Jun 03, 2024 Version 2

Isolation and Characterization of Tissue and Cell-Derived Extracellular Vesicles and Non-Vesicular Extracellular Particles V.2

DOI
dx.doi.org/10.17504/protocols.io.5jyl822k8l2w/v2

Marta Garcia Contreras[1], Emeli Chatterjee[1], Abhik Chakraborty[2], Michail Spanos[1], Kriti Bomb[1], Priyanka Gokulnath[1], Jeffery L. Franklin[3], Erika Duggan[4], John P. Nolan[4], Priyadarshini Pantham[2], Louise C. Laurent[2], Saumya Das[1]

[1]Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA;
[2]Department of Obstetrics, Gynecology & Reproductive Medicine and Sanford Consortium for Regenerative Medicine, University of California San Diego, La Jolla, CA, USA;
[3]Vanderbilt Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA;
[4]Scintillon Institute, La Jolla, CA, USA

Marta Garcia Contreras
MGH/Harvard Medical School

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.5jyl822k8l2w/v2

Protocol Citation: Marta Garcia Contreras, Emeli Chatterjee, Abhik Chakraborty, Michail Spanos, Kriti Bomb, Priyanka Gokulnath, Jeffery L. Franklin, Erika Duggan, John P. Nolan, Priyadarshini Pantham, Louise C. Laurent, Saumya Das 2024. Isolation and Characterization of Tissue and Cell-Derived Extracellular Vesicles and Non-Vesicular Extracellular Particles. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.5jyl822k8l2w/v2**Version created by **Marta Garcia Contreras**

Manuscript citation:
This chapter is for publication in an upcoming volume of “Methods in Molecular Biology”

Protocol status: Working
We use this protocol and it's working

Created: June 03, 2024

Last Modified: June 03, 2024

Protocol Integer ID: 101159

Keywords: extracellular vesicles, Non-vesicular extracellular particles, exomeres, supermeres , derived extracellular vesicle, including extracellular vesicle, extracellular vesicle, vesicle flow cytometry, heterogeneous complement of extracellular biomolecular complex, extracellular biomolecular complex, extracellular complex, types of extracellular complex, lipid bilayer, enclosed membrane particle, biological fluid, evs by size exclusion chromatography, membrane particle, size exclusion chromatography, immunoblotting method, discovered amembranous small multimolecular assembly, amembranous small multimolecular assembly, cell, most cell type, cell culture, characterization of tissue

Funders Acknowledgements:
PP
Grant ID: K99/R00HD096125
LCL
Grant ID: UH3 CA241687
JPN
Grant ID: R44 GM136165
JPN
Grant ID: R44 CA272098
SD
Grant ID: American Heart Association (AHA) SFRN16SFRN31280008
SD
Grant ID: NHLBI R35HL150807
SD
Grant ID: 1RO1HL150401

Disclaimer

This chapter is for publication in an upcoming volume of “Methods in Molecular Biology”

Abstract

Cells produce a heterogeneous complement of extracellular biomolecular complexes, including extracellular vesicles (EVs) and non-vesicular extracellular particles (NVEPs). EVs are lipid bilayer-enclosed membrane particles released by cells. NVEPs are recently discovered amembranous small multimolecular assemblies with distinct RNA and protein cargoes. EVs and NVEPs are released by most cell types and can be found in biological fluids. Both types of extracellular complexes contain unique cargo and have important biological functions. Due to their great potential as diagnostic tools, there is a growing interest in being able to identify and characterize tissue- and cell- derived EVs and NVEPs. Here we described protocols for isolating EVs by size exclusion chromatography (SEC) or an ultracentrifugation method to simultaneously isolate EVs and NVEPs from conditioned media supernatant following tissue or cell culture. In addition, we describe microfluidic resistive pulse sensing, vesicle flow cytometry, and immunoblotting methods for their characterization.

Materials

All solutions must be prepared using ultrapure water and analytical grade reagents.

Processing of Conditioned Culture Media following Tissue or Cell Culture

1. Amicon Ultra centrifugal filters, 10 kDa or 100 kDa MWCO, Millipore.
2. For all serum-containing culture media preparations, exosome-depleted serum (e.g., fetal bovine serum (A2720801, ThermoFisher Scientific) must be used.
3. Centrifuge.

Isolation of Extracellular Vesicles and Non-Vesicular Extracellular Particles

Size Exclusion Chromatography to Isolate Extracellular Vesicles

1. Tissue or cell conditioned culture media (500 µL neat or concentrated to 500µL using Amicon filters).
2. Izon qEV (35 nm) size exclusion chromatography columns (Original Column Gen 2).
3. Izon Automatic Fraction Collector (AFC).
4. 1X Phosphate buffered saline (1X PBS).
5. Disposable plastic 10 mL pipettes.
6. Pipette boy.
7. 1.5 mL Eppendorf tubes.

Differential Ultracentrifugation to Isolate Extracellular Vesicles and Non-Vesicular Extracellular Particles

1. Tissue or cell culture conditioned media, concentrated to 5 mL using Amicon filters.
2. 15 mL centrifuge tubes.
3. Eppendorf 5810R 15 Amp refrigerated benchtop centrifuge 5811F with A-4-81 Rotor.
4. Beckman Optima L-90K ultracentrifuge with a SW 55 Ti rotor.
5. 5 mL, sterile + certified free open-top thin wall ultra-clear tube, 13 × 51 mm, Beckman Coulter.
6. PBS-HEPES (PBS-H, 25 mM) (stored at 4°C)

Characterization of Extracellular Vesicles and Non-Vesicular Extracellular Particles

Vesicle Flow Cytometry to Characterize Extracellular Vesicles in Conditioned Culture Media

1. Neat, unconcentrated tissue or cell culture conditioned culture media.
2. Beckman Coulter Cytoflex S flow cytometer, calibrated.

