Small Particle Detection and Analysis on the ZE5 Cell Analyzer

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Introduction

Exosomes are a type of extracellular vesicle (EV) that contain proteins, RNA, and DNA, and have been implicated in general intracellular communications in health and disease. Exosomes are involved in many processes related to cancer, including tumorigenesis, metastasis, and drug resistance to note just a few (Guo et al. 2017). As messengers, exosomes may contain useful biomarkers, and as such have become a key area of interest in disease research. Due to the small size of exosomes (generally <250 nm), there are limited methods available with which to study them.

The most common method of exosome analysis by flow cytometry is using magnetic beads for capture and detection. Most flow cytometers can be used in this type of analysis because the beads lend detectable size to the samples. The ZE5 Cell Analyzer also has small particle detection capability (forward scatter [FSC] from 405 nm laser), so we wanted to investigate whether we could detect exosomes directly as well as captured on beads.

We studied the presence of common exosomal surface markers CD63 and CD81 (Heijnen et al. 1999), and intravesicular markers ALIX and TSG101 (Lee et al. 2012) in exosomes purified from MCF-7, a breast cancer cell line. We also demonstrated the use of SureBeads to capture exosomes and stain for surface markers. We present both methods of staining and instrument setup, and details for direct exosome detection on a ZE5 using single and dual triggers.

Materials and Methods

Exosome Purification

MCF-7 cells were cultured in 10% FBS and 0.01 mg/ml insulin-supplemented MEM medium until 70–80% confluent. The cells were washed twice with PBS and incubated with exosome-free medium for 12–72 hr. The medium was collected and filtered through a 0.22 µm membrane to remove cells and debris. The filtered medium was mixed thoroughly with 0.5 volumes of the Total Exosome Isolation Reagent (Thermo Fisher Scientific Inc.). 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 exosomes were determined by Zetasizer Nano ZSP (Malvern Panalytical Ltd). Results are shown in Figure 1.

Instrument Setup

In order to minimize the background, ProFlow Sort Grade Water (Bio-Rad Laboratories, Inc.) was used to analyze samples on a ZE5 Cell Analyzer (Bio-Rad). Quality control was performed using ZE Series QC Beads (Bio-Rad). A mixture of yellow fluorescent beads ranging from 0.22–1.35 µm (Spherotech, Inc.) was used to set up the instrument voltages and thresholds. For small particle setup, one or dual thresholds can be used to set up the instrument. For direct exosome detection for surface markers, FSC from 405 nm laser and fluorescent signal were used as thresholds. Only FSC from the 405 nm laser was used to set up the threshold for detection of intravesicular markers and bead-based exosomes. Exosome samples were filtered through a 10 µm filter prior to applying to the instrument. Results are shown in Figure 1.

Flow Cytometry Analysis

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 in 50 µl of PEB buffer (PBS, 5 mM EDTA, and 0.5% BSA) were incubated with FITC Mouse Anti-Human CD63 (Bio-Rad) and APC Anti-Human CD81 (BioLegend) for 30 min at room temperature. After incubation, the samples were washed with PEB and resuspended in 500 µl of PEB buffer. Results are shown in Figure 2.

Intravesicular Staining

eBioscience Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific) was used to fix and permeabilize exosomes for intravesicular staining. Two micrograms of exosomes were fixed in 100 µl of Intracellular 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. The samples were resuspended in 100 µl of Permeabilization Buffer, then incubated with PE Anti-Human TSG101 (Abcam) and ReadiLink 492/516 labeled with Anti-Human ALIX (Bio-Rad) 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. Data were analyzed by Everest Software 2.0 (Bio-Rad) and FlowJo Software 10.0 (FlowJo, LLC). Results are shown in Figure 3.

