A fast, effective, alternative method for exosome isolation from cell culture media

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Abstract

Exosomes are membrane-derived nanovesicles of 30 to 150 nm in diameter that are released by many cell types under both normal and pathological conditions. The influence of exosomes on cellular function has been linked to a wide range of physiological processes including: cell to cell communication, cancer metastasis, immunomodulatory activity, and propagation of infectious agents such as prions and retroviruses. Given this wide range of potential interactions, and considering the fact that these nanovesicles contain RNA, microRNA and proteins from their cells of origin, exosomes represent a burgeoning target for biomarker discovery.

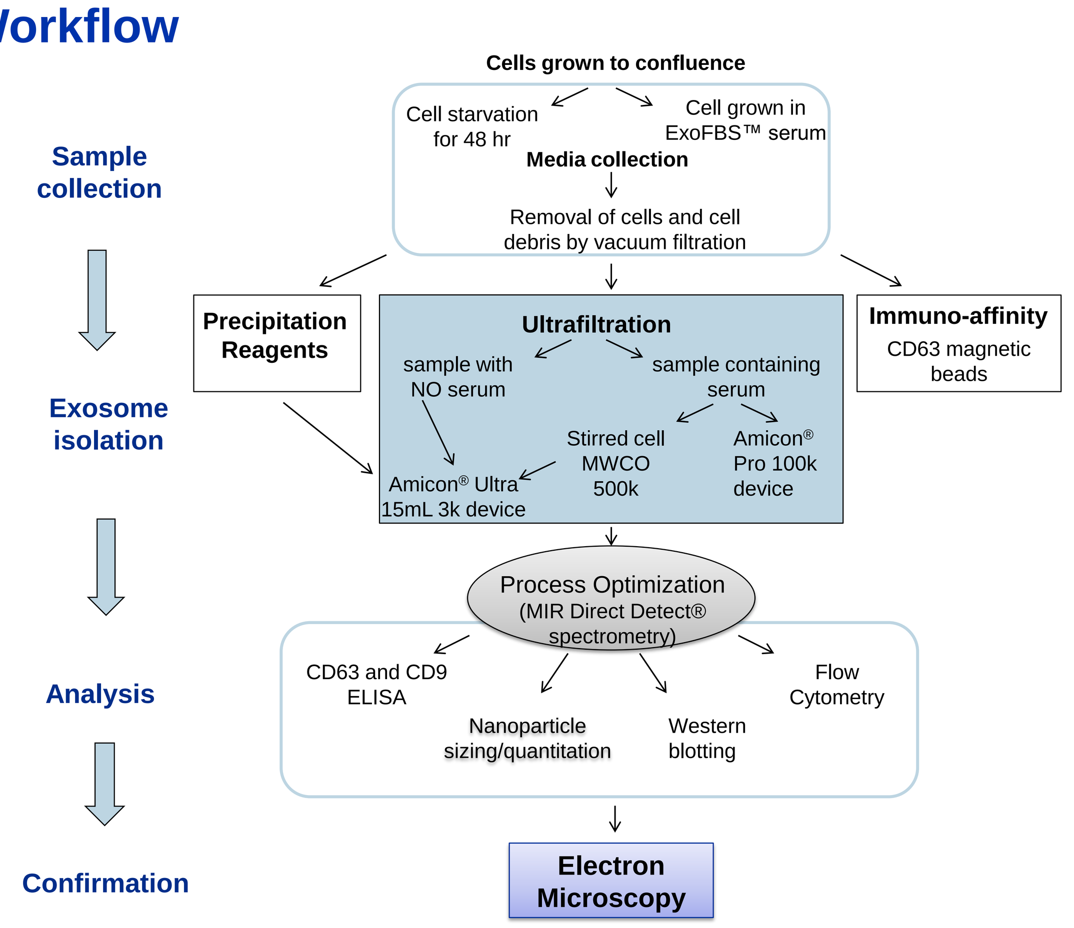
The current gold standard for exosome isolation is differential ultracentrifugation. However, this method requires specific instrumentation, is lengthy, and labor intensive. Here we present a rapid alternative method for the selective fractionation of exosomes from cell culture media using an ultrafiltration device. Optimization of the protocol was aided through use of a mid infrared (MIR)-based spectroscopy platform that permits simultaneous monitoring of lysis conditions, protein quantitation, and analysis of total lipid content during exosome fractionation. The resulting exosome preps were analyzed using a NanoSight platform (to measure size distribution) and by flow cytometry of exosome-bead conjugates (for surface marker expression).