3. V-bottom or U-bottom 96-well plate.
4. vFC staining/dilution buffer.
5. 10X vFRed working solution: vFRed is a membrane stain which can
stain the lipid bilayer membrane of the EVs. The stock solution of vFRed is 100X and should be diluted to 10X by mixing 1 part of vFRed to 9 parts of vFRed dilution buffer (Cellarcus Biosciences).
6. 1000 µL, 200 µL, and 20 µL multichannel pipettes.
7. Barrier 1000 µL, 200 µL, and 20 µL tips.
8. One-time use reagent reservoirs.
9. Sheath fluid: milliQ water (or equivalent).
10. Coulter-cleanse cleaning solution (Beckman Coulter).
11. Fluorescently labelled antibodies: CD9-PEDazzle, CD63-PE and CD81-PECy7 (Cellarcus Biosciences).
12. Positive control EVs for CD9, CD63 and CD81 (Cellarcus Biosciences).
13. Negative control: Synthetic liposome (Lipo-100) (Cellarcus Biosciences).

Store all stock reagents at 4°C. Store positive controls and samples for EV characterization analysis at -80°C. All the reagents must be brought to room temperature, and samples and the positive controls should be thawed on ice before starting the experiment. All the working solutions should be made fresh from the stock reagents on the day of performing experiments.

Microfluidic Resistive Pulse Sensing to Characterize Extracellular Vesicles Purified using Size-Exclusion Chromatography

1. Spectradyne nCS1 system with hardware version 2.5.0.325
(Spectradyne, Signal Hill, CA, USA).
2. Buffer: 0.1% (v/v) Tween-20 in PBS (PBS-T 0.1%).
3. Syringe filters with 0.02 µm pore size (Whatman® Anotop® 10).
4. TS-400 cartridges (particle size range 65 to 400 nm).

Immunoblotting to Characterize Extracellular Vesicles and NonVesicular Extracellular Particles

1. Pierce BCA Protein assay Kit (Thermo Fisher Scientific).
2. RIPA buffer.
3. Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (Thermo Fisher Scientific).
4. Flat-bottom 96-well plates.
5. Microplate reader.
6. 4x Laemmli sample buffer (Bio-rad).
7. Precision Plus Protein Dual Color Standards, 500 µL #1610374 (Biorad).
8. 10X Tris/Glycine/SDS (Bio-rad).
9. Criterion Cell electrophoresis system (Bio-rad).
10. 4–20% Criterion™ TGX Stain-Free™ Protein Gel, 18 well, 30 µL, 5678094 (Bio-rad).
11. Basic Power Supply (Bio-rad).
12. Bio-Rad Trans-Blot Turbo Transfer System (Bio-rad).
13. Trans-Blot® Turbo™ RTA Midi PVDF Transfer Kit, for 40 blots (Bio-rad).

14. Tris-buffered saline with 0.1% Tween® 20 Detergent (TBS-T).
15. 5% Bovine Serum Albumin in TBS-T.
16. Primary Antibodies:

a. BD Pharmingen™ Purified Mouse Anti-Human CD63 (556019, BD Pharmigen).
b.. Purified anti-human CD81 (TAPA-1) Antibody (349502, Biolegend).
c. Purified anti-human CD9 Antibody (312102, Biolegend).
d. Flotillin-1 Antibody (#3253, Cell signaling).
e. Anti-COX IV antibody - Mitochondrial Loading Control (ab16056, abcam).
f. TGFBI / BIGH3 Polyclonal antibody (10188-1-AP, Proteintech).
g. Recombinant Anti-ACE2 antibody [EPR4435(2)] (ab239924, abcam).
h. Recombinant Anti-Argonaute-2 antibody [EPR10411] (ab186733, abcam).
i. GAPDH Antibody (FL-335) (sc-25778, Santacruz).
j. PLAP antibody (CBS36-R670-100T, Cellarcus Biosciences).

17. Secondary antibodies:

a. Peroxidase (HRP) Anti-Mouse IgG Horse Secondary Antibody, (7076S, Cell signaling).
b. Peroxidase (HRP) Anti-Rabbit IgG Goat Secondary Antibody, (7074S, Cell signaling).

18. Digital orbital shaker.
19. Supersignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Thermo Fisher Scientific).
20. iBright Imaging system (Thermo Fisher Scientific).

Troubleshooting

Processing of Conditioned Culture Media following Tissue and Cell Culture

1 Culture DiFi cells11 under the appropriate culture conditions as previously published. BeWo cells were cultured as published earlier12,13 with the modification of addition of 10% exosome-depleted FBS).

Citation

Moore KH, Murphy HA, Chapman H, George EM (2021)
. Syncytialization alters the extracellular matrix and barrier function of placental trophoblasts..

https://doi.org/10.1152/ajpcell.00177.2021 LINK

Citation

Deryabin PI, Borodkina AV (2022)
. Stromal cell senescence contributes to impaired endometrial decidualization and defective interaction with trophoblast cells..

https://doi.org/10.1093/humrep/deac112 LINK

2 Culture placental explant tissue14 and adipose tissue under the appropriate culture conditions as previously published (see Notes 1 and 2).

Citation

Tekkatte C, Lindsay SA, Duggan E, Castro-Martínez A, Hakim A, Saldana I, Zhang Y, Zhou J, Sebastian R, Liu Y, Pontigon DS, Meads M, Liu TN, Pizzo DP, Nolan J, Parast MM, Laurent LC
(2023)
. Identification of optimal conditions for human placental explant culture and extracellular vesicle release..

https://doi.org/10.1016/j.isci.2023.108046 LINK

3 If performing tissue culture, collect conditioned culture media supernatant along with all small pieces of tissues into a fresh 15 mL or 50 mL Falcon tube.

