Methods for EV Enrichment Workflow Characterization

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Abstract

Enrichment of extracellular vesicles (EVs) is required for many studies. Screening methods, functional studies, and analysis of rare subsets of EVs all require some form of EV enrichment. However, it is important to characterize any enrichment method to understand how it impacts the composition of EVs in a given sample and validate the approach to EV enrichment for downstream applications. A rigorous analytical approach for evaluating enrichment methods is needed. In this study, we demonstrate workflows for enrichment method characterization. Vesicle Flow Cytometry (vFC) and BCA measurements were used to characterize media from hollow-fiber bioreactor (HFB) DiFi cell cultures pre- and post-enrichment. There were 5.97E6 total EVs/ul in HFB DiFi cell culture media. 62% of EVs were positive for EGFR above the limit of detection (25 antibodies per vesicle). Enrichment by UF (100k MWCO) resulted in 56% recovery of total EVs with similar EGFR positivity and a significant increase in EV/protein ratio. Other pore sizes differed in total EV recovery (300k MWCO – 24%, 1000k MWCO – 22%). For all pore sizes, UF increased EV and protein concentrations resulting in a decreased EV/protein ratio and did not impact EGFR positivity. SEC enrichment resulted in a high (>80%) recovery of total EVs in in fractions 7-9 (30-52% EGFR+). Most EVs were present in fractions 7-9. SEC enrichment resulted in a substantial increase in EV/protein ratio. This study demonstrates a broadly applicable analytical approach to characterizing enrichment methods for a given sample. Adopting such an approach in workflows requiring EV-enrichment will improve our understanding of input material in any downstream application and will enhance rigor and reproducibility of these studies.

Methods

Bioreactor Culture. DiFi Cells were grown in FiberCell bioreactor according to manufacturer's instructions. EVs were collected following FiberCell's harvest protocol.

Vesicles. Synthetic liposomes (Lipo100) from Cellarcus Biosciences Inc (San Diego, CA) were used as a negative control for EGFR staining and for vFC sizing workflows per manufacturer's instructions. DiFi supernatants were collected from hollow fiber bioreactor cultures. Supernatants were spun at 2500xg for 15 minutes and stored at -80C for further analysis.

EV Enrichment
Ultrafiltration. EVs were concentrated using Sartorius Vivaspin centrifugal ultrafiltration devices. 100, 300, and 1000 kDa MWCO polyethersulfone (PES) filters were compared. Prior to use, filters were pre-rinsed with sterile phosphate-buffered saline (PBS) to minimize membrane binding. EV samples were loaded into the filter units and centrifuged at 4,000 × g for 15 minutes. The concentrated EVs were collected from the upper chamber for downstream analysis.

SEC. EVs were enriched using qEV SEC columns (Izon Science) following the manufacturer's instructions. Columns were equilibrated with sterile-filtered phosphate-buffered saline (PBS) at room temperature prior to sample loading. DiFi supernatants were loaded onto the column, and elution was performed with PBS. The initial void volume was discarded, and all remaining fractions were collected for downstream analysis.

Vesicle Flow Cytometry (Figure 1). EV concentration, size, and surface marker expression were measured by single vesicle flow cytometry(1-5), using a commercial kit (vFC™ Assay kit, Cellarcus Biosciences, La Jolla, CA) and flow cytometer (CytoFLEX, Beckman Coulter). Briefly, samples were stained with the fluorogenic membrane stain vFRed™ and one or more fluorescent antibodies (Table S1) for 1h at RT and analyzed following the CytoFLEX instrument setup protocol. Controls included buffer-only, reagent-only and vesicle standards that have been characterized by orthogonal methods (eg, NTA, RPS, cryo-EM, d-STORM) and serve as positive and negative controls for antigen expression. Spectral compensation was performed using antibody-stained antibody capture beads and validated using single stained controls. Data were analyzed using FCS Express (De Novo Software) and included calibration using a vesicle size and fluorescence intensity standards. The analysis included a pre-stain dilution series to determine the optimal initial sample dilution and multiple positive and negative controls, per guidelines of the International Society for Extracellular Vesicles (ISEV) (6). A detailed description of vFC™ methods and controls.

Protein Measurement. Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, standards were prepared using bovine serum albumin (BSA) in the range of 0–2000 µg/mL. Plates were incubated at 37°C for 30 minutes, and absorbance was measured at 562 nm using a microplate reader.