Introduction

Exosomes and multi-vesicles are released by a wide range of cell types including neurons[1]. The process of exosome release involves: inward budding of endosomes to form multi-vesicular bodies (MVBs), fusion of MVB with the plasma membrane, and release of the sequestered exosomes into the extracellular space[2]. Exosomes contain membrane–derived and cellular proteins, as well as mRNA and microRNA species[4]. Exosomes and multi-vesicles have been associated with the transmission of prions responsible for neurodegenerative diseases such as Creutzfeldt-Jakob disease (CJD) or bovine spongiform encephalopathy (BSE)[5]. In the case of Alzheimer's disease, release of Aβ peptide into the bloodstream, packaged within exosomes, can be traced backed to cleavage of Amyloid Precursor protein (APP) in endosomal vesicles[6].

Exosomes have been isolated from tissue culture media as well as from several biological fluids, including urine, plasma, saliva and breast milk[4,7]. Their presence in routine clinical samples makes exosomes highly desirable sources of potential biomarkers for prognosis and diagnostic of various diseases. While numerous methods of exosome purification exist, including ultracentrifugation, immunoaffinity-based isolation by magnetic beads[7], precipitation by commercial solutions, and ultrafiltration[2,8], all are plagued by sample limitations or require long and tedious workflows to achieve success. Here, we demonstrate that centrifugal diafiltration provides an effective method for the purification of exosomes present in the cell culture supernatant of a neuroblastoma line. Subsequent analyses confirmed size distribution consistent with exosome fractions, presence of exosome-specific CD63 surface expression, and identification of neuronal-specific proteins, including APP and Tau-1, by Western blotting .

Workflow



Materials

Cell culture media and preparation of cell lysates
- SH-SY-5Y (ATCC® CRL-2266™ Neuroblastoma cells) , MDA-MB-231 (ATCC® HTB-26™ breast cancer cells)
- Exo-FBS™ Exosome-depleted FBS (System Biosciences EXO-FBS-250A-1).
- Scepter™ 2.0 Handheld Automated Cell Counter with 60 µm sensors (Merck Millipore PHCC20060).
- CytoBuster™ Protein Extraction Reagent (Merck Millipore 71009-50mL)
- Inhibitor cocktail (Halt™ Proteases & Phosphatase inhibitor cocktail, Thermo Scientific 78440)

Exosome Isolation
- 0.2 µm Steriflip®-GP filter units (Merck Millipore SCGP00525).
- Amicon® Ultra 4 and 15, 3 kDa (Merck Millipore UFC800324, UFC900324)
- Amicon® Pro 100 k Da devices (Merck Millipore ACS500324)
- Amicon® Stirred Cell Model 8050 (Merck Millipore 5122) assembled with Biomax® 500 kDa membrane (Merck Millipore PBVK04710)

Exosome precipitation
- Total Exosome Isolation from cell culture media, (Life Technologies™),
- ExoQuick-TC™ Exosome precipitation Solution (System Biosciences EXOTC10A-1)
- ExoSpin™ Exosome Purification kit (Cell Guidance System EX01-4)

Total protein concentration and lipid content: Direct Detect® spectrometer (Merck Millipore DDHW000-10-WW)