Bead-Based Exosome Detection by Flow Cytometry

Ten micrograms of unconjugated Anti-Human CD63 Antibody (Bio-Rad) were incubated by rotation with 100 µl of SureBeads Protein G Magnetic Beads (Bio-Rad) for 10 min at room temperature. The size of SureBeads is between 2.4–3.4 µm. 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–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). Fifty µl of exosome-coated SureBeads were incubated with FITC Mouse Anti-Human CD63 (Bio-Rad) with or without APC Anti-Human CD81 (BioLegend), depending on treatment condition, for 30 min at room temperature and washed twice with PEB–0.1% Tween 20. Results are shown in Figure 4.

Results

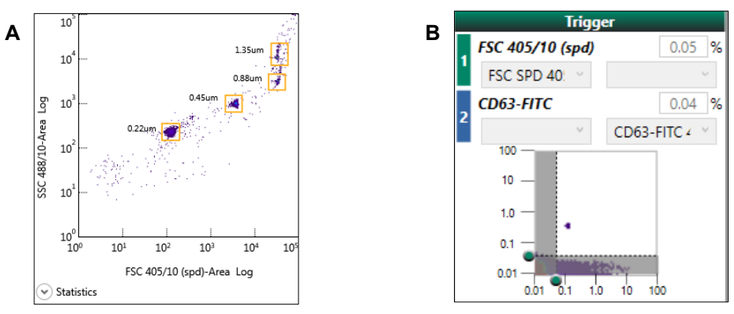

Fig. 1. Exosome size. The size of exosomes purified from MCF-7 cells was determined by Zetasizer Nano ZSP to be in the range of 185 to 300 nm. **A**, yellow beads ranging from 0.22–1.35 µm were used to set up the voltage of a ZE5 Cell Analyzer; **B**, 0.22 µm beads were discriminated from noise when using FSC from a 405 nm laser and fluorescence as triggers.

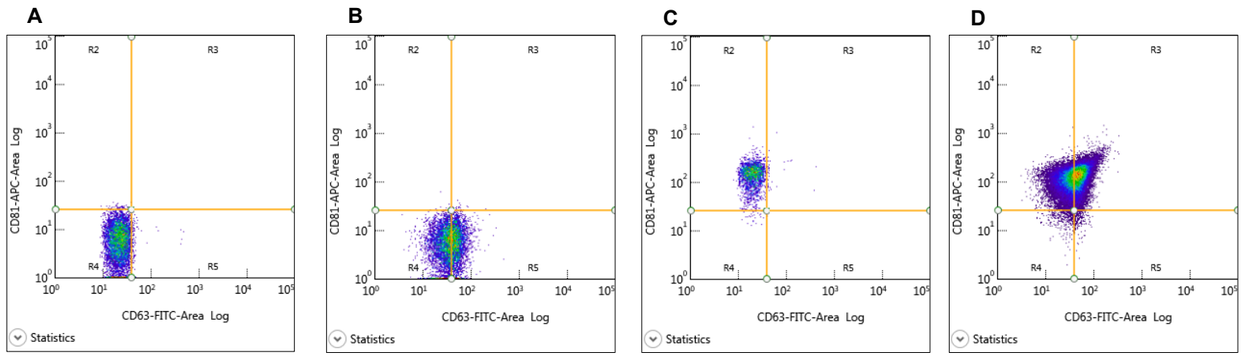

Fig. 2. Single and dual staining of purified exosomes using FSC (405 nm) and CD63-FITC as triggers. First gate was on total population of exosomes (not shown). **A**, unstained; **B**, CD63-FITC; **C**, CD81-APC; **D**, CD63-FITC and CD81-APC.