4 2000 x g Centrifuge at 2,000 xg for 10 minutes at Room temperature (20-22°C).

5 Remove conditioned culture media supernatant carefully without disturbing the pellet and pass it through 0.8 µm syringe filter (optional) to avoid any cellular debris contamination (See Note 3).

6 Aliquot and store filtered media stored at -80°C. -80 °C

Isolation of Extracellular Vesicles and Non-Vesicular Extracellular Particles

7 **Size Exclusion Chromatography to Isolate Extracellular Vesicles**

7.1 Turn on the Izon Automatic Fraction Collector (AFC) by switching on the power button at the rear of the instrument

7.2 Set up the 35 nm qEV Original Gen 2 column on the column mount. Insert the column from above into the column mount, then remove the lower cap and carefully dock the column. Ensure that the IZON logo on the column is facing away from the AFC tower.

7.3 Once the qEV column and the column mount have been attached to the AFC, the column type is determined automatically and is displayed on the AFC touchscreen.

7.4 From the screen, set the fraction number to 4, fraction size 400 µL, and buffer volume to 2.9 mL for collection (See Note 4)

Note

It is important to follow the manufacturer's default settings for the Izon AFC.

7.5 Lock the collection tubes onto the carousel in the appropriate orientation.

7.6 Gently place the carousel onto the carousel plate (See Note 5).

Note

Ensure that the carousel alignment hole engages with the raised carousel alignment pin on the carousel plate. Once the carousel has been loaded, the instrument will pass through the column preparation steps before initiating the collection.

7.7 Add 500 µL of the sample on the top of the qEV original column Gen 2

7.8 Collect fractions 7 to 10 (position 1 to 4) and combine them. The volume can be adjusted by concentrating them according to the downstream experiments. (See Note 6).

Note

Before starting the collection, all collection parameters should be reviewed properly which include default buffer volume, position of fractions and volume of fractions to be collected.

8 Differential Ultracentrifugation to Isolate Extracellular Vesicles and Non-Vesicular Extracellular Particles

8.1 Centrifuge tissue or cell culture conditioned media in 15 mL tubes at 2,000 xg for 20 minutes at 4 °C to remove dead cells. Discard the pellet and transfer the supernatant to a new collection tube

8.2 To isolate large extracellular vesicles (l-EVs), centrifuge the supernatant at 10,000 xg for 40 minutes at 4 °C

8.3 Resuspend the pellet containing l-EVs in PBS-H and store at -80 °C until further analysis (See Note 7). The supernatant is transferred to the corresponding ultracentrifuge tube.

> Note
>
> It is recommended to store the pellets in PBS-H to improve sample preservation 15

8.4 To isolate small extracellular vesicles (s-EVs) centrifuge the supernatant at 167,000 xg for 4 hours at 4 °C

8.5 Resuspend the pellet containing s-EVs in PBS-H and store at -80 °C until further analysis. The supernatant is transferred to the corresponding ultracentrifuge tube

8.6 To isolate exomeres, centrifuge the supernatant at 167,000 xg for 16 hours at 4 °C

8.7 Resuspend the pellet containing exomeres in PBS-H and store at -80 °C until further analysis. The supernatant is transferred to the corresponding ultracentrifuge tube.

8.8 To collect supermeres, centrifuge the supernatant at 367,000 xg for 16 hours at 4 °C .

8.9 The pellet containing supermeres is resuspended in PBS-H and stored at -80 °C until further analysis.

8.10 Optionally, pellets can be washed by resuspending them in PBS and repeating the corresponding centrifugation step.

Characterization of Extracellular Vesicles and Non-Vesicular Extracellular Particles

9 **Vesicle Flow Cytometry to Characterize Extracellular Vesicles in Conditioned Culture Media -**
Determining optimum dilution for the samples for vFC assay

9.1 Thaw neat, unconcentrated conditioned culture media samples on ice (See Note 8).

Note

Repeated-freeze thaw cycles can negatively affect samples. Therefore, the sample should be aliquoted to avoid multiple freeze-thaw cycles

9.2 Prepare vFRed 10X working solution from the 100X stock using vFC buffer. (See Note 9)

Note

For vFC, the vFRed dilution/staining buffer and stock solution should be brought up to room temperature before making the working solution of the 10X vFRed. Detailed Notes for vFC, including Protocols for instrument calibration, sample preparation and staining, flow cytometry, and data analysis are available on the Cellarcus web site (www.cellarcus.com).

9.3 Make serial dilutions of the samples: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320. (See Note 10). Make these dilutions in individual wells of a 96-well plate.

Note

Make a plate map for preparing the sample dilutions

9.4 Add 5µL of 10X vFRed to each of the sample dilution wells and pipette mix them using a 200µL. (See Note 11).

Note

Set the 200 µL multichannel pipette to 45 µL and mix the solution slowly for 4-5 times. Do not fix the pipette to 50 µL (total reaction volume) because it may introduce bubbles.

9.5 Incubate the 96-well plate in the dark for one hour at room temperature

9.6 Post incubation, dilute the sample 1:1000 in vFC dilution/staining buffer. (See Note 12 and Note 13) and mix the wells 4-5 times after the dilutions are made using a 1000 µL multichannel pipette. The sample dilutions are ready to be read using the Cytoflex S (or other suitably sensitive flow cytometer).