Bead-based CD63 detection by flow cytometry
- Dynabeads® Human CD63 (Life Technologies 10606D) PE anti-human CD63 (BioLegend 353003)
- guava easyCyte™ 8HT Flow cytometer (Merck Millipore 0500-4008); analyzed using Guava® ExpressPro 8.1.1 software

Electrophoresis & Western Blotting
- Immobilon®-P membrane (Merck Millipore IPVH08130),
- SNAP i.d.® 2.0 Protein detection system (Merck Millipore SNAP2BASE)
- Antibodies: CD63 (CBL553), CD9 (CBL162), CD81 (CBL 579), AIP1/Alix (ABC40), LAMP3 (MABC44), CDCP1 (ABT180) Amyloid Alzheimer's precursor (MAB348), Tau-1 (MAB3420) all Merck Millipore, HsP70 (Sigma H5147)
- Luminata™ Forte Western HRP Substrate (Merck Millipore WBLVF0500)

Enzyme-Linked ImmunoSorbent Assay (ELISA)
- ExoELISA™ kit for CD63 and CD9 (System Biosciences EXOEL-CD63A-1, EXOEL-CD9A-1)

Nanoparticle Tracking Analysis (NTA): NanoSight NS300 (Malvern Instrument Company)

Electron Microscopy: Whole-mounted exosome TEM with negative staining techniques (9). Core Electron Microscopy Facility, University of Massachusetts Medical School, Worcester, MA.

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Exosome Purification and Detection

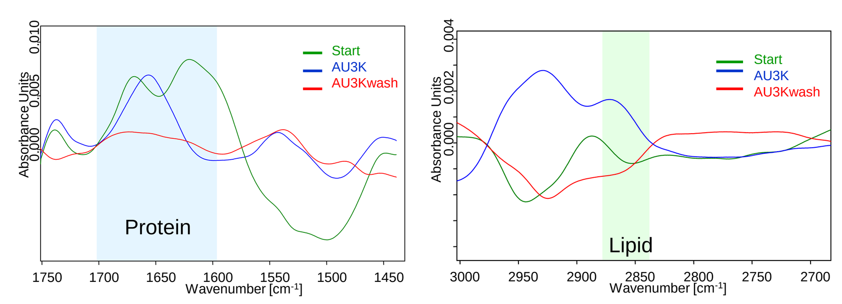
A multidimensional analysis of four SH-SY-5Y cell line-derived exosome samples (with and without serum)

Sample	Serum	Cell density	Volume processed (starting / final)	Protein concentration (mg/mL)	Lipids (relative abundance)	Concentration of exosomes (CD63 ELISA)	Concentration of exosomes (CD9 ELISA)	Exosome diameter (nm) (EM)
A	Yes	NA	15mL/ 0.5-1mL	3.250	0.027	1.97E+08	NA	33.87 (n=20)
B	Yes	2.599E+06	15mlL/0.5-1mL	3.238	0.607	3.9E+08	NA	25.72 (n=13)
C	No	8.64E+04	24mL/1.4 mL	0.376	0.002	1.04E+09	4.72E+08	38.40 (n=12)
D	No	2.5E+05	24mL/1.4mL	0.758	0.002	4.54E+08	1.44E+08	104.37 (n=18)

Comparison of the protein and lipid profile of cell culture media containing exosomes detected by the Direct Detect® spectrometer

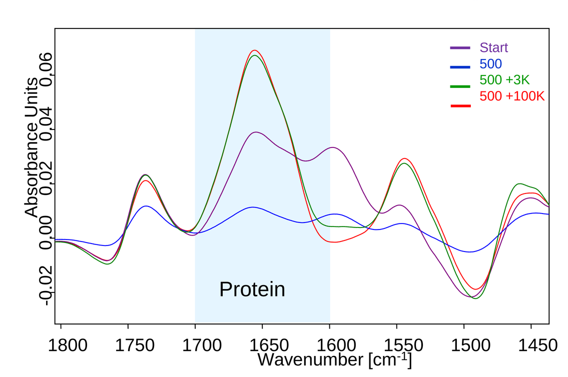
Effectiveness of exosome concentration was monitored using Direct Detect® spectrometer. Full mid-infrared (MIR) spectra were collected for all the samples. The spectra were analyzed for total protein and lipid content in samples with and without serum.

