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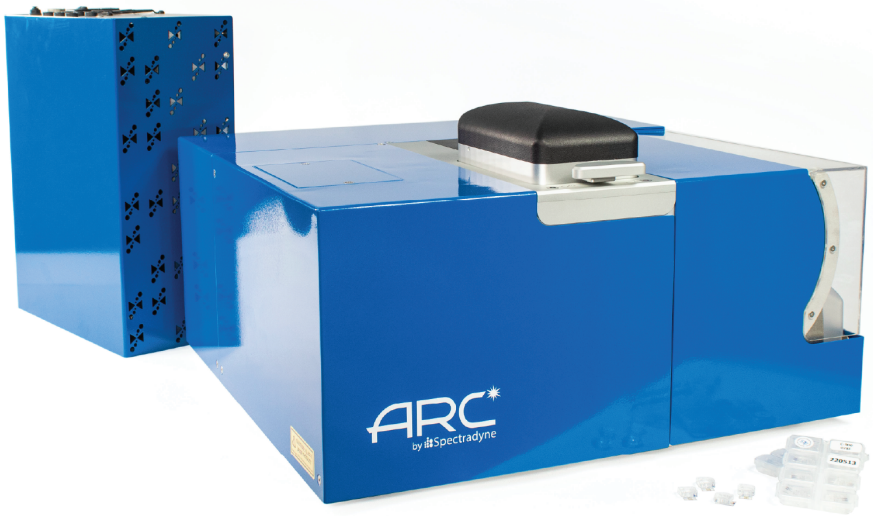
Spectradyne®
PARTICLE ANALYSIS

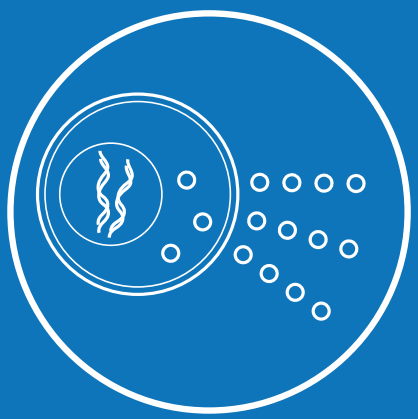
ARC

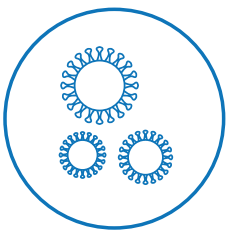
Application Note

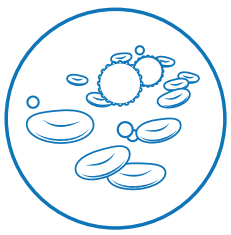
Spectradyne's ARC™ Particle Analyzer compares membrane dyes for extracellular vesicles: ExoBrite™ True EV Membrane Stain and PKH67

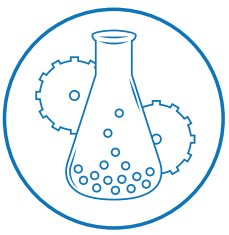
Extracellular Vesicles











Spectradyne PARTICLE ANALYSIS

ARC

Application Note: Extracellular Vesicles

Spectradyne's ARC™ Particle Analyzer compares membrane dyes for extracellular vesicles: ExoBrite™ True EV Membrane Stain and PKH67

Extracellular vesicles (EVs) derived from HEK-293 cells and expressing human CD81 were fluorescently labeled with membrane dyes, and co-labeled with an antibody marker to evaluate:

1. The relative staining efficacy between lipophilic membrane dyes ExoBrite™ True EV Membrane Stain and PKH67.
2. The presence of dye aggregates within the size range of extracellular vesicles.
3. Compatibility of ExoBrite™ True EV Membrane Stain with antibody staining for co-localization analysis with Spectradyne's ARC particle analyzer.

