

Comparative evaluation of L1CAM, NCAM, and ATP1A3 for enrichment and characterization of neuron-enriched extracellular vesicles from human plasma and serum

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Abstract

Aim: Neuron-enriched extracellular vesicles (NEEVs) are promising minimally invasive biomarkers for studying central nervous system biology; however, variability in EV isolation and neuronal enrichment methods limit reproducibility and standardization. Here, we systematically evaluated EV isolation workflows and neuronal immunocapture markers using human plasma and serum samples.

Methods: Total EVs were isolated using precipitation- and size exclusion chromatography (SEC)-based approaches, and NEEVs were enriched using ATP1A3, L1CAM, and NCAM antibodies. EVs and NEEVs were characterized by flow cytometry, microfluidic resistive pulse sensing, super-resolution microscopy, and tetraspanin immunoassays. Data-independent acquisition proteomics was explored for functional total EV and NEEV protein cargo.

Results: Precipitation-based isolation produced the highest EV and NEEV recovery, whereas SEC-based methods demonstrated improved purity. Proteomic analyses identified 297 neuronal-specific proteins in NEEVs, with plasma-derived NEEVs showing greater neuronal protein detection and broader proteome coverage than serum-derived NEEVs. Each workflow showed distinct trade-offs between recovery, purity, and proteomic depth, indicating that optimal method selection should depend on downstream analytical priorities.

Conclusion: EV isolation method was the primary driver of proteomic variability, whereas neuronal capture antibody had comparatively modest effects. Overall, these findings highlight the strong influence of biofluid type and isolation strategy on NEEV characterization and proteomic outcomes.

keywords: Neuron-enriched extracellular vesicles, extracellular vesicles, plasma, serum, isolation methods, proteomics, biomarkers

INTRODUCTION

The field of extracellular vesicles (EVs) is currently grappling with a central question: which neuronal cell membrane marker(s) should be used for isolating EVs that reflect neuronal biology? EVs are nanoparticles that carry diverse cargo, including proteins, lipids, RNA, DNA, and other biologically relevant molecules, and mediate intercellular communication^[1]. Evidence suggests that EVs released from cells within the central nervous system (CNS) cross the blood-brain barrier (BBB) and enter peripheral circulation^[2,3]. This property enables the capture of brain-derived EVs from accessible biofluids such as plasma or serum, positioning them as promising, minimally invasive biomarkers for neurodegenerative, psychiatric, and other CNS disorders.

However, the validity of this approach depends critically on the choice of capture marker. Implicit criteria for an EV-specific neuronal marker includes that the protein must be: (1) expressed in neurons (i.e., exhibit neuronal enrichment or specificity); (2) localized to the plasma membrane with extracellular accessibility (i.e., suitable for immunocapture); and (3) reliably detected in biofluid-derived EVs^[4]. These requirements are not trivial, and failure to meet any one of them can undermine the interpretation of “neuron-specific” EV populations. Therefore, careful selection and validation of markers is essential for the accurate isolation of neuron-enriched EVs (NEEVs; also referred to as neuron-derived EVs or exosomes) in liquid-based biopsy applications.

To date, L1 cell adhesion molecule (L1CAM)^[5], also known as CD171, remains the most widely used neuronal marker for immunocapture of NEEVs from human clinical samples. Several groups studying Alzheimer’s Disease (AD), autism spectrum disorder (ASD), Amyotrophic Lateral Sclerosis (ALS), Parkinson’s Disease, and psychiatric disorders, such as major depressive disorder (MDD), have utilized L1CAM-based immunocapture to isolate

neuronal-origin EVs^[6-12]. *Goetzl et al.* first described the use of L1CAM and demonstrated its utility for enriching neuronal-origin EVs from peripheral blood^[13].

The controversy surrounding L1CAM as a marker for NEEV isolation centers on several key issues: (1) soluble *vs.* EV-associated; (2) lack of co-localization with EV markers; (3) proteolytic cleavage and alternative isoforms; and (4) reproducibility and interpretation of prior findings. A seminal paper by *Norman et al.* demonstrated that L1CAM is not associated with EVs in human plasma or cerebrospinal fluid (CSF), showing that EVs predominantly elute in earlier size exclusion chromatography (SEC) fractions, while L1CAM is found in later fractions (11-14)^[14]. Similarly, *Kadam et al.* reported that the majority of L1CAM in plasma and CSF exists as a free, soluble form rather than being EV-associated^[15]. Given the evidence, caution is warranted when interpreting prior studies using L1CAM for NEEV isolation, as reported signals may reflect co-isolated non-EV cargo.

Conversely, *Nogueras-Ortiz et al.* used single-EV techniques to demonstrate that L1CAM ectodomain epitopes are co-expressed on single EVs alongside neuronal proteins, including GAP43, vesicle-associated membrane protein 2 (VAMP2), and beta-3 tubulin. Notably, 30-63% of L1CAM-positive EVs carried neuronal proteins compared to only 0.8-3.9% of L1CAM-negative EVs, indicating substantial neuronal enrichment^[16]. These findings suggest that despite earlier concerns, L1CAM may still serve as a useful — albeit imperfect — marker for NEEV isolation when coupled with rigorous validation strategies^[17].

Other neuronal markers have been explored, though less extensively, including: (1) neural cell adhesion molecule-1 (NCAM-1/CD56)^[18,19], (2) amyloid beta precursor like protein 1 (APLP1)^[20], (3) growth-associated protein 43 (GAP43) and neuroligin 3 (NLGN3)^[21]; (4) neuroligin 3 (NRXN3)^[22]; and (5) ATPase Na⁺/K⁺ Transporting Subunit Alpha 3 (ATP1A3)^[23]. More recently, next-generation neuronal markers have been proposed to improve the specificity of NEEV isolation. In 2023, *Eitan et al.* suggested a co-capture strategy with GAP43/NLGN3, while *You et al.* proposed ATP1A3 as an alternative marker. The co-capture with GAP43 (an axonal marker) and NLGN3 (a cell surface marker) enabled the evaluation of AD-related biomarkers in NDEVs, including p181-Tau, amyloid beta, pro-

brain-derived neurotrophic factor, and synaptic proteins^[21]. *You et al.* demonstrated that ATP1A3-enriched EVs exhibited greater neuronal specificity, better enrichment of synaptic cargo, and improved detection of AD-related proteins compared to L1CAM- and NCAM-enriched EVs^[23]. Finally, Ter-Ovanesyan reported in a preprint in bioRxiv that NRXN3 was present on EVs in CSF and plasma with SEC and density gradient diffusion^[22].

One might argue that several candidates are well-suited as neuronal markers for NEEVs. For example, L1CAM is crucial for nervous system development and other functions, including neuron-to-neuron adhesion, axonal guidance, and migration^[17,24]. NCAM-1 plays a central role in cell adhesion, neurite outgrowth, synaptic density, and neurodevelopment^[25]. APLP1 is a neuron-enriched type I transmembrane protein that functions as a synaptic cell-adhesion molecule and supports dendritic spine maintenance, synaptogenesis, and basal synaptic transmission^[26]. GAP43 is an intracellular growth-associated protein responsible for neuronal pathfinding and branching during development and regeneration, while NLGN3 is a neuronal adhesion protein known for synaptic organization^[27,28]. ATP1A3 is responsible for maintaining neuronal ion gradients, particularly during recovery after neuronal signaling^[29]. Lastly, NRXN3 plays important roles in synapse formation, differentiation, maturation, and stability^[30].

While these proteins represent promising candidates for NEEV isolation, additional considerations are warranted. The Human Protein Atlas provides estimates of protein abundance in the cerebral cortex, which may inform target selection^[31,32]. For example, although NRXN3 has emerged as a promising neuronal EV surface marker, its reported protein abundance in the cerebral cortex (~90 million protein copies) is substantially lower than that of ATP1A3 (~1 billion copies) and NCAM1 (~1.3 billion copies). Protein abundance alone, however, does not determine suitability for EV immunocapture, as EV incorporation, extracellular epitope accessibility, and cell-type specificity are also critical determinants. Consequently, independent validation across multiple research groups and diverse biofluid cohorts is still needed. Establishing consensus on the optimal neuronal surface marker(s) for EV enrichment will be essential to improving the reproducibility, specificity, and standardization of studies utilizing neuronal-origin extracellular vesicles^[33].

Given this range of biologically relevant markers, a key question remains: which protein markers are appropriate for NEEV isolation. In this study, we systematically compared three neuronal markers, L1CAM, NCAM, and ATP1A3, based on sample and antibody availability. Following MISEV2023 guidelines^[34], we evaluated their performance across (1) clinical sample types (serum and plasma); (2) isolation methods (precipitation and size exclusion); (3) multiple characterization techniques [quantitative tetraspanin assays, microfluidic resistive pulse sensing (MRPS), and super resolution microscopy analysis]; and (4) downstream applications [immune assays and data-independent (DIA) acquisition proteomics]. By integrating biochemical, biophysical, and proteomic analyses, this work aims to establish evidence-based criteria of three currently used neuronal markers for NEEV immunocapture to provide guidance toward standardization in the field.

METHODS

Materials and products have been described in this manuscript to ensure reproducibility by other laboratories. All critical materials, along with manufacturers and catalog numbers, are listed in Supplementary Table 1.

Blood collection

Peripheral venous blood was obtained using BD Vacutainer collection tubes, including EDTA-coated and serum separator tubes. Within two h of blood collection, samples were centrifuged at $1,300 \times g$ for 10 min (min) at room temperature (RT). Serum and plasma were collected, aliquoted, and stored at -80°C until downstream EV isolation. Blood samples were from the Tulsa 1000 study (ClinicalTrials.gov identifier: NCT02450240), carried out at the Laureate Institute for Brain Research in Tulsa, Oklahoma, United States, from January 2015 through December 2018. All participants provided written informed consent and were compensated for participation. The study was approved by the Western Institutional Review Board and conducted in compliance with the Declaration of Helsinki.

Total extracellular vesicle isolation

SEC- and precipitation-based methods are presented below. Given the widespread use of SEC as a standard approach for total EV isolation, several commercially available kits were evaluated including Apex, SmartSEC, and miniPURE-EVs. In addition, precipitation-based methods were evaluated for their potential to provide high EV yield for subsequent immunoaffinity capture, including ExoQuick and ExoQuick Ultra.

Apex size-exclusion chromatography (SEC)

EVs were isolated from plasma or serum using Apex SEC columns (Apex 6B, Everest Biolabs). Plasma or serum samples were thawed on ice and centrifuged at $3,000 \times g$ for 15 min to remove cellular debris and aggregates. Phosphate-buffered saline (PBS) used throughout the procedure was filtered through a 0.22 μm filter.

Prior to sample loading, SEC columns were equilibrated by washing with 17 mL of PBS. A total of 500 μL of clarified plasma or serum was loaded onto the column, and 2 mL of PBS void volume was discarded. Fractions were subsequently collected at 500 μL per fraction. Fractions 1-5 were collected and analyzed for total protein concentration, as well as particle size and concentration.

Based on particle and protein distribution across fractions [Supplementary Figure 1], fractions 1-3 were selected for downstream analysis. Therefore, for the Apex method, fractions 1-3 were combined to yield a total volume of 1,500 μL . In addition, fractions 1-3 were further concentrated to generate the Apex-Concentrated method for comparative analyses.

MiniPURE-EVs spin column

EVs were isolated from plasma or serum using miniPURE-EVs Spin Columns (HansaBioMed Life Sciences), a SEC-based centrifugal method. Plasma and serum samples were thawed on ice and centrifuged at $3,000 \times g$ for 15 min to remove cellular debris, then were further clarified by centrifugation at $10,000 \times g$ for 30 min to remove large particles.

Prior to sample loading, miniPURE-EVs Spin Columns were equilibrated to RT for 30 min. Storage buffer was removed by centrifugation at $200 \times g$ for 3 min, followed by three washes with 400 μ L of 1X PBS, each centrifuged at $200 \times g$ for 4 min. Clarified 200 μ L plasma or serum was then applied directly to the column, which was placed into a new 15 mL collection tube. EVs were eluted by centrifugation at $200 \times g$ for 3 min, and the eluate was collected for downstream analyses.

SmartSEC

Plasma and serum samples were centrifuged at $3,000 \times g$ for 15 min to remove cellular debris. To further eliminate large vesicles and remaining debris, samples underwent an additional differential centrifugation step at $10,000 \times g$ for 15 min.

SmartSEC Single columns (resin stored in buffer; System Biosciences) were prepared by removing the cap and bottom closure, which were retained for later steps. Columns were placed into empty 15 mL collection tubes and centrifuged at $500 \times g$ for 30 seconds (s) to remove storage buffer. Columns were washed by adding 1 mL of column buffer followed by centrifugation at $500 \times g$ for 30 s, after which the collection tube was discarded.

The bottom closure was reinserted, and the prepared sample was applied to the top of the resin bed. PBS was added to adjust the total sample volume to 500 μ L. Columns were capped and incubated at RT for 30 min with rotation.

For EV elution, the cap was loosened halfway, the bottom closure was removed, and the column was immediately placed into a fresh 15 mL collection tube. EVs were collected by centrifugation at $500 \times g$ for 30 s.

SmartSEC-DeLipo

EVs were first isolated using the SmartSEC protocol as described above. Lipoprotein depletion was then performed using the DeLipo column.

DeLipo columns were prepared by removing the cap and bottom closure and placing the column into an empty collection tube. Columns were centrifuged at $500 \times g$ for 30 s to remove storage buffer. The flow-through was discarded, and columns were washed twice with 0.5 mL of column buffer, each followed by centrifugation at $500 \times g$ for 30 s.

Following preparation, the bottom of the column was sealed with the bottom closure. The isolated EV sample obtained from SmartSEC was applied to the resin bed, and the column was capped. Columns were incubated at RT for 5 min with rotation.

For elution, the bottom closure was removed, and the column was immediately transferred to a provided 2 mL Eppendorf tube. The cap was loosened halfway and EVs were collected by centrifugation at $500 \times g$ for 30 s.

ExoQuick

Plasma and serum samples were centrifuged at $3,000 \times g$ for 15 min to remove cellular debris. For plasma samples, a defibrinating step was performed by adding purified thrombin to a final concentration of 5 U/mL. Samples were incubated at RT for 5 min with gentle mixing, followed by centrifugation at 10,000 revolutions per min (rpm) for 5 min. The supernatant was transferred to a new tube for EV isolation.

ExoQuick Exosome Precipitation Solution was added to the clarified plasma or serum at the recommended ratio (e.g., 63 μ L ExoQuick per 250 μ L plasma or serum), mixed thoroughly, and incubated on ice for 30 min. Samples were centrifuged at $1,500 \times g$ for 30 min at 4 °C. The supernatant was aspirated, and residual liquid was removed by an additional centrifugation step at $1,500 \times g$ for 5 min at 4 °C. The resulting EV pellet was resuspended in 250 - 500 μ L of 1X PBS, corresponding to the original plasma or serum input volume.

ExoQuick ULTRA

Plasma and serum samples were clarified by centrifugation at $3,000 \times g$ for 15 min. Plasma samples underwent defibrination using purified thrombin (final concentration 5 U/mL) with

incubation at RT for 5 min, followed by centrifugation at 10,000 rpm for 5 min. The supernatant was transferred to a new tube.

ExoQuick Exosome Precipitation Solution was added at the recommended ratio, and the mixture was incubated on ice for 30 min. Samples were centrifuged at $1,500 \times g$ for 30 min at 4 °C, followed by removal of the supernatant and an additional centrifugation step at $1,500 \times g$ for 5 min at 4 °C to eliminate residual fluid.

The EV pellet was resuspended in 200 μ L of Buffer B, followed by the addition of 200 μ L of Buffer A. Purification columns were prepared by loosening the screw cap, removing the bottom closure, and centrifuging at $1,000 \times g$ for 30 s to remove storage buffer. Columns were washed twice with 500 μ L of Buffer B, each followed by centrifugation at $1,000 \times g$ for 30 s.

The bottom closure was reinserted, and 100 μ L of Buffer B was added to prepare the resin for sample loading. The entire EV suspension was applied to the column, capped, and mixed on a rotating shaker at RT for 5 min.

For elution, the screw cap was loosened, the bottom closure removed, and the column was immediately transferred to a 2 mL Eppendorf tube. Purified EVs were collected by centrifugation at $1,000 \times g$ for 30 s, after which the column was discarded.

Neuron-enriched extracellular vesicles (NEEVs) enrichment

NEEVs were isolated from total EV preparations using an immunoaffinity-based magnetic bead capture approach. Total EVs were first isolated from plasma or serum using the indicated methods (Apex-Concentrated, SmartSEC, or ExoQuick), as described above based on EV yield. Apex fractions 1-3 contain a total EV volume of 1,500 μ L, which dilutes the antibody concentration during immunocapture, therefore, Apex-Concentrated was used for NEEV enrichment. All steps were performed at 4 °C or on ice unless otherwise specified.

Streptavidin-coated magnetic Exo-Flow beads (System Biosciences) were vortexed briefly and aliquoted (100 μ L per reaction) into Protein LoBind tubes. Beads were placed on a magnetic stand for 2 min, and the storage buffer was removed. Beads were washed twice with 500 μ L of 1X bead wash buffer, with each wash followed by 2 min of magnetic separation.

