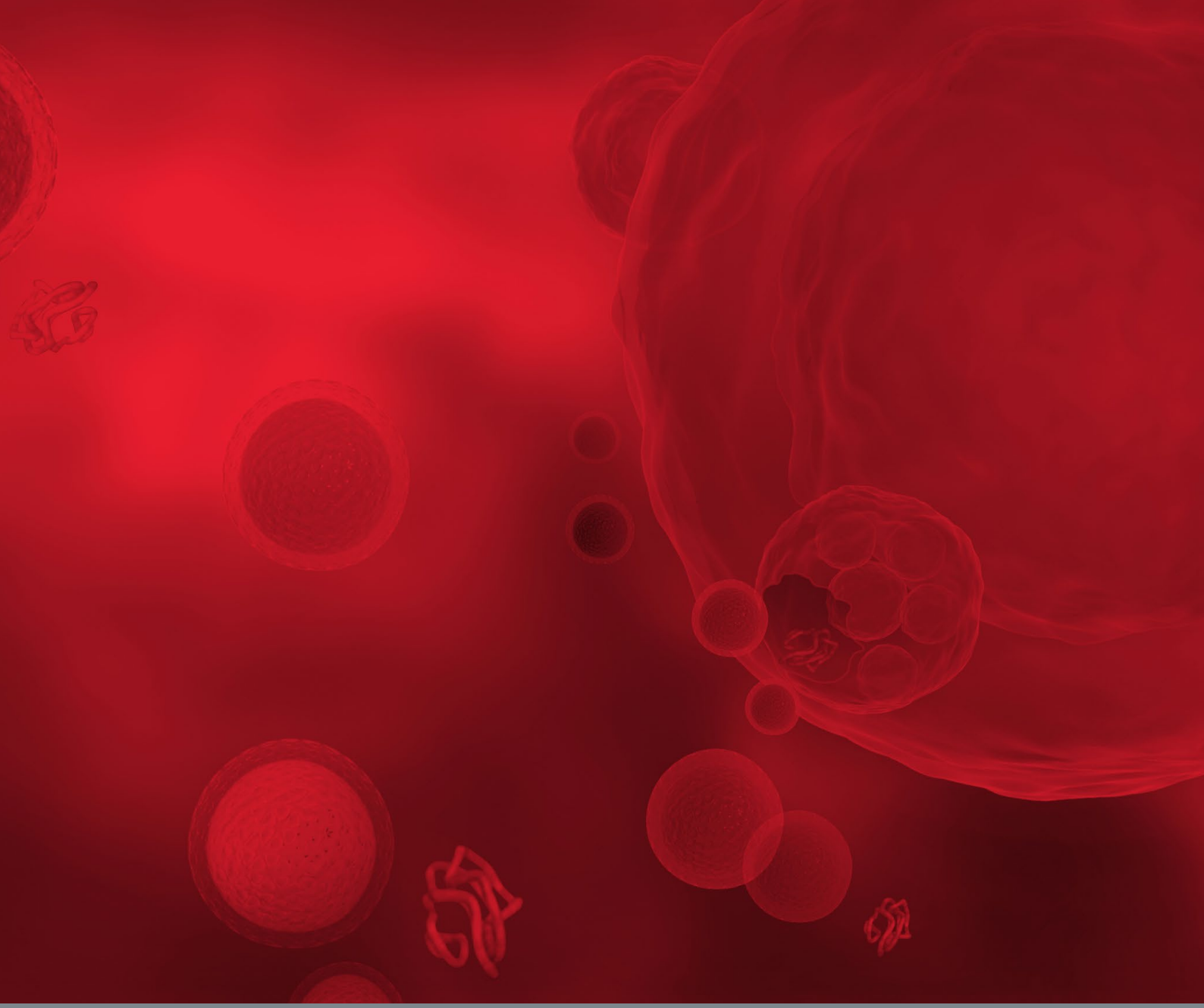

EL*EV*ATE Your Research:

Unlocking the Potential in Extracellular Vesicle Analysis

Expert Insights

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Contents

Introduction

The integration of flow cytometry in the study of extracellular vesicles (EVs) marks a significant advancement in the field of biomedical research, offering a versatile platform for the analysis and characterization of these nanoscale entities. EVs, including exosomes and microvesicles, play critical roles in intercellular communication and are implicated in various physiological and pathological processes, ranging from immune regulation to cancer progression [1].

Flow cytometry enables researchers to analyze EVs at the single-particle level, providing insights into their heterogeneity, surface markers, cargo contents, and functional properties. By labeling EVs with fluorescent probes or antibodies targeting specific surface proteins, flow cytometry allows for the identification and quantification of EV subpopulations within complex biological samples [2]. This capability is particularly valuable for profiling EVs derived from different cell types or physiological conditions, aiding in the elucidation of their biological roles and diagnostic potential.

Key methodologies in EV flow cytometry include sample preparation, staining protocols, and instrument optimization to achieve optimal sensitivity and resolution. Researchers employ various isolation techniques to purify EVs from biological fluids or cell culture supernatants, followed by fluorescent labeling of EV-associated biomolecules for downstream analysis. Additionally, advances in high-dimensional flow cytometry enable multiparametric profiling of EVs, facilitating the simultaneous assessment of multiple phenotypic and functional attributes. Centrifugation is currently the gold standard for EV purification; it allows researchers to employ several different methods to optimize their sample's purity. Sample purification is essential for accurate and timely characterization of formulations.

This Experts Insights eBook begins with a study introducing a novel method for precisely modifying EVs to deliver therapeutics. The research study from Pham *et al.* [3] explores a novel enzymatic technique for covalently attaching peptides and nanobodies onto EVs without genetic manipulation. The method aims to enhance targeted drug delivery by facilitating stable conjugation of EVs with targeting molecules. By utilizing protein-ligating enzymes the study demonstrates a simple and efficient approach to modify EV surfaces which enables specific targeting of EVs to desired cells, potentially improving the efficacy of therapeutic payloads. The research underscores the promise of enzymatic conjugation for precision drug delivery and highlights its versatility in biomedical applications.

The second paper by Welsh *et al.* [4] presents updated guidelines by the International Society for Extracellular Vesicles to standardize EV research. MISEV2023 addresses EVs' nomenclature, isolation, and characterization, incorporating feedback from a broad research community and reflecting the latest scientific advancements. This article plays a crucial role in maintaining methodological uniformity and improving the caliber of EV research, propelling progress in developing diagnostics and treatments based on EVs.

Overall, integrating flow cytometry into the study of EVs holds immense promise for advancing our understanding of intercellular communication, disease pathogenesis, and therapeutic interventions. By providing a robust and quantitative analytical platform, EV flow cytometry contributes to the development of novel diagnostic tools, drug delivery systems, and biomarker discovery strategies with broad implications for translational medicine and personalized healthcare.

Through the methods and applications presented in this Experts Insights eBook, we hope to educate researchers on new technologies and techniques for EV flow cytometry. To gain a deeper understanding of available options for improving your research, we encourage you to visit Beckman Coulter.

Andrew Dickinson, Ph.D.
Content Strategist at Wiley

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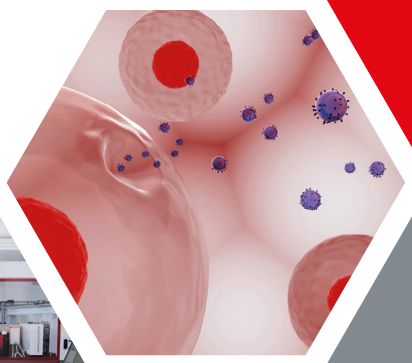

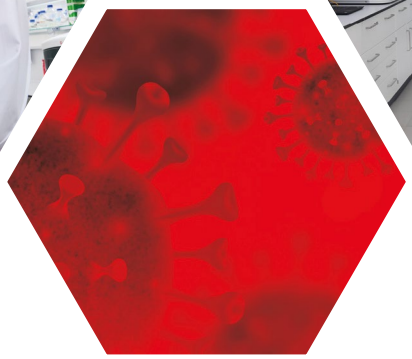

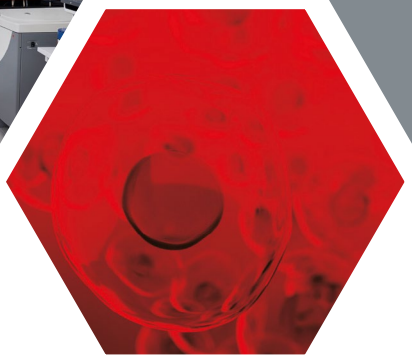

Breaking the boundaries of EV detection

Unveil the full picture of your EV experiment with the CytoFLEX nano flow cytometer. With the ability to count, size, and characterize EVs using a single technique, it propels your research forward. Experience greater sensitivity, consistent instrument performance, and flexibility to study your sample. With the CytoFLEX nano flow cytometer, we have lowered the limits of detection, so you can achieve more.

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Covalent Conjugation of Extracellular Vesicles with Peptides and Nanobodies for Targeted Therapeutic Delivery

Adapted from Pham *et al.*, 2021

Introduction

Extracellular vesicles (EVs) are emerging as novel drug delivery vehicles due to their ability to bypass cellular barriers and facilitate intercellular communication. Human red blood cell-derived EVs (RBCEVs) have shown promise for RNA drug delivery, but their nonspecific uptake may cause unwanted side effects. To address this, researchers have used genetic engineering to equip EVs with targeting peptides or antibodies, but this approach is tedious, costly, and poses risks such as horizontal gene transfer and tumorigenesis. Here, we describe a novel enzymatic method using protein-ligating enzymes (Sortase A and OaAEP1 ligase) to conjugate peptides and nanobodies onto EVs without genetic or chemical modification. This approach is simple, gentle, and efficient, facilitating the stable covalent conjugation of EVs with targeting moieties. We demonstrate the efficacy of this method in delivering therapeutic payloads to target cells, including EGFR-positive lung cancer cells, and highlight its potential for targeted drug delivery with high specificity.

Results

Protein Ligases and RBCEVs are Produced at High Purity

Two protein-ligating enzymes, Sortase A heptamutant and OaAEP1 Cys247Ala ligase, were employed in this study for RBCEV conjugation. Both enzymes were produced at high yield in *E. coli* and highly purified by affinity and size-exclusion chromatography (Fig. 1). RBCEVs were purified using differential ultracentrifugation as described previously [2].

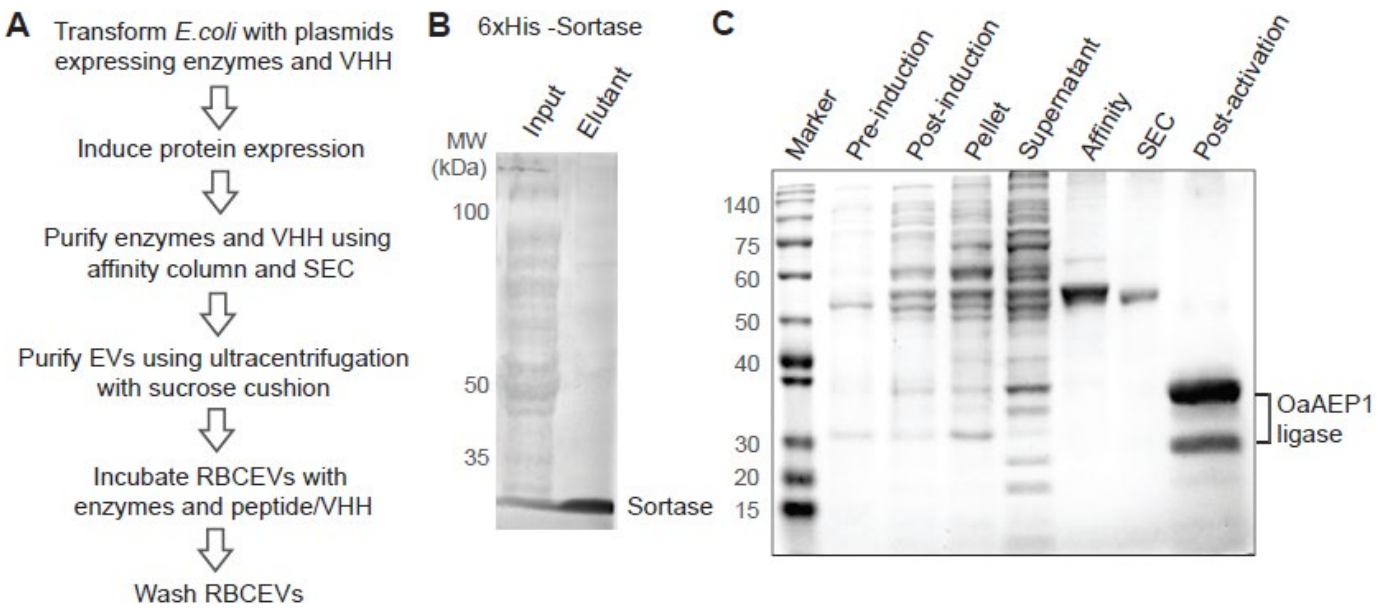

Figure 1. Purification of enzymes and nanobodies for EV conjugation. (A) Experimental workflow for protein purification and conjugation of RBCEVs. **(B)** SDS-PAGE analysis of proteins before (input) and after (eluent) FPLC purification of His-tagged sortase A (18kDa). **(C)** SDS-PAGE analysis of protein fractions obtained over the course of expression and FPLC purification (His-tag affinity and size exclusion chromatography, SEC) of OaAEP1-Cys247Ala ligase.

Sortase A and OaAEP1 Ligase Mediate the Conjugation of EVs with Peptides

We designed biotinylated peptides containing a known EGFR-targeting (ET) site and a recognition motif for SortaseA or OaAEP1 ligase (Fig. 2a, c). Western blotting using HRP-conjugated streptavidin showed multiple biotinylated protein bands after the sortagging or ligation reaction (Fig. 2b, d). Sortagging reactions created two bands as intermediates, while two different bands remained after washing, indicating RBCEV surface proteins conjugated with the biotinylated ET peptide. In the OaAEP1-catalyzed reactions, two intense protein bands were observed, both of which remained after washing. Both the sortagged and ligated products survived denaturing SDS-PAGE, indicating stable covalent bonds between RBCEV membrane proteins and ET peptides. The same results were obtained from three different donor samples (Fig. 2e).

We compared biotin signals from the ET-RBCEVs to a serial dilution of dibiotinylated HRP to quantify the ET peptides conjugated to RBCEVs using OaAEP1 ligase and determined an average of ~380 copies of peptides ligated to each RBCEV (Fig. 2e, f). We also determined an average of ~65 copies of sortagged peptides per EV.

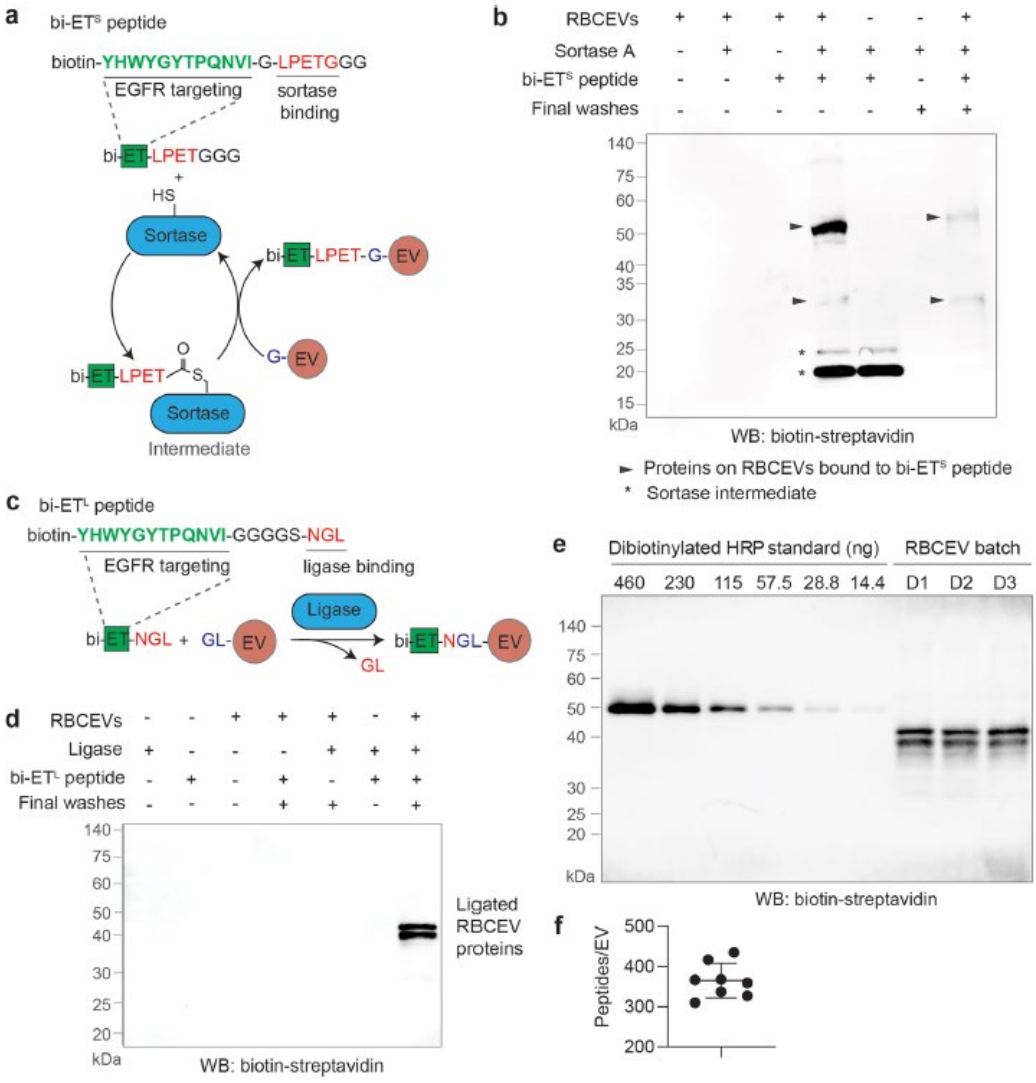

Figure 2: Protein ligating enzymes mediate a covalent conjugation of RBCEVs with peptides. (a) Design of an EGFR-targeting (ET) peptide with a sortase binding site and biotin (bi) conjugation (bi-ETS peptide). Sortagging reaction occurs between the bi-ETS peptide and proteins with N-terminal Glycine (G) on RBCEVs, mediated by Sortase A. **(b)** Western blot (WB) analysis of biotin following an SDS-PAGE separation of RBCEV proteins conjugated with the bi-ETS peptide. Sortase intermediates were removed in three washes with PBS. Biotin was detected using HRP-conjugated streptavidin. Molecular weights (kDa) of protein markers are shown on the left. **(c)** Design of a typical OaAEP1-ligase-mediated reaction between a biotinylated ET peptide with a ligase binding site (bi-ETL peptide) and proteins containing N-terminal GL (preferred but not required) on RBCEVs. **(d)** Western blot analysis of biotin resulted from the OaAEP1-ligase-mediated conjugation of RBCEVs with the bi-ETL peptide, similar to (b). **(e)** Western blot analysis of RBCEVs from three different donors (D1-D3) ligated with a biotinylated control peptide using OaAEP1 ligase. Dibiotinylated HRP was used as a reference for quantification, and a particle analyzer was used to obtain the number of ligated EVs loaded per well. **(f)** Average number of peptides ligated to each EV ± SEM (n = 8 donors).

Transmission Electron Microscopy Showed That Peptide Ligation Did Not Affect EV Structure or Integrity

We analyzed peptide-coated RBCEVs using single-EV flow cytometry according to the MIFlowCyt-EV guidelines and detected biotinylated-peptide (TR5) on the surface of RBCEVs. We identified six proteins ligated to the biotinylated peptide using a biotin-streptavidin pulldown assay, and also detected multiple biotinylated protein bands using EVs from leukemia THP1 cells, indicating membrane proteins that act as ligase substrates. This method demonstrated efficient conjugation of peptides to EVs, potentially enabling targeting and tracking of EVs in various applications.

Conjugation of EVs with EGFR-targeting Peptides Promotes Their Uptake by EGFR-positive Cells

RBCEVs conjugated with an EGFR-specific ET peptide showed significantly higher uptake by H358 cancer cells compared to control peptide-coated RBCEVs. Uptake was specific, as competitive binding with free ET peptide blocked uptake. Immunofluorescence analysis revealed punctate patterns of CFSE (dye)-labeled RBCEVs inside cells, indicating internalization via endocytosis. Inhibitor studies showed that uncoated RBCEVs were taken up through multiple endocytic pathways, while ET-peptide-conjugated RBCEVs relied on caveolin-mediated and lipid raft-mediated endocytosis. These findings demonstrate the potential of peptide-conjugated RBCEVs for targeted drug delivery to EGFR-positive cancer cells.

Conjugation of EVs with a 'Self' Peptide Reduces Their Phagocytosis and Increases EV Circulation

We investigated the surface expression of 'don't eat me' (CD47) and 'eat me' (phosphatidylserine, PS) signals on RBCEVs by flow cytometry. Results showed that PS was more abundant than CD47 on RBCEVs (Fig. 3a, b). To increase the 'don't eat me' signal, we conjugated RBCEVs with a self-peptide derived from CD47. This significantly reduced uptake by monocyte cell lines (Fig. 3c) and increased the circulatory flux of RBCEVs in immunodeficient mice (Fig. 3d). These findings suggest that self-peptide conjugation can reduce nonspecific phagocytosis and enhance the bioavailability of RBCEVs, highlighting a potential strategy for improving EV-based drug delivery.

