A protocol for isolation, purification, characterization and functional dissection of exosomes

Alin Rai[1], Haoyun Fang[1], Monique Fatmous[1,2], Bethany Claridge[1,2], Qi Hui Poh[1,2], Richard J. Simpson[2], David W. Greening[1,2]*

[1]*Baker Heart and Diabetes Institute, Melbourne 3004, Australia.*

[2]*Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne 3086, Australia*

*To whom correspondence should be addressed:

Dr David W. Greening

Molecular Proteomics

Baker Heart and Diabetes Institute

75 Commercial Road, Melbourne, 3004, Australia

Email: David.Greening@baker.edu.au

Keywords: exosome production and purification, proteomics, extracellular vesicles, density-based fractionation, functional assays

Running Head: Exosome isolation, purification and characterization

Abstract

Extracellular vesicles (EVs) are membrane-enclosed vesicles released by cells. They carry proteins, nucleic acids and metabolites which can be transferred to a recipient cell, locally or at a distance, to elicit a functional response. Since their discovery over 30 years ago, the functional repertoire of EVs in both physiological (e.g., organ morphogenesis, embryo implantation) and pathological (e.g., cancer, neurodegeneration) conditions has cemented their crucial role in intercellular communication. Moreover, because the cargo encapsulated within circulating EVs remains protected from degradation, their diagnostic as well as therapeutic (such as drug delivery tool) applications have garnered vested interest. Global efforts have been made to purify EV subtypes from biological fluids and *in vitro* cell culture media using a variety of strategies and techniques, with a major focus on EVs of endocytic origin called exosomes (30-150 nm in size). Given that the secretome comprises of soluble secreted proteins, protein aggregates, RNA granules and EV subtypes (such as exosomes, shed microvesicles, apoptotic bodies), it is imperative to purify exosomes to homogeneity if we are to perform biochemical and biophysical characterization and importantly, functional dissection. Besides understanding the composition of EV-subtypes, defining molecular bias of how they reprogram target cells also remains of paramount importance in this area of active research. Here, we outline a systematic "how to" protocol (along with useful insights/tips) to obtain highly purified exosomes and perform their biophysical and biochemical characterization. This protocol employs a mass spectrometry-based proteomics approach to characterize the protein composition of exosomes. We also provide insights on different isolation strategies and their usefulness in various downstream applications. We outline protocols for lipophilic-labelling of exosomes to study uptake by a recipient cell, investigating cellular reprogramming using proteomics and studying functional response to exosomes in the Transwell-Matrigel^{TM} Invasion assay.

1. Introduction

Extracellular vesicles (EVs) are lipid encapsulated membranous vesicles that are released by cells into their extracellular space. EVs carry proteins, nucleic acids and metabolites which they can transfer between cells. While they function in cell–cell and cell–microenvironment communication [1,2], EVs are also emerging as universal agents in intra- and cross-organism communication for mammals [3-7], viruses [8,9], bacteria [10], archaea [11], microbes [12,13], parasites [14], fungi [15,16] and plants [17]. Cells have been shown to release different types of EVs which include exosomes, shedding microvesicles (sMVs) [18] or oncosomes [19], apoptotic blebs (ABs) and gesicles [20]. Exosomes originate by inward budding of endosomal membrane as intraluminal vesicles (ILVs) encapsulated within the larger multivesicular bodies (MVBs). These MVBs then fuse with the plasma membrane and release the ILVs into the extracellular space as "exosomes" [21,1,22,23].

Since their discovery over 30 years ago [24,25], it has become clear that exosomes contribute to many aspects of physiology and disease by reprogramming recipient cells and regulating cellular niche [26,27,18,28,29]. Circulating exosomes also have diagnostic potential and their therapeutic use as a drug delivery vehicle is currently an active area of research [30]. Although numerous studies have catalogued form, function, and biology of exosomes [31-41], much of this information has been obtained from heterogeneous or impure exosome preparations, which potentially confound interpretation of findings [42,43]. Moreover, function of a given EV subtype may be limited or masked by the presence of the other EV subpopulations. Hence, there is an urgent need to better define exosome preparations, not only to understand composition [31,37] but also to infer function [21,43,5,7,44]. This is particularly important when embarking on large-scale production of exosomes as a clinical-grade reagent.

Here, we expand on our previous guideline protocol [45] to outline a systematic "how to" protocol (along with useful insights/tips) to obtain highly purified exosomes and perform their biophysical and biochemical characterization. This protocol employs a mass spectrometry-based proteomic approach to characterize the protein composition of exosomes [23]. We provide insights on different isolation strategies and their usefulness in various downstream applications. We outline protocols for lipophilic-labelling of exosomes to study uptake by a recipient cell [7], investigating cellular reprogramming using proteomics [5] and

80 studying functional response to exosomes in Transwell-Matrigel™ Invasion assay. This
81 detailed protocol will enable generation, isolation, purification, and characterization of highly
82 purified exosomes, and understand how exosomes elicit a functional response through
83 exosome-mediated molecular reprogramming of the recipient cell.

2. Materials

2.1. Small-scale exosome production

1. Cell culture medium (e.g., RPMI-1640, DMEM) with cell culture supplements (see steps 2-4).
2. 5% (v/v) Foetal calf serum (FCS) or EV-depleted FCS in cell culture medium; i.e., 25 mL FCS in 500 mL. EV-depleted FCS is prepared following ultracentrifugation at 100,000 × *g*, 16 h, 4 °C.
3. 1% (v/v) Penicillin/Streptomycin (Pen/Strep) in cell culture medium; i.e., 5 mL Pen/Strep in 500 mL.
4. 0.6-1% (v/v) Insulin-Transferrin-Selenium (ITS) in cell culture medium; i.e., 600-1000 µL ITS (100x) in 500 mL.
5. Phosphate-buffered saline (PBS).
6. Tissue culture plates (e.g., 10-15 cm^2) / flasks (e.g., T75-175).
7. 50 mL polypropylene centrifuge tubes.
8. Refrigerated centrifuge.
9. Polyallomer tubes or polycarbonate bottles, appropriate for the ultracentrifuge rotor (**Table 1**).
10. Incubator (37 °C with 5% CO_2).

2.2. Large-scale exosome production

1. CELLine *adhere* bioreactor flask (CELLine AD 1000, Integra Biosciences)
2. Cell culture medium (e.g., RPMI-1640, DMEM) with cell culture supplements (see steps 3-5).
3. 5% FCS or EV-depleted FCS (see step 2 in Section 2.1) in cell culture medium.
4. 1% (v/v) Pen/Strep in cell culture medium.
5. 0.6-1% (v/v) ITS in cell culture medium.
6. 50 mL polypropylene centrifuge tubes.
7. Refrigerated centrifuge.

2.3 Protein quantitation

2.3.1 Micro BCA assay

1. Micro BCA Protein assay kit (Thermo Fisher Scientific).
2. Bovine Serum Albumin (BSA) as standard protein. BSA purity > 98%.

3. Working reagent (WR): 100 µL for each standard/sample. Mix 25 parts Reagent A, 24 parts Reagent B, 1 part Reagent C.
4. 1% (w/v) SDS in ultrapure water.
5. Exosome or cell lysate sample for measurement.
6. 1.5 mL microcentrifuge tubes.
7. Flat-bottom, clear 96-well plates.
8. Incubator (37 °C).
9. Microplate reader with 562 nm filter.

2.3.2 Protein staining densitometry

1. 2x SDS sample buffer: 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 125 mM Tris-HCl, pH 6.8. If reducing conditions are required, supplement 2x SDS sample buffer with dithiothreitol (DTT) to a final concentration of 2% (w/v).
2. Exosome or cell lysate sample for measurement.
3. NuPAGE Bis-Tris Precast gels: 1 mm thickness, 10- or 12-well, 4-12% gradient.
4. 1 × MES running buffer: 50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3. Do not use acid or base to adjust the pH.
5. XCell Surelock™ gel tank, with compatible power supply (e.g., Bio-Rad Laboratories, Hoefer, Thermo Fisher Scientific).
6. BenchMark™ Protein Ladder standard of known protein concentration: 1.7 µg/µL.
7. SYPRO® Ruby staining solution.
8. SYPRO® Ruby fixation solution: 40% (v/v) methanol, 10% (v/v) acetic acid in water.
9. SYPRO® Ruby destaining solution: 10% (v/v) methanol, 6% (v/v) acetic acid in water.
10. Orbital shaker.
11. Typhoon 9410 variable mode imager with green (532 nm) excitation laser and 610BP30 emission filter.
12. ImageQuant software or suitable densitometry-based analysis software.

2.4 Ultracentrifugation exosome isolation

1. Conditioned medium.
2. Sterile/filtered PBS.

3. Optima XPN Ultracentrifuge and matched rotor (**Table 1**).
4. SW28/SW32 Ti swinging-bucket (large scale spins) with 38.5 mL Open-Top Thinwall Ultra-Clear Tube, 25 x 89mm.
5. TLA-55 fixed angle (small scale/washing) with 1.5 mL Polypropylene Tube with Snap-on Cap, 9.5 x 38mm.

2.5 OptiPrep™ density gradient exosome isolation

1. Conditioned medium.
2. Optima XPN Ultracentrifuge and matched rotor (**Table 1**).
3. SW 41 Ti swinging-bucket (large scale) with 13.2 mL Thinwall Polypropylene Tubes, 14 x 89mm.
4. TLA-55 fixed angle (small scale/washing) with 1.5 mL Polypropylene Tube with Snap-on Cap, 9.5 x 38mm.
5. OptiPrep™ stock solution: 60% (w/v) aqueous iodixanol.
6. 0.25 M sucrose in 10 mM Tris-HCl, pH 7.5.
7. Sterile/filtered PBS.

2.6 Cushion-based separation of exosomes

1. Conditioned medium.
2. Optima XPN Ultracentrifuge and matched rotor (**Table 1**).
3. SW28/SW32 Ti swinging-bucket (large scale spins) with 38.5 mL Open-Top Thinwall Ultra-Clear Tube, 25 x 89mm.
4. SW 41 Ti swinging-bucket (large scale) with 13.2 mL Thinwall Polypropylene Tubes, 14 x 89mm.
5. TLA-55 fixed angle (small scale/washing) with 1.5 mL Polypropylene Tube with Snap-on Cap, 9.5 x 38mm.
6. OptiPrep™ stock solution: 60% (w/v) aqueous iodixanol.
7. Sterile/filtered PBS.

2.7 EpCAM immunoaffinity capture (IAC) exosome isolation

1. Conditioned medium.
2. EpCAM (CD326) magnetic microbeads (Miltenyi Biotec, Auburn, CA)
3. 3 mL LS Microcolumn for the gentle isolation of MicroBead-labeled cells.
4. Solid support magnet (SSM).

5. IAC Rinsing Solution: MACS® BSA Stock Solution diluted 1:20 with autoMACS® Rinsing Solution.
6. Optima XPN Ultracentrifuge and matched rotor (**Table 1**).
7. TLA-55 fixed angle (small scale/washing) with 1.5 mL Polypropylene Tube with Snap-on Cap, 9.5 x 38mm.
8. Sterile/filtered PBS.
9. IAC Elution buffer: 0.2 M glycine, Tris-HCl, pH 2.8.
10. 2x SDS sample buffer (see step 1 in Section 2.3.2).

2.8 Western blot analysis

1. Exosome, cell lysate preparations (~10-20 µg protein).
2. 2 × SDS sample buffer (see step 1 in **Section 2.3.2**).
3. NuPAGE Bis-Tris Precast gel (see **Section 2.3.2**).
4. 1 × MES running buffer (see **Section 2.3.2**).
5. XCell Surelock™ gel tank, with compatible power supply (see **Section 2.3.2**).
6. See blue plus 2
7. Pre-stained Protein Standard (e.g., SeeBlue™ Plus2, or Dual Color Standard).
8. iBlot™ Dry Blotting System and nitrocellulose transfer membranes.
9. TTBS solution: 0.05% Tween® 20 in Tris-Buffered Saline (TBS).
10. Blocking buffer: 5% (w/v) skim milk powder in TTBS.
11. Primary antibody Mouse anti-TSG101 (#612696; BD Biosciences): 1:500 in TTBS.
12. Primary antibody Mouse anti-Alix (#2171, Cell Signaling Technology): 1:1000 in TTBS.
13. Secondary antibody IRDye 800 goat anti-mouse IgG: 1:15,000 in TTBS.
14. Orbital shaker.
15. Odyssey Infrared Imaging System, v3.0.

2.9 Nanoparticle tracking analysis (NTA)

1. Exosome preparation (~1-2 µg protein).
2. NanoSight NS300 system.
3. Ultra-pure water.
4. Disposable 1 mL syringe for sample loading.

2.10 Aldehyde/sulfate latex (ALS) bead-based capture

1. Exosome preparations (~2-5 µg protein).
2. 1.5 mL Polypropylene Tube with Snap-on Cap.
3. 4% w/v Aldehyde/Sulfate Latex Beads, 4 µm.
4. Blocking buffer: 0.2% (v/v) Triton X-100, 2% (w/v) BSA in PBS.
5. Wash buffer: 0.2% (v/v) Triton X-100 in PBS.
6. 1 M glycine in PBS.
7. Primary antibody Mouse anti-Alix (#2171; Cell Signaling Technology): 1:1,000 in blocking buffer.
8. Secondary antibody IRDye800 goat anti-mouse IgG: 1:15,000 in blocking buffer.
9. Benchtop centrifuge.
10. Microscope slide.
11. Zeiss AxioObserver Z1 microscope.

2.11 Electron microscopy (EM)

2.11.1 Transmission EM

1. Exosome preparations (~2 µg protein).
2. Sterile/filtered PBS.
3. Fixing solution: 1% (v/v) glutaraldehyde.
4. Formvar coated 200 mesh copper grids.
5. 1% (w/v) aqueous uranyl acetate.
6. Gatan UltraScan 1000 (2k × 2k) CCD camera coupled to a Tecnai F30 electron microscope.

