Separation Of Blood-Derived Extracellular Vesicles Using Droplet-Based Sorting

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INTRODUCTION

Extracellular vesicles (EVs) are cell-derived, membrane-bound, small particles measuring less than a micron in size, that demonstrate potential as valuable biomarkers and therapeutic agents in various disease environments. Due to their small size and heterogeneity, EVs can be difficult to characterize;[1] however, single vesicle flow cytometry is a proven method to detect and analyze EVs.[2,3,4] Growing interest exists for using high-speed, droplet-based sorting to isolate specific EV populations for further characterization in both functional assays and to explore their use in therapeutics. The work described herein outlines a proof-of-concept assay that demonstrates the ability to effectively enrich specific EV populations using a droplet-based cell sorter.

MATERIALS AND METHODS

Cell Sorter Setup

A Cytek Aurora™ CS system was equipped with 5 lasers (355 nm, 405 nm, 488 nm, 561 nm, 640 nm) and outfitted with the Enhanced Small Particle™ (ESP™) Detection Option to increase side scatter sensitivity by directing 20x more light to the violet scatter detector. The Cytek Aurora CS system was qualified using a set of hard-dyed beads (vCal™ nanoRainbow beads) and calibrated with antibody capture beads (vCal™ nanoCal beads) and a synthetic vesicle size standard (Lipo100™) labeled with vFRed™. Cytek ESP Detection Option settings were used as shown in **Figure 1A**. Nozzle settings for sorting EVs were: 70 µm nozzle at default settings (**Figure 1B**). The settings were optimized for this instrument and nozzle.

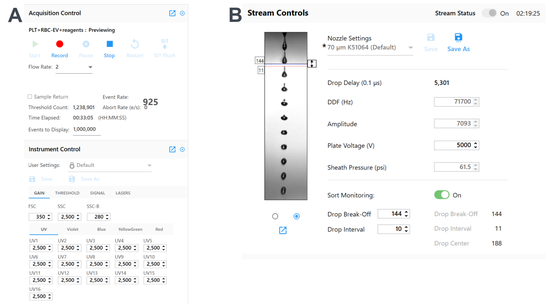

Figure 1. Cytek ESP Detection Option instrument settings (A), and 70 µm nozzle settings (B).

EV Sorting with Single Vesicle Flow Cytometry (vFC™)

The three-color vFC assay (Cellarcus Biosciences, Inc.) was performed according to the manufacturer's workflows and protocols, following MIFlowCyt-EV guidelines. Pre-stained vCal nanoCal beads (4-peak, 800 nm, calibrated antibody capture beads labeled with the APC or BV421 antibodies), and EVs (platelet-derived EVs (PLT-EV) and red blood cell-derived EVs (RBC-EV)) labeled with vFRed were used as single stained controls to perform live unmixing using SpectroFlo® CS software.

A mixture of platelet- and red blood cell-derived EVs was analyzed using the vFC assay. PLT-EVs were stained with anti-CD41 (vTag™ antibody conjugated to BV421), and RBC-EVs were stained with CD235ab (vTag antibody conjugated to APC). In addition, PLT- and RBC-EVs were labeled with the membrane dye vFRed. EV samples and assay controls were acquired for 2 minutes using a low flow rate. EV samples and assay controls are collected for time at the same settings for direct comparison. To minimize coincident detection of particles, as indicated by the frequency of events in the double positive gate (<0.5%), the event rate for sorting was limited to 2000 events per second.

Final sort gates were drawn using assay controls (buffer-only, buffer+reagents, EVs+reagents, data not shown) to isolate CD41-positive platelet-derived EVs and CD235ab-positive red blood cell-derived EVs, as shown in **Figure 2**. One million events were sorted using multiway sort mode into 5 mL polystyrene tubes containing 50 µL 0.01% bovine serum albumin (BSA) in phosphate buffered saline (PBS) collection buffer. Multiway sort mode prioritizes purity over yield of target particles.

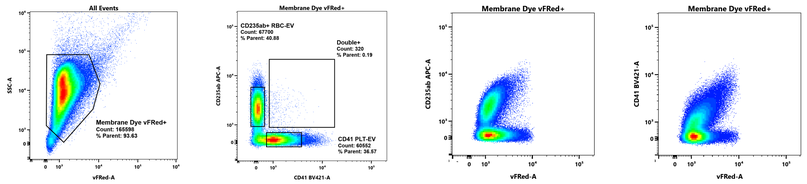

Figure 2. Pre-sort gating strategy to separate CD41+ PLT-EVs and CD235ab+ RBC-EVs.