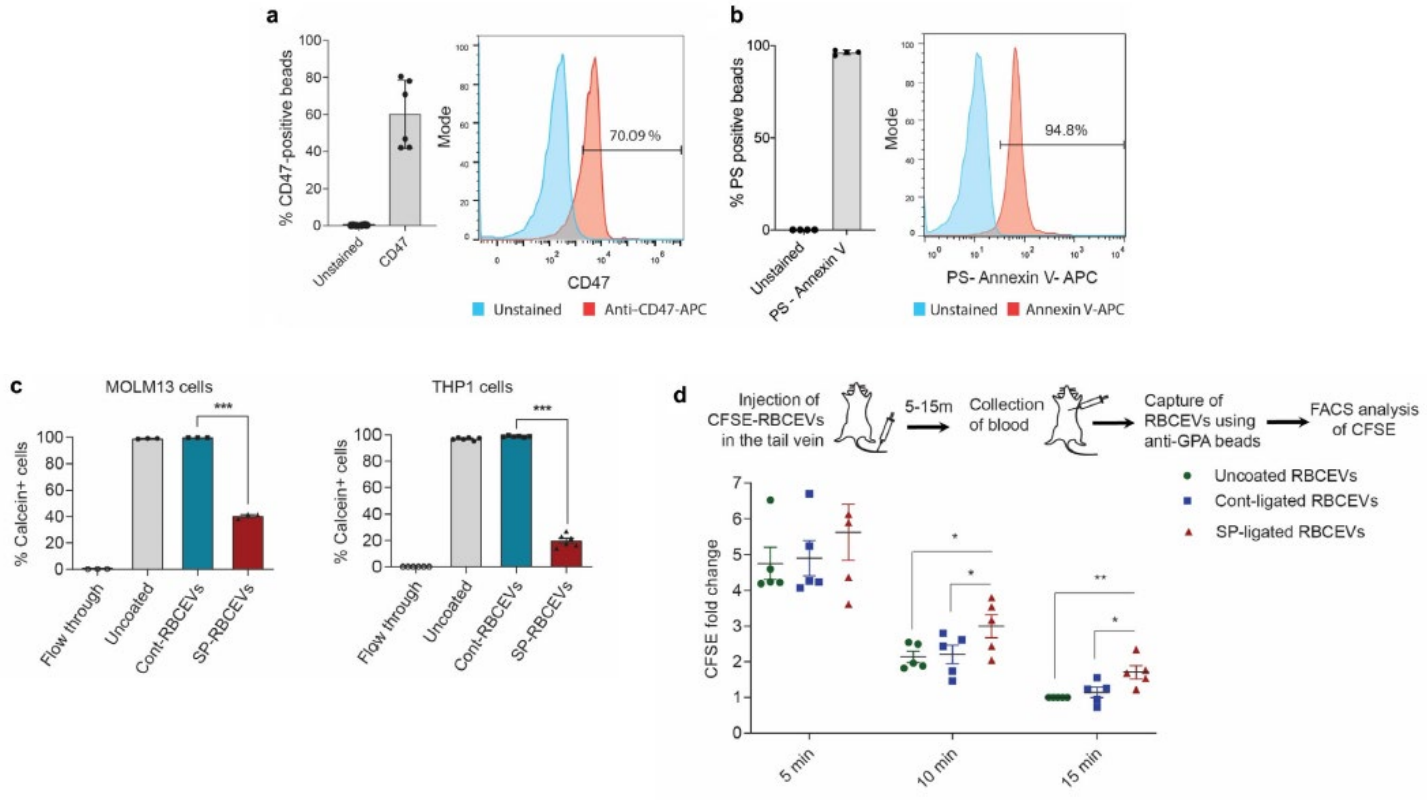

Figure 3: Conjugation with self-peptide prevents phagocytosis of RBCEVs and enhances the availability of RBCEVs in the circulation. **(a)** Flow cytometry analysis of CD47 on RBCEV-bound beads. **(b)** Flow cytometry analysis of Annexin V binding to PS on RBCEVs that were immobilized on latex beads. **(c)** FACS analysis of Calcein AM in MOLM13 and THP1 monocytes that were treated with control or self-peptide (SP) ligated Calcein-labelled RBCEVs. 200,000 cells were incubated with 5 μg RBCEVs (2.5×10^9 particles) at 37°C for 2 h. The graphs present the average percentage of Calcein-positive cells ± SEM (n = 3 to 6 donors). **(d)** FACS analysis of CFSE-labelled RBCEVs that were captured by anti-GPA-antibody-coated streptavidin beads from the plasma of NSG mice, 5–15 min after a tail vein injection of 0.5 mg CFSE-labelled human RBCEVs (2.5×10^{11} particles). RBCEVs were uncoated or ligated with the control or self-peptide. The graph presents the mean intensity of CFSE ± SEM (n = 5 mice). Student's one-tailed *t*-test $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Conjugation of Nanobodies to EVs Using a Two-step Ligation Method

We explored using nanobodies to target RBCEVs to specific cell types. We purified an anti-EGFR camelid biparatopic nanobody (VHH) and designed a linker peptide to bridge the nanobody to RBCEVs, allowing sequential ligation (Fig. 4). The linker peptide enabled efficient conjugation of the nanobody to RBCEVs, with an estimated 49 copies per EV. FACS analysis confirmed a large fraction of RBCEVs were conjugated to the nanobody. Nanobody conjugation was less efficient than peptide conjugation, likely due to size limitations.

Conjugation of EVs with Nanobodies Promotes Their Specific Uptake by Target Cell Types

We observed a significant increase in RBCEV uptake by HCC827 cells when conjugated with α-EGFR VHH via a linker peptide (Fig. 4c). The increase was comparable to that observed with ET peptide conjugation, likely due to the high affinity of α-EGFR VHH for EGFR. Conjugation with α-mCherry VHH and α-HER2 VHH also promoted uptake by mCherry-expressing and HER2-expressing cells, respectively (Fig. 4d, e).

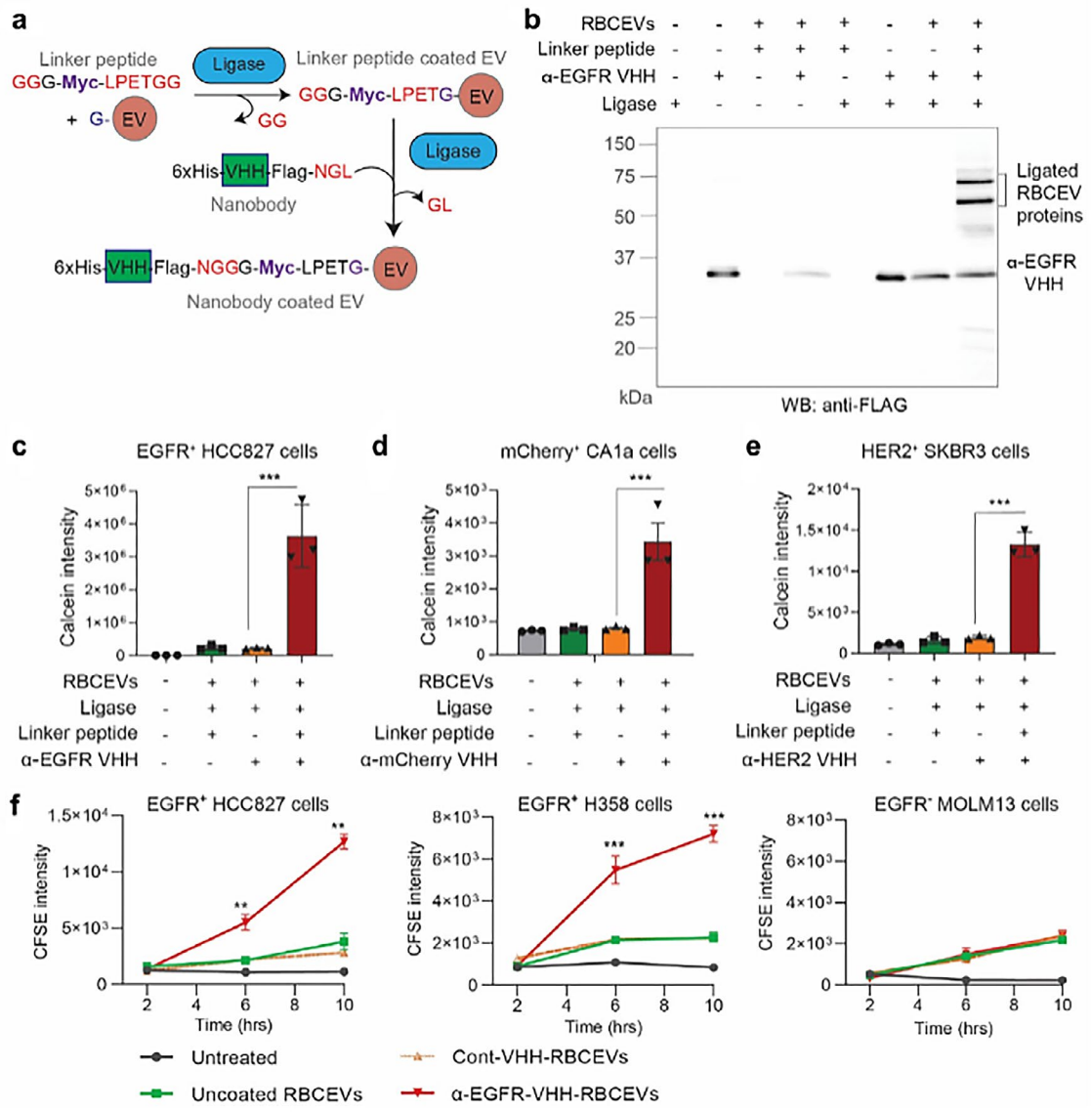

Figure 4: Nanobodies were conjugated to RBCEVs via a linker peptide, increasing the specific uptake of RBCEVs. (a) Two-step conjugation of RBCEVs with nanobodies: EVs were first ligated with a linker peptide which was then ligated to a VHH nanobody. **(b)** Western blot analysis of α-EGFR VHH (using α-FLAG-tag antibody), with or without conjugation to RBCEVs, after SDS-PAGE separation. **(c)** Uptake of Calcein-labelled α-EGFR-VHH-ligated RBCEVs by EGFR+ lung cancer HCC827 cells. **(d)** Uptake of Calcein-labelled α-mCherry-VHH-ligated RBCEVs by mCherry-expressing breast cancer CA1a cells. **(e)** Uptake of Calcein-labelled α-HER2-VHH-ligated RBCEVs by HER2-expressing breast cancer SKBR3 cells. (f) Uptake of CFSE-labelled RBCEVs ligated with α-EGFR or control (α-mCherry) VHH after 2–10 h of incubation with EGFR-positive HCC827 or H358 cells versus EGFR-negative MOLM13 cells. Graphs in (c) – **(f)** present the mean intensity of Calcein AM or CFSE ± SEM (n = 3 donors), analyzed using FACS. Student's one-tailed *t*-test: $^{**}P < 0.01$, $^{***}P < 0.001$.

α-EGFR VHH exhibited high affinity for EGFR-positive cells, and conjugated RBCEVs bound specifically to these cells as determined using FACS analysis (Fig. 5), followed by uptake (Fig. 4f). Immunofluorescence analysis confirmed the uptake, suggesting endocytosis as the main uptake mechanism.

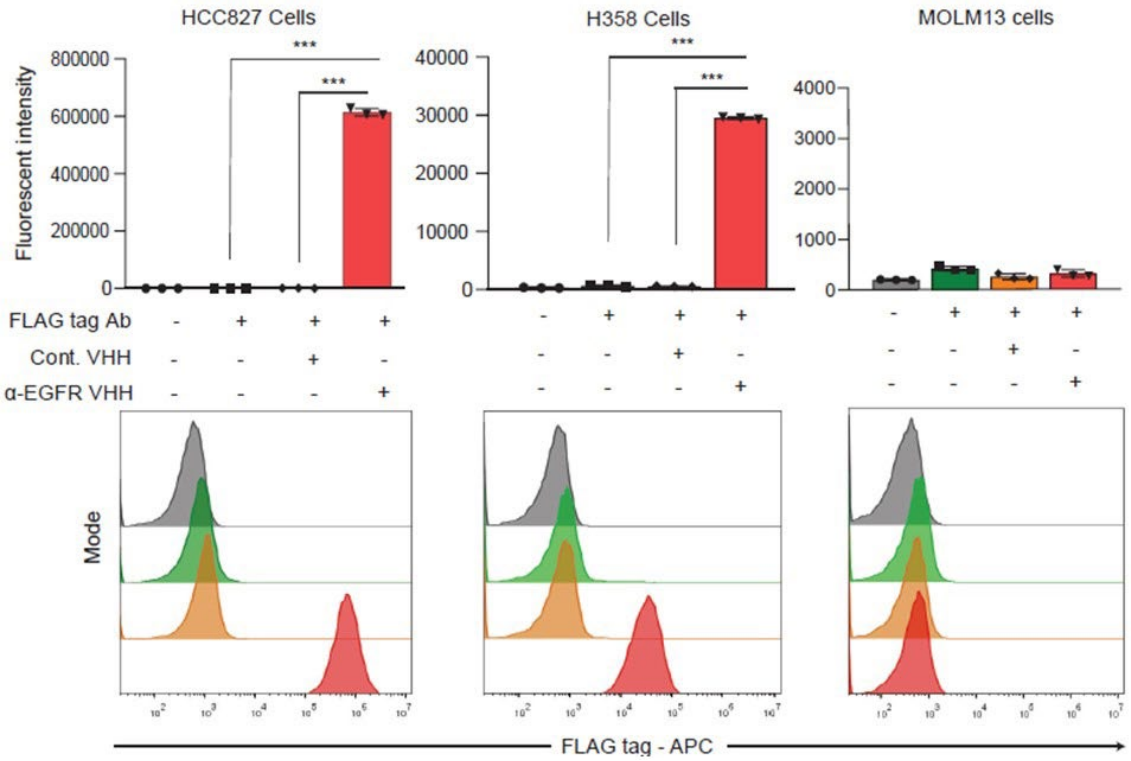

Figure 5: FACS analysis of FLAG tag, an epitope tag on the a-EGFR VHH or control (α-mCherry) VHH nanobody that bound to EGFR-positive HCC827 and H358 cells versus EGFR-negative MOLM13 cells, using an APC α-FLAG tag antibody (Ab). Student's *t*-test: ***$P < 0.001$ (n = 3 EV donors).

Surface-modified RBCEVs Promote Specific Delivery of RNA and Chemotherapeutic Payloads

We investigated whether conjugating RBCEVs with nanobodies affects RNA loading and delivery. We found that conjugating RBCEVs with α-EGFR VHH enhanced the efficiency of mRNA delivery to EGFR-positive cancer cells (Fig. 6a). Additionally, we optimized a protocol for loading paclitaxel into RBCEVs using sonication, achieving a loading capacity of 5-6% and efficiency of ~25% (Fig. 6b). Treating EGFR-positive cancer cells with paclitaxel-loaded RBCEVs showed enhanced efficacy at lower doses, suggesting that conjugated RBCEVs can improve the effectiveness of chemotherapy.

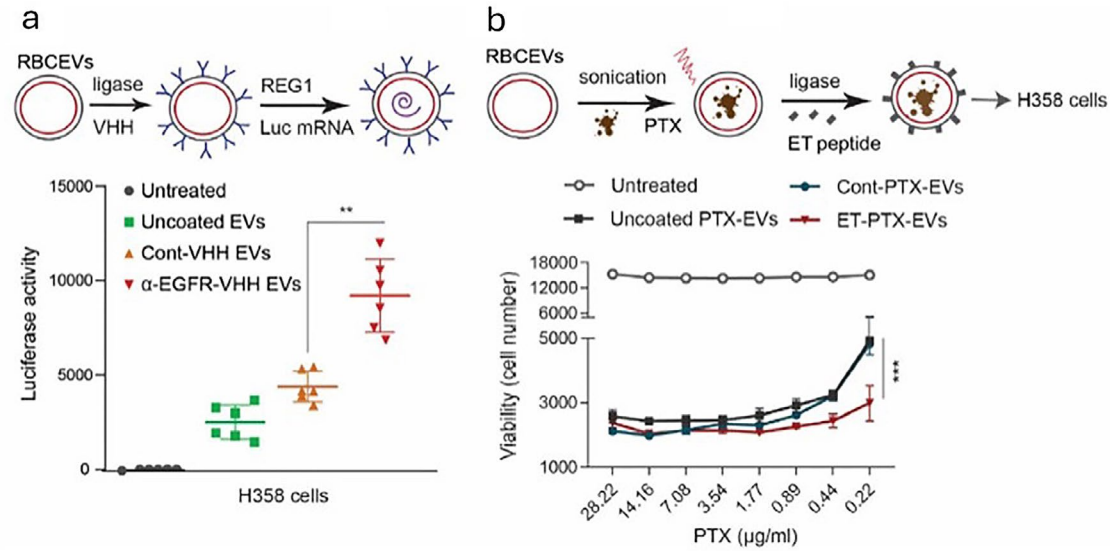

Figure 6: Delivery of luciferase-expressing (luc) mRNA using control (α-mCherry-VHH) or α-EGFR-VHH-ligated RBCEVs, quantified based on luciferase activity in H358 cells after 24-h incubation with mRNA-loaded RBCEVs (uncoated or ligated with VHH). Luciferase mRNA was loaded in RBCEVs using REG1 loading reagent. Graph presents luciferase signal ± SEM (n = 6 repeats). Student's one-tailed *t*-test: $^{**}P < 0.01$.

EGFR-targeting RBCEVs Accumulate in Xenografted EGFR-positive Lung Cancer Cells

We established a lung cancer mouse model by injecting luciferase-mCherry-labelled H358 cells into immunodeficient mice (Fig. 7a). After three weeks, we treated the mice with DiR (dye)-labeled RBCEVs and observed biodistribution. ET-peptide-conjugated RBCEVs showed increased accumulation in the lung and reduced liver uptake compared to control-peptide-conjugated RBCEVs (Fig. 7a, b). We analyzed specific uptake of RBCEVs by tumor cells *in vivo* by injecting CFSE (dye)-labeled peptide/VHH-conjugated RBCEVs into mice bearing mCherry-H358 lung tumors (Fig. 7c). Specific uptake of RBCEVs was confirmed by FACS analysis, with ET peptide-conjugated RBCEVs performing better than α-EGFR-VHH-conjugated RBCEVs. The ET peptide increased CFSE-positive tumor cells from 3-6% to 20-30%, while α-EGFR VHH also increased CFSE-positive cells to ~20%. These findings suggest that ET-peptide-conjugated RBCEVs can target EGFR-positive lung tumor cells *in vivo*, making them a promising tool for drug delivery in lung cancer treatment.

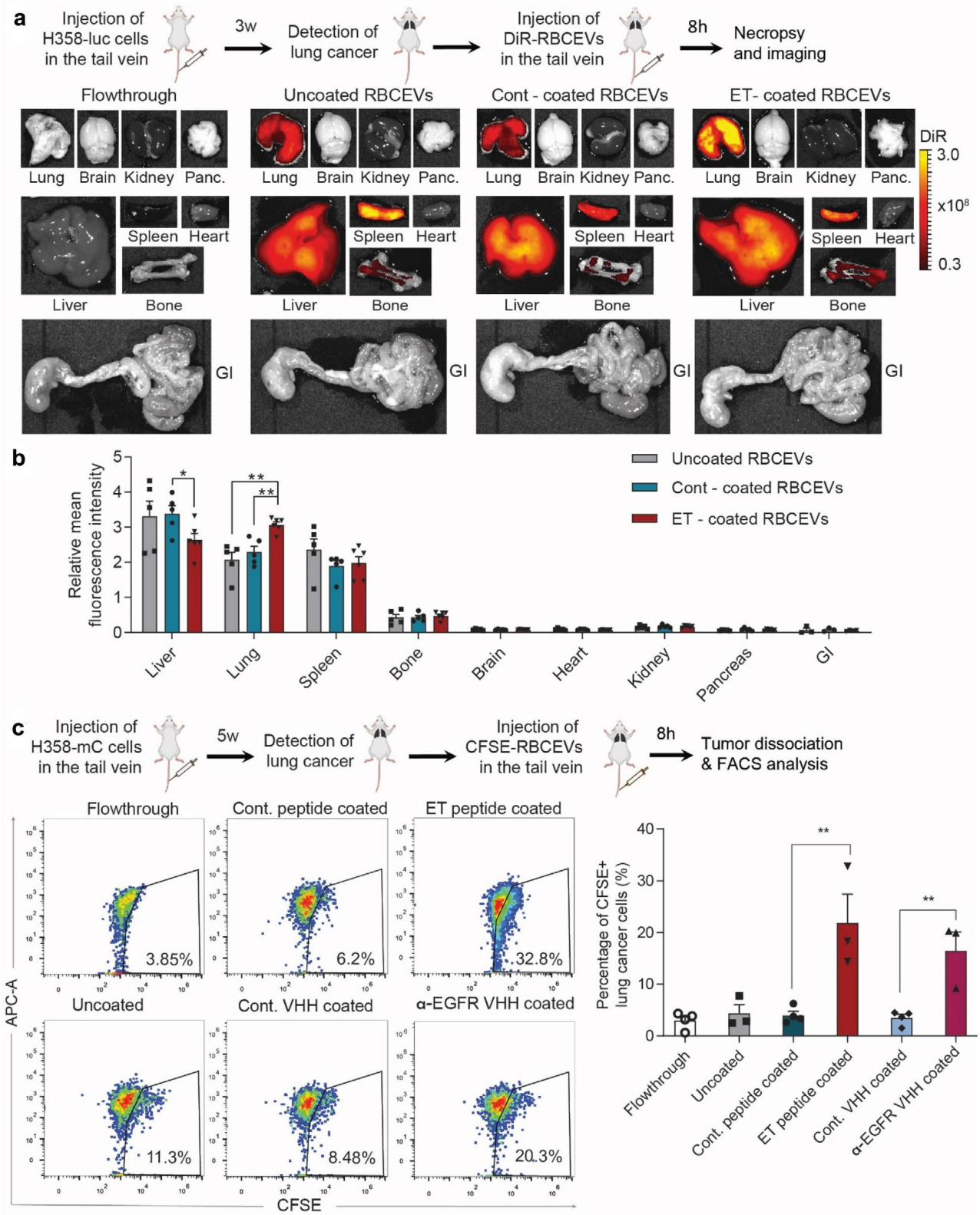

Figure 7: EGFR-targeting RBCEVs accumulate in xenografted EGFR-positive lung cancer cells. (a) Biodistribution of DiR-labelled RBCEVs in NSG mice bearing EGFR+ H358 lung cancer. Shown are representative DiR fluorescent images of organs from lung-cancer-bearing mice preconditioned and injected with uncoated RBCEVs, control/ET-RBCEVs, or with the flowthrough of the RBCEV wash. **(b)** Average DiR intensity in each organ relative to the average mean intensity of all organs, subtracted by signals detected in flowthrough controls. Abbreviations: Panc, pancreas; GI, gastrointestinal tract. **(c)** *In vivo* uptake of CFSE-labelled RBCEVs by mCherry-positive H358 cancer cells, gated based on mCherry expression, in the lung of the mice that were treated with cont/ET peptide or cont/*α*-EGFR-VHH ligated RBCEVs, analyzed using FACS. Student's one-tailed *t*-test: $*P < 0.05$, $**P < 0.01$ (n = 3 to 5 mice).

