Extracellular Vesicles (EVs) are nanometer scale, membrane bound compartments that contain proteins, RNAs and metabolites endogenous to their cell of origin1. As such, the content of EVs, collected in biofluids, can serve as a molecular snapshot of the parent cell. A preponderance of EV research has focused on isolating EVs from specific cell types, or from tumors, utilizing transmembrane proteins that can allude to cell origin2. While this has led to some success, as in the case of monitoring prostate cancer3, studies that have sought to capture brain-derived EVs have been hampered by methodological challenges. Specifically, proteins that have been cited as transmembrane or internal to EVs have in fact been shown to be predominantly cleaved and secreted4. It is therefore critical to develop methods to differentiate EV associated proteins from those that are secreted and cleaved in biofluids.

EVs can be easily separated from membrane free proteins by size exclusion chromatography (SEC) or density gradient chromatography (DGC)4. Still, analyzing the proteomic content of the EV and membrane free protein fractions with a single biochemical technique can be challenging because the membrane free protein fractions contain several orders of magnitude more protein than the EV fractions. Unbiased techniques like mass spectrometry cannot be effectively employed in membrane free protein fractions as abundant proteins like albumin confound the detection of low abundance proteins. Similarly, these protein rich fractions cannot be analyzed by Western blots because the abundance of protein clogs the gel. ELISAs have thus far been the best method of assessing EV fractionation pattern5. In an effort to optimize total EV isolation, our lab has previously developed highly sensitive ELISA assays to quantify both canonical EV proteins (CD9, CD63, CD81, alix) and potential contaminants to EV preparations (Apo lipo B, albumin) using the Simoa platform invented in our lab4-6. Here, we sought to apply unbiased methods to assess a larger number of proteins to identify targets that can be used for immunocapture and for internal validation of brain-derived EVs.

To that end, we used a highly multiplexed proximity extension ELISA platform, OLink, which can analyze thousands of proteins from microliters of biofluid with high specificity (ref). To target EVs coming from the brain, we fractionated cerebrospinal fluid (CSF) using SEC to separate proteins that localize to the EV fractions (7-10) and membrane free fractions (>13) (See online methods). We reserved x uL of each fraction and quantified CD81 using our previously validated Simoa assay to demonstrate that EVs predominantly eluted in fractions 9 and 10 (Figure 1a)4-6. The remainder of each sample was analyzed using the OLink Explore platform which quantifies 3072 proteins (SI table 1). To further define the EV fractions, we analyzed the fractionation pattern of CD63 using data from the OLink assay and demonstrated that the highest signal was again in fractions 9 and 10 (Figure 1b).

To identify targets that could be effective for EV immunocapture or for analysis of EV cargo we selected all proteins whose median value across eight CSF samples was greater in fractions 9 and 10 compared to both fraction 7 and 12 (SI table 2). As many proteins can be found as both EV bound and membrane free isoforms, we did not choose proteins based on being greater in the early (7-10) compared to late fractions (>13), but rather selected proteins where a definable EV peak could be demonstrated. We overlayed the Uniprot database over the 3072 proteins and identified z proteins that were annotated as transmembrane7. As would be expected, the majority of these proteins had an EV peak. x/272? of these proteins were in our dataset that had an EV peak while y/2800? of these were proteins that did not have an EV peak (Figure 1c).

To be of utility for the study of healthy brain development as well as for understanding cell-type specific changes in brain diseases, we pursued proteins whose fractionation pattern was stable both in healthy brains and in brains where proteolytic cleavage of transmembrane proteins is pathopneumonic8. Therefore, our 8 CSF samples were comprised of four healthy controls, two patients with mild cognitive impairment and two patients with Alzheimer’s Disease (AD) (SI table 3). X proteins had higher levels in fractions 9 and 10 compared to fraction 7 and 12 in healthy and AD spectrum CSF, while y proteins only demonstrated a peak in the healthy brains and z proteins only demonstrated a peak in the AD brains (Figure 1d). The remainder of our analysis focused on the X proteins that showed an EV peak regardless of disease state.

We overlayed these X proteins first with the Uniprot data to identify those that were predicted to be transmembrane (a) or cytosolic (b)7. Next, we overlayed this with the BrainRNASeq atlas and selected proteins which were at least 3-fold enriched in a specific cell type; neurons, astrocytes, microglia, or oligodendrocytes (figure 2a and 2b)9. Thus, we identified transmembrane proteins which can potentially be used in CSF to isolate cell-type specific EVs using antibodies (figure 2a) as well as cytosolic proteins which can be used to confirm cell-type specificity after proteinase protection assay (figure 2b).

Utilizing a highly sensitive and specific multiplexed ELISA platform on fractionated healthy and AD spectrum CSF, we were able to identify cell type specific EV associated proteins both for potential immunocapture and internal analysis. Several important caveats are of note: First, while we were able to analyze 3072 proteins, this remains only a fraction of the ~20,000 known proteins expressed in humans10. Second, there are many proteins which are known to be both secreted and transmembrane. In some cases, the abundance of the secreted form can mask an EV peak. However, without the ability to separate the EV peak, these proteins are likely not useful for EV analysis unless they have a unique extracellular epitope not present on secreted forms2,4. Finally, while proximity extension assays lower the chance of nonspecific binding in ELISAs, the membrane free protein fractions have substantially more protein, increasing the chance for nonspecific binding to occlude an EV peak. Thus, our analysis is useful for identifying potential EV associated proteins but cannot rule out EV association for proteins that did not meet criteria or were not included in the OLink dataset. Substantial additional work is required to assess cell-type origin and EV association for those that did meet criteria displayed in figure 2. Nevertheless, this dataset is an important step towards validating brain-derived extracellular vesicles to enable a future liquid biopsy of the brain.

Figure 1

1. Graph of median CD81 in 8 CSF samples quantified using Simoa
2. Graph of median CD63 in 8 CSF samples quantified using olink
3. Bar graph of transmembrane protein % in the protein list that met criteria for an EV peak (left) and the proteins that did not (right)
4. Percentage overlapping vs unique proteins for EV peaking proteins between healthy and AD spectrum.

Figure 2

1. List of membrane associated proteins enriched >1:3 in a given brain cell type (list 4 columns and colorcode for degree of enrichment)
2. List of cytosolic proteins enriched >1:3 in a given brain cell type (list 4 columns and colorcode for degree of enrichment red to blue)

**Supplementary Information**

Table 1: Entire dataset table from OLink

Table 2: List of proteins that have an EV peak, as defined by proteins with higher values in fractions 9 and 10 compared to fractions 7 and 12

Table 3: Demographic information for samples utilized

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