Protein and lipid profile of SH-SY-5Y media collected after 48 hr cell starvation.



Comparison between the starting material, the material concentrated with Amicon® Ultra 3 kDa device and the PBS device wash.

Protein profile of SH-SY-5Y media of cells grown in the presence of Exo-FBS™ serum



Comparison of the protein profile of a sample containing serum from the collection point (start) through the process in stirred cell with 500kDa membrane and further concentration using 3kDa or 100kDa membrane.

High protein concentration and higher relative absorbance units for lipids when the 3 kDa device was used indicated that this device was a better choice for exosome sample preparation.

Device/MWCO	Protein (mg/mL)	Lipids (Absorbance Units)
Start	3.25	0.027
Stirred cell 500 kDa	0.963	0.009
500 kDa + 3 kDa	5.39	0.047
500 kDa+100 kDa	5.577	0.038

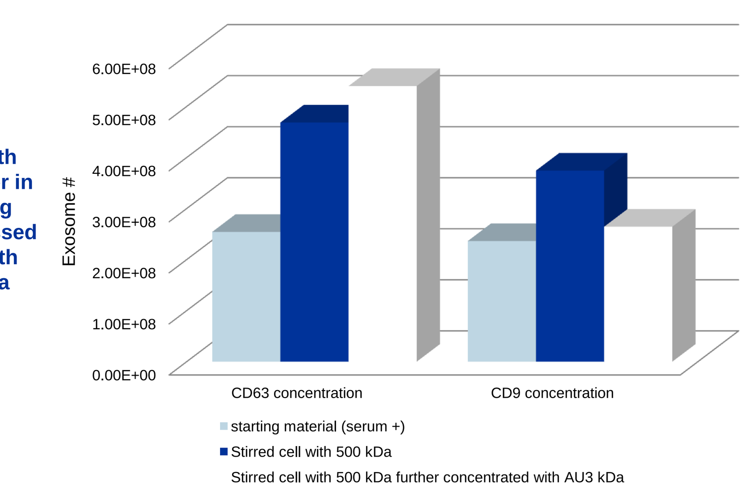
Enzyme-linked Immunosorbent Assay (ELISA) for the detection and quantification of CD9 and CD64 in various exosome sample preps.

Sample	Concentration of exosomes with CD9 marker (ELISA*)
Starting material (exosome collected media with serum)	2.63 E+08
ExoQuick-TC™ Exosome precipitation solution	6.12 E+08
Total Exosome Isolation reagent	3.17 E+08
ExoSpin™ Exosome Purification reagent	2.17 E+09
Amicon® Pro with 100 kDa Amicon® Ultra	1.48 E+09
Amicon® Pro with 100 kDa Amicon® Ultra AND precipitation using Total Exosome Isolation from cell culture media reagent	3.85 E+09

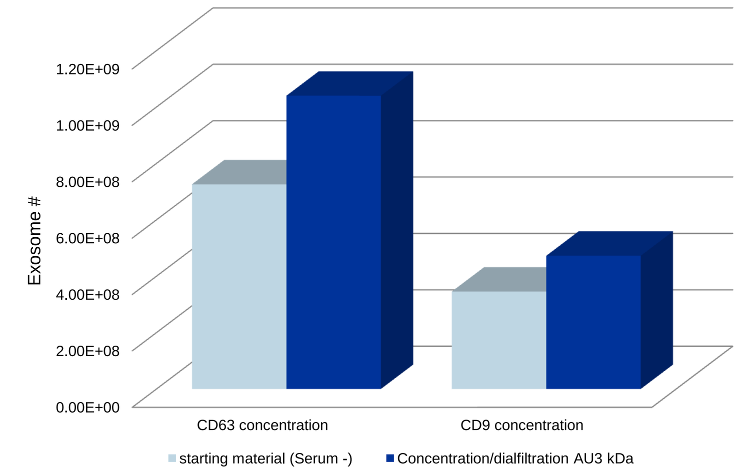
*Exosome ELISA kit from SBI uses standards calibrated by NanoSight and the units are referred as number of exosomes

