, Open-Top Thickwall Polycarbonate Tube	Beckman Coulter	Cat# 343778
Nanosight LM10	Malvern Panalytical, UK	N/A

MATERIALS AND EQUIPMENT

This protocol uses TOMY MX-307 High Speed Refrigerated Microcentrifuge with centrifugation rack, AR510-04 and AR015-24 for pelleting l-EVs. Beckman Coulter Optima™ L-90K Ultracentrifuge with Ti45 Beckman Rotor and Ultracentrifuge Beckman Coulter Optima™ TLX 120,000 RPM with Beckman TLA 120.2 rotor are used for pelleting s-EVs.

10 × Phosphate-buffered saline (PBS)	Final concentration	Amount
NaCl	1.37 M	40 g
$Na_2HPO_412H_2O$	81 mM	14.5 g
KCL	26.8 mM	1 g
KH_2PO_4	14.7 mM	1 g
ddH_2O	-	Bring volume up to 500 mL
Total	-	**500 mL**

Note: 1 × PBS buffer can be prepared by diluting 10 × PBS buffer with ddH_2O. Both of them can be stored at 25°C for up to 24 months.

Alternatives: Commercial ready PBS buffer available in different companies such as Sigma (Cat# P5368-10PAK) and Thermo Fisher (Cat# 14190144) can also be used.

5 × protein sample buffer	Final concentration	Amount
2M Tris-HCL pH6.8	0.25 M	1.25 mL
SDS	10% (w/v)	1 g
2-mercaptoethanol	30% (v/v)	3 mL
Glycerol	50% (v/v)	5 mL
1% Bromophenol blue (BPB)	0.02% (w/v)	0.2 mL
Total		**10 mL**

Note: 1 × protein sample buffer can be prepared by diluting 5 × protein sample buffer with ddH_2O. Both of them can be stored at −30°C for up to 24 months.

Alkaline phosphatase (AP) buffer	Final concentration	Amount
Tris	100 mM	12 g
NaCl	100 mM	5.8 g
$MgCl_2 \cdot 6H_2O$	5 mM	1 g
ddH_2O		Adjust pH to 9.5 and volume up to 1 L
Total		1 L

Note: AP buffer can be stored at 25°C for up to 24 months.

STEP-BY-STEP METHOD DETAILS

Day 1: Preparation of FreeStyle™ 293-F cell for transfection

Timing: 30 min for seeding cells; 48 h of growing cells before transfection

The EVs are secreted into FreeStyle 293 Expression Medium during culture, and the resulting medium is referred to as the conditioned medium.

Caution: Perform all steps described in this section in a sterilized biosafety cabinet.

1. Grow and expand suspension cell number in culture flask, such that they will reach the density of $0.6 – 1.0 \times 10^6$ cells/mL after 48 h. In the case of FreeStyle™ 293-F cells, culture 2×10^5 cells/mL in a 125 mL polycarbonate, disposable, nonpyrogenic Erlenmeyer flask, in final volume of 15–30 mL of FreeStyle 293 Expression Medium. Maintain the culture in 37°C, 8% CO_2 incubator with constant agitation on an orbital shaker rotating at 135 rpm.

   Note: To assure the cell density is sufficient for transfection at 48 h later, the cells at this point should be in the log phase, and the cell viability should be more than 90%.

2. Use trypan blue exclusion to determine cell density and cell viability.
   a. Aliquot 100 μL of cell suspension into a microcentrifuge tube.
   b. Add equal part of 0.4% trypan blue solution (100 μL in this case) to the cell suspension to achieve 2 times dilution and pipetting several times to mix completely.
   c. Load 8 μL of the trypan blue/cell mixture to a hemocytometer chamber.
   d. View the hemocytometer under light microscope with low magnification.
   e. Count the stained (nonviable) and unstained (viable) cells from the four corner squares of hemocytometer.
   f. Calculate the percentage of viable cells as follows:
   g. Calculate the cell density as follows:

   ⚠ CRITICAL: To ensure that the amount of EVs collected from the conditioned medium is sufficient for downstream analysis (Please refer to the expected outcome for the typical yield of this protocol), it is recommended that a minimum of 15 mL cell suspension at the density of 2×10^5 cells/mL is used as a starter culture at this point.

   Note: The cell number can be scaled up or down by modifying the culture volume based on experimental needs.

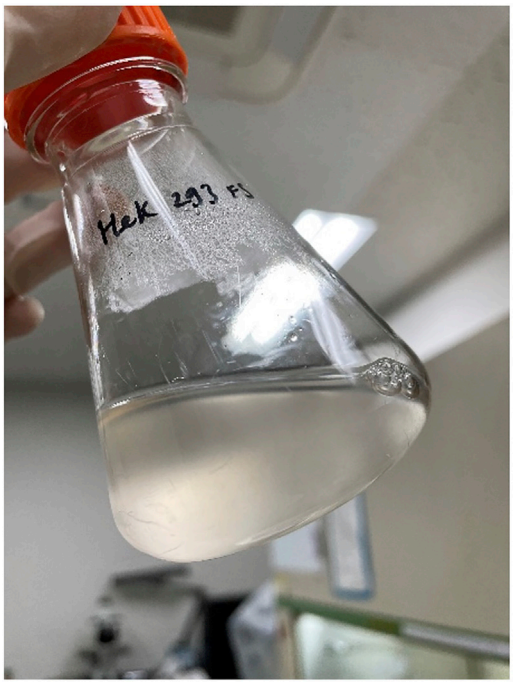

Figure 1. Appearance of the Freestyle 293-F cell suspension culture in a flask

Day 3: Transfection of FreeStyle™ 293-F cell

Timing: 1 h to transfect cells; 48 h for plasmid-DNA or siRNA-transfected cell culture before EV collection

This section aims to transfect the cells with desired plasmid DNA or siRNA to determine the effect of a gene of interest on EV secretion. For the manufacturer's protocol of FreeStyle™ 293-F cell transfection, please visit https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0007819_293fectinTransfectionReagent_UG.pdf&title=VXNlciBHdWlkZTogMjkzZmVjdGluIFRyYW5zZmVjdGlvbiBSZWFnZW50.

Caution: Perform all steps described in this section in a sterilized biosafety cabinet.

3. Aliquot 100 μL of cell suspension into microcentrifuge tube. Use trypan blue exclusion method (see step 2) to determine cell viability and examine the clumping of the cells. To achieve high transfection efficiency, the percentage of cell viability must be more than 90%.

   Note: For good transfection efficiency, it is important to ensure the cells are in single-cell suspension. Vortex the cell suspension vigorously at 2,500–3,000 rpm for about 30 s to dissociate cell clumping. The cell suspension should appear turbid and homogenous, as shown in Figure 1.

4. Count the cell number by using hemocytometer. The cell density at this point should be around $0.6–1.0\times10^6$ cells/mL. FreeStyle™ 293-F cells usually will achieve approximately 0.8×10^6 cells/mL.
5. Pellet down the cells at 200 × *g* for 5 min at 25°C and resuspend them in fresh, pre-warmed medium. For each 30 mL transfection, resuspend the cells in 28 mL of the medium. Keep the cells in culture flask under constant agitation to avoid cell clumping for the transfection later.