Washed beads were incubated with 20 μ g of biotinylated anti-human antibodies targeting neuronal markers: ATP1A3 (Rabbit anti-Human ATP1A3 Polyclonal Antibody, Biotin Conjugated, Cat# MBS7049718, MyBioSource.com), L1CAM (CD171 Monoclonal Antibody (eBio5G3 (5G3)), Biotin, Cat# 13-1719-82, eBioscience), or NCAM1 (CD56 Antibody, anti-human, Biotin, Cat# 130-113-311, Miltenyi Biotec, Inc.). Antibodies were added directly to the beads and incubated on ice for 2 h with gentle mixing by intermittent inversion every 30 min to allow antibody-bead conjugation. Following incubation, antibody-bead complexes were washed once with 300 μ L and twice with 500 μ L of 1X bead wash buffer and resuspended in 500 μ L of wash buffer.

Total EV suspensions (approximately 250 μ L for Apex-Concentrated samples and 500 μ L for ExoQuick or SmartSEC samples) were added to the antibody-bead complexes and incubated overnight at 4 °C with end-over-end rotation at 12 rpm to allow selective capture of NEEVs. Following incubation, bead-antibody-EV complexes were collected using a magnetic stand for 2 min, and the supernatant was removed. Complexes were washed three times with 500 μ L of 1X bead wash buffer to remove unbound and non-specific associated vesicles.

For protein extraction, bead-bound NEEVs were lysed using 250 μ L of cold M-PER Mammalian Protein Extraction Reagent supplemented with cOmplete™ Mini Protease Inhibitor Cocktail. Lysis was performed at 4 °C with rotation at 12 rpm for 15 min. Samples were briefly centrifuged at 500 $\times$ g for 1 min at 4 °C to collect liquid from tube lids and ensure homogeneous mixing.

Lysates were transferred to 0.45 μm Spin-X centrifuge tube filters and centrifuged at 3,000 $\times g$ for 3 min at 4 $^{\circ}\text{C}$ to remove beads and insoluble material. The clarified flow-through, containing NEEV protein lysates, was immediately placed on ice and stored at -80 $^{\circ}\text{C}$ until downstream analyses.

Characterization

Flow cytometry

To select the optimal bead type for immunocapture, five magnetic bead preparations were evaluated for the enrichment of L1CAM-positive NEEVs: (1) EXO-FLOW beads (9.1 μm diameter); (2) Dynabeads M-280 Streptavidin beads (2.8 μm); (3) Dynabeads M-270 Streptavidin beads (2.8 μm); (4) Dynabeads MyOne Streptavidin C1 beads (1.0 μm); and (5) Dynabeads MyOne Streptavidin T1 beads (1.0 μm). Briefly, EVs were isolated from 250 μL of human serum using the ExoQuick precipitation method. L1CAM-positive NEEVs were subsequently enriched using biotinylated anti-L1CAM antibodies coupled to streptavidin-coated magnetic beads. The captured particles were evaluated by flow cytometry for the EV-associated markers CD63 and CD81 and the neuronal enrichment marker L1CAM.

For comparing different antibodies, a Cytex Aurora spectral flow cytometer was used to confirm EV populations using fluorescently labeled antibodies (CD81-FITC and EXO-FITC). Samples were washed three times with 500 μL of 1X wash buffer. After the third wash, 50 μL of each sample was placed on a magnetic stand, the supernatant was removed, and 300 μL of stain buffer was added. A 100 μL aliquot was then distributed into four tubes (diluted NEEV). The stains were prepared as follows: 1) 100 μL diluted NEEV + 4 μL stain buffer (unstained control); 2) 100 μL diluted NEEV + 4 μL CD81-FITC; 3) 100 μL diluted NEEV + 4 μL EXO-FITC. Negative controls included beads only, PBS-enriched NEEV (PBS used in place of EV during enrichment), and EV-depleted plasma NEEV (EV-depleted plasma used during enrichment).

Flow cytometry data were analyzed using FlowJo software. The unstained bead sample was used to define the negative population and establish gating via histogram analysis. Fluorescent channels were then overlaid to compare expression profiles. Histograms and

mean fluorescence intensity (MFI) for each fluorophore were subsequently generated using all described methods for both serum and plasma NEEV samples.

Meso scale diagnostics (MSD) assays and bicinchoninic acid (BCA) assay

Meso Scale Diagnostics (MSD) assays were used to quantify EV-associated tetraspanins in total EVs and NEEVs. Total EV samples were lysed in M-PER buffer supplemented with 1X protease inhibitor cocktail (PIC) at a 1:2 volume ratio, while NEEV samples were lysed in 250 μ L M-PER buffer containing 1X PIC. All lysates were rotated for 15 min at 4 °C to ensure complete protein extraction. Following lysis, samples were clarified and prepared according to MSD assay requirements.

Quantification of EV tetraspanins was performed using the U-PLEX Human CD9 (EV), CD63 (EV), and CD81 (EV) assays (MSD). Assays were conducted according to the manufacturer's instructions, including plate blocking, sample and detection antibody incubation, washing steps, and signal development. Electrochemiluminescent signals were detected using a Meso QuickPlex SQ120 instrument, and analyte concentrations were calculated based on standard curves generated for each tetraspanin. All samples were run under identical assay conditions to ensure comparability across EV populations.

Total protein concentration was determined using the bicinchoninic acid (BCA) assay. Total EV samples were diluted 1:2 in M-PER buffer containing 1X PIC, whereas NEEV samples were measured undiluted following lysis. Twenty-five microliters of each sample were assayed using separate standard curves prepared for total EV and NEEV. The working reagent was prepared at a 50:1 ratio of BCA Reagent A to Reagent B, samples were incubated at 37 °C for 30 min, and absorbance was measured at 570 nanometers (nm) using a microplate reader.

Microfluidic resistive pulse sensing (MRPS)

Size distribution and particle concentration for total EV and NEEV were determined using MRPS on a Spectradyn nCS1TM instrument. All measurements were performed in accordance with the manufacturer's recommended operating procedures. EV collection

buffers were analyzed in parallel and served as negative controls to account for background particle counts.

Prior to analysis, total EV samples were diluted 1:10 and NEEV samples were diluted 1:2 in sample buffer consisting of PBS supplemented with 1% Tween-20. For each measurement, 5 μ L of diluted sample was loaded onto C-400 microfluidic cartridges and analyzed using the nCS1 system. Particle detection and data acquisition were conducted using nCS1 Viewer software. Particle concentrations were calculated after applying the appropriate dilution factors, and background signal was subtracted prior to data export for downstream analysis and visualization.

The MRPS running buffer (150 mL) was prepared by supplementing 1X PBS with 1% Tween-20 and mixing continuously on a magnetic stir plate to ensure homogeneity. The running buffer was filtered through a 0.22 μ m bottle-top filter and stored at 4 °C prior to use. Sample dilution buffer was prepared by further filtering the running buffer through a 0.02 μ m syringe filter to minimize particulate contamination.

ONI super-resolution microscopy

Total EVs and NEEVs were detected using the ONI EV profiler 2 kit and assessed using direct stochastic optical reconstruction microscopy (dSTORM) on the ONI super-resolution Nanoimager. Samples were immobilized by phosphatidylserine (PS) capture on assay chips prior to the washing of unbound capture antibodies. EVs were fixed and stained with a 3-color tetraspanin antibody panel (CD9-488 nm, CD63-561 nm, CD81-640 nm) with laser powers set at 55%, 40%, and 25% for 488, 561, and 640 nm, respectively. The quantitative evaluation of EVs was conducted by using EV profiling module in CODI software, incorporating drift correction, signal filtering, DBSCAN clustering, cluster filtering, and particle counting analyses (<https://alto.codi.bio/>). This workflow enabled quantification of EV populations positive for individual (CD9, CD63, or CD81), dual, or triple tetraspanin expression. EVs were detected at a scale of 100 nm.

Two NEEV elution strategies were evaluated prior to ONI analysis: (1) using elution buffers provided in the Basic Exo-Flow kit and (2) elution using an acid-based glycine-HCl method. For glycine-HCl elution, a 0.05 M glycine-HCl solution (pH 3.0) was prepared by combining 7.5 mL deionized distilled (DD) water with 2.5 mL of 0.2 M glycine buffer (pH 3.0). To neutralize the eluate, 25 μ L of 10% bovine serum albumin (BSA) and 10 μ L of 1 M Tris-HCl (pH 8.0) were pre-aliquoted into labeled tubes for each sample. Following immunocapture of NEEVs on antibody-conjugated beads, the supernatant was removed, and 100 μ L of cold 0.05 M glycine-HCl (pH 3.0) was added to the bead-antibody-EV complex. Samples were gently mixed for 30 s to dissociate NEEVs from the beads. Tubes were then placed on a magnetic stand for 2 min to separate the beads. The supernatant containing eluted NEEVs was carefully transferred to the pre-prepared tubes containing BSA and Tris-HCl for immediate neutralization, yielding a final volume of 135 μ L. Eluted NEEVs from both methods were subsequently subjected to ONI Nanoimager analysis for single-particle characterization.

Proteomics

Proteomics for total EV

Total EV proteomics were performed using data-independent acquisition (DIA) mass spectrometry (MS) on an Orbitrap Exploris 480 with 15 Th windows. Total protein from each sample was reduced, alkylated, and digested using single-pot, solid-phase-enhanced sample preparation^[35] with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were then separated by reverse phase XSelect CSH C18 2.5 μ m resin (Waters) on an in-line 150 $\times$ 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 65:35 buffer A(0.1% formic acid, 0.5% acetonitrile):B (0.1% formic acid, 99.9% acetonitrile) ratio. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275 $^{\circ}$ C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific). Precursor spectra were acquired with a scan from 385-1,015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 40 $\times$ 15 Th isolation windows from 394-1,009 Th, normalized AGC target 500%,

maximum injection time 40 ms. A second DIA was acquired in a staggered window (15 Th) pattern with optimized window placements from 400-1,015 Th.

Following data acquisition, data were searched using Spectronaut (Biognosys version 20.2) against the UniProt Homo sapiens database (Proteome ID: UP000005640, Taxon ID: 9606, 3rd version of 2025) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to Quant 2.0, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Fixed Modifications were set to Carbamidomethyl (C), and variable modifications were set to Acetyl (Protein N-term), Oxidation (M). Protein MS2 intensity values were assessed for quality using ProteiNorm^[36]. The data was normalized using Cyclic Loess^[37] and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors. Proteins with a false discovery rate (FDR) adjusted (adj) p -value < 0.05 and a $|\log_2 \text{fold-change} (\log_2 \text{FC})| > 1$ were considered significant.

Proteomics for NEEV

NEEV proteomics were performed using DIA-MS on an Orbitrap Astral. Total protein from each sample was reduced, alkylated, and digested using single-pot, solid-phase-enhanced sample preparation^[35] with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were then separated by a reverse phase Ion-Opticks-TS analytical column (25 cm $\times$ 75 μm with 1.7 μm C18 resin) supported by an EASY-Spray nano-source and stabilized with a Heater THOR Controller (Ion-Opticks) at 60 °C. Peptides were trapped and eluted from a (PepMap Neo, 300 μm $\times$ 5mm Trap) using a Vanquish Neo UHPLC nano system (Thermo Scientific) which kept the samples at 11 °C before injection. Peptides were eluted at a flow rate of 0.350 $\mu\text{L}/\text{min}$ using a 35 min gradient from 98% Buffer A (0.1% formic acid, 0.5% acetonitrile in water): 2% Buffer B (80% acetonitrile, 20% water, 0.1% formic acid) to 94.5:5.5 at 0.1 min to 56:44 at 27.1 min followed by a column wash of 45:55 at 29.7 min to 1:99 at 35 min followed by equilibration back to 98:2. Eluted peptides were ionized by electrospray (2.5 kV) followed by mass spectrometric analysis on an Orbitrap Astral mass

spectrometer (Thermo). Precursor spectra were acquired from 380-980 Th, 240,000 resolution, normalized AGC target 200%, maximum injection time 3 ms. DIA acquisition on the Orbitrap Astral was configured to acquire 199, 3 Th window from 380-980 Th, 25% HCD Collision Energy, normalized AGC target 100%, maximum injection time 3 ms. Fragment (MS2) scan range from 150-2,000 Th with an RF Lens (%) set to 40.

Following data acquisition, data were searched using Spectronaut (Biognosys version 20.5) against the UniProt Homo sapiens database (Proteome ID: UP000005640, Taxon ID: 9606, 4th version of 2025) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to MaxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Fixed Modifications were set to Carbamidomethyl (C), and variable modifications were set to Acetyl (Protein N-term), Oxidation (M). Protein MS2 intensity values were assessed for quality using ProteiNorm^[36]. The data was normalized using Cyclic Loess^[37] and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors. Proteins with an FDR-adj *p*-value < 0.05 and a $|\log_2FC| > 1$ were considered significant.

Neuronal specific protein abundance

Proteins with neuronal cell specificity were identified from brain single-cell RNA expression clusters in the Human Protein Atlas (HPA, proteinatlas.org)^[38,39]. Proteins quantified by DIA-MS in total EV and NEEV samples were each intersected with the HPA neuronal set to derive analysis-specific subsets. Detection rates were calculated as the percentage of neuronal proteins with non-missing Cyclic Loess-normalized abundance per sample, grouped by sample matrix and EV isolation methods for total EV analyses, and sample matrix, capture antibody, and EV isolation method for NEEV analyses. PCA was performed on the normalized abundance of the neuronal protein subset across all EV or NEEV samples, with centering and scaling applied. Neuronal protein detection overlap across experimental groups was assessed with UpSet plots, evaluated at three individual factor levels (sample matrix, EV isolation method, and capture antibody) or across all combined groups.

Group-level abundance was calculated as the median normalized expression across replicates within groups defined by matrix $\times$ EV isolation method for total EV, and antibody $\times$ matrix $\times$ EV isolation method for NEEV. Abundance distributions were visualized as violin plots with median and interquartile range, separated by sample matrix. For heatmap visualization, missing values were set to zero prior to z-scaling, and unsupervised hierarchical clustering was applied to both neuronal proteins and sample groups using pheatmap (v1.0.12), annotated by EV isolation method, sample matrix, and capture antibody. Supplementary tables of median protein abundance per group are provided for the neuronal proteins, with missingness denoted as “—” and row and column order corresponding to the heatmap clustering for direct reference. Visualizations were generated using ggplot2 (v3.5.2), with ggforce (v0.5.0) for PCA cluster ellipses and UpSetR (v1.4.0) for intersection plots.

RESULTS

The graphical abstract^[40] shows the comparative evaluation workflow of total EV isolation and L1CAM, ATP1A3, and NCAM1 neuron-enriched EV immunocapture.

Total EV isolation

Buffer and storage test for plasma and serum EV

To optimize buffer and EV storage conditions for downstream analysis, plasma and serum EVs isolated by SEC were evaluated for total protein and tetraspanin (CD9, CD63, CD81) concentrations across various conditions. EV protein was extracted using either MPER with PIC or RIPA with PIC. EVs were suspended in PBS or PBS with PIC. Storage conditions included -80 °C for two weeks, 4 °C for one or two weeks, fresh EVs, and EV protein lysates stored at -80 °C for two weeks. Results showed that both M-PER and RIPA extracted similar total protein amounts, but M-PER yielded tetraspanin concentrations 16-45 times higher than RIPA buffer in both plasma and serum EVs [Figure 1A]. EVs stored in PBS or PBS with PIC showed no difference in total protein or tetraspanins concentrations [Figure 1B]. Storage at 4 °C or -80 °C for up to two weeks did not affect total protein or tetraspanins concentrations, though a slight decrease in tetraspanins was observed in EV protein lysates stored at -80 °C [Figure 1C]. Based on these findings, all subsequent experiments used M-PER with PIC for

EV protein extraction, PBS for EV resuspension, and EV storage at 4 °C for up to two weeks, with EV samples stored at -80 °C for extended storage.

