Flow cytometry

Exosome detection and sorting using 405 nm small particle forward scatter on the Bigfoot Spectral Cell Sorter

Introduction

Gaining significant attention in cancer research, extracellular vesicles (ECVs), including exosomes, microvesicles, autophagic ECVs, and matrix vesicles, are potential vehicles for delivering therapeutic agents in clinical trials. To help ensure accurate sorting of these small particles, adhering to specific guidelines is crucial. Here we focus on a protocol to sort and characterize particles that encompass a range of ECVs. The ability to isolate subpopulations of ECVs by sorting provides additional benefits compared to magnetic bead isolation. Along with markers to identify the origin of and characterize these vesicles, tetraspanins CD9, CD63, and CD81 are emerging as significant markers to allow the understanding of the properties and functions of ECVs. Tetraspanins are integral components of the vesicle membrane that play key roles in exosome biogenesis, cargo sorting, and interaction with target cells. The presence or absence of tetraspanins can provide valuable insight into the origin, composition, and functional characteristics of ECVs to help advance basic research and clinical applications [1].

Materials and methods

Instrument setup

To accurately observe and sort ECVs, a cell sorter must be clean, free from debris and noise, and display a clear threshold plot when the sheath alone is running. Full instrument decontamination is recommended prior to conducting ECV experiments to eliminate potential contaminants that could interfere with the analysis. Use a 100 µm or 70 µm nozzle tip for optimal detection and sorting of ECVs. The instrument quality control (QC) procedure, by which the appropriate mean fluorescence intensity (MFI) voltages are selected, helps ensure accurate and reliable measurements and is imperative to complete prior to experimentation. The 405 nm small particle detector (SPD) and side scatter (SSC) detectors can help reduce interference from drop drive noise often seen in the forward scatter (FSC) detector, especially when using the 70 µm nozzle. Utilizing the 70 µm nozzle allows for higher event rates and a smaller sort drop volume. As an optional step, depending on the specific experimental requirements, researchers can modify the optical filters to allow dim events to reach the photomultiplier tube (PMT) detectors by inserting an empty filter holder. This can increase the light captured by the PMT, thus enhancing the sensitivity and detection capabilities of the instrument. By following these guidelines, researchers can enable the proper function and reliability of the instrument for ECV studies.

Sample preparation

To enhance fluorescence signal to noise when staining ECVs, use the optimal titration of the markers based on the results of staining index (SI) calculations. The SI determines the optimal concentration of the staining reagents for accurate and reliable testing [2]. In this study, incubating ECVs with the staining reagents on ice for one hour, protected from light, minimized potential degradation or photobleaching [3]. Markers used for ECV staining include Invitrogen™ CD9 Monoclonal Antibody (MEM-61), FITC (Cat. No. MA1-19557); CD63 Monoclonal Antibody (H5C6), PE, eBioscience™ (Cat. No. 12-0639-42); and CD81 Monoclonal Antibody (1D6-CD81), APC, eBioscience™ (Cat. No. 17-0819-42). Following the manufacturing standards on staining is imperative.

Exosome swarming is a phenomenon in which ECVs, particularly exosomes, aggregate or cluster together, forming larger structures. High concentrations of exosomes in a sample or specific conditions that promote exosome interaction can cause aggregation. Exosome swarming complicates the analysis and characterization of individual exosomes due to inaccurate quantification and assessment of their properties. To prevent exosome swarming and minimize aggregation, the sample was diluted 5 times with filtered phosphate buffered saline (PBS) plus 1% exosome-depleted fetal bovine serum (FBS), to achieve an event rate of 2,000 events per second (eps). Performing a dilution curve can optimize the prevention of exosome swarming. Diluting enables the adequate dispersion of the ECVs for better analysis and characterization of individual vesicles as it reduces coincident events, providing more accurate insights into the composition, function, and role in intercellular communication of ECVs [3].

Results

Sort setup

Single-color antibody capture bead controls produce a brighter signal than is possible to detect with ECV controls. Therefore, for this ECV protocol, Invitrogen™ UltraComp eBeads™ Compensation Beads (Cat. No. 01-2222-4) were used as antibody capture bead controls to help achieve accurate spectral unmixing and subsequent construction of matrices. Employing other essential controls after spectral unmixing is complete helps ensure the reliability and accuracy of the experiment. Essential controls include single-color, isotype, and fluorescence minus one (FMO) controls, among others, depending on the specific experimental requirements (Table 1). The sample buffer control is imperative to distinguish the nominal buffer signal from the ECVs in the sample. Figure 1 demonstrates the optimization of ECV analysis using the 2D threshold plot to precisely set the threshold and establish the negative gate.