6. Prepare the lipid-DNA or siRNA complex. In the FreeStyle™ 293-F cells system, we use 293fectin reagent for siRNA transfection according to the manufacturer's protocol. As for plasmid DNA transfection, we use 293fectin or polyethyleneimine (PEI MAX).
   a. Dilute 30 μg of plasmid DNA or 72 μL of 20 μM siRNA in 1 mL Opti-MEM reduced-serum medium in a tube. Dilute 60 μL of 293fectin in 1 mL Opti-MEM medium in another tube. For plasmid DNA transfection, 90 μL of 1 mg/mL PEI MAX can be used instead of 293fectin. Incubate them for 5 min at 25°C.
   b. Mix the solutions and incubate for 30 min at 25°C to allow the formation of lipid-DNA or siRNA complex.
   c. Add the complex to the previously prepared 28 mL cell suspension to form total 30 mL cell suspension culture.
7. Incubate the cells in 37°C, 8% CO_2 incubator with constant agitation on an orbital shaker rotating at 135 rpm for 48 h.

⚠ CRITICAL: For siRNA transfection, optimal cell concentration at the transfection is 0.6–0.7×10^6 cells/mL.

Note: When transfecting cells with nucleic acid, the possible contamination of nucleic acid in the EV preparation is unavoidable. Hence, extra caution should be taken for analysis of nucleic acid contents by next-generation sequencing (NGS) and so on.

Day 5: Collecting whole cell lysate and conditioned medium

Timing: 1–1.5 h

The cell suspension is collected in 50 mL conical tube and centrifuged twice at low speed (1000 × *g* and 3000 × *g*, each for 10 min) to pellet down the cell body and eliminate cellular debris from the conditioned medium. This partially clarified medium will be subjected to differential ultracentrifugation in the next section to separate the l-EVs and s-EVs. An overview of the whole-cell lysate (WCL) and EV preparation is illustrated in Figure 2.

8. Observe the cell suspension under light. As shown in Figure 1, you should see a homogenous cell suspension without cell clumping. Alternatively, aliquot a small amount of cell suspension and observe under microscope. If cell clumping is observed, vortex the cell suspension vigorously at 2,500–3,000 rpm for about 30 s to dissociate the clump.

⚠ CRITICAL: It is important to dissociate cell clumping as it will affect the cell counting, which will be used to calculate the number of EV secretion per cell and immunoblotting normalization later.

9. Aliquot 100 μL of the cell suspension to microcentrifuge tube for trypan blue exclusion assay and cell counting using hemocytometer (see step 2). Both the viable cell number and dead cell number should be counted.

Note: We recommend examining the number of dead cells, which can be fragmented into the apoptotic bodies. The dead cell number should be similar between comparisons. The larger fraction of dead cells in the culture will cause the contamination of apoptotic bodies in the EV fractions.

10. Aliquot another 500 μL cell suspension to a microcentrifuge tube to prepare WCL fraction.
    a. Centrifuge the cell at 1,000 × *g* at 4°C for 10 min.
    b. Discard the supernatant and resuspend the pellet in 1× sample buffer (dilute 5× sample buffer with ddH_2O). Pipette the WCL up and down several times.

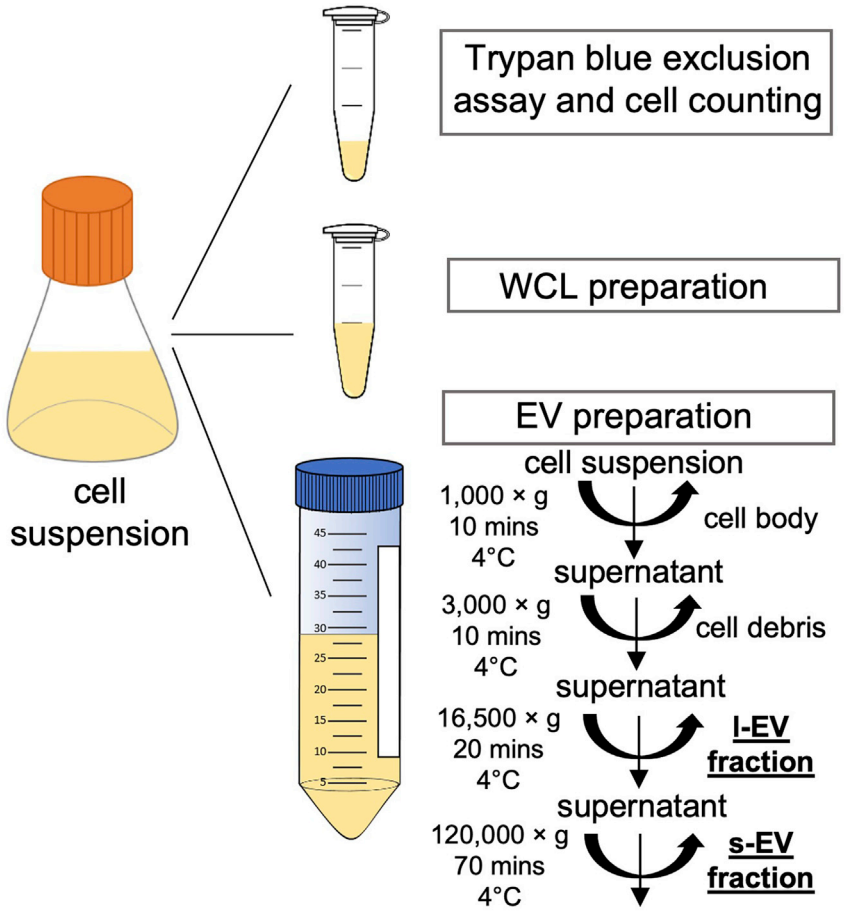

Figure 2. Scheme of the preparation of WCL, l-EVs, and s-EVs by stepwise centrifugation from HEK 293 cell culture media

c. Sonicate the lysate.

Note: We sonicate the cell lysate for 30 s at the intensity of output 4 using an ultrasonic cell homogenizer (Taitec Co., Model VP-5S). Other model of homogenizer with similar output can be used as well. The purpose here is to reduce the viscosity caused by the nucleic acids in the samples for electrophoresis, therefore, moderate sonication is sufficient.

d. Heat the sample at 95°C for 5 min.

e. Keep the sample at −30°C for further analysis.

11. Transfer the rest of the homogenous cell suspension to a 50 mL conical tube for EV separation.
12. Perform 2-steps low-speed centrifugation to eliminate the cell body and debris from the conditioned medium.
    a. Centrifuge the cell at 1,000 × *g* at 4°C for 10 min to pellet down the cell body.
    b. Transfer the supernatant to a new 50 mL conical tube and centrifuge again at 3,000 × *g* at 4°C for 10 min to pellet down the cell debris.
    c. Transfer the supernatant to a new 50 mL conical tube for EV fractionation. Keep the tube on ice to avoid EV degradation.

⚠ CRITICAL: Gently open the lid of the conical tube and carefully transfer the supernatant without disturbing the pellet.