2.11.2 Cryo EM

1. Exosome preparations (~2 µg protein).
2. Aurion Protein-G gold 10 nm.
3. Sterile/filtered PBS.
4. Glow-discharged C-flat holey carbon grids.
5. Vitrobot or automated sample preparation device.
6. Liquid ethane.
7. Liquid nitrogen.
8. Gatan cryoholder.

9. Tecnai G2 F30 electron microscope.

2.12 Proteomics: sample preparation

2.12.1 In-solution reduction, alkylation and digestion

1. Exosome and cell lysate preparations (~5-10 µg protein).
2. 1.5 mL Protein LoBind Tubes or Protein LoBind deep 96 well Plates (1000 µL).
3. 1 M Tetraethylammonium bromide (TEAB) stock solution in LC-MS grade water.
4. 50 mM TEAB, pH 8.0, in LC-MS grade water. For pH adjustment use 1 M hydrochloride in LC-MS grade water.
5. 100 mM TEAB, pH 8.0, in LC-MS grade water. For pH adjustment use 1 M hydrochloride in LC-MS grade water.
6. 100% (v/v) acetone (LC grade). Stored at -20 °C.
7. 90% (v/v) acetone in water (LC grade). Stored at -20 °C.
8. Lysis buffer: 1% (w/v) SDS, 50 mM TEAB, pH 8.0.
9. 500 mM DTT stock solution in 100 mM TEAB, pH 8.0. Prepare fresh before the reduction step.
10. 1 M iodoacetamide (IAA) stock solution in 100 mM TEAB, pH 8.0. Prepare fresh before the alkylation step.
11. Trypsin, sequencing grade. Store lyophilized or frozen at -80 °C.
12. Formic acid (LC-MS grade), prepare 1.5% (v/v) stock.
13. Water (LC-MS grade).
14. ThermoMixer with 1.5 mL microfuge tube capacity.
15. Thermostat oven or incubator at 37 °C.
16. pH strip, range 1-14.
17. Minsox S-4000 600W Sonicator with microtip (or related sonicator).

2.12.2 StageTip sample cleanup

1. Empore SDB-RPS (Styrene Divinyl Benzene–Reversed Phase Sulfonate) solid-phase extraction disks for StageTip preparation.
2. SDB-RPS StageTip loading buffer: 1% (v/v) trifluoroacetic acid (TFA) in acetonitrile (ACN).
3. SDB-RPS StageTip wash buffer 1: 1% (v/v) TFA in ACN.
4. SDB-RPS StageTip wash buffer 2: 0.2% (v/v) TFA in 5% (v/v) ACN.

5. SDB-RPS StageTip elution buffer: 20 µL of NH_4OH in 4 mL of 60% (v/v) ACN. Elution buffer must be prepared fresh (within 1 h of use) because the pH will begin to increase due to its high volatility, thereby reducing its elution strength.
6. Water (LC-MS grade).
7. Vacuum centrifuge (lyophilizer).
8. MS loading buffer: 0.07% (v/v) TFA in LC-MS water. This buffer is stable for >6 months at RT.

2.13 Fluorometric peptide assay

1. Quantitative Fluorometric Peptide Assay kit (Thermo Scientific #23290).
2. MS loading buffer (see step 9 in **Section 2.12.2**).
3. Peptide samples for analysis.
4. Fluorescence compatible 96 well microplate (black).
5. Fluorescent plate reader compatible with Ex 390 nm / Em 475 nm.

2.14 UHPLC-MS/MS

1. MS loading buffer: 0.07% (v/v) TFA in LC-MS water.
2. Transparent MS sample vials, 300µL: snap ring vial with PP insert.
3. UHPLC System coupled to a Q Exactive HF-X Hybrid Quadrupole-Orbitrap MS System.
4. Load column (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size)
5. Analytical/separation column (50-cm fused-silica emitter reversed-phase PepMapRSLC C18, 75 µm inner diameter, 2 µm resin with 100 Å pore-size)
6. Mobile Phase A: 0.1% formic acid.
7. Mobile Phase B: 0.1% formic acid in acetonitrile.

2.15 Data analysis

1. MaxQuant software.
2. Microsoft Office Excel and Perseus software [46] (Max-Planck Institute of Biochemistry, Munich).
3. Online web-based bioinformatics resources such as gProfiler (https://biit.cs.ut.ee/gprofiler/gost), STRING (https://string-db.org/) and Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/).

2.16 Phenotypic reprogramming of cells by exosomes: dissecting function

2.16.1 Labelling exosomes with lipophilic tracer

1. 1-2.5 mg/mL Dil (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) stock solution in DMSO.
2. Dil staining solution: 1 µM in PBS (10 mL).
3. OptiPrep™ density gradient reagents (see **Section 2.5**)
4. 96 well plate (black).
5. Fluorescence plate reader (Ex 549 nm / Em 565 nm).

2.16.2 Exosome uptake

1. Dil stained exosomes.
2. Recipient cells of choice (~70% confluency).
3. 1 µM DiO or DiR staining solution in PBS.
4. 6-well plate or 8-well microscopy cover slides.
5. 10 µg/mL Hoechst nuclei stain in PBS.
6. PBS.
7. Phenol-red free media.
8. Formaldehyde.
9. Dako fluorescence mounting media.
10. Fluorescence microscope.

2.16.3 Cell activation assay

1. Recipient cells of choice (e.g., fibroblasts (5×10^4)).
2. 96-well plate.
3. Serum-free culture media.
4. Purified exosomes.
5. PBS.

2.16.4 Transwell-Matrigel™ invasion assay

1. Recipient cells of choice (e.g., fibroblasts (5×10^4)).
2. Purified exosomes.
3. PBS.

4. Growth factor-reduced Matrigel™ matrix.
5. Transwell inserts, 8 µm pore size.
6. 24-well plate.
7. Incubator at 37 °C.
8. 4% (v/v) Formaldehyde.
9. 10 µg/µL Hoechst nuclei stain in PBS (or other nuclei stain).
10. Cotton swab.
11. Fluorescence microscope.
12. ImageJ image analysis tool.

2.16.5 Molecular reprogramming of cells by exosomes

1. 70-80% confluent cells.
2. Ice cold PBS.
3. Cell scraper.
4. 2% (w/v) SDS sample buffer.
5. 1.5 mL, Polypropylene Tube with Snap-on Cap.
6. Benchtop Centrifuge (up to 20,000 × *g* spin).
7. Heat block (up to 95 °C).
8. Minsox S-4000 600W Sonicator with microtip (or related sonicator).

(optional for ultracentifugation approach)

- *1.5 ml ultracentrifuge tubes, polypropylene (#41121703, Beckman Coulter).*
- *TLA-55 rotor (Beckman Coulter).*
- *Ultracentrifuge (Optima MAX-MP Tabletop Ultracentrifuge, #393315, Beckman Coulter).*

3. Methods

3.1 Small-scale exosome production (2-20 μg)

Typically, cells grown to 80% confluency on 15 cm^2 tissue culture plate yield ~1-2 μg (24 h incubation) of exosomes. This yield is cell type-dependent and should be determined for each cell type.

1. Culture cells to 60-70% confluency on 15 cm^2 tissue culture plate in suitable culture media with supplements as required: 5% (v/v) FCS, 1% (v/v) Pen/Strep) at 37 °C with 5% CO_2 (see **Note 1**).
2. Gentle wash cells three times with 10 mL (pre-warmed at 37 °C) of PBS.
3. Culture for 24-48 h in cell culture medium (see **Note 2**) containing either 5% (v/v) EV-depleted FCS, or 0.6-1% (v/v) ITS.
4. After 24-48 h incubation, collect conditioned medium (CM) into 50 mL polypropylene tubes and centrifuge: 480 × *g* for 5 min followed by 2,000 × *g* for 10 min) at 4 °C to remove intact cells and cell debris. Carefully transfer the supernatant into a new tube and subject to desired exosome isolation protocol or store at -20 °C until further use. This supernatant contains both soluble and vesicle components (see **Note 3**).
5. Cell viability should be assessed in cell culture medium (i.e., 24 or 48 h incubation) (see **Note 4**).

3.2 Large scale exosome production

Bioreactor flasks contain a lower cell-cultivation chamber separated from an upper nutrient supply chamber by a semi-permeable membrane with a molecular weight cut-off of 10 kDa (see **Note 5**). This membrane allows continuous diffusion of nutrients from the upper to the lower chamber, and waste elimination from the lower to the upper chamber. Exosomes released by cells are retained in the lower chamber. For detailed notes on large scale exosome production (see **Note 6**).

1. Add 500 mL of pre-warmed (37 °C) DMEM with 5% (v/v) FCS, 1% (v/v) Pen/Strip) to the nutrient-supply chamber.
2. Prepare cells (15-30 × 10^6 cells) in 15 mL of DMEM medium with 5% (v/v) FCS and 1% (v/v) Pen/Strep.
3. Transfer cells to the cultivation chamber and allow to seed/expand for 3-4 days.

4. Remove the cultivation chamber medium (the cells have now adhered to cultivation chamber).
5. Wash the cultivation chamber three times with serum-free DMEM medium.
6. Add 15 mL of DMEM medium with 0.6-1% (v/v) ITS, 1% (v/v) Pen/Strep to the cultivation chamber.
7. Replace media in the nutrient chamber every 4-7 days (cell type dependent).
8. Replace media in the cultivation chamber every 1-2 days (cell type dependent).
9. Subject the conditioned medium from the cultivation chamber to exosome isolation protocol (see **Sections 3.4-3.7**).
10. Collect CM into 50 mL polypropylene tubes and centrifuge at 480 × *g* for 5 min followed by 2,000 × *g* for 10 min at 4 °C to remove intact cells and cell debris (see **Note 3**).
11. Carefully transfer the supernatant into a new tube and subject to desired exosome isolation protocol or store at -20 °C until further use. This supernatant contains both soluble secreted and EV components.

3.3 Protein quantitation (see **Note 7**)

3.3.1 Micro BCA assay

1. Prepare a set of standard dilutions with BSA, starting with 200 µg/mL, performing dilutions in 1% SDS solution to 0.5 µg/mL and place them at RT (1% SDS solution can crystallize on ice).
2. Thaw protein sample (CM, exosomes, or cell lysates samples) and place on ice.
3. In a flat-bottom 96-well plate, load 100 µL of each standard BSA dilution/blank. The blank is dependent on what stock/lysis solution exosomes are prepared in.
4. Load 1-4 µL of CM, exosomes, or cell lysate samples into wells, making up the total volume to 100 µL using 1% SDS solution.
5. Prepare working reagent (WR) by mixing reagent A, B and C (as provided in the commercial kit) with the ratio of 25:24:1.
6. Add 100 µL of WR to each sample/standard.
7. Incubate at 37 °C for 1.5-2 h.
8. Cool plate to RT.
9. Measure absorbance at 562 nm.

10. Prepare standard curve, subtract the blank (abs) from the sample (abs) and determine the concentration of starting sample.

3.3.2 Protein staining densitometry

1. Solubilize samples (2-5 µL of CM, exosomes, or cell lysates) in reducing 2x SDS sample buffer (10 µL), and heat at 95 ºC for 5 min (closed cap). (*Optional but recommended to ultracentrifuge cell lysate at 400,000 × g for 30 mins (TLA-100.2 rotor) to remove genomic DNA*).
2. Briefly centrifuge samples using benchtop centrifuge and resolve on NuPAGE™ 4-12% (w/v) Bis-Tris Precast gels.
3. Load BenchMark™ Protein Ladder (2-5 µL, same volume as sample; 1.7 µg/µL) for quantitation. An empty lane will serve as background control.
4. Perform electrophoresis at 150 V for 1 h in 1 × MES running buffer.
5. Remove the gel (and plastic casing) and place it in 50 mL fixing solution for 30 min on an orbital shaker and stain with 30 mL SYPRO® Ruby for 40 min, followed by destaining in SYPRO® Ruby destaining solution for 1 h.
6. Image gel on a Typhoon 9410 variable mode imager, using a green (532 nm) excitation laser and a 610BP30 emission filter at 100 µm resolution.
7. Perform densitometry quantitation using ImageQuant software to determine protein concentration relative to a BenchMark™ Protein Ladder standard of known protein concentration (1.7 µg/µL). Normalize by subtracting background signal from empty lane.

3.4 Ultracentrifugation exosome isolation

1. Centrifuge CM (~30 mL) at 10,000 × *g* (SW28/ SW32 Ti, swing bucket) for 30 min at 4 °C to pellet shed microvesicles. Retain the supernatant which contains exosomes.
2. Centrifuge the supernatant at 100,000 × *g* (SW28/ SW32 Ti, swing bucket) for 1 h at 4 °C to pellet exosomes (see **Notes 8, 9, 10, 11**).
3. Resuspend exosome pellet in 1 mL sterile/filtered PBS and re-centrifuge at 100,000 × *g* (TLA 55 rotor) for 1 h to obtain crude exosomes.
4. Resuspend crude exosomes in 50 µL PBS and either use immediately or store at -80 °C.

