Flow Cytometry–Based Exosome Detection and Analysis Using the ZE5 Cell Analyzer

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Flow Cytometry

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Abstract

Analysis of small particles such as exosomes can be extremely challenging and frustrating. Electronic noise, voltage adjustment, sheath purification, and population separation are all aspects of exosome detection by flow cytometry that pose significant challenges. However, utilizing the exceptional range of the ZE5 Cell Analyzer alleviates these challenges. Here, we compare flow cytometry–based exosome detection methods using the ZE5 and demonstrate that exosomes can be directly detected without being attached to beads. We also highlight further advantages of the ZE5 that lead to experimental reliance.

Introduction

Exosomes are a type of secreted membrane vesicle of approximately 50–100 nm in diameter. They are mainly involved in intracellular communication but also have key roles in several processes such as metastasis and antigen presentation. Since exosomes are secreted in all major bodily fluids, including blood and urine, and carry important cellular information, they have recently emerged as a novel source of potential biomarkers. However, because of their small size in comparison to cells, they can be difficult to characterize and study.

Traditional exosome analysis by flow cytometry requires manual hardware adjustments, advanced instrument calibration, hours of sheath purification, and data manipulation. The ZE5 Cell Analyzer eliminates these hurdles of current exosome research by creating a streamlined process for easier analysis (Figure 1). By using the built-in capabilities of the ZE5, any lab can study exosomes and other small particles. Regardless of the staining method, whether for surface or internal markers, the ZE5 displays optimal sensitivity for small particle identification.

Here, we show that surface exosome marker proteins such as CD63 and CD81, as well as intravesicular markers ALIX and TSG101 can easily be detected using the ZE5 Cell Analyzer. The ZE5 has many unique features that, in addition to studying large cancer cells, allow it to be used in small particle analysis.

Methods and Results

Sample Preparation

MCF-7 cells were cultured in minimum essential media (MEM) supplemented with 10% FBS and 0.01 mg/ml insulin until they were 70–80% confluent. The cells were washed twice with PBS and incubated with exosome-free media for 12–72 hr. The media was collected and filtered through a 0.22 µm membrane to remove cells and debris. The filtered media was mixed thoroughly with 0.5 volumes of Total Exosome Isolation Reagent (Life Technologies). The mixture was incubated at 4°C overnight followed by centrifugation at 10,000 x g for 1 hr at 4°C. The supernatant was aspirated, and the pellet was washed with PBS filtered through a 0.1 µm membrane. The protein concentration and size of the exosomes were determined by the NanoDrop Spectrophotometer (Thermo Scientific Inc.) and Zetasizer Nano ZSP Dedicated Zeta Analyzer (Malvern Pananalytical) as 185–300 nm.

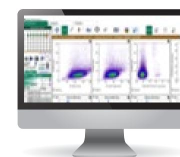

Fig. 1. Exosome detection workflow with the ZE5 Cell Analyzer

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Instrument Setup

ProFlow Sort Grade Water (Bio-Rad Laboratories) was used to analyze samples on the ZE5 Cell Analyzer (Bio-Rad Laboratories). QC was performed using ZE Series QC Beads (Bio-Rad Laboratories). A mixture of yellow fluorescent size beads ranging from 0.22–1.35 µm (Spherotech) was used to set up instrument voltages and thresholds (Figure 2). For small particle setup, single or dual thresholds can be used to set up the instrument. For direct exosome detection of surface markers, forward side scatter (FSC) from the 405 nm laser and fluorescent signal were used as thresholds. FSC from the 405 nm laser was used to set up the threshold for intravesicular marker and bead-based exosome detection.