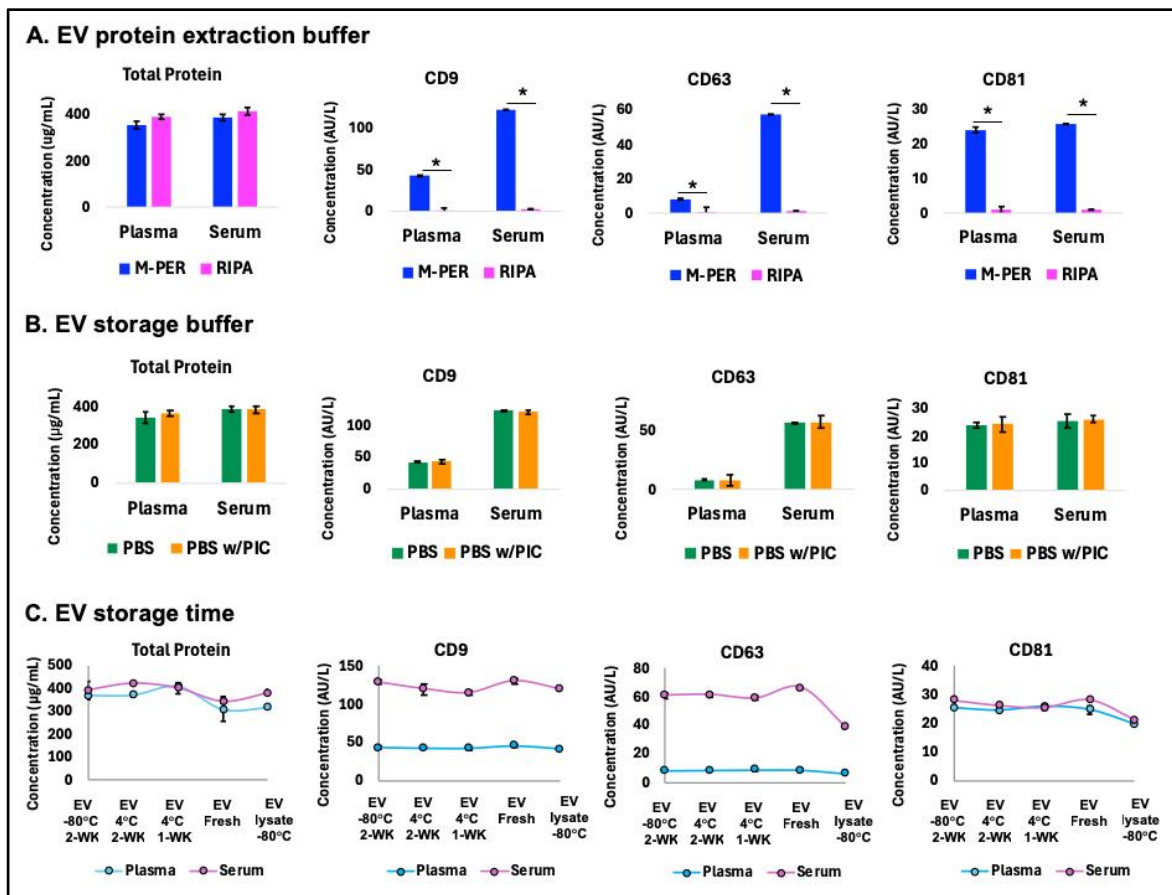


Figure 1. Comparison of plasma and serum extracellular vesicle (EV) total protein and tetraspanins (CD9, CD63, CD81) under different conditions. A) Comparison of EV protein extraction buffer ($n = 8$). Independent t -test was used to test the difference between MPER and RIPA on total protein, CD9, CD63, and CD81 concentrations within plasma or serum EVs. $*p < 0.001$; B) Comparison of EV storage buffer ($n = 5$). Independent t -test was used to test the difference between PBS and PBS w/PIC on total protein, CD9, CD63, and CD81 concentrations within plasma or serum EVs. No significant differences were observed; C) Comparison of EV storage time ($n = 2$). Note, Tetraspanin markers were measured using the U-PLEX Human CD9/CD63/CD81 (EV) assay, AU - Arbitrary Units. All samples in panel B and C were extracted with MPER. All comparisons were performed using blood from the same individual. n – number of EV samples isolated from plasma or serum. PIC - protease inhibitor cocktail.

Plasma and serum EV characterization

MRPS was used to characterize EV size distribution and concentration in plasma- and serum-derived EV samples across multiple isolation methods [Figure 2]. In plasma, EV populations were predominantly detected within the expected small EV size range (~50-150 nm), with notable variability in particle concentration across methods. ExoQuick and Apex yielded the highest particle concentrations, with ExoQuick showing a pronounced peak around ~70 nm. SmartSEC and SmartSEC-DeLipo produced comparatively lower particle counts, while ExoQuick ULTRA and miniPURE-EVs exhibited moderate yields with broader size distributions. Across all plasma isolation methods, particle counts declined sharply beyond ~150 nm, indicating minimal contribution from larger vesicles. Serum EV samples had a similar size distribution profile centered within ~50-150 nm. Apex demonstrated the highest particle yield, followed closely by ExoQuick, SmartSEC, and miniPURE-EVs. SmartSEC-DeLipo and ExoQuick ULTRA showed lower particle recovery. As observed in plasma, particle counts in serum samples decreased substantially beyond ~150 nm.

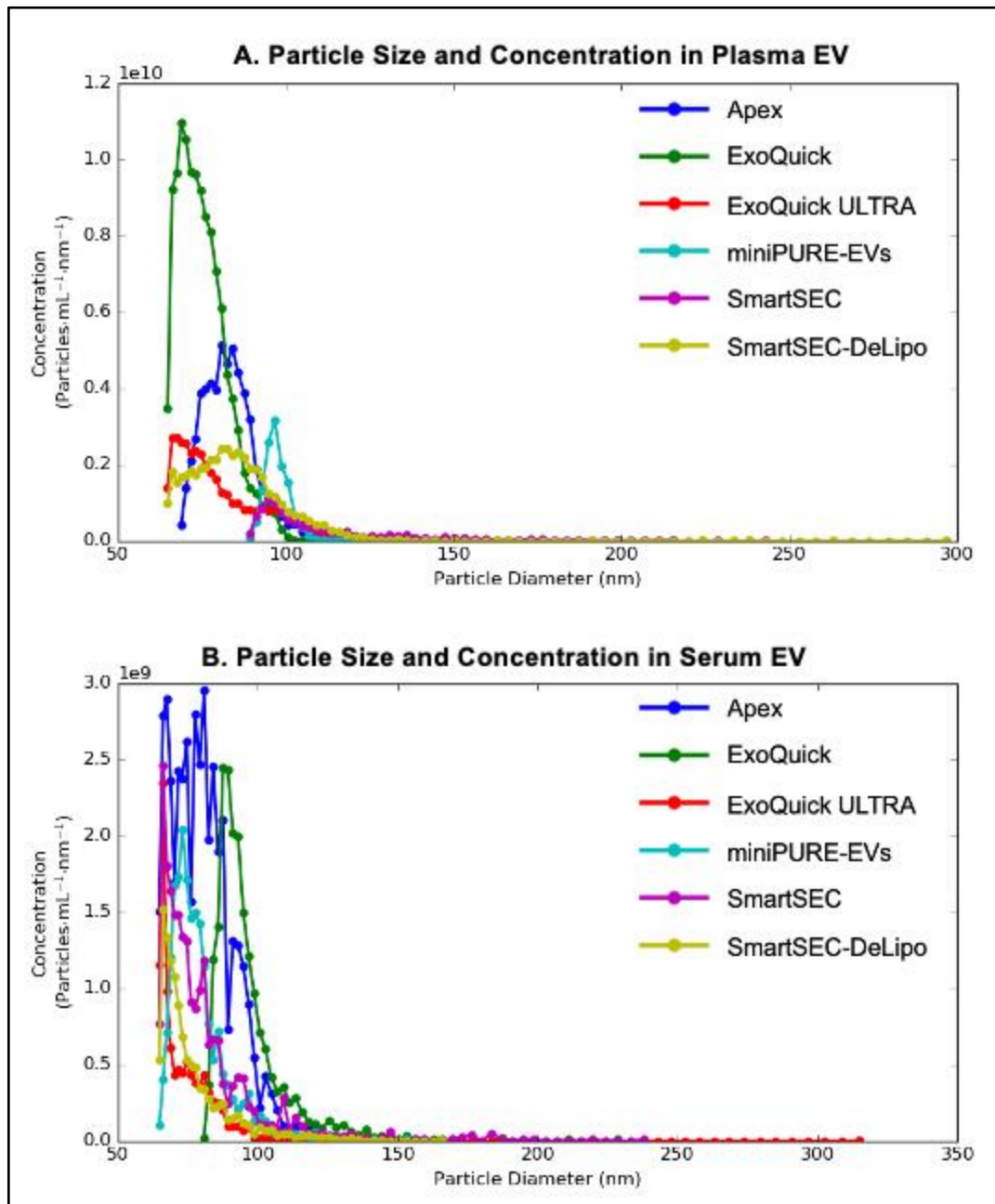


Figure 2. Microfluidic resistive pulse sensing (MRPS) analysis of particle size distribution and concentration for extracellular vesicles (EVs) isolated from plasma and serum. MRPS was used to measure particle concentration and size distribution for all EV isolation methods. A) Plasma EVs from all isolation methods; B) Serum EVs from all methods. All comparisons were performed using blood from the same individual.

To further quantify total EV yield, ONI Nanoimager analysis was performed [Figure 3]. In plasma-EVs, both ExoQuick and miniPURE-EVs methods demonstrated significantly higher total EV yields than other isolation techniques ($p < 0.05$) [Figure 3A]. Other methods, including Apex, SmartSEC, ExoQuick ULTRA, and SmartSEC-DeLipo, showed comparatively reduced yields. In serum-EVs, ExoQuick consistently yielded the highest number of EVs among all methods ($p < 0.05$) [Figure 3B]. Statistical analysis using one-way ANOVA confirmed significant differences in EV yield across isolation methods for both plasma and serum samples.

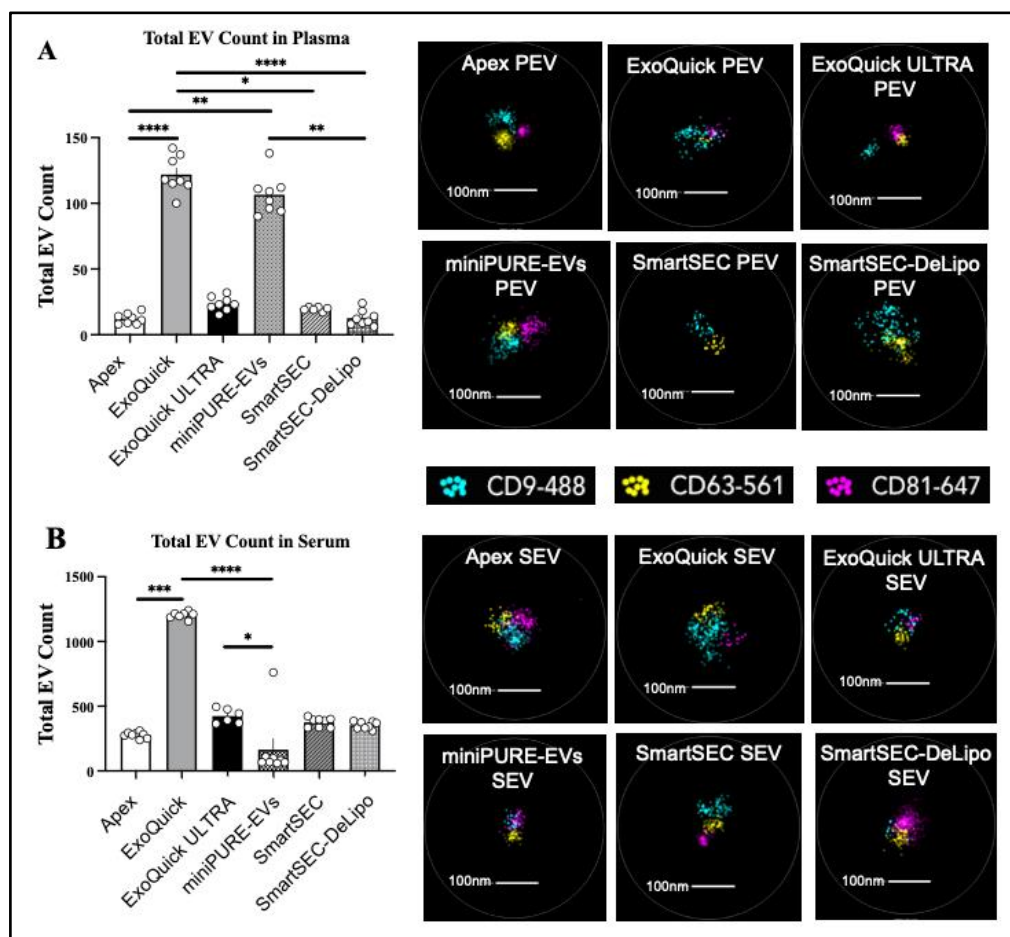


Figure 3. Comparison of total extracellular vesicle (EV) yield across various isolation techniques using ONI Nanoimager analysis. Representative ONI Nanoimager super-resolution microscopy images illustrate the distribution of canonical EV tetraspanin markers CD9-488 (cyan), CD63-561 (yellow), and CD81-647 (magenta) in plasma-derived EVs (PEVs) and serum-derived EVs (SEVs). Scale bars measure 100 nm. Quantitative bar graphs

represent EV counts measured per field of view (FOV) acquired on the ONI super-resolution microscope. Each bar represents an EV isolation method with one biological sample, and each individual dot corresponds to a single independent imaged FOV ($n = 8$ FOVs/sample). EV counts per FOV were quantified to assess relative vesicle abundance across isolation platforms. A) Total EV in plasma; B) Total EV in serum. The isolation groups were compared using one-way ANOVA with statistical significance determined by p -value < 0.05 and results expressed as mean $\pm$ SEM. Statistical significance is indicated as: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant. All comparisons were performed using blood from the same individual.

Comparison of plasma and serum EV tetraspanins across isolation methods

Next, a comparison of plasma and serum EV yields [arbitrary units (AU) per 500 μ L of plasma or serum input] and purity (AU per g of protein) for tetraspanins (CD9, CD63, CD81) using different EV isolation methods. CD9, CD63, and CD81 concentrations were obtained from MSD Human EV immunoassays [Figure 4].

In Figure 4A, plasma EVs from ExoQuick produced the highest yields for all three tetraspanins. Apex, and SmartSEC were after ExoQuick for CD9, CD63, and CD81. ExoQuick Ultra was the lowest yield across all tetraspanins.

For plasma EV purity, SmartSEC exhibited the highest purity across all three tetraspanins. ExoQuick Ultra was after SmartSEC in purity for CD63 and CD81, while Apex for CD9. ExoQuick showed the lowest purity.

In Figure 4B, serum EVs from Apex yielded the highest levels of CD9 and CD63, while ExoQuick yielded the highest for CD81. Apex-Concentrated and SmartSEC for CD9, SmartSEC and ExoQuick for CD63, and SmartSEC and Apex for CD81 were the second and third highest yielded methods. ExoQuick Ultra was the lowest.

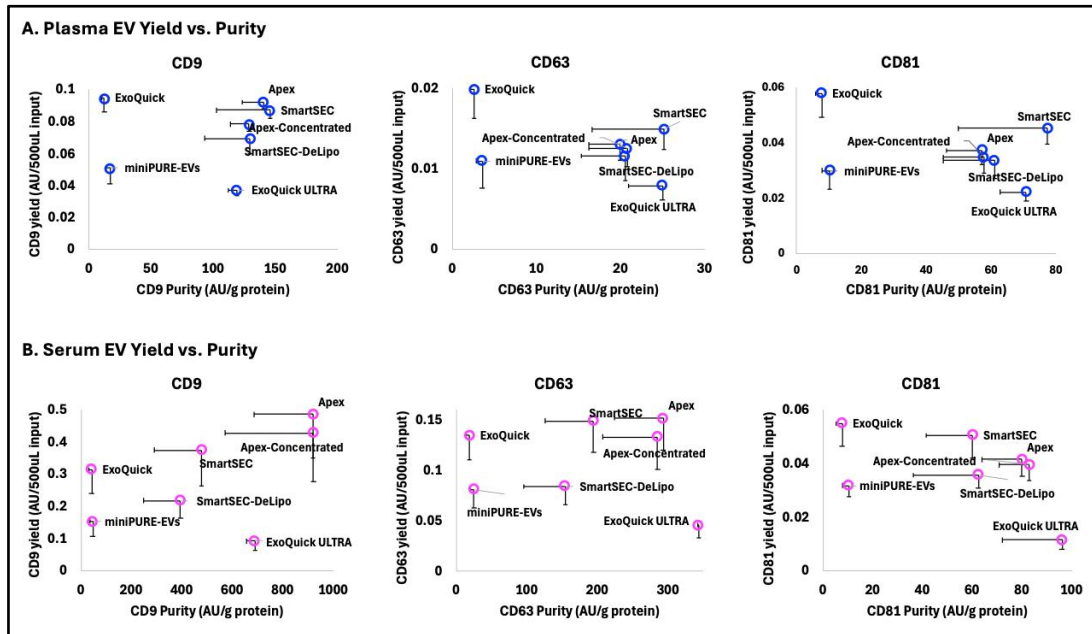


Figure 4. Comparison of plasma and serum extracellular vesicle (EV) tetraspanins (CD63, CD81, CD9) on different isolation methods. Total EV from plasma and serum were analyzed for EV tetraspanin concentration and purity. Note, Tetraspanin markers were measured using the U-PLEX Human CD9/CD63/CD81 (EV) Assays. AU - Arbitrary Units. $n = 3$ (number of subjects analyzed, all EV were isolated using blood from the same 3 individuals).

Apex and Apex-Concentrated for CD9, and ExoQuick Ultra for C63 and CD81 provided the highest purity. ExoQuick and miniPURE-EVs were the lowest across all three tetraspanins. See Supplementary Table 2 for yield and purity values. In addition, Supplementary Table 3 listed the feasibility for EV isolation with different methods.