3.5 OptiPrep™ density gradient exosome isolation

1. Prepare OptiPrep™ density gradient by diluting a stock solution of 60% (w/v) OptiPrep™ with 0.25 M sucrose/10 mM Tris-HCl, pH 7.5, to obtain 40% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) solutions of iodixanol. These solutions are made immediately prior to centrifugation and allow up to 30 min mixing time. The gradient is formed by adding 3 mL of 40% iodixanol solution, followed by careful layering of 3 mL each of 20% and 10% solutions, and 2 mL of the 5% solution (see **Note 11**).
2. Overlay crude exosomes (from **Section 3.4**) (500 µL) on top of the discontinuous iodixanol gradient.
3. Overlay 500 µL of 0.25 M sucrose/10 mM Tris-HCl, pH 7.5, on top of the discontinuous iodixanol gradient (control experiment).
4. Centrifuge at 100,000 × *g* (SW41Ti swinging bucket) for 18 h at 4 °C (**Table 1**).
5. After centrifugation, collect twelve individual 1 mL gradient fractions (with increasing density – top-bottom collection) using a 1 mL pipette. Isolation from the meniscus is imperative for this careful procedure (see **Note 12**).
6. Dilute fractions with 2 mL PBS and centrifuge at 100,000 × g for 1 h at 4 °C followed by washing with 1 mL PBS (repeat 100,000 × g for 1 h at 4 °C), and re-suspend in 50 µL PBS.
7. Determine density of each fraction of the control OptiPrep™ gradient run in parallel (see step 3). Collect 12 fractions from the control gradient, serially dilute 1:10,000 with distilled water, and determine the iodixanol concentration based on absorbance at 244 nm using a molar extinction coefficient of 320 $L.g^{-1}cm^{-1}$ [47] (see **Note 12**).
8. Use exosomes immediately or store at -80 °C (see **Note 13**).

3.6 Cushion-based separation of exosomes

Exosome pellet obtained following ultracentrifugation potentially contains protein aggregates sticking to the surface of exosomes [48]. Thus, interpretation of down-stream biochemical, biophysical and functional characterization may be compromised. Although IP-based isolation of exosomes can overcome these limitations, it only enables enrichment of a specific subset of exosomes (see **Note 14**). Here, we outline ultracentrifugation coupled to cushion-based separation of exosomes to gently obtain exosomes and prevent their

pelleting/aggregation. We highly recommend using CM from CELLine *adhere/ classic* bioreactor flasks, as the concentration of exosomes in the CM is high.

3.6.1 Cushion-based separation of exosomes

1. Add 1 mL of 40% OptiPrep™ solution (prepared as described in **Section 3.5**) to Open-Top Thinwall Ultra-Clear Tube (38.5 mL).
2. Gently overlay with 30 mL conditioned media (post 10,000 × *g* spin) (see **Section 3.4** step 1).
3. Centrifuge at 100,000 × *g* for 1 h at 4 °C (SW28/ SW32 Ti swing bucket).
4. Exosomes float at CM and OptiPrep™ solution interphase as a band.
5. Aspirate out the CM, being careful not to disturb the exosome-band.
6. Collect the exosome band.
7. Dilute exosomes in 30 mL PBS and repeat ultracentrifugation-cushion separation or subject to density-based separation.
8. Use exosomes immediately or stored at -80 °C.

3.6.2 Ultracentrifugation-density gradient separation

1. Prepare 4 mL 5-40% OptiPrep™ gradient: 1 mL, 5%; 1 mL, 10%; 1 mL, 20%; 1 mL, 40%) in an Open-Top Thinwall Ultra-Clear tube (38.5 mL).
2. Gently overlay with 8 mL conditioned media (post 10,000 × *g* spin) (see **Section 3.4** step 1).
3. Centrifuge at 100,000 × *g* for 18 h at 4 °C (SW28/ SW32 Ti swing bucket).
4. Just as in regular OptiPrep™ density gradient exosome isolation (see **Section 3.5**), exosomes enter the gradient, float at ~1.07-1.09 g/mL, and visible often as a band if the yield is high.
5. Aspirate out the CM, being careful not to disturb the OptiPrep™ gradient.
6. Carefully collect twelve 330 µL fractions.
7. Dilute 330 µL fraction with 1 mL PBS. Ultracentrifuge 10% of the sample at 100,000 × *g* for 1 h and subject the pellets to western blotting (ALIX and TSG101 primary antibodies) to determine exosome-containing fraction(s) (see **Section 3.8**).
8. Pool and dilute exosome containing fraction(s) in 10 mL PBS and overlay on 13.2 mL tube containing 500 µL 40% OptiPrep™ solution. Fraction pooling is based on density and exosome marker presence.
9. Ultracentrifuge at 100,000 × *g* for 1 h (SW41Ti swing bucket).

10. Exosomes float atop the OptiPrep™-solution as a band.
11. Aspirate out the supernatant, being careful not to disturb the exosome-band.
12. Collect the exosome band.
13. Use exosomes immediately or store at -80 °C.

3.7 EpCAM immunoaffinity capture exosome isolation (see **Note 15**)

1. Incubate concentrated CM with 100 µL EpCAM magnetic microbeads for 4 h at 4 °C [18,49].
2. Place 3 mL LS Microcolumn in a solid support magnet (SSM) and rinse three times with Rinsing Solution.
3. Pipette exosome-bound microbeads into the LS column and wash three times with 1 mL Rinsing Solution.
4. Remove LS column from the SSM and recover exosome-bound microbeads by rinsing the LS column at room temperature (RT) with 3 × 1 mL Rinsing Solution.
5. Wash exosome-bound microbeads twice with 1 mL PBS, place in ultracentrifuge microfuge vials, and centrifuge at 100,000 × *g* for 1 h at 4 °C (**Table 1**).
6. Remove the supernatant and elute IAC-Exosomes (yield ~195 µg) from the microbeads with either 100 µL of IAC elution buffer for EM imaging, or lyse with 100 µL of 2× SDS sample buffer (without DTT).

3.8 Western blot analysis (see **Note 16**)

1. Lyse the samples (~10-15 µg protein) in 2x SDS sample buffer with or without DTT (depending on the primary antibody reducing/non-reducing compatibility, respectively) and heat for 5 min at 95 °C (closed cap, although cap can be opened for evaporation to reduce load volume).
2. Perform electrophoresis on lysed samples at 150 V for 1 h.
3. Following electrophoresis, electro-transfer proteins onto nitrocellulose membranes using iBlot™ Dry Blotting System.
4. Incubate membranes with blocking buffer for 1 h at RT. Care should be taken to not touch and disrupt the membrane (only the immediate corners/edges).
5. Probe membranes with primary antibody solution for 1 h in TTBS followed by incubation with secondary antibody solution for 1 h in dark. Carry out antibody incubations using gentle orbital shaking at RT.

6. Wash western blots three times in TTBS for 10 min after each antibody incubation step and subsequently visualize at 800 nm using the Odyssey Infrared Imaging System.

3.9 Nanoparticle tracking analysis (NTA) (see **Note 17**)

1. Dilute sample (exosome or CM) to a final volume of 1 mL (final concentration 1 µg/µL in ultrapure water (1:10,000 dilution)).
2. Load sample onto NS300 flow-cell top plate using syringe pump.
3. Set analysis settings as per [6]: Detection threshold: 10; Flow rate: 100; Temperature: 25 °C.
4. Adjust camera level and screen gain until particles are visible.
5. For video capture, set conditions as follows: Number of capture: 3 (for triplicate); capture duration: 60 s.
6. For acquisition, change base file name to preferred destination folder.
7. Create and run script to begin capture and analysis with NTA software. Ensure that detection threshold is set to include as many particles as possible with the restriction that 10-100 red crosses were counted while only <10% were not associated with distinct particles (see **Note 17**). Repeat dilution and analysis as required ensuring particle counts are within 20-100 particle/frame.
8. Set report details as prompted and view NTA report in destination folder: PDF graphs and batch summaries show size distribution profile data and statistics; CSV files contain raw data for further processing; AVI files contain video capture.

3.10 Aldehyde/sulfate latex bead-based capture

1. Incubate 4 µL of exosomes with 1 µL of latex beads to a final volume of 1 mL with PBS.
2. Incubate overnight at 4 °C with constant but gentle shaking.
3. Add 110 µL of 1 M glycine in PBS and allow mixture to stand for 45 min at RT.
4. Wash beads twice with 1 mL blocking buffer by centrifugation at 1500 × *g* for 3 min at RT.
5. Incubate beads with primary antibody for 1 h at RT with constant but gentle shaking.
6. Perform negative control using ASL bead-bound exosomes with matched IgG antibody.
7. Wash beads twice with wash buffer.

8. Incubate beads with appropriate secondary antibody for 20 min at RT with constant but gentle shaking.
9. Wash beads twice with wash buffer.
10. Resuspend beads in PBS, transfer to microscope slide and cover with glass slip.
11. Image with AxioObserver Z1 microscope and process with software.

3.11 Electron microscopy (EM) (see **Note 18**)

3.11.1 Transmission EM

1. Fix exosome preparations (~2 µg protein) in fixation solution, layered onto formvar-coated copper grids, and allow drying at RT.
2. Wash grids twice with PBS for 5 min, and stain with uranyl acetate solution for 10 min.
3. Image at an acceleration voltage of 200 kV using a CCD camera coupled to an electron microscope.
4. Typically, 10-20 fields of view are obtained.

3.11.2 CryoEM

1. *Optional:* Mix exosome preparations (~2 µg protein) with Aurion Protein-G gold at a ratio of 1:3. This will allow vesicle diameters to be determined and the possibility for tomographic data collection.
2. Dilute samples as required in PBS.
3. Transfer samples onto glow-discharged C-flat holey carbon grids and blot off excess liquid (*Optional,* use of Vitrobot allows automated specimen preparation).
4. Immediately plunge-freeze grids in liquid ethane.
5. Mount grids in a cryoholder in liquid nitrogen.
6. Acquire images at 300 kV using a Tecnai G2 F30 in low dose mode.
7. Typically, 10-20 fields of view are obtained.

3.12 Proteomics: sample preparation

3.12.1 In-solution reduction, alkylation and digestion (see **Note 19**)

1. On ice, normalize exosome samples to 10 µg in 50 µL lysis buffer (see **Note 20**) in protein LoBind tubes/plates.
2. Prepare fresh 500 mM DTT stock, vortex briefly until dissolved (see **Note 21**).

3. For sample reduction, pre-heat ThermoMixer to 50 °C and add 1 µL DTT stock to each tube (10 mM final concentration), vortex and microfuge briefly. Incubate all samples in the thermomixer at 50 °C for 45 min (350 rpm). Keep the remaining DTT stock on ice for quenching (Step 7).
4. Cool down all samples to RT and reset the ThermoMixer to 25°C to avoid non-specific alkylation reaction.
5. Prepare fresh 1 M IAA stock and ensure minimal light exposure of IAA. Vortex briefly until dissolved.
6. For sample alkylation, add 1 µL IAA stock to each tube (final concentration 20 mM IAA), vortex and microfuge briefly. Incubate samples in the ThermoMixer at 25°C for 30 min (350 rpm) in the dark.
7. To quench the alkylation reaction, add 1 µL DTT stock to all samples (final concentration 20 mM), exposing to light.
8. Add 500 µL ice-cold acetone to all samples, vortex briefly. Store the samples in -20 °C freezer overnight. Samples can be stored at -20 °C for up to three days.
8. Prior to tryptic digestion centrifuge all samples at 16,000 ×g for 10 min at 4 °C. Carefully discard supernatant without disturbing the protein pellet.
9. Resuspend protein pellets in 50 µL 90% (v/v) ice-cold acetone, vortex briefly.
10. Centrifuge all samples at 16,000 × *g* for 10 min at 4 °C. Carefully discard supernatant without disturbing the pellet. Air-dry the samples under the fume hood for 5 min.
11. Resuspend protein pellets in 50 µL 50 mM TEAB, pH 8.0 (see **Note 22**).
12. Add 0.2 µg equivalent trypsin enzyme (1:50 enzyme-to-substrate ratio) to each sample. It is often easier to make the trypsin working solution (calculate adding 50 µL per sample; 50 mM TEAB, pH 8.0, on ice), add required amount of trypsin to the trypsin working solution, and aliquot to each resuspended protein pellet (see **Note 22**).
13. Set the ThermoMixer to 37 °C and incubate all samples for 18 hours at 350 rpm.
14. Acidify all samples with 0.75 µL formic acid (final concentration 1.5% (v/v)) to pH 2, test with pH strips.
15. Centrifuge all samples at 16,000 × *g* for 5 min. Transfer supernatant to a protein LoBind tubes if insoluble precipitate is spotted at the bottom of the tube.

16. Place all samples in -80 °C freezer until frozen. Peptides can be stored in -80 °C for an extended period before lyophilization. Skip this step if StageTip Cleanup is required.

3.12.2 StageTip cleanup (see **Note 23**)

1. SDB-RPS StageTips are prepared as described [50], using Empore solid-phase extraction disks. Prepare one SDB-RPS StageTip for each sample.
2. We use two plugs of SDB-RPS material for the respective StageTips, punched out by using a blunt-end 14-gauge syringe to pierce the ends of 200 µL pipette tips (see **Note 23**).
3. Add 30 µL of SDB-RPS loading buffer to the top of each sample. Place the StageTips into centrifuge adapters and centrifuge the sample through to dryness (1,500 × *g*, for ~1 min at RT).
4. Wash the SDB-RPS StageTips using 30 µL of SDB-RPS wash buffer 1 to each sample and centrifuge for ~1 min. Repeat.
5. Transfer each peptide sample to the top of an SDB-RPS StageTip.
6. Place the StageTips into same centrifuge adapter and centrifuge the sample through to dryness (1,500 × *g* for ~5-8 min at RT) (see **Note 24**).
7. Wash the StageTips with 100 µL of SDB-RPS wash buffer 1 and centrifuge the sample through to dryness (1,500 × *g* for ~5-8 min at RT). Repeat with SDB-RPS wash buffer 2.
8. Elute the peptides by adding 60 µL of SDB-RPS elution buffer of the StageTip, and centrifuge through to dryness (1,500 × *g* for ~5 min at RT). Collect the eluates into clean protein LoBind tubes.
9. Immediately place the tubes into an evaporative concentrator and concentrate under vacuum at 45 °C to dryness. The time to dryness will depend on the number of samples being processed. This typically takes 40-60 min; however, after the first 30 min have elapsed, samples should be checked at intervals of 10 min and concentrated until no liquid remains.
10. Reconstitute peptide by adding 12 µL of MS loading buffer and either shaking for 2 min at 2,000 rpm or optionally incubating for 5 min in a sonicating water bath on low power, then centrifuge the samples at 2,000 × *g* for 1 min at RT.

