Beyond Tetraspanin Markers: Strengthening Extracellular Vesicle Characterisation Using Aco-Dyes™

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Introduction

Accurate characterisation of extracellular vesicles (EVs) is challenging as surface protein markers alone do not confirm whether the detected particles are membrane-bound vesicles or non-vesicular contaminants. According to the "Minimal information for studies of extracellular vesicles" (MISEV2023), EVs are defined as particles released from cells that are delimited by a lipid bilayer [1]. This lipid bilayer membrane distinguishes EVs from other extracellular particles and contaminants. Traditionally, EV characterisation predominantly relies on tetraspanin protein markers such as CD9, CD63 and CD81. However, this method has limitations:

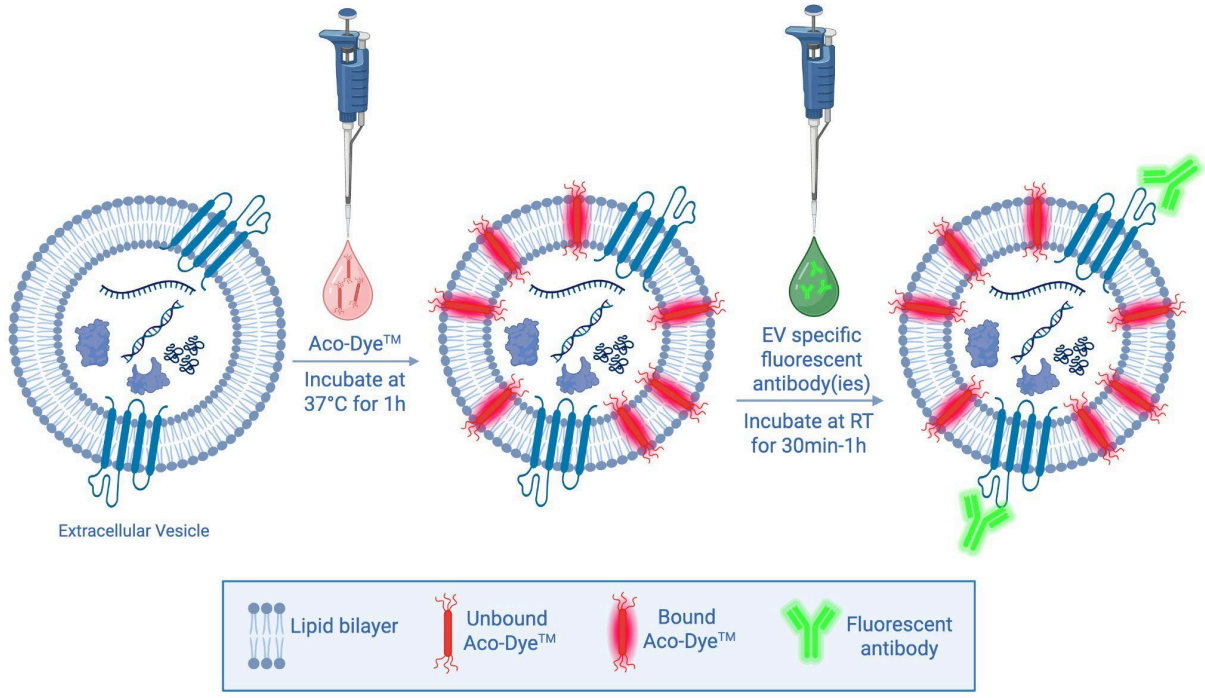
- Not all EVs express the same tetraspanin markers [2].
- Protein marker detection alone cannot confirm the presence of an intact lipid bilayer, the defining feature of EVs.

Keywords: *Extracellular Vesicles (EVs), characterisation, co-labelling, lipophilic, fluorogenic, water-soluble membrane dye, nano flow cytometry*

- Detected protein signals may originate from co-isolated non-vesicular contaminants [3], complicating accurate EV identification.

