Comparison of Extracellular Vesicle Isolation Methods from Human Biofluids using Single Molecule Arrays

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Abstract

Extracellular vesicles (EVs) are released by all cells into biofluids and hold great promise as reservoirs of disease biomarkers. One of the main challenges in studying EVs and using them for diagnostics is a lack of methods to quantify EVs that are sensitive enough and can differentiate EVs from similarly sized lipoproteins and protein aggregates. We demonstrate the use of ultrasensitive assays to quantify EVs by immuno-isolating and detecting EV transmembrane proteins in microwell arrays. We developed single molecule array (Simoa) assays for the quantification of EVs using three widely expressed transmembrane proteins: the tetraspanins CD9, CD63, and CD81. Using Simoa to measure these three EV markers, as well as albumin to measure protein contamination, we were able to compare the relative efficiency and purity of several commonly used EV isolation methods in plasma and cerebrospinal fluid (CSF): ultracentrifugation, precipitation, and size exclusion chromatography (SEC). We further used these assays to rapidly optimize EV isolation using SEC from plasma and CSF. Our results highlight the utility of quantifying EVs using Simoa and provide a rapid framework for comparing and improving EV isolation methods from biofluids.

Introduction

Extracellular vesicles (EVs) are released by all cell types and are found in biofluids such as plasma and CSF. EVs contain contents from their donor cells, providing broad non-invasive access to molecular information about cell types in the human body inaccessible to biopsy (1). Despite the diagnostic potential of EVs, there are several challenges that have hampered their utility as biomarkers. First, EVs are heterogeneous and difficult to quantify (2, 3). Second, EVs and their contents are present at low amounts in clinically relevant samples (4). Finally, there is a lack of consensus about the best way to isolate EVs from biofluids (5).

Several techniques have been used in attempts to quantify EVs. These methods, such as nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and tunable resistive pulse sensing (TRPS), aim to measure both particle size and concentration (3). A major limitation of these methods is that they cannot discriminate lipoproteins or particles of aggregated proteins from EVs (5). Since biofluids, and plasma in particular, contain an abundance of lipoproteins and protein aggregates at levels higher than those of EVs (6, 7), these methods are ill-suited for quantifying EVs (2). Lipid dyes have also been used to label and measure EVs (8, 9), but these dyes also bind to lipoproteins, however, and lack sensitivity (2). A feature of EVs that distinguishes them from both lipoproteins and free protein aggregates is the presence of transmembrane proteins that span the phospholipid bilayer (6). The tetraspanins CD9, CD63, and CD81 are transmembrane proteins that are widely expressed and readily found on EVs, often referred to as "EV markers" (2). Although there is not one transmembrane protein present on all EVs,

simultaneously measuring several highly expressed proteins in a sample should minimize the possibility that a rare subset of EVs is being analyzed. Therefore, measuring the three tetraspanin markers should be reliable proxy for EV abundance in many contexts.

Our lab has previously developed single molecule array (Simoa) technology, now commercially available, which converts the enzyme-linked immunosorbent assay (ELISA) into a digital readout by distributing single protein-antibody complexes into femtoliter wells (10). Simoa assays can be 100 to 1000 times more sensitive than traditional ELISAs (11), which is essential for EV analysis. The levels of EV proteins are often low in clinical samples and undetectable using conventional techniques (12). We developed Simoa assays EVs by immuno-isolating and detecting EV transmembrane proteins (CD9, CD63, and CD81) in microwell arrays. We previously used these assays, as well as a Simoa assay for the putative neuron-derived EV marker, L1CAM, to show that L1CAM is not associated with EVs by size exclusion chromatography (SEC) or density gradient centrifugation (DCG) (10).

Simoa can count individual antigen-positive vesicles with a specific transmembrane protein and the ratio of "on wells" to "off wells" can then be mapped against a standard calibration curve of recombinant protein of known concentration. As this mapping allows for quantitative measurements, we reasoned that Simoa could be used to perform relative quantification of EVs between different EV isolation methods. In this study, we demonstrate the application of Simoa for relative EV quantification by comparing different EV isolation methods from human biofluids. In particular, we applied Simoa to compare EV isolation methods from

human plasma and cerebrospinal fluid (CSF) using three of the most commonly used isolation techniques: ultracentrifugation, precipitation, and size exclusion chromatography (SEC). By measuring levels of albumin using Simoa, we were also able to determine the relative purity for each technique in the same experiment. These comparisons were enabled by Simoa's high sensitivity, wide dynamic range, and high-throughput capabilities. We were then able to use these Simoa assays to guide the development of improved SEC protocols for EV isolation in plasma and CSF, demonstrating the utility of Simoa for EV quantification and analysis in biological fluids.

Results

EV analysis using single molecule arrays (Simoa)

We employed single molecule array (Simoa) technology, an ultrasensitive digital ELISA, as a novel platform for EV quantification. Although Simoa is generally used to quantify free proteins, we applied Simoa to EVs by immuno-isolating single vesicles into microwell arrays labeled with antibodies to transmembrane proteins on the EV surface. As in conventional ELISA, Simoa uses two antibodies: a capture antibody conjugated to a magnetic bead, and a detector antibody that is labeled with an enzyme creating a fluorescent product. Because EVs have more than one copy of each protein molecule on their surface, the detector antibody can be either the same protein as the capture antibody, or another protein on the same vesicle. Unlike traditional ELISA, individual immuno-complexes are isolated into femtoliter wells that fit only one bead per well. In a given sample, there are many more antibody-

bound beads than target proteins, and therefore Poisson statistics dictate that only a single immuno-complex is present per well. This allows counting "on wells" as individual protein molecules, or, in the case of EVs, antigen-positive vesicles (Figure 1a) for markers such as CD9, CD63, or CD81. We previously developed Simoa assays for these proteins and showed that they are one to three orders of magnitude more sensitive than the corresponding standard ELISA assays with the same pairs of antibodies (10).