Table 1. Optimizing flow cytometry analysis of ECVs: a step-by-step protocol for setting controls and thresholds.

Control	Objective
1. Sizing beads	Establish photomultiplier tube (PMT) voltages and threshold settings to set the 405 nm SPD and SSC regions
2. On-board sheath	Modify threshold if needed and adjust the 405 nm SPD and SSC regions
3. Sample buffer	Adjust the 405 nm SPD and SSC regions if needed
4. Unstained ECVs	Set the positive gates using 405 nm SPD detector and fluorescence detectors as defined
5. Free antibody controls	Adjust the positive gates
6. Single-color ECV controls	Adjust the positive gates
7. FMOs (optional)	Adjust the positive gates
8. All stain with detergent (e.g., 1% Triton™ X-100 detergent)	Confirm the detergent degrades the small particles, otherwise the result could mean that the particles are something other than ECVs

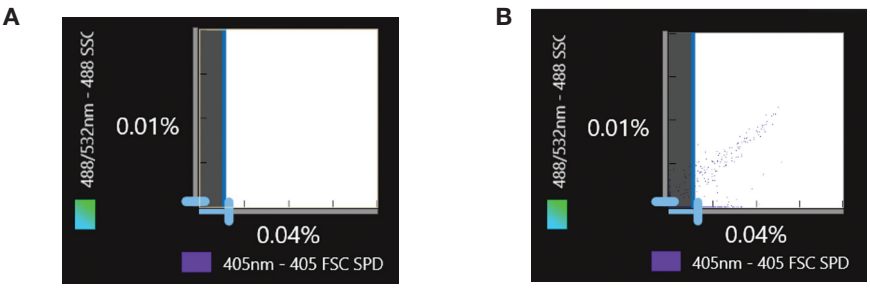

Figure 1. Optimizing flow cytometry analysis of ECVs utilizing the 2D threshold plot for precise threshold setting and negative gate establishment. (A) Illustration of paused sample demonstrates the absence of drop drive noise interference with the threshold. **(B)** Example of the threshold plot while running the stained ECV sample.

Sorting methods

Adjust the 2D trigger with 405 nm FSC, SPD, and SSC parameters to set the threshold above the background signal as shown in Figure 1 to reference the 2D trigger above. After running all controls and adjusting gates appropriately, record the FCS files (Figure 2). Optimize resolution by modifying the axis options to visualize the data, including the scale settings. Sort the desired samples into appropriate media and collection vessels to help ensure accurate and efficient collection of the sorted particles.

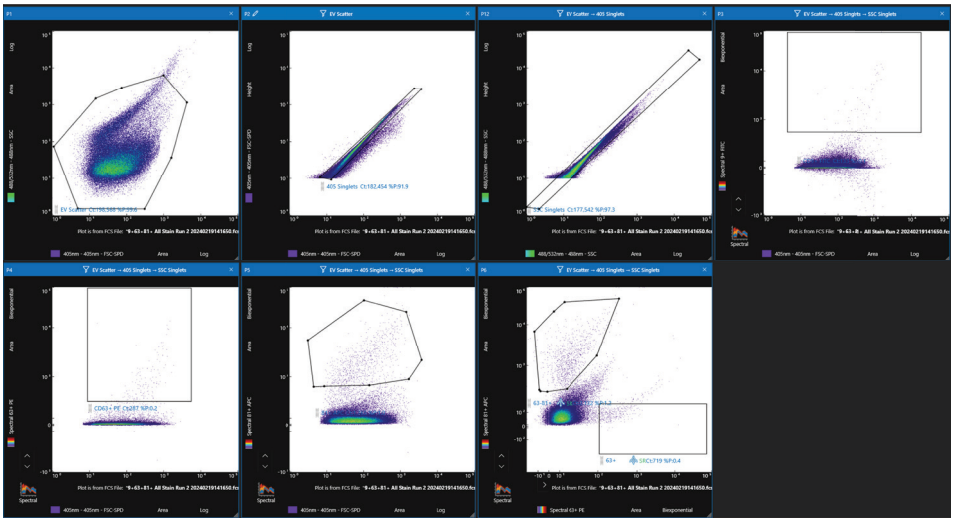

Figure 2. Optimizing sorting of extracellular particles utilizing 2D threshold triggering from Figure 1B. To set up the sort, adjust the threshold to the 2D option as shown in Figure 1 with 405 nm FSC on the x-axis and SSC on the y-axis. Then, set the threshold above the background signal.