To address these limitations, this application note presents a co-labelling workflow (Fig. 1) using Acoerela's Aco-Dyes™, a family of conjugated oligoelectrolytes (COEs) that intercalate into lipid bilayers. Aco-Dyes are water-soluble and do not form micelles, thereby minimising the risk of false positive signals. They are also fluorogenic, exhibiting maximum fluorescence only upon intercalation into a lipid bilayer. By combining Aco-Dye labelling, which confirms the presence of a lipid bilayer membrane, with antibody-based detection of EV-specific tetraspanin markers, this approach improves the accuracy of EV identification and ensures MISEV2023-compliance.

Figure 1. Co-labelling workflow



Schematic workflow of extracellular vesicle (EV) co-labelling, where Aco-Dye intercalates into the lipid bilayer membrane and fluorescent antibodies target EV-specific tetraspanin markers.

In addition, this application note outlines the key considerations and best practices for optimising EV co-labelling using Aco-Dyes, to help researchers implement this strategy effectively and achieve reliable, reproducible results.

Materials and Methods

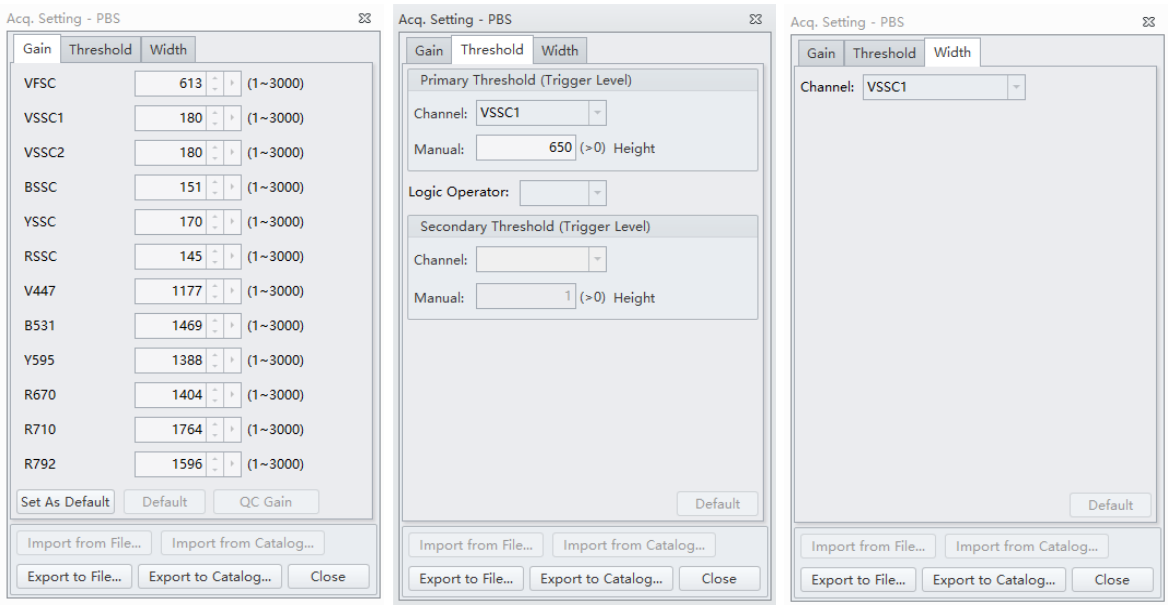
Sample preparation: Aco-800™ dye was reconstituted and sonicated as per the datasheet. Red Blood Cell-derived EVs (RBCEVs) and pancreatic EVs were quantified and adjusted to a final concentration of 1e10 particles/mL with filtered 1X phosphate buffered saline (PBS). Each reaction consisted of 45µL of EVs (1e10 particles/mL) and 5 µL of 10 µM Aco-800 dye (1µM final concentration) incubated for 1 hour at 37°C, followed by a 1 hour incubation at room temperature with the respective antibodies: CD235a-PE (Glycophorin A from BioLegend Cat#: 349105, working concentration: 0.004mg/mL) for RBCEVs, and CD9-FITC (BioLegend Cat#: 312104, working concentration: 0.004mg/mL) and CD63-Alexa Fluor® 647 (BioLegend Cat#: 353016, working concentration: 0.004mg/mL) for pancreatic EVs. RBCEVs have been reported to express CD235a [4], while pancreatic EVs have been shown to express CD9 and CD63 [5], validating the choice of tetraspanin markers used for the respective EV identification. Appropriate controls were also prepared and incubated under the same conditions.