Application of Simoa for comparison of existing EV isolation methods

We used Simoa to directly compare EV isolation from 0.5 mL samples of human plasma and CSF using commonly used EV isolation methods. For each method, we used identical samples of plasma or CSF that were pooled and aliquoted, allowing us to directly compare the different methods. To separate EVs from cells, cell debris, or large vesicles, all samples were first centrifuged and then filtered through a 0.45 μm filter. We compared ultracentrifugation (with and without a wash step), two commercial precipitation kits (ExoQuick and ExoQuick ULTRA), and two commercially available SEC columns (Izon qEVoriginal 35nm and 70nm). SEC separates EVs from free proteins based on size; proteins enter porous beads and elute from the column later than the EVs, which are much larger and less likely to enter the beads (Figure 1b). Whereas the ultracentrifugation and precipitation conditions each yielded a single sample, for the SEC conditions, we collected several fractions and analyzed each fraction to assess the distribution of EVs relative to albumin.

We were able to quantify EVs by measuring the levels of CD9, CD63, and CD81 across the different EV isolation methods in both plasma and CSF (Figure 1c). Since we are interested in all EVs, as opposed to subsets with a specific marker, we quantified EV yield by averaging the levels of the three tetraspanins. We first used the Simoa measurement (in picomoles, determined relative to a corresponding recombinant protein standard) to calculate EV recovery for each individual marker by normalizing the level of tetraspanin in each condition to the amount of that tetraspanin in fractions 7-10 of the Izon qEV 35nm SEC column (the condition with highest EV levels in plasma). Next, we averaged the relative tetraspanin recovery values across the three tetraspanins to calculate relative EV recovery (Figure 2).

After determining combined relative EV recovery and albumin concentration for each EV isolation method, we could directly compare EV recovery and purity in both plasma and CSF. In plasma, we found that the Izon qEVoriginal 35nm SEC column (collecting fractions 7-10) yielded both the highest recovery of EVs and the highest purity of EVs (Figure 2a-e). Meanwhile, in CSF, ExoQuick yielded the highest recovery of EVs while Izon qEVoriginal 70nm yielded the highest purity (Figure 2f-j).

Application of Simoa for custom SEC column optimization

We next took advantage of Simoa's high throughput nature to optimize EV isolation from 0.5 mL samples of plasma and CSF using SEC. We prepared our own SEC columns and systematically tested several parameters of the SEC isolation procedure in an effort to improve EV yield and purity beyond that of the

commercially available columns. To accomplish this goal, we tested three Sepharose resins (CL-2B, CL-4B and CL-6B) at two column heights (10 and 20 mL). This comprehensive comparison led us to several conclusions. First, we found that resins with smaller pore sizes led to higher yield. Sepharose CL-6B, which has the smallest pore size, gave the highest yield of EVs, although it was accompanied by higher albumin contamination, as expected. For all SEC columns, higher purity could also be achieved by taking a smaller number of fractions (e.g. 7-9 instead of 7-10), albeit at the expense of lower EV yield. We quantified this difference for all columns and found that doubling the height of any given column from 10 to 20 mL resulted in better separation between EVs and free proteins, leading to higher purity but lower EV recovery (Figures 3 & 4). When we compared different volumes of plasma and CSF for a 10 mL Sepharose CL-6B column, we found that the sample volume loaded had an effect on purity, with larger loading volumes leading to lower purity (Supplementary Figure 1).

Direct comparison of all EV isolation methods

Combining all of the Simoa data we generated, we were able to perform a direct, quantitative comparison of the relative yield and purity of EVs across all methods tested. We normalized the level of each tetraspanin recovered using a given method by the level measured across all fractions (6-21) in a 10 mL Sepharose CL-6B column. We then averaged the values of the three tetraspanins to obtain relative EV recovery for each method. Similarly, albumin contamination was calculated by normalizing the amount of albumin in each isolation method to the

total albumin detected in all fractions of a 10 mL Sepharose CL-6B column (Figure 5).

For plasma, the techniques that demonstrated the highest recovery were Sepharose CL-6B 10 mL, Izon qEVoriginal 35nm column, and ExoQuick (Figure 5a). Of these three methods, Sepharose CL-6B 10 mL demonstrated the highest yield whereas the Izon qEVoriginal 35nm column demonstrated the highest purity. For CSF, we observed the highest recovery with 10 mL Sepharose CL-6B, Sepharose CL-4B, and ExoQuick (Figure 5b). Sepharose CL-6B 10 mL demonstrated the highest yield, while Sepharose CL-4B demonstrated the highest purity of these three methods. These findings suggest that the best isolation method depends both on the biofluid of interest and downstream application since there is a tradeoff between EV yield and purity.