Comparison of 5 different methods for the purification of exosome, demonstrated a higher concentration of exosomes with CD9 marker in samples that were concentrated first with Amicon® Ultra device followed by precipitation.

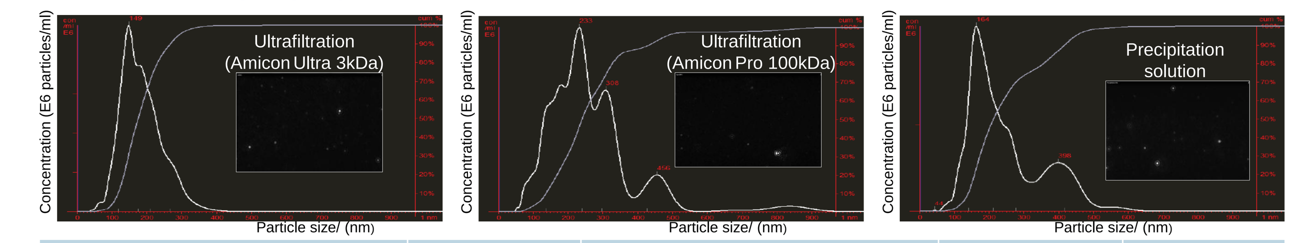
Quantitation of exosomes with CD63 and CD9 protein marker in harvested samples containing serum. Samples were processed in an Amicon® stirred cell with further concentration in 3 kDa Amicon® Ultra devices



Quantitation of exosomes with CD63 and CD9 protein markers in samples without serum. Samples were concentrated and dialfiltered with3 kDa Amicon® Ultra devices



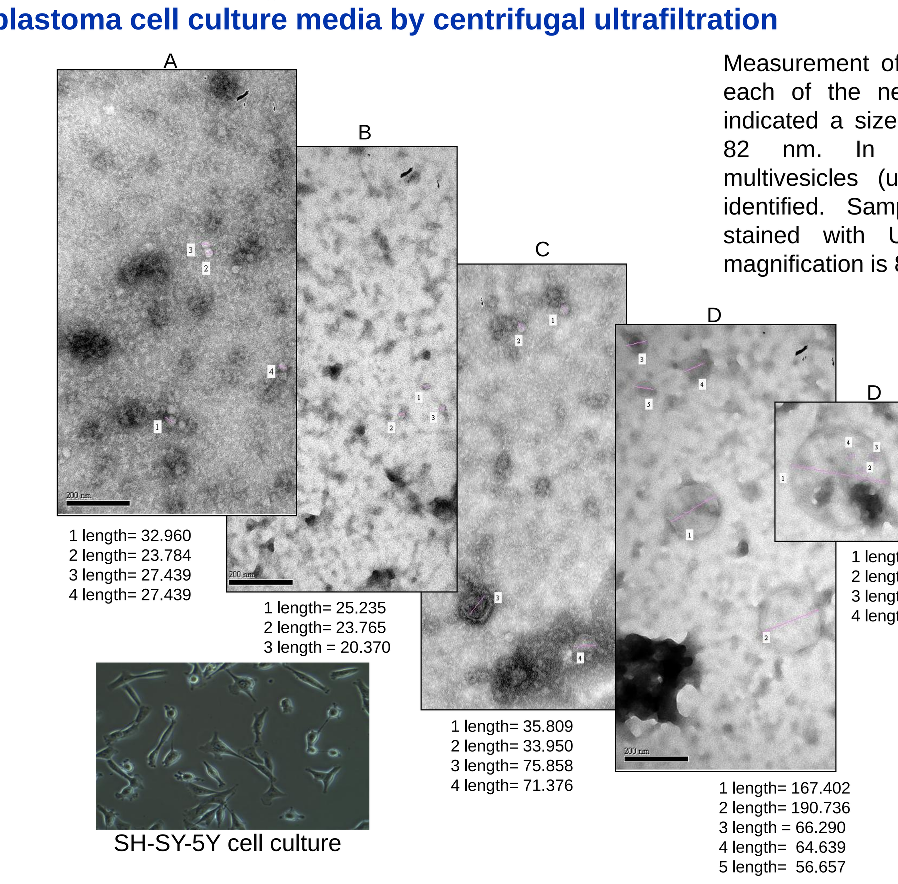
NanoSight analysis: concentration measurement and size profiling for various exosome sample preparations