Experimental Design:

HBM-HEK-BFP-81 EVs (HansaBioMed Life Sciences, Estonia) are extracellular vesicles expressing the human tetraspanin CD81, that are designed to serve as a reference material for diverse particle characterization platforms. In these experiments, the BFP-81 EVs were first analyzed without modification to characterize their native size, concentration, and fluorescence brightness.

For the evaluation of the membrane dyes the EVs were stained with ExoBrite™ 515/540 True EV Membrane Stain (Biotium, USA, Cat. No. 30129) and PKH67 green fluorescent membrane stain (MilliporeSigma, USA). Dye-only controls were also analyzed to quantify dye aggregates within the size range of interest.

To demonstrate the compatibility of ExoBrite™ 515/540 True EV Membrane Stain with immunostaining, the EVs were membrane labeled prior to immunolabeling with APC-conjugated anti-human CD81 antibody (BioLegend, USA). Particle size, concentration and fluorescence of all samples were measured using Spectradyne's ARC particle analyzer configured for colinear fluorescence excitation at 488 nm and 638 nm, and detection bands defined by the filters below:

FL1 (ExoBrite™/PKH67 - FITC)	FL2 (PE)	FL3 (AF647)
488 nm – 525/39	488 nm – 575/19	638 nm – 698/70

Results:

Figure 1 shows the particle size distribution of all particles in the unlabeled BFP-81 EVs (red), ExoBrite™ 515/540 True EV Membrane Stain-stained EVs (blue), and PKH67 membrane-stained EVs (green). The size distribution in each of the membrane labeled samples contains a peak population with a mean diameter of 80nm, consistent with the size distribution of the unlabeled sample. Neither membrane dye significantly impacts the particle size distribution of vesicles.

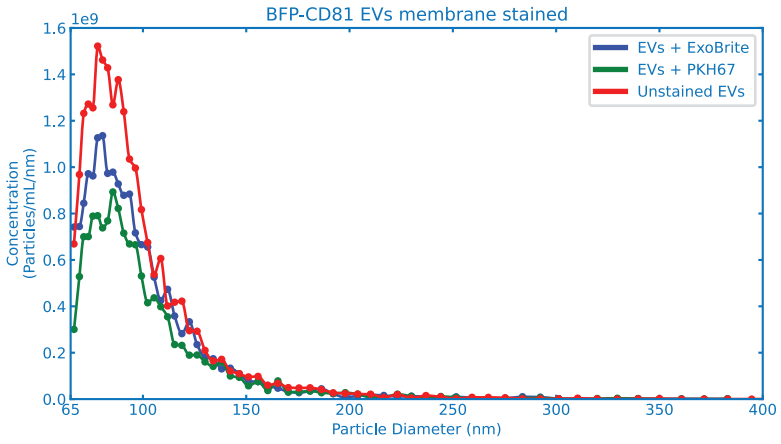


Figure 1

Particle size distributions for the unlabeled BFP-81 EVs (red), ExoBrite™ 515/540 True EV Membrane Stain-stained EVs (blue), and PKH67 membrane-stained EVs (green).

Results (cont.):

The top panel of Figure 2 shows the FITC fluorescence scatter plot (FITC brightness vs. particle diameter) for particle detection events in each sample. The fluorescence limit of detection for the ARC in the FITC channel is indicated by the horizontal line on the scatterplot at 25 FITC-ERF. As expected, no significant fluorescence from the unlabeled EVs was observed in the FITC detection band (red points). For the EVs stained with membrane dye, 62% of all particles detected on the measured size range were brighter than 25 ERF-FITC when labeled with ExoBrite™ (blue) compared to only 49% when labeled with PKH67 (green). By this metric, the ExoBrite™ 515/540 True EV Membrane Stain labels EVs more effectively than PKH67.

The bottom panel of Figure 2 shows the particle size distribution of the gated population of fluorescent particles in each sample. The ExoBrite™-stained sample (blue) reveals a population of EVs positive for ExoBrite™ having a mean diameter of 96nm. Similarly, the PKH67-stained sample (green) shows a peak population of EVs positive for PKH67 with a mean diameter of 92nm.