Delivery of Paclitaxel by EGFR-targeting EVs Enhances Treatment Efficacy in Xenografted Mice

We investigated the *in vivo* efficacy of paclitaxel-loaded RBCEVs in a lung cancer mouse model. Low-dose paclitaxel (1 mg/kg) was administered every three days, and bioluminescent imaging revealed that ET-RBCEVs significantly enhanced tumor inhibition compared to free paclitaxel or control RBCEVs (Fig. 8a, c). Histological analysis confirmed reduced tumor size and increased apoptosis with ET-RBCEV treatment (Fig. 8b, d). These findings suggest that EGFR-targeted RBCEVs can enhance specific delivery of anti-cancer drugs to tumor cells *in vivo*.

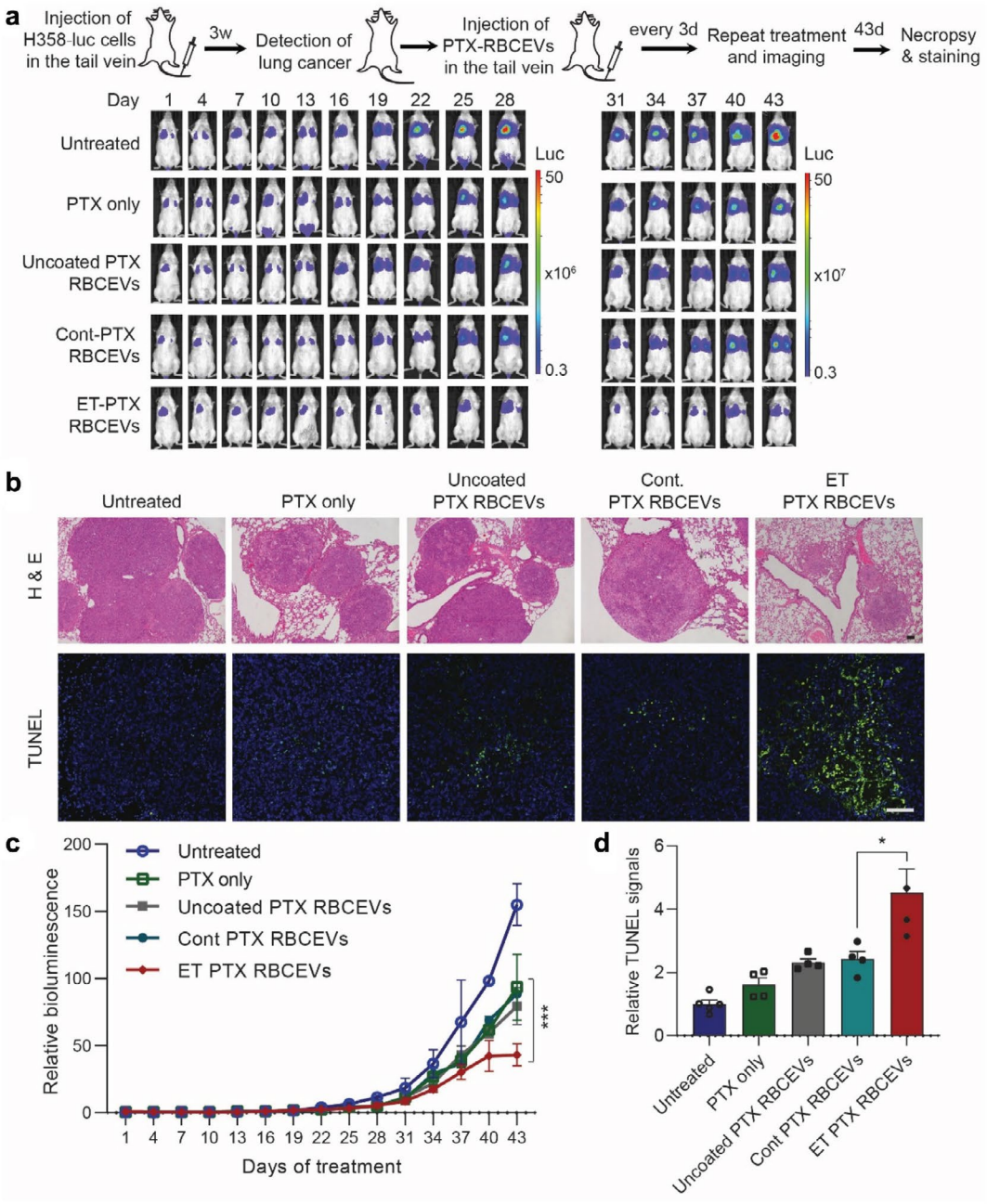

Figure 8: Delivery of paclitaxel (PTX) using EGFR-targeting RBCEVs increases the treatment efficacy in an EGFR-positive lung cancer mouse model. (a) Representative bioluminescent images of NSG mice with EGFR+ luciferase-expressing H358 cancer cells in the lung during a course of systemic (i.v.) treatments with 1mg/kg PTX only or the same dose of PTX loaded in cont/ET-RBCEVs. Treatments were repeated every three days and images were taken one day after every treatment. Colors indicate bioluminescent signals (photon/s) in two scales (the images are divided into two groups, day 1–28 and day 31–43 from the treatment start date, to avoid saturated signals). **(b)** Representative images of H&E staining and TUNEL assay (green fluorescence) of lung sections from the lung cancer mice treated with PTX, with or without RBCEV-mediated delivery. Nuclei were stained with DAPI (blue). Scale bar, 100 *μm*. **(c)** Average bioluminescent signals quantified in the lung area during the development of H358 lung tumors (photons/s), normalized by the signals at the start of the treatments, and presented as mean ± SEM. (d) Average fold change in TUNEL staining signals relative to the untreated control} SEM. Two-way ANOVA test **(c)** and Student's one-tailed *t*-test **(d)**: *P < 0.05, ***P < 0.001 (n = 3 to 4 mice)

Discussion

This report describes a protein ligase-mediated method for conjugating peptides and nanobodies to EVs, to create targeted therapies. This reproducible approach offers advantages over existing methods, including reduced cost and time, no risk of transformation, stable covalent bonds, and the ability to inexpensively scale up production of high-purity products. This method also allows for enhanced delivery of therapeutic molecules to cancer cells and versatility in targeting multiple ligands *in vivo*. Additionally, modifying EV surfaces with self-peptide decreases phagocytosis and increases bioavailability. It is conceivable that conjugating antigens or biomarker-binding (poly)peptides to EVs may facilitate future therapeutics and diagnostics. Limitations include limited nanobody and peptide libraries, and potential immunogenicity risks, but methods under development for stable conjugation of monoclonal antibodies to EVs may address these limitations.

Materials and Methods

Purification of EVs from RBCs and THP1 Cells

RBCs from healthy donors were purified by ultracentrifugation and RBCEVs were isolated from the resulting supernatant, via ultracentrifugation steps similar to those described in Usman et al. [2]. Leukemia EVs were purified from THP1 (leukemic) cell cultures by differential ultracentrifugation. To enrich for EVs the supernatant was passed through a 0.45 µM filter, and centrifuged using sequential sucrose cushions in SW32 and SW41 rotors (Beckman Coulter). RBCEV and leukemia EV size distribution and concentration were determined using a NanoSight analyzer.

Peptide and Nanobody Design

Peptides were designed with non-targeting sequences as negative controls or with an EGFR-targeting sequence or 'self' sequence. A sortase- or ligase-binding motif was added to the C-termini and a biotin was added to the N-termini of the peptides.

EGFR-VHH (variable homodimer), α-mCherry, and α-HER2 nanobody sequences were each cloned with a 6xHis tag, a FLAG tag, and an NGL ligase-binding site in this order: 6xHis-GSG-VHH-GSG-FLAG-NGL.

Conjugation of EVs with peptides using Sortase A or OaAEP1 ligase

Purified EVs were incubated with peptide and Sortase A (for sortagging) or ligase (for ligation) and then purified by size-exclusion chromatography. Conjugated EVs were analyzed by western blotting with horseradish peroxidase-conjugated anti-FLAG antibody detection.

Loading RNAs and Drugs into RBCEVs

Luciferase RNA was loaded into RBCEVs using a commercial transfection agent, while paclitaxel was loaded via sonication with uncoated RBCEVs, which were subsequently coated with peptides as described above.

Flow Cytometry (FACS) Analysis and Single-EV Flow Cytometry

FACS analysis of latex beads or cells in FACS buffer was performed using a CytoFLEX-S cytometer (Beckman Coulter) and analyzed using Flowjo V10 (Flowjo, USA). The beads or cells were first gated by FSC-A versus SSC-A, excluding debris and dead cells. Single cells were then gated by FSC-width versus FSC-height, excluding doublets and aggregates. The fluorescent-positive population of beads or cells was subsequently gated by targeted fluorescent channels.

Single-EV flow cytometry was carried out using a CytoFLEX LX flow cytometer (Beckman Coulter).

Generation of *in vivo* Cancer Models and Treatment with RBCEVs

All experiments in mice were conducted according to IACUC-approved protocols.

mCherry-luciferase-tranduced H358 cells were injected into immunodeficient mice and bioluminescence was monitored after three weeks.

For RBCEV biodistribution analysis, tumor-bearing mice were injected with DiR-labelled RBCEVs, subsequently sacrificed, and organ fluorescence was measured.

To analyze the specific uptake of RBCEVs by tumor cells, tumor-bearing mice were injected with CFSE-labeled RBCEVs, and the lung tissue was later subjected to FACS analysis.

For drug treatment, mice were treated i.v. with paclitaxel alone or an equivalent dose in RBCEVs with or without ET peptide ligation and bioluminescence was monitored.

Quantification of RBCEVs in the Circulation

CFSE-labeled peptide-ligated RBCEVs were injected in immunodeficient mice and blood was later collected. Plasma was incubated with anti-GPA antibody and streptavidin beads and then analyzed by FACS.

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Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches

Adapted from Welsh *et al.*, 2024

Extracellular vesicles (EVs) hold significant promise as biomarkers and therapeutic agents, however, despite progress in EV measurement and application, challenges persist in nomenclature, separating EVs from non-vesicular particles, and conducting characterization and functional studies. To address these issues, the International Society for Extracellular Vesicles (ISEV) has updated its guidelines to produce MISEV2023. MISEV2023 aims to provide a comprehensive overview of current methodologies and their benefits and limitations for EV production, separation, and characterization from various sources. Notably, MISEV2023 introduces new sections on EV release and uptake and briefly discusses *in vivo* approaches for studying EVs. Incorporating feedback from ISEV task forces and over 1000 researchers, MISEV2023 reflects the latest advances in EV research. Its goal is to provide basic biological and clinical researchers with the necessary tools and knowledge to overcome existing challenges.

Introduction to ISEV and MISEV2023

The International Society for Extracellular Vesicles (ISEV) is a professional organization dedicated to promoting research and understanding of extracellular vesicles (EVs), including exosomes, microvesicles, and other membranous structures released by cells. ISEV brings together scientists, clinicians, and industry experts to share knowledge, standardize methods, and advance the field of EV biology and its applications in human health and disease. As EVs play crucial roles in intercellular communication, biomarker discovery, and therapeutic development, ISEV's efforts are crucial for harnessing the potential of EVs in diagnostics, drug delivery, and regenerative medicine.

Why has ISEV Updated the Minimal Information for Studies of Extracellular Vesicles (MISEV2023)?

Important advances have been made in EV metrology and our understanding and application of EV biology. However, there are still obstacles to fully realizing the potential of EVs in various domains, from basic biology to clinical applications. These hurdles include challenges in EV nomenclature, distinguishing them from non-vesicular extracellular particles, characterizing them, and conducting functional studies.

The current document, MISEV2023, aims to offer researchers an updated overview of the available approaches for the production, separation, and characterization of EVs from different sources, such as cell culture, body fluids, and solid tissues. It aims to outline the advantages and limitations of these approaches.

In addition to covering the latest advancements in the fundamental principles of EV research, MISEV2023 explores advanced techniques and approaches that are pushing the boundaries of the field. MISEV2023 also includes new sections on EV release and uptake, as well as a brief discussion on *in vivo* methods for studying EVs.

While not every co-author may agree with every section or recommendation, MISEV2023 represents the consensus position of the EV community at present. As such, it seeks to provide recommendations and guidance for EV-related studies. It encourages researchers to enhance their research design and reporting of experimental details, building upon the criteria and guidelines established in the previous two iterations.

MISEV2023 aims to answer the following questions:

- What terms do you use, and what do they mean?
- From what/where did you obtain your EVs?
- How did you separate, concentrate, characterize, and store them?
- How confidently can you attribute a function or biomarker to EVs versus other components?
- Have you shared data and reported methods in sufficient detail to enable others to replicate or reproduce your results?

Defining EVs

The term 'extracellular vesicles' (EVs) refers to particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own (i.e., do not contain a functional nucleus; Table 1). The terms exosome, ectosome, or microvesicle may not be used unless such an EV population is specifically separated and characterized.

The term 'EV mimetics' can be used to denote EV-like particles that are produced through direct disruption of cells, by *de novo* synthesis from molecular components, or by fusion of native EVs with, for example, liposomes.

Non-vesicular extracellular particles (NVEPs) are non-EV particles derived from cell components, such as proteins and nucleic acids, without a lipid bilayer membrane (Fig. 1). NVEPs and EVs can have similar physicochemical properties, and NVEPs may be more abundant than EVs in biological samples. Consequently, many methods for isolating EVs also co-isolate NVEPs. Additionally, some characterization methods do not specifically identify EVs, and smaller NVEPs may go undetected. In situations where EVs and NVEPs cannot be clearly distinguished, the term "EP" or "EV preparation" may be appropriate.

Regarding size, although 'small' might generally refer to EVs <200 nm in diameter, there is no strict consensus on upper and lower size cut-offs. It has also become clear that many separation methods, such as differential ultracentrifugation, yield EV populations with overlapping size profiles.

Table 1 Reference card on EV nomenclature and related terms.

Term	Definition	Usage
Extracellular vesicles (EVs)	Particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own.	Recommended
Non-vesicular extracellular particles (NVEPs)	Multimolecular assemblies that are released from cells and do not have a lipid bilayer (non-vesicular extracellular particle fraction).	Recommended
Extracellular particles (EPs)	Umbrella term for all particles outside the cell, including EVs and NVEPs.	Recommended
EV mimetic	EV-like particles that are produced through direct artificial manipulation. This term is preferred over 'exosome-like vesicles' and similar terms that imply specific biogenesis-related properties.	Recommended
Artificial cell-derived vesicles (ACDVs)	EV mimetics that are produced in the laboratory under conditions of induced cell disruption, such as extrusion.	Recommended
Synthetic vesicles (SVs)	EV mimetics that are synthesized *de novo* from molecular components or made as hybrid entities, e.g., fusions between liposomes and native EVs.	Recommended
Small EVs (operational term)	Based on the diameter of the separated particles, small EVs are often described as <200 nm in diameter. However, measured diameter is related to the specific characterization method.	Recommended, but caution required
Large EVs (operational term)	Based on the diameter of the separated particles, large EVs are often described as >200 nm in diameter. However, measured diameter is related to the specific characterization method.	Recommended, but caution required
Other 'operational terms'	Physical characteristics: e.g., diameter: small extracellular vesicles (sEVs), large EVs (lEVs), density: low, medium, high (defined ranges). Biochemical composition: e.g., contains a specific (macro)molecule, such as a protein. Cellular origin and/or conditions under which EVs were generated: terms that highlight specific aspects of biogenesis such as molecular mechanisms, energy-dependence (or lack thereof) and functional state of the parent cell related to stress or death.	Recommended, but caution required
Exosome	Biogenesis-related term indicating origin from the endosomal system. Unless subcellular origin can be demonstrated, it is likely that a broad population of EVs is being studied, not exosomes specifically. Exosomes represent a subtype of small EVs: the diameter of intraluminal vesicles of endosomes is generally smaller than 200 nm.	Discouraged unless subcellular origin can be determined
Ectosome	Biogenesis-related term indicating origin from the plasma membrane. Unless subcellular origin can be demonstrated it is likely that a broad population of EVs is being studied, not ectosomes specifically. Ectosomes can have a wide range of sizes, including sizes similar to those of exosomes.	Discouraged unless subcellular origin can be determined
Microvesicle	Biogenesis-related term indicating origin from the plasma membrane. However, historically, the term has often been used to designate large EVs or all EVs, whatever their subcellular origin. This term can therefore lead to confusion.	Discouraged
Exosome-like vesicles	As 'exosome' is a biogenesis-related term indicating origin from the endosomal system, this and similar terms are discouraged for synthesized EV mimetics.	Discouraged

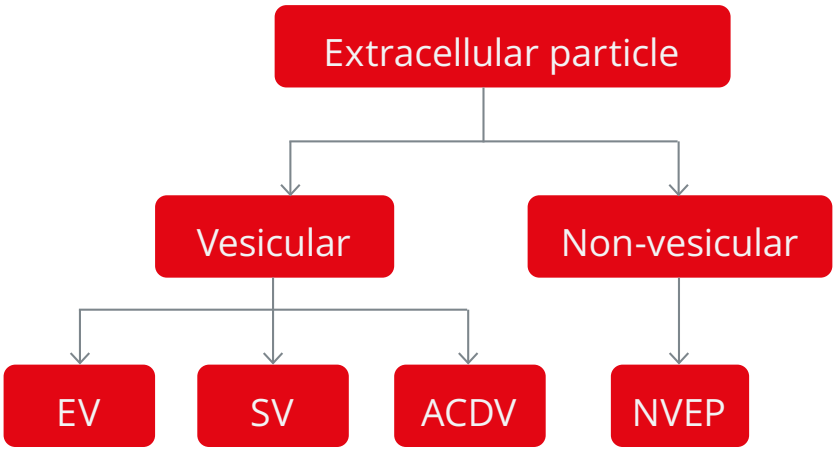


Figure 1. Hierarchy of EP nomenclature. Extracellular particles include vesicular and non-vesicular particles. This figure presents several distinctions that can be made between classes of EPs, as well as examples of possible nomenclature. EP: extracellular particle; EV: extracellular vesicle; SV: synthetic vesicle; ACDV: artificial cell-derived vesicle; NVEP: non-vesicular extracellular particle.

EV Collection and Pre-processing: Pre-analytical Variables Through to Storage

Generally applicable recommendations for reporting details of sample collection and pre-processing (i.e., before specific EV separation/concentration steps) of EVs are provided in MISEV2023 and include describing the source, quality, and quantity of EV-containing materials, sample collection and storage procedures, isolation procedures, and quality control measures used.

MISEV2023 also provides detailed recommendations regarding cell culture conditions and isolation of EVs from specific source materials (e.g., blood, urine, cerebrospinal fluid, saliva, and other complex samples such as synovial fluid, milk, and solid tissues).

EV Separation and Concentration Methods

EVs are usually characterized and utilized following one or more separation or concentration procedures, based on their size, density, charge, or surface composition. These processes are termed “enrichment”, “purification”, or “isolation”, resulting in an “EV-containing preparation” or “EV preparation.” Selection of a separation method should consider the specific EV source properties and the desired yield and specificity (Fig. 2). In complex biofluids, quantifying yield and specificity is challenging, often relying on surrogate measurements of EV abundance.

Direct study or use of EVs in their source matrix is sometimes feasible, particularly in biomarker studies where sufficient specificity and sensitivity are achievable without separation. However, to confirm the exclusive association of a biomarker or function with EVs, initial separation may be necessary. Guidance on these methods is provided in MISEV2023, but for comprehensive insights, refer to sources such as Hendrix *et al.* (2023) [1], which offer extensive details on EV preparation methods.

Concentration in EV studies involves increasing the particle number relative to sample volume and is often necessary for processing large volumes of source materials like cell culture media, urine, or milk before separating EVs from other EPs. Various concentration methods exist, each with unique benefits and limitations (Fig. 2).

