Flow Cytometry and Imaging Technologies

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

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Introduction

This study evaluated the accuracy of the Invitrogen™ Bigfoot™ Spectral Cell Sorter in detecting and sorting exosomes, which are crucial in current cancer research. The identification and sorting of exosomes are essential for downstream applications, particularly in understanding tumor microenvironments. Extracellular vesicles (ECVs), including exosomes, microvesicles, autophagic exosomes (ECVs), and matrix vesicles, have gained significant attention in cancer research and are being explored in clinical trials as potential vehicles for delivering therapeutic agents. Therefore, it is important to adhere to specific guidelines to ensure accurate sorting of these small particles. In our study, we sorted and characterized microparticles that were stained with CD9, CD63, and CD81, which have emerged as important tetraspanin markers. Tetraspanins are integral components of the vesicle membrane and play key roles in exosome biogenesis, cargo sorting, and interaction with target cells (1). CD9, CD63, and CD81 are widely utilized as markers in research studies to identify and understand the properties and functions of extracellular vesicles. The presence or absence of tetraspanin markers can provide valuable insight into the origin, composition, and functional characteristics of ECVs, which will likely contribute to the advancement of basic research and clinical applications.

Materials and Methods

1. Marker Titrations: Titrate the markers used for staining the ECVs based on the results of staining indexes calculations. This will help determine the optimal concentration of staining reagents for accurate and reliable staining. Common markers for ECV staining are CD9 FITC, CD63 PE, and CD81 APC.
2. Staining ECVs: Perform the staining procedure on ice for one hour to maintain the integrity of the ECVs. Protect the samples from light to minimize potential degradation and photobleaching.
3. Dilute the Sample: Add media with serum, such as PBS+1% FBS, (filtered through a 0.22 µm filter) to the sample at least five times to dilute the sample and prevent exosome swarming. This step ensures that the ECVs are adequately dispersed to prevent coincident events for better analysis.
4. Maintain Quality Control: Ensure that all steps are performed with strict adherence to quality control measures to obtain accurate and reproducible results.
5. Sort Setup: Set up the Bigfoot Spectral Cell Sorter with the 70 µm or 100 µm nozzle tip. The 70 µm tip allows the user to run at a higher frequency, which results in a more concentrated sorted sample.

Table 1. Sort settings for the 70 µm and 100 µm nozzle tip sizes

Sort setting	70 µm nozzle tip	100 µm nozzle tip
Frequency (Hz)	85000 Hz – 91500 Hz	34500 Hz – 38000 Hz
Amplitude (V)	9 V – 20 V	20 V
Events per second (EPS)	700 EPS – 2500 EPS	500 EPS – 1300 EPS
Sort Rate (sorts per second, SPS)	1 SPS – 38 SPS	1 SPS – 20 SPS
Efficiency (Collected cell targets divided by available cell targets)	94.5% – 99.4%	94.7% – 96%

Results

Table 2. Controls were used to accurately determine gating scheme outlined in the table below.

Sample	Objective
Sizing beads	Establish PMT voltages, threshold, and set the scatter region
On-board sheath	Threshold modification if needed and adjust scatter region
Sample buffer	Adjust scatter region if needed
Unstained ECVs	Set positive gates using 405 nm small particle detector (SPD) and fluorescent detectors as defined
Free-antibody controls	Adjust the positive gates
Single-color extracellular vesicle controls	Adjust the positive gates
Fluorescence minus one (FMO) controls (optional)	Adjust the positive gates
All stained ECVs with detergent (i.e. 1% Triton-X-100)	Confirm the detergent degrades the small particles

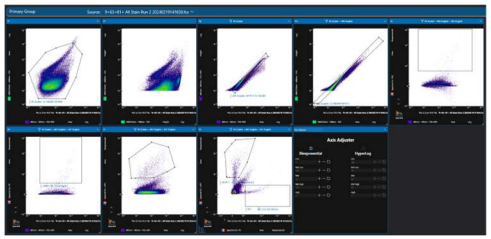

Figure 1. Sort setup with the lower-right gate showing the two sort populations (CD63+ and CD81+)

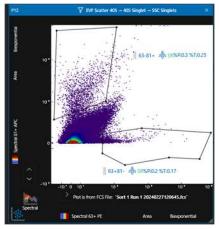

Figure 2. Sort gating shows the two sort populations (CD63+ and CD81+)

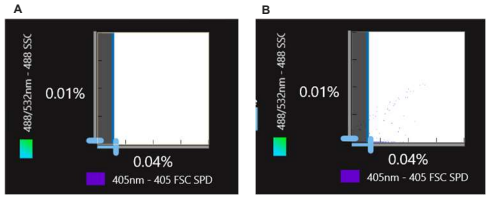

Figure 3. Dual sort trigger plots are shown. Using the 405 nm small particle detector (SPD) and side scatter (SSC) allows for optimal resolution and the ability to use the 70 µm or 100 µm nozzle tip. (A) Sample is paused to show lack of background signal. (B) Sample is running.

Validation

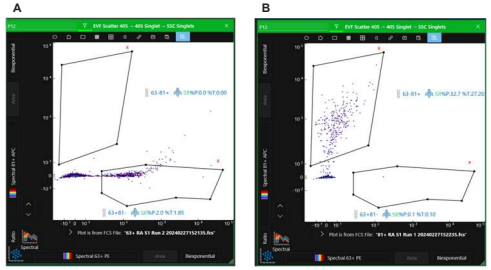

Figure 4. Sorted Samples. (A) CD63+ PE sorted sample was reanalyzed on the Bigfoot Spectral Cell Sorter, (B) CD81+ APC sorted sample was reanalyzed on the Bigfoot Spectral Cell Sorter.

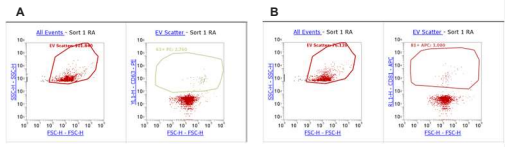

Figure 5. Attune CytPix Plots. (A) CD63+PE sorted sample is reanalyzed on the Attune CytPix Flow Cytometer showing positive signal for the particles. (B) CD81+ APC sorted sample is reanalyzed on the Attune CytPix Flow Cytometer showing positive signal for the particles.

Transmission Electron Microscopy

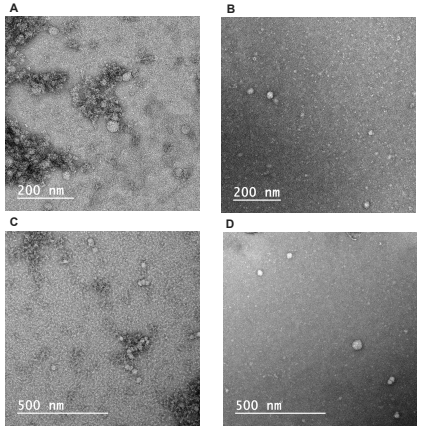

Figure 6. TEM Images. (A) Unsorted ECVs (B) Sorted ECVs. (C) Unsorted ECVs. (D) Sorted ECVs

Conclusions

- Bigfoot has features highly suited for isolation of ECVs, many controls enable proper gating, and multi-instrument validation confirms results.
- Employing controls as outlined in Table 2 is essential for appropriate gating.
- The Bigfoot Spectral Cell Sorter includes many features such as 405 nm FSC SPD, interchangeable filters, and a visual threshold that support ECV detection and sorting.

Acknowledgements

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