Figure 3 compares the particle size distribution of total aggregates in dye-only control samples prepared identically to the stained EV samples above. Dye aggregates are evident in both dye control samples within the full measured size range, however, the ExoBrite™ membrane dye sample generates a significantly lower concentration of aggregates (red curve, 1.1 x 10^8 particles/mL), compared to the PKH67 (green curve, 6.4 x 10^8 particles/mL). The ExoBrite™ 515/540 True EV Membrane Stain is less susceptible to dye aggregation.

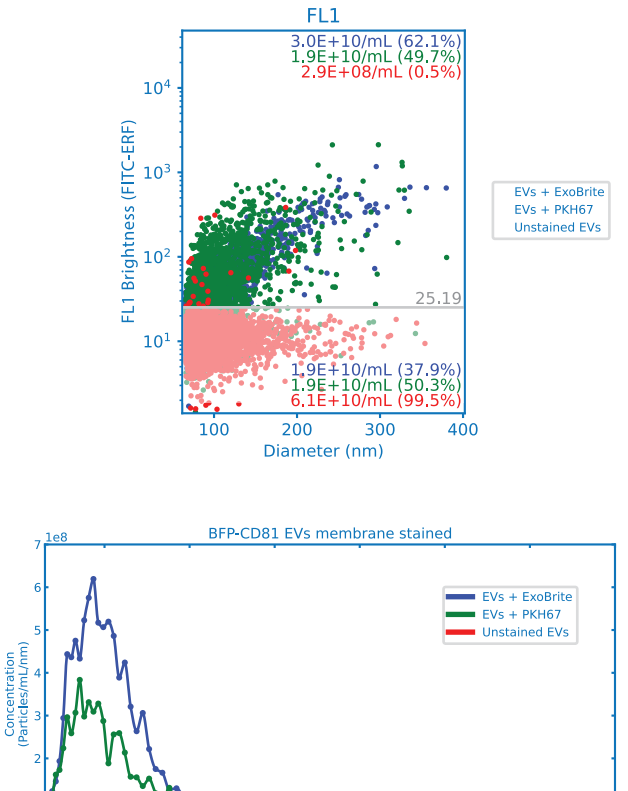


Figure 2

Fluorescence analysis of the membrane-stained EVs. Top panel shows the brightness of each particle detected in FITC vs. its true particle diameter. Bottom panel shows the fluorescence subpopulations of ExoBrite™+ and PKH67+ EVs. The results indicate that the ExoBrite™ 515/540 True EV Membrane Stain is more effective at staining EVs than PKH67 membrane dye (62% vs. 49%, respectfully).