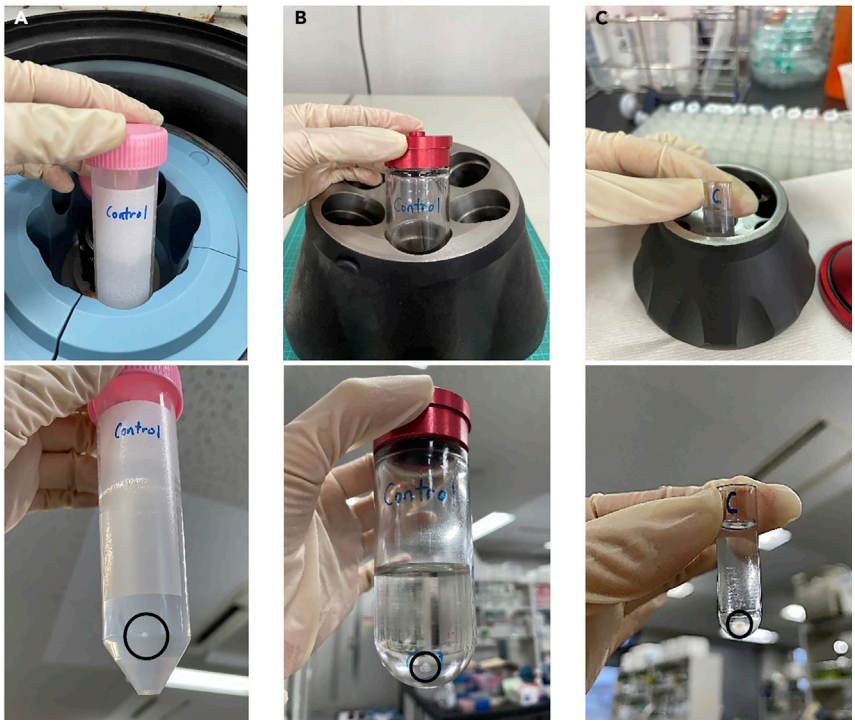

Figure 3. Setting of the centrifuge tubes in rotors and the appearance of the pellet after the centrifugation for each preparation

(A) Top: setting of the conical tube in AR510-04 rotor. Bottom: the appearance of the l-EV pellet after centrifugation.

(B) Top: setting of polycarbonate ultracentrifuge bottle in Ti45 Beckman rotor. Bottom: the appearance of the s-EV pellet after centrifugation.

(C) Top: setting of 1mL open-top thick-wall polycarbonate ultracentrifuge tube in the Beckman TLA 120.2 rotor. Bottom: the appearance of the s-EV pellet after centrifugation.

Day 5: Extracellular vesicle fractionation

Timing: 4–5 h

Differential high-speed centrifugation is applied to the conditioned medium to separate the l-EVs and s-EVs. The stepwise ultracentrifugation is depicted in Figure 2.

Note: The ultracentrifugation parameters in this protocol are partly adapted (Lasser et al., 2012).

13. The partially clarified conditioned medium in 50 mL conical tube now contains l-EVs and s-EVs. To separate l-EVs, we use TOMY MX-307 high-speed refrigerated microcentrifuge.

Note: As shown in Figure 3A, while using fixed angle rotor, place the conical tube in the rotor so that the marking area of the conical tube is facing outward. This eases the finding of the pellet, which usually accumulates at the lower portion of the tube at the side of the marked area. In this way, even if the pellet is too small to be seen, we can still know which side of the tube the pellet is on.

a. Subject the conditioned medium to 16,500 × *g* centrifugation for 20 min at 4°C (TOMY AR510-04 rotor; k-factor: 880; 13,500 rpm) to pellet down the l-EV.

b. Mark the position of the l-EV pellet on the conical tube and gently decant the supernatant to a clean polycarbonate ultracentrifuge bottle (Beckman, 355622). Immediately keep the

Figure 4. Decantation of the supernatant to a polycarbonate ultracentrifuge bottle with the part of the tube with EV pellet (red circle) facing upward

bottle on ice as the supernatant at this point contains s-EV. (Please go to step 14 for s-EV separation)

Note: An off-white to transparent EV pellet is usually visible in the lower portion of the tube at the marking side. The pellet might be hard to be seen if the size is too small. Please refer to troubleshooting problem 1 if unable to visualize the EV pellet.

⚠ CRITICAL: Open the lid of the conical tube carefully without disturbing the pellet. While decanting, keep the pellet side of the tube facing upward so that it is not transferred to the new tube (Figure 4).

c. Resuspend the l-EV pellet in 500 μL of sterile ice-cold filtered PBS. Pipet up and down against the wall of the conical tube at the marking area to fully dislodge and dissociate the pellet.
d. Move the resuspended l-EV to a low-protein-binding microcentrifuge tube (Watson, PK-15C-500).
e. Transfer another 500 μL sterile ice-cold PBS to the conical tube and pipette against the wall to collect the remaining EV in the tube.
f. Move the remaining EV suspension to the low-protein binding microcentrifuge tube to make a total of 1 mL l-EV resuspended in sterile ice-cold PBS.
g. Centrifuge the tube at 16,500 × *g* centrifugation for 20 min at 4°C (TOMY AR015-24; k-factor: 643; 13,500 rpm) to precipitate the l-EV.
h. Use p1000 to remove 800 μL of the supernatant.

⚠ CRITICAL: Pipette out the supernatant gently. Leave 200 μL of supernatant in the micro-centrifuge tube to avoid disturbing the pellet.

i. Centrifuge the tube again at 16,500 × *g* centrifugation for 3 min at 4°C (TOMY AR015-24; k-factor: 643; 13,500 rpm).
j. Use p200 to remove the remaining supernatant completely.
k. Resuspend pellet in a suitable buffer for downstream application. Keep the tube on ice.

Note: We suggest to resuspend the l-EVs in 75–150 μL cold-sterile PBS buffer, and dispense some amount to sterile tubes for downstream experiments. For example, aliquot 5–10 μL of l-EV suspension for NTA, 3–5 μL for Qubit protein quantification assay, 10 μL for cell migration assay, and 5–10 μL for lamellipodia formation assay. For immunoblot analysis, add 5× sample buffer to 1/4 of the sample volume to lyse the EVs. Besides PBS buffer, other neutral pH buffers such as HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer can also be used for l-EVs suspension.

Note: L-EVs might form clusters due to their "sticky" nature. Gently resuspend the pellet by pipetting up and down for 20–30 times to dissociate the clump adequately.

Note: The l-EVs suspended in PBS buffer can be kept frozen at −80°C to be used for protein quantification, immunoblotting and functional study later on. However, multiple freeze-thaw cycles might deteriorate the sample quality. For NTA, we suggest storing the l-EV suspension at 4°C and analyze within 24 h in order to get stable and reproducible results.

14. Weigh the polycarbonate ultracentrifuge bottles containing supernatant from step 13b. Equate their weight by using sterile ice-cold PBS.

⚠ CRITICAL: It is important to ensure that all the bottles are properly balanced. An imbalance can damage the ultracentrifuge equipment.

15. Place the bottles into the pre-cooled Ti45 Beckman rotor. We use Beckman Coulter Optima™ L-90K Ultracentrifuge to separate s-EVs.

Note: Similar to the previous step, while using the Ti45 fix-angle rotor, it is important to label the side of the bottle facing outward of the rotor to indicate the pelleting side. This step is also shown in Figure 3B.

a. Centrifuge the bottles at 120,000 × *g* for 70 min at 4°C (Ti45 Beckman Rotor; k-factor: 259; 32200 rpm).
b. Immediately pull out the bottles from the rotor. Use a marker to circle the EV pellet.

Note: An off-white to transparent EV pellet is usually visible in the bottom portion of the bottles at the marking side. The pellet might be hard to be seen if it is too small. Hence, it is important to indicate the side where the pellet will attach.

c. Gently open the lid of the bottle and carefully decant the supernatant. The supernatant is now free of l-EVs and s-EVs.

Optional: The vesicle-free supernatant can be collected in a conical tube to be analyzed for its contents, such as soluble factors. The soluble factors in the vesicle-free medium can be concentrated by using centrifugal concentrator and analyzed by using immunoblotting (Wang et al., 2021).