3.13 Fluorometric peptide assay (see **Note 25**)

1. Prepare peptide standards from the Assay Kit starting with 1000 µg/mL and performing serial dilutions in MS loading buffer down to 7.8 µg/mL as per the manufacturer's instructions (8 standards plus MS loading buffer as blank).
2. Load the 10 µL of each standard/blank into the black 96 well plate.
3. Load 2 µL of each sample into the plate and make up to 10 µL by adding 8 µL MS loading buffer.
4. Add 70 µL of Fluorometric Peptide Assay Buffer to each well.
5. Add 20 µL of Fluorometric Peptide Assay Reagent to each well.
6. Incubate at RT for 5 min.
7. Measure fluorescence using Ex/Em at 390 nm/475 nm.
8. Use the standard curve to calculate the concentrations of your samples (ensuring to subtract blank (abs) from all standards/samples (abs)).

3.14 UHPLC-MS/MS

1. Perform UHPLC-MS/MS analysis of trypsin digested exosome protein samples at a flow rate of 300 nL min^{-1} by using the following UHPLC gradient.

Time interval (min)	Gradient (% buffer B)
0	2
95	28
98	80
100	80
104	2
110	2

2. Use the following MS parameters on the **Q Exactive HF-X** for protein identification.

Instrument	
Polarity	Positive
S-lens/ion-funnel RF level	45
Capillary temperature	300 °C
Full MS	
Microscans	1
Resolution	60,000
Automatic gain control target	3×10^6 ion counts
Maximum ion time	128 ms
Scan range	300–1650 m/z
dd-MS^2 (data dependent)	
Microscans	1
Resolution	30,000

Automatic gain control (AGC) target	1×10^5 ion counts
Maximum ion time	60 ms
Loop count	25
Isolation window	1.3 m/z
Isolation offset	0
Fixed first mass	120 m/z
Normalized collision energy	25
Spectrum data type	Profile
Data Dependent settings	
Minimum AGC target	1.2×10^4 ion counts
Apex trigger	-
Charge exclusion	Unassigned, 1, ≥6
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	30 s

3.15 Data analysis

1. Analyse raw proteomics data using MaxQuant [51] (see **Note 26**) and perform downstream bioinformatics analysis using the Perseus platform [46] (described below). However, several other suitable programs are freely or commercially available that can be used to process proteomics data [52-55].
2. Specify the parameters used for searching label-free proteomics data using MaxQuant. Default settings in MaxQuant are typically optimal and can normally be left unchanged, with a few exceptions such as defining experiment, modifications, and enzyme as described below. Typical parameters are outlined in the table below.

Setting	**Value**
Raw data	
Parameter group	All samples: Group 0
Experiment	Enter unique name, suffixed by biological replicate (e.g., 'Control_1')
Fraction	All samples: 1
PTM	FALSE
Group-specific parameters	
Type	Standard
Multiplicity	1
Labels	None
Variable modifications	Oxidation (M), Acetyl (protein N-term)
Digestion mode	Specific
Enzyme	Trypsin/P
Label free quantitation (LFQ)	TRUE, maxLFQ
Maximum missed (cleavages)	2
Database	Specify protein database (i.e., reference proteome) in FASTA

	format
Global parameters	
FASTA files	Specify protein sequence databases
Fixed modifications	Carbamidomethyl (C)
Second peptide	TRUE
Protein FDR, peptide spectral match	0.01 / 0.01
Match between runs	TRUE
Match time window	0.7 min
Alignment time window	20 min
Advanced site intensities	Yes

737

738 3. Perform data analysis using Microsoft Office Excel and Perseus software [46] (see **Note**
739 **26**).
740 4. Use ProteinGroup.txt file for protein grouping and identification, with contaminants
741 (CON_) and reverse (REV_) identifications manually removed.
742 5. Perform Hierarchical clustering in Perseus using Euclidian distance and average linkage
743 clustering.
744 6. Perform functional enrichment analyses (Gene Ontology (GO), KEGG, and Reactome
745 pathways) using gProfiler web-based bioinformatics resource [56].
746 7. Pathway enrichment map analysis can also be performed using Cytoscape [57] and
747 protein-protein interaction networks generated using STRING [58].
748

749 **3.16 Phenotypic reprogramming of cells by exosomes: Dissecting function**

750 Design of functional assays are directly dictated by the biology and the question raised.
751 Here, we questioned whether cancer exosomes can be taken up and activate fibroblasts
752 and confer them with cancer-promoting ability. We will then outline how we gained
753 insight into underlying mechanism using mass spectrometry-based proteomics.
754 Importantly, time points cataloguing activation assays and functional response will be
755 important in deciding time points for mass spectrometry-based proteomics.
756

757 **3.16.1 Labelling exosomes with lipophilic tracer** (see **Note 27**)

758 1. Prepare Dil staining solution.
759 2. Add crude exosome (300 μg) in 300 μL of PBS to the staining solution.
760 3. Incubate at 37 °C for 15 min.
761 4. Centrifuge at 100,000 × *g* for 1 h to obtain Dil-labelled crude exosomes

5. Resuspend the pelleted exosomes in 300 µL PBS and subject to OptiPrep™ density gradient exosome isolation (see **Section 3.5**) to separate labelled exosomes from the unbound dye that co-pellets (see **Note 28**).
6. To assess labelling, aliquot 100 µL of 12 fractions from the OptiPrep™ density gradient to black 96 well plate and measure fluorescence using a plate reader (excitation/emission 549/565 nm).
7. Confirm exosome-containing fraction(s) by western blotting (see **Section 3.8**).
8. Reconstitute labelled exosomes in PBS and store at -80 °C until further use.

3.16.2 Exosome uptake (see **Note 29**)

1. Culture recipient cells to ~70% confluency on glass cover slips in wells of 6 well plate or 8-well culture chambers (see **Note 29**).
2. Prepare 100 µL of cell culture growth medium supplemented with Dil-labelled exosomes (between 5-10 µg).
3. Remove the spent media from the 6-well plate or the 8-well chamber and gently overlay with media containing labelled exosomes.
4. Incubate at 37 °C for 2 h (see **Note 30**).
5. Stain the nuclei with Hoechst stain at 37 °C for 30 min.
6. Gently wash cells three times with warm (37 °C) PBS.
7. Perform live cell imaging of cells in phenol-red free medium.
8. Alternatively, cells can be fixed in 5% formaldehyde for 10 min at RT and imaged.
9. For long term storage, mount the slide with Dako mounting media (see **Note 31**).

3.16.3 Cell activation assay

1. Culture recipient cells (e.g., neonatal human foreskin fibroblasts) in 96-well plate to 100% confluency.
2. Wash cells and maintain in 100 µL serum-free culture medium for 72 h at 37 °C
3. Supplement the medium with exosomes to a final concertation of 15 µg/mL or PBS vehicle alone.
4. Culture further for 72 h at 37 °C.
5. Wash cells three times with PBS and perform western blotting analysis for activation marker (e.g. smooth muscle actin as a marker of activated fibroblasts).
6. The timing of the activation assay should be determined for each experiment and should be a good guide for downstream mass spectrometry-based analysis.

3.16.4 Invasion assay

1. Thaw stock Matrigel matrix on ice.
2. Coat Transwell insert with 100 µL of 1 mg/mL Matrigel matrix in PBS.
3. Allow to polymerize for 4 h at 37 °C.
4. In the meantime, stimulate recipient cells (50,000 cells) in 500 µL culture medium (serum-free) with exosomes (15 µg/mL) or PBS vehicle alone for 2h at 37 °C.
5. Centrifuge cells at 500 × *g* for 5 min, resuspend in 100 µL serum-free medium and carefully overlay onto the Matrigel-coated insert.
6. Place the insert in the companion plate that contain growth medium (containing serum) supplemented with exosomes 15 µg/mL or PBS vehicle alone.
7. Incubate at 37 °C for 16 h (or up to 48 h).
8. Wash inserts with PBS, fix cells with 4% formaldehyde and stain nuclei with Hoechst dye.
9. Remove non-invading cells (from the upper side of the insert) using cotton swab.
10. Wash insert with PBS.
11. Image nuclei of the cells invaded to the lower side of the insert using a fluorescence microscope (image up to 5 fields of view per insert).
12. Quantify using Image J software or by manual counting.

3.16.5 Molecular reprogramming of cells by exosomes

1. Activate recipient cells with exosomes in 24 well plate as described in **Section 3.16.4**
2. Wash exosome-treated cells three times with 500 µL ice cold PBS.
3. Lyse cells with 200 µL SDS sample buffer.
4. Scrape the lysed cells and transfer into a centrifuge tube.
5. Heat denature at 95 °C for 5 min.
6. Sonicate to sheer the genomic DNA. Place all samples on ice. Ensure to immerse the microtip into the lysate without touching the microtube. Sonicate samples with 23 amplitudes for 10 s. If insufficient sonication is observed, rest the sample on ice for at least 30 s and repeat the sonication step. Remove un-solubilized material by centrifugation at 16,000 × *g* for 20 min.
7. Alternatively, to remove DNA and insoluble cellular debris, lysates are centrifuged at 400,000 × *g* for 30 min (TLA-100.2 rotor), with supernatants collected and stored at -80 °C (up to 6 months).

8. Quantify the protein amount (see **Section 3.3**).
9. Subject protein samples to mass-spectrometry-based proteomics (see **Section 3.12**)
10. Perform pathway enrichment analysis using gprofiler [56], or Cytoscape (enrichment map plug in) [57] to gain insight into molecular players of recipient cell reprogramming by exosomes.

4. Notes

1. Cells should be grown to reach 60-70% confluency for adherent or cells grown in suspension. Utilizing as many cells as possible ensures a concentrated CM that is enriched in EVs/exosomes.
2. Standard growth medium for most cells in culture require FCS as a growth supplement to DMEM/RPMI. FCS is derived from bovine serum and contains exosomes from this source [59]. As such, exosome presence in FCS can interfere or cause significant protein background issues when studying exosomes secreted from cells of interest in cell culture [60]. For exosome production, we typically modify the growth medium to either serum-free media or a combination of serum-free, exosome-depleted FCS and ITS (0.2-1.5% (w/v)) to ensure cells remain viable during exosome production. Any changes to cell growth medium supplements should be evaluated over the time-course of exosome production (typically 24-48 h). Depending on the cell type, further methods to modify the cell growth medium include 1% (w/v) BSA to replace FCS, and depletion of FCS-containing exosomes using ultracentrifugation (100,000 × *g*, 16 h), or filtration/ immunodepletion ($CD63^+$, exosome-sized vesicles removed, no measurable bovine microRNAs) [61]. Further, exosome-depleted FCS growth supplements are commercially available (e.g., EXO-FBS-250A-1, System Biosciences; A2720801, Life Technologies) as adjunct approaches.
3. For sample and exosome sterility, it is only necessary to use sterile conditions if the application requires sterility (e.g., functional *in vivo* or *in vitro* assays). Collection tubes may be sterilized by rinsing with 70-80% (v/v) ethanol, air dried, and incubated under UV light for 30 min. If only biophysical and biochemical characterization will be performed (e.g., electron microscopy, immunoblotting, -omic profiling), sterility is not required. The use of filtered and sterile PBS is often used to ensure no contaminants are introduced during preparation and solubilisation. If sterility is required, sterile centrifuge and ultracentrifuge tubes must be used, with centrifuge tube holders and all related steps performed in an enclosed tissue culture hood. To

sterilize ultracentrifuge tubes, wash the clean tubes and their lids briefly in 70-80% (v/v) ethanol, rinse twice in sterile PBS, and remove PBS. All rotor lids and caps, centrifugal ultrafilter units should also be washed with 70-80% ethanol and air dried (depending on compatibility with filter membranes, check with manufacturer requirements).

4. Cell viability should be assessed in cells grown in serum-free medium. For example, Trypan Blue dye assay includes treating cells with Trypan Blue dye, preparing a 1:1 dilution of the cell suspension using a 0.4% (w/v) Trypan Blue solution, and performed as per manufacturer instruction. Further assay methods may include but not limited to different classes of colorimetric tetrazolium reagents, resazurin reduction and protease substrates generating a fluorescent signal, the luminogenic ATP assay, metabolic assays such as the MTT and XTT assays, and a novel real-time assay to monitor live cells for days in culture [62], https://www.abcam.com/kits/cell-viability-assays; https://www.cellsignal.com/contents/_/synopsis-of-cell-proliferation-metabolic-status-and-cell-death/cell-viability-and-survival.
5. For large-scale exosome preparation we have employed the use of the two-compartment bioreactor CELLine tank system for continuous culture (exosome yield in mg quantities). This is attained by separating the bioreactor into an upper nutrient chamber and lower cultivation chamber. CELLine doesn't require any specific adaptation of cell culture techniques or media composition and is suitable for applications based on serum-supplemented or serum free cultures. We have utilized both CELLine *classic* (CELLine CL 1000, #90005, INTEGRA Biosciences) and CELLine *adhere* (CELLine AD 1000, #90025) for suspension or anchorage-dependent cells to be grown in the bioreactor and extensive EV yields generated [6]. It has been reported CELLine culture system for exosome production from cell cultures significantly increased exosome yield ~12 fold than conventional flasks to ~10.1 μg/mL [63-65].
6. We would recommend washing the cell compartment (every 2nd day, 3-washes in media) to remove poorly adherent, non-viable cells/dead cells/debris before addition of medium to both compartments. We also advise removing any bubbles from the cell compartment chamber. Take an aliquot of media from the cell compartment chamber to check for possible contamination. For non-adherent cells, floating cell organoids can be reintroduced back into the cell compartment chamber following CM collection; spheroids can be pelleted at 150 × *g* for 2-5 min. We recommend changing

media in the nutrient chamber every 4-7 days (cell type dependent), which can be guided by media colour change based on pH.

