Quantitative analysis of molecular cargo transfer from cells to EVs

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Abstract

Introduction. EVs carry molecular cargo from their cell of origin, but the mechanisms of cargo selection and loading into EVs are not well understood. We used quantitative single cell and vesicle flow cytometry to measure membrane protein expression on cells and EVs.

Methods. PC3 cells were cultured, media collected, and EVs concentrated using ultrafiltration (100K MWCO). Cell surface markers were measured by flow cytometry (FC). EV concentration, size, and cargo were measured by single vesicle flow cytometry (vFC). Instruments were calibrated and intensity reported in units of antibodies per cell or EV.

Results. PC3 cells express surface markers at high (>250K median copies/cell: CD71, CD29, CD44, CD54), medium (50K-250K copies: CD9, CD63, CD49f) and low (<50K copies: CD81, EPCAM, EGFR, STEAP-1) abundances. Cell permeabilization reduced staining for CD9 and CD81 (due to disruption of the plasma membrane), and increased staining for CD63 and STEAP1 (due to accessibility of internal antigen). EVs expressed detectable (>~10 PE MESF) CD9, CD63, CD81 and CD29, with a fraction (~50%) also staining with AnnV. Expression was proportional to EV surface area, with surface densities ranging from a background of ~10 molecules/um^2 to >1000 molecules/um^2 for high abundance targets. Several high abundance markers (CD71, CD44, CD54) were not detectable on EVs, suggesting differential packaging of cell surface cargo into released EVs. CD63 was expressed at low abundance overall, but a subset of smaller EVs (<100 nm) expressed CD63 at high surface density (~1000 um2).

Conclusions. We find that the abundance and surface density of cargo on vesicles can be higher or lower than on the cell of origin. Some abundant cell surface molecules (CD71, ICAM, CD44) were undetectable on EVs, while others (CD9, CD81, CD29) were present at surface density similar to cells. CD63 was present at high density on smaller EVs, consistent with enrichment of CD63 on small exosomes formed inside the cell.

Methods

Vesicles. Synthetic vesicle standard (Lipo100™) and PLT EVs were from Cellarcus Biosciences Inc (San Diego, CA). PC3 EVs were prepared by differential ultracentrifugation (10Kxg, 30' > 100kxg, 60').

Cell Flow Cytometry. Cell surface marker expression was measured using PE-conjugated antibodies and a calibrated flow cytometer (CytoFlex, Beckman Coulter). Single cells were gated on forward vs side scatter.

Vesicle Flow Cytometry. 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 Calibrated flow cytometers (CytoFlex, Beckman Coulter; CellStream, Luminex Corp.). Briefly, samples were stained with the fluorogenic membrane stain vFRed™ and fluorescent antibodies for 1h at RT and analyzed using flow cytometry with detection triggered by vFRed fluorescence. 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). Marker surface density was estimated for each EV as the surface marker abundance (MESF) divided by the surface area (um^2).

Results

Cell surface marker expression. PC3 cells express surface markers at high (>250K median copies/cell: CD71, CD29, CD44, CD54), medium (50K-250K copies: CD9, CD63, CD49f) and low (<50K copies: CD81, EPCAM, EGFR, STEAP-1) abundances. Cell permeabilization reduced staining for CD9 and CD81 (due to disruption of the plasma membrane), and increased staining for CD63 and STEAP1 (due to accessibility of internal antigen). Prior measurements suggest that PC3 cells have a diameter of ~18 um, and a surface area of ~1000 um^2, assuming a spherical geometry, allowing estimate of cell surface density

EV surface marker expression. EVs expressed detectable (>~10 PE MESF) CD9, CD63, CD81 and CD29, with a fraction (~50%) also staining with AnnV. Expression was proportional to EV surface area, with surface densities ranging from a background of ~10 molecules/um^2 to >1000 molecules/um^2 for high abundance targets. Several high abundance markers (CD71, CD44, CD54) were not detectable on EVs, suggesting differential packaging of cell surface cargo into released EVs. CD63 was expressed at low abundance overall, but a subset of smaller EVs (<100 nm) expressed CD63 at high surface density (~1000 um2).

Conclusions

EVs bear surface cargo from their cell of origin, but a predictive understanding of cargo packaging required quantitative measurements of cargo on both cells and EVs. Quantitative flow cytometry can provide these measurements on both cells and EVs.