Comparison of plasma and serum EV proteomics across isolation methods

Based on the EV yield and purity, four methods (Apex, Apex-Concentrated, SmartSEC-DeLipo, and ExoQuick ULTRA) were selected for EV proteomics. Differential protein abundance was assessed using multiple-testing-corrected statistics, and only proteins meeting both an adj p -value threshold adj $p < 0.05$) and a magnitude cutoff $|\log_2FC| > 1$ were considered significantly different.

Apex and Apex-Concentrated showed similar results, with no different abundance proteins observed [Supplementary Figure 2], therefore, only three methods (Apex, SmartSEC-DeLipo, and ExoQuick ULTRA) were used for proteomics comparisons.

In plasma-derived EVs, clear proteomic differences were observed across isolation methods [Figure 5]. Comparison of ExoQuick ULTRA versus Apex identified 186 proteins with significantly different abundance ($\text{adj } p < 0.05$ and $|\log_2\text{FC}| > 1$), indicating substantial method-dependent effects on EV protein composition. Similarly, ExoQuick ULTRA versus SmartSEC-DeLipo revealed 140 differentially abundant proteins, whereas SmartSEC-DeLipo versus Apex identified 137 significant proteins that met both statistical and fold-change thresholds. Supplementary Table 4-6 showed the protein names, $\log_2\text{FC}$, and $\text{adj } p$ -value of differentially expressed proteins in plasma EV using different methods. These findings demonstrate that although the overall plasma EV proteome is largely shared across methods, a substantial subset of proteins exhibits large, statistically robust differences in abundance depending on the isolation approach.

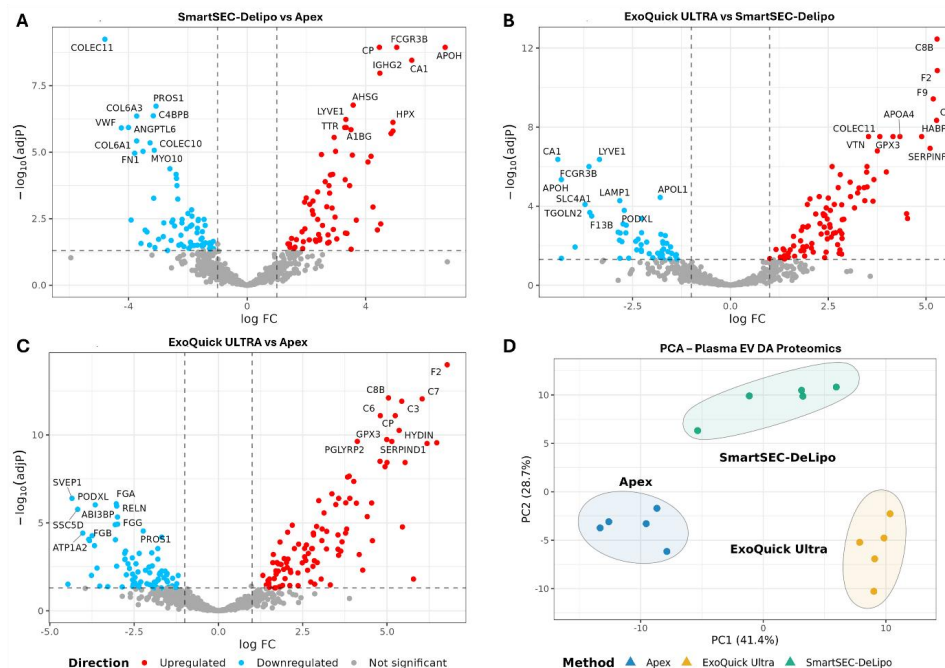


Figure 5. Comparison of EV proteomics using different EV isolation methods (Apex, SmartSEC-DeLipo, and ExoQuick ULTRA) in plasma EV. Volcano plots of differential abundance (DA) for proteins upregulated ($+\log_2\text{FC}$, red) or downregulated ($-\log_2\text{FC}$, blue)

across contrasts: A) SmartSEC-DeLipo vs. Apex; B) ExoQuick ULTRA vs. SmartSEC-DeLipo, and C) ExoQuick ULTRA vs. Apex. Significance determined at threshold $\text{adj } p < 0.05$ and $|\log_2\text{FC}| > 1$, with annotation for top ten lowest $\text{adj } p$ -value in upregulated and downregulated proteins. D) Venn diagram of significant DA protein overlap between EV isolation methods. $n = 5$ (number of subjects analyzed, all EV were isolated using blood from the same 5 individuals).

In serum-derived EVs, proteomic differences between isolation methods were similarly pronounced [Figure 6]. The ExoQuick ULTRA versus Apex comparison identified 181 proteins with significantly different abundance. Comparison of ExoQuick ULTRA versus SmartSEC-DeLipo yielded 129 significant proteins, whereas SmartSEC-DeLipo versus Apex showed the largest divergence with 211 proteins meeting significance and fold-change criteria. These results indicate that serum EV proteomes are strongly influenced by isolation strategy. See Supplementary Table 7-9 for protein names, $\log_2\text{FC}$, and $\text{adj } p$ -values of differently expressed proteins in serum EV using different methods.

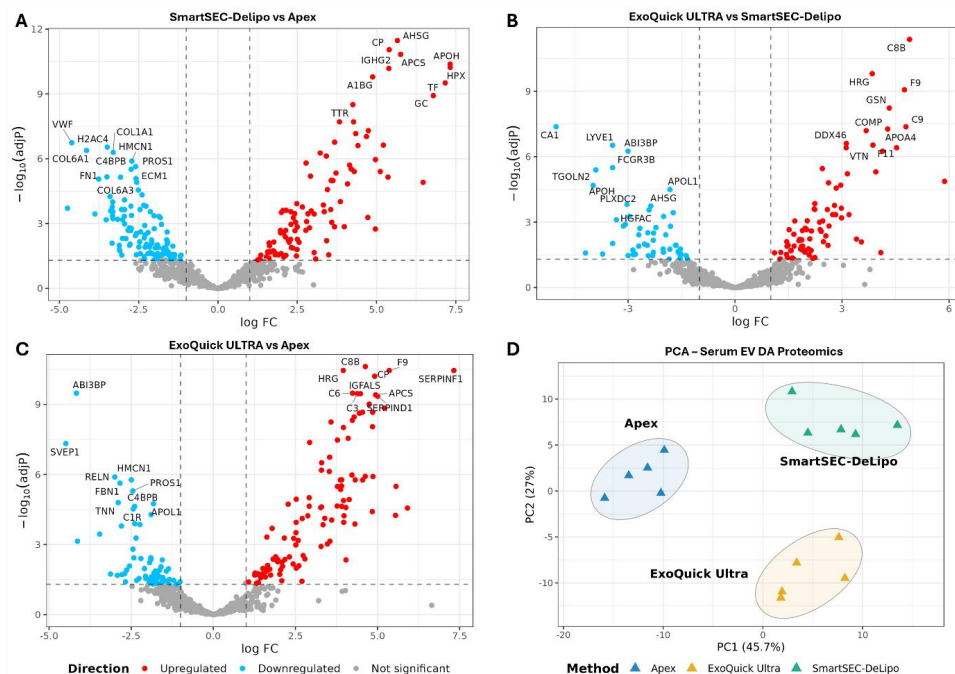


Figure 6. Comparison of EV proteomics using different EV isolation methods (Apex, SmartSEC-DeLipo, and ExoQuick ULTRA) in serum EV. Volcano plots of differential

abundance (DA) for proteins upregulated ($+\log_2\text{FC}$, red) or downregulated ($-\log_2\text{FC}$, blue) across contrasts: A) SmartSEC-DeLipo vs. Apex, B) ExoQuick ULTRA vs. SmartSEC-DeLipo, and C) ExoQuick ULTRA vs. Apex. Significance determined at threshold $\text{adj } p < 0.05$ and $|\log_2\text{FC}| > 1$, with annotation for top ten lowest $\text{adj } p$ -value in upregulated and downregulated proteins. D) Venn diagram of significant DA protein overlap between EV isolation methods. $n = 5$ (number of subjects analyzed, all EV were isolated using blood from the same 5 individuals).

To assess matrix-specific effects, plasma and serum EV proteomes were compared using identical isolation methods [Figure 7]. Figure 7A shows that EV isolation using Apex revealed 57 proteins that differed significantly between plasma and serum EVs. In contrast, fewer differences were observed for SmartSEC-DeLipo (24 proteins, Figure 7B) and ExoQuick ULTRA (29 proteins, Figure 7C). See Supplementary Table 10-12 for protein names, $\log_2\text{FC}$, and $\text{adj } p$ -values of differently expressed proteins in plasma vs. serum EV using the same method. These results indicate that plasma and serum EVs share highly similar proteomic profiles when processed with the same isolation method, and that the isolation strategy contributes more strongly to proteomic variability than biofluid type.

In addition, principal component analysis (PCA) supported these findings [Figure 7D], with the EV isolation method accounting for the largest source of variance (PC1, 22.4%), separating ExoQuick ULTRA and SmartSEC-DeLipo from Apex samples, and PC2 (19.9%) further distinguishing Apex and SmartSEC from ExoQuick ULTRA.

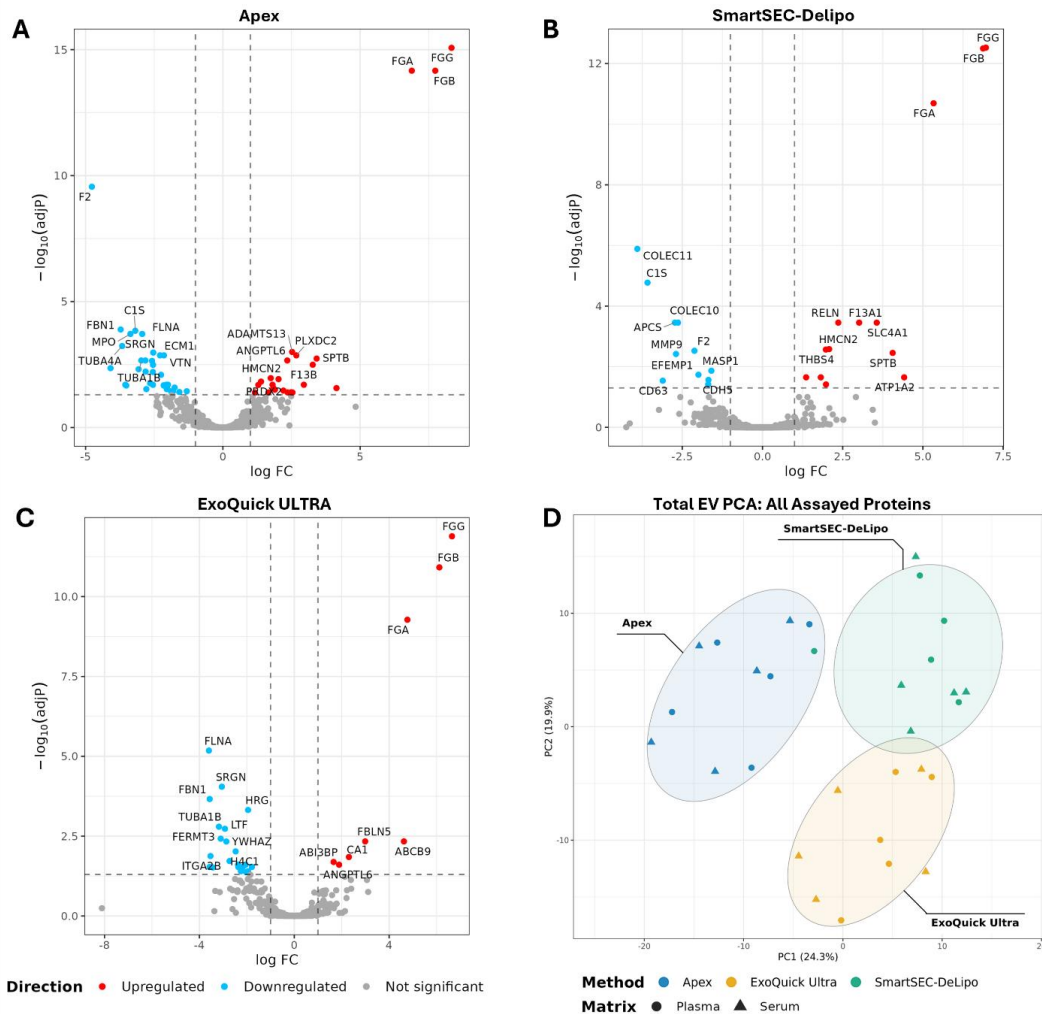


Figure 7. Comparison of plasma vs. serum EV proteomics using same EV isolation method (Apex, SmartSEC-DeLipo, or EXOULTRA). Volcano plots of differential abundance (DA) for proteins upregulated ($+\log_2FC$, red) or downregulated ($-\log_2FC$, blue) across contrasts in plasma vs serum for isolation methods: A) Apex; B) SmartSEC-DeLipo; and C) ExoQuick ULTRA. D) PCA of all ($n = 785$) assayed proteins in total EVs, annotated for sample matrix or isolation method. PC1 (24.3%) and PC2 (19.9%) separate EV isolation method, with no distinct differentiation of sample matrix within methods, except for one outlying SmartSEC-DeLipo sample ($PC1 = -2.89$). Significance determined at threshold adj $p < 0.05$ and $|\log_2FC| > 1$, with annotation for top ten lowest adj p -value in upregulated and downregulated proteins. Plasma and serum samples are from the same blood draw ($n = 5$ subjects).

Neuronal protein profiles across EV isolation groups in total EV

The neuronal protein subset acquired from the HPA and intersection proteins quantified by DIA-MS in total EV revealed 209 observed neuronal proteins, with 178 proteins shared across all six groups for matrix $\times$ EV isolation method [Supplementary Figure 3A-C]. Detection rates were consistent across both sample matrices, with plasma and serum showing broadly similar coverage across EV isolation methods [Supplementary Figure 4A-B]. Abundance distributions of detected neuronal proteins were similarly comparable across groups, with no systemic differences by matrix or isolation method [Supplementary Figure 4C-D].

Unsupervised hierarchical clustering revealed that EV isolation method was the primary factor driving separation of neuronal protein profiles, with Apex, SmartSEC-DeLipo, and ExoQuick ULTRA samples forming distinct clusters [Figure 8A]. PCA supported these findings, with PC1 (27.8%) separating Apex from SmartSEC-DeLipo and ExoQuick ULTRA, and PC2 (20.9%) further distinguishing ExoQuick ULTRA from the remaining two methods [Figure 8B]. Sample matrix showed no clear separation within any cluster. Median abundance values for all 209 neuronal proteins are provided in Supplementary Table 13, with row and column order directly corresponding to the heatmap clustering for direct reference.

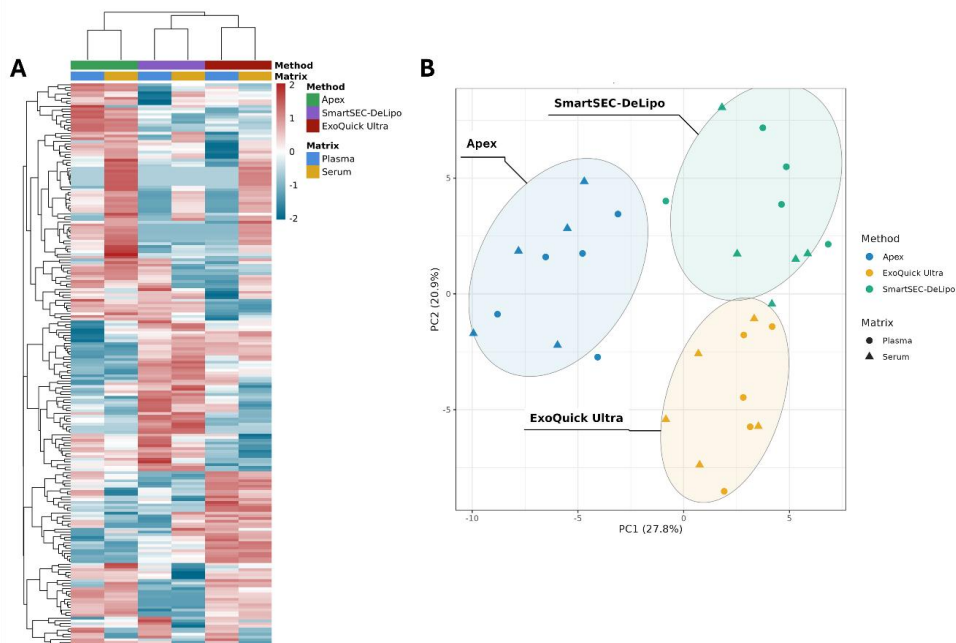


Figure 8. Neuronal protein profiles across EV isolation groups in total EV. A) Heatmap of z-scaled expression for 209 proteins with neuronal cell specificity (Human Protein Atlas) in EV. Rows represent individual neuronal proteins; columns represent median protein abundance across replicates per group, with unsupervised hierarchical clustering applied to both. Missing values were set to zero prior to z-scaling. Annotations indicate EV isolation method (Apex, SmartSEC-DeLipo, ExoQuick ULTRA) and sample matrix (plasma, serum); B) PCA of Cyclic Loess normalized ($\log_2$) abundance for the same neuronal proteins across all individual samples. Points are colored by EV isolation method; shape indicates sample matrix. Ellipses highlight three clusters corresponding to matrix $\times$ method. PC1 (27.8%) and PC2 (20.9%) separate EV isolation method, with no distinct differentiation of sample matrix within methods. Plasma and serum samples are from the same blood draw ($n = 5$ subjects).