7. For protein quantitation we typically employ different methods based on sample quantity and buffer compatibility, including the micro BCA assay (2-5 µL sample), or protein densitometry (2-5 µL sample). The micro BCA is a sensitive detergent-compatible bicinchoninic acid formulation for colorimetric detection and quantitation of total protein. Importantly, it is optimized for use with dilute protein samples (0.5-20 µg/mL), with a strong absorbance at 562 nm that is linear with increasing protein concentrations. It is important to restrict non-compatible components including reducing agents (i.e., DTT), lipids, phenol red, and EGTA. If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, refer Bradford-based Protein Assay. For detailed comparison of compatible concentrations for different protein assays please refer to https://assets.thermofisher.com/TFS-Assets/LSG/Application-Notes/TR0068-Protein-assay-compatibility.pdf. For samples employing detergents, or other reagents which affect absorbance, we would recommend using the protein densitometry method. The protein densitometry method is reproducible, has a linear quantitation range over three orders of magnitude [66], and is compatible with GeLC-MS/MS [67,18,68].
8. Large-scale instruments and centrifuges (due to the concentration of exosomes in culture medium, large culture volumes; >500-1000 mL) are often utilized in order to generate exosome yields for characterisation/functional studies. This introduces issues with the capacity of centrifugation, availability (rotors, instrumentation), and time. If researchers are limited by large-scale ultracentrifugation, then the CM preparation approach, with IAC, or commercial kits to isolate EVs would be suggested.
9. Requirement to isolate shed microvesicles for analysis (using the CM preparation vesicles >0.1 µm are not retained and therefore restricting their subsequent analysis). Shed microvesicles are heterogeneous ~150 - ~1000 nm diameter vesicles, distinct in their biogenesis and marker expression composition to exosomes [69]. If researchers are interested in the isolation of shed microvesicles, then this fraction can be easily isolated from culture medium in the process of obtaining the crude exosome pellet. For the isolation of shed microvesicles, a direct ultracentrifugation approach is employed. Following 10,000 × *g* centrifugation, the shed microvesicle pellet is resuspended in 1 mL of PBS and 10,000 × *g* centrifugation performed to obtain the

washed shed microvesicle fraction (resuspend in 50-100 µL PBS). Further use of density-based separation can purify this EV type [6]. The isolation and proteomic characterization of shed microvesicles has been described previously [18,44,6,70].

10. For ultracentrifugation, mark each ultracentrifuge tube and orient the tube in the rotor with the mark facing up. The mark should be used as a reference for the location of a pellet following centrifugation. For swing bucket rotors, the pellet is at the bottom of the tube. For fixed-angle rotors, the pellet is on the side of the tube near the base.
11. The ultracentrifuge tubes are thin walled and will collapse if improperly filled. All tubes should be at least 80% capacity to ensure the tube isn't flexible (add PBS if required). Tube specifications should be checked with the manufacturer prior to use. Ensure that the tubes are sealed (no condensation which can directly affect vacuum) and rotor is properly seated within the centrifuge.
12. Exosomes float at densities ranging from 1.09-1.15 g/mL on continuous sucrose or iodixanol density gradients following centrifugation [49,18,71,70]. OptiPrep™ density gradients have low viscosity, isosmotic gradients that provide rapid and efficient separation of extracellular vesicles. OptiPrep™ density gradients have been shown to efficiently separate exosomes from HIV-1 particles [72]. Following OptiPrep™ density gradient, isolated protein fractions (in this case 12 fractions) can be assessed for density (e.g., range 1.01-1.30 g/mL), and protein yield, and assessing expression of exosomal markers using immunoblot analysis. As an example, OptiPrep™ density gradient fractions (10 µg per fraction) revealed exosomal marker Alix detected/enriched in density fraction 1.09 g/mL (fraction 7) [73,7,70].
13. Exosome stability during different storage conditions has been examined (i.e., at -20 °C, 4 °C, and 37 °C) [74]. The size of exosomes decreases at 4 °C (3-4 days) and 37 °C (from 2 days), indicating a possible structural change or degradation. Multiple freezing to -20 °C and thawing does not affect exosome size based on nanoparticle tracking analysis (NTA) [74]. In our studies, exosome degradation has been monitored at 4 °C (within 72 h) and 37 °C (within 24 h) (data not shown). We recommend storage of exosomes at 4 °C on ice for short-term use (within 3 days), and for long periods at either -20 °C/-80 °C or lyophilisation (over 12 months at 4 °C). Exosome samples should be stored in small (50-100 µL) aliquots to avoid repeated freezing and thawing.
14. The availability and suitability of exosome markers for IAC is dependent on their target specificity and application [21,43]. Currently, several markers for exosomes

have been used for IAC including Glycoprotein A33$^+$ exosomes derived from human epithelial colon cancer cells [75], EpCAM$^+$ exosomes derived from human epithelial colon cancer cells [49], sequential Glycoprotein A33$^+$ and EpCAM$^+$ exosomes from human epithelial colon cancer cells to reveal specific subpopulations of exosomes [18], MHC II$^+$ exosomes derived from dendritic cells [76], HER2$^+$ exosomes derived from BT-474 breast cancer cells [77], and CD45$^+$ exosomes derived from Jurkat and SupT1/CCR5 cells [78]. Commercially, Dynabeads®-based CD63-specific reagent (#10606D, Life Technologies), and streptavidin reagent with choice of a biotinylated antibody to purify a specific vesicle population based on a surface antigen (#10608D, Life Technologies) are available. The use of density-based separation provides significant advantages for exosome isolation when the use of IAC is limited (due to availability/suitability of exosome markers and their localization on exosomes, i.e., surface capture).

15. CD326 (EpCAM)$^+$ exosomes are bound with EpCAM MicroBeads, with the CM loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator (SSM). The magnetically labelled EpCAM$^+$ exosomes are retained within the column. The EpCAM$^-$ vesicles are passed through; this fraction is depleted of EpCAM$^+$ exosomes. After removing the column from the magnetic field, the magnetically retained EpCAM$^+$ exosomes are eluted as the positively selected exosome fraction. For this application 100 µL of CD326 (EpCAM) MicroBeads were used for 500 mL CM (1.5 mg CCM) generated from 2×10^9 cells. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific vesicle binding.
16. To characterize extracellular vesicles as exosomes, it is important to demonstrate the expression of common exosomal proteins using immunoblotting. The commonly used markers may include Alix (PDCD6IP, programmmed cell death 6 interacting protein), TSG101 (tumor susceptibility gene 101), CD63 (tetraspanin CD63), and CD81 (tetraspanin CD81). As recommended by MISEV guidelines [43]; Western blotting should be performed by loading side-by-side extracellular vesicles as exosomes and source material lysates either in specified protein amount or in cell-equivalent amounts to determine if the analyzed proteins are enriched in extracellular vesicles as exosomes as compared with their producing cells. Further, to specify small EV subtype (including exosomes), further proteins identification should be performed in/on intracellular compartments of eukaryotic secreting cells other than the plasma

membrane and endosomes (i.e. components of the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, autophagosomes, peroxisomes) not enriched in the smaller EVs of plasma membrane or endosomal origin [43].

17. Nanosight analysis settings and sample dilution may be adjusted to obtain ideal particle per frame value (20-100 particle/frame). Dilution should be made in ultrapure water free from particle contamination (*i.e.,* dust).
18. We provide two methods (TEM and cryoEM) for electron microscopy exosome sample preparation. For TEM analysis, exosomes are fixed using glutaraldehyde and negatively stained using uranyl acetate. This approach typically has a rapid sample preparation approach (<20 min) although it provides exosome images of low resolution. Significant shortcomings being that the sample preparation and imaging technique require dehydration, chemical fixation and/or staining of the biological specimens. This has the potential to underrepresent the diameter of vesicles due to dehydration and chemical fixation. In contrast, cryo-EM does not use staining or chemical fixation procedures and samples are directly applied onto an EM grid, vitrified and visualized. Vitrification is a cryo-fixation method to preserve biological specimens to near-atomic resolution [79], while water is transformed into a glass-like state without formation of ice crystals. Cryo-EM of vitrified whole cells or EVs enables observation of biological structures in a near-native state [80,81]. Further, cryo-EM allows tomographic data collection and the ability for spatial visualization of more complex structures. Therefore, cryo-EM has the capacity to reveal highly textured morphology of exosomes [82].
19. Proteomics sample preparation can be conducted with alternative urea-based lysate with several amendments; urea lysis buffer contains 8 M urea in 50 mM TEAB, pH 8, and reduction performed without heat. All samples need to be diluted 8-fold (<1 M urea) with 50 mM TEAB (pH 8) before adding trypsin.
20. For efficient reduction, alkylation and digestion, ensure pH of the MS SDS lysis buffer is adjusted between 7.9-8.0 immediately prior to use. This also applies to enzymatic (tryptic) digestion; it is important to adjust the pH between 7.9-8.0.
21. It is important to avoid exposure to moisture when storing DTT, and to protect IAA from light in storage, stock preparation and alkylation reaction to prevent degradation of the IAA. To avoid non-specific alkylation, allow the both sample and ThermoMixer cool down to room temperature before adding IAA [83]. Several

solutions (i.e., TFA and formic acid) are corrosive. Prepare the solutions in a fume hood and handle with gloves. This buffer is stable for >3 months at RT.

22. We recommend the trypsin solution is diluted in ice-cold 50 mM TEAB (pH 8) immediately before enzymatic digestion if dealing with small amounts of starting material. Other proteases can be used on top of or replace trypsin (i.e. Lys-C, Arg-C). To ensure optimal peptide digestion, adjust the working pH and the temperature of reaction accordingly when using other proteases for protein digestion [84,85].
23. StageTips can be prepared in advance and stored in a covered pipette-tip box for several months at RT. Commercial alternatives are available; however, buffers may need to be adapted. Ensure that the discs are securely wedged in the bottom of the tip (i.e., no gap between inserts), however do not use too much force to compress StageTip material into the pipette tips as this increases the time required for sample/solvent to flow through. Slow-flowing StageTips can be used but may require higher centrifugation speeds for longer duration (see below).
24. If a StageTip flow is slow or stops entirely during capture, lipid or other insoluble material may have been present before enrichment. This should be avoided. This can be resolved by centrifugation of peptide sample (2,000 × *g*, 15 min, RT) and careful transfer of supernatants to prepared StageTips.
25. Ensure compatibility of buffers and reagents with the peptide assay. If peptides have been tandem mass tag (TMT) labelled, the fluorescence peptide assay is not suitable and a colorimetric (e.g., Life Technologies, #23275) alternative must be used.
26. Current MaxQuant release (http://maxquant.org) or other software for analyzing raw proteomics data, installed on a suitable workstation or server. For in-depth details on MaxQuant setup and minimum system requirements, and analysis pipelines including Perseus (https://maxquant.net/perseus/) see [46,86].
27. Exosomes donor cells can alternatively be labelled with Dil and exosomes released by these labelled cells can then be purified. Exosome-donor cells can also be engineered to stably express recombinant proteins (such as GFP targeting the plasma membrane) [87].
28. Aggregation and micelle formation of lipophilic dyes may yield false signal of EVs [88]. It should also be noted that several EV labelling dyes like PKH lipophilic membrane dyes has been reported to have an *in vivo* half-life ranging from 5 to > 100 days [89,90].

29. Optimal concentration and time length must be determined for different recipient cells. From our experience using live fluorescence imaging, exosome uptake (10-30 µg/mL) can start within the first 15 min [91,92].
30. It is recommended to label the plasma membrane of recipient cells with DiO or DiR or to stain intracellular structures (such as organelle probes; e.g., https://www.thermofisher.com/au/en/home/references/molecular-probes-the-handbook/tables/molecular-probes-organelle-selective-probes.html).
31. Important: Do not subject the samples to detergent permeabilization as the Dil dye labelling the exosomes will be washed away with the lipids resulting in loss of signal.
32. Resource for rotor speed, conversion, tubes and adapters can be found at https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/centrifugation/rotors/index.htm?t=3 and https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/centrifugation/tubes-and-adapters/index.htm

Acknowledgements

This work was funded by NHMRC project grants (#1057741 and #1139489; DWG), Helen Amelia Hanis Fellowship (DWG), Australian Government Research Training Program Scholarship (MF, BC), BrightSparks Scholarship (BC), and La Trobe University-Baker Institute Postgraduate Scholarship (QP).

The authors declare no conflict of interest.