Discussion

We developed Simoa assays that can be used for ultrasensitive quantification of EVs in biological fluids and applied these assays to the analysis of EV isolation methods. Although several techniques have previously been applied to EV detection (3), Simoa technology has a unique set of features that makes it particularly well-suited for measuring EVs, overcoming several limitations of other methods. In addition to differentiating EVs from other particles such as lipoproteins or aggregated proteins - since transmembrane proteins are present on EVs but should not be present in other particles - Simoa also offers high dynamic range, throughput, and sensitivity. This sensitivity is achieved by converting ELISA to a digital readout

via immuno-capture and counting of individual protein molecules in a microwell array. The high-throughput nature of Simoa is enabled by the automated liquid handling of the commercially-available Quanterix HD-X instrument, although our lab has developed other digital ELISA methods that do not rely on commercial instrumentation (13-15), which could be similarly applied to EVs.

We used Simoa to directly compare yield and purity of EV isolation methods. To obtain the purest EVs possible (and separate EVs from lipoproteins), it has been shown that it is necessary to combine several techniques sequentially, such as density gradient centrifugation (DGC) and SEC (16, 17). However, techniques such as DGC are not scalable to many samples and therefore not amenable to biomarker studies. Thus, we focused on several commonly-used EV isolation methods that are amenable to biomarker studies and used Simoa to assess their recovery and purity in both plasma and CSF.

Although several previous reports have previously compared EV isolation methods (18-28) and yielded important insights, the lack of available quantitative methods for EV measurement has made these studies difficult to interpret (2, 5, 29). We introduce Simoa as a new analytical tool to rapidly provide a quantitative comparison of EV isolation methods. Although not every EV will necessarily contain a tetraspanin protein, by detecting three different tetraspanins per sample with Simoa, we minimize the probability that we are measuring a rare subset of EVs. In the experiments reported here, we observed a strong correlation of the relative levels of the three tetraspanins in different SEC fractions. Since we compared isolation methods from the same starting sample, we were able to provide a direct

quantitative comparison of tetraspanin levels between the different isolation methods. By also developing a Simoa assay to measure free albumin (the main contaminant of EV preparations), we were able to assess the purity of EV preparations with respect to unwanted co-purification of free proteins.

We compared ultracentrifugation (with and without a wash), the commonly used commercial EV precipitation reagent ExoQuick (as well as the newer version, ExoQuick Ultra), and SEC using two different commercial Izon qEV columns. Our results show that there are significant differences between the performance of various methods between plasma and CSF. These differences could be due to a much higher protein concentration in plasma than CSF, which may affect the yield and purity of the different isolation methods. Of the existing methods, commercial SEC columns gave the best combination of yield and purity in plasma. In CSF, EV precipitation by ExoQuick gave higher yield but lower purity than SEC.

Given the high-throughput capabilities of Simoa, we compared several resins and column volumes to improve EV isolation by SEC. Analyzing the tetraspanin and albumin levels in each fraction, we directly compared homemade columns using two different bed volumes and three different resins to the most commonly-used commercial SEC columns. Our results demonstrate that the custom columns significantly improve EV yield over that of the commercial columns. In particular, we find that Sepharose CL-6B, which has not been previously used for EV isolation, yields considerably higher levels of EVs than Sepharose CL-2B or Sepharose CL-4B. This is likely due to Sepharose CL-6B beads having a smaller average pore size, leading to a lower probability that EVs will enter the beads.

In SEC, we find that there is a tradeoff between EV yield and albumin contamination. Collecting more EVs generally means more albumin contamination, as the later EV fractions become contaminated with free protein. Thus, we envision different SEC columns will be suited for different applications. Using a 10 mL Sepharose CL-6B column for EV isolation from plasma or CSF is the best choice for downstream applications where maximum EV yield is needed and where some free protein contamination is not detrimental. Use of this column would also be preferable for analyzing rare EV cargo or when further purification of EVs will be performed (such as immuno-isolation). On the other hand, if isolating EVs from plasma where minimal free protein contamination is desired (for example, in EV protein analysis by western blot), a larger 20 mL column with Sepharose CL-6B or Sepharose CL-4B would yield better results. For CSF, we found that larger 20 mL columns demonstrated poor yield; therefore, using 10 mL columns is preferable - Sepharose CL-6B for maximum yield or Sepharose CL-4B for maximum purity.

We used Simoa to quantify EVs after using the different isolation methods, but we think Simoa should be useful for a much broader set of applications. We analyzed EV subpopulations with capture and detector antibodies against one transmembrane protein at a time, but one could also analyze individual EVs in microwells with antibodies against more than one protein, as has been recently demonstrated (30). Since the goal of our study was to directly compare different EV isolation methods in biological fluids, we were less concerned with measuring intact EVs, and more concerned with finding assay conditions that maximized sensitivity and specificity. Thus, our assays were performed in the presence of detergents such

as Tween, which may affect whether EVs are intact. Although it has been previously reported that EVs remain intact in the presence of low concentrations of Tween (31), we did not confirm the integrity of EVs in our studies. Future studies could ensure that assay conditions preserve the full EV structure, and then apply Simoa to the study of EV heterogeneity at the individual vesicle level.

The general experimental framework presented here could be easily applied to evaluate new EV isolation methods in plasma, CSF, or other biological fluids, such as urine or saliva. One could also add Simoa assays for lipoproteins to evaluate how well various methods separate EVs from lipoproteins. While we limited our studies in this paper to EVs from biological fluids, these methods could also be applied to the study of EVs in cell culture. The sensitivity and high-throughput nature of Simoa allows for relative EV quantification in cell culture media from small numbers of cells, for example, after genetic or cellular perturbations. As sensitivity of EV detection and specificity in differentiating EVs from contaminants are obstacles in all EV studies, we envision that ultrasensitive protein detection of EV surface proteins will be applicable to both the study of basic EV biology and the development of diagnostic methods.