Note

After staining, a post-stain dilution is performed to reduce background from unbound stain. For plate-based assays where well capacities are ~300 uL, the 1000X post-stain dilution is performed in two steps. After incubating the staining mixture in dark conditions for 1 hour at room temperature, 8µL of staining mixture is mixed with 237 µL of vFC staining/dilution buffer (Dilution-1). After pipette mixing the contents of Dilution-1 well, 8µL of Dilution-1 is mixed with 237 µL of vFC staining/dilution buffer (Dilution-2). Dilution-2 is read by the cytometer.

Note

This protocol has been adapted for use with the CytoFlex instrument in the authors' lab. For other instruments, detailed protocols are available on the Cellarcus web site (www.cellarcus.com)

9.7 Before loading the sample into the cytometer, clean the flow cytometer by running 250µL the Coulter-cleanse cleaning solution followed by 250µL of a 0.22µm-filtered deionized water wash.

9.8 The sample can be loaded into the flow cytometer in two ways: a) manually: by using FACS tubes/Ria vials for individual samples; or b) semiautomated using a 96-well plate-loader. The semi-automatic plate loader can be used to load the samples into the flow cytometer since the number of sample tubes is >10.

9.9 Open a new experiment in the CytExpert software using a pre-saved template used for the assay (See Note 14).

Note

The CytoFlex cytometer acquisition settings are as follows:
Scatter gains (VSSC, SSC, and FSC) = 500
Active channels = All channels
Primary threshold =vFRed-H
Manual Threshold = 1900
Width parameter = vFRed (690/50)
Time to record = Checked, 120 seconds
Events to record = Unchecked
Events to display = 50,000
Sample flow rate = Fast (60 µL/min)

9.10 The .fcs files generated by the flow cytometer can be analyzed in the FCS Express software package (See Note 15).

Note

The instrument setup Protocols (www.cellarcus.com) use calibration particles to perform spectral and intensity calibrations, which are stored as ".comp" (spillover and compensation matrices for the fluorophores being measured), ".cal" (expressing fluorescence in units of molecules per particle), and "fcf" files (used for calibration of EV size [surface area, diameter, etc], scattering cross-section, and fluorophore surface density) which are loaded in the data analysis Layout to apply these calibrations.

9.11 The dilution that generates 25,000-50,000 total events is considered the optimum dilution for the vFC assay.

9.12 **Vesicle Flow Cytometry to Characterize Extracellular Vesicles in Conditioned Culture Media -** ***Staining of Optimally Diluted EVs with Fluorescently Labelled Antibodies***

9.13 Thaw neat, unconcentrated conditioned culture media samples on ice

9.14 The total staining reaction volume should be 50 µL .
- For single surface marker staining, add 5 µL of pre-titrated antibodies to each tube of the reaction mixture.
- Add 5 µL of 10X vFRed to each tube of the reaction mixture.
- The sample input must be calculated as per the optimal dilution decided in the previous experiment.

9.15 The total vFC staining/dilution buffer needs to be adjusted as per the volume of sample input, total volume of antibody/antibodies, and the volume of vFRed added to the reaction mixture

9.16 After the total reaction mixture is made, pipette the contents of the well to mix using a 50 µL multichannel pipette

9.17 Incubate the staining reaction mixture in dark for 1 hour at room temperature.

9.18 Post-stain, dilute the sample 1000X in vFC dilution/staining buffer, (See Note 12 and Note 13), pipette 4-5 times to mix the wells after the dilutions are made using a 1000 µL multichannel pipette. The sample dilutions are ready to be read using the Cytoflex S.

9.19 Before loading the sample into the flow cytometer, clean the cytometer by running a cleaning reagent followed by a 0.22 µm-filtered deionized water

wash.

9.20 Open a new experiment in the flow cytometer using a pre-saved template used for the assay (See Note 14)

Note

The CytoFlex cytometer acquisition settings are as follows:
Scatter gains (VSSC, SSC, and FSC) = 500
Active channels = All channels
Primary threshold =vFRed-H
Manual Threshold = 1900
Width parameter = vFRed (690/50)
Time to record = Checked, 120 seconds
Events to record = Unchecked
Events to display = 50,000
Sample flow rate = Fast (60 µL/min)

9.21 Analyze the .fcs files generated by the flow cytometer using the FCS express software package (See Note 15) to calculate the diameter of the vesicles and the number of vesicles that are positive for each surface marker, and each combination of surface markers (Figure 1a-c).