Results

vFC™ Assay Performance. Prior to measuring EV concentration and cargo by vFC, a dilution series was run to identify an optimal pre-stain dilution and demonstrate single EV resolution and the absence of coincidence. Similarly, a detergent treatment was run to examine specificity. EV counts decreased proportional to dilution while size (calculated from vFRed™ intensity) remained constant. Buffer and reagent only show acceptable numbers of background events. A 40X dilution was selected for cargo measurements. The majority of events were detergent labile. Overall, the assay exhibited single EV resolution and adequate EV specificity.

Ultrafiltration. vFC™ analysis revealed EVs produced from DiFi cells in HfB bioreactors cultures were present at 5.97E6 total EVs/ul prior to enrichment. 62% of EVs were positive for EGFR. MWCO increased both EV concentration and protein concentration but had a greater impact on protein concentration resulting in decreased EV/protein ratios (20-44% of crude). Although recovery was low (22-56%), There is no observed bias introduced as reflected in EV size or EGFR+ EV number or EGFR brightness **(Figure 2).**

Size Exclusion Chromatography. vFC™ analysis revealed EVs produced from DiFi cells in HfB bioreactors cultures were present at 7.89E6 total EVs/ul prior to enrichment. 59% were positive for EGFR. EV concentrations and protein concentrations are reported across fractions in **Figure 3**. Most EVs were found in fractions 6-9. Protein concentration was 412.90 ug/ml in the crude fraction and was reduced to between 5 and 35 ug/ml in fractions 7-9 resulting in a 11-15-fold enrichment of EVs relative to proteins in separate SEC runs. Recoveries were consistently above 80%. EVs were 0.087-0.014 as concentrated as in the crude sample. There is no observed bias introduced as reflected in EV size or EGFR+ EV number or EGFR brightness.

Conclusions

EV enrichment is often required to prepare samples for downstream applications including screening, functional studies, and rare subpopulation measurement. This study demonstrates application of standardized methods, including EV-specific count, size, and surface marker measurements to evaluate EV recovery and enrichment relative to total protein to enable empirical examination of enrichment method suitability for a given downstream application. We examined two common enrichment methods, SEC and UF. Characterization of UF revealed robust sample concentration, but up to 78% sample loss depending upon MWCO selected. 100 kDa MWCO provides higher concentration and recovery than other pore sizes tested. Characterization of SEC revealed robust EV enrichment relative to total protein and >80% recovery, but with significant sample dilution. Most EVs were contained in fractions 6-9.
The measurement workflows described provide a general approach that could be combined with measurements of specific EV subpopulations (by vFC) and additional contaminants of concern. In this example comparison of UF and SEC, characterization of UF and SEC revealed very different effects of these two enrichment methods on sample recovery and composition. Similar characterization of both crude and enriched samples should be used when designing any study requiring EV enrichment to examine suitability of an enrichment workflow for a given experiment.

Acknowledgements

Supported by National Cancer Institute of the National Institutes of Health under Award Number R44CA272098 and the NIH Common Fund Extracellular RNA Communication Consortium (ERCC) through the Office of Strategic Coordination/Office of the NIH Director. The authors acknowledge contributions from Robert Raffai and Martin Ng at UCSF in performance of SEC enrichment and Jeff Franklin and Robert Coffee at Vanderbilt University Medical Center for the DIFI HFB CM.

This experiment describes standardized methods for evaluating EV enrichment workflows.