Materials and Methods

Human Sample Handling

Pre-aliquoted pooled human plasma and CSF samples were ordered from BioIVT. The same pools were used for all main figures throughout the paper in order to ensure comparable analysis of methods. For all EV isolation technique comparisons,

one 0.5 mL sample was used for each isolation method. Plasma or CSF was thawed at room temperature. After sample thawing, 100X Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology) was added to 1X. The sample was then centrifuged at 2000 x *g* for 10 minutes. The supernatant was subsequently centrifuged through a 0.45 μm Corning Costar SPIN-X centrifuge tube filter (Sigma-Aldrich) at 2000 x *g* for 10 minutes to get rid of any remaining cells or cell debris.

Simoa Assays

Simoa assays were developed and performed as previously described (10). Capture antibodies were coupled to Carboxylated Paramagnetic Beads from the Simoa Homebrew Assay Development Kit (Quanterix) using EDC chemistry (Thermo Fisher Scientific). Detection antibodies were conjugated to biotin using EZ-Link NHS-PEG4 Biotin (Thermo Fisher Scientific). The following antibodies were used as capture antibodies for tetraspanins: ab195422 (Abcam), MAB5048 (R&D Systems), and ab79559 (Abcam). The following antibodies were used as detector antibodies for tetraspanins: ab58989 (R&D Systems), 556019 (BD) and 349502 (BioLegend). For albumin, DY1455 (R&D Systems) was used as both capture and detector antibody. The following recombinant proteins were used for CD9, CD63, CD81, and albumin: ab152262 (Abcam), TP301733 (Origene), TP317508 (Origene), ab201876 (Abcam).

On-board dilution was performed with 4X dilution for each of the tetraspanins, while manual 20X dilution was used for albumin. All samples were raised to 160 μL per replicate in sample diluent. For tetraspanin assays, samples

were incubated with immunocapture beads (25 μL) and biotinylated detection antibody (20 μL) for 35 minutes. Next, six washes were performed, and the beads were resuspended in 100 μL of Streptavidin labeled β-galactosidase (Quanterix) and incubated for 5 minutes. An additional six washes were performed, and the beads were resuspended in 25 μL Resorufin β-D-galactopyranoside (Quanterix) before being loaded into the microwell array on the Quanterix HD-X instrument.

For the albumin assay, samples were incubated first with immunocapture beads (25 μL) for 15 minutes and then washed six times. Subsequently, 100 μL detection antibody was incubated with the beads for 5 minutes. Next, six washes were performed, and the beads were resuspended in 100 μL of Streptavidin labeled β-galactosidase (Quanterix) for a final 5 minute incubation. After an additional six washes, the beads were resuspended in 25 μL Resorufin β-D-galactopyranoside (Quanterix) and then loaded into the microwell array on the Quanterix HD-X instrument.

Preparation of Custom SEC Columns

The resins Sepharose CL-2B, Sepharose CL-4B, and Sepharose CL-6B (all from GE Healthcare/Cytiva) were washed in PBS. The volume of resin was washed with an equal volume of PBS in a glass container and then placed at 4 °C in order to let the resin settle completely (several hours or overnight). The PBS was then poured off, and an equal volume of PBS was again added two more times for a total of three washes. Columns were prepared fresh on the day of use. Washed resin was poured into an Econo-Pac Chromotography column (Bio-Rad) to bring the bed volume (the

resin without liquid) to 10 mL or 20 mL. When the desired amount of resin filled the column and the liquid dripped through, the top frit was immediately placed at the top of the resin without compressing the resin. PBS was then added again before sample addition.

Collection of Size Exclusion Chromatography Fractions

Once prepared, all columns were washed with at least 20 mL of PBS in the column. Immediately before sample addition, the column was allowed to fully drip out and, after last drop of PBS, sample (filtered plasma or CSF) was added to the column. As soon as sample was added, 0.5 mL fractions were collected in individual tubes. As soon as the plasma or CSF completely entered the column (below the frit), PBS was added to the top of column 1 mL at a time. Fraction numbers correspond to 0.5mL increments collected as soon as sample is added. For Izon and 10 mL columns, fractions 6-21 were collected (since first few fractions correspond to void volume). For 20 mL columns, fractions 12-27 were collected (since void volume is larger for 20 mL columns than 10 mL columns).

Ultracentrifugation

Samples of filtered 0.5 mL plasma or CSF were added to 3.5 mL Open-Top Thickwall Polycarbonate ultracentrifuge tubes (Beckman Coulter), and PBS was added to fill tubes to the top. Samples were ultracentrifuged at 120,000 x *g* for 90 minutes at 4 °C in an Optima XPN-80 ultracentrifuge (Beckman Coulter) using a SW55 Ti swinging-bucket rotor (Beckman Coulter). Afterwards, all supernatant was aspirated. Pellets

were resuspended in PBS for the "Ultracentrifugation" condition. For the "Ultracentrifugation with wash" condition, the ultracentrifuge tubes were filled to the top with PBS, and samples were ultracentrifuged again at 120,000 x *g* for 90 minutes. Supernatant was then aspirated, and pellets were resuspended in 500 uL PBS. For all ultracentrifugation samples, isolation was performed on two separate days and then resulting Simoa values were averaged.