Expected result

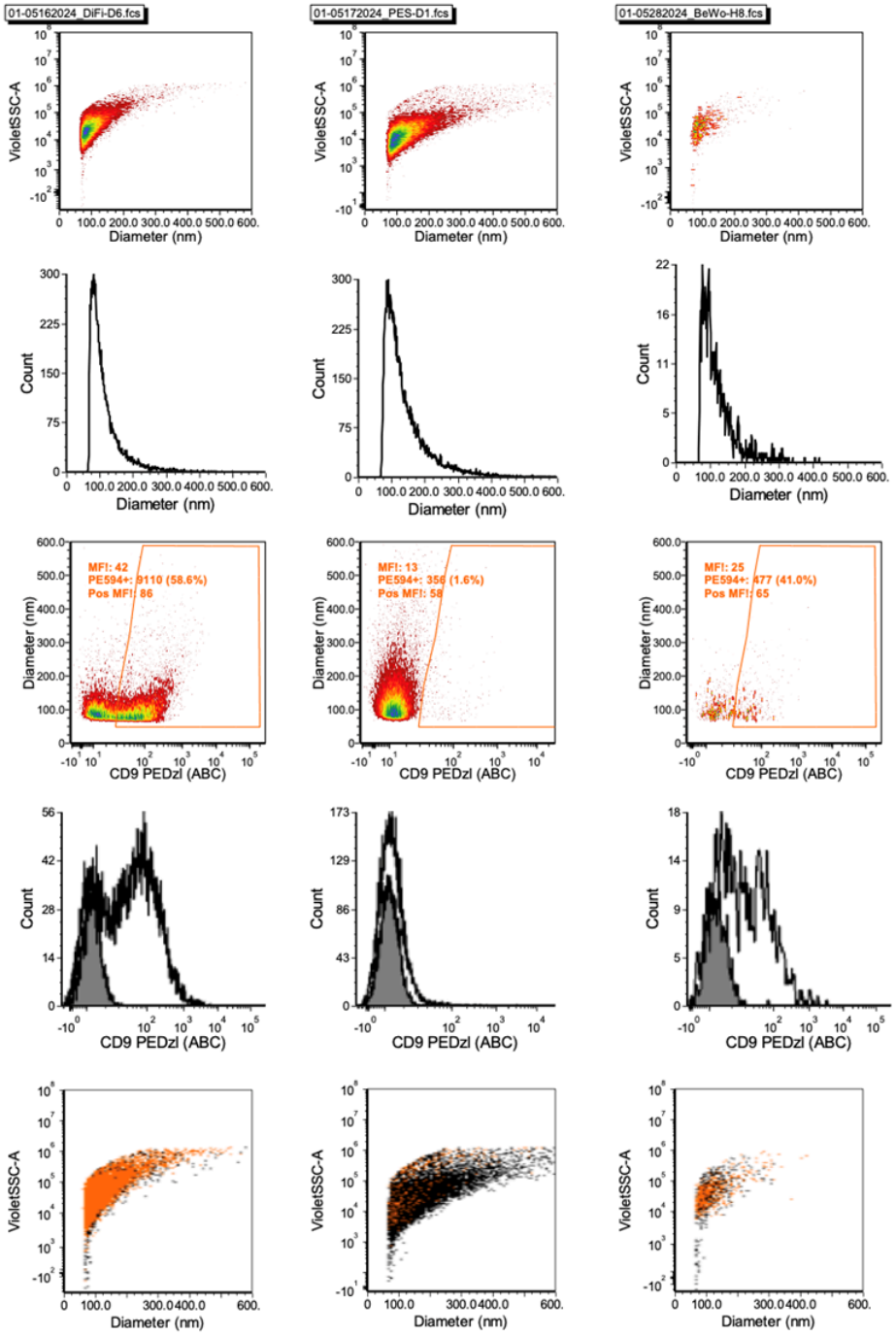

Figure 1: Representative images showing CD9$^+$ extracellular vesicles in neat, unconcentrated conditioned culture media using vesicle flow cytometry (vFC). (A) DiFi conditioned culture media stained with CD9 showing 58.6% CD9$^+$ EV population, (B) Placental explant supernatant (PES) showing 1.6% CD9$^+$ EV population, and (C) BeWo conditioned culture media showing 41% CD9$^+$ EV population.

10 **Microfluidic Resistive Pulse Sensing to Characterize Fractions Collected from Size-Exclusion Chromatography**

10.1 Begin by priming the microfluidic system of the Spectradyne nCS1 with PBS-T 0.1%.

10.2 Filter the diluent using the 0.02µm syringe filters to remove any particulate matter that could cause false-positive counts.

10.3 Dilute the EV samples 1:100 in the prepared PBS-T 0.1% buffer to align with the sensitivity range of the TS-400 cartridge.

10.4 Load 7 µL of the diluted sample into the cartridge. Ensure the sample is applied smoothly to prevent air bubbles, which can affect the measurement accuracy

Note

For Spectradyne analysis, in this protocol, we used a TS-400 cartridge. These cartridges are designed for analyzing particles ranging from 65 to 400 nm, suitable for small extracellular vesicles. If the EVs are collected via a different method and thus may be larger in diameter, different cartridges are recommended (e.g., C900/TS-900 for 130-900 nm particle diameters, or C-2000/TS-2000 for 250-2000 nm particle diameters.

10.5 Start the measurement process. The nCS1 system will automatically adjust the pressure and voltage settings to optimize the detection of particles within the sample

10.6 Continue data acquisition until the standard error of the mean particle count is less than 2%, indicating reliable data collection. This may require continuous acquisitions over several minutes.

10.7 Upon completion of the measurements, the data collected are processed using the nCS1 Data Viewer software.

10.8 Apply peak filters and perform background subtraction as directed by the manufacturer to ensure accurate particle size distribution is obtained (Figure 2).