Analysis

In this study, controls, all stained samples, and sorted samples were analyzed using both the Invitrogen™ Attune™ CytPix™ Flow Cytometer and the Invitrogen™ Bigfoot™ Spectral Cell Sorter. Various controls, as well as post-sort samples, were analyzed. Purity was not considered for analysis due to the overlap of the sheath background signal and the small particles. The Bigfoot Spectral Cell Sorter (Figure 3) and Attune CytPix Flow Cytometer (Figure 4) both showed similar post-sort results with the particles falling in the appropriate fluorescence gates as expected.

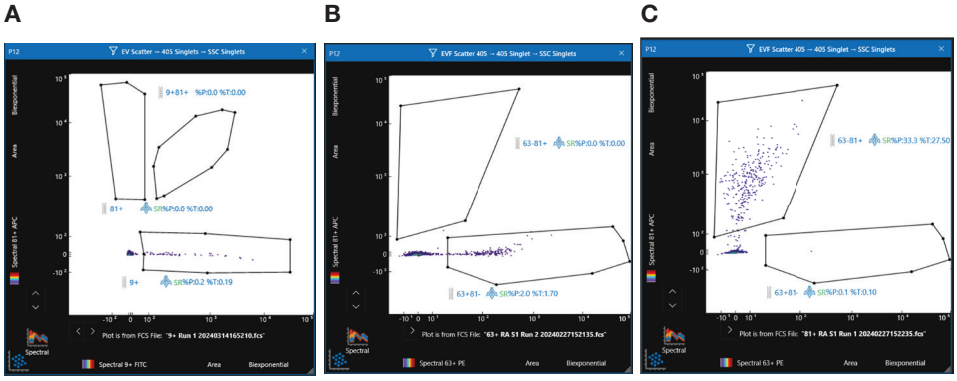

Figure 3. Reanalysis of (A) CD9, (B) CD63, and (C) CD81 expression on the Bigfoot Spectral Cell Sorter.

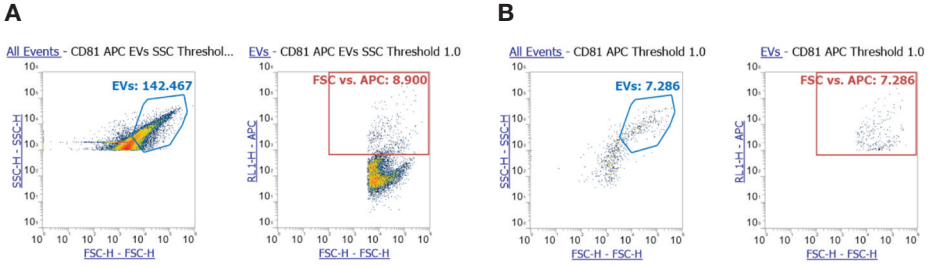

Figure 4. Attune CytPix Flow Cytometer reanalysis with the on-plot statistics representing concentration per µL. (A) Unsorted sample from the Bigfoot Spectral Cell Sorter (CD81 APC) reanalyzed on the Attune CytPix Flow Cytometer. **(B) Sorted** sample from the Bigfoot Spectral Cell Sorter (CD81 APC) reanalyzed on the Attune CytPix Flow Cytometer.

TEM imaging

Transmission electron microscopy (TEM) imaging is critical in delivering high-resolution imaging of exosomes that contain valuable information about size, morphology, and surface characteristics. Validation by TEM confirmed that the sorted sample contained ECVs with membranes as opposed to aggregation of antibodies or debris (Figure 5). By visualizing exosomes using TEM, researchers can gain insights into the structural features of ECVs, such as the presence of membrane-bound vesicles and the arrangement of surface proteins, for a deeper understanding of the biological functions and potential applications of exosomes. Overall, TEM imaging plays a critical role in advancing our understanding of exosomes and their roles in various biological processes and diseases [4].