Data acquisition: To minimise swarming, samples were diluted in filtered 1X PBS to achieve an event rate of 1000–1500 events/second, typically requiring dilution up to 1:2000. Once optimised, the same dilution was applied to all samples and controls, which were then acquired on the CytoFLEX nano flow cytometer using the acquisition settings shown in Fig. 2.

Data analysis: For data analysis, the gating strategy was applied as follows: singlets were gated first with VSSC1-Width on y-axis and VSSC1-H Height on x-axis and used as the parent population for the subsequent plots. In the unlabelled EV sample, the population negative for both Aco-Dye and antibodies was defined with a quadrant gate (Fig. 3g, 4m), and in the labelled samples and controls, the respective populations were identified using the same gating strategy.

Sizing: EV sizes were measured using the CytoFLEX nano flow cytometer with FCM_{PASS} software, following the manufacturer's (Beckman Coulter Life Sciences) recommended protocol. NIST-traceable size standards (nanoViS Nanoscale Sizing Standards) were used to convert arbitrary VSSC signals into nanometer units, allowing determination of EV size distributions. Sizes were compared before and after labelling with Aco-Dye and antibody(ies).

Figure 2: Data acquisition settings for the CytoFLEX nano



All samples and controls were acquired using the same acquisition settings of gain, threshold and width to ensure consistency across all measurements.

Results

RBCEVs co-labelled with Aco-800 and CD235a-PE (Fig. 3c) displayed a well-defined dual-positive population with nearly all $CD235a^+$ EVs being positive for Aco-800. Additionally, a subset of membrane-bound EVs that were positive for Aco-800 but negative for CD235a was observed, indicating that these EVs may not express CD235a. The negative controls (Fig. 3d-h) had minimal background, while the single-colour controls (Fig. 3a-b) individually labelled with either Aco-800 or CD235a showed clear positive signals for their respective markers.

For pancreatic EVs, three-colour co-labelling with Aco-800, CD9-FITC and CD63-Alexa Fluor® 647 revealed dual-positive populations of Aco-800$^+$CD9$^+$ and Aco-800$^+$CD63$^+$ (Fig. 4a). Two-colour samples (Fig. 4c-d: EVs + Aco-800 + CD9-FITC, or EVs + Aco-800 + CD63-Alexa Fluor® 647) and single-colour samples (Fig. 4g-i: EVs + Aco-800, CD9-FITC or CD63-Alexa Fluor® 647) displayed their respective positive signals. All corresponding controls (Fig. 4b, e-f, j-n) had minimal background, indicating no interference with the true signal.

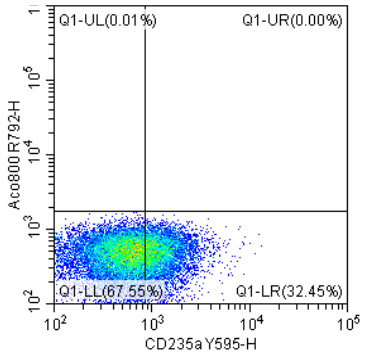
Comparison of sizes (Table 1) showed that labelled RBCEVs and pancreatic EVs were slightly larger than their unlabelled counterparts. The lower average sizes of unlabelled samples are inclusive of non-membranous particles, whereas labelling with Aco-Dye preferentially highlights membrane-bound EVs, with both the dye and antibody(ies) contributing to the labelled EV size.