ExoQuick & ExoQuick ULTRA

Samples of plasma or CSF were mixed with ExoQuick Exosome Precipitation Solution (System Biosciences) or ExoQuick ULTRA EV Isolation Kit for Serum and Plasma (System Biosciences), and protocols were performed according to manufacturer's instructions. For ExoQuick, 0.5 mL of plasma or CSF was mixed with 126 uL of ExoQuick and incubated at 4 °C for 30 minutes, followed by centrifugation at 1500 x *g* for 30 minutes. Supernatant was removed, and samples were centrifuged at 1500 x *g* for an additional 5 minutes. Residual supernatant was removed, and pellets were resuspended in 500 uL PBS. For ExoQuick ULTRA, 250 uL of plasma or CSF was used in accordance with instructions, and Simoa values were corrected by multiplying by two to match 0.5 mL volume used for other samples. For each sample, 500 uL of EVs was eluted per column. For all precipitations, isolation was performed on two separate days and then resulting Simoa values were averaged.

Author Contributions

DT and MN conceived of study and designed experiments. DT, MN, WT, JHL, and RL performed experiments. DT, MN, and DRW analyzed data and wrote the manuscript with input from all authors. GMC and DRW supervised the study and provided funding support.

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Competing Interests

DRW is the scientific founder and a board member of Quanterix Inc. GMC commercial interests: http://arep.med.harvard.edu/gmc/tech.html The authors have filed a provisional patent on some of the work described in this manuscript.

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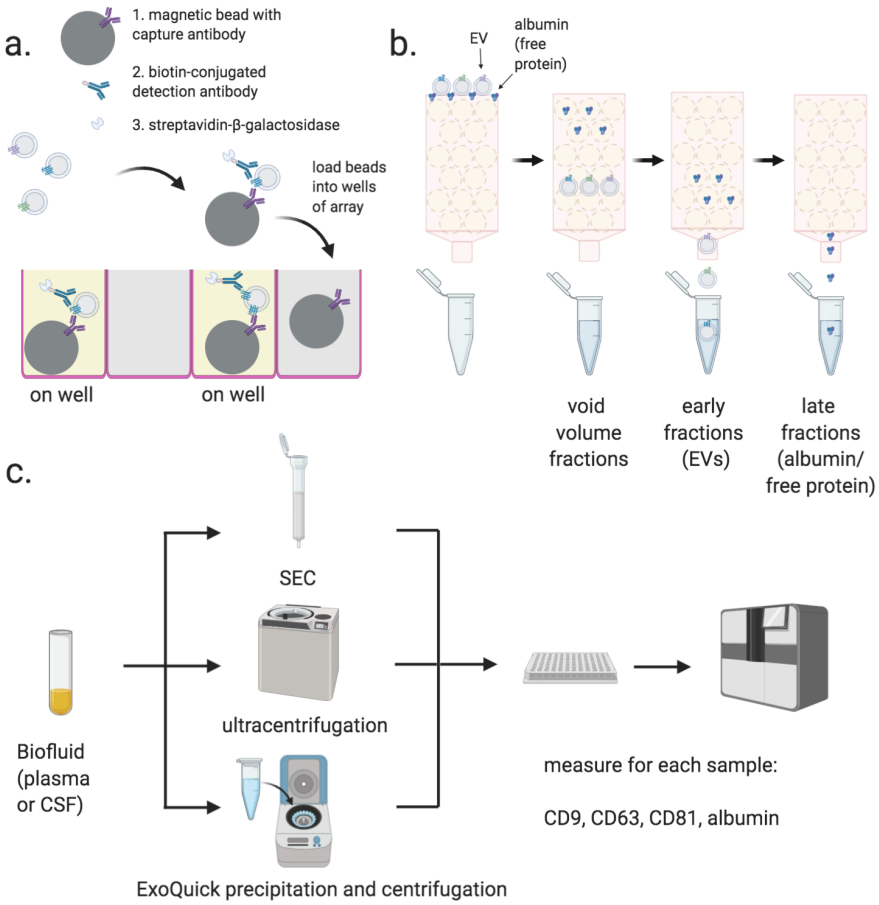

Figure 1. Overview of EV detection using Single Molecule Arrays (Simoa) and experimental design

a. Single immuno-complexes are formed by binding the target tetraspanin protein on EVs to a magnetic bead conjugated to a capture antibody and a biotin-labeled detection antibody. Detection antibodies are then labeled with a streptavidin-conjugated enzyme. The beads are then loaded into individual wells of a microwell array where each well matches the size of the magnetic bead limiting a maximum of one bead per well. Wells with the full immuno-complex produce fluorescent signal upon conversion of substrate. Wells that are "on" are then counted by fluorescence imaging. In the case of detecting transmembrane proteins such as tetraspanins, an immuno-complex can be formed by capture and detection antibodies binding to either the same individual protein or to different tetraspanin proteins on the same EV. b. EV and free proteins such as albumin in a biofluid sample are separated by size exclusion chromatography (SEC). Free proteins elute from the column in later fractions than EVs because free proteins are smaller than the pore size of the beads while EVs are larger and are excluded from entering the beads

c. Aliquots of 0.45 μm filtered plasma or CSF are isolated using SEC, ultracentrifugation, or ExoQuick precipitation. Samples from each isolation method are then loaded into a 96-well plate into a Quanterix HD-X instrument for Simoa analysis of CD9, CD63, CD81, and albumin.