NEEV results

For the selection of the optimal bead type for immunocapture, L1CAM-positive EVs were captured by all five bead types, although the intensity and separation of the positive signals varied among the beads [Supplementary Figure 5]. EXO-FLOW produced the strongest and most clearly separated signals across CD63, CD81, and L1CAM, indicating efficient EV capture and consistent neuronal enrichment. Dynabeads M-280 beads also generated distinct positive signals, whereas Dynabeads M-270 showed comparatively weaker separation. The smaller MyOne C1 and T1 beads produced less distinct signals, particularly for CD81 and L1CAM. Based on their consistently strong performance across all three markers, EXO-FLOW beads were selected for subsequent NEEV immunocapture experiments.

Based on total EV yield and purity assessments, Apex-Concentrated, ExoQuick, and SmartSEC EV isolates were selected for initial NEEV marker enrichment evaluation. As shown in Supplementary Figure 6, the Apex-L1CAM combination yielded the highest levels of CD9, CD63, and CD81 in plasma NEEVs. ExoQuick-L1CAM EV isolates showed the highest levels of CD9 and CD63 in serum NEEVs. Apex-ATP1A3 showed highest level of CD81 in serum NEEVs. In contrast, SmartSEC NEEVs did not exhibit the highest marker levels in any condition. Accordingly, subsequent NEEV comparisons were restricted to EVs isolated using Apex and ExoQuick methods.

Flow cytometry characterization of NEEV

Flow cytometry was used to characterize NEEVs using both a general EV marker (EXO-FITC) and the tetraspanin marker CD81-FITC [Figure 9]. Representative histograms demonstrated clear rightward shifts in fluorescence intensity for both EXO-FITC and CD81-FITC compared to controls, indicating successful labeling and detection of EVs [Figure 9A-B].

Quantitative analysis of mean fluorescence intensity (MFI) revealed substantially higher EXO-FITC signal compared to CD81-FITC across all conditions [Figure 9C-D, Supplementary Table 13]. For EXO-FITC, plasma NEEVs isolated using the Apex method exhibited the highest MFI, particularly for ATP1A3-positive vesicles (mean MFI: 1,440,508), followed by L1CAM (793,984) and NCAM (611,212). In contrast, ExoQuick isolation yielded lower but still robust EXO-FITC signals in plasma samples (e.g., ATP1A3: 525,563). [Supplementary Table 14].

For CD81-FITC, MFI values were markedly lower than EXO-FITC but followed similar trends [Figure 9D]. ExoQuick plasma NEEVs showed the highest CD81 signal for ATP1A3 (54,859), followed by L1CAM (39,769) and NCAM (33,987), whereas Apex yielded moderately lower values. Serum NEEVs exhibited reduced CD81-FITC MFI across all markers and methods. Importantly, EV-depleted and PBS controls showed substantially lower MFIs for both EXO-FITC and CD81-FITC, confirming assay specificity and minimal background signal.

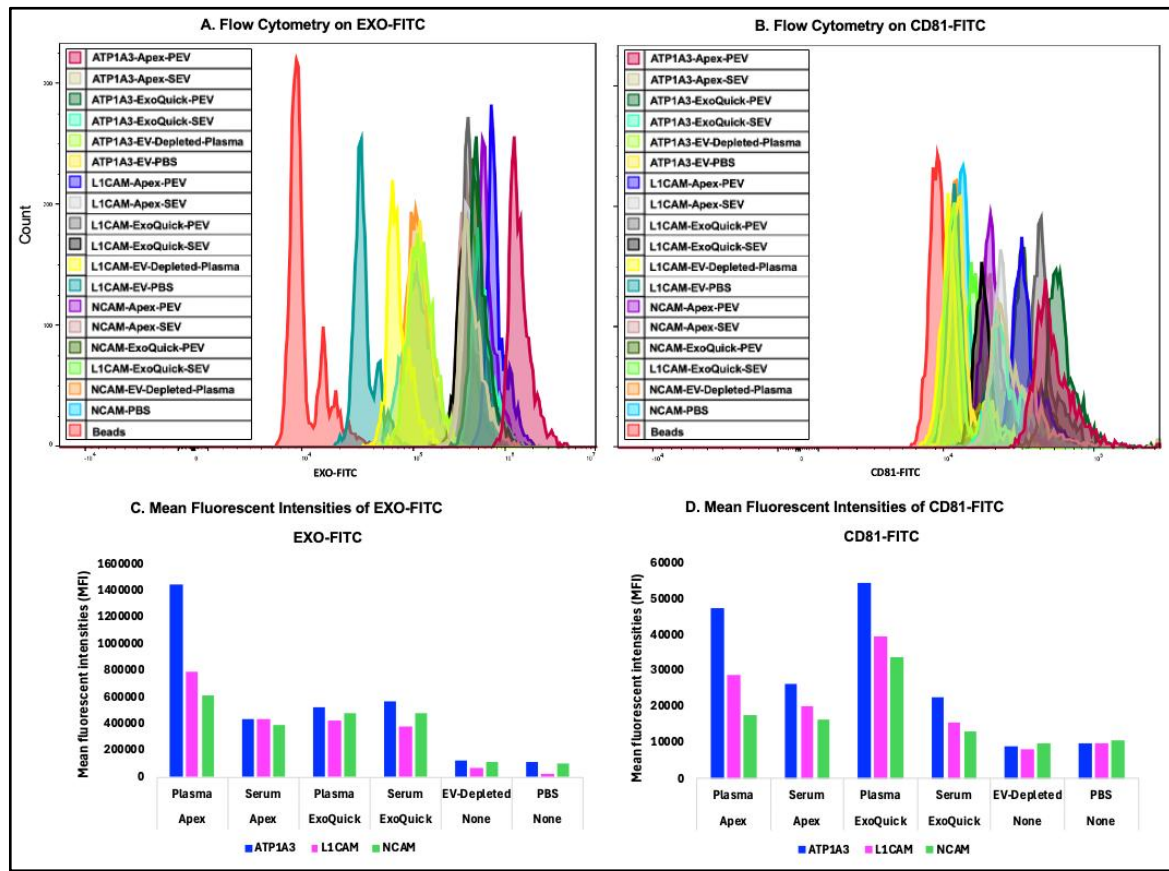


Figure 9. Flow cytometry characterization of neuronal-enriched extracellular vesicles (NEEV). A) Representative flow cytometry histograms showing EXO-FITC signal for NEEVs; B) Representative histograms of CD81-FITC labeling for NEEVs; C) Quantification of mean fluorescence intensity (MFI) for EXO-FITC; D) Quantification of mean fluorescence intensity (MFI) for CD81-FITC. All samples were isolated using blood from the same individual.

Across all neuronal markers (ATP1A3, L1CAM, and NCAM), plasma NEEVs consistently showed higher fluorescence intensity compared to serum counterparts, indicating greater enrichment or recovery of neuronal EVs in plasma samples. Additionally, Apex preferentially enhanced EXO-FITC signal intensity, whereas ExoQuick demonstrated stronger CD81-FITC labeling, suggesting method-dependent differences in vesicle composition or surface marker accessibility.

Together, these results confirm the successful detection and characterization of NEEVs and highlight distinct differences in fluorescence intensity profiles by biofluid source and isolation method.

Microfluidic resistive pulse sensing (MRPS) on NEEV

MRPS was performed to assess particle size distribution and concentration of NEEVs isolated from plasma and serum [Figure 10]. Across both sample types, NEEVs exhibited size distributions predominantly within the expected exosome range (~50-150 nm), confirming successful enrichment of small EV populations.

In Figure 10A, plasma NEEV particle concentrations were generally higher compared to serum, with distinct peaks observed within the ~70-120 nm range. The size distribution profiles were relatively narrow, indicating a more homogeneous vesicle population following neuronal enrichment. In contrast, Figure 10B show that serum NEEVs displayed slightly broader size distributions, although most particles remained within the exosome size range. Notably, the abundance of larger particles (> 150 nm) was minimal in both plasma and serum NEEV samples, suggesting limited contamination with larger vesicles or aggregates following enrichment. Overall, MRPS analysis demonstrates that neuronal enrichment yields a consistent population of small EVs.

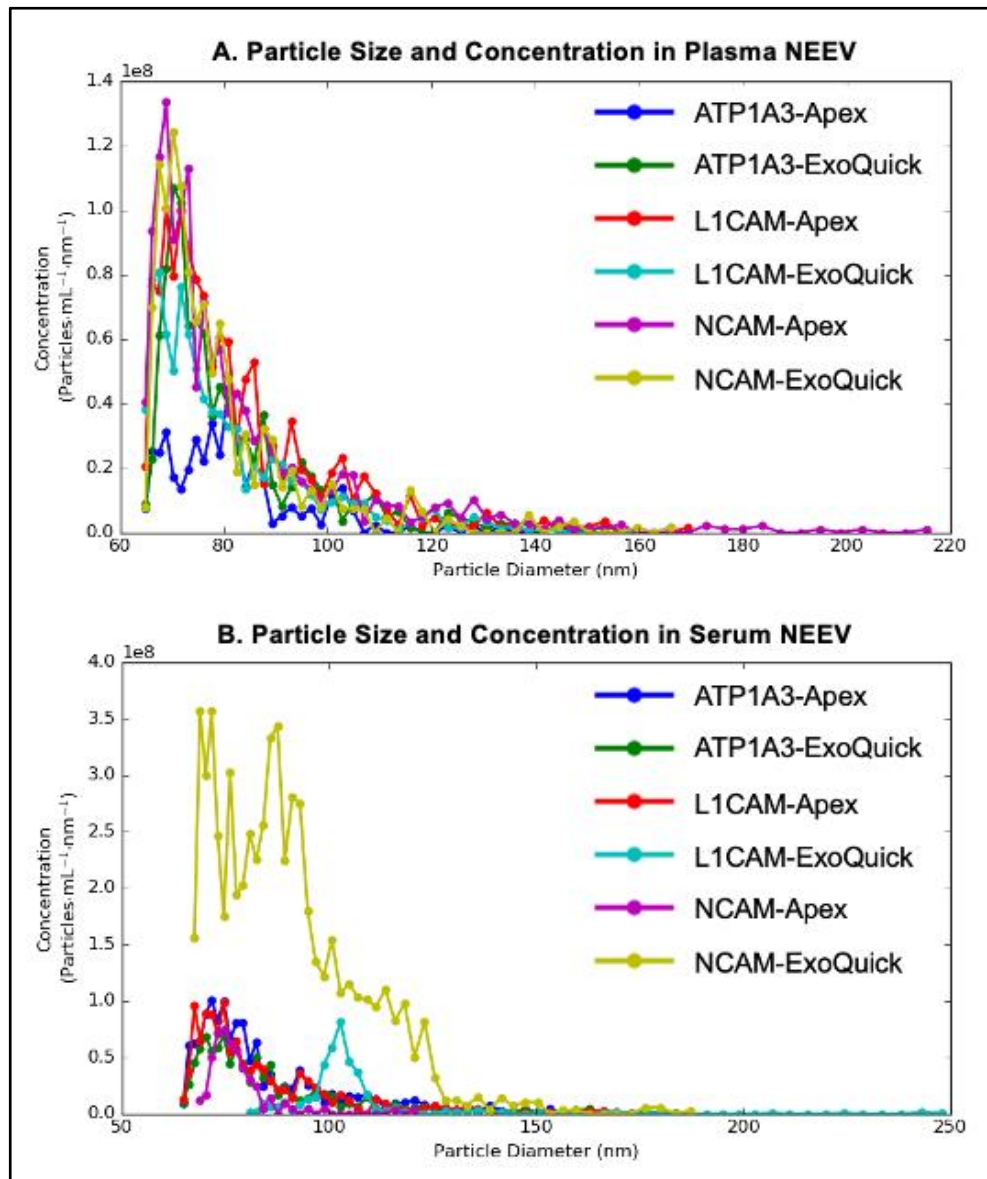


Figure 10. Microfluidic resistive pulse sensing (MRPS) analysis of particle size distribution and concentration for neuronal-enriched extracellular vesicles (NEEVs) isolated from plasma and serum. MRPS was used to measure particle concentration and size distribution for all NEEV types. A) Plasma and B) serum NEEVs from all antibody-method combinations. Concentration was detected as particles (mL)/nm and size distribution in particle diameter (nm). All samples were isolated using blood from the same individual.

ONI characterization of NEEV

ONI analysis showed that the glycine-HCl method (NEEV 2) produced higher NEEV yield compared to the kit elution buffer (NEEV 1), as indicated by increased particle counts [Supplementary Figure 7]. Both methods yielded signals above negative controls; however, the kit buffer showed lower overall recovery, suggesting less efficient release of vesicles from the capture complex. Despite differences in yield, both methods produced vesicles within the expected exosome size range (~50-150 nm), indicating that vesicle size distribution was preserved. Overall, the glycine-HCl elution method improved NEEV recovery without affecting size characteristics and was therefore selected for subsequent ONI-based NEEV comparisons.

NEEVs were characterized using the ONI Nanoimager platform following isolation. In plasma-NEEVs, ATP1A3-ExoQuick method demonstrated highest total NEEV yield, followed by L1CAM-ExoQuick method, than other enrichment techniques ($p < 0.05$) [Figure 11A]. In serum-NEEVs, L1CAM-Apex yielded the highest number of NEEVs counts [Figure 11B].

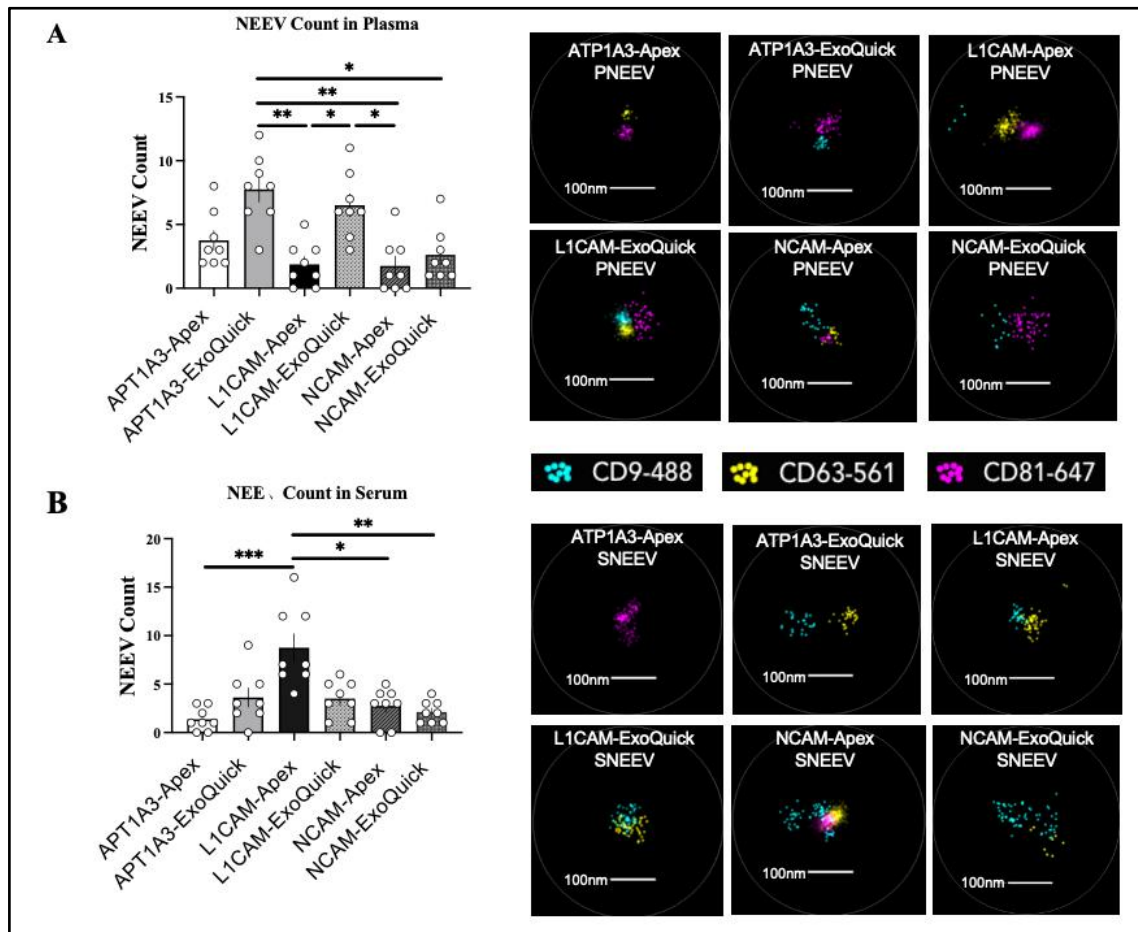


Figure 11. Comparison of NEEV yield across various isolation techniques using ONI Nanoimager analysis. The distribution and colocalization of canonical EV tetraspanin markers CD9-488 (cyan), CD63-561 (yellow), and CD81-647 (magenta) in plasma NEEVs and serum NEEVs are illustrated in corresponding ONI super resolution microscopy images. Scale bars measure the value of 100 nm. Each bar represents EV isolation method with one biological sample, and each individual dot corresponds to a single imaged field of view (FOV) ($n = 8$ FOVs/sample). NEEV counts per FOV were quantified to determine relative vesicle abundance among antibody-isolation platforms. A) Plasma NEEV; B) Serum NEEV. The groups were compared using one-way ANOVA with statistical significance determined by p -value < 0.05 and results expressed as mean $\pm$ SEM. Statistical significance is indicated as: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant. All samples were isolated using blood from the same individual.