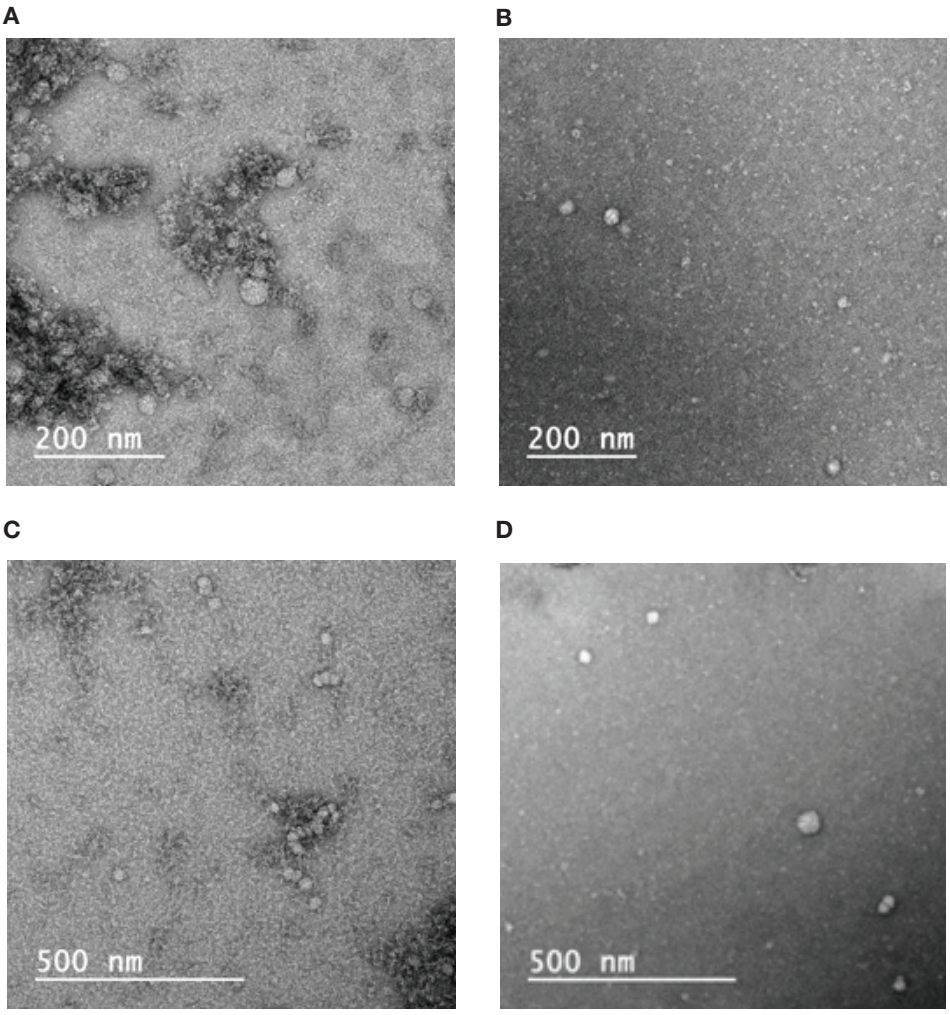

Figure 5. TEM images. (A) Unsorted sample. **(B)** Sorted extracellular vesicles from the Bigfoot Spectral Cell Sorter. Samples from **(A)** and **(B)** were imaged on a transmission electron microscope on a 200 nm scale. **(C)** Unsorted sample. **(D)** Sorted extracellular vesicles from the Bigfoot Spectral Cell Sorter. Samples from **(C)** and **(D)** were imaged on a transmission electron microscope on a 500 nm scale.

Conclusions

This application note highlights several important considerations and steps in the analysis of ECVs using flow cytometry. The use of appropriate controls, including size beads, unstained ECVs, and free antibody controls, helps ensure accurate gating and proper adjustment of thresholds for reliable analysis. Utilizing the Bigfoot Spectral Cell Sorter with specific parameters and triggers tailored for small particle sorting allows for the isolation of subpopulations as opposed to other methods like magnetic bead isolation. Evaluation and consideration of alternative imaging methods were utilized to verify the results. Overall, this paper emphasizes the importance of thorough experimental design, proper controls, and careful analysis techniques to help ensure accurate and reliable characterization of ECVs.

References

1. Jankovičová J, Sečová P, Michalková K, et al. (2020) More than markers of extracellular vesicles in reproduction. *Int J Mol Sci* 21(20):7568. doi.org/10.3390/ijms21207568
2. Kalser S (2023) Stain index for flow cytometry—explained. FluoroFinder. fluorofinder.com/stain-index-flow-cytometry/
3. Buntsma NC, Shahsavari M, Gąsecka A, et al. (2023) Preventing swarm detection in extracellular vesicle flow cytometry: a clinically applicable procedure. *Res Pract Thromb Haemost* 7(4):100171. doi.org/10.1016/j.rpth.2023.100171
4. Gonzalez-Villasana V, Rashed M, Gonzalez-Cantú Y, et al. (2019) Presence of circulating miR-145, miR-155, and miR-382 in exosomes isolated from serum of breast cancer patients and healthy donors. *Disease Markers*. doi.org/10.1155/2019/6852917