Expected result

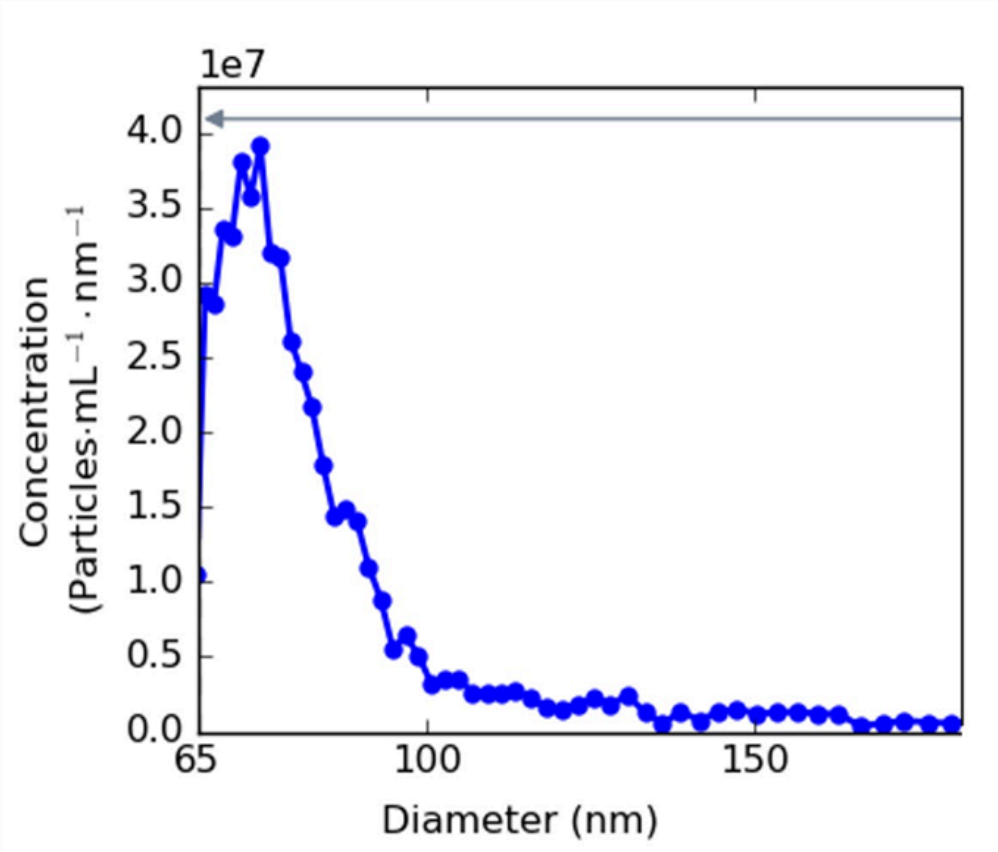

Figure 2: Representative image showing size range of placental extracellular vesicles isolated using size exclusion chromatography (SEC) from placental explant supernatant (PES). Microfluidic resistive pulse sensing conducted using the Spectradyne nCS1 shows that the size range of EVs isolated from Amiconconcentrated PES ranges from 75-100nm in size.

11 Immunoblotting to Characterize Extracellular Vesicles and NonVesicular Extracellular Particles

11.1 Lyse EVs and NVEPs purified using SEC or ultracentrifugation using 20 uL of 10X RIPA buffer (fractions are collected on 200 uL of PBS-H) containing protease and phosphatase inhibitors.

11.2 To remove debris, centrifuge lysates at 14,000 xg for 15 minutes and collect and store supernatants at -80°C (up to 6 months).

11.3 Prepare BSA standards, and store up to 2 weeks at 4°C.Conduct BCA assay according to manufacturer's instructions.

11.4 Prepare 30 µg of sample lysate diluted in 4x Laemmli sample buffer and heat them at 95 °C for 5 min (closed cap, to avoid evaporation which might reduce the load volume). Load protein ladder (5 µL) and 30 µg of samples on 4–20% Criterion™ TGX Stain-Free™ precast gels

11.5 Perform electrophoresis at a constant 120 V for 1 h in 1x Tris/Glycine/SDS running buffer

11.6 Following electrophoresis, transfer proteins onto PVDF membranes using a Bio-Rad Trans-Blot Turbo Transfer System

11.7 . Block the membrane in TBS-T+5% BSA for 1h shaking at RT.

11.8 Incubate membranes with primary antibodies (15 mL- 1:1000, or other optimized dilution) in TBS-T+5% BSA overnight shaking at 4°C.

11.9 Wash membranes 3 times in TBS-T (20 mL for 10 minutes shaking at RT. Incubate membranes with secondary antibodies at optimized dilution in TBS-T+5% BSA for 1 hour, shaking at RT

11.10 Wash membranes 3 times,10 minutes each in TBS-T shaking at RT.

11.11 Prepare the substrate working solution by mixing equal parts of the Substrate and Stable Peroxide components. Use a sufficient volume to ensure that the blot is covered with the substrate and the blot does not become dry

11.12 Incubate the membrane with the substrate working solution for 5 minutes.

11.13 Image the blot on an iBright Imaging system (Figures 3 and 4).

Expected result

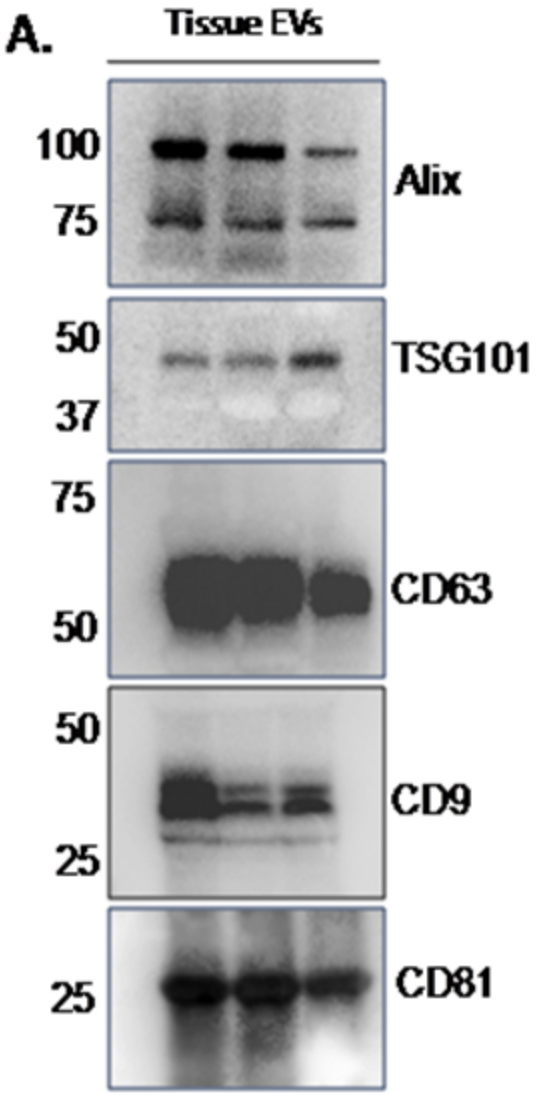

Figure 3: Representative images of adipose tissue-derived extracellular vesicles isolated using size exclusion chromatography (SEC) characterized by immunoblotting. Representative Western blot of the expression of Alix, TSG101, CD63, CD9, and CD81 as determined in pooled adipose tissue-derived EVs isolated by SEC.