Table 1: Size measurements using nanoViS Nanoscale Sizing Standards

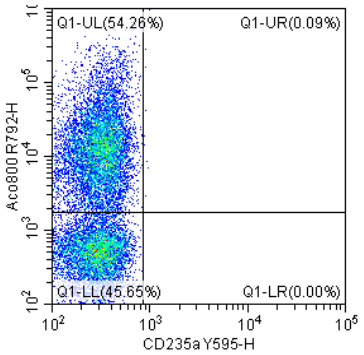
EV Type	Sample	Mean EV Diameter (nm) [Average RI]-H
RBCEV	Unlabelled EVs (singlets)	86.1
	EVs + Aco-800	117.3
	EVs + CD235a-PE	98.9
	EVs + Aco-800 + CD235a-PE	136.4
Pancreatic EV	Unlabelled EVs (singlets)	88.7
	EVs + Aco-800	125.4
	EVs + CD9-FITC	141.7
	EVs + CD63-Alexa Fluor® 647	105.2
	EVs + Aco-800 + CD9-FITC + CD63-Alexa Fluor® 647	200.2

Figure 3. Two colour co-labelling of RBCEVs with Aco-800 and CD235a-PE

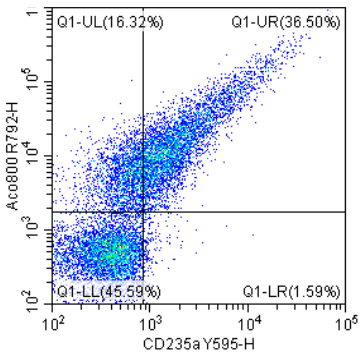
a. EVs + CD235a-PE



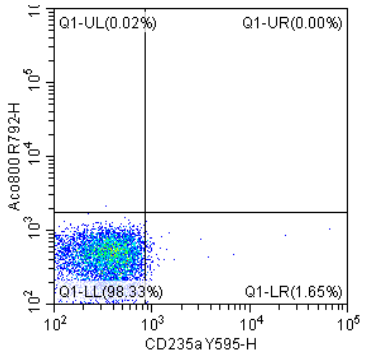
b. EVs + Aco-800



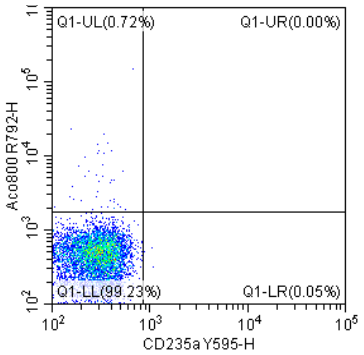
c. EVs + CD235a-PE + Aco-800



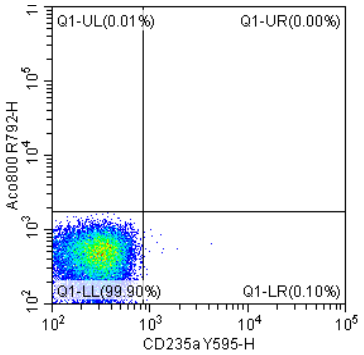
d. PBS + CD235a-PE (antibody control)



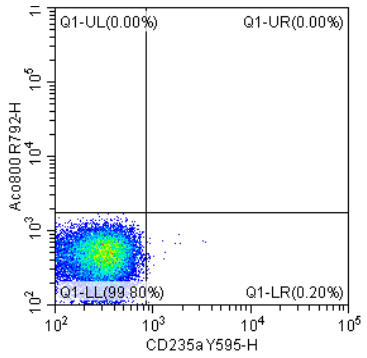
e. PBS + Aco-800 (dye control)



f. PBS + CD235a-PE + Aco-800 (antibody + dye control)



g. Unlabelled EVs (EV control)



h. PBS (buffer control)