Product / device	Serum content	Concentration of exosomes (Particles/mL)	Mean particle size (nm)	Standard deviation
3 kDa Amicon® Ultra	(-)	5.72E+08	186	57
Amicon® Pro system with 100 kDa	(+)	2.69E+08	266	129
Precipitation by Total Exosome Isolation from cell culture media (Life Tech)	(+)	3.18E+08	248	105

The histograms plot particle size (nm) *vs.* concentration (10^6/mL). More nanoparticles (see table above) and more uniform particle distribution was observed in samples with no serum concentrated in Amicon® Ultra 3 kDa devices.

Electron microscope images of whole mounted exosomes purified from SH-SY-5Y neuroblastoma cell culture media by centrifugal ultrafiltration



Measurement of several exosomes in each of the neuroblastoma samples indicated a size range between 16 to 82 nm. In addition, several multivesicles (up to 485 nm) were identified. Samples were negatively stained with Uranyl Acetate. Total magnification is 87,000 x.

Exosome Phenotyping

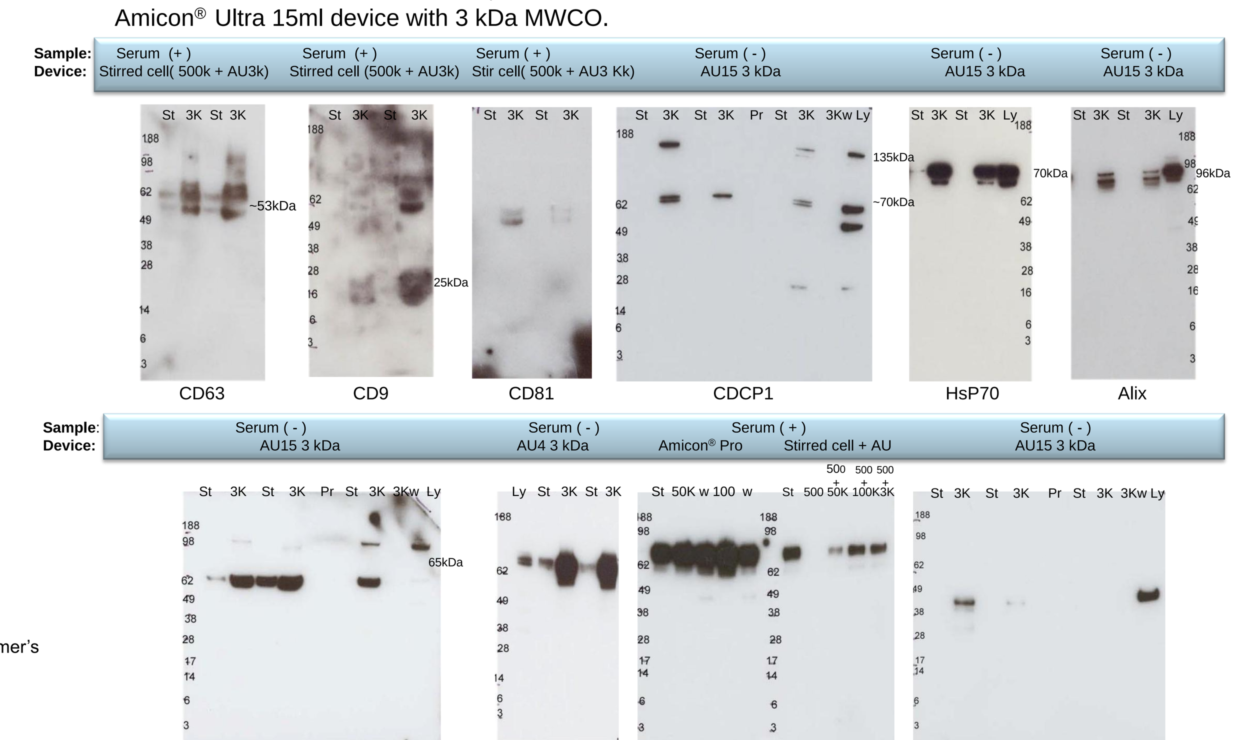
Immunodetection of various neuroblastoma culture exosome samples prepared by ultrafiltration