We find that some cargos are released on EVs with surface densities comparable to their expression on cells, while others have higher (CD29) or lower (CD71) surface densities compared to cells. Such measurements provide a quantitative basis on which to understand the mechanisms of cargo packaging into EVs released by cells.

These measurements involve some assumptions and uncertainties that limit interpretation. Cell surface area estimates assume cell sphericity, which is likely too simplistic, while single EV measurements are limited by the sensitivity of the instrument with respect to vesicle size and fluorescence detection. In addition, many cell surface markers are also present on internal membrane that are also a source of EVs.

Current work is directed towards measuring the abundance of surface markers present on internal membranes in cells, which are also the source of EVs (eg exosomes) and the effects of cell culture and treatment conditions that might modulate cargo packaging.

Selected References

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EVs bear cargo from their cell of origin, but different cell surface cargo molecules are packaged into EVs in different amounts.

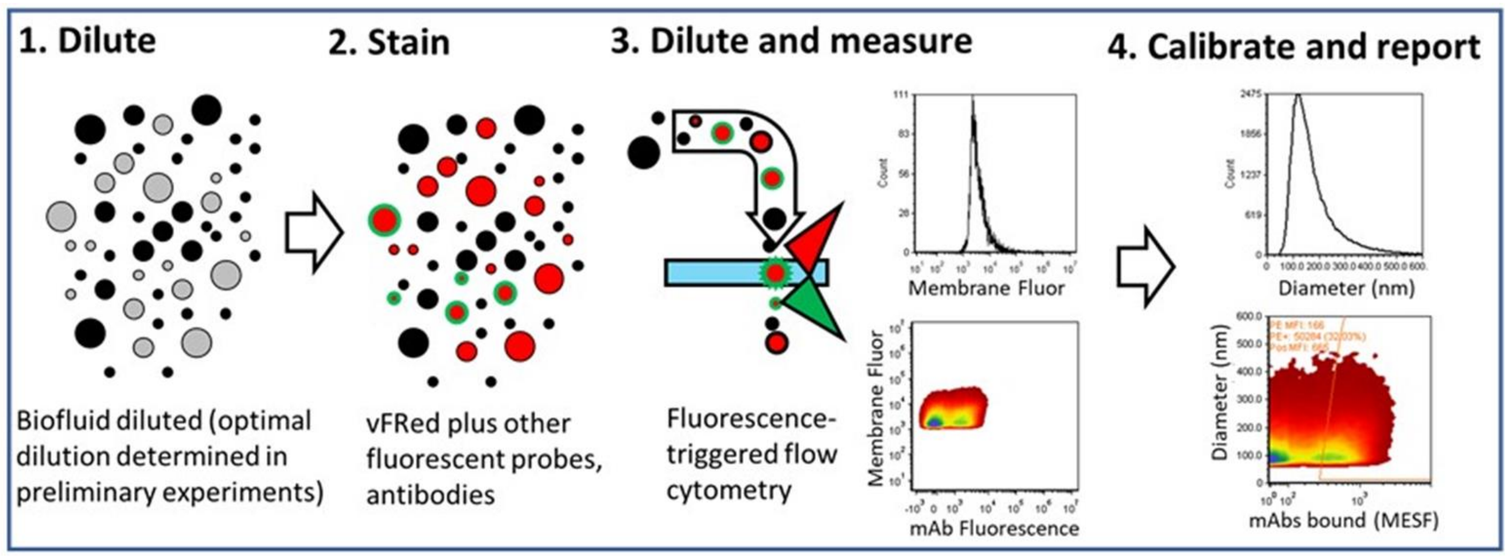

Figure 1. Schematic of VFC workflow. Vesicle flow cytometry (vFC™) is a homogeneous assay in which a cell-free sample, prepared by centrifugation, is stained with a fluorogenic membrane stain and one or more additional fluorescence probes then analyzed by flow cytometry with detection triggered by membrane fluorescence. The size distribution of a synthetic vesicle standard (Lipo100™), determined by NTA, is used to calibrate membrane fluorescence in terms of vesicle surface area, while fluorescence intensity and antibody capture standards are used to calibrate fluorescence intensity and antibody binding in units of MESF (mean equivalent soluble fluorochromes) or ABV (antibodies bound per vesicle).
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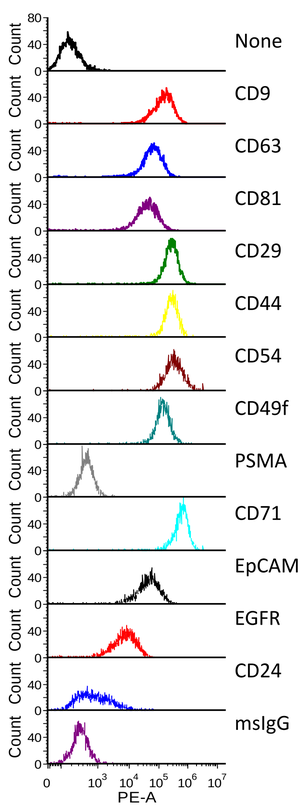
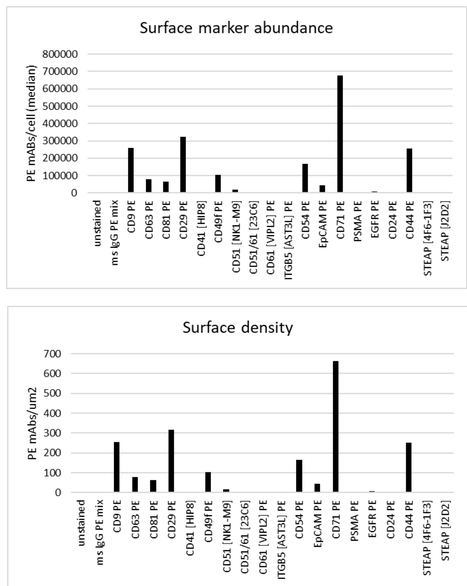
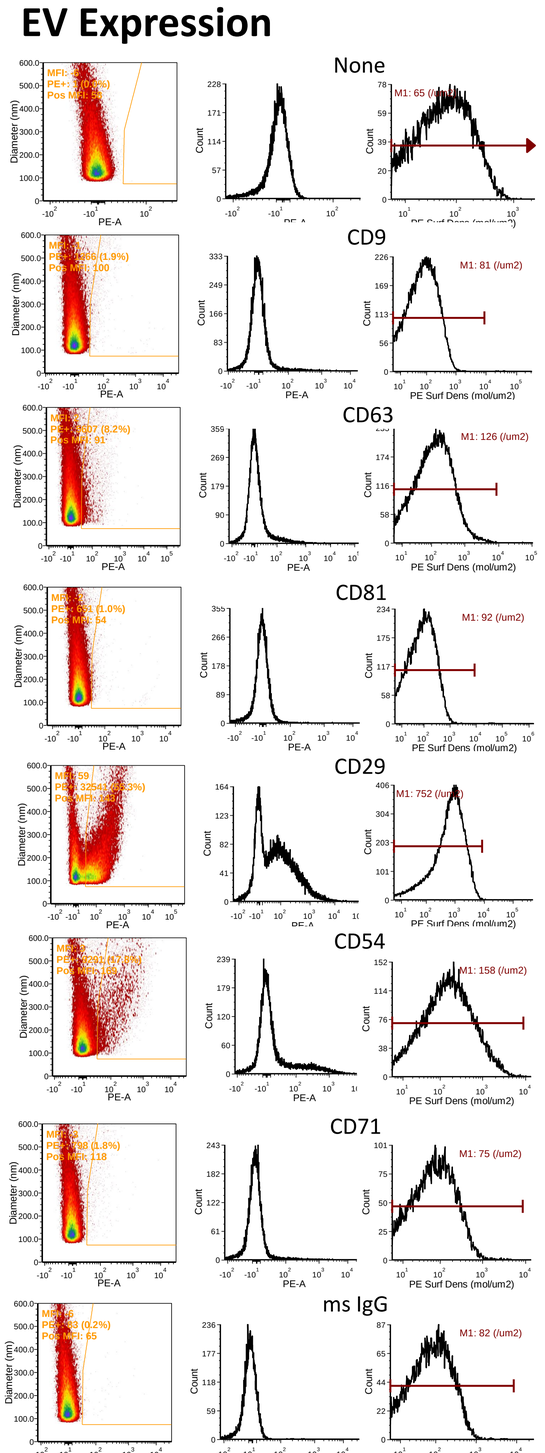
Cell Expression





EV Expression



EV Surface Density