Note: While decanting, keep the pellet side of the tube facing upward so that it is not disturbed.

d. Immediately keep the bottles containing the s-EV pellet on ice.
e. Wash the s-EV pellet with 400 μL sterile ice-cold PBS. Gently resuspend the pellet by pipetting up and down several times.
f. Transfer the resuspended s-EV to the labeled, 1 mL open-top thick-wall polycarbonate ultracentrifuge tube (Beckman, 343775), which is placed on ice.
g. Collect remaining EV using another 400 μL sterile ice-cold PBS.
h. Weigh the tubes to ensure that they are balanced. Add sterile ice-cold PBS to equate their weight if necessary.

i. Place the tube into the pre-cold Beckman TLA120.2 fixed-angle rotor (Figure 3C).
j. Centrifuge at 120,000 × *g* for 70 min at 4°C (Beckman TLA 120.2 rotor; k-factor: 34.8; 58,000 rpm) using ultracentrifuge Beckman Coulter Optima™ TLX 120,000 RPM.
k. Immediately pull out the tube from the rotor and decant the supernatant.

Note: While decanting, keep the pellet side of the tube facing upward so that it is not disturbed.

l. Remove the remaining medium in the tube completely by turning them upside down on a paper towel. Use p200 to remove the residual medium in the tube if necessary.
m. Resuspend pellet in a suitable buffer for downstream application and move the EV suspension to a low-protein-binding microcentrifuge tube (Watson, PK-15C-500). Keep the tube on ice.

Note: We suggest to resuspend the s-EVs in 75–150 μL cold-sterile PBS buffer, and dispense some amount to sterile tubes for downstream experiments. For example, aliquot 5–10 μL of s-EV suspension for NTA, 3–5 μL for Qubit protein quantification assay, 10 μL for cell migration assay, and 5–10 μL for lamellipodia formation assay. For immunoblot analysis, add 5× sample buffer to 1/4 of the sample volume to lyse the EVs. Besides PBS buffer, other neutral pH buffers such as HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer can also be used for s-EVs suspension.

Note: The s-EVs suspended in PBS buffer can be kept frozen at −80°C to be used for protein quantification, immunoblotting and functional study later on. However, multiple freeze-thaw cycles might deteriorate the sample quality. For NTA, we suggest storing the s-EV suspension at 4°C and analyze within 24 h in order to get stable and reproducible results.

Day 5: Measurement of the protein concentration of EVs

Timing: 25 min

The protein concentration of the l-EV and s-EV fractions should be quantified. The protein concentration of EV will be used to normalize the sample loading in the immunoblotting assay later. In this protocol, we use Qubit™ protein quantification assay for the measurement. This assay is easy to perform and provides accurate results. For the manufacturer's protocol, please visit https://assets.thermofisher.com/TFS-Assets/BID/manuals/MAN0017210_Qubit_4_Assays_QR.pdf

Note: To quantify the protein amount inside EVs, add 0.2% SDS to the EVs suspension to lyse the EVs (Vergauwen et al., 2017). To measure EV surface protein, no detergent is required.

16. Prepare 3 Qubit™ assay tubes for 3 standards and 1 Qubit™ assay tube for each sample. Label the standards and the sample according to their orders at the tube lids.

Note: Always label the tube lids only. Labeling at the side of the tube could interfere with the measurement.

17. Prepare 200 μL Qubit™ working solution for each standard and sample.
    a. Dilute the Qubit™ reagent in the Qubit™ buffer in a ratio of 1:200. For example, if there are 2 samples, prepare 1000 μL working solution by diluting 5 μL Qubit™ reagent in 995 μL Qubit™ buffer.
    b. Vortex the working solution for 5–10 s to mix well.
18. Dispense 1–5 μL of EV samples suspended in sterile PBS into Qubit assay tube and dilute with the Qubit™ working solution to a total volume of 200 μL.

Note: Qubit™ protein quantification assay can detect the protein concentration range from 12.5 μg/mL to 5 mg/mL. Sample volume can be increased up to 20 μL depending on the protein concentration.

⚠ CRITICAL: The presence of KPO_4 and NaCl in the PBS could interfere with the measurement. For an accurate result, it is necessary to add an equal amount of PBS to the standard tubes. For other different contaminants in the buffer of choice, please visit the user guide at http://tools.thermofisher.com/content/sfs/manuals/Qubit_Protein_Assay_UG.pdf

19. Dispense 10 μL of each standard #1, #2, and #3 into clean tubes and add same volume of PBS (1–5 μL) used to load the samples. Dilute the standards with Qubit™ working solution to a total volume of 200 μL.
20. Vortex all tubes for 2–3 s to mix well.
21. Incubate them at 25°C for 15 min.

⚠ CRITICAL: The Qubit™ Protein Assay works best at room temperature (22°C–28°C). Warm up the working solution to around 25°C for accurate measurements. Avoid holding the sample before measurement, as raising in temperature could lower the reading.

22. Insert the tube in the Qubit™ Fluorometer and take the reading.

Day 5: Measurement of the size distribution and concentration of EVs

Timing: 15 min per sample

NTA is used to measure the size and amount of EVs. NTA enables the tracking and quantification of EVs by capturing the movies of particles moving under Brownian motion. NTA tracks the center of each particle, calculates the average distance moved per time, and converts it into a size distribution profile. The concentration of EVs measured in this section will be used to normalize the sample loading in the immunoblotting assay later. In this protocol, Nanosight LM10 (Malvern Panalytical, UK) and NTA 3.1 analytical software (Malvern Panalytical, UK) are used for the analysis.

⚠ CRITICAL: Always keep the EVs on ice or in 4°C fridge for storage. Perform the NTA measurement within 48 h after EV collection to reduce EV lysis and aggregation.

23. Dilute the EVs sample in 300–500 μL of sterile ice-cold PBS and keep on ice.

Note: For initial measurement, it is recommended to start with low dilution (e.g., 40× dilution for l-EVs and 100× dilution for s-EVs) and then use further dilution to get an optimum particle concentration for the measurement. The optimum particle range should within 10^8–10^9 particles/mL.

24. Clean the LM10 viewing unit using a deionized-water-dampened optical lens grade cleaning tissue to wipe over the inside surface of the top-plate window and the optical plate surface. Dry the surface by wiping with the tissue and blowing with an air stream from an air compressor. The viewing unit components are depicted in Figure 5.

Caution: Water could seep into the laser module and cause damage. Do not wipe the surface by using soggy tissue or pour any liquid over the surface.

25. Assemble the LM10 viewing unit and attach the power lead.

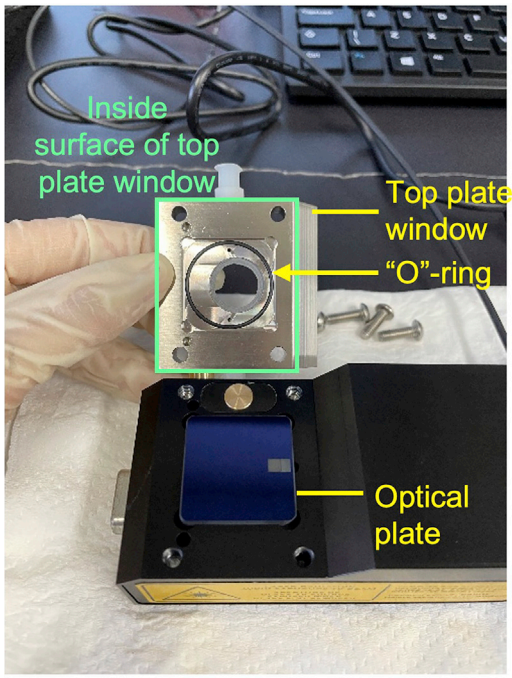

Figure 5. Nanosight LM10 viewing unit components

Caution: Make sure the "O"-ring is placed correctly in the groove on the inside surface of the top plate (Figure 5). It is important to avoid any leakage of liquid to the laser inside.