(Ultra)centrifugation enables efficient EV concentration using various techniques including increasing relative centrifugal force (differential ultracentrifugation; dUC) and sucrose gradients or cushions.

dUC isolates EVs based on their sedimentation coefficients. The technique applies increasing relative centrifugal forces to sequentially pellet EVs of decreasing size and density. While larger and denser EVs are typically pelleted at medium speeds (10,000-20,000 × g for 10-90 min), smaller and lighter EVs require higher speeds (100,000-200,000 × g for 45-150 min). However, perfect separation is not achievable, resulting in overlapping properties between pellets. Challenges include low yield of smaller EVs in protein-rich fluids and potential EV aggregation at high speeds. Therefore, intermediate centrifugation pellets should be analyzed alongside the final high-speed pellet. Reporting centrifugation parameters is crucial, including speed, rotor type, time, temperature, and acceleration/deceleration settings. These factors allow calculation of

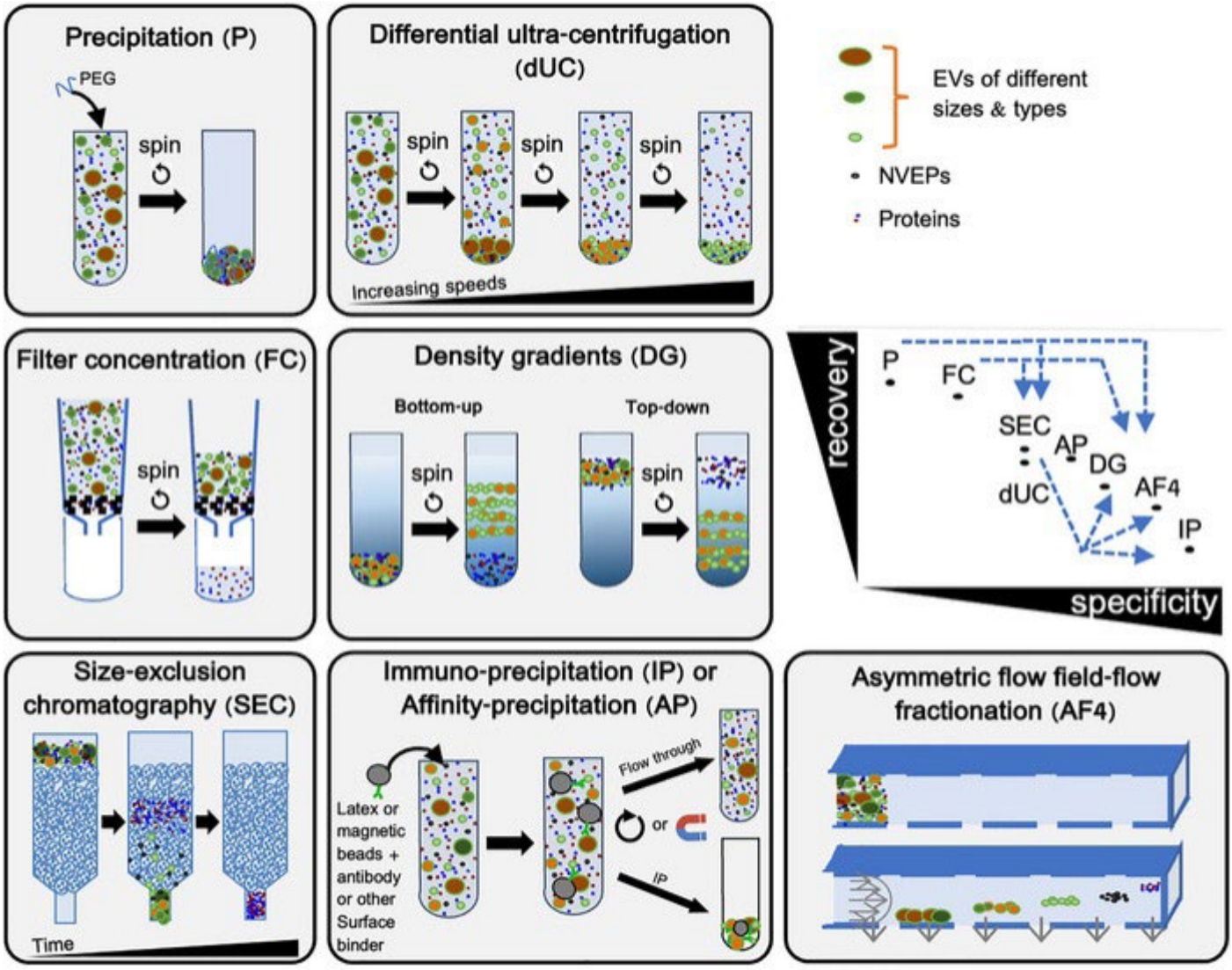


Figure 2. Position of some EV separation and concentration methods on a recovery (yield) versus specificity grid. Dashed blue arrows indicate combinations of methods resulting in increased specificity. Specificity can be of different types: Size exclusion chromatography (SEC) separates EVs by size from many (but not all) NVEPs, but all EV types are recovered together, while differential ultracentrifugation (dUC) separates EV subtypes based on their size/weight, but also co-isolates NVEPs at high speeds. Note that many 'exosome purification' kits use precipitation (P), thus do not isolate pure exosomes or even EVs but a mixture of EPs, while some use affinity precipitation (AP), which may be more specific to EVs but not exosomes. Those who develop new methods should consider positioning their EV outcomes on such a graph.

sedimentation coefficients, theoretically ranging from 15-150 S for larger EVs and 2-5 S for smaller EVs.

Density gradients or cushions employ dense media like sucrose or iodixanol in decreasing density layers to separate EPs based on their characteristic densities. Two approaches are used: bottom-up, where samples are loaded beneath the gradient, and top-down, where samples are placed on top of the gradient or cushion. In bottom-up ultracentrifugation, particles float upwards to their buoyant density, with smaller EVs traveling slower. Top-down approaches allow particles to travel into the gradient until reaching equilibrium buoyant density. Cushions separate particles by a density threshold at the interface. Gradient ultracentrifugation duration varies, ranging from 1-2 hours to over 48 hours for optimal separation. Post-separation, fractions must be carefully collected and density confirmed. The dense medium is typically removed before downstream analysis, often by dilution and ultracentrifugation or size-exclusion chromatography. However, particle recovery after these processes is relatively low.

EV Characterization Methods

EV characterization is essential for quantifying EVs, confirming their presence, and evaluating non-EV

contributions to preparations. Challenges include small particle size, heterogeneity, and the absence of universal identification methods. No single measurement technique meets all characterization requirements, necessitating the use of orthogonal methods to overcome individual method limitations.

Characterization needs vary by sample source, often requiring additional steps and reporting to account for preanalytical variables. The composition of EVs—including proteins, lipids, nucleic acids, and other biomolecules—varies by source, and while these molecular class measurements can estimate EV abundance, they do not universally correlate with EV concentration.

No universal molecular markers for EVs or their subtypes exist. Markers should be selected based on source- and type-specific evidence. Proposed markers, such as Annexin A1, SLC3A2, BSG for ectosomes, and Lamp1 for exosomes, lack universal acceptance. Affinity-based protocols using tetraspanins CD9, CD63, and CD81 do not specifically identify exosomes and do not capture all EVs, highlighting the need for careful marker selection.

Employing orthogonal methods is critical to ensure co-isolates are not responsible for biomarker or functional findings. Transparent reporting of methods and results is crucial for reproducibility, as outlined in EV-TRACK guidelines.

Recommendations for EV characterization, include:

- Each EV preparation should be defined by quantitative measures of the source of EVs (e.g., number of secreting cells, volume of biofluid, mass of tissue).
- Approximations of the abundance of EVs should be made (particle number, protein, and/or lipid content). EV preparations should be tested for the presence of components associated with EV subtypes or EVs generically.
- Establish the degree to which non-vesicular, co-isolated components are present.
- Provide an indication of the instrument/method limit of detection (LOD) when EVs are characterized with quantitative metrics.

The Following Sections Offer Recommendations for Various Specific EV Characterization Approaches

Quantification of Particle Number Concentration

EV number concentration (in particles/mL) is a widely used metric for assay standardization and *in vivo* dosing but often lacks reliability due to limited specificity and sensitivity in measurement techniques. The ISEV Rigor and Standardization EV Reference Material Task Force emphasizes the need to report assay limits of detection (LOD) for validation of findings to allow others to validate findings irrespective of the sensitivity limit.

As an example, EV concentration in blood plasma varies greatly depending on the measurement method, spanning six orders of magnitude. Greater accuracy can be achieved using orthogonal methods with defined LODs, such as light scattering intensity, fluorescence intensity, and physical size, which provide complementary data. For instance, resistive pulse sensing (RPS) reports LOD in diameter, which is influenced by pore size, while flow cytometry reports LOD based on light-scattering models or molecules of equivalent soluble fluorophores (MESF). Techniques like nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), or imaging flow cytometry currently lack methods to derive traceable LODs due to numerous detectability variables. Thus, concentration measurements without phenotypic characterization can lead to overestimation due to dye aggregation.

For methods unable to differentiate EVs from contaminants, concentrations should be reported as “particle or EP concentration” rather than “EV concentration”.

Quantification of Particle Size

Measurements of EV size often assume sphericity or mobility and can be influenced by upstream variables. High-throughput methods like flow cytometry, NTA, RPS, multi-angle light scattering, and DLS assume spherical EVs and measure hydrodynamic diameter, which can overestimate size compared to cryo-EM imaging. No method measures the entire EV size range accurately, and high-resolution cryo-EM, though precise, is low-throughput and may not quantify larger, less abundant EVs. Single-particle techniques reveal that many EV preparations show an asymmetric, right-skewed size distribution, with most EVs <100 nm. The full diameter distribution should be shared, as summary metrics like mean or mode can be skewed by the LOD and size distribution asymmetry. Software variability

and refractive index assumptions can also introduce measurement variations. For techniques that cannot differentiate EVs from contaminants, size should be reported as 'particle' or 'EP' diameter.

Quantification of Total Protein, Lipids, and RNA

Total protein in EV preparations (in µg or µg/mL) can be estimated using a variety of assays, each varying in sensitivity and accuracy. Measurement details, such as the physical disruption method and detergent used, should be provided. Using protein concentration as a surrogate for EV concentration is generally not recommended due to variability in protein enrichment across cellular phenotypes. Absolute protein and particle concentrations should be reported separately if ratios are given.

Total lipid quantification of EV samples can be achieved using a variety of assays, however, these methods may lack sensitivity for small EV amounts and often require specialized equipment. Lipid measurements may overestimate EVs due to co-isolated non-vesicular extracellular particles (NVEPs).

Total RNA quantification is crucial for EV characterization but is not recommended as a surrogate for EV concentration due to abundant extra-EV RNA. RNA quantification methods may not distinguish between RNA and DNA, and pre-treatment with RNase-free DNase may improve accuracy.

Characterization of EVs by Morphology and Protein Composition

High-resolution imaging techniques such as electron microscopy (EM) and atomic force microscopy (AFM), are best for assessing smaller EV morphology. Larger EVs (≥200 nm) can be assessed by conventional light microscopy. These methods are not interchangeable and may produce varying image quality, e.g., desiccated conditions can cause artefactual shapes not seen in hydrated conditions.

MISEV2023 endorses the five-component framework from MISEV2018 for reporting EV protein content (Table 2). MISEV2023 also offers a few marker examples and suggests using databases like Uniprot for additional markers. Ideally, marker enrichment or depletion in EVs versus source material should be shown. Although these categories apply to all EVs, some markers may not be usable in single-EV analysis techniques, requiring alternative controls.

Flow Cytometry-based Methods

EVs can be labeled with antibodies to detect specific markers on their surface or to assess their heterogeneity by using multiple markers whenever possible. Another approach is to genetically label EV proteins by introducing a genetic construct that co-translates a tag, such as GFP, with the protein or protein domain of interest. Flow cytometry is commonly used to detect the fluorescence associated with these labeled EVs

Bead-based Flow Cytometry

Bead-based flow cytometry is extensively used to analyze EV surface proteins. Large beads, such as surfactant-free aldehyde/sulfate beads, capture particles regardless of surface composition, while antibody-conjugated beads capture particles exposing specific antigens. Commercial EV multiplex kits enable the interrogation of over 30 surface antigens. Bead-associated particles are labeled with fluorescent affinity reagents for detection. Differences in staining intensity are semi-quantitative due to signals from multiple particles per bead, reflecting variations in particle concentration, epitope density, diameter distribution, or EV subset abundance. Controls should include isotypes for detection antibodies, isotype-conjugated capture beads, and capture beads with detection antibodies alone. Multiple EV input concentrations are recommended to demonstrate signal titration and rule out non-specific binding.

Normalized bead median fluorescence intensities should be reported instead of stained bead percentages, and data should be reported in molecules of equivalent soluble fluorophore (MESF) from singlet-gated beads for standardization. For in-house bead preparation, reagents and conjugation chemistry should be detailed, while for commercial beads, catalog and lot numbers should be provided. Additional reporting parameters include total bead number, sample-bead incubation time, post-incubation wash methodology, detection reagent staining time, and post-staining wash methodology.

Single-EV Flow Cytometry

Flow cytometry is capable of detecting vesicles as small as ~40 nm in specialized cases and ~100 nm using modern conventional cytometers through light scatter and fluorescence. Calibration of flow cytometry data allows characterization of particle diameter, epitope abundance, epitope density, effective refractive index, and number concentration within standardized size ranges. A 2023 tri-society working group published a compendium for developing single-EV flow cytometry assays [2].

Table 2

Protein Content-based EV Characterization. At least one protein of categories 1, 2, and 3 should be analyzed as EV hallmarks to assess the presence of NVEPs in an EV preparation. Analysis of proteins of category 4 is optional, as they may be present in some subtypes of EVs, or under certain conditions, with no general rule. Proteins of category 5 may bind to EVs after their release and may be part of the recently described EV 'corona'. ***Please note that this table provides a limited number of examples only*** for proteins commonly found in mammalian cell-derived EVs. Other proteins that fall into the given categories may be equally valid, particularly for analysis of EVs from prokaryotic (bacteria) or non-mammalian eukaryotic sources (including parasites and plants). For most proteins of interest, their subcellular location in intracellular compartments (for categories 1 and 4), or their transmembrane or lipid-anchored nature (for categories 1 and 2), is provided in the Uniprot database (https://www.uniprot.org/). *XX* = human gene names. *XX** or *XX*** used for families of multiple proteins, for example, for integrins: *ITGA** indicates any integrin alpha chain.

Category				
1- Transmembrane (or GPI-anchored) proteins associated with plasma membrane and/or endosomes All EVs Non-exhaustive examples, categorized a, b, c: by decreasing strength of membrane association.	**2- Cytosolic proteins in EVs** All EVs	**3- Major components of non-EV co-isolated structures (NVEPs)** All EVs as purity control	**4- Transmembrane, lipid-bound and soluble proteins associated with intracellular compartments other than PM/endosomes** Subtypes of EVs and/or pathologic/atypical state, and/or novel separation method	**5- Secreted proteins recovered with EVs** Corona or functional component of EVs
1a: multi-pass transmembrane proteins. Tetraspanins (CD9, CD63, CD81, CD82); other multi-pass membrane proteins (CD47; heterotrimeric G proteins GNA*, TSAP6)	**2a: with lipid or membrane protein-binding ability.** ESCRT-I/II/III (TSG101, CHMP*) and accessory proteins: ALIX (PDCD6IP), VPS4A/B; ARRDC1; Flotillins (FLOT1/2); caveolins (CAV*); syntenin (SDCBP)	**3a: lipoproteins.** Produced mostly by liver, abundant in plasma, serum. Apolipoproteins	**4a: nucleus.** Histones (HIST1H**); Lamin A/C (LMNA/C)	**5a: blood-derived corona proteins.** Partially overlapping with 3a/3b: apolipoproteins, complement, fibrinogen
1b: single-pass transmembrane proteins. Major Histocompatibility Class I or II, Integrins (ITGA*/ITGB*), transferrin receptor (TFR2); LAMP1/2; heparan sulphate proteoglycans including syndecans (SDC*); EMMPRIN (BSG); ADAM10	**2b: promiscuous incorporation into EVs (and possibly NVEPs).** Heat shock proteins HSC70 (HSPA8), and HSP84 (HSP90AB1) note that both are abundant also in NVEPs; cytoskeleton: actin (ACT*), tubulin (TUB*); enzymes (GAPDH)	**3b: protein and protein/nucleic acid aggregates.** Immunoglobulins (blood); Tamm-Horsfall protein (Uromodulin/UMOD; urine); albumin. YWAH* (14-3-3*) and AGO* (can be present in EVs but generally more abundant in NVEPs).	**4b: mitochondria.** VDAC, cytochrome C (CYC1); TOMM20	**5b: cytokines and growth factors.** e.g., TGFB1/2; IFNG, VEGFA, FGF1/2, PDGF*, EGF, interleukins (IL*)
1c: GPI- or lipid-anchored proteins. Glypicans (GPC1), 5′nucleotidase CD73 (NT5E), complement-binding protein CD59		**3c: exomere or supermere-enriched components.** HSP90AA/B, TGFBI, HSPA13, LDHA/B	**4c: secretory pathway.** Endoplasmic reticulum, Golgi apparatus: calnexin (CANX); Grp94 (HSP90B1); BIP (HSPA5), GM130 (GOLGA2)	**5c: adhesion and extracellular matrix proteins.** Fibronectin (FN1), Collagens (COL**), MFGE8; galectin3-binding protein (LGALS3BP), CD5L; fetuin-A (AHSG)
			4d: others. Autophagosomes, cytoskeleton… LC3 (MAP1LC3A), Actinin1/4 (ACTN1/4)	

Calibration of fluorescent and light scatter parameters is crucial for the interpretation and replication of single-EV flow cytometry results. If particle concentrations are reported using single-EV flow cytometry, it is important to define the upper and lower LOD to allow for data replication and interpretation using orthogonal techniques. However, determining the lower LOD can be challenging to define and standardize, particularly for imaging cytometers that use dynamic triggering methods.

The MIFlowCyt-EV framework, developed in 2020, provides comprehensive guidelines for flow cytometry experiments and reporting, including preanalytical variables, experimental design, sample preparation, assay controls, instrument calibration, data acquisition, EV characterization, and data sharing [3]. Resources for implementing MIFlowCyt-EV are available on the EV Flow Cytometry Working Group website (http://www.evflowcytometry.org/). The framework applies to all flow cytometers, including conventional, spectral, imaging, and single-photon-detecting cytometers.

Other Techniques to Characterize EVs

Additional methodologies that are used to characterize EV are listed in Table 3.

Conclusions

MISEV2023 provides guidelines and recommendations for EV research, spanning basic to advanced state-of-the-art technologies and methodologies. It represents the current best practice in the field and is the current consensus position of the EV community.

References

[1] Hendrix, A., *et al.* (2023). Extracellular vesicle analysis. *Nature Reviews Methods Primers*. DOI: 10.1038/s43586-023-00248-5

[2] Welsh, J. A., *et al.* (2023). A compendium of single extracellular vesicle flow cytometry. *Journal of Extracellular Vesicles*. DOI: 10.1002/jev2.12299

[3] Welsh, J. A., *et al.* (2020). MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments. *Journal of Extracellular Vesicles*. DOI: 10.1080/20013078.2020.1713526

Table 3 **Techniques Used to Characterize EVs.**

Technique	Description/Application
Mass spectrometry (MS)	commonly used in EV studies to detect and characterize EV-associated proteins, both in discovery and targeted applications.
Atomic Force Microscopy (AFM)	enables label-free imaging of individual EVs and co-isolated nanoparticles, providing information on size distribution, ultrastructural details, and the presence of contaminants.
Total Internal Reflection Microscopy (TIRF-M)	confocal microscopy and light-sheet microscopy are utilized for studying cell-EV interactions, EV release and uptake, and the composition of single EVs.
Dynamic Light Scattering (DLS)	determines the hydrodynamic diameter of monodisperse particles in aqueous dispersions.
Electron microscopy (EM)	EM variants can detect EVs irrespective of size, but larger EVs may be statistically underestimated compared to smaller EVs due to throughput limitations.
Nanoparticle Tracking Analysis (NTA)	estimates particle size, concentration, and effective refractive index, although interpretation should be cautious in complex biofluids due to co-isolates and difficulty quantifying larger EVs.
Single-Particle Interferometric Reflectance Imaging Sensing (SP-IRIS)	derives size and number of captured particles from interference patterns, but variations in refractive index may impact measurements.
Super-resolution microscopy	provides high-resolution imaging for detecting and characterizing individual EVs and their components.
Reverse Transcription Real-Time Quantitative PCR (qPCR)	widely used for nucleic acid detection in EVs, but biases can be introduced during RNA purification and pre-assay preparations.
Raman Spectroscopy (RS)	resolves chemical composition based on scattered photons, but calibration is essential to address inter-device variability.
Resistive Pulse Sensing (RPS)	determines particle concentration and diameter using the Coulter principle, but it cannot differentiate EVs from co-isolates in complex biofluids.
Western blotting	commonly employed to detect proteins in EV-containing preparations, but suitable loading controls may be challenging in biofluids compared to cell cultures.