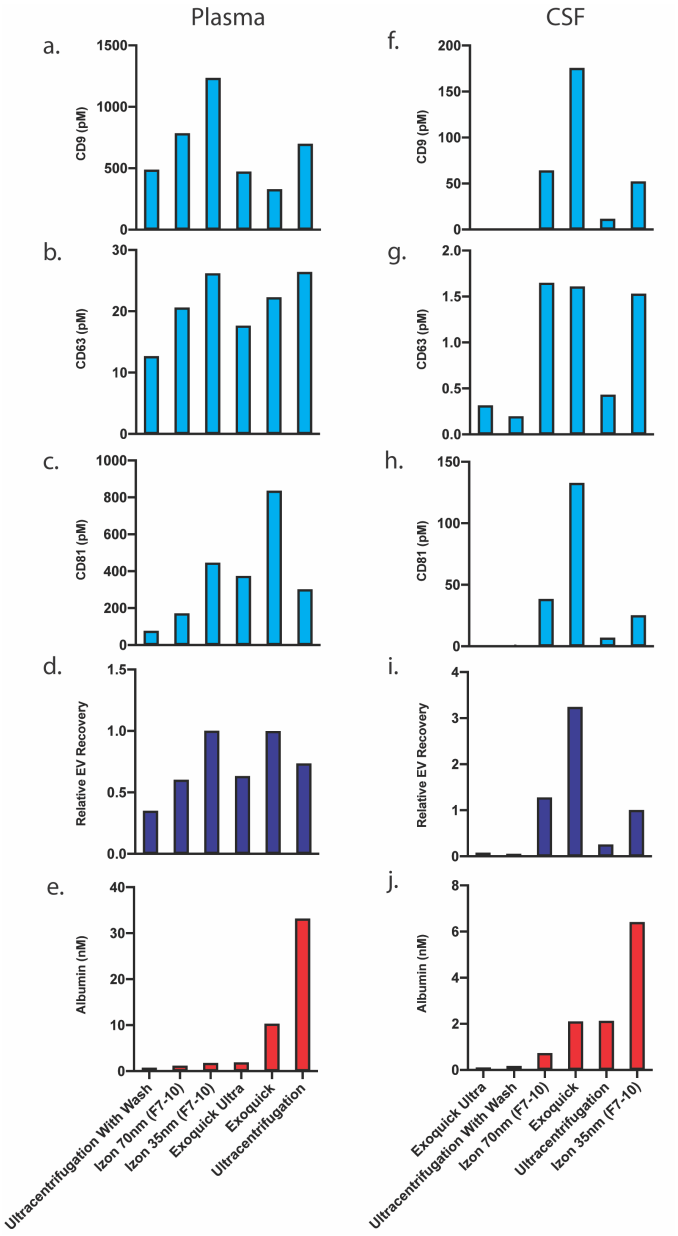

Figure 2. Comparison of existing methods for EV isolation in plasma and CSF
All methods are listed in order of increasing albumin levels.
a-c. Individual tetraspanin yields using different isolation methods from plasma.
d. Relative EV recovery from plasma was calculated by first normalizing individual tetraspanin values (in pM) in each technique to that of Izon qEVoriginal 35nm EV fractions (7-10) and then averaging the three tetraspanin percentages.
e. Albumin levels using different EV isolation methods from plasma.
f-h. Individual tetraspanin yield using different isolation methods from CSF.
i. Relative EV recovery in CSF was calculated by first normalizing individual tetraspanin values (in pM) in each technique to that of Izon qEVoriginal 35nm fractions (7-10) and then averaging the three tetraspanin percentages.
j. Albumin levels using different EV isolation methods from CSF.