Several tetraspanins, as well as other exosome markers were identified in samples purified from neurobastoma cells harvested in the presence or absence of serum. Choice of ultrafiltration device and membrane molecular weight cut off (MWCO) was based on sample type and volume.

For sample volumes above 30 mL containing serum, the best choice for concentration was the Amicon® stirred cell with 500 kDa membrane with further concentration using the Amicon® Ultra 15 mL device with 3 kDa membrane. Smaller volumes of serum can be concentrated and dialfiltered using the Amicon® Pro device with 100 kDa membrane. For samples with no serum and volumes around 15ml, the best choice was the Amicon® Ultra 15ml device with 3 kDa MWCO.

Legend

St	Starting material
AU15 3k	Amicon® Ultra 15ml with 3kDa
3 kw	PBS Wash post-concentration
Ly	Lysate
Pr	Precipitation solution



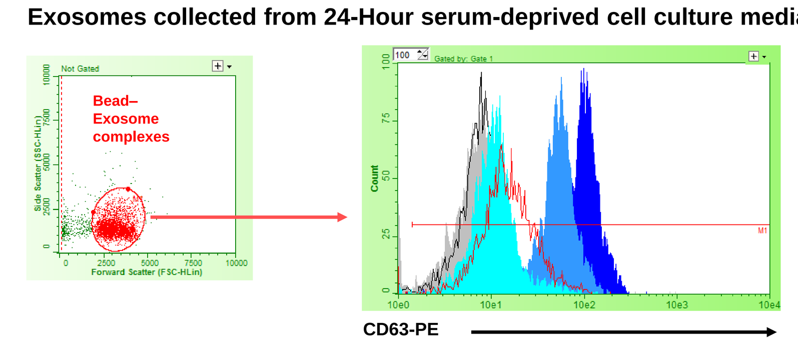
Proteins implicated in Alzheimer's disease such as Amyloid Alzheimer's precursor and Tau-1 protein were identified in samples with and without serum. Detection of these proteins in exosome samples has been reported previously in neuronal cells[6].

Bead-based detection of CD63+ exosomes using a guava® easyCyte™ flow cytometer

Understanding exosomes, and their role in biological function, requires recovery of sufficient quantities for multilevel analyses. Three different approaches for exosome pre-enrichment from MDA-MB-231 cell culture media were assessed for the detection of CD63: (1) Precipitation with Total Exosome Isolation solution (TEI), (2) Concentrated by Amicon® Ultra device then precipitated with TE, and (3) Concentrated by Amicon® Ultra device alone.

