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Isolation and Characterization of Tissue and Cell-Derived Extracellular Vesicles and Non-Vesicular Extracellular Particles V.1

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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 x 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).

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).

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

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 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.

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).

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).

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).

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.

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).

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

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.

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

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).

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).

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

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)

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).

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

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).

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. (See Note 16)

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).

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.

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

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