Comparison of NEEV CD9, CD63, and CD81 concentrations and purity

A comparison of NEEV tetraspanin (CD9, CD63, CD81) yield (AU/ μ L) and purity (AU per mg of protein) using different antibodies, sample types, and EV isolation methods is shown in [Figures 12 and 13].

To evaluate which experimental factors were associated with the concentrations or purity of tetraspanins, a three-factor analysis of variance (ANOVA) model was fitted with antibody (ATP1A3, L1CAM, NCAM), sample type (plasma and serum), and EV isolation methods (Apex, ExoQuick) as main effects. When significant main effects were detected, Tukey's HSD post-hoc tests were used to estimate adj pairwise differences and 95% confidence intervals.

Figure 12A shows that in the CD9 assay, sample type and isolation method were both significant predictors of NEEV concentration, whereas antibody was not. Specifically, CD9 concentration did not differ among antibodies ($F(2,55) = 0.151, p = 0.860$). In contrast, CD9 concentration was significantly higher in serum NEEV than in plasma NEEV ($F(2,55) = 17.906, p < 0.001$) and differed significantly by EV isolation method ($F(2,55) = 15.361, p < 0.001$). Tukey's HSD post-hoc tests confirmed that serum NEEV had higher CD9 concentration than plasma NEEV (serum-plasma mean difference = 31.11 AU/L, 95% CI [16.38, 45.84], adj $p < 0.001$) and that ExoQuick NEEV produced higher CD9 concentration than Apex (ExoQuick – Apex mean difference = 28.81 AU/L, 95% CI [14.08, 43.54], adj $p < 0.001$). No pairwise antibody comparisons were significant (all adj $p \geq 0.86$).

Figure 12B shows that in the CD63 assay, antibody was not a significant predictor of NEEV concentration ($F(2,55) = 0.212, p = 0.810$), indicating no meaningful differences among ATP1A3, L1CAM, and NCAM. In contrast, both sample type and EV isolation method were significant. CD63 concentration was significantly higher in serum NEEV than in plasma NEEV ($F(1,55) = 23.488, p < 0.001$) and differed significantly by isolation method ($F(1,55) = 10.400, p = 0.002$). Tukey's HSD post-hoc tests confirmed that serum NEEV had a higher CD63 concentration than plasma NEEV (serum-plasma mean difference = 5.66 AU/L, 95% CI [3.32, 8.00], adj $p < 0.001$). Additionally, ExoQuick isolation yielded significantly higher CD63 concentrations than Apex (ExoQuick- Apex mean difference = 3.76 AU/L, 95% CI

[1.43, 6.10], adj $p = 0.002$). No pairwise comparisons among antibodies were significant (all adj $p \geq 0.81$).

Figure 12C shows that in the CD81 assay, antibody again was not a significant predictor of NEEV concentration ($F(2,55) = 0.466$, $p = 0.630$). Unlike CD9 and CD63, sample type was not statistically significant for CD81 ($F(1,55) = 2.764$, $p = 0.102$), although serum showed a non-significant trend toward higher concentrations than plasma (serum-plasma mean difference = 0.29 AU/L, 95% CI [-0.06, 0.64], adj $p = 0.102$). In contrast, the EV isolation method was a strong predictor of CD81 concentration ($F(1,55) = 26.094$, $p < 0.001$). Tukey's HSD post-hoc test indicated a significant difference between ExoQuick and Apex (ExoQuick- Apex mean difference = -0.89 AU/L, 95% CI [-1.24, -0.54], adj $p < 0.001$), reflecting higher CD81 concentrations in Apex relative to ExoQuick. No significant pairwise differences were observed among antibodies (all adj $p \geq 0.65$).

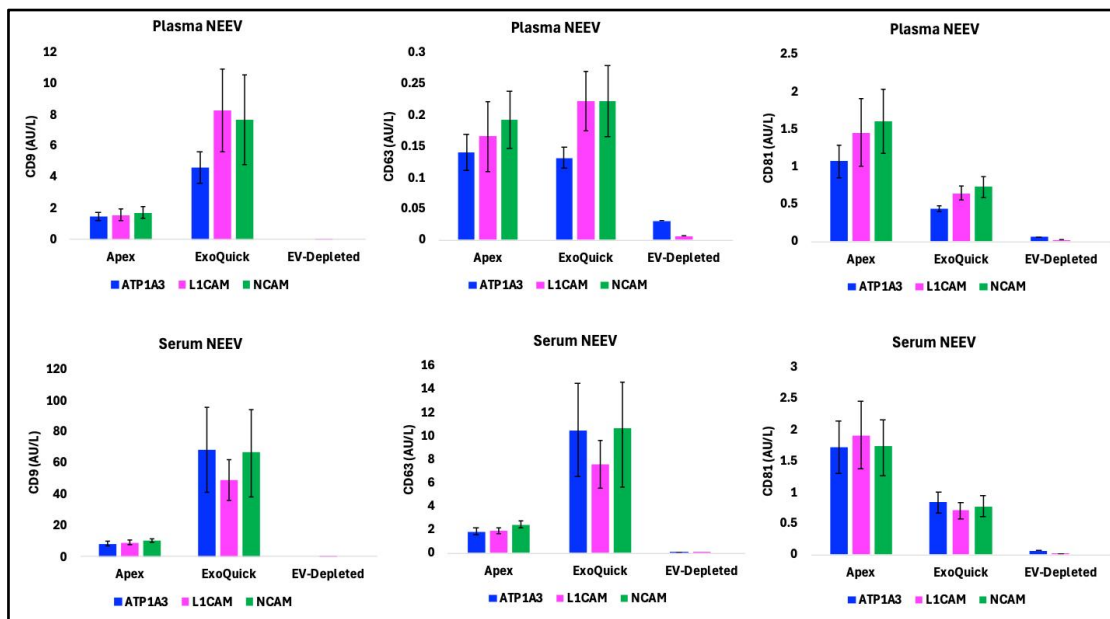


Figure 12. Comparison of CD9, CD63, and CD81 concentrations on NEEV. NEEVs from plasma and serum were analyzed for tetraspanin concentrations. Note, Tetraspanin markers were measured using U-PLEX human CD9/CD63/CD81 (EV) Assays. AU - Arbitrary Units. $n = 5$ (number of subjects analyzed, all EV were isolated using blood from the same 5 individuals). Data shown as Mean $\pm$ SE.

Overall, across the three tetraspanins, antibody selection did not significantly influence NEEV concentration in any assay. Sample type significantly affected CD9 and CD63 concentrations, with serum consistently yielding higher values than plasma, but did not significantly influence CD81. EV isolation method significantly affected all three tetraspanins; however, the direction of effect differed for CD81 compared with CD9 and CD63. ExoQuick yielded higher CD9 and CD63 concentrations, whereas Apex yielded higher CD81 concentrations. These findings underscore the strong impact of pre-analytical factors, particularly isolation method, on measured NEEV tetraspanin levels.

Purity analyses (AU per mg protein) showed a more selective pattern of effects than concentration outcomes [Figure 13].

For CD9 purity, the antibody was not a significant predictor ($F(2,55) = 1.575, p = 0.216$), and none of the pairwise antibody comparisons were significant (all adj $p \geq 0.19$). The isolation method was also not significant ($F(1,55) = 0.040, p = 0.843$). In contrast, sample type significantly influenced CD9 purity ($F(1,55) = 5.639, p = 0.021$). Tukey's HSD post-hoc test confirmed that serum-derived NEEV had significantly higher CD9 purity than plasma-derived NEEV (serum-plasma mean difference = 17.46 AU/mg protein, 95% CI [2.73, 32.20], adj $p = 0.021$)- Figure 13A.

Similarly, for CD63 purity, the antibody was not significant ($F(2,55) = 1.684, p = 0.195$), no significant pairwise differences (all adj $p \geq 0.17$). The isolation method again was not significant ($F(1,55) = 0.531, p = 0.469$). However, sample type significantly predicted CD63 purity ($F(1,55) = 7.005, p = 0.011$). Post-hoc testing showed that serum NEEV had higher CD63 purity than plasma NEEV (serum-plasma mean difference = 3.43 AU/mg, 95% CI [0.83, 6.02], adj $p = 0.011$)- Figure 13B.

In the CD81 purity assay, the antibody remained non-significant ($F(2,55) = 0.699, p = 0.502$), and no antibody pairwise comparisons were significant (all adj $p \geq 0.48$). Sample type showed a trend but did not reach statistical significance ($F(1,55) = 2.827, p = 0.098$), with serum tending toward higher purity than plasma (mean difference = 1.11 AU/mg, 95% CI [–

0.21, 2.44], adj $p = 0.098$). In contrast to CD9 and CD63, isolation method significantly affected CD81 purity ($F(1,55) = 6.607$, $p = 0.013$). Tukey's HSD post-hoc test indicated that ExoQuick had significantly lower CD81 purity than Apex (ExoQuick- Apex mean difference = -1.70 AU/mg, 95% CI [-3.03, -0.38], adj $p = 0.013$), indicating greater CD81 purity with Apex isolation [Figure 13C].

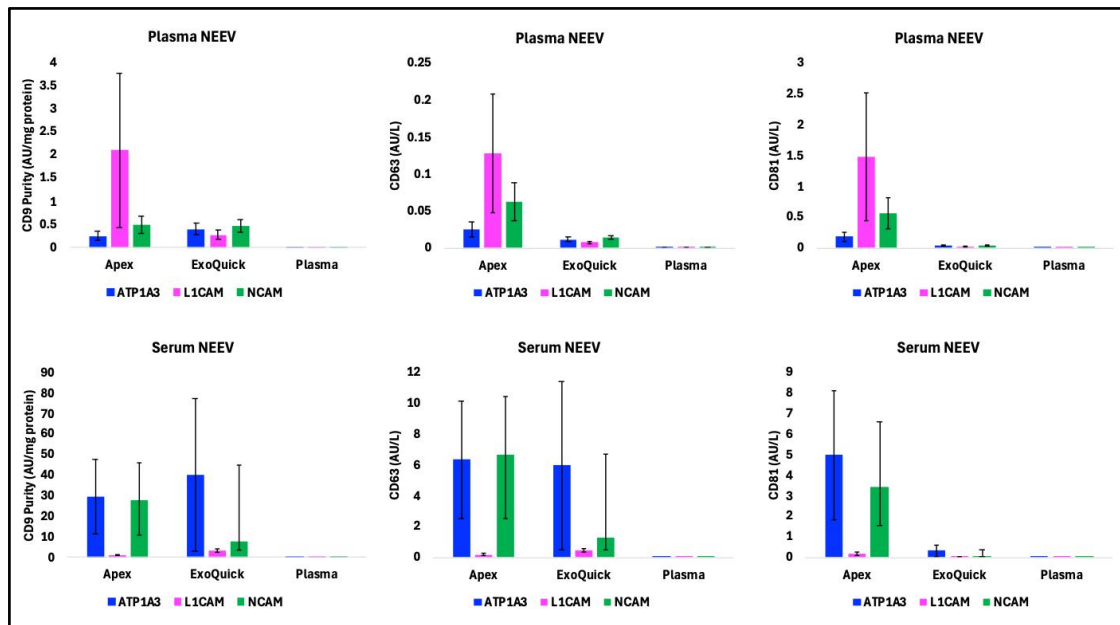


Figure 13. Comparison of CD9, CD63, and CD81 purity on NEEV. NEEVs from plasma and serum were analyzed for tetraspanins purity. Note, Tetraspanin markers were measured using U-PLEX human CD9/CD63/CD81 (EV) Assays. AU - Arbitrary Units: Human EV Calibrator Standard 1 (to of Standard curve) = 1,000 AU/L. $n = 5$ (number of subjects analyzed, all EV were isolated using blood from the same 5 individuals). Data shown as Mean $\pm$ SE. Apex: EV isolated using Apex method; ExoQuick: EV isolated with ExoQuick method; Plasma: Plasma was directly used for NEEV enrichment without EV isolation; Serum: Serum was directly used for NEEV enrichment without EV isolation.

Taken together, antibody selection did not significantly influence purity for any of the three tetraspanins measured in NEEVs. Sample type significantly affected CD9 and CD63 purity, with serum consistently demonstrating higher purity than plasma. In contrast, the isolation method significantly influenced only CD81 purity, with Apex yielding higher CD81 purity

than ExoQuick. These findings suggest that, while serum enhances both yield and purity for CD9 and CD63, the choice of isolation method differentially affects CD81 purity, further underscoring the importance of pre-analytical methodological factors in NEEV characterization.

NEEV proteomics results

1. Differential protein abundance in plasma NEEV

To evaluate the impact of EV isolation method and neuronal EV capture antibody on the plasma NEEV proteome, differential protein abundance analyses were performed using pairwise comparisons between Apex and ExoQuick isolation workflows and among ATP1A3-, L1CAM-, and NCAM1-enriched vesicle populations [Figure 14]. Volcano plot analyses identified numerous proteins that differed significantly between methods and capture strategies ($\text{adj } p < 0.05$ and $|\log_2\text{FC}| > 1$), indicating that both EV isolation workflow and neuronal EV enrichment target substantially influence the resulting proteomic profiles. Comparisons between Apex and ExoQuick preparations within the same capture antibody revealed widespread proteomic differences. ATP1A3-enriched NEEVs isolated by Apex versus ExoQuick demonstrated extensive differential abundance, with a large subset of proteins enriched in the ExoQuick fraction and fewer proteins preferentially enriched in Apex isolates [Figure 14A]. Similar trends were observed for L1CAM- and NCAM1-captured NEEVs, where ExoQuick-derived samples showed markedly greater enrichment of multiple proteins relative to Apex isolates [Figures 14B,C]. These findings suggest that ExoQuick recovery yields a broader or compositionally distinct plasma EV proteome compared with Apex-based isolation.

Direct comparisons among neuronal EV capture antibodies within the same isolation platform demonstrated that capture target selection also strongly influenced detected protein composition. Within Apex-isolated NEEVs, ATP1A3 versus L1CAM and ATP1A3 versus NCAM1 comparisons showed relatively few significantly altered proteins, indicating substantial overlap among Apex-derived neuronal EV populations [Figures 14D-F]. Notably, ATP1A3 and L1CAM co-isolated significantly less albumin than NCAM.

Among ExoQuick-isolated NEEVs, comparisons between ATP1A3 and L1CAM demonstrated the greatest degree of proteomic separation, with large numbers of proteins significantly enriched in both directions [Figure 14G]. ATP1A3 versus NCAM1 and L1CAM versus NCAM1 comparisons also revealed distinct protein abundance patterns, although with fewer significantly altered proteins than observed for ATP1A3 versus L1CAM [Figures 14H,I]. Collectively, these results demonstrate that both EV isolation strategy and neuronal EV capture antibody selection substantially shape the detectable plasma NEEV proteome, highlighting methodological considerations that may influence downstream biomarker discovery and biological interpretation.