Table 1 – Ultracentrifuge, Rotor, and Consumable Information for Exosome and Shed Microvesicle Purification (see Note 32)

Exosome preparation

	Speed (*g*)	**Volume** (per tube)	**Rotor**	**Polyallomer tubes or polycarbonate bottles (Beckman Coulter)**
Large scale processing	100,000	32.5 mL	SW 28 / SW 32 Ti swinging bucket	Open-Top Thinwall Ultra-Clear Tube (#3440558)
OptiPrep™	100,000	13.2 mL	SW41 Ti swinging-bucket	Thinwall Polypropylene Tube (#331372)
Small scale (washing)	100,000	1.5 mL	TLA-55 fixed angle	Microcentrifuge Polypropylene Tube (#357448)

Shed microvesicle preparation

	Speed (*g*)	**Volume** (per tube)	**Rotor**	**Polyallomer tubes or polycarbonate bottles (Beckman Coulter)**
Large scale processing	10,000	32.5 mL	SW 28 / SW 32 Ti swinging bucket	Open-Top Thinwall Ultra-Clear Tube (#3440558)
Small scale (washing)	10,000	1.5 mL	TLA-55 fixed angle	Microcentrifuge Polypropylene Tube (#357448)

1096 **Table 2 – Overview of commonly used exosome isolation methods**
1097
1098

Isolation method	Mechanism	Advantage(s)	Disadvantage(s)
Differential ultracentrifugation (DC)	Sedimentation velocity (size, volume, density). Typically used to isolate crude EVs from conditioned media and various biological fluids[93,94] with the potential to purify with wash steps and DGC[6]. Stepwise DC approach includes: initial 500 × *g* /2000 × *g* centrifugation (remove cells, membrane debris, apoptotic bodies), membrane filtration including 0.1 µm [49] or 0.22 µm membrane filtration [61], 10-14,000 × g to isolate crude sMVs [18,95,96], 100,000 × *g* to isolate crude exosomes [6].	- Low/medium recovery yield - Scalability	- High heterogeneity/ low purity - Co-purification with non-EV components - Yield dependent on sample viscosity and concentration [97] - Reproducibility may be influenced by rotor type and G-force [98] - Additional wash steps and handling may decrease yield [99,100]
Density-gradient centrifugation (DGC)	Buoyant density (density, size). Used for further purification of EV populations by a discontinuous gradient of sucrose (or less-viscous iodixanol, OptiPrep™ [87,6]) [71,82,45]. Iodixanol gradients are readily measured by refractive index [47], less toxic than sucrose in downstream functional cell assays [101], forms iso-osmotic solutions at all densities (preserves vesicle size) [102], and allow non-vesicular components to be differentially fractionated [72]. Typically, use of DC (ultracentrifugation) at 100,000 × *g* to establish gradients. Different variations of DGC include float-down. Use to separate subpopulations of EVs, including exosomes of low (1.12-1.19 g/mL) and high density (1.26-1.29 g/mL) [103,104], and sMVs of low (1.09 g/mL) and high density (1.12 g/mL) [103].	- High purity - Potential for EV subtype isolation - Applicable to clinical settings [105,106]	- Time consuming - Unable to separate different EVs based on density - Low recovery (sample loss due to additional handling) - Can co-purify with different density lipoproteins [107,108]
Density-cushioned ultracentrifugation (DCGC)	Sedimentation velocity and buoyant density. Layer of 40% iodixanol below CM, EVs and aggregates are concentrated in this cushion during ultracentrifugation. Can be followed by DGC for purification.	- Preservation of physical integrity and biological activity - Avoids aggregates	- Requires concentrated CM
Affinity isolation	Surface marker selectivity (protein or peptide epitope target). The tag may be a biospecific surface protein, such as a monoclonal antibody (mAb), that targets an EV-surface antigen[109] (e.g., mAb 763.74 specific CSPG4 epitope uniquely expressed on melanoma cells), biospecific peptide (e.g., designer synthetic peptides with high affinity for HSPs [110]), or proteoglycan affinity reagent (e.g., heparin [111-113]). mAbs that have been successfully employed include those directed against A33 [75], EpCAM	- High purity - Potential to purify different EV (sub)populations - Ability of coupling with other methods of characterization (i.e., flow cytometry, western blotting and rt-PCR)	- Expensive (if antibody based) - EV elution might damage surface proteins and functionality - Typically, dependent on availability of suitable mAbs directed to specific EV-surface antigens - Low scalability - Low yield (binding

	[114,49], MHC-II antigens [115,116], CD45 [78,117], CD63 [118,119], CD81 [119], CD9/CD1b/CD1a/CD14 [120], CD9 [121], HER2 [77], and L1CAMb[122]. Heparin affinity-based affinity capture [113] is generally applicable for EV isolation from cell culture media and biofluids, given it overcomes limitations with availability of suitable mAbs directed to specific EV-surface antigens.		capacity)
Size exclusion and gel permeation chromatography	Size, molecular weight. This approach has been widely applied for isolating EVs from plasma samples [123] and adapted (using commercially-available columns) for high-throughput clinical samples [124]. Gel permeation chromatography overcomes many of the problems associated with EV isolation from plasma/serum using DC/DGC – e.g., co-isolation of EVs with large-M_r protein aggregates and lipoproteins [124,125,123].	- High scalability	- Dilution in elution buffer
Precipitation	Salting out using a polyethylene glycol/salt solution. This approach provides rapid, but impure EV preparations, and is therefore unsuitable for detailed biophysical/ functional assay purposes. However, the method acts as an isolation/concentration step for crude EV preparation for diagnostic assays of known EV-associated biomarkers. Recent developments using sequential polyethylene glycol precipitation and adsorption to immobilized lectin concanavalin A [126] have demonstrated both exosomes and sMVs can be selectively enriched.	- Applicable for large volumes - Recent advances in sequential precipitation/ absorption have indicated potential for select types of EVs to be differentially isolated	- Low purity - PEG chain might envelope the EVs, possibly interfering with their functionality
Sequential filtration	Membrane filtration (size, molecular weight) of proteins and other macromolecules. Nanomembrane ultrafiltration spin devices equipped with low protein binding membranes (e.g., polyether sulfone or hydrophilic polyvinylidene difluoride (PVDF)) can be used for EV isolation [127-129]. In combination with DC and DGC, nanomembrane ultrafiltration has enabled fractionation of EV subpopulations; sMVs and exosomes from the same cancer cell origin [44].	- Medium scalability - Time of separation (efficiency) [130] - No direct requirement for ultracentrifugation (i.e., preserves integrity of exosomes)	- EV can clog the membrane filter - Sample loss (yield) - Does not discriminate between specific subtypes of EVs which are similar in size

1099
1100
1101
1102

References

1. van Niel G, D'Angelo G, Raposo G (2018) Shedding light on the cell biology of extracellular vesicles. Nature Reviews Molecular Cell Biology 19:213-228. doi:10.1038/nrm.2017.125
2. Mathieu M, Martin-Jaular L, Lavieu G, Théry C (2019) Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nature Cell Biology 21:9-17. doi:10.1038/s41556-018-0250-9
3. Bidarimath M, Khalaj K, Kridli RT, Kan FWK, Koti M, Tayade C (2017) Extracellular vesicle mediated intercellular communication at the porcine maternal-fetal interface: A new paradigm for conceptus-endometrial cross-talk. Scientific reports 7:40476-40476. doi:10.1038/srep40476
4. Zhang X, Hubal MJ, Kraus VB (2020) Immune cell extracellular vesicles and their mitochondrial content decline with ageing. Immunity & Ageing 17 (1):1. doi:10.1186/s12979-019-0172-9
5. Evans J, Rai A, Nguyen HPT, Poh QH, Elglass K, Simpson RJ, Salamonsen LA, Greening DW (2019) Human Endometrial Extracellular Vesicles Functionally Prepare Human Trophectoderm Model for Implantation: Understanding Bidirectional Maternal-Embryo Communication. Proteomics. doi:10.1002/pmic.201800423
6. Suwakulsiri W, Rai A, Xu R, Chen M, Greening DW, Simpson RJ (2019) Proteomic profiling reveals key cancer progression modulators in shed microvesicles released from isogenic human primary and metastatic colorectal cancer cell lines. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1867 (12). doi:10.1016/j.bbapap.2018.11.008
7. Rai A, Greening DW, Chen M, Xu R, Ji H, Simpson RJ (2019) Exosomes Derived from Human Primary and Metastatic Colorectal Cancer Cells Contribute to Functional Heterogeneity of Activated Fibroblasts by Reprogramming Their Proteome. PROTEOMICS 19 (8):1800148. doi:10.1002/pmic.201800148
8. Nolte-'t Hoen E, Cremer T, Gallo RC, Margolis LB (2016) Extracellular vesicles and viruses: Are they close relatives? Proceedings of the National Academy of Sciences of the United States of America 113 (33):9155-9161. doi:10.1073/pnas.1605146113
9. Yuan Z, Petree JR, Lee FE-H, Fan X, Salaita K, Guidot DM, Sadikot RT (2019) Macrophages exposed to HIV viral protein disrupt lung epithelial cell integrity and mitochondrial bioenergetics via exosomal microRNA shuttling. Cell Death Dis 10 (8):580-580. doi:10.1038/s41419-019-1803-y
10. Cheng Y, Schorey JS (2019) Extracellular vesicles deliver Mycobacterium RNA to promote host immunity and bacterial killing. EMBO Rep 20 (3):e46613. doi:10.15252/embr.201846613
11. Johnson TB, Mach C, Grove R, Kelly R, Van Cott K, Blum P (2018) Secretion and fusion of biogeochemically active archaeal membrane vesicles. Geobiology 16 (6):659-673. doi:10.1111/gbi.12306
12. Schatz D, Rosenwasser S, Malitsky S, Wolf SG, Feldmesser E, Vardi A (2017) Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga. Nat Microbiol 2 (11):1485-1492. doi:10.1038/s41564-017-0024-3
13. Lin W-C, Tsai C-Y, Huang J-M, Wu S-R, Chu LJ, Huang K-Y (2019) Quantitative proteomic analysis and functional characterization of Acanthamoeba castellanii exosome-like vesicles. Parasit Vectors 12 (1):467-467. doi:10.1186/s13071-019-3725-z

14. Atayde VD, da Silva Lira Filho A, Chaparro V, Zimmermann A, Martel C, Jaramillo M, Olivier M (2019) Exploitation of the Leishmania exosomal pathway by Leishmania RNA virus 1. Nat Microbiol 4 (4):714-723. doi:10.1038/s41564-018-0352-y
15. Zhao K, Bleackley M, Chisanga D, Gangoda L, Fonseka P, Liem M, Kalra H, Al Saffar H, Keerthikumar S, Ang C-S, Adda CG, Jiang L, Yap K, Poon IK, Lock P, Bulone V, Anderson M, Mathivanan S (2019) Extracellular vesicles secreted by Saccharomyces cerevisiae are involved in cell wall remodelling. Commun Biol 2:305. doi:10.1038/s42003-019-0538-8
16. Zarnowski R, Sanchez H, Covelli AS, Dominguez E, Jaromin A, Bernhardt J, Mitchell KF, Heiss C, Azadi P, Mitchell A, Andes DR (2018) Candida albicans biofilm–induced vesicles confer drug resistance through matrix biogenesis. PLOS Biology 16 (10):e2006872. doi:10.1371/journal.pbio.2006872
17. Teng Y, Ren Y, Sayed M, Hu X, Lei C, Kumar A, Hutchins E, Mu J, Deng Z, Luo C, Sundaram K, Sriwastva MK, Zhang L, Hsieh M, Reiman R, Haribabu B, Yan J, Jala VR, Miller DM, Van Keuren-Jensen K, Merchant ML, McClain CJ, Park JW, Egilmez NK, Zhang H-G (2018) Plant-Derived Exosomal MicroRNAs Shape the Gut Microbiota. Cell Host Microbe 24 (5):637-652.e638. doi:10.1016/j.chom.2018.10.001
18. Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ (2013) Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. Molecular & cellular proteomics : MCP 12 (3):587-598. doi:10.1074/mcp.M112.021303
19. Morello M, Minciacchi VR, de Candia P, Yang J, Posadas E, Kim H, Griffiths D, Bhowmick N, Chung LW, Gandellini P, Freeman MR, Demichelis F, Di Vizio D (2013) Large oncosomes mediate intercellular transfer of functional microRNA. Cell Cycle 12 (22):3526-3536. doi:10.4161/cc.26539
20. Mangeot PE, Dollet S, Girard M, Ciancia C, Joly S, Peschanski M, Lotteau V (2011) Protein transfer into human cells by VSV-G-induced nanovesicles. Mol Ther 19 (9):1656-1666. doi:10.1038/mt.2011.138
21. Greening DW, Simpson RJ (2018) Understanding extracellular vesicle diversity - current status. Expert Rev Proteomics 15 (11):887-910. doi:10.1080/14789450.2018.1537788
22. Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ (2018) Extracellular vesicles in cancer - implications for future improvements in cancer care. Nat Rev Clin Oncol 15 (10):617-638. doi:10.1038/s41571-018-0036-9
23. Greening DW, Xu R, Gopal SK, Rai A, Simpson RJ (2017) Proteomic insights into extracellular vesicle biology - defining exosomes and shed microvesicles. Expert Rev Proteomics 14 (1):69-95. doi:10.1080/14789450.2017.1260450
24. Harding C, Stahl P (1983) Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing. Biochem Biophys Res Commun 113 (2):650-658
25. Pan BT, Johnstone RM (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell 33 (3):967-978
26. Valencia K, Luis-Ravelo D, Bovy N, Anton I, Martinez-Canarias S, Zandueta C, Ormazabal C, Struman I, Tabruyn S, Rebmann V, De Las Rivas J, Guruceaga E, Bandres E, Lecanda F (2014) miRNA cargo within exosome-like vesicle transfer influences metastatic bone colonization. Mol Oncol 8 (3):689-703. doi:10.1016/j.molonc.2014.01.012
27. Record M, Carayon K, Poirot M, Silvente-Poirot S (2014) Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. Biochimica et biophysica acta 1841 (1):108-120. doi:10.1016/j.bbalip.2013.10.004