Note: To further ensure the beam path is free of impurities, users are encouraged to rinse the beam path twice by injecting 1 mL of PBS (or any buffer used to resuspend the EVs) into the chamber slowly and steadily using a syringe.

26. Load the EV sample into the chamber. Similar steps can be taken for PBS buffer (or any buffer used in EV suspension) as a control for the measurement.
    a. Vortex the EV sample gently at 500–1,000 rpm for 5 s.
    b. Use 1 mL syringe to withdraw 0.5 mL of sample. Ensure no bubbles are introduced into the syringe. If there is, hold the syringe upright and slowly eject the air bubbles from the tip.
    c. Introduce the sample slowly and steadily into the chamber. Rapid injection might increase the pressure in the chamber and cause leakage. After the introduction, withdraw the sample by pulling the plunger and then introduce it again. Repeat these 3 times to make the sample distribute evenly in the chamber.

Alternatives: For smoother flow of sample loading, the Malvern Panalytical NanoSight syringe pump can be used instead of manual injection.

27. Place the viewing unit on the microscope stage and turn on the laser power.
28. Adjust the imaging position, focus, and camera level on the capture setting to obtain a clear and distinguishable particle image on the screen.

⚠ CRITICAL: EV aggregates can affect the NTA analysis. To reduce the aggregates, add more PBS to resuspend the EVs and pipette up and down several times. If aggregates are still visible, gently vortex the suspension at 500–1,000 rpm for 10–15 s.

29. Capture the movie of the particles under Brownian motion for 30–60 s.

30. Change to a different observation field by slowly inject more sample into the chamber and record another movie. Repeat this step another 3 times to capture a total of 5 different movies.
31. Adjust the detection threshold to the level that most of the particles are properly identified. Proceed to the NTA analysis to determine the particle concentration and size distribution.

Day 5-6: Identification of protein marker in EVs by immunoblotting

Timing: 2 days

The total cell number, EV protein concentration, and EV number quantified previously can be used to normalize the immunoblotting analysis in this section. The protein of interest and specific markers in WCL, l-EV and s-EV fractions can be detected using various antibodies, including those listed in the key resources table. The protein amount can be quantified by using ImageJ software (NIH, USA).

32. Add 5× sample buffer of one-fourth volume into the l-EV and s-EV samples obtained previously to make 1× sample buffer concentration and heat them at 95°C for 5 min.
33. Load the equal cell number, equal protein concentration, and equal EV number per sample to SDS-PAGE. For cell-number normalization, load 1×10^4 cells for WCL and 4×10^6 cells for EV; for total-protein normalization, load 500 ng protein; for EV-number normalization, load 6×10^8 EVs.
34. Run the SDS-PAGE and resolve the protein based on their size.
35. Transfer the protein resolved in the gel onto polyvinylidene difluoride (PVDF) membrane using a semi-dry western blot transfer system for 45 min to 1 h at 24 V.
36. Block the membrane in 20 mL of 5% skim milk/PBS-0.05% Tween 20 (PBS-T) solution and rock it on a shaker for 1 h at 25°C.
37. Wash the membrane with PBS-T once.
38. Incubate the membrane in primary antibody diluted in PBS-T/0.01% NaN_3 and rock it for 12–16 h at 4°C. The antibody dilution varies according to manufacturer recommendation. For the antibodies listed in the key resources table and used in Figure 7:
    a. Calnexin and GM130 antibodies can be used as negative marker for EVs at 1:1000 dilution.
    b. CD81, CD63, TSG101 and ALIX antibodies can be used as s-EV markers at 1:1000 dilution.
    c. Annexin A1 antibody can be used as l-EV marker at 1:1000 dilution.
    d. MIM, Integrin α2, IRS4, and GAPDH antibodies can be used at 1:000 dilution.
    e. Nectin-2 antibody can be used at 1:2000 dilution.
    f. Rac1 and GFP antibodies can be used at 1:4000 dilution.

    Caution: Many antibodies for tetraspanins, including the anti-CD63 antibody listed in the key resources table, do not bind to antigens treated with sample buffers containing reducing agents such as 2-mercaptoethanol or dithiothreitol. In case you use those antibodies, you should omit reducing agents from the sample buffer. The anti-CD81 antibody in the list can be used for samples treated with these reagents. See also troubleshooting problem 5.

39. Wash the membrane with PBS-T for 10 min at 25°C for 3 times.
40. Incubate the membrane with AP- or HRP- labeled secondary antibody diluted in PBS-T for 10,000× at 25°C for 1 h.
41. Wash the membrane with PBS-T for 10 min at 25°C for 3 times.

For detection by AP, go to step 42, and for detection by HRP, go to step 46.

42. For AP detection, wash the membrane with AP buffer at 25°C once.
43. Dilute 37.5 μL BCIP solution (50 mg/mL in dimethylformamide) and 50 μL NBT solution (100 mg/mL in 70% dimethylformamide) in 10 mL AP buffer. Add 4–5 mL to each membrane and shake at 25°C until the protein bands appear.
44. To stop the reaction, discard the solution and wash the membrane with tap water.

45. Dry the membrane and quantify the signals by using ImageJ software.
46. For HRP detection, add 5 mL of ECL Prime western blotting detection reagents and detect the chemiluminescence using the image detector, such as LAS 4000 (GE Healthcare).

Day 5-6: The assay for the EV-induced cell migration

Timing: 2 days

Some EVs promote the migration of cells in an autocrine or paracrine manner, which may cause some diseases such as cancer cell metastasis (Becker et al., 2016). Cell migration can be measured by live cell imaging in the presence of EVs and subsequent cell tracking analysis.

Lamellipodia formation is one of the key features of cell migration (Krause and Gautreau, 2014). Lamellipodia are fan-like structures of actin filaments assembled at the cell periphery, especially at the leading edge of the migrating cells (Krause and Gautreau, 2014). Lamellipodia are mainly generated by the activation of the Wiskott-Aldrich syndrome protein-family verprolin homologous protein 2 (WAVE2) complex, which causes the Actin-related protein 2/3 (Arp2/3) complex-mediated actin polymerization (Oikawa et al., 2004). WAVE2 complex is activated by a small G protein Rac1, which activation leads to the lamellipodia formation (Miki et al., 1998). Lamellipodia formation can be assessed by the staining of actin filaments and WAVE2, which is the major component of WAVE2 complex.

Note: It is important to control the amount of EVs added to the cells. By adding the same amount of EVs to the cells, we can compare the differential effects of EVs. The amount can be adjusted by the number of the cells that produce EVs, the number of EV particles, the amount of proteins that EVs have, and so on. For example, 2–10 μg/mL of EVs can lead to cell migration in our hand. However, the amount is subjected to cell type and experimental condition. In this section, we describe the effect of the EV on cell migration, lamellipodia formation, and Rac1 activity, but other analyses can be done in a similar way.

47. Cell migration assay
    a. Mix 293-F cells (8×10^4 cells) suspended in 1.2 mL of FreeStyle 293 Expression Medium with the EVs and seed them in the 3.5 cm culture dishes (Corning).