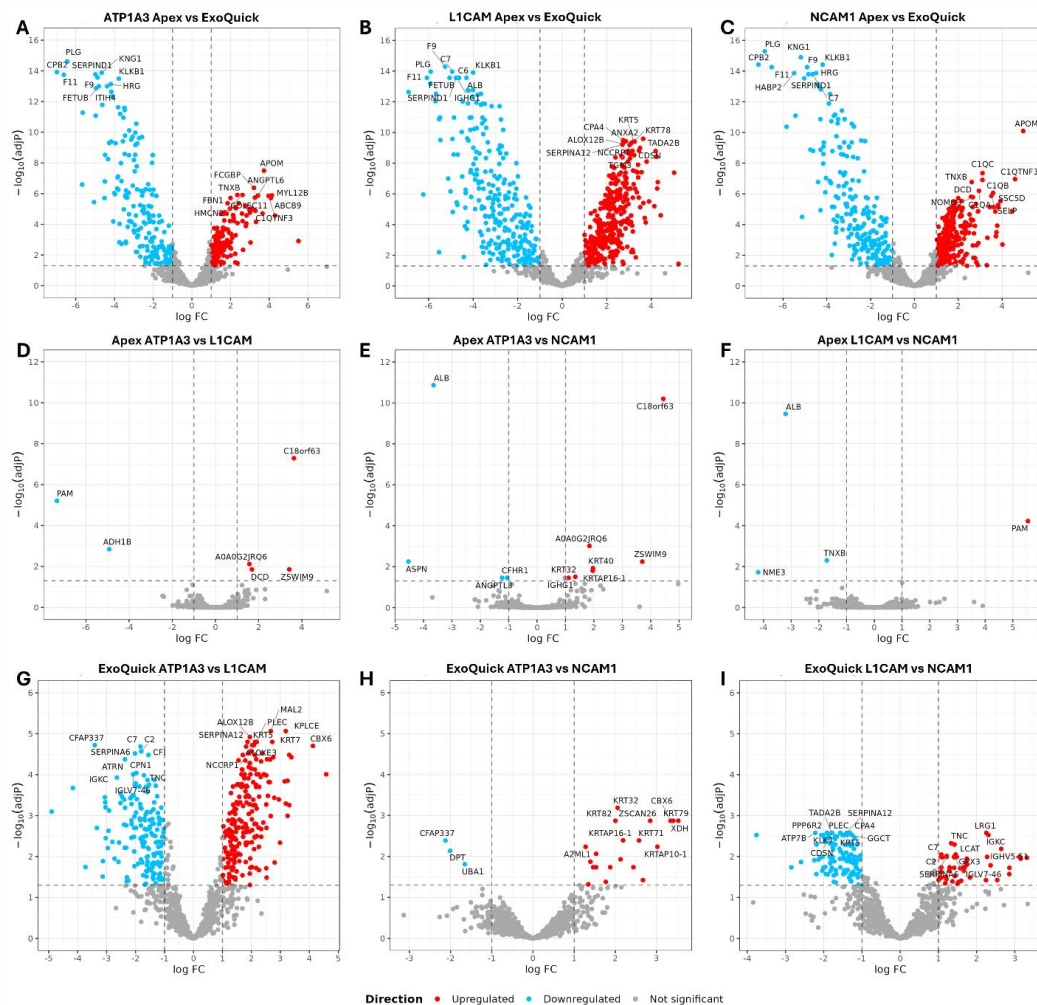


Figure 14. Differential protein abundance across plasma NEEVs by isolation methods and capture antibodies. Volcano plots showing differential protein abundance from mass

spectrometry analysis of plasma NEEVs across pairwise comparisons of EV isolation methods (Apex vs. ExoQuick) and capture antibodies (ATP1A3, L1CAM, NCAM). Each panel represents a specific group comparison (e.g., ATP1A3 Apex vs. ExoQuick). The x-axis indicates log fold change ($\log_2FC$), and the y-axis shows statistical significance ($-\log_{10} p$ -value). Proteins meeting significance thresholds ($\text{adj } p < 0.05$ and $|\log_2FC| > 1$) are highlighted in red (upregulated) and blue (downregulated), while non-significant proteins are shown in gray. Plasma samples are from the same blood draw ($n = 5$ subjects).

2. Differential protein abundance in serum NEEV

Similar differential protein abundance analyses were performed to evaluate the effects of EV isolation method and neuronal EV capture antibody selection on the serum NEEV proteome [Figure 15]. Pairwise volcano plot comparisons demonstrated that both isolation workflow and capture strategy influenced the abundance of detected proteins, although the magnitude of these differences appeared more limited in serum than observed in plasma-derived NEEVs. Comparisons between Apex and ExoQuick isolation methods within the same capture antibody group identified multiple significantly altered proteins. ATP1A3-enriched serum NEEVs showed numerous proteins with differential abundance between Apex and ExoQuick preparations, including both proteins enriched in ExoQuick isolates and proteins preferentially detected in Apex samples [Figure 15A]. Similar patterns were observed for L1CAM- and NCAM1-captured serum NEEVs, where several proteins demonstrated significantly increased abundance in ExoQuick isolated NEEVs relative to Apex isolated NEEVs [Figures 15B, C]. Overall, ExoQuick preparations exhibited a broader distribution of significantly enriched proteins compared with Apex, suggesting differences in vesicle recovery efficiency or co-isolated protein composition between the two isolation approaches.

Within Apex-isolated serum NEEVs, comparisons among capture antibodies demonstrated relatively limited differential protein abundance. ATP1A3 versus L1CAM and ATP1A3 versus NCAM1 comparisons identified very few significantly altered proteins, indicating substantial overlap in the proteomic composition of these Apex-derived NEEV populations [Figures 15D, E]. Likewise, the L1CAM versus NCAM1 comparison revealed minimal

proteomic separation and few proteins meeting statistical significance thresholds [Figure 15F]. Notably, ATP1A3 co-isolated significantly less albumin than NCAM.

In contrast, ExoQuick-isolated serum NEEVs demonstrated modest but detectable differences among capture antibody groups. ATP1A3 versus L1CAM and ATP1A3 versus NCAM1 comparisons showed only a small number of significantly altered proteins [Figures 15G, H]. However, the ExoQuick L1CAM versus NCAM1 comparison revealed a greater number of differentially abundant proteins, with several proteins enriched in the L1CAM-captured fraction relative to NCAM1 isolates [Figure 15I]. These findings indicate that capture antibody selection exerts a measurable but comparatively modest influence on the serum NEEV proteome, whereas EV isolation methodology contributes more substantially to differences in detected protein abundance profiles.

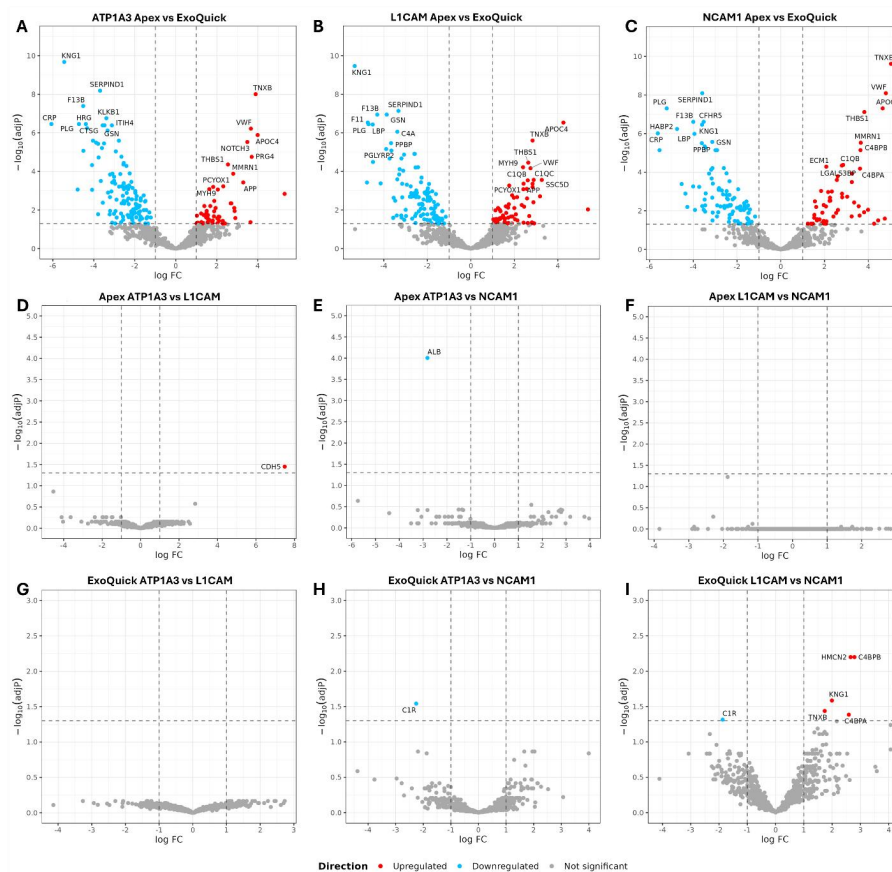


Figure 15. Differential protein abundance across serum NEEVs by isolation method and capture antibody. Volcano plots showing differential protein abundance from mass

spectrometry analysis of serum NEEVs across pairwise comparisons of EV isolation methods (Apex vs. ExoQuick) and capture antibodies (ATP1A3, L1CAM, NCAM). Each panel represents a specific group comparison (e.g., ATP1A3 Apex vs. ExoQuick). The x-axis indicates log fold change ($\log_2FC$), and the y-axis shows statistical significance ($-\log_{10} p$ -value). Proteins meeting significance thresholds ($\text{adj } p < 0.05$ and $|\log_2FC| > 1$) are highlighted in red (upregulated) and blue (downregulated), while non-significant proteins are shown in gray. Serum samples are from the same blood draw ($n = 5$ subjects).

3. Differential protein abundance between plasma and serum NEEV

To assess the impact of biofluid source, differential protein abundance analyses were performed to compare plasma- and serum-derived NEEVs isolated using the same EV isolation workflow and neuronal EV capture antibody [Figure 16]. Volcano plot analyses demonstrated substantial proteomic differences between plasma and serum NEEVs across all capture antibodies and isolation methods, indicating that blood matrix source strongly influences the detectable neuronal EV proteome.

Among Apex-isolated NEEVs, ATP1A3-captured plasma versus serum comparisons revealed numerous significantly altered proteins in both directions, with distinct subsets preferentially enriched in plasma- or serum-derived NEEVs [Figure 16A]. Similar patterns were observed for L1CAM- and NCAM1-enriched Apex NEEVs, where multiple proteins showed significantly greater abundance in either plasma or serum preparations [Figures 16B, C]. Notably, several complement-associated and extracellular matrix-related proteins were among the significantly altered features, suggesting compositional differences in circulating EV-associated protein cargo between plasma and serum sources.

Comparisons of ExoQuick-isolated NEEVs demonstrated even broader proteomic separation between plasma and serum samples. ATP1A3 ExoQuick plasma versus serum comparisons identified many significantly differentially abundant proteins distributed across both positive and negative $\log_2FC$ values [Figure 16D]. L1CAM- and NCAM1-captured ExoQuick isolates likewise exhibited extensive differential abundance patterns, with numerous proteins enriched selectively in plasma- or serum-derived NEEVs [Figures 16E, F]. Compared with

Apex-isolated NEEVs, ExoQuick preparations demonstrated a greater density of significantly altered proteins, suggesting that precipitation-based isolation may capture a broader range of matrix-associated or co-isolated proteins that differ between plasma and serum.

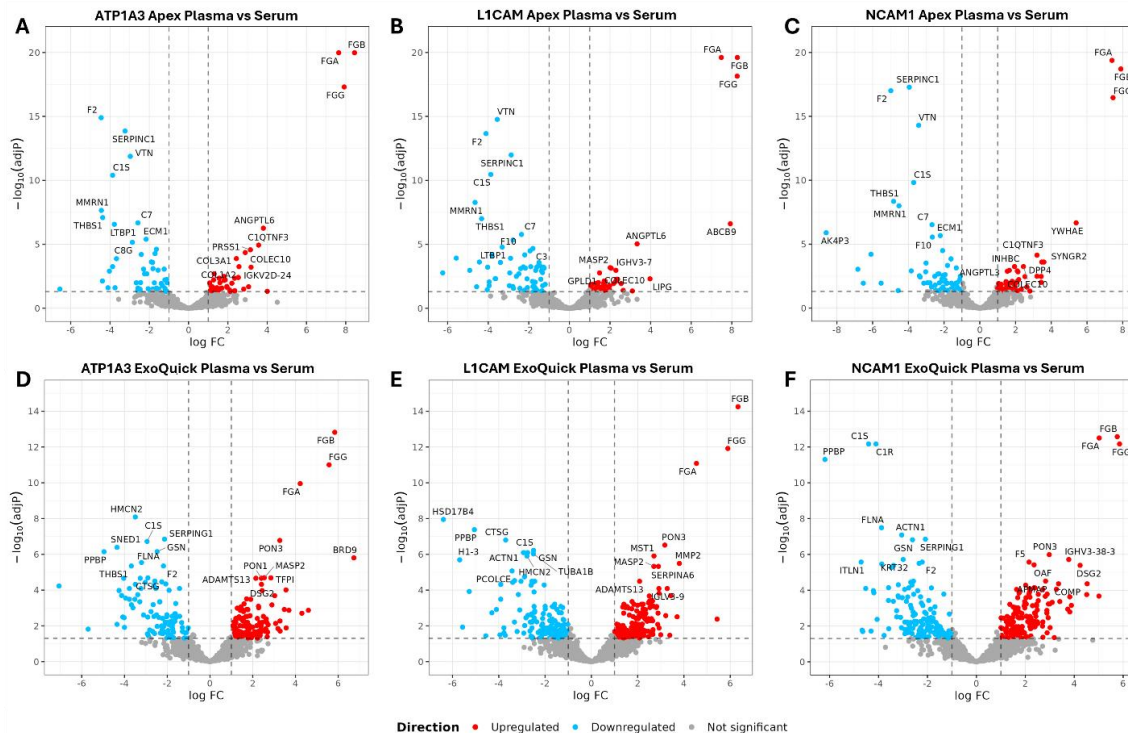


Figure 16. Differential protein abundance comparing plasma and serum NEEVs by same isolation method and capture antibody. Volcano plots showing differential protein abundance from mass spectrometry analysis of plasma and serum NEEVs across pairwise comparisons of same EV isolation methods (Apex and ExoQuick) and capture antibodies (ATP1A3, L1CAM, NCAM). Each panel represents a plasma vs. serum NEEV using a specific method and capture antibody (ATP1A3 Apex Plasma vs. Serum). The x-axis indicates log fold change (log₂FC), and the y-axis shows statistical significance (-log₁₀ *p*-value). Proteins meeting significance thresholds (adj *p* < 0.05 and |log FC| > 1) are highlighted in red (upregulated) and blue (downregulated), while non-significant proteins are shown in gray. Plasma and serum samples are from the same blood draw (*n* = 5 subjects).

Across both isolation methods, serum-derived NEEVs frequently demonstrated enrichment of coagulation- and complement-related proteins, whereas plasma-derived NEEVs showed relative enrichment of proteins associated with cytoskeletal organization, membrane trafficking, and neuronal signaling. These findings indicate that specimen type exerts a major influence on the detectable NEEV proteome independent of isolation strategy or neuronal EV capture antibody. Collectively, the data highlight the importance of maintaining consistency in blood processing workflows for EV biomarker studies, as plasma and serum NEEVs represent compositionally distinct proteomic populations despite being derived from matched blood draws from the same subjects.

4. Neuronal protein abundance in NEEV

297 neuronal proteins were identified from the HPA overlap in NEEV DIA-MS and evaluated across experimental groups. Detection rates of neuronal proteins were consistently higher in plasma NEEVs compared to serum NEEVs. Plasma samples showed robust detection (approximately 70-95%) with low variability across groups, whereas serum samples exhibited lower and more variable detection rates (approximately 20-85%) [Supplementary Figure 8A,B]. Across both matrices, ExoQuick isolation outperformed Apex, with ExoQuick plasma samples demonstrating the highest neuronal protein coverage. Despite these differences in detection, the overall abundance distributions of detected neuronal proteins were similar across all groups, indicating that differences were primarily driven by protein coverage rather than expression level [Supplementary Figure 8C, D].

Unsupervised hierarchical clustering revealed that the EV isolation method was the primary factor driving the separation of neuronal protein profiles, with ExoQuick and Apex samples forming distinct clusters [Figure 17A]. Within these clusters, samples were further segregated by matrix, with plasma groups, particularly those isolated using ExoQuick, showing higher relative abundance of neuronal proteins. Capture antibody had a comparatively minor effect on clustering.

Principal component analysis (PCA) supported these findings, with the EV isolation method accounting for the largest source of variance (PC1, 46.3%), clearly separating ExoQuick and

Apex samples [Figure 17B]. Sample matrix contributed to secondary variation (PC2, 15.8%), distinguishing plasma from serum, while antibody effects were limited to within-group variability.