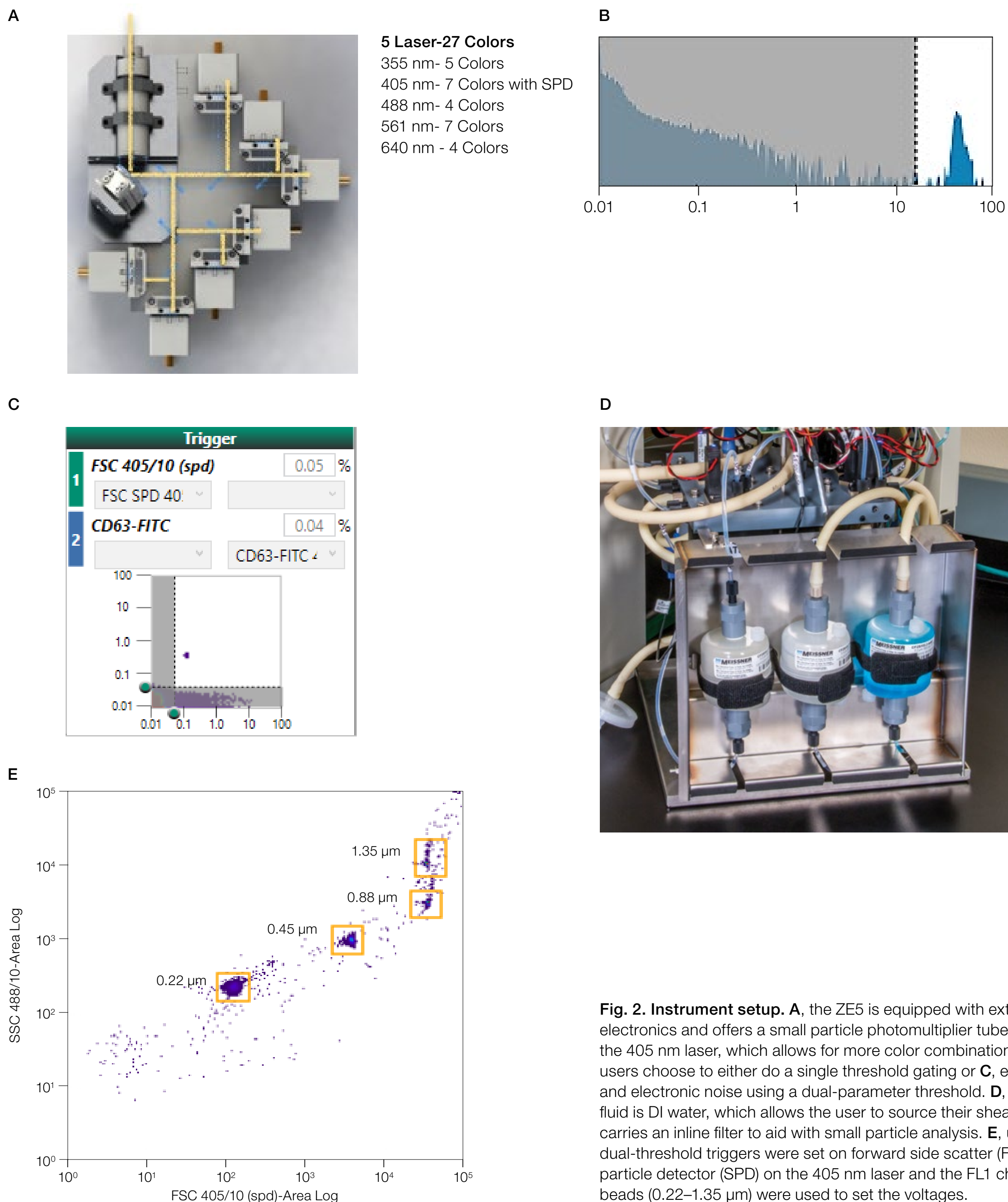

Fig. 2. Instrument setup. A, the ZE5 is equipped with extremely low-noise electronics and offers a small particle photomultiplier tube (PMT) detector using the 405 nm laser, which allows for more color combinations. **B**, the ZE5 lets users choose to either do a single threshold gating or **C**, exclude more debris and electronic noise using a dual-parameter threshold. **D**, the ZE5 sheath fluid is DI water, which allows the user to source their sheath. The system also carries an inline filter to aid with small particle analysis. **E**, using 0.22 µm beads, dual-threshold triggers were set on forward side scatter (FSC) from the small particle detector (SPD) on the 405 nm laser and the FL1 channel (C). Yellow size beads (0.22–1.35 µm) were used to set the voltages.