Expected result

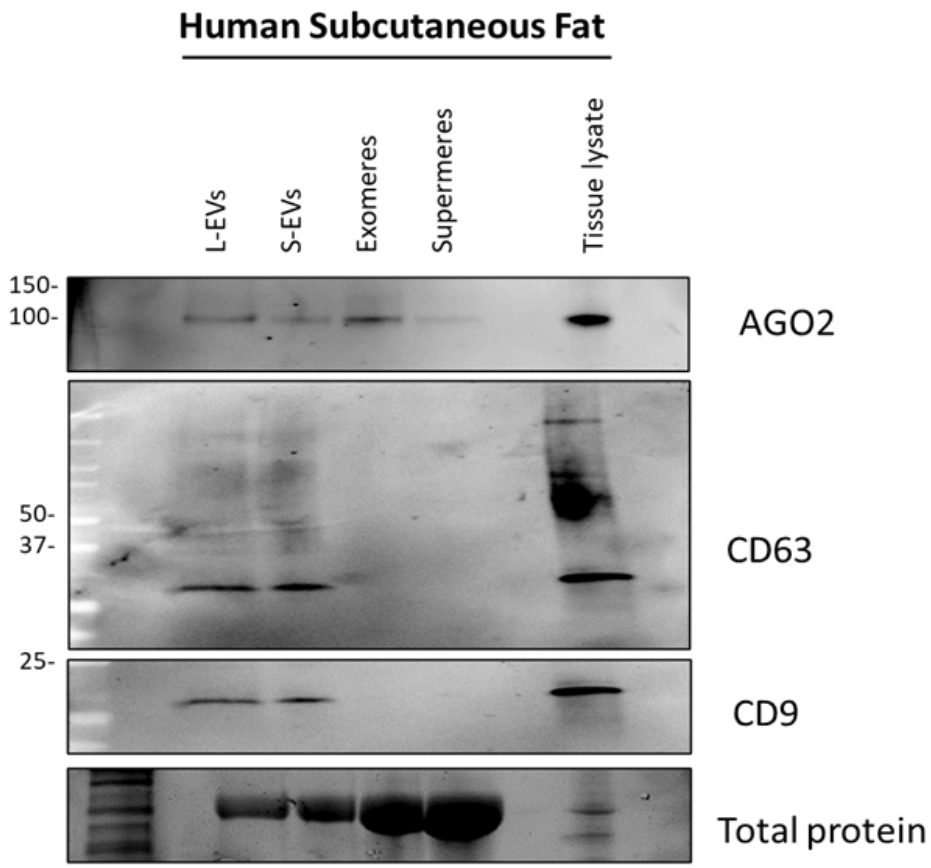

Figure 4: Representative images of adipose tissue-derived extracellular vesicles and non-vesicular extracellular particles (NVEPs) characterized by immunoblotting. Representative Western blot of the expression of AGO2, CD63, and CD9 as determined in adipose tissue-derived EV and NVEP samples isolated using ultracentrifugation.

Notes

12 1. We recommend using tissue as soon as possible after harvest to avoid cell death which might interfere with the EV and NVEPs preparation

13 2. It is critical to pay attention to the amount of medium/weight of sample to maintain a constant ratio for consistency and reproducibility.

14 3. It is critical to collect the culture supernatant without disturbing the pellet as the pellet might have contaminating red blood cells.

15 4. It is important to follow the manufacturer's default settings for the Izon AFC.

16 5. Ensure that the carousel alignment hole engages with the raised carousel alignment pin on the carousel plate. Once the carousel has been loaded, the instrument will pass through the column preparation steps before initiating the collection.

17 6. Before starting the collection, all collection parameters should be reviewed properly which include default buffer volume, position of fractions and volume of fractions to be collected

18 7. It is recommended to store the pellets in PBS-H to improve sample preservation 15

Citation

Görgens A, Corso G, Hagey DW, Jawad Wiklander R, Gustafsson MO, Felldin U, Lee Y, Bostancioglu RB, Sork H, Liang X, Zheng W, Mohammad DK, van de Wakker SI, Vader P, Zickler AM, Mamand DR, Ma L, Holme MN, Stevens MM, Wiklander OPB, El Andaloussi S (2022)
. Identification of storage conditions stabilizing extracellular vesicles preparations..

https://doi.org/10.1002/jev2.12238 LINK

19 8. Repeated-freeze thaw cycles can negatively affect samples. Therefore, the sample should be aliquoted to avoid multiple freeze-thaw cycles.

20 9. For vFC, the vFRed dilution/staining buffer and stock solution should be brought up to room temperature before making the working solution of the 10X vFRed. Detailed Notes for vFC, including Protocols for instrument calibration, sample preparation and staining, flow cytometry, and data analysis are available on the Cellarcus web site (www.cellarcus.com).

21 10. Make a plate map for preparing the sample dilutions.

22 11. Set the 200 µL multichannel pipette to 45 µL and mix the solution slowly for 4-5 times. Do not fix the pipette to 50 µL (total reaction volume) because it may

introduce bubbles.