Note: The amount of EVs to be used in the assay can be adjusted by cell number, EV number, or EV protein concentration. For example, we perform the assay by using EVs prepared from 9×10^6 of HEK293-F cells.

    b. Set the dishes on the CytoSMART Lux2 (Zutphen, Netherlands) apparatus in the 8% CO_2 incubator at 37°C. After 30 min, take pictures of the cells every 15 min for 48 h. For CytoSMART Lux2 information, please visit the following link: https://www.cytosmart.com/products/lux2.
    c. Track the migration of cells manually by ImageJ (NIH, USA) using Cell Tracker plug-ins such as MTrackJ or Manual Tracking (NIH, USA). Select 10 cells per region and track the cell migration every 15 min for 48 h. Calculate the average migration speed for every 12 h and compare them with the first 12 h migration speed.

48. Lamellipodia formation assay
    a. Suspend HEK 293-F cells (8×10^3 cells) in 0.1 mL of FreeStyle 293 Expression Medium, seed them in glass-based dishes (IWAKI), and incubate in 8% CO_2 incubator at 37°C for 3 h.
    b. Replace the FreeStyle 293 Expression Medium with fresh medium containing EVs and incubate at 37°C, 8% CO_2 for 15 min.

Note: The amount of EVs to be used in the assay can be adjusted by cell number, EV number, or EV protein concentration. For example, we perform the assay by using EVs prepared from 9×10^6 of HEK293-F cells.

c. Immediately after removing the medium, fix the cells with 4% (wt/vol) paraformaldehyde (PFA) in the FreeStyle 293 Expression Medium at 25°C for 5 min. Then, wash the cells with tris-buffered saline (TBS) once.

Caution: After removing the medium, the cells should be fixed immediately and quickly to avoid the disassembly of actin filaments, including lamellipodia.

d. Permeabilize cells with 0.1% Triton X-100 in 1% bovine serum albumin (BSA)/TBS for 5 min. Then, wash the cells with TBS once.
e. Block the cells with 3% (wt/vol) BSA in TBS at 4°C for 16 h. Then, wash the cells with TBS once.
f. Incubate the cells with anti-WAVE2 antibody or an antibody to any other lamellipodia localizing protein in 1% BSA/TBS (90 μL) at 25°C for 2 h.

Note: The rabbit anti-WAVE2 antibody recommended in key resources table can be used at 1:100 dilution.

g. Wash the cells 3 times with TBS.
h. Incubate cells with secondary fluorochrome-labeled antibodies and fluorochrome-labeled phalloidin in 1% BSA/TBS at 25°C for 1 h.

Note: Please refer to the key resources table for the recommended antibodies. Dilute the Alexa Fluor 647-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-rabbit IgG at 1:400. For rhodamine-labelled phalloidin, dilute at 1:1000.

i. Wash the cells 3 times with TBS.
j. Soak the cells in TBS containing 0.02% (w/v) NaN_3.
k. Observe cells under a confocal microscope.

49. Rac1 activity measurement

Note: Because Rac1 was enriched in the missing in metastasis (MIM)-induced l-EVs, we describe this measurement (Nishimura et al., 2021). Rac1 activity in l-EVs was measured using Rac1/Cdc42 Activation Assay Kit (Merck) according to the manufacturer's protocol with slight modifications as follows. https://www.merckmillipore.com/JP/en/product/Rac1-cdc42-Activation-Magnetic-Beads-Pulldown-Assay,MM_NF-17-10394?ReferrerURL=https%3A%2F%2Fwww.bing.com%2F#overview.

a. Lyse l-EVs prepared from 3.6×10^7 cells with 300 μL of lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol containing protease inhibitors) on ice and then centrifuge at 14,000 × *g* for 5 min at 4°C.
b. Transfer the supernatant to GST-PAK1-PBD-conjugated resin (bed volume: 10 μL) and rotate at 4°C for 45 min.
c. Wash 3 times with 0.5 mL of lysis buffer and analyze the protein bound to resin by immunoblotting.

Note: Only GTP-bound active form of Rac1, but not GDP-bound inactive form, can bind to the GST-PAK1-PBD-conjugated resin.

EXPECTED OUTCOMES

This protocol illustrates the l-EV and s-EV separation by differential ultracentrifugation from FreeStyle™ HEK 293-F suspension cell culture and indicates some downstream analysis methods. The medium after 120,000 × *g* ultracentrifugation are considered to be vesicle-free, as this g-force can pellet most of the small particles such as exosomes, exomeres and non-vesicular particles

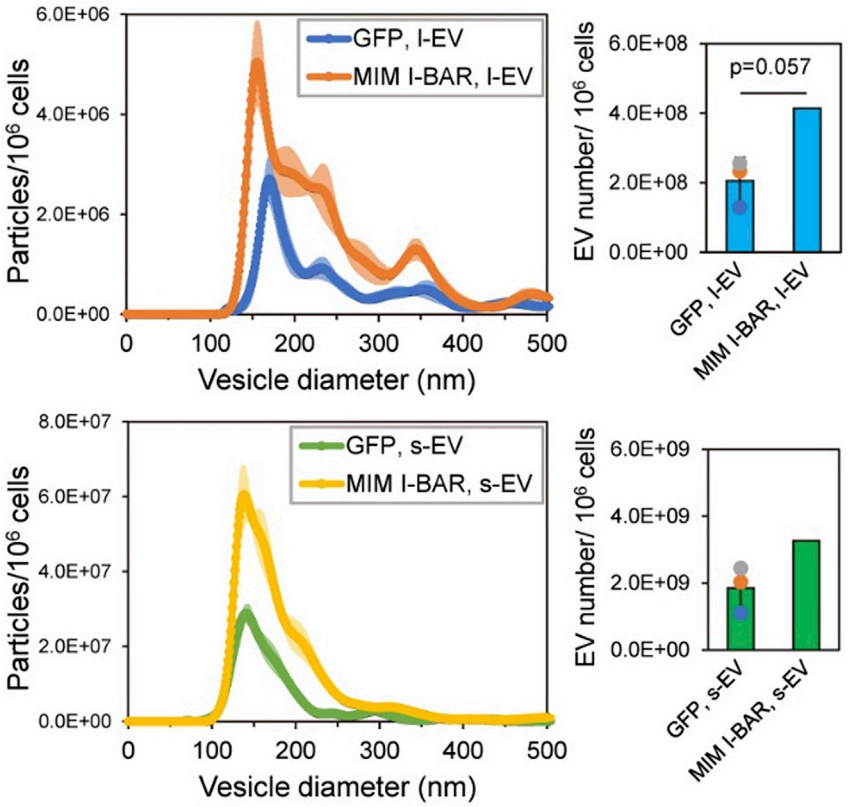

Figure 6. Size distribution and concentration of the EVs

Adapted from Nishimura et al. (2021).

EVs were prepared from the culture media per 1×10^6 of HEK 293 cells expressing GFP or MIM I-BAR-GFP and then analyzed by nano-tracking analysis. p values were determined by paired two-tailed Student's t-tests (n = 3–4).

(Jeppesen et al., 2019). The vesicle-free medium contains soluble factors that can be concentrated and analyzed by using immunoblotting (Wang et al., 2021). Apart from the analyses mentioned in this protocol, we encourage users to further characterize the morphology and cargo content of EVs by using transmission electron microscopy and mass spectrometry.