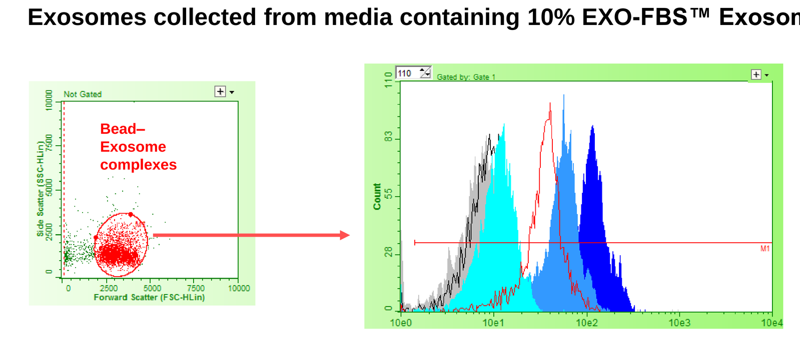
Processed samples (50, 25 or 5 µL) were incubated for 24 hours with CD63 magnetic beads to form bead-exosome complexes. Following incubation, complexes were washed. Samples were stained for 1 hour with anti-Human CD63–PE labeled antibody, washed, and acquired using the guava® easyCyte™ flow cytometer. All data was analyzed using the Guava Express® Pro software module.

Exosomes collected from 24-Hour serum-deprived cell culture media



Prep Method	MFI
Beads alone - control	8.64
TEI alone - 50 µL	8.3
AU0.5 + TEI - 50µL	99.68
AU0.5 + TEI - 25µL	52.78
AU0.5 + TEI - 5µL	13.15
AU0.5 alone - 50 µL	19.37

Exosomes collected from media containing 10% EXO-FBS™ Exosome-depleted FBS



Prep Method	MFI
Beads alone - control	8.64
TEI alone - 50 µL	9.07
AU0.5 + TEI - 50µL	109.02
AU0.5 + TEI - 25µL	53.8
AU0.5 + TEI - 5µL	11.9
AU0.5 alone - 50 µL	38.71

Data are shown for MDA-MB-231 cells cultured under two conditions. The dot plots demonstrate gating specifically on the bead-exosome complex in each sample (**Far Left**). CD63-PE signal for gated fractions are displayed in histogram overlays; Mean Fluorescent Intensity (MFI) for each peak is presented in the table (**Far Right**). For both conditions, exosome samples prepared with concentration yielded more intense signal than precipitation alone.

Conclusions

- **The presence of exosomes obtained by ultrafiltration methods was verified by Transmission Electron Microscopy (TEI). Sample interrogation via nanoparticle tracking analysis (NTA) and exosome-specific ELISAs, further validate the fractionation technique.**
- **Albumin content in serum-containing supernatants can be significantly reduced by a 2-step diafiltration process. For culture media lacking serum, ultrafiltration with one buffer exchange using the 3 kDa membrane was the most effective method.**
- **The ultrafiltration method is not only fast and efficient, but it is also compatible with subsequent purification via precipitation or bead-based affinity techniques.**
- **The use of a mid-infrared-based spectroscopic analysis permits monitoring of protein and lipid content for process optimization during exosome sample preparation.**
- **Flow cytometry, in combination with affinity capture beads, provides a platform for exosomal phenotyping. The method is also amenable to multiparameter assessment of surface markers.**

Summary

A 2-step sample prep workflow involving size-selective cell and debris removal followed by ultrafiltration provides a fast and easy method for exosome purification from cell culture media. Samples containing serum were dialfiltered using a high MWCO membrane followed by a 3k MWCO membrane for concentration. Sample preparation was efficiently monitored for protein and lipid content using mid-infrared spectroscopy. Purified fractions were analyzed by Western blotting using the SNAP i.d.® 2.-0 system; several tetraspanins, as well as other protein markers like Amyloid Precursor Protein and Tau-1 implicated in Alzheimer's disease, were identified. CD9 and CD63 surface marker expression was confirmed and quantified by both ELISA and flow cytometry. Total particle concentration and size distribution was performed by nanoparticle tracking analysis. Electron microscopy confirmed the presence of exosomes and microvesicles in the samples prepared by ultrafiltration.

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