Non-Bead–Based Surface Staining of Exosomes

All buffers used for exosome detection were filtered through a 0.1 µm membrane. The titrated amount of fluorochrome-labeled antibody was used for surface and intravesicular staining. For exosome surface staining, 2 µg of exosomes were added to 50 µl of PEB buffer (PBS, 5 mM EDTA, and 0.5% BSA) incubated with FITC anti-human CD63 (Bio-Rad Laboratories) and APC anti-human CD81 (BioLegend) for 30 min at room temperature. After incubation, the samples were washed with PEB and re-suspended in 500 µl of PEB. Exosome samples were filtered through a 10 µm filter prior to loading on the instrument. As shown in Figure 3, exosome surface markers were easily detected compared to an unstained control.

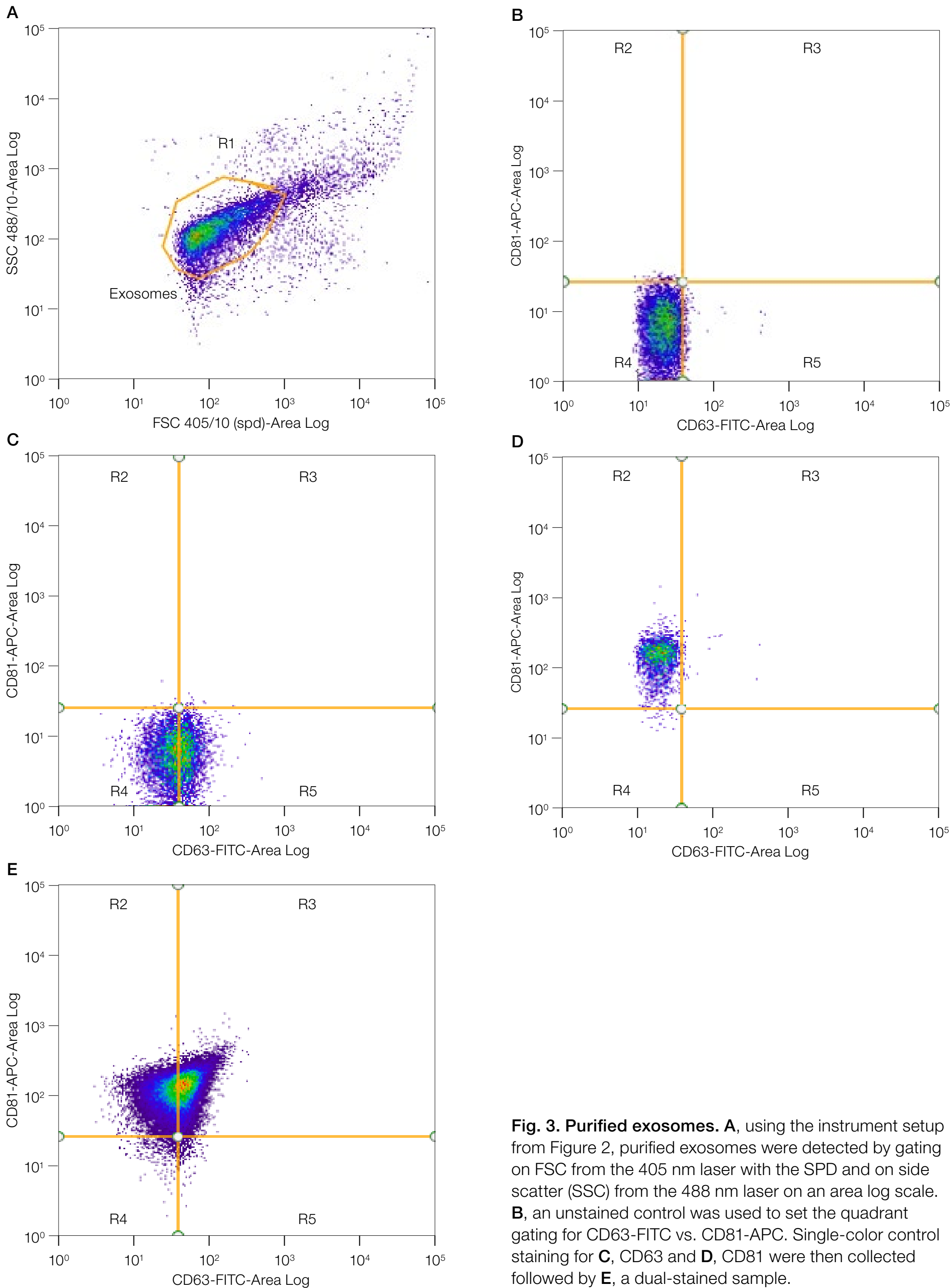

Fig. 3. Purified exosomes. A, using the instrument setup from Figure 2, purified exosomes were detected by gating on FSC from the 405 nm laser with the SPD and on side scatter (SSC) from the 488 nm laser on an area log scale. **B**, an unstained control was used to set the quadrant gating for CD63-FITC vs. CD81-APC. Single-color control staining for **C**, CD63 and **D**, CD81 were then collected followed by **E**, a dual-stained sample.

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Intravesicular Staining

The Intracellular Fixation and Permeabilization Buffer Set (eBioscience) was used to fix and permeabilize exosomes for intravesicular staining. Exosomes (2 µg) were fixed in 100 µl of IC Fixation Buffer and incubated in the dark at room temperature for 20 min. After incubation, the samples were washed twice with 1 ml of permeabilization buffer and then resuspended in 100 µl of the same buffer. The samples were then incubated with PE anti-human TSG101 (Abcam) and anti-human ALIX conjugated to 494/516 excitation/emission labeling dye, using the ReadiLink Antibody Labeling Kit (Bio-Rad Laboratories), for 30 min at room temperature. After incubation, the samples were washed twice with 1 ml of permeabilization buffer and resuspended in 500 µl of PEB buffer (Figure 4).