28. Mahaweni NM, Kaijen-Lambers ME, Dekkers J, Aerts JG, Hegmans JP (2013) Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma. Journal of extracellular vesicles 2. doi:10.3402/jev.v2i0.22492
29. El Andaloussi S, Lakhal S, Mager I, Wood MJ (2013) Exosomes for targeted siRNA delivery across biological barriers. Adv Drug Deliv Rev 65 (3):391-397. doi:10.1016/j.addr.2012.08.008
30. Johnsen KB, Gudbergsson JM, Skov MN, Pilgaard L, Moos T, Duroux M (2014) A comprehensive overview of exosomes as drug delivery vehicles - Endogenous nanocarriers for targeted cancer therapy. Biochim Biophys Acta 1846 (1):75-87. doi:10.1016/j.bbcan.2014.04.005
31. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, Liebler DC, Ping J, Liu Q, Evans R, Fissell WH, Patton JG, Rome LH, Burnette DT, Coffey RJ (2019) Reassessment of Exosome Composition. Cell 177 (2):428-445 e418. doi:10.1016/j.cell.2019.02.029
32. Larios J, Mercier V, Roux A, Gruenberg J (2020) ALIX- and ESCRT-III-dependent sorting of tetraspanins to exosomes. J Cell Biol 219 (3). doi:10.1083/jcb.201904113
33. Wu D, Yan J, Shen X, Sun Y, Thulin M, Cai Y, Wik L, Shen Q, Oelrich J, Qian X, Dubois KL, Ronquist KG, Nilsson M, Landegren U, Kamali-Moghaddam M (2019) Profiling surface proteins on individual exosomes using a proximity barcoding assay. Nat Commun 10 (1):3854. doi:10.1038/s41467-019-11486-1
34. Rai A, Greening DW, Chen M, Xu R, Ji H, Simpson RJ (2019) Exosomes Derived from Human Primary and Metastatic Colorectal Cancer Cells Contribute to Functional Heterogeneity of Activated Fibroblasts by Reprogramming Their Proteome. Proteomics 19 (8):e1800148. doi:10.1002/pmic.201800148
35. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, Yu Z, Yang J, Wang B, Sun H, Xia H, Man Q, Zhong W, Antelo LF, Wu B, Xiong X, Liu X, Guan L, Li T, Liu S, Yang R, Lu Y, Dong L, McGettigan S, Somasundaram R, Radhakrishnan R, Mills G, Lu Y, Kim J, Chen YH, Dong H, Zhao Y, Karakousis GC, Mitchell TC, Schuchter LM, Herlyn M, Wherry EJ, Xu X, Guo W (2018) Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature 560 (7718):382-386. doi:10.1038/s41586-018-0392-8
36. Rodrigues G, Hoshino A, Kenific CM, Matei IR, Steiner L, Freitas D, Kim HS, Oxley PR, Scandariato I, Casanova-Salas I, Dai J, Badwe CR, Gril B, Tesic Mark M, Dill BD, Molina H, Zhang H, Benito-Martin A, Bojmar L, Ararso Y, Offer K, LaPlant Q, Buehring W, Wang H, Jiang X, Lu TM, Liu Y, Sabari JK, Shin SJ, Narula N, Ginter PS, Rajasekhar VK, Healey JH, Meylan E, Costa-Silva B, Wang SE, Rafii S, Altorki NK, Rudin CM, Jones DR, Steeg PS, Peinado H, Ghajar CM, Bromberg J, de Sousa M, Pisapia D, Lyden D (2019) Tumour exosomal CEMIP protein promotes cancer cell colonization in brain metastasis. Nat Cell Biol 21 (11):1403-1412. doi:10.1038/s41556-019-0404-4
37. Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, Mark MT, Molina H, Martin AB, Bojmar L, Fang J, Rampersaud S, Hoshino A, Matei I, Kenific CM, Nakajima M, Mutvei AP, Sansone P, Buehring W, Wang H, Jimenez JP, Cohen-Gould L, Paknejad N, Brendel M, Manova-Todorova K, Magalhaes A, Ferreira JA, Osorio H, Silva AM, Massey A, Cubillos-Ruiz JR, Galletti G, Giannakakou P, Cuervo AM, Blenis J, Schwartz R, Brady MS, Peinado H, Bromberg J, Matsui H, Reis CA, Lyden D (2018) Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat Cell Biol 20 (3):332-343. doi:10.1038/s41556-018-0040-4

38. Sansone P, Savini C, Kurelac I, Chang Q, Amato LB, Strillacci A, Stepanova A, Iommarini L, Mastroleo C, Daly L, Galkin A, Thakur BK, Soplop N, Uryu K, Hoshino A, Norton L, Bonafe M, Cricca M, Gasparre G, Lyden D, Bromberg J (2017) Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc Natl Acad Sci U S A 114 (43):E9066-E9075. doi:10.1073/pnas.1704862114
39. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D (2015) Tumour exosome integrins determine organotropic metastasis. Nature 527 (7578):329-335. doi:10.1038/nature15756
40. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, Becker A, Hoshino A, Mark MT, Molina H, Xiang J, Zhang T, Theilen TM, Garcia-Santos G, Williams C, Ararso Y, Huang Y, Rodrigues G, Shen TL, Labori KJ, Lothe IM, Kure EH, Hernandez J, Doussot A, Ebbesen SH, Grandgenett PM, Hollingsworth MA, Jain M, Mallya K, Batra SK, Jarnagin WR, Schwartz RE, Matei I, Peinado H, Stanger BZ, Bromberg J, Lyden D (2015) Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol 17 (6):816-826. doi:10.1038/ncb3169
41. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, Garcia-Santos G, Ghajar C, Nitadori-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 18 (6):883-891. doi:10.1038/nm.2753
42. Xu R, Greening DW, Zhu HJ, Takahashi N, Simpson RJ (2016) Extracellular vesicle isolation and characterization: toward clinical application. J Clin Invest 126 (4):1152-1162. doi:10.1172/JCI81129
43. Tkach M, Kowal J, Théry C (2018) Why the need and how to approach the functional diversity of extracellular vesicles. Philos Trans R Soc Lond B Biol Sci 373 (1737):20160479. doi:10.1098/rstb.2016.0479
44. Xu R, Greening DW, Rai A, Ji H, Simpson RJ (2015) Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct. Methods 87:11-25. doi:10.1016/j.ymeth.2015.04.008
45. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ (2015) A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods in Molecular Biology 1295:179-209
46. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13 (9):731-740. doi:10.1038/nmeth.3901
47. Schroder M, Schafer R, Friedl P (1997) Spectrophotometric determination of iodixanol in subcellular fractions of mammalian cells. Anal Biochem 244 (1):174-176. doi:10.1006/abio.1996.9861

48. Linares R, Tan S, Gounou C, Arraud N, Brisson AR (2015) High-speed centrifugation induces aggregation of extracellular vesicles. J Extracell Vesicles 4:29509. doi:10.3402/jev.v4.29509
49. Tauro BJ, Greening DW, Mathias RA, Ji H, Mathivanan S, Scott AM, Simpson RJ (2012) Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. Methods 56 (2):293-304. doi:10.1016/j.ymeth.2012.01.002
50. Rappsilber J, Mann M, Ishihama Y (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc 2 (8):1896-1906. doi:10.1038/nprot.2007.261
51. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26 (12):1367-1372. doi:10.1038/nbt.1511
52. Ramus C, Hovasse A, Marcellin M, Hesse AM, Mouton-Barbosa E, Bouyssie D, Vaca S, Carapito C, Chaoui K, Bruley C, Garin J, Cianferani S, Ferro M, Van Dorssaeler A, Burlet-Schiltz O, Schaeffer C, Coute Y, Gonzalez de Peredo A (2016) Benchmarking quantitative label-free LC-MS data processing workflows using a complex spiked proteomic standard dataset. J Proteomics 132:51-62. doi:10.1016/j.jprot.2015.11.011
53. Hakkinen J, Vincic G, Mansson O, Warell K, Levander F (2009) The proteios software environment: an extensible multiuser platform for management and analysis of proteomics data. J Proteome Res 8 (6):3037-3043. doi:10.1021/pr900189c
54. Ma B, Zhang K, Hendrie C, Liang C, Li M, Doherty-Kirby A, Lajoie G (2003) PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. Rapid Commun Mass Spectrom 17 (20):2337-2342. doi:10.1002/rcm.1196
55. Sturm M, Bertsch A, Gropl C, Hildebrandt A, Hussong R, Lange E, Pfeifer N, Schulz-Trieglaff O, Zerck A, Reinert K, Kohlbacher O (2008) OpenMS - an open-source software framework for mass spectrometry. BMC Bioinformatics 9:163. doi:10.1186/1471-2105-9-163
56. Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, Vilo J (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res 47 (W1):W191-W198. doi:10.1093/nar/gkz369
57. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13 (11):2498-2504. doi:10.1101/gr.1239303
58. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV (2019) STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47 (D1):D607-D613. doi:10.1093/nar/gky1131
59. Kornilov R, Puhka M, Mannerstrom B, Hiidenmaa H, Peltoniemi H, Siljander P, Seppanen-Kaijansinkko R, Kaur S (2018) Efficient ultrafiltration-based protocol to deplete extracellular vesicles from fetal bovine serum. J Extracell Vesicles 7 (1):1422674. doi:10.1080/20013078.2017.1422674
60. Shelke GV, Lasser C, Gho YS, Lotvall J (2014) Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. J Extracell Vesicles 3. doi:10.3402/jev.v3.24783
61. Thery C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol Chapter 3:Unit 3 22. doi:10.1002/0471143030.cb0322s30

62. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L (2004) Cell Viability Assays. In: Sittampalam GS, Grossman A, Brimacombe K et al. (eds) Assay Guidance Manual. Bethesda (MD),
63. Mitchell JP, Court J, Mason MD, Tabi Z, Clayton A (2008) Increased exosome production from tumour cell cultures using the Integra CELLine Culture System. Journal of immunological methods 335 (1-2):98-105. doi:10.1016/j.jim.2008.03.001
64. Guerreiro EM, Vestad B, Steffensen LA, Aass HCD, Saeed M, Ovstebo R, Costea DE, Galtung HK, Soland TM (2018) Efficient extracellular vesicle isolation by combining cell media modifications, ultrafiltration, and size-exclusion chromatography. PloS one 13 (9):e0204276. doi:10.1371/journal.pone.0204276
65. Palviainen M, Saari H, Karkkainen O, Pekkinen J, Auriola S, Yliperttula M, Puhka M, Hanhineva K, Siljander PR (2019) Metabolic signature of extracellular vesicles depends on the cell culture conditions. J Extracell Vesicles 8 (1):1596669. doi:10.1080/20013078.2019.1596669
66. Steinberg TH, Lauber WM, Berggren K, Kemper C, Yue S, Patton WF (2000) Fluorescence detection of proteins in sodium dodecyl sulfate-polyacrylamide gels using environmentally benign, nonfixative, saline solution. Electrophoresis 21 (3):497-508. doi:10.1002/(SICI)1522-2683(20000201)21:3<497::AID-ELPS497>3.0.CO;2-I
67. White IR, Pickford R, Wood J, Skehel JM, Gangadharan B, Cutler P (2004) A statistical comparison of silver and SYPRO Ruby staining for proteomic analysis. Electrophoresis 25 (17):3048-3054. doi:10.1002/elps.200405947
68. Tauro BJ, Mathias RA, Greening DW, Gopal SK, Ji H, Kapp EA, Coleman BM, Hill AF, Kusebauch U, Hallows JL, Shteynberg D, Moritz RL, Zhu HJ, Simpson RJ (2013) Oncogenic H-ras reprograms Madin-Darby canine kidney (MDCK) cell-derived exosomal proteins following epithelial-mesenchymal transition. Mol Cell Proteomics 12 (8):2148-2159. doi:10.1074/mcp.M112.027086
69. Cocucci E, Racchetti G, Meldolesi J (2009) Shedding microvesicles: artefacts no more. Trends Cell Biol 19 (2):43-51. doi:10.1016/j.tcb.2008.11.003
70. Xu R, Simpson RJ, Greening DW (2017) A Protocol for Isolation and Proteomic Characterization of Distinct Extracellular Vesicle Subtypes by Sequential Centrifugal Ultrafiltration. Methods Mol Biol 1545:91-116. doi:10.1007/978-1-4939-6728-5_7
71. Ji H, Greening DW, Barnes TW, Lim JW, Tauro BJ, Rai A, Xu R, Adda C, Mathivanan S, Zhao W, Xue Y, Xu T, Zhu HJ, Simpson RJ (2013) Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. Proteomics 13 (10-11):1672-1686. doi:10.1002/pmic.201200562
72. Cantin R, Diou J, Belanger D, Tremblay AM, Gilbert C (2008) Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants. J Immunol Methods 338 (1-2):21-30. doi:10.1016/j.jim.2008.07.007
73. Chen M, Xu R, Ji H, Greening DW, Rai A, Izumikawa K, Ishikawa H, Takahashi N, Simpson RJ (2016) Transcriptome and long noncoding RNA sequencing of three extracellular vesicle subtypes released from the human colon cancer LIM1863 cell line. Sci Rep 6:38397. doi:10.1038/srep38397
74. Sokolova V, Ludwig AK, Hornung S, Rotan O, Horn PA, Epple M, Giebel B (2011) Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids Surf B Biointerfaces 87 (1):146-150. doi:10.1016/j.colsurfb.2011.05.013
75. Mathivanan S, Lim JW, Tauro BJ, Ji H, Moritz RL, Simpson RJ (2010) Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon