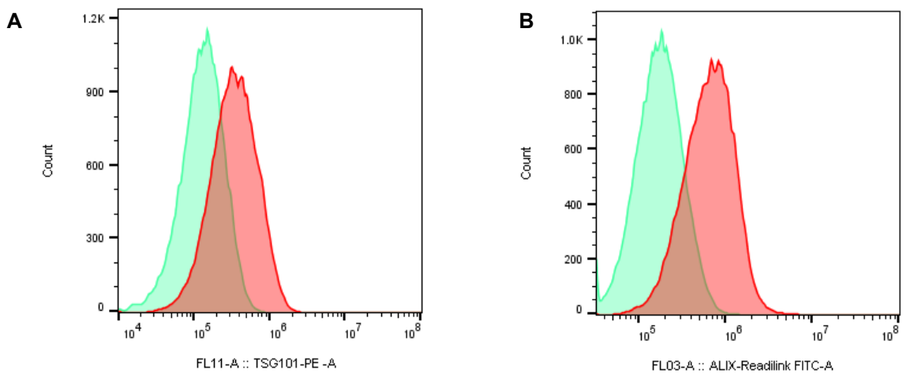

Fig. 3. Intravesicular staining using fluorescent trigger. A, TSG101-PE; **B**, ALIX–ReadiLink 492/516.

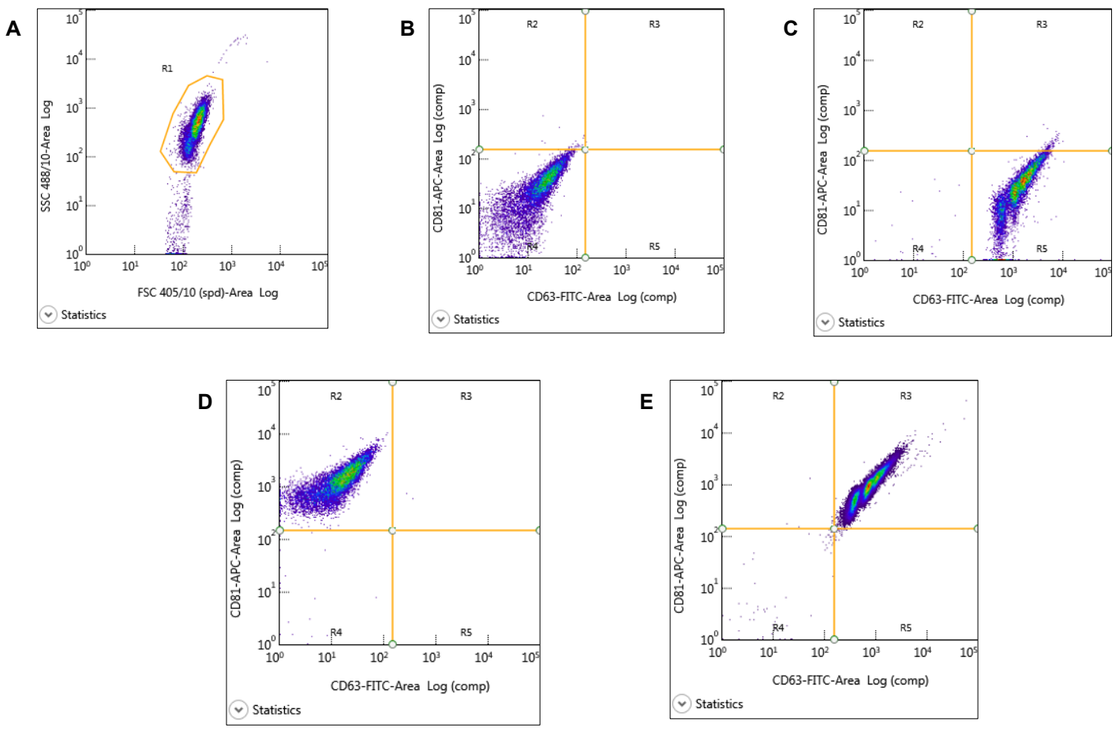

Fig. 4. Bead-based exosome capture. Exosomes were captured using SureBeads Protein G Magnetic Beads coated with unlabeled CD63 and detection by FSC (405 nm). **A**, total population R1; **B**, unstained; **C**, CD63-FITC; **D**, CD81-APC; **E**, CD63-FITC and CD81-APC.

Conclusions

We used a ZE5 Cell Analyzer to detect exosomes using several different methods:

- Direct detection of surface-stained exosomes using dual triggers: FSC (405 nm) and fluorescence
- Intravesicular-stained exosomes using FSC (405 nm) as the trigger
- SureBeads captured exosomes using FSC (405 nm) as the trigger

Acknowledgements

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References

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