1116
doi:10.1017/S1431927619006317
Microsc. Microanal. 25 (Suppl 2), 2019

Isolation and Characterization of Cell- and Size-Specific Extracellular Vesicles by Nanoscale High Resolution Flow Cytometry

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Cells release extracellular vesicles (EVs) into the circulation that range in size from exosomes to larger microvesicles and apoptotic fragments. EVs arise from the plasma membrane and contain cell-specific surface antigens (e.g. placental alkaline phosphatase [PLAP] in pregnant women) and various tetraspanins (e.g. CD9). Cell-specific surface antigens provide a means to isolate cell-specific EVs by affinity capture, or to test for antigen enrichment in EV preparations like ultracentrifugation and density gradient fractions. Currently, EV quantitation, sizing, and isolation are hindered by the lack of an approach that can provide *both* cell- and size- specificity.

We report an approach to reproducibly image, count, and isolate cell- and size-specific EVs from human plasma by nanoscale high resolution flow cytometry (HRFC) using multiplexed fluorophore-conjugated antibodies. We employ uniform blood collection, processing, and standardization of flow settings with commercially available polystyrene beads. In addition, all plasma/media samples are prepared using *de novo* 200nm sorted bead buffer to provide an internal size- and concentration control (e.g. 5000 beads/ul of 0.1um filtered PBS dilution buffer). Similarly, we describe an internal EV flow sorting control (100nm FITC-conjugated liposomes) that provides a means to adjust for simultaneously sorted background contamination for EV characterization studies, including CryoEM and *–omics* analyses.

With this increased specificity, sorted EVs reveal differences between groups that were not evident in plasma or total mixed EV populations. Our data suggest that our HRFC sorting can reliably and reproducibly provide cell- and size-specific EV counts in human plasma. This is possible because of tightly controlled standardization protocols. Innovations include employment of sorted 200nm bead buffer as a universal size and count standard "spiked" into the dilution buffer used to test plasma samples, and similarly, the use of sorted 100nm liposomes as a FACs control during simultaneous sorting of exosome-sized cell-specific EVs. Liposome sorting controls adjust for technical "hitchhikers", contaminating background proteins, nucleic acids, and membrane fragments that inevitably confound the precision of any EV preparation method. Certainly this is a significant improvement over the analysis of centrifugation gradient fractions or affinity capture aliquots that cannot be similarly quality controlled.

References:

[1] T Morgan, Trans Res. **201** (2018), p. 40.
[2] A Mitrugno et al., AJP-Cell Phys. **316** (2019), C264.

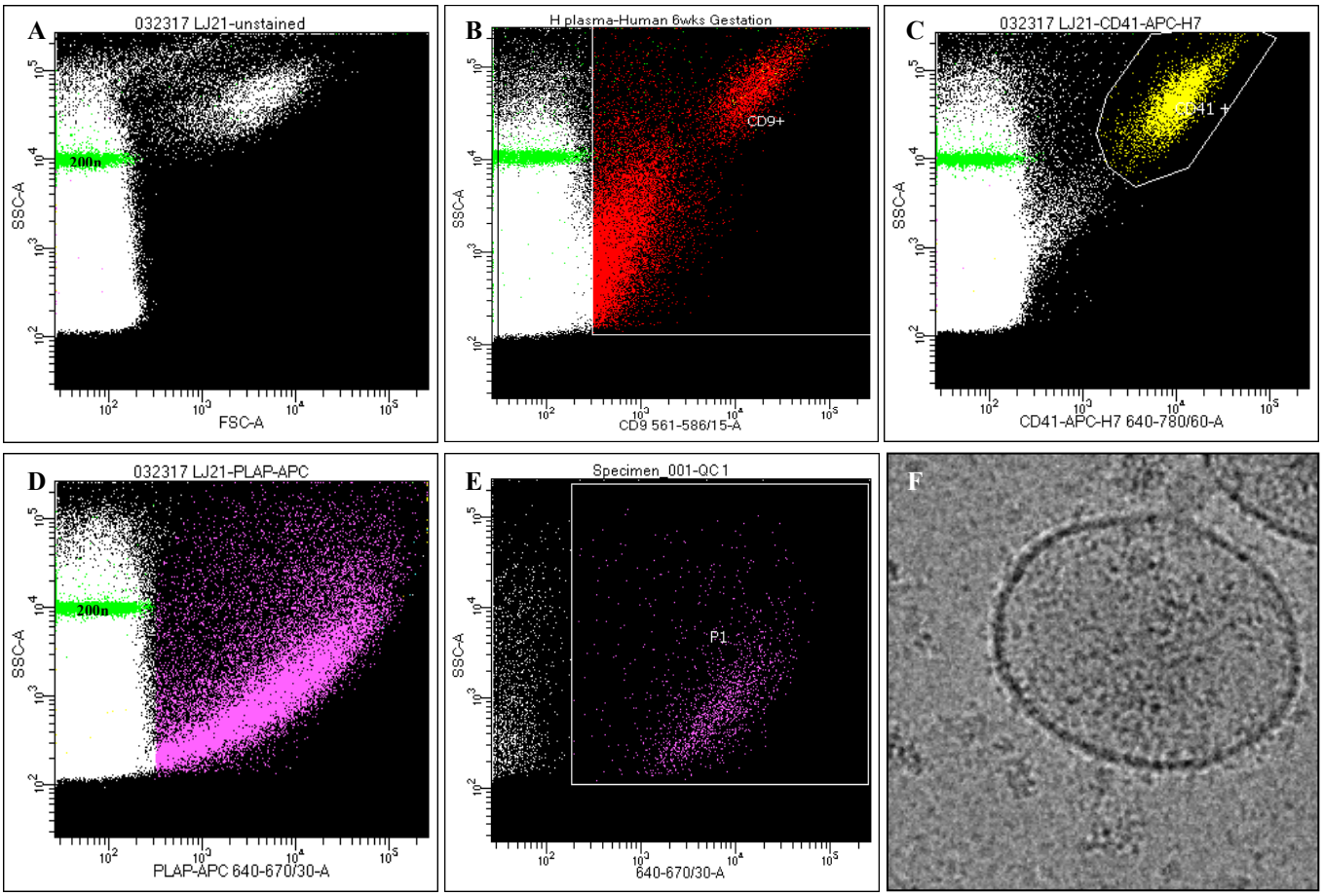

Figure 1. Human plasma multiplexed with fluorophore-conjugated monoclonal antibodies against CD9, C41, PLAP, and sorted to isolate placental exosomes for Cryo-EM validation. Unstained plasma from woman at 6 weeks gestation diluted 1:100 in sorted 200nm bead buffer showing forward light scatter (FSC) cannot reliably distinguish particles smaller than 200nm using a *BD FacsAria Fusion* flow cytometer (A). Imaging CD9 (B), CD41 (C), and placental PLAP (D) suggests the larger CD9+ population may also be positive for CD41**.** PLAP+ exosomes are smaller than the 200 nm control beads (D) and have less FSC than CD41+ platelet MVs (E). Sorted PLAP+ EVs confirmed to be exosomes by *Cryo-EM* (F), revealing a relatively uniform population (75-125nm in size).

https://doi.org/10.1017/S1431927619006317 Published online by Cambridge University Press