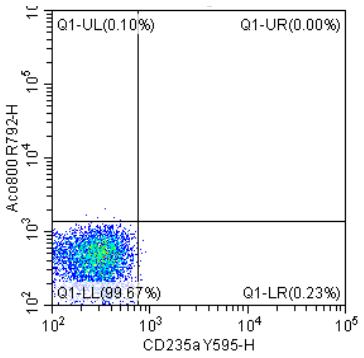
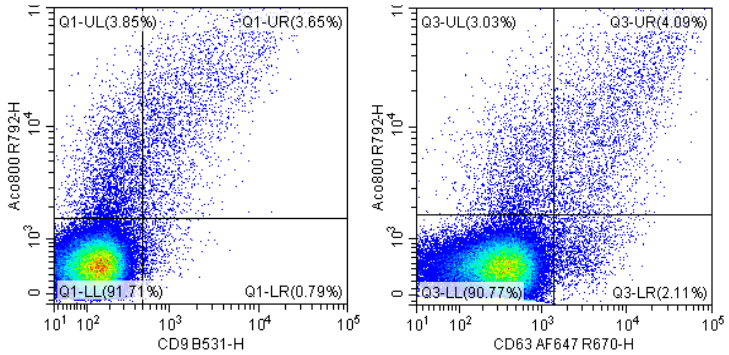


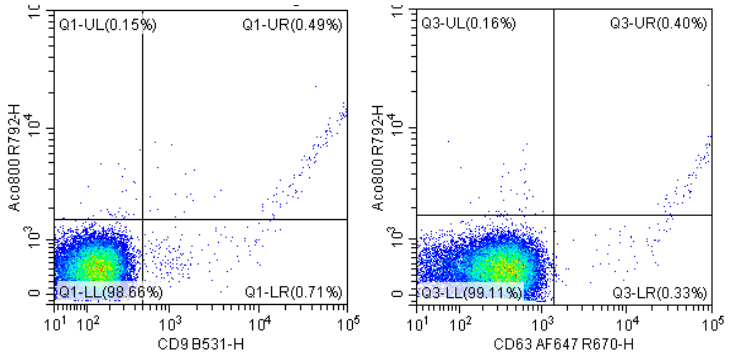
Figure 4. Three colour co-labelling of pancreatic EVs with Aco-800, CD9-FITC and CD63-Alexa Fluor® 647

Three colour data:

a. EVs + Aco-800 + CD9-FITC + CD63-Alexa Fluor® 647

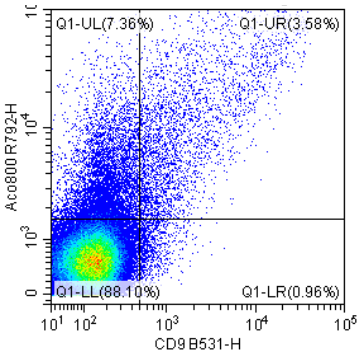


b. PBS + Aco-800 + CD9-FITC + CD63-Alexa Fluor® 647



Two colour data:

c. EVs + Aco-800 + CD9-FITC



d. EVs + Aco-800 + CD63-Alexa Fluor® 647

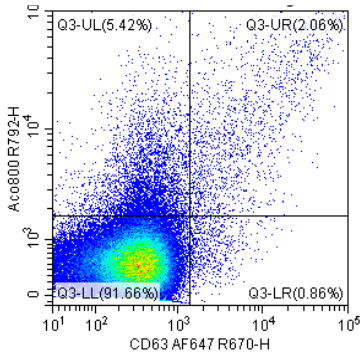
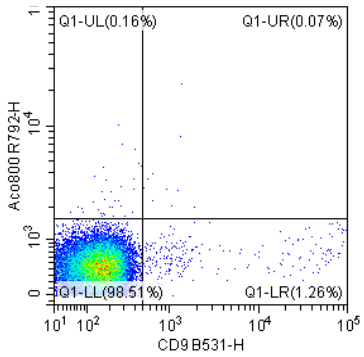
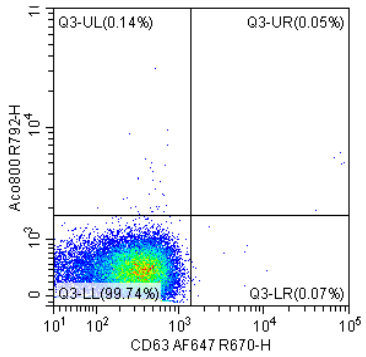