23 12. After staining, a post-stain dilution is performed to reduce background from unbound stain. For plate-based assays where well capacities are ~300 uL, the 1000X post-stain dilution is performed in two steps. After incubating the staining mixture in dark conditions for 1 hour at room temperature, 8µL of staining mixture is mixed with 237 µL of vFC staining/dilution buffer (Dilution-1). After pipette mixing the contents of Dilution-1 well, 8µL of Dilution-1 is mixed with 237 µL of vFC staining/dilution buffer (Dilution-2). Dilution-2 is read by the cytometer

24 13. This protocol has been adapted for use with the CytoFlex instrument in the authors' lab. For other instruments, detailed protocols are available on the Cellarcus web site (www.cellarcus.com).

25 14. The CytoFlex cytometer acquisition settings are as follows:
Scatter gains (VSSC, SSC, and FSC) = 500
Active channels = All channels
Primary threshold =vFRed-H
Manual Threshold = 1900
Width parameter = vFRed (690/50)
Time to record = Checked, 120 seconds
Events to record = Unchecked
Events to display = 50,000
Sample flow rate = Fast (60 µL/min)

26 15. The instrument setup Protocols (www.cellarcus.com) use calibration particles to perform spectral and intensity calibrations, which are stored as ".comp" (spillover and compensation matrices for the fluorophores being measured), ".cal" (expressing fluorescence in units of molecules per particle), and "fcf" files (used for calibration of EV size [surface area, diameter, etc], scattering cross-section, and fluorophore surface density) which are loaded in the data analysis Layout to apply these calibrations

27 16. For Spectradyne analysis, in this protocol, we used a TS-400 cartridge. These cartridges are designed for analyzing particles ranging from 65 to 400 nm, suitable for small extracellular vesicles. If the EVs are collected via a different method and thus may be larger in diameter, different cartridges are recommended (e.g., C900/TS-900 for 130-900 nm particle diameters, or C-2000/TS-2000 for 250-2000 nm particle diameters.

Protocol references

1. Jeppesen, D. K., Zhang, Q., Franklin, J. L. & Coffey, R. J. Extracellular vesicles and nanoparticles: emerging complexities. Trends in Cell Biology 33, 667–681 (2023).
2. Welsh, J. A. et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. Journal of Extracellular Vesicles 13, e12404 (2024).
3. Valadi, H. et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9, 654–659 (2007).
4. Zhang, H. et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat Cell Biol 20, 332–343 (2018).
5. Zhang, Q. et al. Supermeres are functional extracellular nanoparticles replete with disease biomarkers and therapeutic targets. Nat Cell Biol 23, 1240–1254 (2021).
6. Zhang, Q. et al. Transfer of Functional Cargo in Exomeres. Cell Reports 27, 940-954.e6 (2019).
7. Wang, G. et al. Tumour extracellular vesicles and particles induce liver metabolic dysfunction. Nature 618, 374–382 (2023).
8. Jeppesen, D. K., Zhang, Q., Franklin, J. L. & Coffey, R. J. Are Supermeres a Distinct Nanoparticle? J Extracell Biol 1, e44 (2022).
12
This chapter is for publication in an upcoming volume of “Methods in Molecular Biology”
9. Crescitelli, R., Lässer, C. & Lötvall, J. Isolation and characterization of extracellular vesicle subpopulations from tissues. Nat Protoc 16, 1548–1580 (2021).
10. Vella, L. J. et al. A rigorous method to enrich for exosomes from brain tissue. Journal of Extracellular Vesicles 6, 1348885 (2017).
11. Higginbotham, J. N. et al. Identification and characterization of EGF receptor in individual exosomes by fluorescence-activated vesicle sorting. J Extracell Vesicles 5, 29254 (2016).
12. Moore, K. H., Murphy, H. A., Chapman, H. & George, E. M. Syncytialization alters the extracellular matrix and barrier function of placental trophoblasts. Am J Physiol Cell Physiol 321, C694–C703 (2021).
13. Deryabin, P. I. & Borodkina, A. V. Stromal cell senescence contributes to impaired endometrial decidualization and defective interaction with trophoblast cells. Human Reproduction 37, 1505–1524 (2022).
14. Tekkatte, C. et al. Identification of optimal conditions for human placental explant culture and extracellular vesicle release. iScience 26, 108046 (2023).
15. Görgens, A. et al. Identification of storage conditions stabilizing extracellular vesicles preparations. J Extracell Vesicles 11, e12238 (2022).

Citations

Step 1

Moore KH, Murphy HA, Chapman H, George EM. Syncytialization alters the extracellular matrix and barrier function of placental trophoblasts.
https://doi.org/10.1152/ajpcell.00177.2021

Step 1

Deryabin PI, Borodkina AV. Stromal cell senescence contributes to impaired endometrial decidualization and defective interaction with trophoblast cells.
https://doi.org/10.1093/humrep/deac112

Step 18

Görgens A, Corso G, Hagey DW, Jawad Wiklander R, Gustafsson MO, Felldin U, Lee Y, Bostancioglu RB, Sork H, Liang X, Zheng W, Mohammad DK, van de Wakker SI, Vader P, Zickler AM, Mamand DR, Ma L, Holme MN, Stevens MM, Wiklander OPB, El Andaloussi S. Identification of storage conditions stabilizing extracellular vesicles preparations.
https://doi.org/10.1002/jev2.12238

Step 2

Tekkatte C, Lindsay SA, Duggan E, Castro-Martínez A, Hakim A, Saldana I, Zhang Y, Zhou J, Sebastian R, Liu Y, Pontigon DS, Meads M, Liu TN, Pizzo DP, Nolan J, Parast MM, Laurent LC. Identification of optimal conditions for human placental explant culture and extracellular vesicle release.
https://doi.org/10.1016/j.isci.2023.108046