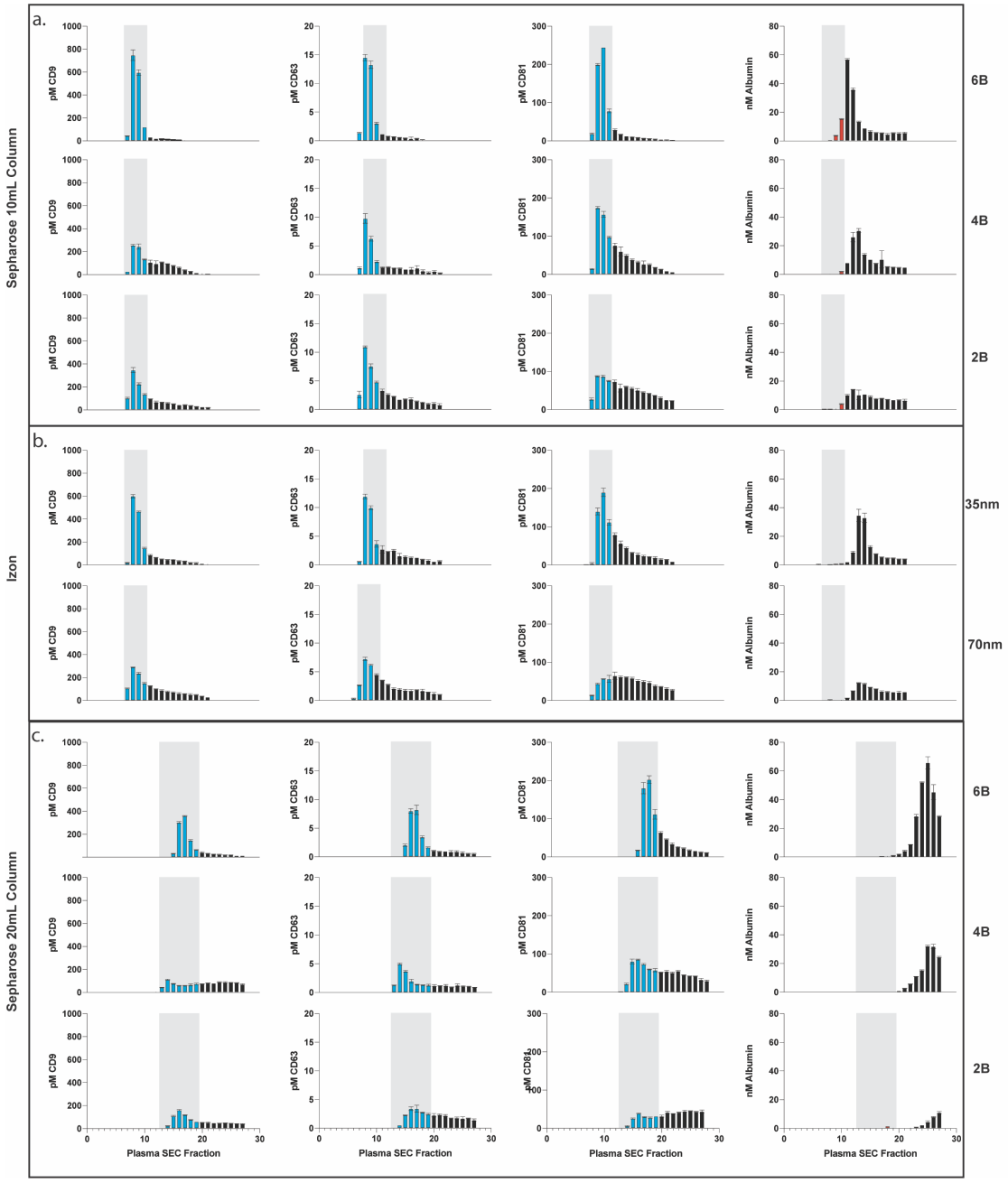

Figure 3: Comparison of SEC methods for EV isolation in plasma
Error bars represent the standard deviations from two technical replicates.
a. Levels of tetraspanins and albumin in plasma after fractionation with 10 mL custom columns filled with: Sepharose CL-6B (top), Sepharose CL-4B (middle), and Sepharose CL-2B (bottom).
b. Levels of tetraspanins and albumin in plasma after fractionation with Izon qEVoriginal 35nm column (top) and Izon qEVoriginal 70nm column (bottom).
c. Levels of tetraspanins and albumin in plasma after fractionation with 20mL custom columns; Sepharose CL-6B (top), Sepharose CL-4B (middle), Sepharose CL-2B (bottom).