Figure 4 (continued). Three colour co-labelling of pancreatic EVs with Aco-800, CD9-FITC and CD63-Alexa Fluor® 647

e. PBS + Aco-800 + CD9-FITC

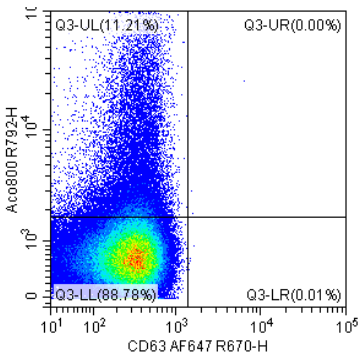


f. PBS + Aco-800 + CD63-Alexa Fluor® 647

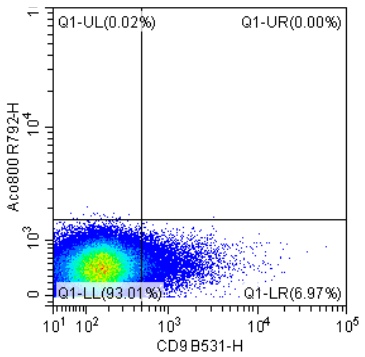


Single colour data:

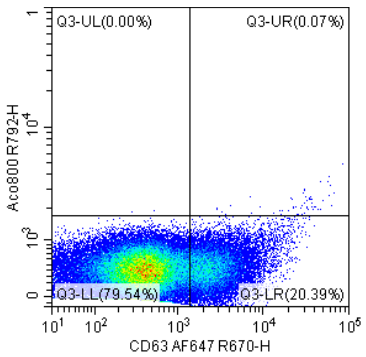
g. EVs + Aco-800



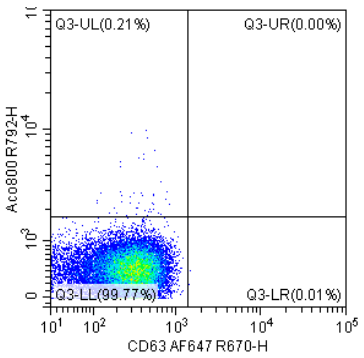
h. EVs + CD9-FITC



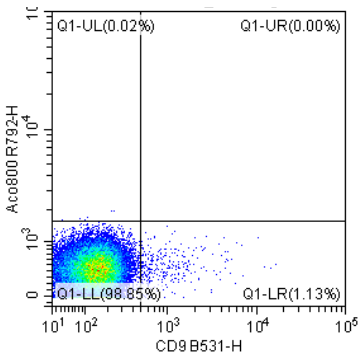
i. EVs + CD63-Alexa Fluor® 647



j. PBS + Aco-800



k. PBS + CD9-FITC



l. PBS + CD63-Alexa Fluor® 647

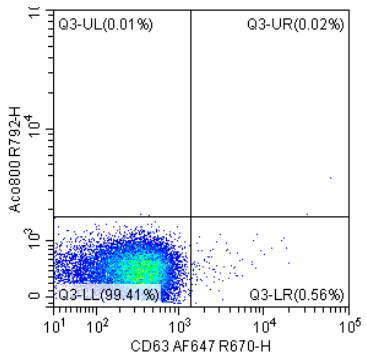
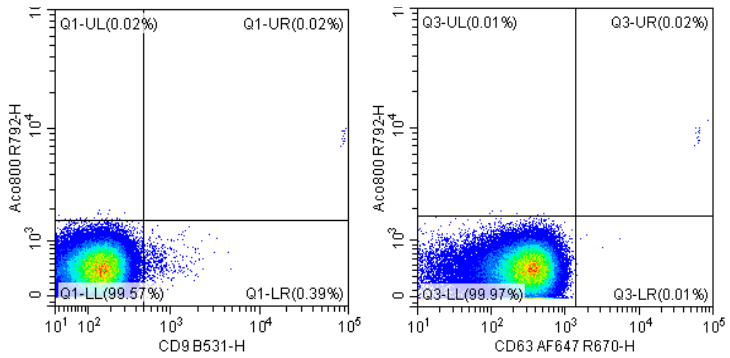