Pioneering Advances in Extracellular Vesicle Research

Interview with Dr. Luca Musante

Website: LinkedIn

Dr. Luca Musante is a renowned extracellular vesicle (EV) research expert. He holds a B.S. in Biological Sciences, an M.S. in Biochemistry and Analytical Chemistry, and a Doctorate in Experimental Medicine. Dr. Musante has, to date, authored 69 peer-reviewed publications and developed the innovative Hydrostatic Filtration Dialysis (HFD) method to optimize EV recovery. His work focuses on understanding the role of EVs in diseases such as diabetes and hypertension, utilizing state-of-the-art analytical platforms. Dr. Musante promotes rigorous, reproducible research and fosters scientific collaboration.

Dr. Luca Musante's journey into EV research began in 2009, focusing on urinary extracellular vesicles (uEVs) as potential biomarkers for diseases such as diabetes and hypertension. His work led to the development of the Hydrostatic Filtration Dialysis (HFD) method, a novel technique designed to optimize the recovery of uEVs during centrifugation. This method addresses the biological variability inherent in biofluids, allowing for the maximization of representative vesicle populations without the need for large volumes of urine for biobanking. The HFD method has since become a valuable tool in the study of uEVs, enhancing the reliability and reproducibility of research findings.

Throughout his career, Dr. Musante has acquired extensive experience in EV separation techniques, including centrifugation, density, and size exclusion chromatography. He is proficient in utilizing advanced analytical platforms such as Microfluidic/Tunable Resistive Pulse Sensing (MRPS and TRSP), Nano Track Analysis (NTA), Flow Cytometry, and Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS). These technologies enable detailed analysis and multiplexed phenotyping of EVs with minimal sample processing, pushing the boundaries of what is possible in EV research.

Dr. Musante is a passionate educator and mentor, having trained numerous scientists and students at various levels, from undergraduates to senior Ph.D. candidates. He emphasizes the importance of strict analysis protocols for the preparation and characterization of EV samples to ensure the production of reliable, replicable results. His commitment to maintaining high standards in EV research is reflected in his adherence to guidelines outlined in positional papers issued by the Journal of Extracellular Vesicles and endorsed by the International Society of Extracellular Vesicles.

In this interview, we delve into Dr. Musante's work, his insights into the future of EV research, and his vision for fostering collaboration and innovation within the scientific community. Join us as we explore the fascinating world of extracellular vesicles and the impact of Dr. Musante's contributions to this dynamic field.

What motivated you to focus your research on Extracellular Vesicles?

The EV field caught my attention after Dr. Knepper's group demonstrated that urinary exosomes, as they were called at the time, contain hundreds of well-known disease-related proteins [1,2], back in 2004. I was conducting research in the field of nephrology at the time and I was intrigued by the potential of urinary EVs as a new source of biomarkers related to kidney disease and urogenital disorders. That was the beginning of my journey into the world of EVs. It was 2009, and I was a postdoctoral researcher at Dublin City University (DCU), Ireland, working in Professor Harry Holthofer's laboratory. Back then, only a few tools for analyzing EVs were available. Besides Electron Microscopy (EM), most

of the analysis was protein-based and carried out by Gel Electrophoresis (SDS_PAGE), Western Blot (WB), and Mass Spectrometry (MS). As a result, my experience with these analysis tools and my interest in protein analysis proved valuable for the development of the project I was responsible for.

With the development of the field and the emergence of new instruments and technologies, I was fortunate to have the opportunity to learn the use of Tunable Resistive Pulse Sensing (TRPS) at DCU. In 2016, I joined Dr. Uta Erdbrugger's lab at the University of Virginia and continued to acquire new skills in EV analysis tools, such as Nanoparticle Tracking Analysis (NTA), Flow Cytometry (FCM), and Single-Particle Interferometric Reflectance Imaging Sensing (SP-IRIS), as new instruments were gradually acquired by either the lab I was a part of or the Flow Cytometry Core at the University. This allowed me to gain experience with virtually every particle analyzer that was available at that time. In my current position as Director, I devote my time exclusively to the isolation and analysis of EVs, along with other types of lipids and non-lipid particles. Our laboratory is equipped with several EV isolation and analysis instruments, including the last generation of Nanoflow Cytometers (nFCM), which allow me to continue exploring this fascinating nano-cosmos.

Could you share a specific research project that you found particularly exciting or impactful?

In the laboratory of Dr. Uta Erdbrugger at the University of Virginia, we collaborated with Dr. Steven Mallin from the Department of Kinesiology. The main goal of the study was to determine whether circulating EV levels in subjects with metabolic syndrome changed following physical exercise or metabolic challenges induced by either insulin infusion or oral glucose administration. Along with their role as novel biomarkers of Type 2 diabetes and cardiovascular disease (CVD) risk, EVs have also been proposed as potential mediators of exercise-induced cardiometabolic health. As a result of preliminary findings, it was postulated that physical exercise may reduce CVD risk because of the interaction between EV and insulin. More research is needed to fully understand how physical exercise and diet can affect the repertoire of circulation EVs, as well as the molecular benefits.

Anyway, as someone who has always trained in the gym balancing strength and cardiovascular workouts and focusing on my diet, I appreciate the fact that EV research also demonstrates that eating a balanced, healthy diet and exercising regularly is truly the way to administer to the body the best medicine to cure, heal, and prevent all co-morbidities associated with metabolic syndrome. As a result of this regimen, the body is healthier, since lymph circulation is sustained by vascular smooth muscles, contraction of skeletal muscles, respiratory movements, and arterial pulsations, thus playing an important role in immune surveillance in addition to the other functions carried out by the lymphatic system [3]. In this context, it surely would be interesting to know more about the role of lymph-circulating EVs.

Can you discuss any collaborations or partnerships you've had with other researchers or institutions?

As Director, I have been exposed to a wide range of projects and studies focused on the entire animal kingdom, including eukaryotic and prokaryotic organisms and plant EVs. Normally, the moment I meet with scientists to discuss their new projects, I am hooked and feel deeply involved in the discussion as if I were part of their team. A recent collaboration - still in progress - aims to understand how UV radiation can induce inflammation of the derma. The hypothesis is that UVB-irradiated mice produce keratinocyte-derived EVs that penetrate the basement membrane and promote inflammation. As of now, we have finished setting up the protocol to isolate EVs from the derma and performed a basic characterization of the EV population to identify EVs that carry keratinocyte markers. Following the establishment of the method, the study will then compare EVs from irradiated mice with those from non-irradiated controls.

What techniques or methodologies do you find most valuable in your work?

It is difficult to give a straight answer, since it depends on the goal of the analysis and the question we want to address. At present, I am in a privileged position since I can access a variety of particle analyzers, so I can choose and suggest which instrument is most appropriate for each project. Each analytical platform has its advantages and limitations due to its design. For example, if I have a mixture of homogeneous particles such as 70, 100, and 150 nm polystyrene beads, TRPS/MRPS will provide a higher and better separation than NTA (Fig. 1). NTA can measure every single population (Fig. 1A) but when we pool and mix the three sets of beads in equal volume,

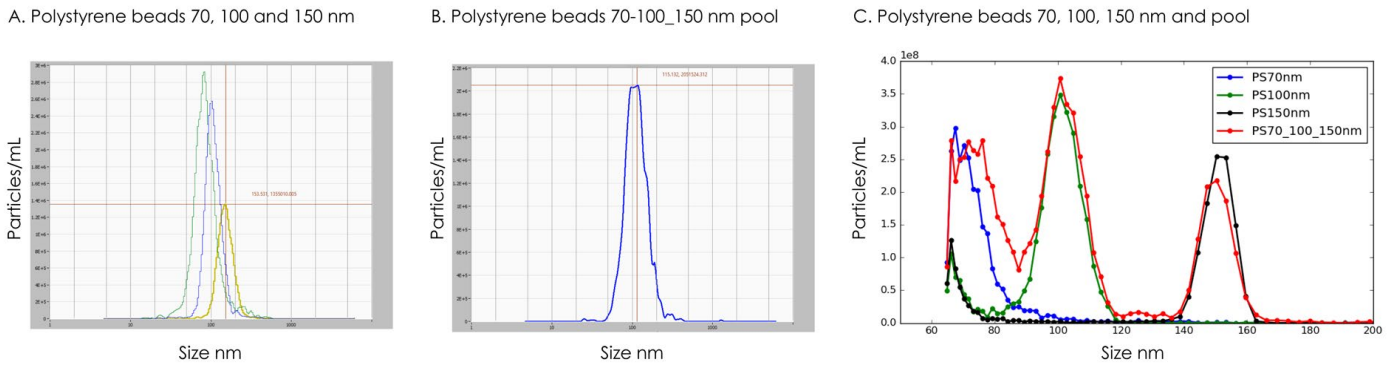


Figure 1

the size distribution merges into one population of particles (Fig. 1B). On the contrary, MRPS can separate the three populations when the beads are pooled together (Fig. 1C).

As far as marker detection by fluorescence reporters is concerned, Nanoflow Cytometers (nFCMs) are considerably more sensitive than other platforms available in the Core Facility. If, however, the aim is to detect an intraluminal protein, such as Syntenin-1, using SP-IRPS would make the entire analysis less labor intensive than with nFCM, also resulting in a lower loss of EVs. To remove excess reagents from fixation, permeabilization, and staining, one must either centrifuge the sample after each step [4] or likewise use desalt Size Exclusion Chromatography (SEC) columns. Finally, my experience brings me to believe that one of the most valuable methods to examine EVs is to look at their protein pattern by electrophoresis (SDS-PAGE).

Users in the Core Facility often ask me how to test the purity of an EV preparation. I merely apply the same concept and approach used in protein purification. In other words, I check all the fractions generated during the isolation protocol, beginning with raw material and continuing until the final product is obtained. As we progress through the protein purification protocol, the complexity of the protein pattern progressively decreases until only a few bands are detected in SDS-PAGE gels depending on the protein structure. The EV proteome is, of course, composed of several hundreds if not thousands of proteins, as reported by the proteomic profiling by Mass Spectrometry. If we apply this approach, however, we can still identify how protein profiles change during a separation procedure, such as differential centrifugation to sediment particles at different Relative Centrifugation Forces (RCF), as shown in Figure 2.

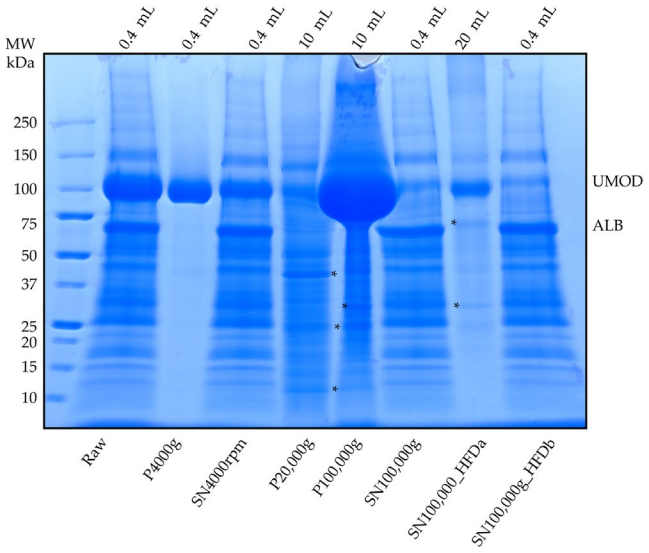


Figure 2

This image clearly illustrates how the protein pattern changes following differential centrifugation using urine as a specimen. The equivalent volume of urine loaded to have good staining and to visualize the protein distribution for each fraction generated in the protocol. Some bands (*) are visible in the centrifugation pellets but not in the supernatants. Conversely, we can observe that some proteins like albumin (ALB) are mostly present in the supernatant at 67 kDa, and so is the partitioning of uromodulin (UMOD) at 100 kDa. Uromodulin is a very interesting protein because as soon as it gets released into the lumen of the tubules, it starts to associate, forming dimers, trimers, oligomers, and polymers that can sediment at any speed, depending on the size of the complexes. In the Hydrostatic Filtration Dialysis paper [5], Figures 5 and 6 illustrate a more in-depth analysis, also corroborated by Western Blots, to investigate EV enrichment and/or purity grade.

How did you develop the Hydrostatic Filtration Dialysis (HFD) method, and what impact has it had on EV research?

As often occurs, the HFD method was a stroke of serendipity. Back in 2009, urinary Extracellular Vesicles (uEVs) were a very promising source for the identification of new biomarkers for the diagnosis and prognosis of kidney disease, particularly diabetic kidney disease. At that time, we did not have access to any of the particle analyzers we can avail of today. Our first attempt to measure the particle size was by Dynamic Light Scattering (DLS). There were different ways to export the results, such as by size, expressed in nanometers (nm), and by molecular weight, expressed in Dalton, and it was fascinating to learn that the size of EVs we were measuring was in the order of mega Dalton. As a result of the decision to focus exclusively on uEVs for biomarker discovery, we decided to introduce a dialysis step using membranes with the largest Molecular Weight Cutoff (MWCO) available on the market, which was 1,000 kDa.

The objectives were:

1. To equalize the urine composition so that when the enrichment began all samples were in the same condition.
2. To eliminate the excess of all molecules smaller than the MWCO, particularly albumin, that was present in different amounts in the study groups that included normo-micro and macroalbuminuric samples.

In the first set of dialysis, we did not have any clips to seal the dialysis tube. Therefore, we tied a simple knot at the very end of the membrane. We filled the tube with water and applied some pressure with our fingers to check for leaks from the knot. Interestingly, that little pressure was sufficient to push water through the dialysis membrane. First, it appeared that the knot had not been sufficiently tightened to seal, as water dripped profusely. We then realized that water was “sweating” from the whole surface of the dialysis membrane, just with a small amount of pressure. After that, we connected one end to a funnel and clipped the other end. The hydrostatic pressure of the liquid sitting on top of the dialysis membrane in the funnel was sufficient to force water through the membrane, resulting in the birth of HFD [5].

HFD offers an alternative method of enriching EVs which does not require any expensive equipment, but only basic tools commonly found in any laboratory, such as parafilm, a cylinder, and a funnel. Only some dialysis membranes and clips need to be purchased. The method can be used with virtually any biofluid and type of conditioned media. It has been applied to isolate outer membrane vesicles from bacterial culture media [6] and immortalized podocyte cell lines [7].

Could you elaborate on the use of advanced analytical platforms like MRPS, TRSP, NTA, Flow Cytometry, and SP-IRIS in EV analysis? How do these tools enhance our understanding of EVs?

They are all important tools for characterizing particles and EVs. I often remind our young, and sometimes less young, scientists in the Core Facility about the fact that EVs are particles, but not every particle is an EV. Having said that, one of the most useful tools is the SP-IRS, which is designed to capture CD9, CD63, and CD81 in the basic tetraspanin kit, CD41a capture antibody in the plasma kit, and finally an isotype control that is part of the chip to verify non-specific binding. The detection antibody cocktail usually consists of the same clones of Abs conjugated with a fluorescence dye, such as AF488 for CD9, AF555 for CD81, and AF647 for CD63. Over the last few years, more kits have become available that allow the capture chip to be customized with antibodies of interest, and reagents have been added to stain intraluminal proteins, such as syntenin -1. As it is highly sensitive, it usually does not require any enrichment steps, so we can check the tetraspanin profiles in the native sample. However, when analyzing plasma EVs, a size exclusion chromatography step is strongly recommended to minimize some unidentified background noise.

In addition to its flexibility, this technique has also a wide range of other applications. We can evaluate the relative abundance of tetraspanin in our EV population, check what markers are co-expressed on the same EV, and find out whether different enrichment protocols are more effective at isolating certain types of tetraspanins, as an example. Nano-flow cytometers can detect particles by side scatter as well as EVs, based on the staining dye's properties, such as antibodies, lipids, and Annexin-V, to name a few. It is common practice to detect EVs using a lipid dye but the dye stains any lipid particles, including lipoproteins, and it is also possible that large hydrophobic proteins or complexes of proteins are stained, as we can sometime see in the buffer + reagent control made of a lipid dye and an antibody. Therefore, it is necessary to consider the EV source, such as plasma EVs versus urinary EVs or serum-free conditioned media EVs versus serum-supplemented conditioned media. By detecting specific markers, nFCM may be able to provide some additional information not only about EVs, but also

about the presence of other types of particles and the ratio between particles and EVs.

In addition to a comprehensive general characterization of our EV preparation, this is also essential for successful staining. FCMs have an optimal particle concentration range to avoid swarming effects, which can be estimated by the event rate. For this reason, it is very important to analyze good EV prep with minimal carryover of other types of particles that are detected by side scatter. A nFCM provides a multiparametric analysis of several targets on the same sample simultaneously. In terms of particle and EV characterization, the nFCM is unquestionably a very sensitive and powerful tool. As NTA and RPS only detect particles, EV characterization requires additional studies to provide more supporting evidence. Because some instruments are equipped with fluorescence detectors, the same considerations made for the nFCM can be applied to these instruments. The tools are user-friendly, the results are straightforward, and they offer valuable information about particle size distribution and concentration in a timely manner. Nominating a winner is difficult and probably unfair. All of them are effective in delivering what they were designed for. To learn more about it, I recommend reading the paper by Tanina Arab and colleagues [8].

Finally, super-resolution microscopy analysis of EVs is becoming increasingly popular thanks to the introduction of PALM and STORM techniques. These types of microscopes can provide imaging with a resolution as low as 20 nm, so it is possible to see the EV markers distribution both on the surface and in the EV lumen and determine the size, shape, and location of the molecule of interest in fine detail.

In your work to optimize the recovery of representative vesicle populations, what novel techniques or strategies have you explored?

It is becoming increasingly important to sort out specific EV populations and there is a high demand for tools designed to do so. The use of flow cytometry for sorting EVs is still limited, although several publications have demonstrated its feasibility [9,10]. A larger use of the system is, however, restricted by several obstacles, including the limited amount of sorted material for downstream applications, the long sorting time, and associated costs. No doubt sorting by flow cytometry will become a possibility, more easily accessible, and more cost-effective in the near future.

Recently, I have focused my attention on the isolation and recovery of vesicles using the affinity capture technique. I tested several commercial kits, both antibody and non-antibody-based systems. Our first goal has been to isolate high-purity, high-yield EVs from plasma for proteomic and transcriptomic studies. The ultimate goal is to enrich EVs with as few lipoproteins and other abundant proteins in plasma as possible. This is not an easy task. A major aspect of the complexity of plasma is its protein composition, as demonstrated by proteomic analysis by MS, as well as its particle-protein interaction, which can form supramolecular structures that can make EV isolation harder, a matter that, in my opinion, has been understudied. Moreover, lipoproteins overlap with EVs in size and/or density, are more abundant than EVs by several orders of magnitude [11], and carry microRNAs [12]. This greatly complicates the analysis, particularly if we want to link the candidate biomarker(s) specifically to EVs. Most kits work, but their utility is limited by the presence of these contaminants.

Another objective of the affinity capture strategy is to reduce the complexity of the EV population, thereby making it easier to detect low-expressed EV markers using nFCMs. The detection of low-expressed markers such as PD-L1 can be hindered by the presence of excessive particles that are not EVs and also by EVs that do not carry PD-L1. The idea is to perform a negative subtraction removing – for example – EVs positive for tetraspanin, usually very abundant in an EV prep, to lower the detection limit. We must emphasize that our marker should not be co-expressed on the same particle, otherwise it will be lost during the capture process. Working with these affinity kits I am intrigued by the fact that in many commercial kits' protocols, as documented in the literature [13], EVs or - more precisely - EV markers (e.g., microRNA) are eluted by solutions containing either organic solvents or detergents. In native conditions, eluting EVs is extremely challenging.

Considering the growing interest in EVs as biomarkers for disease, how do you envision the future of EV analysis? Are there any specific challenges you're actively addressing?

Most analytical assays are antibody-based, such as flow cytometry, super-resolution microscopy, or SP-IRIS, for example. Currently, we are using clones of antibodies and their conjugated forms generated for cell biology applications. Of course, an antibody staining on a cell that is several microns in size is not the same as staining a particle that is 40-150 nanometers in size. A variety of factors must be considered when detecting and/or visualizing EV targets using flow cytometry or super

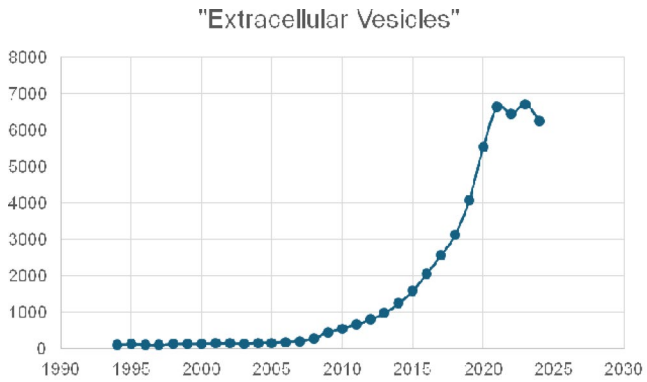
resolution microscopy, including hysteric hindrance, copy number of the antigen, epitope distance, type and size of fluorescence dyes (phycoerythrin PE versus Alexa Fluor AF-555, as an example). Some of the experiments I have conducted at different times aimed to use the same probe such as antibodies, annexin V, and streptavidin conjugated with different fluorescence molecules. The results indicate that the same binder has different effects depending on the size nature of the reporter. Moreover, in FCM experiments, it is not uncommon that the same clone of antibody stains differently depending on whether it is in a single staining tube or an antibody cocktail tube. Detecting low-represented markers, as described in the previous question, can be challenging, and that is the reason why I started testing some commercially available kits to find a solution. So, these are currently some of the main challenges. I wouldn't be surprised if, in a few years, we will have reagents such as antibodies designed specifically to detect EV markers with a selected type of reporter and perhaps introduce spacer arms to extend the distance between fluorescence dyes. The same concept applies to the affinity capture of EV on solid phases, such as chips or magnetic beads. The spacer arm can confer some flexibility to the capture antibody, improving the affinity and capture yield eventually.