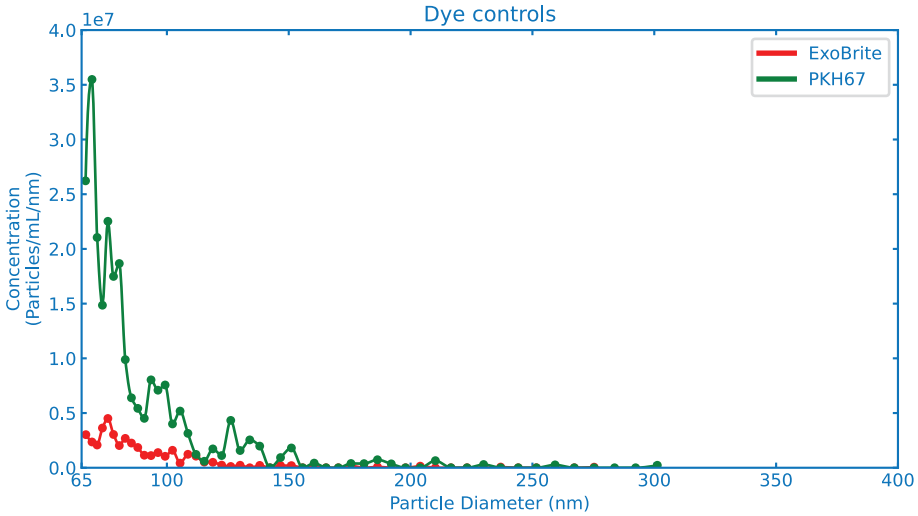


Figure 3

Particle size distribution of dye particles in the dye controls showing a much lower concentration of dye aggregates in the ExoBrite™ 515/540 True EV Membrane Stain sample (red) than in the PKH67 sample (green).

Results (cont.):

Figure 4 shows measurements of the BFP-81 EVs after staining with both ExoBrite™ 515/540 True EV Membrane Stain and APC-conjugated anti-CD81 antibody. Immunostaining of the EVs was performed after the EVs had been labeled with the membrane dye.

Left panel: A clear increase in the concentration of APC fluorescent particles is observed for the double-labeled EVs (red) when compared to the single membrane-labeled EVs (blue). The population of BFP-81 EVs carrying the CD81 tetraspanin marker accounts for 12.5% of the total particle concentration.

Right panel: Colocalization analysis of the double-labeled EVs reveals that 8% of BFP-81 EVs are positive for both ExoBrite™ membrane dye and CD81.

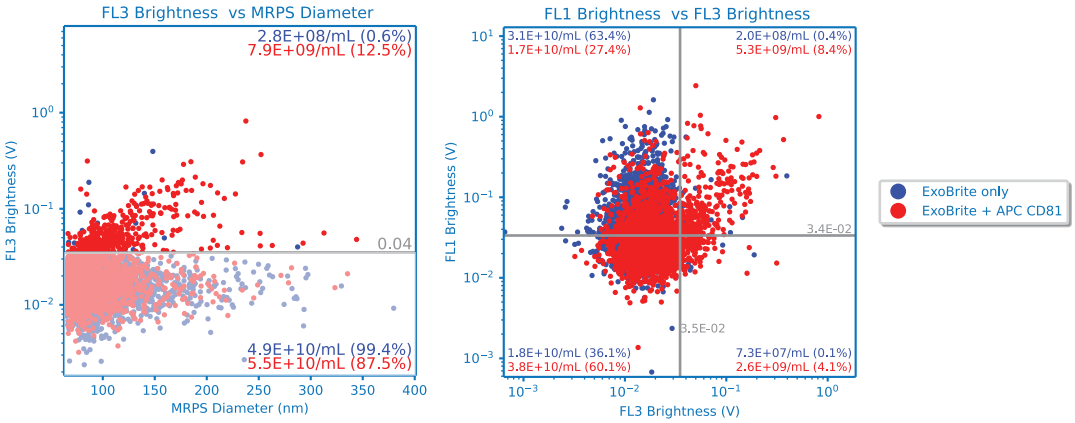


Figure 4

Fluorescence scatter plots for single-labeled EVs with ExoBrite™ 515/540 True EV Membrane Stain (blue) and dual-labeled EVs with both ExoBrite™ and APC anti-CD81 antibody (red).
Left panel: Red channel fluorescence data (FL3 brightness vs. particle diameter) showing 12.5% of EVs in dual-labeled EV sample are positive for CD81.
Right panel: Colocalization analysis reveals 8% of EVs are double positive for ExoBrite™ and CD81 (top right quadrant).

Discussion:

This example demonstrates the ARC particle analyzer's ability to deliver accurate, quantitative subpopulation analysis of extracellular vesicles using fluorescent labeling. The results reveal that the ExoBrite™ 515/540 True EV Membrane Stain is more effective at staining EVs and is less susceptible to dye aggregation than PKH67 membrane dye. These measurements also confirm that membrane staining with ExoBrite™ membrane dye did not interfere with the subsequent immunolabeling of EVs.