vFC Overview

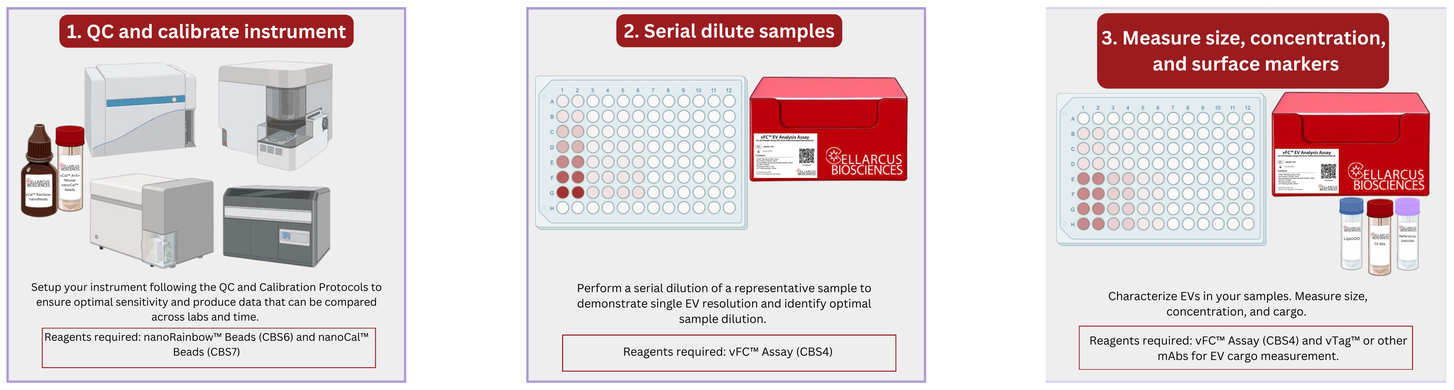

Figure 1. Schematic of VFC workflow. Vesicle flow cytometry (vFC™) is a homogeneous assay in which a cell-free sample is stained with a fluorogenic membrane stain and one or more additional fluorescence probes then analyzed by flow cytometry with detection triggered by membrane fluorescence. The size distribution of a synthetic vesicle standard (Lipo100™), determined by orthogonal methods, is used to calibrate membrane fluorescence into surface area (nm^2), nanoCal™ antibody capture beads are used to calibrate fluorescence intensity in the remaining channels to report fluorescence as antibodies per EV.

Ultrafiltration

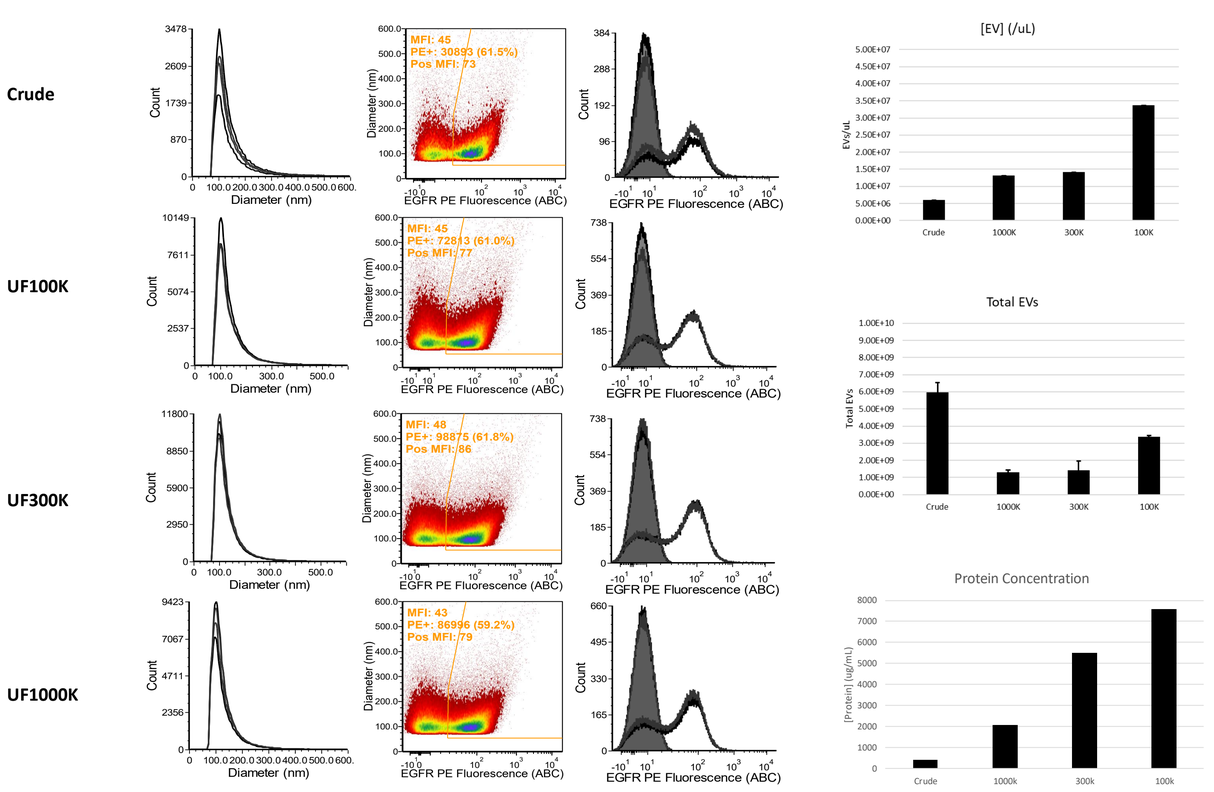

Figure 2. Characterization of EV enrichment by Ultrafiltration. Comparison of EV size, EGFR expression, and total protein concentration (BCA) pre- and post-sample processing by ultrafiltration. There is no observed bias introduced at any MWCO as reflected in EV size (left) or EGFR+ EV number or EGFR brightness (shown as density plots and histograms). Sample concentration, recovery, and protein concentration measurements by condition are summarized in the bar graphs to the right.