Could you elaborate on any recent breakthroughs or discoveries in your field?

There is no easy answer to this question. First of all, there has been a dramatic increase in the number of publications on EVs. A PubMed search using the keyword 'extracellular vesicles' demonstrates this growth (Figure 3).

It seems that the number of publications per year has stabilized on an average of over 6500 in the last four years, which is equivalent to nearly 18 new papers per day on average. As a result of using more specific search keywords such as ‘exosomes’, ‘microvesicles’, ‘microparticles’, and ‘extracellular vesicles’ with different search engines (Pub Med, Web for Science and Google), I am now receiving notifications of new publications every day, and it becomes increasingly difficult to keep up with this pace.

As there have been many excellent papers published, deciding on one would inevitably diminish many others, so it is a difficult decision. With that in mind and in response to the question, I have selected two papers by Zhong W and colleagues [14] and Gong N and colleagues [15]. These two papers illustrate how you may cross paths with EVs at some point, regardless of whether they are the subject of your project or not.



Search query: extracellular vesicles

Year	Count
2025	20
2024	6243
2023	6719
2022	6445
2021	6648
2020	5538
2019	4079

Figure 3

In both papers, EVs secreted in the tumor microenvironment play a protective defensive role to resist CAR T cell therapy and prevent LNP from delivering their cargo redirecting them to the liver for degradation. Therefore, a new chapter in pharmacokinetics has begun, which may lead to new strategies to evade tumor EV-mediated surveillance so that therapy can be effective.

What challenges do you face in balancing lab work, teaching, and other responsibilities?

Being in charge of a Core Facility has been a completely new experience, and I have been learning how to navigate this new world. Although the EV field has been growing considerably, we are still a small Core Facility and we have to optimize our resources to achieve our goals. Currently, I do not have any technical assistance and I manage many aspects of the lab single-handedly, integrating the scientific and technical responsibilities with most of the administrative aspects of running a Core Facility.

I'm very fortunate to have the support of our Finance and Legal teams for complex financial and legal matters. However, the Core wouldn't exist if it wasn't for my Principal Investigator. He has been an unwavering

advocate for the EV Core Facility since he founded it in 2019, making it the first on the East Coast to exclusively focus on EV isolation and characterization. His relentless pursuit of support has been instrumental in enabling us to offer essential services such as training, project planning consultation, and guidance on EV analysis and characterization in alignment with ISEV and MISEV standards.

The need to maintain all instruments' uptime and accuracy also requires considerable effort. So, instruments that are in high demand receive priority in terms of being covered by service contracts, and over time one learns to identify which are the ones it is possible to gamble upon, counting on their robustness. Sometimes users come to the lab with high hopes, such as wanting to isolate EVs from minimal plasma volumes with high yield and purity in a single step. That's when guidance and experience can be of help to bridge the gap between what is feasible and what is not. I address these challenges with dedication and hard work, consistently striving to provide the best service to our users and clients.

What advice would you give to students or researchers interested in pursuing a career in Extracellular Vesicle research?

To begin with, it should be noted that science is not easy in general and that some branches of science are more challenging than others. According to my experience, EV research is a very challenging field as there are no tools for EV amplification, and we can only scale up volumes to obtain the quantities of EVs necessary for basic characterization and downstream analysis. As a result, it can sometimes be frustrating, requiring a great deal of patience, dedication, and perseverance. It is, however, one of the most fascinating biological subjects with a virtually endless number of potential applications. It provides a good grounding in biochemistry, physics, and cell biology. All aspects of the "omics" analysis, as well as engineering, physiology, and pathophysiology can be covered.

I would recommend not fearing backfires and failures, as there are no negative results. It is not uncommon for experimental results to differ from what we expected. Therefore, I would also encourage them to be flexible and ready to revise their hypothesis if their experimental results are "unexpected". Very importantly, I would advise them to introduce as many controls as possible into their experiment and never take anything for granted. This job requires being prepared to play a sort of mind game to solve the mystery and complete the puzzle after every experiment. Discovering something new is extremely satisfying, especially as their research might directly contribute to the advancement of science in a way that benefits humanity, our animal companions, livestock well-being, and ultimately the quality of our food. By adopting this mindset, they can remain dedicated to their work in spite of obstacles they may encounter. I am a firm believer in the "never ever give up" motto.

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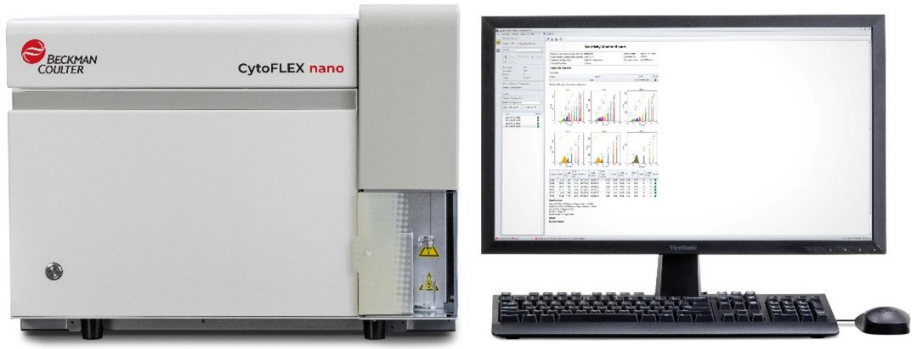
APPLICATION NOTE

A new approach to nanoscale flow cytometry with the CytoFLEX nano analyzer



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Background

Nanoscale flow cytometry is a cutting-edge technique that combines the principles of flow cytometry with nanotechnology. It enables the analysis of particles at the nanoscale, providing valuable information about their size, composition, and surface properties. Nanoscale flow cytometry has numerous applications in various fields, including biology, medicine, and materials science. It allows researchers to analyze and characterize nanoparticles, extracellular vesicles (EVs), and other small particles with great precision and sensitivity. In addition to its research applications, nanoscale flow cytometry also holds promise in the development of diagnostic tools and targeted drug delivery systems. The ability to analyze nanoparticles based on their characteristics opens new possibilities for personalized medicine and nanomedicine.

While nanoscale flow cytometry is a powerful technique with numerous advantages, it also has some limitations, including:

Detection sensitivity: there are still limits to the smallest particles that can be detected reliably. Currently, flow cytometers are not designed to detect and characterize EVs smaller than 100 nm. The detection threshold for nanoparticles is influenced by factors such as background noise, autofluorescence, and the efficiency of labeling or detection probes.

Size resolution: Flow cytometry can characterize particles in the nanoscale range, but accurately resolving nanoparticles of similar sizes can be challenging. Distinguishing particles with very small size differences, such as distinguishing between 50 nm and 60 nm particles, may be difficult due to limitations in the detection and resolution capabilities of the instrument.

Sample preparation: Preparing samples for nanoparticle or EV detection can be complex. Sample preparation methods need to consider factors such as aggregation, stability, and potential alterations of particle properties during the process. Obtaining a representative and homogeneous sample can be crucial for accurate analysis.

Standardization: Nanoscale flow cytometry is a relatively new and evolving field, and the field is still working on standardizing protocols and reference materials. This can lead to variability in data collection and analysis across different laboratories, making it challenging to compare results and establish consistent methodologies.

Data analysis complexity: Flow cytometry generates complex and multidimensional datasets, which require sophisticated data analysis techniques. Analyzing and interpreting large datasets can be time-consuming, requiring expertise in data processing, visualization, and statistical analysis.

In this application note, we will introduce the CytoFLEX nano Flow Cytometer and its workflow. This new analyzer is the first purpose-built nanoscale flow cytometer that can detect nanoparticles, such as extracellular vesicles (EVs) at least as small as 40 nm, while simultaneously performing multiparameter fluorescent detection. Furthermore, it enables counting, characterization, and particle size definition, all within a single instrument, setting a new standard and overcoming current limitations for nanoparticle research. The CytoFLEX nano software interface, CytExpert nano, provides the sophistication to explore the unknown at the nanoscale range while providing the ease-of-use characteristics of the CytoFLEX platform. This way, getting answers to challenging research questions becomes easier than ever for EV researchers.

The CytoFLEX nano Flow Cytometer offers:

1. High sensitivity to detect and characterize nanoparticles at least as small as 40 nm (based on polystyrene beads).
2. High resolution to accurately distinguish particles of similar size within 10 nm difference (based on silica beads), and ability to characterize low-abundance targets in a heterogeneous population.
3. Consistency in instrument performance and data analysis, thanks to a very detailed QC process and fluorescence Sensitivity Monitor, as well as multiple options for on-board cleaning.
4. Flexibility to design experiments, thanks to 5 side scatter parameters and 6 fluorescence channels.

Introduction

Extracellular vesicles (EVs) are small membrane-bound particles released by cells into the extracellular environment. They play important roles in intercellular communication and are involved in various physiological and pathological processes. EVs are classified into different subtypes based on their biogenesis and size, including exosomes, microvesicles, and apoptotic bodies.

Exosomes or small EVs typically range in size from 30 to 150 nm. They are formed through the inward budding of multivesicular bodies (MVBs) within the cell, which then fuse with the plasma membrane, releasing the exosomes into the extracellular space. Exosomes contain various bioactive molecules, such as proteins, lipids, nucleic acids (DNA, RNA), and microRNAs, which can be transferred to target cells, influencing their function and behavior.

Microvesicles, also known as large EVs or microparticles or ectosomes, are larger than exosomes, ranging from 100 to 1000 nm in size. Unlike exosomes, microvesicles are formed by the outward budding and shedding of the plasma membrane directly. They also carry a diverse cargo of proteins, lipids, and nucleic acids, and can transfer these molecules to recipient cells.

Apoptotic bodies are the largest EVs, typically ranging from 1 to 5 µm. They are released from dying cells during the process of programmed cell death (apoptosis). Apoptotic bodies contain cellular fragments, organelles, and nuclear material, and are recognized and engulfed by phagocytic cells to facilitate their clearance.

EVs have gained significant attention in recent years due to their potential as biomarkers for disease diagnosis and prognosis, as well as their roles in cell-to-cell communication and their therapeutic applications. Researchers are studying EVs in various biological fluids, including blood, urine, and cerebrospinal fluid, to gain insights into their cargo and functions.

Technologies such as nano flow cytometry, electron microscopy, and molecular profiling techniques like RNA sequencing and proteomics are used to study and characterize EVs.

Understanding EV biology, cargo, and functions holds great promise for advancing our knowledge of cellular communication and their potential applications in diagnostics, therapeutics, and regenerative medicine.

Currently, EV analysis is critical and challenging. Isolation and purification methods can suffer from low yield, contamination from other particles, and difficulties in standardization. Heterogeneity of EV populations in terms of size, cargo, and biogenesis complicates their study. EVs can exhibit diverse biological activities and functions depending on their cellular origin and cargo. However, deciphering the specific functions and mechanisms of action of EVs in different contexts is still a challenge. The functional heterogeneity of EVs requires more comprehensive characterization and standardized functional assays.

Researchers in the field of EVs are actively working to address these limitations by developing improved isolation techniques, standardizing protocols, and advancing our understanding of EV biology. As the field progresses, overcoming these challenges will help unlock the full potential of EV research and its applications in various biomedical areas.

One of the biggest limitations is the need for multiple techniques to accurately count, characterize and determine the size of EVs, resulting in time-consuming and laborious workflows with poor repeatability.

The CytoFLEX nano Flow Cytometer combines everything in only one instrument, enabling count, characterization, and size determination, thanks to the following features:

Performance		
Violet Side scatter sensitivity	VSSC1: 40 nm relative to polystyrene nanoparticles VSSC2: 80 nm relative to polystyrene nanoparticles	
Scatter Detection dynamic range	VSSC1 for small range 40-150 nm polystyrene nanoparticles VSSC2 for large range 80-1000 nm polystyrene nanoparticles	
Violet forward scatter sensitivity	300 nm relative to polystyrene nanoparticles	
Fluorescence sensitivity and resolution	Simultaneous detection of fluorescence on six fluorescence detectors, using 500 nm CytoFLEX nano Multi-intensity Fluorospheres V447: 8 peaks B531: 8 peaks Y595: 8 peaks R670: 6 peaks R710: 5 peaks R792: 4 peaks	
Fluorescence rCV	rCV <10% (using QC Fluorospheres at 1 µL/min)	
Carryover between samples	≤ 1%[a]	
Acquisition speed	Maximum electronic acquisition speed	16000 events/second with ≥95% yield
	Recommended maximum sample acquisition speed	5000 events/second to avoid possible swarming or coincidence situation
Volumetric counting accuracy	≥90%[b]	

[a] tested with polystyrene beads, [b] tested with 144nm QC Scatterspheres on 3 units under the conditions: record 3 minutes at the sample flow rates of 1 µL/min, 2 µL/min, 3 µL/min, 4 µL/min, 5 µL/min, 6 µL/min respectively, repeat 5 times, then calculated the average total events for each speed, compared the calculated the volumetric counting accuracy with the theoretical total events.

Figure 1. The CytoFLEX nano Flow Cytometer performance specifications.

Protocol

1. System Startup

After comfirming that the sheath fluid and the cleaning reagent are sufficient for the day, and waste container is empty, turn on the CytoFLEX nano Flow Cytometer and start the CytExpert nano software, using the link on the desktop. Select the System Startup procedure. The procedure will take about 6 minutes, during which the system automatically purges the sheath damper, executes debubble for the sheath filter, the sheath line, the flow cell and, the piston pump, and cleans the sample line, ensuring the fluidics system is set to start.

2. Configuration setting

The CytoFLEX nano Flow Cytometer is equipped with 2 Wavelength Division Multiplexers (WDMs), one for scatter optical filters (VioletSSC1, VioletSSC2, BlueSSC, YellowSSC and RedSSC) and one for fluorescence optical filters (V447, B531, Y595, R670, R710, R792), as shown in Figure 2 below.

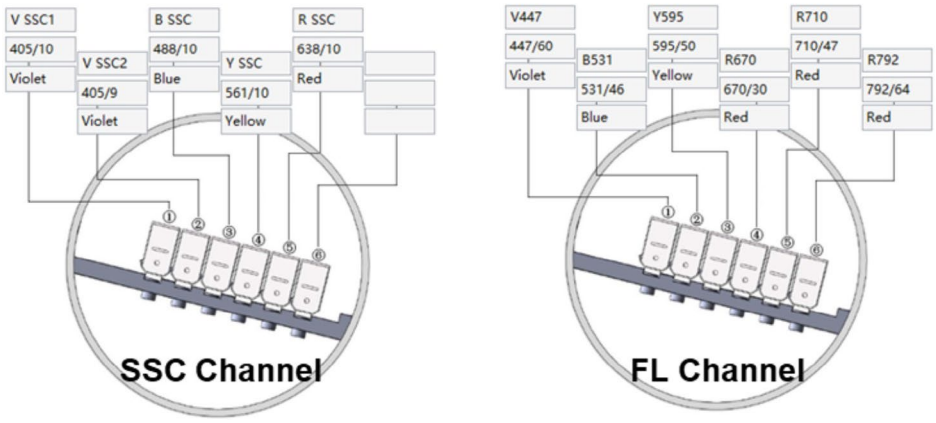

Figure 2. The CytoFLEX nano Flow Cytometer default detector configuration.

3. Perform Daily QC

Select the QC/Sensitivity menu and input the bead lots. Running QC will ensure the CytoFLEX nano Flow Cytometer will provide adequate signal strength and precision.

The QC process will start evaluating laser powers and sheath flow rate.

Next, the process is divided in three steps:

- **Instrument background assessment:** using 5 nm filtered CytoFLEX sheath the system assesses background noise in both sheath and sample line, very close to the limit of optical and electrical noise, to ensure it will achieve the lowest limit of detection.
- **Scatter performance assessment:** using CytoFLEX nano Daily QC Scatterspheres the system will monitor event rate and signal strength.
- **Fluorescence performance assessment:** using CytoFLEX nano Daily QC Fluorospheres the system will monitor event rate, laser delay and fluorescence detection performance. Between each tube the system will trigger an automatic backflush cycle with sheath fluid. The generated QC report confirms success at each step. If a failure occurs, it clearly indicates what needs attention.

4. Sensitivity Monitor

When fluorescence sensitivity is key, the sensitivity monitor will add confidence, showing the number of fluorescence peaks the CytoFLEX nano Flow Cytometer can resolve and the distance between the noise and dimmest resolvable peak. Select Sensitivity monitor in the QC/Sensitivity menu and input the lot you will be using. This process will run the Multi-fluorescent Fluorospheres, which are a mixture of multi-intensity multi-fluorescent 500 nm polystyrene beads. See Figure 3 for an example of the QC and Sensitivity Monitor Reports.

QC Report

Scatterspheres Lot No.: 2692901
Scatterspheres Expires: 2024-10-02
Fluorospheres Lot No.: BAQ03
Fluorospheres Expires: 2024-10-02
Detector Configuration: Default-Configuration
QC Date: 2024-01-16 09:44
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano
Sample Flow Rate: 1µL/min

Laser

Laser	Delay(µs)	Default Delay(µs)	Difference Delay(µs)	Power(mW)	Target Power(mW)	Result
Violet	0.00	0.00	0.00	116	108-132	✓
Blue	0.00	0.00	0.00	49	40-60	✓
Yellow	0.00	0.00	0.00	30	25-45	✓
Red	-0.48	0.00	-0.48	108	90-110	✓

Sheath Flow Rate/Background

Item	Value	Target	Unit	Result
Sheath Flow Rate	1801	1600-2000	µL/min	✓
Threshold (VSSC1-H)	398	≤600	-	✓
Sheath Noise	6	≤80	Events/Sec	✓
Sample Noise	5	≤150	Events/Sec	✓

Side Scatter Assessment

Event Rate

Value	Target	Unit	Result
197	≥ 50	Events/Sec	✓

Side Scatter Assessment Result

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	FD	Target FD	Result
VSSC1	86	89	-3.54	1429937.0	1420000.0	0.70	8.50	1.00	✓
VSSC2	181	181	0.00	41014.0	40000.0	2.53	8.20	1.00	✓
BSSC	68	68	0.00	465571.3	460000.0	1.21	7.19	1.00	✓
YSSC	108	108	0.00	265961.9	260000.0	2.29	6.08	1.00	✓
RSSC	170	170	0.00	865046.6	860000.0	0.59	4.59	1.00	✓

Fluorescence Assessment

Event Rate

Value	Target	Unit	Result
187	≥ 50	Events/Sec	✓

Fluorescence Assessment Result (Single Laser)

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	Result
VFSC	1241	1241	0.00	1011360.0	1000000.0	1.14	-	-	✓
V447	839	839	0.00	426396.9	410000.0	4.00	3.40	10.00	✓
B531	1123	1052	6.75	91220.6	90000.0	1.36	4.46	10.00	✓
Y595	1279	1201	6.49	167632.5	170000.0	-1.39	4.52	10.00	✓

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Sensitivity Monitor Report

Multi-intensity Fluorospheres Lot No.: BAQ04_SM
Multi-intensity Fluorospheres Expires: 2024-11-03
Sample Flow Rate: 1µL/min
Detector Configuration: Default-Configuration
Monitor Date: 2024-01-16 10:04
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano

Fluorescence Assessment

Event Rate

Value	Target	Unit	Result
1246	≥ 50	Events/Sec	✓

Fluorescence Assessment Result (All Lasers)

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	FD	Peaks	Target Peaks	Result
V447	818	818	0.00	418218.7	410000.0	2.00	3.22	10.00	2.66	8	8	✓
B531	1229	1229	0.00	551101.0	560000.0	-1.59	3.34	10.00	4.57	8	8	✓
Y595	768	768	0.00	380877.8	390000.0	-2.34	3.53	10.00	2.49	8	8	✓
R670	535	535	0.00	156710.6	160000.0	-2.06	3.79	10.00	3.33	6	6	✓

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QC Report

Scatterspheres Lot No.: 2692901
Scatterspheres Expires: 2024-10-02
Fluorospheres Lot No.: BAQ03
Fluorospheres Expires: 2024-10-02
Detector Configuration: Default-Configuration
QC Date: 2024-01-16 09:44
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano
Sample Flow Rate: 1µL/min

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	Result
R670	473	514	-7.98	80352.4	80000.0	0.44	4.17	10.00	✓
R710	1105	1223	-9.68	560552.2	560000.0	0.10	4.27	10.00	✓
R792	758	856	-11.48	201150.1	200000.0	0.58	4.18	10.00	✓

Specifications

Delay: -4.00µs ≤ Difference Delay ≤ 4.00µs.
Gain: -20.00% ≤ %Difference Target Gain ≤ 20.00%.
Median: -5.00% ≤ %Difference Target Median ≤ 5.00%.
rCV: rCV(%) ≤ Target rCV(%).
FD: FD ≥ Target FD.

Result

QC Passed.

Sensitivity Monitor Report

Multi-intensity Fluorospheres Lot No.: BAQ04_SM
Multi-intensity Fluorospheres Expires: 2024-11-03
Sample Flow Rate: 1µL/min
Detector Configuration: Default-Configuration
Monitor Date: 2024-01-16 10:04
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	FD	Peaks	Target Peaks	Result
R710	983	983	0.00	493619.2	510000.0	-3.21	4.06	10.00	2.27	6	5	✓
R792	850	850	0.00	211389.6	220000.0	-3.91	4.21	10.00	10.63	4	4	✓

Specifications

Gain: -20.00% ≤ %Difference Target Gain ≤ 20.00%.
Median: -5.00% ≤ %Difference Target Median ≤ 5.00%.
rCV: rCV(%) ≤ Target rCV(%).
Peaks: Peaks ≥ Target Peaks.