To get adequate yield for the analysis, we suggest starting with a 15–30 mL FreeStyle™ 293-F suspension cell at the density range from $0.6–0.8 \times 10^6$ cells/mL. From our experience, by culturing 30 mL of control cells at the density of 0.8×10^6 cells/mL for 2 days, an approximate protein amount of 10–35 μg of l-EVs and 40–110 μg of s-EVs can be obtained. As for the EV number, approximately $1–3 \times 10^{10}$ l-EVs and $1–3 \times 10^{11}$ s-EVs can be recovered. This recovery is sufficient to run the western blot for three different normalization methods at the same time. The representative l-EV and s-EV concentration and distribution analyzed by NTA are presented in Figure 6. The l-EVs have size distribution range from 150–400 nm, with majority peak at 150–200 nm. Compared to l-EVs, s-EVs have smaller size distribution, which range from 100–250 nm, with the peak at 130–150 nm.

For the immunoblotting analysis, EV fractions devoid of contamination of intracellular compartment are successfully purified by this protocol. According to the minimal information for studies of extracellular vesicles (MISEV), there is no universal negative marker for EVs (Thery et al., 2018; Witwer and Thery, 2019). As shown in Figure 7, the recovered EVs from this protocol are free from the negative markers, Calnexin and GM130 (Lotvall et al., 2014; Thery et al., 2018). To distinguish l-EVs and s-EVs, we use l-EV marker, Annexin A1 (Jeppesen et al., 2019), and s-EV markers, ALIX and CD81 (Thery

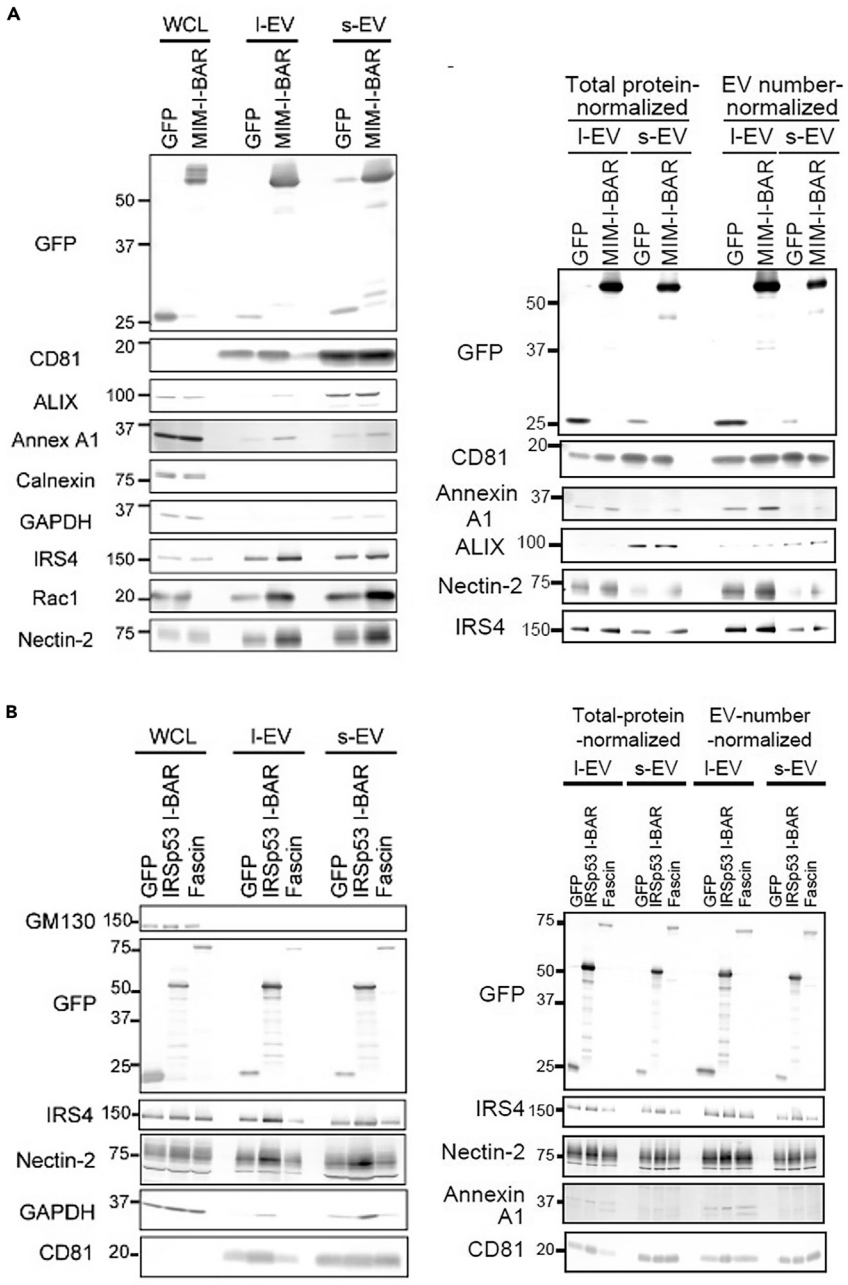

Figure 7. Western blot analysis of EVs

Adapted from Nishimura et al. (2021).

(A) Western blots of EV fractions from the HEK 293 cells expressing GFP or MIM I-BAR-GFP.

(B) Western blots of EV fractions from the HEK 293 cells expressing GFP, GFP-tagged IRSp53 I-BAR, or Fascin. Proteins of interest and s-EV markers, CD81 and ALIX, and an l-EV marker, Annexin A1, in the WCL, l-EVs and s-EVs were analyzed. The loading amounts were normalized to the cell number used for the EV preparation (left panel) and the total protein and total number of EV (right panel).

et al., 2018). The immunoblotting of EV sample shows that most of Annexin A1 accumulates in the l-EV fraction while ALIX and CD81 accumulate at the s-EV fraction (Figure 7).

The three kinds of normalization methods in this protocol (depicted at step 33) are used to carefully examine the distribution of proteins based on cell number, EV protein concentration, and EV