Size Exclusion Chromatography

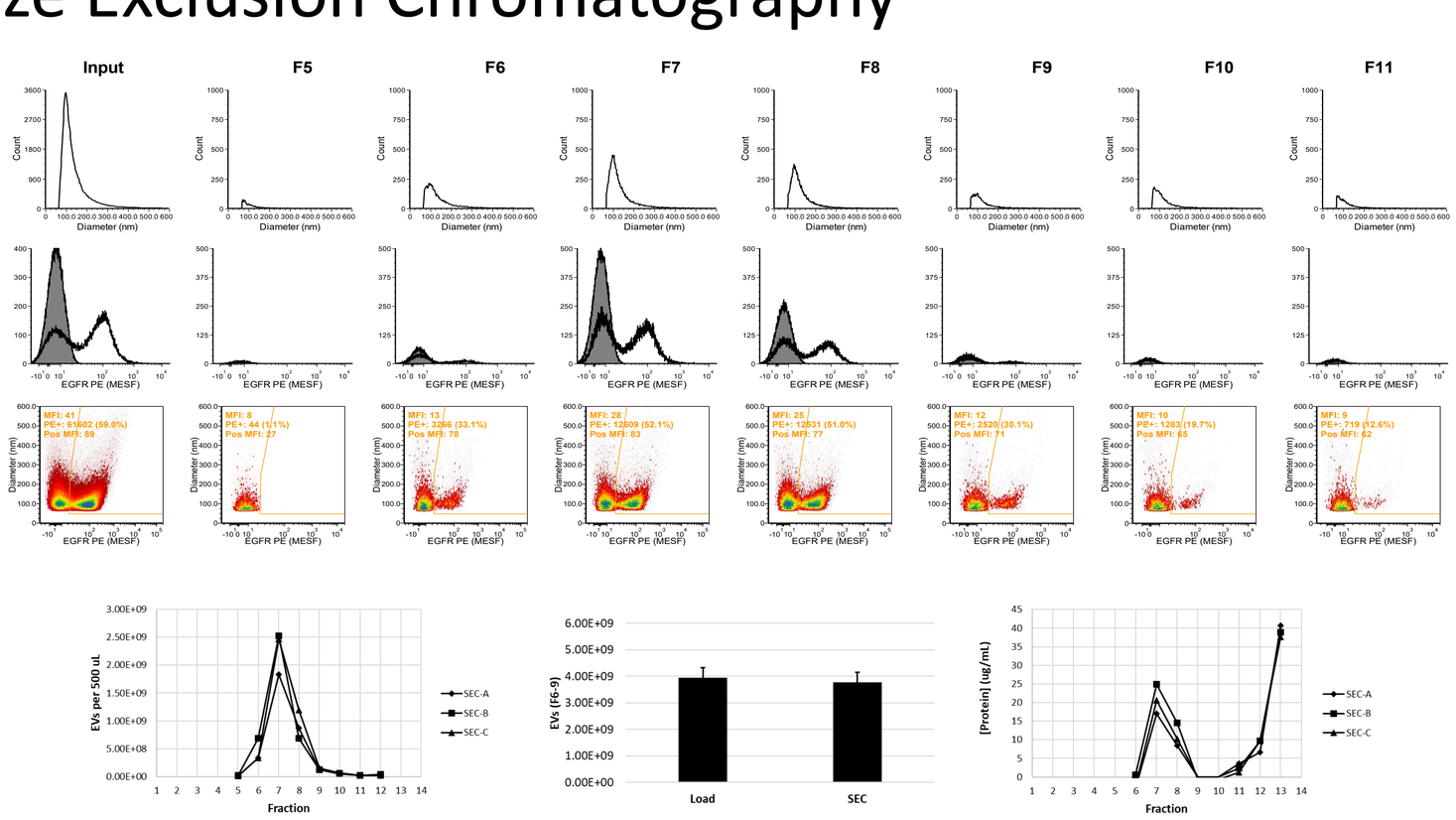

Figure 3. Characterization of EV enrichment by Size Exclusion Chromatography. Comparison of EV size, EGFR expression, and total protein concentration (BCA) pre- and post-sample processing by SEC. There is no observed bias introduced at any MWCO as reflected in EV size (top) or EGFR+ EV number or EGFR brightness (shown as density plots and histograms in rows 2 and 3). EV concentration by fraction to assess sample dilution is in the line graph on the left. EV number to assess recovery is summarized in the middle bar chart. Protein concentration (BCA) is summarized across fractions in the line chart on the right.