Result

Monitor Passed.

Figure 3. Example of QC and Sensitivity Monitor Reports.

5. The CytoFLEX nano Flow Cytometer on-board clean processes

The CytoFLEX nano Flow Cytometer offers multiple automated cleaning options to meet different sample and workflow needs. Each selected workflow ensures that researchers can accurately and reproducibly characterize EVs and other biological nanoparticles, confirming that the background noise does not impact EV/nanoparticle evaluations.

Workflows available:

- Backflush is automatically integrated into Unload process, and it will run after QC and Sensitivity Monitor. It can also be triggered by clicking on Backflush in the Acquisition Control Panel. In the Cytometer menu, select Cytometer Configuration and select the number of backflushes the instrument will run when Backflush will be initiated.
- On-board Clean has 3 pre-set options in the Cytometer Configuration menu and a customized number of back and forth (BFF) with CytoFLEX cleaner at the end:
  - **Option 1:** 1 BFF (cycle time: 7min 19s)
  - **Option 2:** 5 BFF (cycle time: 10min 26s)
  - **Option 3:** 10 BFF (cycle time: 14min 18s)
  - **Option 4:** Custom - Select Back & Forth Cycles, BFF from 1 to 10. Corresponding time will appear.
- Manual Clean is a cleaning process similar to what the CytoFLEX instruments Daily Clean offers. When the time of Manual Clean is set similar to On-Board Clean, the results are comparable. Selecting Manual Clean from the Cytometer menu, the following steps are to define the time with cleaner and time with water or sample buffer.
- Shutdown Clean
  - **Option 1:** is to perform the selected On-board Clean and shutdown automatically afterwards.
  - Overnight (recommended 8 hours) soaking of the sample line is expected to completely clear the line. To extend flexibility, soaking can be stopped at any time.
- Flow Cell Clean is necessary only in extreme cases, which a service representative can help define. The process run with freshly prepared 10% Contrad 70 solution, placed in its onboard bottle, in the front of the CytoFLEX nano Flow Cytometer.
  - Select Flow Cell Clean on the Cytometer Menu. The system rinses and soaks the Flow Cell with 10% Contrad 70 solution. The soaking is suggested to be at least 30min. Following that the instrument proceeds to rinse out Contrad 70 with multiple Flow Cell Primes.
  - Flow Cell Prime (in the Cytometer menu) can be selected and used outside of the Flow Cell Clean, and it is useful when detergents are used.

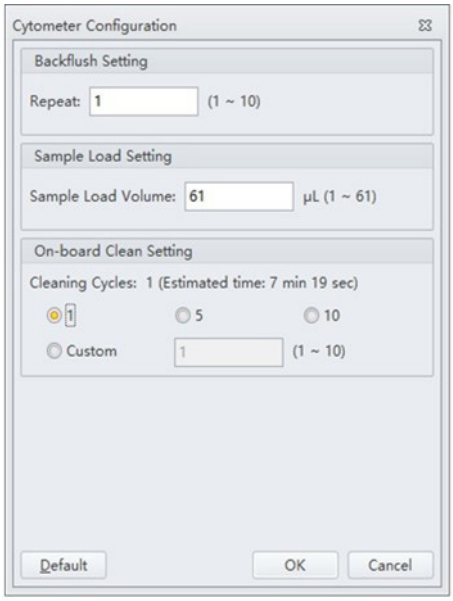

Figure 4. Backflush and On-Board Cleaning Setting.

6. Experiment Setting

- Select File and open a new experiment.
- On Cytometer Menu, select Cytometer configuration and input the volume of sample that will be collected by the sample pump from the sample tube. There is 32 µL of dead volume to consider, and the minimum volume in the sample tube is 100 µL.
- On Cytometer Menu, select Settings and Options. Sample volume is enabled by default, so the system will monitor sample consumption and stop automatically when there is less than the define volume to collect. The sample volume monitor can be disabled by unchecking that box.
- On the Acquisition Control Panel on the left, select:
  - How many events to display and the stopping rule for recording. It can be by a number of events in a specific gate, by time or by volume. If multiple options are selected the instrument will stop whichever will come first.
  - Sample flow rate. 1 µL/min is suggested for beads acquisition and 3 µL/min is suggested for biological samples. Other options available are 6 µL/min and customized between 1 and 6 µL/min.
- Use the icons on icon top bar to generate the type of histograms and plots needed for the experiment. On that menu, there are also statistics, hierarchical gating, manual and automated gates, scaling, gain and compensation tools.

7. Sizing calibration

To obtain reliable and accurate measurements, sizing calibration is needed. NanoVis product is a multi-size mix of polystyrene beads, including 44 nm, 80 nm, 100 nm 144 nm, 300 nm, 600 nm, and 1 µm. Thresholding in VSSC1, the mix results assessed by a reference calibration method will enable you to generate calibrated data with the CytoFLEX nano Flow Cytometer.

8. Sample Buffer and Baseline Monitor Setting

- Select a new tube, using the icon in the Acquisition Control Panel on the left, and load a tube of sample buffer. Click run and proceed to Acquisition Setting. From this menu, adjust threshold and gain of all the interested channels for the ongoing experiment. This step will create a baseline for the future samples that will be run.
- The sample buffer tube can be used to generate baseline monitor Settings. Select a new Blank, using the icon in the Acquisition Control Panel on the left. Right click on the tube and select Baseline Monitor. In the menu select:
  - **Blank sample type:** sample tube will require a sample buffer tube in the sample holder every time baseline monitor will be run, where sample line will trigger a pull in of 5 nm sheath in the sample line.
  - **Acceptance criteria:** events/sec or a number of events in all events or a specific gate drawn. The gate can be drawn at the right of the noise in the most sensitive scatter channel, VSSC1. This specific gate will monitor any residue in the sample line, other than the noise coming from the sample buffer, allowing a measure of the instrument readiness for the following sample.
  - **Cleaning Cycle Settings:** if the acceptance criteria are not met, define how many cycles of backflush and how many backflushes per cycle will be run. Checking On-Board Clean box will include a cycle of selected On-Board Clean. Between each cleaning cycle the instrument will reassess baseline and if the acceptance criteria are met. If yes, the monitor will stop; if not the following defined cleaning cycle will be run.
  - **Baseline monitor will be saved as fcs file:** All Cycles Data will merge all the baseline monitor run in one fcs file, where Last Cycle Data will save only the last run.
  - Once set, Baseline Monitor, with these settings, can be initiated during the experiment by generating a new Blank tube and clicking Run. Setting can be modified by right clicking on the blank tube and selecting Baseline Monitor.

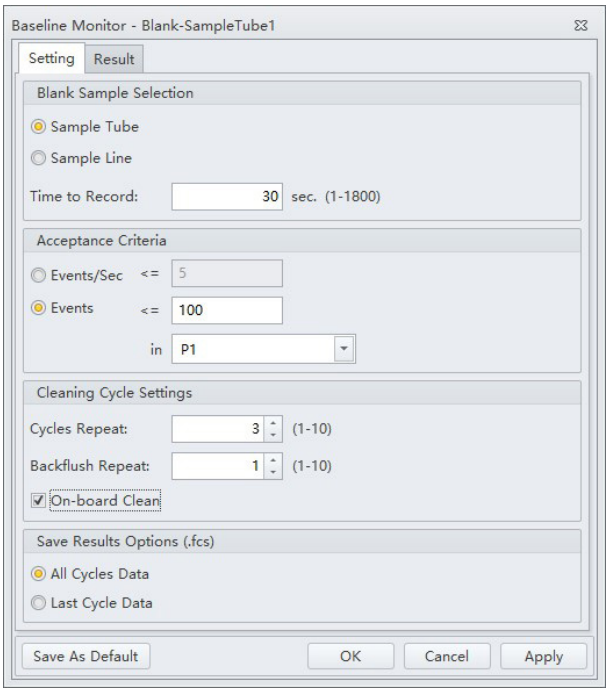

Figure 5. Baseline Monitor Setting.

9. Sample acquisition

- Using the icon in the Acquisition Control Panel on the left, create a tube with the same setting of the sample buffer, and load a sample tube.
- When working with a new sample type, start with a sample titration, running from the lowest to the highest concentration. A slight modification of gain and threshold can be applied to best resolve the sample. If that is the case, we suggest repeating the sample buffer run and changing the Baseline Monitor Setting.
- Coincidence and swarming, as cellular flow cytometry, depend on sample concentration but for the CytoFLEX nano Flow Cytometer, it is also dependent on particle size. For samples with a size higher than 100-150 nm, the order of 104 particles/µL can be considered a good concentration and a limit before swarming. The more representation of small particles (and/or higher the small EV/large EV ratio) allows a higher concentration to be run. If swarming conditions are too high the Events Processed % in the Acquisition Control Panel will be lower than 100%.
- Once the best sample dilution is selected, based on the number of events per second or in a specific gate, and sample resolution and representation in the plots of interest, start running single-color stained samples and adjust fluorescence gains in the acquisition setting menu.
- When a panel of color will be run, compensation can help correct for fluorescence spillover, removing undesired signal. Compensation can be done using the Compensation Matrix, selecting the icon in the Acquisition control panel, or done manually using the icon in the top bar.
- CytoFLEX nano Flow Cytometer offers fluorescent triggering options that can help when focusing on specific stained/labeled populations.
- Baseline Monitor is suggested between different sample types, or when a lower concentration will be run, or when an unstained sample will be run after a stained one.
- Always run all controls as suggested by MIFlowCyt-EV position paper (ref #1). If a detergent-treated sample will be run, we suggest a detergent titration first, starting from the lowest concentration. This will evaluate the detergent background on CytoFLEX nano Flow Cytometer. When multiple tubes of detergent or a high concentration will be run, perform debubble options and Prime to eliminate possible nano- and micro-bubble formation.

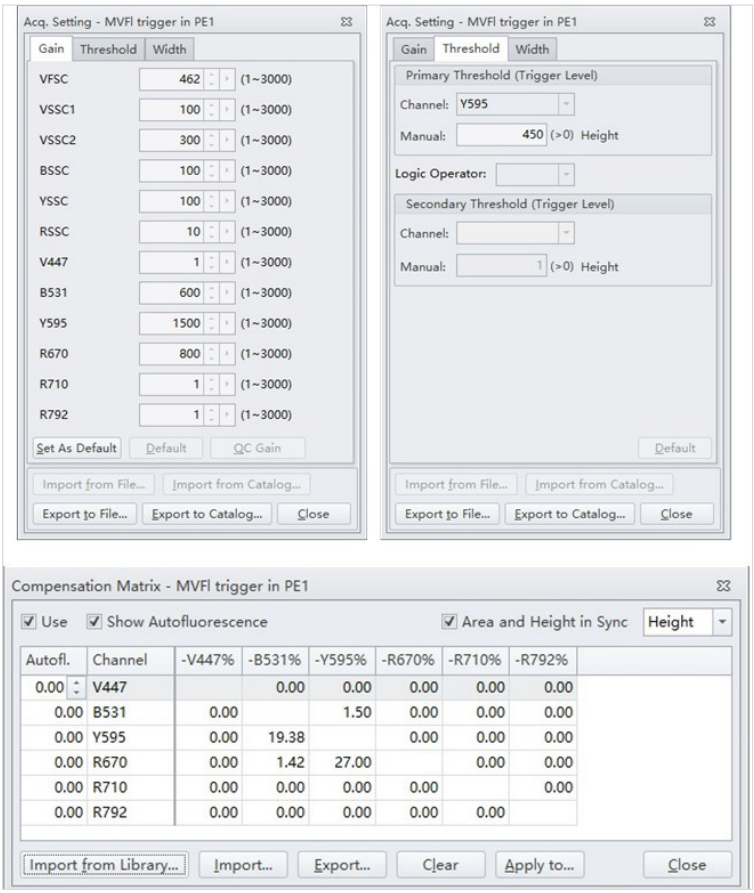

Figure 6. Acquisition setting and Compensation Matrix.

10. Cleaning and System Shutdown Program

- Between different experiments, an On-Board Clean is suggested. Select Cytometer in the menu bar, followed by On-Board Clean.
- At the end of the day, select Cytometer in the menu bar, followed by System Shutdown program. The program provides three options for different needs:
  - Clean the sample line with cleaner with the selected On-Board clean cycle, prior to the System Shutdown.
  - Running long-term soaking prior to the System Shutdown.
  - System Shutdown only.

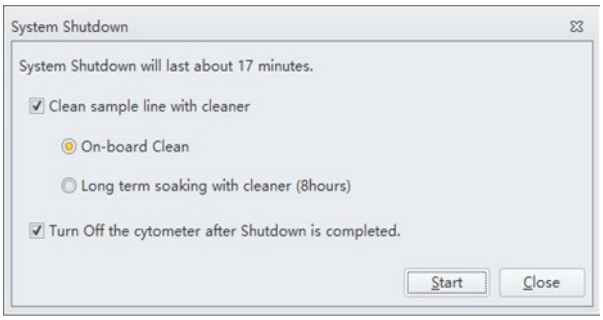

Figure 7. System Shutdown.

Conclusions

The CytoFLEX nano Flow Cytometer is the first flow cytometer that can clearly demonstrate detection of extracellular vesicles down to a size of 40 nm (measured with polystyrene beads). Its high sensitivity and resolution for small particles, combined with automated cleaning, extensive QC process and Fluorescence Sensitivity monitor, will undoubtedly propel the field of research forward. The capabilities and features of this instrument will enable researchers to explore previously uncharted territories and obtain more comprehensive and accurate data. With this cutting-edge tool, we anticipate significant advancements in research findings and a deeper understanding of extracellular vesicles, and their applications.

References

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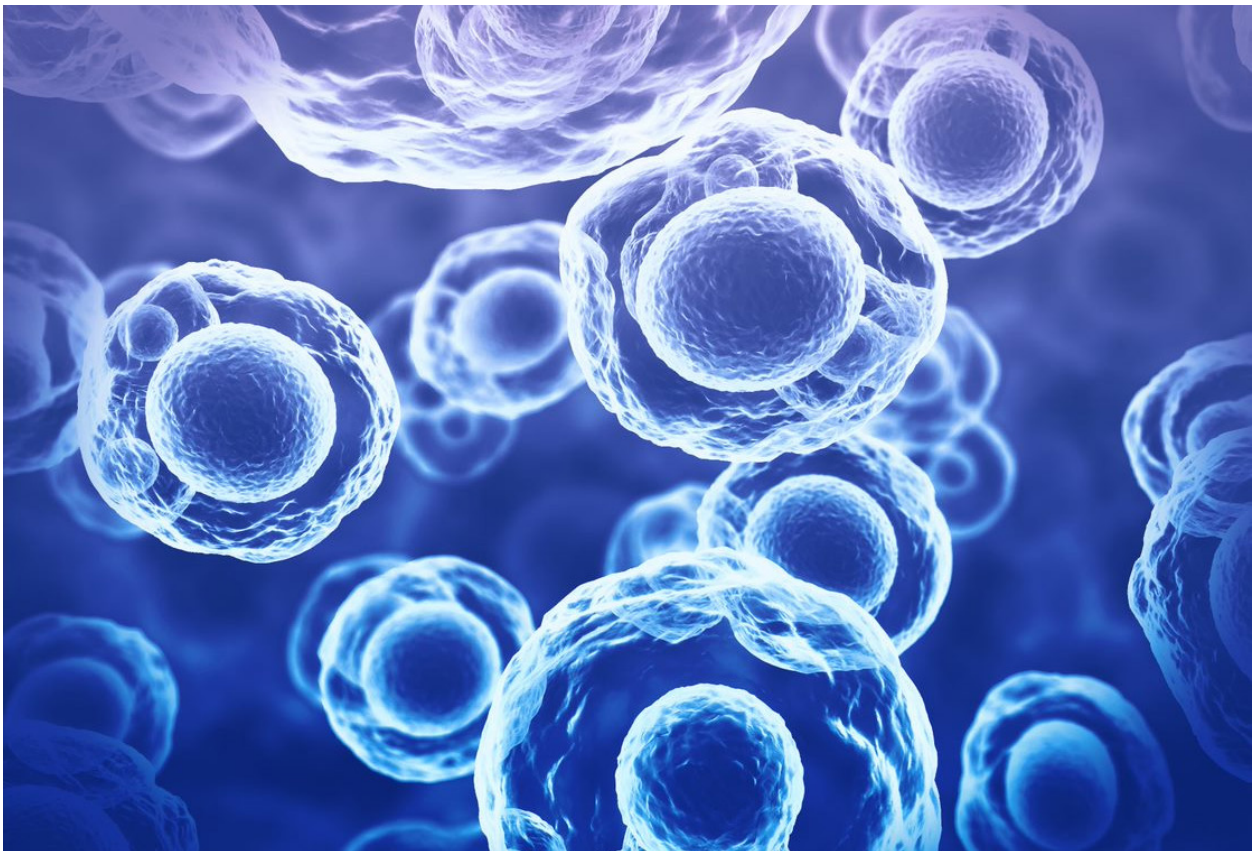
APPLICATION NOTE

Purifying High-Quality Extracellular Vesicles using Ultracentrifugation



Background

Extracellular vesicles (EVs) are lipid bilayer vesicles secreted by cells. They carry a variety of cargoes and play critical roles in intercellular communications and physiological processes. EVs show tremendous promise in the clinic as biomarkers and therapeutics.[2-4]

Challenge

Despite the rapid growth in EV research, it is still challenging to produce samples with both high purity and yield due to the heterogeneity of EVs with respect to size, composition and function. This results in tradeoffs and challenges as researchers subsequently proceed with characterization and applications.[1]

Solution

With the high resolution and separating power of ultracentrifugation (UC), specific EV subpopulations can be reproducibly isolated. UC is the most used EV purification technique and is broadly considered the gold standard.[5,6] This application note highlights the available UC approaches, including differential UC (DUC) and density gradient UC (DGUC), and explains how different rotors and tubes can reduce run times and improve purity levels. It also highlights how the analytical ultracentrifuge (AUC) can help improve EV characterization by detecting different populations and loading states via multiwavelength analysis.

Centrifugation methods for EV purification

Ultracentrifugation (UC) is not a single technique; methods can be optimized based on the desired output.

There are two UC separation methods typically used for purification. The first separates analytes based on sedimentation coefficients (S-values). With this method, successive centrifugation steps are typically used to selectively pellet materials of different sizes.

The second method uses density gradients to separate particles based on their buoyant density.

Having the ability to separate particles by different properties enables researchers to optimize their sample purification methods based on the requirements for their downstream processes.

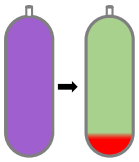
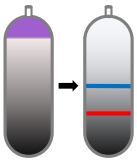
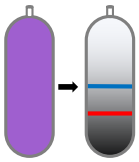
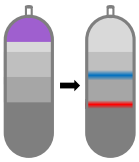
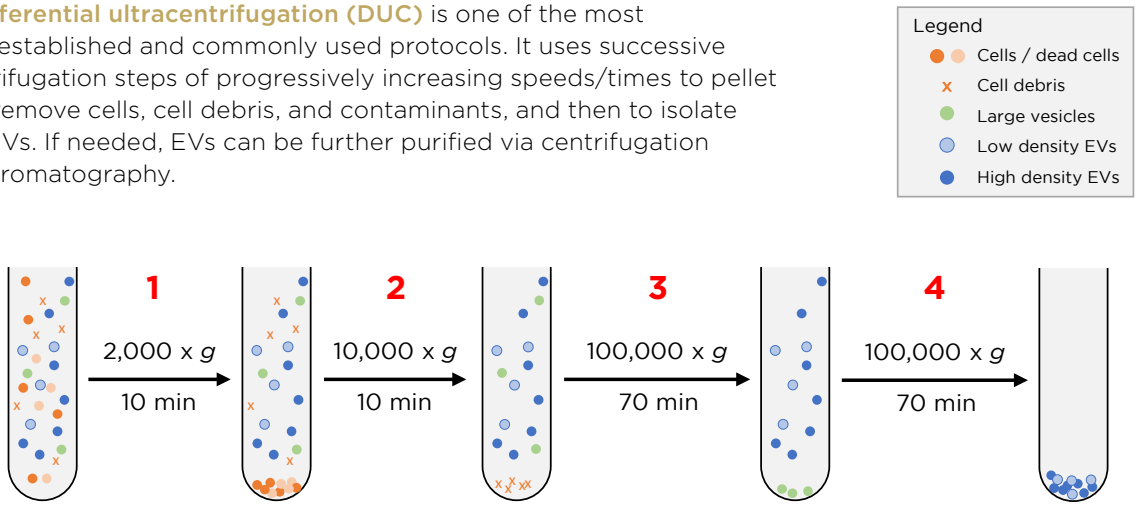
	Differential Centrifugation Pelleting	Rate Zonal	Isopycnic	Equilibrium Zonal
Separation basis	Materials separate by S-value in buffer. Sucrose cushions can be used to prevent hard pelleting.	Materials separate by S-value (size & mass) in a pre-formed density gradient.	Materials separate by buoyant density in a self-forming (continuous) density gradient.	Materials separate by buoyant density in a pre-formed density gradient.
Typical density gradient	No density gradient used	Continuous gradient (e.g., linear sucrose gradients)	Continuous gradient (e.g., CsCl gradients)	Sample separated into discrete segments of density (or steps)
Common gradient material(s)	N/A	Sucrose, Iodixanol	CsCl, Iodixanol	Iodixanol (more common for viral separations), sucrose
Advantages	Fast, simple separation of materials with widely different S-values	High-resolution rate-based separation of materials with similar S-values	Highest-resolution separation by density	One-step purification & concentration by density
	 Before After	 Before After	 Before After	 Before After

Table 1: Overview of centrifugation methods

Protocols for purifying EVs based on sedimentation coefficient

1. Differential ultracentrifugation (DUC) is one of the most well-established and commonly used protocols. It uses successive centrifugation steps of progressively increasing speeds/times to pellet and remove cells, cell debris, and contaminants, and then to isolate the EVs. If needed, EVs can be further purified via centrifugation or chromatography.