Post-Sort Analysis

Sorted samples were acquired for 2 minutes on a Cytek Aurora™ cell analyzer fitted with 5 lasers (355 nm, 405 nm, 488 nm, 561 nm, 640 nm) and ESP Detection Option. Gates were drawn using assay controls (buffer-only, buffer+reagents, EVs+reagents, data not shown). These data provide initial sorted-product verification, such as sort purity and size range reporting.

The data were calibrated for size (nm) using Lipo100 vesicle size standards stained with vFRed. Fluorescent units (antibody bound per vesicle, ABV) were calibrated with nanoCal beads (Cellarcus Biosciences) using FCS Express™ software (Dotmatics).

RESULTS

The two populations were sorted using the multiway sort mode at an average sort rate of ~450 events per second. The results of three independent experiments were similar, with an average sort efficiency of 97% (**Figure 3**) and an average purity of 97% (**Figure 4**). The average time to sort one million EVs was 30 minutes.

Figure 3. Post-sort report from Cytek Aurora CS system with ESP Detection Option, indicating average sort rates and average efficiency.

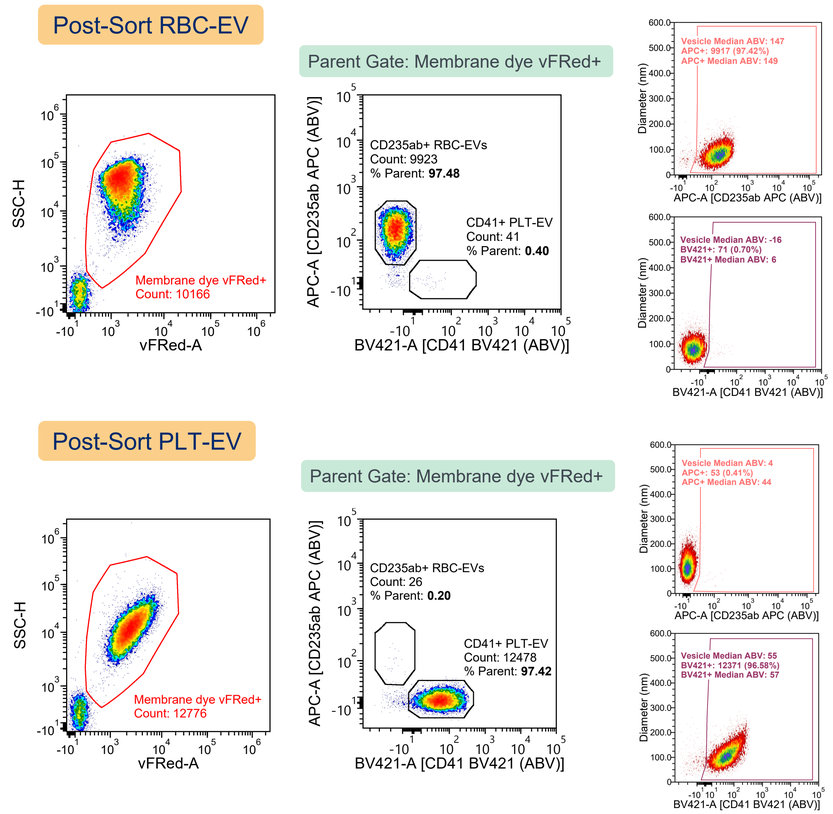

Figure 4. Sorted product verification on Cytek Aurora cell analyzer with ESP Detection Option, indicating size range and sorted-product purity (data in calibrated units).

CONCLUSION

Here, we describe a feasibility study that demonstrates the enrichment of two EV populations using fluorescent cell-of-origin surface markers and a high-sensitivity cell sorter. The droplet-based sorting of EVs may help address the challenges of EV analysis and opens novel research avenues for advancements in therapy and diagnostic development. This endeavor provides a starting point for the technique; future work will involve optimizing droplet settings to reduce sorted volumes and verifying the results with orthogonal and downstream applications.

REFERENCES

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