tumor cell line LIM1215 reveals a tissue-specific protein signature. Mol Cell Proteomics 9 (2):197-208. doi:10.1074/mcp.M900152-MCP200
76. Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z (2008) Human tumor-derived exosomes down-modulate NKG2D expression. J Immunol 180 (11):7249-7258
77. Koga K, Matsumoto K, Akiyoshi T, Kubo M, Yamanaka N, Tasaki A, Nakashima H, Nakamura M, Kuroki S, Tanaka M, Katano M (2005) Purification, characterization and biological significance of tumor-derived exosomes. Anticancer Res 25 (6A):3703-3707
78. Coren LV, Shatzer T, Ott DE (2008) CD45 immunoaffinity depletion of vesicles from Jurkat T cells demonstrates that exosomes contain CD45: no evidence for a distinct exosome/HIV-1 budding pathway. Retrovirology 5:64. doi:10.1186/1742-4690-5-64
79. Grigorieff N, Harrison SC (2011) Near-atomic resolution reconstructions of icosahedral viruses from electron cryo-microscopy. Curr Opin Struct Biol 21 (2):265-273. doi:10.1016/j.sbi.2011.01.008
80. Koning RI, Koster AJ (2009) Cryo-electron tomography in biology and medicine. Ann Anat 191 (5):427-445. doi:10.1016/j.aanat.2009.04.003
81. Tatischeff I, Larquet E, Falcon-Perez JM, Turpin PY, Kruglik SG (2012) Fast characterisation of cell-derived extracellular vesicles by nanoparticles tracking analysis, cryo-electron microscopy, and Raman tweezers microspectroscopy. Journal of extracellular vesicles 1. doi:10.3402/jev.v1i0.19179
82. Greening DW, Nguyen HP, Elgass K, Simpson RJ, Salamonsen LA (2016) Human Endometrial Exosomes Contain Hormone-Specific Cargo Modulating Trophoblast Adhesive Capacity: Insights into Endometrial-Embryo Interactions. Biol Reprod 94 (2):38. doi:10.1095/biolreprod.115.134890
83. Suttapitugsakul S, Xiao H, Smeekens J, Wu R (2017) Evaluation and optimization of reduction and alkylation methods to maximize peptide identification with MS-based proteomics. Mol Biosyst 13 (12):2574-2582. doi:10.1039/c7mb00393e
84. Tsiatsiani L, Heck AJ (2015) Proteomics beyond trypsin. Febs j 282 (14):2612-2626. doi:10.1111/febs.13287
85. Wu Z, Huang J, Huang J, Li Q, Zhang X (2018) Lys-C/Arg-C, a More Specific and Efficient Digestion Approach for Proteomics Studies. Anal Chem 90 (16):9700-9707. doi:10.1021/acs.analchem.8b02448
86. Tyanova S, Cox J (2018) Perseus: A Bioinformatics Platform for Integrative Analysis of Proteomics Data in Cancer Research. Methods Mol Biol 1711:133-148. doi:10.1007/978-1-4939-7493-1_7
87. Xu R, Greening DW, Chen M, Rai A, Ji H, Takahashi N, Simpson RJ (2019) Surfaceome of Exosomes Secreted from the Colorectal Cancer Cell Line SW480: Peripheral and Integral Membrane Proteins Analyzed by Proteolysis and TX114. PROTEOMICS 19 (8):1700453. doi:10.1002/pmic.201700453
88. Wallace PK, Tario JD, Jr., Fisher JL, Wallace SS, Ernstoff MS, Muirhead KA (2008) Tracking antigen-driven responses by flow cytometry: monitoring proliferation by dye dilution. Cytometry A 73 (11):1019-1034. doi:10.1002/cyto.a.20619
89. Skardelly M, Gaber K, Burdack S, Scheidt F, Hilbig H, Boltze J, Forschler A, Schwarz S, Schwarz J, Meixensberger J, Schuhmann MU (2011) Long-term benefit of human fetal neuronal progenitor cell transplantation in a clinically adapted model after traumatic brain injury. J Neurotrauma 28 (3):401-414. doi:10.1089/neu.2010.1526
90. Nowacki M, Nazarewski L, Pokrywczynska M, Kloskowski T, Tyloch D, Pietkun K, Jundzill A, Rasmus M, Warda K, Gagat M, Grzanka A, Bodnar M, Marszalek A, Krawczyk M, Habib SL, Drewa T (2015) Long-term influence of bone marrow-derived mesenchymal stem cells on liver ischemia-reperfusion injury in a rat model. Ann Transplant 20:132-140. doi:10.12659/aot.892364

91. Feng D, Zhao WL, Ye YY, Bai XC, Liu RQ, Chang LF, Zhou Q, Sui SF (2010) Cellular internalization of exosomes occurs through phagocytosis. Traffic 11 (5):675-687. doi:10.1111/j.1600-0854.2010.01041.x
92. Ofir-Birin Y, Abou karam P, Rudik A, Giladi T, Porat Z, Regev-Rudzki N (2018) Monitoring Extracellular Vesicle Cargo Active Uptake by Imaging Flow Cytometry. Frontiers in Immunology 9 (1011). doi:10.3389/fimmu.2018.01011
93. Bryzgunova OE, Zaripov MM, Skvortsova TE, Lekchnov EA, Grigor'eva AE, Zaporozhchenko IA, Morozkin ES, Ryabchikova EI, Yurchenko YB, Voitsitskiy VE, Laktionov PP (2016) Comparative Study of Extracellular Vesicles from the Urine of Healthy Individuals and Prostate Cancer Patients. PloS one 11 (6):e0157566. doi:10.1371/journal.pone.0157566
94. Campoy I, Lanau L, Altadill T, Sequeiros T, Cabrera S, Cubo-Abert M, Pérez-Benavente A, Garcia A, Borrós S, Santamaria A, Ponce J, Matias-Guiu X, Reventós J, Gil-Moreno A, Rigau M, Colas E (2016) Exosome-like vesicles in uterine aspirates: a comparison of ultracentrifugation-based isolation protocols. Journal of Translational Medicine 14 (1):180. doi:10.1186/s12967-016-0935-4
95. Pospichalova V, Svoboda J, Dave Z, Kotrbova A, Kaiser K, Klemova D, Ilkovics L, Hampl A, Crha I, Jandakova E, Minar L, Weinberger V, Bryja V (2015) Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. J Extracell Vesicles 4:25530. doi:10.3402/jev.v4.25530
96. Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G, D'Souza-Schorey C (2009) ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Current biology : CB 19 (22):1875-1885. doi:10.1016/j.cub.2009.09.059
97. Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, Kuo WP (2012) Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. Front Physiol 3:162. doi:10.3389/fphys.2012.00162
98. Abramowicz A, Widlaka P, Pietrowska M (2016) Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation Molecular BioSystems 12 (5):1407-1419. doi:10.1039/C6MB00082G
99. Livshits MA, Khomyakova E, Evtushenko EG, Lazarev VN, Kulemin NA, Semina SE, Generozov EV, Govorun VM (2015) Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. Scientific Reports 5. doi:10.1038/srep17319
100. Webber J, Clayton A (2013) How pure are your vesicles? Journal of extracellular vesicles 2:10.3402/jev.v3402i3400.19861. doi:10.3402/jev.v2i0.19861
101. Ford T, Graham J, Rickwood D (1994) Iodixanol: a nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. Anal Biochem 220 (2):360-366. doi:10.1006/abio.1994.1350
102. Dettenhofer M, Yu XF (1999) Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. J Virol 73 (2):1460-1467
103. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, Thery C (2016) Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci U S A 113 (8):E968-977. doi:10.1073/pnas.1521230113
104. Willms E, Johansson HJ, Mager I, Lee Y, Blomberg KE, Sadik M, Alaarg A, Smith CI, Lehtio J, El Andaloussi S, Wood MJ, Vader P (2016) Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep 6:22519. doi:10.1038/srep22519

105. Dai S, Wei D, Wu Z, Zhou X, Wei X, Huang H, Li G (2008) Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. Mol Ther 16 (4):782-790. doi:10.1038/mt.2008.1
106. Besse B, Charrier M, Lapierre V, Dansin E, Lantz O, Planchard D, Le Chevalier T, Livartoski A, Barlesi F, Laplanche A, Ploix S, Vimond N, Peguillet I, Thery C, Lacroix L, Zoernig I, Dhodapkar K, Dhodapkar M, Viaud S, Soria JC, Reiners KS, Pogge von Strandmann E, Vely F, Rusakiewicz S, Eggermont A, Pitt JM, Zitvogel L, Chaput N (2016) Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. Oncoimmunology 5 (4):e1071008. doi:10.1080/2162402X.2015.1071008
107. Poliakov A, Spilman M, Dokland T, Amling CL, Mobley JA (2009) Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. Prostate 69 (2):159-167. doi:10.1002/pros.20860
108. Yuana Y, Levels J, Grootemaat A, Sturk A, Nieuwland R (2014) Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. J Extracell Vesicles 3. doi:10.3402/jev.v3.23262
109. Sharma P, Ludwig S, Muller L, Hong CS, Kirkwood JM, Ferrone S, Whiteside TL (2018) Immunoaffinity-based isolation of melanoma cell-derived exosomes from plasma of patients with melanoma. J Extracell Vesicles 7 (1):1435138. doi:10.1080/20013078.2018.1435138
110. Ghosh A, Davey M, Chute IC, Griffiths SG, Lewis S, Chacko S, Barnett D, Crapoulet N, Fournier S, Joy A, Caissie MC, Ferguson AD, Daigle M, Meli MV, Lewis SM, Ouellette RJ (2014) Rapid isolation of extracellular vesicles from cell culture and biological fluids using a synthetic peptide with specific affinity for heat shock proteins. PloS one 9 (10):e110443. doi:10.1371/journal.pone.0110443
111. Atai NA, Balaj L, van Veen H, Breakefield XO, Jarzyna PA, Van Noorden CJ, Skog J, Maguire CA (2013) Heparin blocks transfer of extracellular vesicles between donor and recipient cells. Journal of neuro-oncology 115 (3):343-351. doi:10.1007/s11060-013-1235-y
112. Christianson HC, Svensson KJ, van Kuppevelt TH, Li JP, Belting M (2013) Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. Proc Natl Acad Sci U S A 110 (43):17380-17385. doi:10.1073/pnas.1304266110
113. Balaj L, Atai NA, Chen W, Mu D, Tannous BA, Breakefield XO, Skog J, Maguire CA (2015) Heparin affinity purification of extracellular vesicles. Sci Rep 5:10266. doi:10.1038/srep10266
114. Yoo CE, Kim G, Kim M, Park D, Kang HJ, Lee M, Huh N (2012) A direct extraction method for microRNAs from exosomes captured by immunoaffinity beads. Anal Biochem 431 (2):96-98. doi:10.1016/j.ab.2012.09.008
115. Clayton A, Court J, Navabi H, Adams M, Mason MD, Hobot JA, Newman GR, Jasani B (2001) Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. J Immunol Methods 247 (1-2):163-174
116. Keryer-Bibens C, Pioche-Durieu C, Villemant C, Souquere S, Nishi N, Hirashima M, Middeldorp J, Busson P (2006) Exosomes released by EBV-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9. BMC cancer 6:283. doi:10.1186/1471-2407-6-283
117. Mercier SK, Donaghy H, Botting RA, Turville SG, Harman AN, Nasr N, Ji H, Kusebauch U, Mendoza L, Shteynberg D, Sandgren K, Simpson RJ, Moritz RL, Cunningham AL (2013) The microvesicle component of HIV-1 inocula modulates

dendritic cell infection and maturation and enhances adhesion to and activation of T lymphocytes. PLoS pathogens 9 (10):e1003700. doi:10.1371/journal.ppat.1003700
118. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C (2005) Exosomal-like vesicles are present in human blood plasma. International immunology 17 (7):879-887. doi:10.1093/intimm/dxh267
119. Oksvold MP, Kullmann A, Forfang L, Kierulf B, Li M, Brech A, Vlassov AV, Smeland EB, Neurauter A, Pedersen KW (2014) Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. Clinical therapeutics 36 (6):847-862 e841. doi:10.1016/j.clinthera.2014.05.010
120. Wiley RD, Gummuluru S (2006) Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection. Proc Natl Acad Sci U S A 103 (3):738-743. doi:10.1073/pnas.0507995103
121. Ueda K, Ishikawa N, Tatsuguchi A, Saichi N, Fujii R, Nakagawa H (2014) Antibody-coupled monolithic silica microtips for highthroughput molecular profiling of circulating exosomes. Sci Rep 4:6232. doi:10.1038/srep06232
122. Goetzl EJ, Boxer A, Schwartz JB, Abner EL, Petersen RC, Miller BL, Kapogiannis D (2015) Altered lysosomal proteins in neural-derived plasma exosomes in preclinical Alzheimer disease. Neurology 85 (1):40-47. doi:10.1212/wnl.0000000000001702
123. Muller L, Hong CS, Stolz DB, Watkins SC, Whiteside TL (2014) Isolation of biologically-active exosomes from human plasma. J Immunol Methods 411:55-65. doi:10.1016/j.jim.2014.06.007
124. Welton JL, Webber JP, Botos LA, Jones M, Clayton A (2015) Ready-made chromatography columns for extracellular vesicle isolation from plasma. J Extracell Vesicles 4:27269. doi:10.3402/jev.v4.27269
125. Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R (2014) Single-step isolation of extracellular vesicles by size-exclusion chromatography. J Extracell Vesicles 3. doi:10.3402/jev.v3.23430
126. Jackson CE, Scruggs BS, Schaffer JE, Hanson PI (2017) Effects of Inhibiting VPS4 Support a General Role for ESCRTs in Extracellular Vesicle Biogenesis. Biophys J 113 (6):1342-1352. doi:10.1016/j.bpj.2017.05.032
127. Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PS, Star RA (2007) Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. American journal of physiology Renal physiology 292 (5):F1657-1661. doi:10.1152/ajprenal.00434.2006
128. Merchant ML, Powell DW, Wilkey DW, Cummins TD, Deegens JK, Rood IM, McAfee KJ, Fleischer C, Klein E, Klein JB (2010) Microfiltration isolation of human urinary exosomes for characterization by MS. Proteomics Clin Appl 4 (1):84-96. doi:10.1002/prca.200800093
129. Grant R, Ansa-Addo E, Stratton D, Antwi-Baffour S, Jorfi S, Kholia S, Krige L, Lange S, Inal J (2011) A filtration-based protocol to isolate human plasma membrane-derived vesicles and exosomes from blood plasma. J Immunol Methods 371 (1-2):143-151. doi:10.1016/j.jim.2011.06.024
130. He L, Zhu D, Wang J, Wu X (2019) A highly efficient method for isolating urinary exosomes. Int J Mol Med 43 (1):83-90. doi:10.3892/ijmm.2018.3944