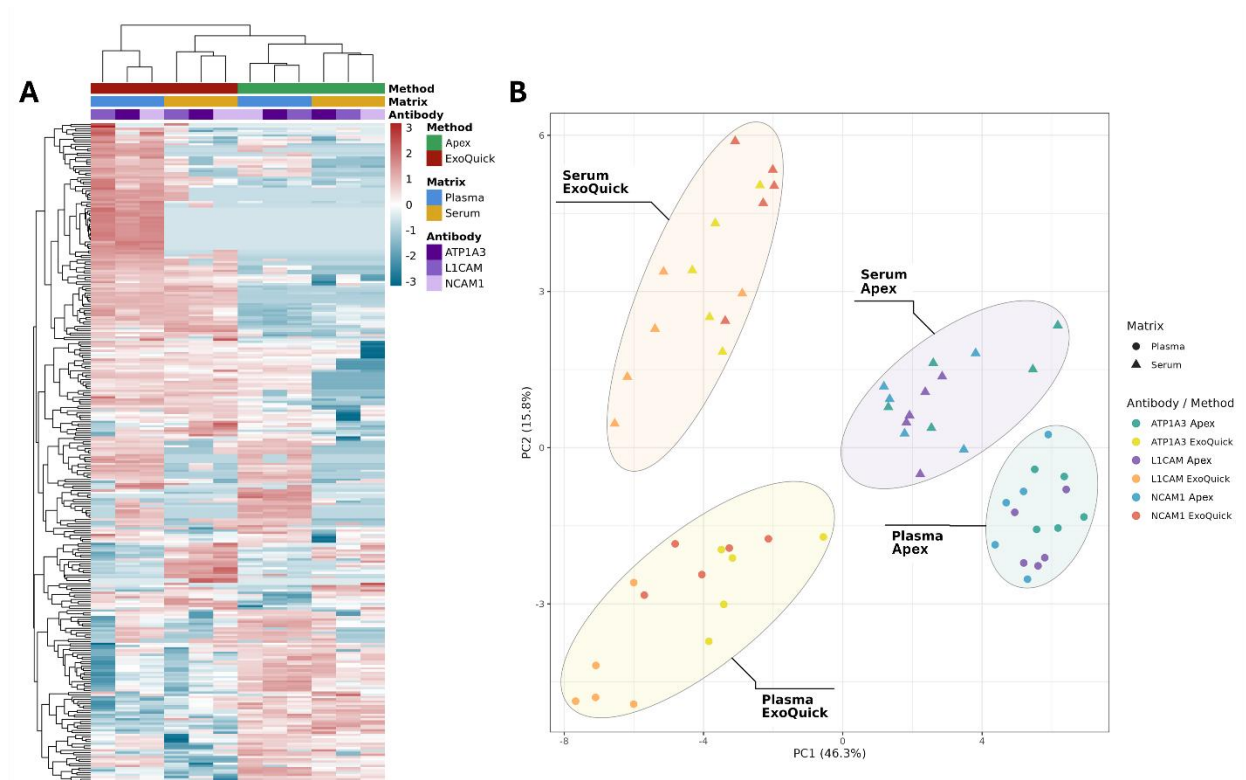


Figure 17. Neuronal protein profiles across EV isolation groups in NEEV. A) Heatmap of z-scaled expression for 297 proteins with neuronal cell specificity (Human Protein Atlas) in NEEV. Rows represent individual neuronal proteins; columns represent median abundance across replicates per group, with unsupervised hierarchical clustering applied to both. Missing values were set to zero prior to z-scaling. Annotations indicate EV isolation method (Apex, ExoQuick), sample matrix (plasma, serum), and capture antibody (ATP1A3, L1CAM, NCAM); B) PCA of Cyclic Loess normalized (log₂) abundance for the same neuronal proteins across all 60 individual samples. Points are colored by antibody and EV isolation method; shape indicates sample matrix. Ellipses highlight four clusters corresponding to matrix × method. PC1 (46.3%) separates EV isolation method; PC2 (15.8%) separates sample matrix, with greater plasma–serum separation observed in ExoQuick than Apex.

Overlap analysis demonstrated that most neuronal proteins were shared across experimental conditions. Specifically, 287 of 297 proteins were detected in both plasma and serum, 285 were shared between isolation methods, and all 297 proteins were detected across capture antibodies [Supplementary Figure 9A-C]. At the combined group level, 176 proteins were consistently detected across all conditions, while the remaining proteins exhibited group-specific patterns primarily influenced by the isolation method and sample matrix [Supplementary Figure 9D].

Overall, these results indicate that plasma NEEVs, particularly those isolated using ExoQuick, provide the highest neuronal protein coverage, while differences in abundance are minimal across methods. Antibody choice had a modest impact, with ATP1A3 and NCAM showing slightly improved performance compared to L1CAM. Median protein abundance values for all neuronal proteins are provided in Supplementary Table 15.

5. Neuronal protein abundance between NEEV and total EV

To evaluate the enrichment of neuron-specific proteins in NEEV relative to total EV cohorts, proteins with neuronal cell specificity annotated by the HPA and detected without missing values were compared across plasma, serum, and combined datasets [Figure 18]. UpSet plot analyses demonstrated substantial overlap between total EV and NEEV cohorts while also identifying a large subset of neuronal proteins uniquely detected in NEEVs.

In plasma samples, 160 neuronal proteins were shared between total EV and NEEV cohorts, while 132 neuronal proteins were uniquely detected in NEEVs and 48 proteins were unique to total EV isolates [Figure 18A]. Similar overlap patterns were observed in serum-derived samples, where 160 neuronal proteins were shared between total EV and NEEV datasets, 132 proteins were unique to NEEVs, and 49 proteins were unique to total EV isolates [Figure 18B].

When all experimental groups were analyzed collectively, 161 neuronal proteins were consistently detected in both total EV and NEEV cohorts, while 136 neuronal proteins were

unique to NEEVs and 48 were unique to total EV samples [Figure 18C, Supplementary Table 16-18].

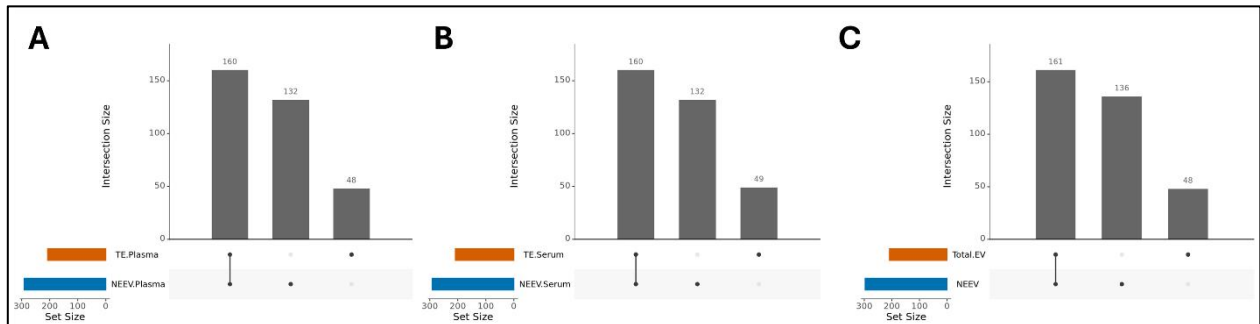


Figure 18. Overlap of detected neuronal proteins across experimental groups between total EV and NEEV cohorts. UpSet plots showing intersection of 209 proteins in total EV (TE) and 297 proteins in NEEV with neuronal cell specificity (Human Protein Atlas) detected (non-missing) between A) plasma; B) serum; and C) overall. 161 neuronal proteins were detected across both TE and NEEV cohorts, with 136 in NEEV alone and 48 in TE alone.

These findings indicate that neuronal enrichment strategies substantially expand the detectable repertoire of neuron-specific proteins beyond those observed in unfractionated total EV isolates. The greater number of neuronal proteins uniquely identified in NEEVs supports the effectiveness of neuronal capture approaches for enhancing detection of brain-related EV cargo and improving analytical sensitivity for neuronal proteomic profiling.

DISCUSSION

Here, we systematically evaluated (1) total EVs derived from plasma and serum using two precipitation-based and five SEC-based isolation methods; and (2) neuron-enriched EV immunocapture with three neuronal markers – L1CAM, NCAM1, and ATP1A3. Total EVs and NEEVs, characterized in accordance with MISEV2023^[34], were within the optimal range for exosomes or small EVs (50-150 nm) as measured with MRPS, and confirmed expression of tetraspanins with multiple characterization techniques including flow cytometry, super-resolution microscopy, and high-sensitivity immunoassays.

Overall, EVs consistently expressed canonical tetraspanin markers (CD63, CD81, CD9) in both plasma and serum, with levels influenced by the extraction buffer. Yield and purity varied depending on the isolation approach with precipitation favoring higher recovery and SEC greater purity across biofluids. Previous research has extensively detailed a comparison of EV isolation methods from serum and plasma^[41-51]. Consistent with *Suresh and Zhang* and *Figueroa-Hall et al.*, we showed that total EVs isolated with ExoQuick and SmartSEC from plasma yielded the highest concentration of CD9^[42,44].

Immunocapture of brain-enriched EVs offers an advantageous approach to probing brain-specific biological processes via EV cargo. NEEVs are of particular importance given the central roles of neurons in electrical and chemical signaling, neuroplasticity, network formation, and maintenance of brain homeostasis^[52-54]. For NEEVs, tetraspanin levels and overall performance were influenced by both the isolation strategy and the neuronal marker used for immunocapture. Certain combinations of methods and markers yielded higher purity or more consistent results, though performance varied between plasma and serum. Proteomic analysis identified a substantial number of neuronal-associated proteins, with plasma-derived NEEVs generally providing greater protein coverage. Together, these findings highlight the importance of carefully selecting both the starting biofluid and isolation strategy when designing EV-based studies. Notably, ATP1A3-enriched plasma NEEVs demonstrated the strongest fluorescence intensities across both EXO-FITC and CD81-FITC flow cytometry assays, supporting the findings that ATP1A3-based enrichment combined with plasma EV isolation provides robust EV recovery.

At the same time, the differing fluorescence profiles observed between EXO-FITC and CD81-FITC highlight the importance of interpreting EV characterization assays within the context of the specific marker and analytical platform used. SEC-based isolation preferentially enhanced general EV-associated fluorescence, whereas precipitation-based isolation demonstrated stronger CD81-associated signals, suggesting method-dependent differences in vesicle composition, surface marker accessibility, or co-isolated EV subpopulations. Together, these findings emphasize that both biofluid source and isolation methodology substantially influence NEEV yield, surface marker detection, and downstream

proteomic outcomes. Importantly, no single workflow uniformly optimized all performance metrics, underscoring the need to balance recovery, purity, and neuronal protein coverage according to the intended downstream application.

Although there is ongoing debate regarding the optimal neuronal marker(s) for EV immunocapture, continued evaluation of commonly used and emerging candidates remains an active area of research. Assessment of plasma abundance and brain specificity using mass spectrometry data (e.g., Protein Atlas) shows that NCAM1 is the most abundant in plasma, followed by L1CAM, ATP1A3, GAP43, and SNAP25^[55]. However, despite its relatively high abundance, NCAM1 shares key limitations with L1CAM, including limited neuronal specificity—due to expression in immune cells—and the presence of soluble forms^[56,57]. Other candidates, such as synaptosome-associated protein 25 (SNAP25) and glycoprotein M6A (GPM6A), have been evaluated using postmortem brain tissue and demonstrate high neuronal specificity without detectable soluble forms^[58]. However, their low abundance in plasma suggests they may yield limited recovery of neuron-enriched EVs^[55].

Proteomics analyses in this study further demonstrated that the EV isolation method is the primary driver of variability in protein composition, exceeding the effects of biofluid type and neuronal marker selection. Across both total EV and NEEV datasets, substantial numbers of differentially abundant proteins were observed between Apex and ExoQuick isolation methods, whereas comparisons between antibodies within the same isolation method yielded relatively few differences. These findings are consistent with prior reports indicating that isolation strategy strongly influences EV proteomic profiles, reflecting differences in vesicle recovery, co-isolated contaminants, and physicochemical properties of the isolated material^[44,48,49,59,60].

Precipitation-based approaches such as ExoQuick consistently yielded higher overall protein recovery and broader proteome coverage, particularly in plasma-derived samples. This increased yield likely contributes to the higher number of detected neuron-associated proteins observed in ExoQuick plasma NEEVs. However, this advantage may be accompanied by increased co-isolation of non-EV components, including lipoproteins and soluble proteins,

which can influence downstream proteomic interpretation^[41,49,56]. In contrast, SEC-based methods such as Apex provided more selective enrichment of EV populations, resulting in distinct protein subsets with reduced background signal but lower overall coverage. These differences highlight the trade-off between sensitivity and specificity that is inherent to EV isolation strategies^[44,48].

Notably, despite these methodological differences, a large proportion of proteins, including neuron-associated proteins, were shared across all conditions, indicating the presence of a conserved core EV and NEEV proteome. Differences between groups were primarily driven by protein detection frequency rather than large shifts in abundance, suggesting that isolation methods influence the depth of proteomic sampling rather than fundamentally altering the biological identity of EV populations^[44,49]. This observation is further supported by clustering and principal component analyses, which consistently identified the isolation method as the dominant source of variance, with biofluid type contributing secondary effects and neuronal marker selection having comparatively minimal impact.

Comparison of plasma and serum NEEV proteomes revealed that plasma samples consistently achieved higher rates of neuronal protein detection and more robust coverage. This finding is in line with previous studies showing that clotting during the serum sample preparation can alter EV composition and reduce recovery of certain vesicle subpopulations^[41,50]. Furthermore, the greater divergence observed between plasma and serum under precipitation-based isolation suggests that such methods may amplify matrix-specific differences, whereas SEC-based approaches yield more comparable profiles across biofluids^[44,48].

Importantly, although ATP1A3 and NCAM1 showed modest improvements in neuronal protein detection relative to L1CAM, antibody choice did not substantially alter global proteomic patterns. This suggests that current immunocapture strategies enrich overlapping EV populations rather than distinct neuronal subsets. These findings reinforce the concept that NEEVs obtained through single-marker immunocapture should be interpreted as “neuron-enriched” rather than strictly neuron-specific populations^[55]. Future studies

incorporating multi-marker or combinatorial capture strategies may improve specificity and enable more refined isolation of neuronal EV subtypes^[45].

Together, these proteomic results underscore the critical importance of methodological considerations in EV research. The choice of isolation method has a profound impact on both protein yield and composition, directly influencing biomarker discovery and biological interpretation. Standardization of proteomic workflows, including consistent isolation protocols, normalization strategies, and reporting practices, will be essential for improving reproducibility and facilitating cross-study comparisons in the field.

An additional important finding from this study was the substantial expansion of detectable neuronal-specific proteins following neuronal EV enrichment compared with unfractionated total EV preparations. Across plasma, serum, and combined datasets, NEEVs consistently demonstrated a greater repertoire of neuronal proteins than total EV cohorts, with more than 130 neuronal proteins uniquely identified in NEEVs. These findings support the concept that immunocapture-based enrichment substantially improves detection sensitivity for neuron-associated cargo in peripheral biofluids. Previous studies have similarly reported that neuronal EV enrichment increases recovery of synaptic, axonal, and neuron-specific proteins that are otherwise difficult to detect within heterogeneous circulating EV populations^[6,12,16,55,58].

Despite this enrichment, a large proportion of neuronal proteins remained shared between total EV and NEEV cohorts, indicating that neuronal-associated cargo is detectable within unfractionated circulating EV populations. This observation aligns with increasing evidence that brain-derived EVs contribute measurably to the peripheral EV pool despite the predominance of EVs originating from hematopoietic and peripheral tissues^[2,3,55]. However, the markedly greater neuronal protein coverage observed following immunocapture suggests that targeted enrichment enhances proteomic depth and analytical sensitivity, which may be particularly important for detecting low-abundance CNS-derived biomarkers in neurological and psychiatric disorders.

The observation that a subset of neuronal proteins remained uniquely detectable in total EV isolates may reflect biological and technical factors. Total EV preparations likely contain neuronal EV subpopulations not captured by the selected immunocapture antibodies, as well as EVs derived from other neural cell types expressing overlapping neuronal-associated proteins. In addition, immunocapture using a single marker may preferentially isolate only specific neuronal EV subsets, thereby excluding vesicles carrying distinct neuronal proteins. These findings further support the interpretation that current NEEV approaches enrich, but do not fully encompass, the complete diversity of neuron-derived EV populations^[45,55].

Together, these results demonstrate that neuronal enrichment strategies provide complementary advantages to total EV analyses by substantially increasing detection of brain-related proteins while preserving a shared core EV proteome. The enhanced neuronal protein coverage observed in NEEVs supports their potential utility for biomarker discovery and mechanistic studies of CNS disorders, particularly in applications requiring improved sensitivity for low-abundance neuronal cargo.

LIMITATIONS

Several limitations should be considered when interpreting the findings of this study. First, immunocapture using a single neuronal marker likely targets only a subset of neuronal EV populations, as different markers are associated with distinct neuronal subtypes, cellular compartments, or activation states. As a result, the isolated NEEVs should be interpreted as neuron-enriched rather than exclusively neuron-derived, and important subpopulations may not be captured.

Second, co-isolation of non-neuronal vesicles and non-EV components remains challenging, particularly with precipitation-based isolation methods. Lipoproteins, protein aggregates, and EVs from non-neuronal cell types may be co-enriched, which can confound interpretation of neuronal-specific signals and contribute to variability in both tetraspanin and proteomic measurements.

Finally, this study did not exhaustively evaluate all available EV isolation techniques or neuronal markers. While multiple commonly used methods and markers were systematically compared, emerging technologies and additional candidate markers may offer improved specificity, yield, or reproducibility. Future studies incorporating a broader range of isolation strategies and combinatorial marker approaches will be important for further optimization and standardization of NEEV workflows.

CONCLUSIONS

As the search for optimal neuronal markers continue, it is possible that no single marker will fully satisfy all desired criteria. Our findings highlight that EV characterization, and downstream outcomes are influenced by both the starting biofluid and the isolation approach. Moving forward, it will be essential to prioritize transparency and standardization in experimental reporting, including detailed documentation of biofluid type, antibody clones used for immunocapture, isolation workflows, and downstream analytical methods.

DECLARATIONS

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Authors' contributions

Contributed to research design, data collection, data analysis, manuscript writing, preparation, and review: Burrows K;

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Availability of data and materials

Data available upon request and approval by the Director of the Laureate Institute for Brain Research.

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Conflict of interest

All authors report no conflicts of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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