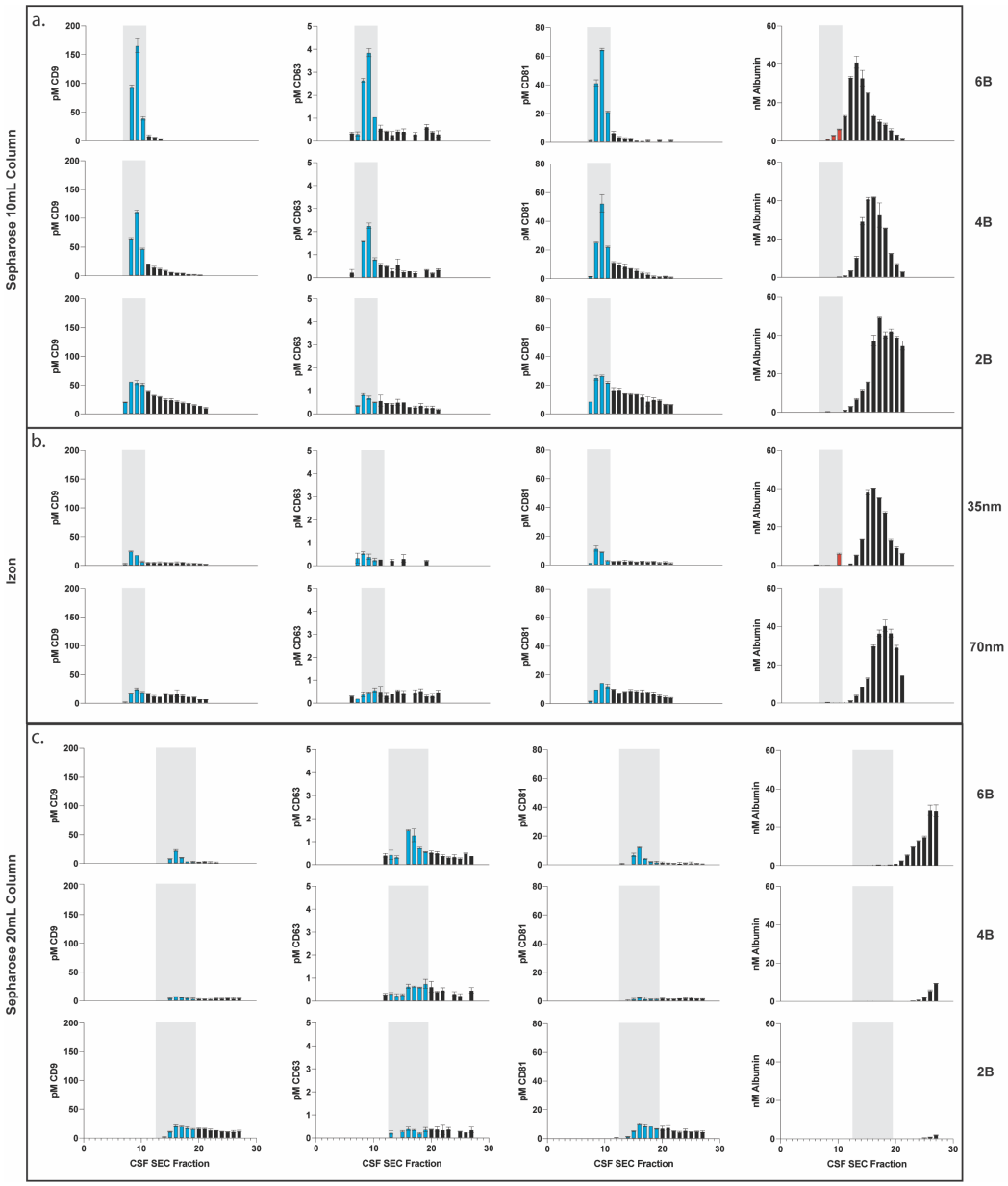

Figure 4: Comparison of SEC methods for EV isolation in CSF
Error bars represent the standard deviations from two technical replicates.
a. Levels of tetraspanins and albumin in CSF after fractionation with 10 mL custom columns filled with: Sepharose CL-6B (top), Sepharose CL-4B (middle), and Sepharose CL-2B (bottom).
b. Levels of tetraspanins and albumin in CSF after fractionation with Izon qEVoriginal 35nm column (top) and Izon qEVoriginal 70nm column (bottom).
c. Levels of tetraspanins and albumin in CSF after fractionation with 20mL custom columns; Sepharose CL-6B (top), Sepharose CL-4B (middle), Sepharose CL-2B (bottom).

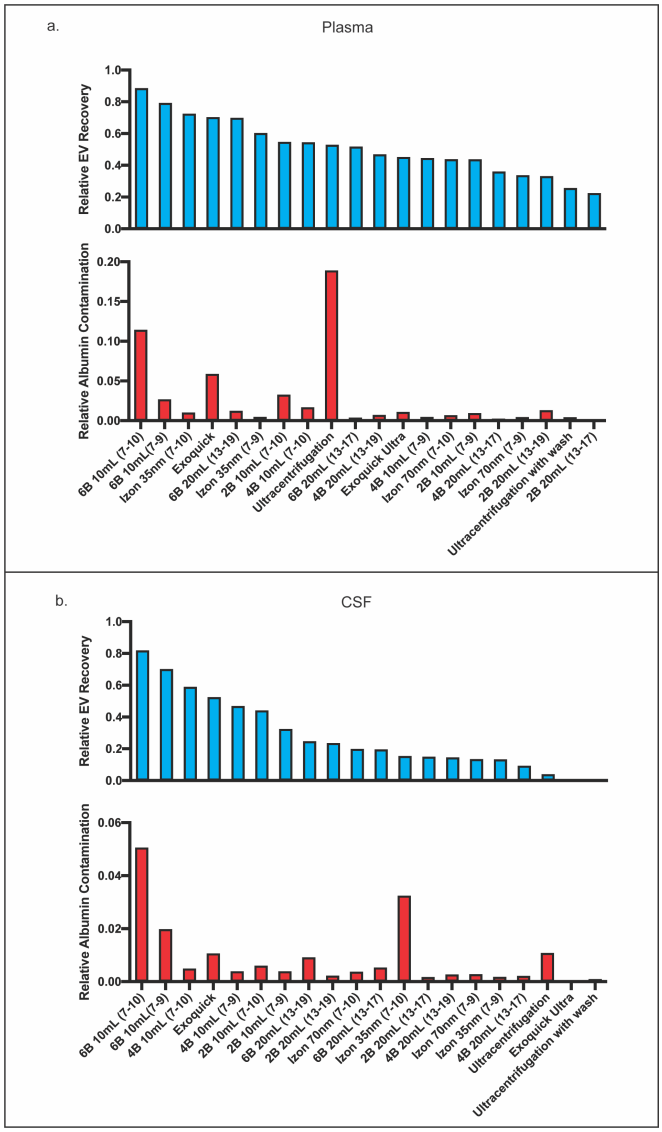

Figure 5: Comparison of EV recovery and albumin contamination across all tested methods in plasma and CSF

a. Comparison of plasma EV recovery (top) and albumin contamination (bottom) across all tested methods ranked by EV recovery. Relative EV recovery was calculated by individually normalizing each tetraspanin to the sum of that tetraspanin in all fractions (6-21) in the 10mL Sepharose CL-6B condition. The three tetraspanin percentages were then averaged to calculate the relative EV recovery. Similarly, albumin for each condition was calculated as a fraction of the albumin found in all fractions (6-21) in the 10mL Sepharose CL-6B condition.

b. Comparison of CSF EV recovery (top) and albumin contamination (bottom) across all tested methods ranked by EV recovery. Relative EV recovery was calculated by individually normalizing each tetraspanin to the sum of that tetraspanin in all fractions (6-21) in the 10mL Sepharose CL-6B condition. The three tetraspanin percentages were then averaged to calculate the relative EV recovery. Similarly, albumin for each condition was calculated as a fraction of the albumin found in all fractions (6-21) in the 10mL Sepharose CL-6B condition.