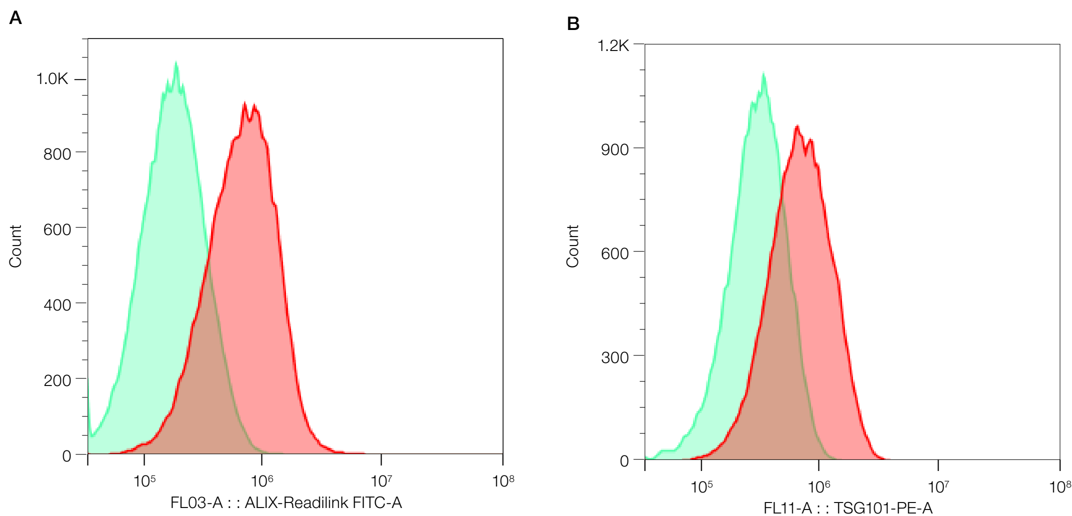

Fig. 4. Exosome intravesicular detection. The marker proteins ALIX and TSG101 are used to define exosome populations and are detectable by flow cytometry. Overlay histograms for **A**, ALIX and **B**, TSG101 are shown in red and the unstained control in green.

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Traditional Bead-Based Exosome Detection

Anti-human CD63 antibody (10 µg, Bio-Rad Laboratories) was incubated with 100 µl of SureBeads Protein G Magnetic Beads (Bio-Rad Laboratories) with rotation for 10 min at room temperature. Coated SureBeads were then incubated with 10 µg of exosomes for 1 hr at room temperature. Exosome-coated SureBeads were washed three times with PBS containing 0.1% Tween 20, and resuspended in 250 µl of PEB-0.1% Tween 20 (PBS, 5 mM EDTA, 0.5% BSA, and 0.1% Tween 20). Exosome-coated SureBeads (50 µl) were incubated with FITC anti-human CD63 (Bio-Rad Laboratories) and APC anti-human CD81 (BioLegend) for 30 min at room temperature and washed twice with PEB-0.1% Tween 20. Figure 5 shows successful characterization of exosome surface markers using bead-based flow cytometry on the ZE5 Cell Analyzer.