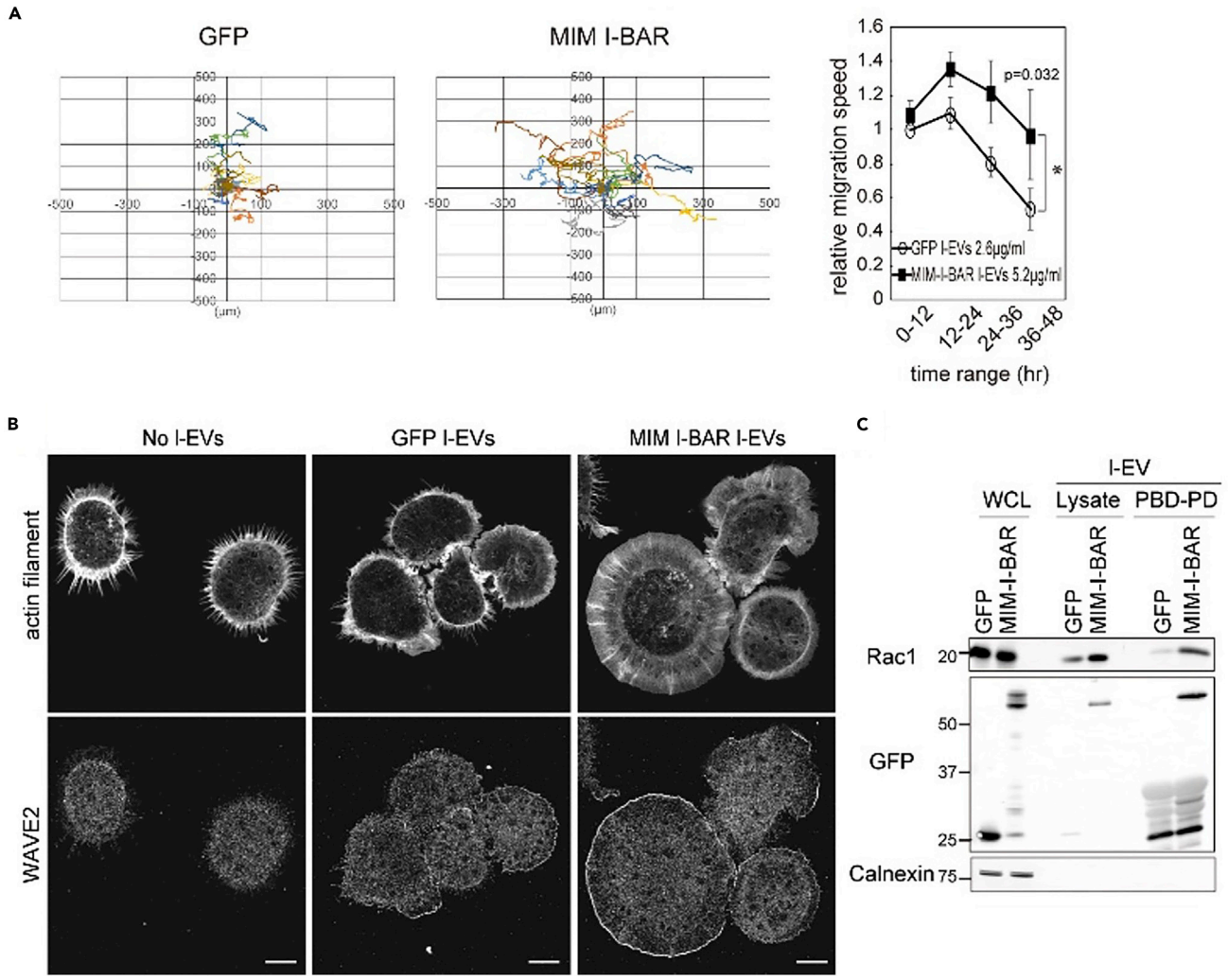

Figure 8. Examples of the comparative functional experiments using EV fractions prepared by this protocol

Adapted from Nishimura et al. (2021).

(A) HEK 293 cell migration after adding l-EVs obtained by MIM I-BAR-expressing HEK 293 cells. Left: trajectories of 10 cells selected in the field. Right: the average migration speed during 12 h interval was calculated for each experiment. The averages of 3 experiments were shown with SD.

(B) Lamellipodia formation of HEK 293 cells induced by adding l-EVs prepared from MIM I-BAR-expressing HEK 293 cells. Scale bars: 10μm

(C) Rac1 activity in MIM I-BAR l-EVs. GFP and MIM I-BAR l-EVs prepared from the same number of cells were lysed and pulled down by PAK-PBD resin to collect active Rac1.

number. For example, the protein of interest, IRS4, distributes differently depending on the normalization methods, verifying the dependency of the protein on the EV number or the nature of EV itself (Figure 7).

Using the EVs prepared by this protocol, many functional studies can be performed, including cell migration assay and lamellipodia formation assay we have shown here (Figure 8). Since EV components may differ due to the originating cells, their conditions, and how they were produced, various EV functions specific to these features are expected to be elucidated.

LIMITATIONS

Differential ultracentrifugation employs high g-force to separate the EVs with different sedimentation rates based on their size and density. However, due to the overlapping size range of l-EVs

and s-EVs (Camussi et al., 2010; Raposo and Stoorvogel, 2013; van Niel et al., 2018), this technique is unable to perfectly distinguish these two populations of vesicles. As shown in Figure 7, there is no perfect separation of the s-EV marker, CD81 and l-EV marker, Annexin A1.

The advantages of differential ultracentrifugation approach in separating EVs are no potential interferences, such as chemical additives, will be included in the yield, and applicable on large volume of biological fluid. However, in comparison to other approaches such as size exclusion chromatography and immunoaffinity isolation, this technique is relatively labor intensive and requires larger sample volume (Sun and Meckes, 2018). In addition, the EVs separated by differential ultracentrifugation have lower purity due to the aggregation of proteins or EVs and unable to further distinguish the subpopulation of EVs. Thus, using chemical additives such as sucrose or iodixanol in combination with ultracentrifugation could further separate the EVs classes based on their size and density (Jeppesen et al., 2019).

This protocol is optimized for the EV preparation from serum-free suspension cell culture. A similar procedure for the ultracentrifugation could be applied on adherent cell culture, but one should be reminded that the amount of starting material (i.e., initial cell number) is crucial in order to get the desired yield. For adherent cell culture, multiple 10–15 cm dishes will be required to achieve the expected yield. Additional steps such as cells trypsinization is required to obtain the cell count of adherent cells. For a cell line that is adapted in a serum-containing culture medium, additional steps are required to pre-clear the EVs in the serum. Up to 95% of RNA-containing EVs in fetal bovine serum (FBS) can be removed by subjecting the serum to 120,000 × *g* ultracentrifugation at 4°C for 18 h (Shelke et al., 2014).

TROUBLESHOOTING

Problem 1

Unable to visualize the l-EV and s-EV pellet after ultracentrifugation (steps 13 and 15).

Potential solution

Observe the bottom part of the tube carefully under the light (Figure 3) over a dark background. Do not exert large movement when retrieving the tube from the rotor or while opening the cap. Insufficient yield also could cause the pellet to be difficult to be seen. To solve the problem of low yield, please refer to the next potential solution.

Problem 2

The EV yield is too low and not sufficient for downstream analysis (step 33).

Potential solution

We realized that cell number is a critical factor for EV yield. By following the cell concentration mentioned in the protocol, we encourage users to purify EVs from 30–45 mL of culture to achieve sufficient yield. It could be further scaled up using an additional flask for the culture, depending on the requirement for analysis.

Problem 3

Inconsistent EV yield (steps 22, 31, 45, and 46).

Potential solution

The using of early- or late-passage cells could affect the transfection efficiency and EV secretion. Hence, please use middle-passage cell, such as within passage 4–25 for FreeStyle™ 293-F cell. In addition, use consistent g-force, k-factor and centrifugation time for EV collection as all these factors could contribute to EV pelleting efficiency.

Problem 4

"Measurement error" messaged displayed during Qubit protein concentration measurement (step 22).

Potential solution

There are 2 possibilities if error occurs. First is when the sample concentration is out of range. Adjust the sample concentration so that it is within the range from 12.5 μg/mL to 5 mg/mL. Second possibility is when the contaminant concentration is too high in the sample. Visit Qubit protein assay kits user guide at https://assets.fishersci.com/TFS-Assets/LSG/manuals/Qubit_Protein_Assay_UG.pdf to adjust the contaminant according to the acceptable range.

Problem 5

The s-EV markers CD63, CD9, and CD81 are not detected in s-EV fractions by immunoblotting.

Potential solution

Tetraspanins, such as CD63, CD9 and CD81, are abundantly included in s-EVs and used as markers for s-EVs. However, many antibodies raised against tetraspanins do not bind to antigens treated with immunoblotting sample buffers that contain reducing agents such as 2-mercaptoethanol or dithiothreitol. If you cannot detect the expected bands using those antibodies, try the sample buffer eliminating those reducing agents.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shiro Suetsugu (suetsugu@bs.naist.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

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AUTHOR CONTRIBUTIONS

H.T.H. and T.N. performed the biochemical and cell biological analyses. H.T.H., T.N., and S.S. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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