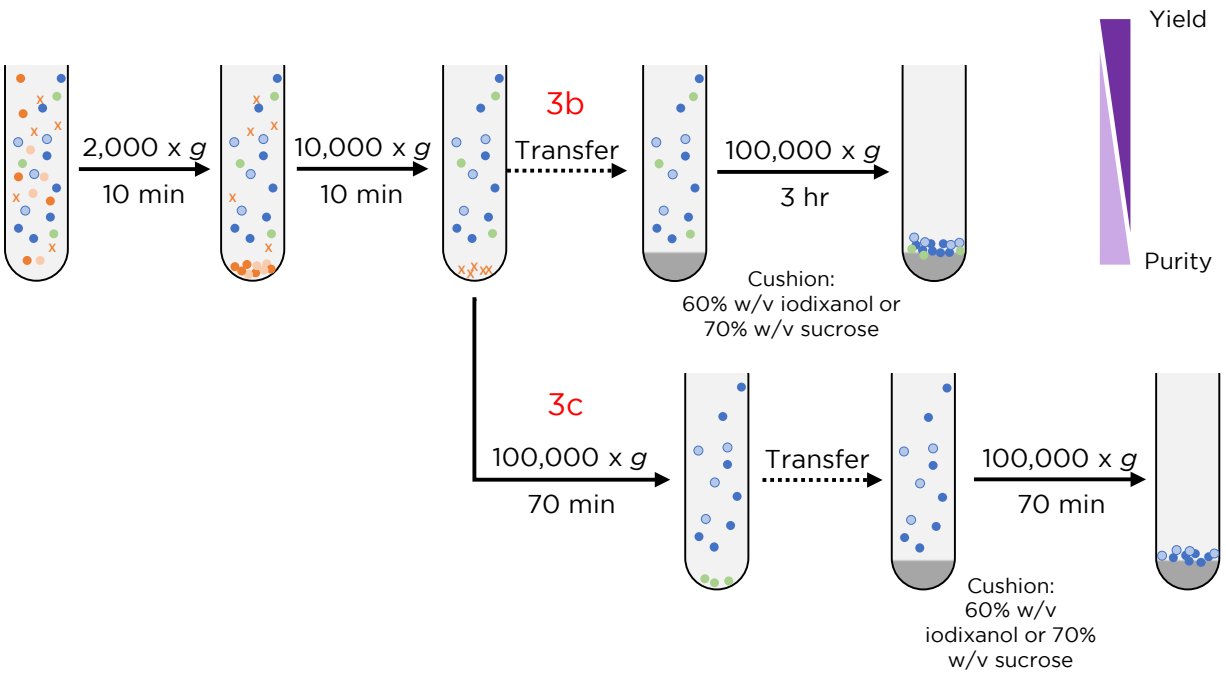


Protocol modified from Xie 2022[7] and Gall 2020.[8]

1. Removal of whole cells. Thus, speed depends heavily on EV source (e.g., CHO cell culture, blood). Refer to the literature to determine the appropriate speed to remove specific cell types.
2. Removal of cell debris.
3. Removal of apoptotic bodies and microvesicles.
4. Isolation of EVs. Sample may still contain some larger vesicles at this stage but should be enriched for EVs.

2. Cushioned differential ultracentrifugation is similar to standard DUC, except the EVs are pelleted on a high-density cushion to better preserve morphology and function. This protocol is a "sweet spot" in terms of hands-on time and product quality. The cushion separates particles based on a threshold of density; particles less dense than the cushion will remain at the interface, while particles denser than the cushion continue into and through the cushion.

Notably, two versions are presented, with (3b) offering better yield and (3c) providing higher purity.



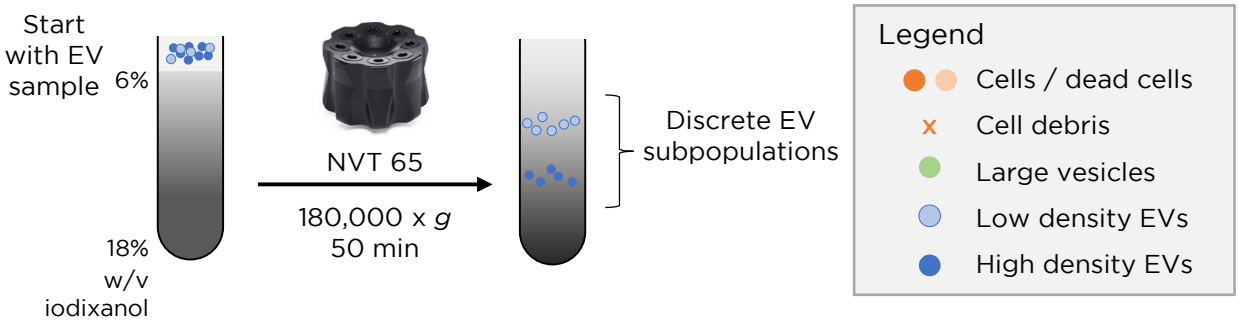
Protocol modified from Duong 2019.[9]

Potential improvements to DUC and cushion protocols:

- Shorter pathlength (better k-factor)
- Conical tubes may provide enhanced pellet recovery

These DUC protocols can be run in all standard fixed-angle and swinging-bucket rotors.

3. Rate Zonal Density Gradient Ultracentrifugation uses a density gradient but still separates particles based on their sedimentation coefficients. The crude sample is placed on top of a pre-formed linear density gradient. Upon centrifugation, particles will sediment at different rates depending on their individual sedimentation coefficients.



Protocol modified from Vaillancourt 2021.[14]

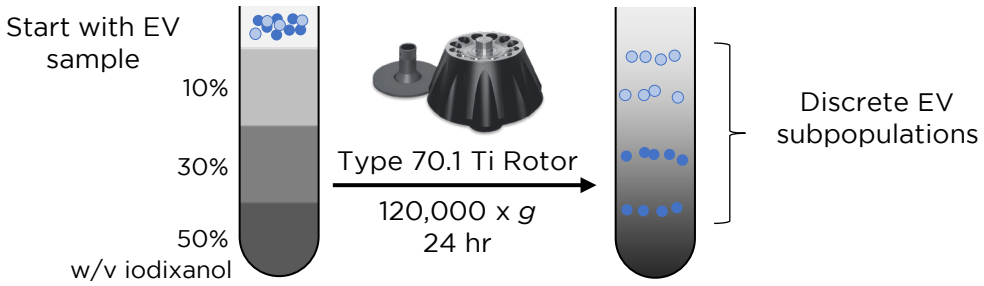
Potential improvements to rate zonal purification:

- Using a swinging-bucket rotor to increase pathlength and boost resolution (this extends run time, however)
- Increased centrifugal speed to reduce run time*

Protocols for purifying EVs based on density

Density gradient ultracentrifugation (DGUC) methods use density gradients to separate particles based on their buoyant densities. EV buoyant densities are usually between 1.08 and 1.20 g/mL,[11,12] depending on the density gradient-forming material used.

4. Isopycnic density gradient ultracentrifugation uses a dense material such as iodixanol to separate crude samples or further purify EV samples based on their density differences. Upon centrifugation, the particles migrate to a position in the gradient equal to their buoyant density.



Protocol modified from Onodi 2018.[10]

Several modifications can be made to improve sample purity, including:

- Number/volume of gradient steps
- Placement of the sample (top/middle/bottom of tube)
- Starting density of sample
- Run speed/time *
- Rotor type

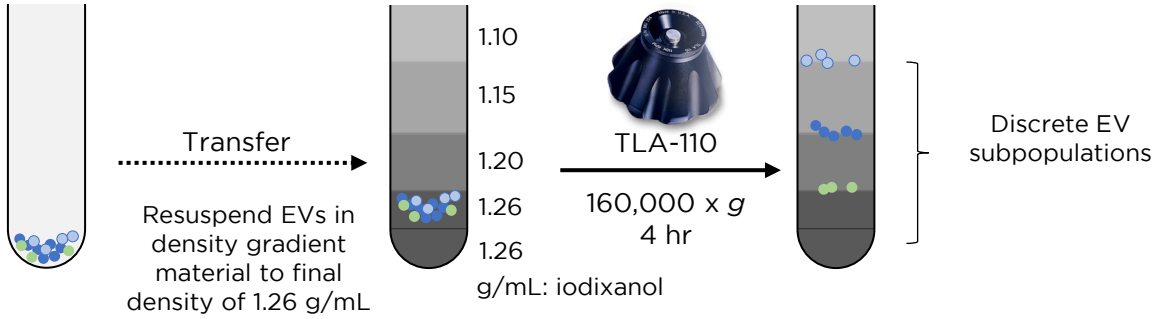
If the experiment is spun until equilibrium is reached, a linear density gradient is formed, as illustrated here.

Potential improvements to isopycnic centrifugation:

- Use a vertical rotor to shorten the pathlength and reduce run time
- Multispeed protocols to boost efficiency (high speed first to reach equilibrium quickly, then lower speed to flatten the gradient and increase resolution)
- Reduce run time by starting with more layers (trade off with hands-on prep time)

Isopycnic DGUC protocols can be run in all standard fixed-angle and swinging-bucket rotors.

5. Equilibrium zonal density gradient ultracentrifugation uses the same principle as isopycnic DGUC (i.e., the distinct steps are maintained), but the experiment is stopped before a linear density gradient is formed. Upon centrifugation, the particles will migrate to the position in the gradient equal to their buoyant density, typically at an interface between two steps.



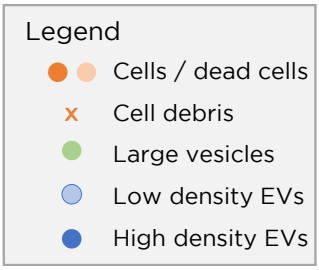
Protocol modified from Iwai 2016.[13]

Much like isopycnic DGUC, there are many variations of this separation approach. In this specific example, EVs are pelleted in a previous step and resuspended directly into a buffered solution at a specific density, but this is not required.

Performing centrifugation until a linear gradient is reached, as seen in the isopycnic DGU example, typically increases sample purity but requires longer centrifugation times.

Potential improvements to this protocol:

- Use a vertical (or near vertical) rotor to shorten pathlength and reduce run time
- Spin faster to reduce run time *



Summary

Centrifugation is a robust, scalable and cost-effective method for purifying EVs while maintaining their integrity and biological function. Several different methods can be applied to separate particles based on their density or sedimentation coefficient, and methods can be modified to either enhance for purity or for volume, depending on processing requirements.

	DUC	Cushioned DUC	Isopycnic DGUC	Equilibrium Zonal DGUC	Rate Zonal DGUC
Purity					
Yield					
Ease of use					
Run time					
Separation basis	Size & mass (S-value)	Size & mass (S-value)	Buoyant density	Buoyant density	Size & mass (S-value)
Ideal Rotor	FA or SW	FA or SW	VT	VT	SW

Table 2: Comparing ultracentrifuge methods for EV purification

Ratings in this table are meant to serve only as directionally useful estimates using standard protocols.

Rotors for EV purification

The angle of a rotor during centrifugation affects the pathlength over which particles sediment. This, in turn, affects centrifugation time required and purity levels that can be achieved. Table 3 highlights where each type of rotor is best utilized.

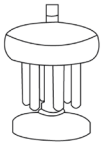
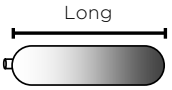
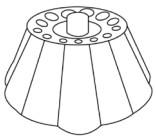
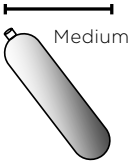
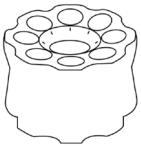
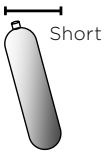
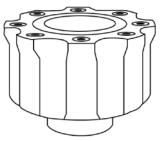
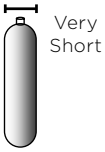
	Use cases	Angle	Example	Pathlength (at speed)
Swinging-bucket (SW)	With the longest pathlength, SW rotors are the best option available for rate zonal separations. SW rotors are also beneficial for pelleting very small sample masses to maximize visibility and pellet stability.	90°		
Fixed-angle (FA)	Highly versatile, FA rotors are applicable for all purification methods except rate zonal. FA rotors are preferable for larger-scale pelleting, especially when there is sufficient sample to allow for visualization.	20-30°		
Near-vertical (NVT)	NVT rotors are preferred for density-based separation with less pure samples that may have a small amount of floating or sedimented contaminants.	7-10°		
Vertical (VT)	VT rotors are the most preferable option for density gradient formation and high-resolution density-based separations.	0°		

Table 3: Rotors for EV purification

Tubes for EV purification

Most tubes are available in multiple materials, with polypropylene and Ultra-Clear being the preferred options. Polypropylene tubes enable marginally easier piercing while Ultra-Clear tubes offer improved visibility. When dealing with EVs, the transparent Ultra-Clear tubes are recommended.

Quick-Seal
Heat-sealed easily and reliably for a robust seal

OptiSeal
Plug-based seal provides simplicity & reliability

Open Top
Simplest option, no closure at all

Open Top
Simplest option, no closure at all

* For all DGUC methods, the density and rotor used will dictate the speed at which the run can be safely performed; please refer to the rotor instructions for use (IFU) to determine safe operating speeds.

Choosing the right ultracentrifuge setup for sample purification†

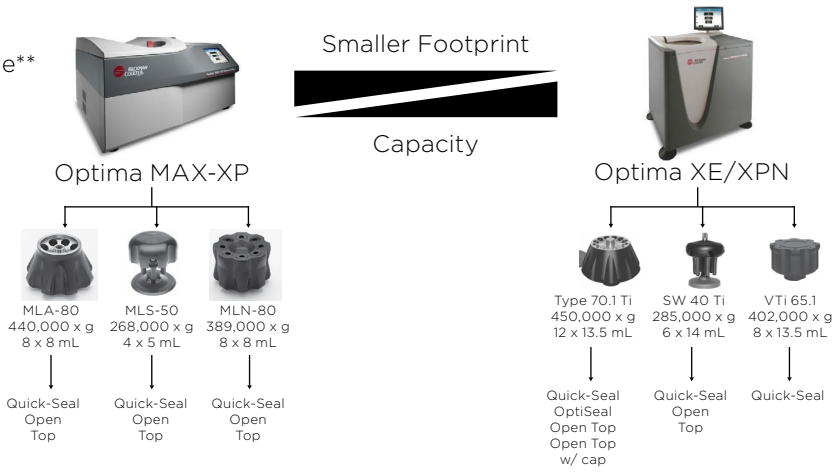
1. Floor-standing or tabletop

The Optima MAX-XP ultracentrifuge** has a small footprint, while Optima XE & XPN models offer higher capacities & more rotor/tube options

2. Rotor type

- Fixed-angle (FA)
- Swinging-bucket (SW)
- Vertical (VT)
- Near-vertical (NVT)

3. Tube type



†Rotors/tubes shown are recommendations only. Please see the Ultracentrifuge Catalog for a complete list of compatible rotors/tubes. Some tubes may require adapters to work with specific rotors.

Reach ultracentrifuge speeds in the Avanti JXN-30 centrifuge

Avanti JXN-30

Cell harvest and lysate clarification

JLA-9.1000
16,780 x g
4 x 1000 mL

JLA-12.500
26,890 x g
6 x 500 mL

Exosome purification

JA-30.50 Ti
108,860 x g
8 x 50 mL

JS-24.15
110,510 x g
6 x 15 mL

As part of our high-performance line of centrifuges, the Avanti JXN-30 instrument excels at large-volume centrifugation. However, the JXN-30 is unique in that it can also reach ultracentrifugation speeds required to purify EVs. Consider the Avanti JXN-30 as a one-stop-shop solution to enable EV research.

Use J-LITE JLA-9.1000 or JLA-12.500 high-capacity rotors to spin down cell cultures, then switch to the JA-30.50 Ti or JS-24.15 rotors to easily perform EV isolation.

When maximum versatility is needed, look no further than the Avanti JXN-30 centrifuge.

Analytical Ultracentrifugation (AUC) for characterizing EVs

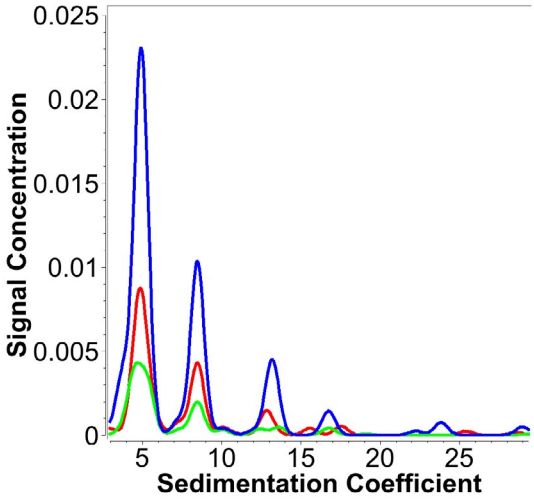
AUC enables analysis of samples in their native state and offers an extremely large dynamic range, with the ability to characterize particles from peptides to intact viruses. It is an in-solution, label-free method that does not require standards.

Data is collected while the sample is separated during centrifugation, allowing for high-resolution detection of many different species in the sample.

Understanding population distributions

Sedimentation coefficient results provide an in-depth understanding of the different EV populations in solution, and can identify contaminants and aggregates if present.

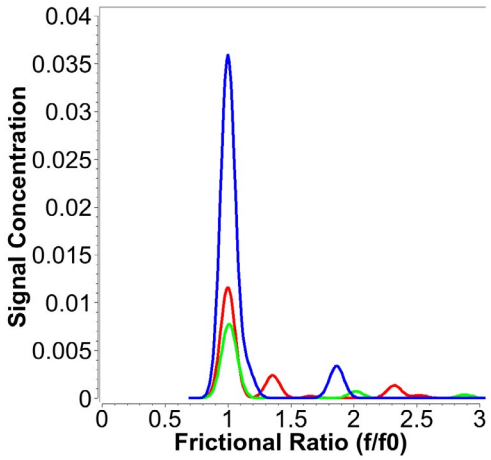
For example, AUC facilitated determination that an umbilical EV sample contained 4 prominent species, with the smallest (5 S species) being the most abundant.



Understanding the shape of EVs

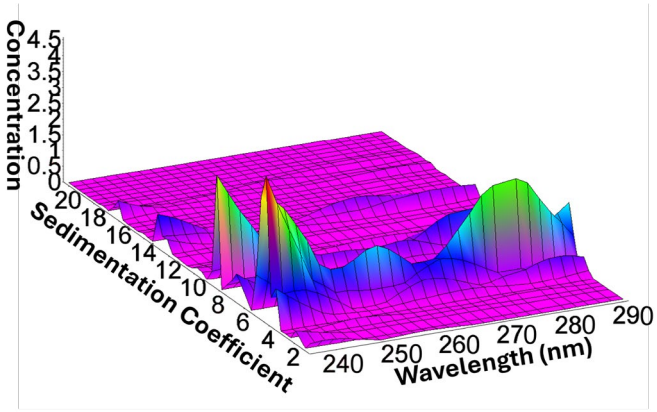
AUC can expand an understanding of analyte shapes in solution. This is done using the frictional ratio (f/f0), which is calculated based on the diffusion coefficient measured by AUC. The f/f0 describes the anisotropy (or relative shape) of analytes in solution.

A frictional ratio of 1, as seen for the majority of the EVs here, indicates a sphere, while larger f/f0 indicate a more extended or linear structure.



Identification of EV composition

When the sample is measured at multiple wavelengths in AUC, insights into the molecular content of the EV sample can be developed. Multiwavelength AUC can identify and quantify proteins, nucleic acids, lipids, and other biomolecules based on their absorption profiles.



Summary

The Optima AUC analytical ultracentrifuge** enables the quantitative identification of EV populations and provides crucial insights into their molecular content, shape, and purity.

Such comprehensive characterization can greatly enhance the understanding of EVs and their potential applications in various fields such as medicine, biology and biotechnology.

All figures were generated using UltraScan III software. Third-party analysis software products, including UltraScan, have not been validated by Beckman Coulter for use with an analytical ultracentrifuge. Beckman Coulter does not endorse any third-party analysis software. Beckman Coulter warranties and/or performance guarantees that may apply to our analytical ultracentrifuge instruments do not apply to any third-party software.

Please see the relevant centrifuge brochures and rotor manuals (instructions for use) for a complete list of available instruments, hardware and consumables.

For more information about our centrifuges, please visit: **https://www.beckman.com/centrifuges**

For more information about exosomes, please visit: **https://www.beckman.com/resources/sample-type/extracellular-vesicles/exosomes**

Further reading and resources

- **CytoFLEX nano Flow Cytometer**
- **Advancing EV research – First Experiences with the CytoFLEX nano Flow Cytometer**
- **Extracellular Vesicles**
- **Into The World Of Extracellular Vesicles (EVs)**
- **The Discovery of Extracellular Vesicles (EVs): Tracing the Journey from Inception to Present Day**
- **A comprehensive guide to extracellular vesicle counting, size determination, and characterization**
- **Why should I choose ultracentrifugation (UC) as the preferred method for extracellular vesicle purification**

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