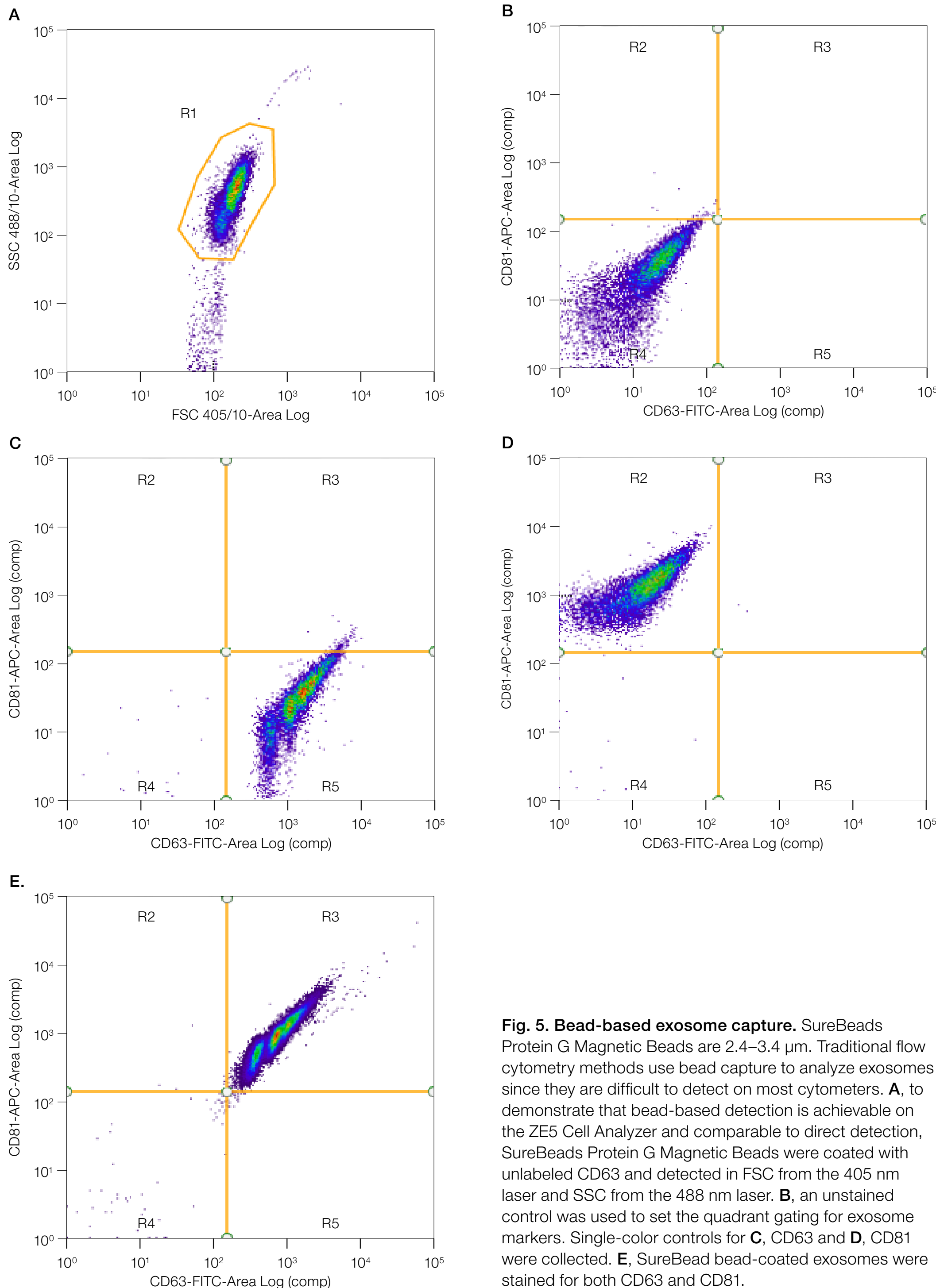

Fig. 5. Bead-based exosome capture. SureBeads Protein G Magnetic Beads are 2.4–3.4 µm. Traditional flow cytometry methods use bead capture to analyze exosomes since they are difficult to detect on most cytometers. **A**, to demonstrate that bead-based detection is achievable on the ZE5 Cell Analyzer and comparable to direct detection, SureBeads Protein G Magnetic Beads were coated with unlabeled CD63 and detected in FSC from the 405 nm laser and SSC from the 488 nm laser. **B**, an unstained control was used to set the quadrant gating for exosome markers. Single-color controls for **C**, CD63 and **D**, CD81 were collected. **E**, SureBead bead-coated exosomes were stained for both CD63 and CD81.

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Conclusions

- The direct detection of exosomes is possible on the ZE5 Cell Analyzer
- Traditional bead detection methods for flow cytometry can also be performed on the ZE5
- Distinct intravesicular exosome marker proteins such as ALIX and TSG101 can be quantified on the ZE5
- The exosome workflow is made simpler by eliminating preparation steps on the ZE5

Advantages of Using the ZE5 Cell Analyzer

Exosome research can be challenging, from sheath purification to gating in data analysis. To address these challenges in an easy-to-use system, the ZE5 was designed with the input of flow cytometry researchers. As a result, it contains built-in capabilities that allow any lab to study exosomes and other small particles. The ZE5 is equipped with extremely low noise electronics and offers a small particle PMT detector using a 100 mW 405 nm laser. Together, this aspect, coupled with dual parameter thresholding, solves the problem of distinguishing exosomes or other small particles from debris and noise. Furthermore, the ZE5 uses DI water as its sheath and has an inline sheath filter to eliminate the need to pre-filter. This, together with filtering of samples leaves few other cellular particulates to populate the plots. This boosts confidence in gating on the exosome population. Although Bio-Rad offers bead conjugation methods, the ZE5 Cell Analyzer is able to detect exosomes without beads. Using size beads, the ZE5 can quickly be set up to easily collect purified exosomes.

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