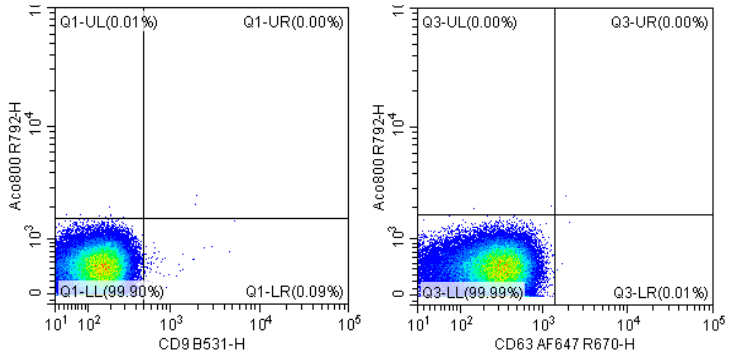
Figure 4 (continued). Three colour co-labelling of pancreatic EVs with Aco-800, CD9-FITC and CD63-Alexa Fluor® 647

Other controls:

m. Unlabelled EVs (EV control)



n. PBS (buffer control)



Conclusion

Combining Aco-Dyes with tetraspanin markers provides a highly synergistic approach for EV characterisation using flow cytometry. Aco-Dyes serve as a robust universal identifier for true EV populations, independent of their cargo, allowing for an accurate count and size estimation, while tetraspanin markers deliver specific phenotyping information to identify and quantify distinct EV subpopulations.

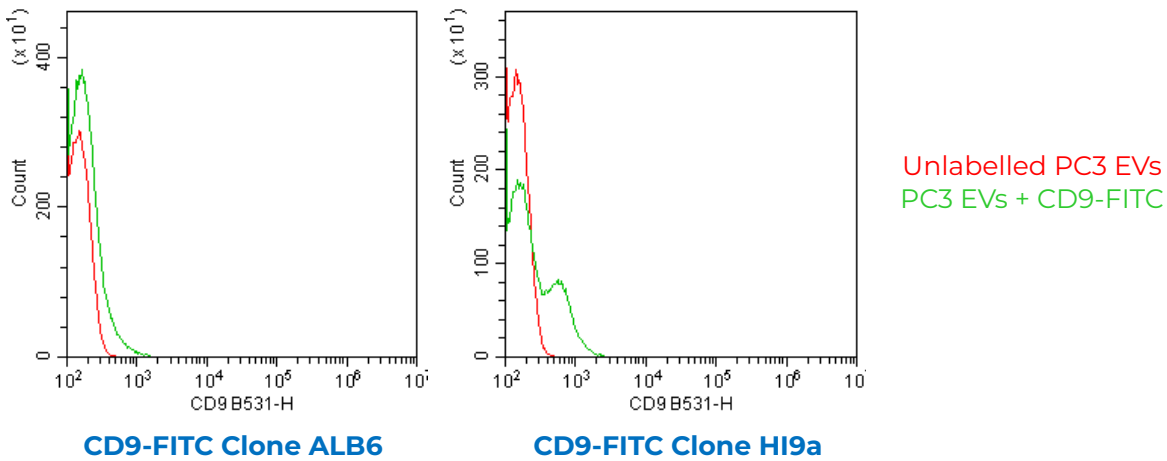
Moreover, the low background fluorescence of Aco-Dyes simplifies gating and background subtraction, allowing for confident and reproducible identification of EV populations without the risk of false positives.

This co-labelling strategy advances EV research by moving beyond simple enumeration and towards a sophisticated analysis of EV heterogeneity, deepening our understanding of EV composition and function in biological samples. It forms a strong foundation for both basic research and the development of EVs as biomarkers or therapeutic agents across diverse applications.

Considerations for Effective EV Co-Labelling

1. **Antibody clone selection:** Not all monoclonal antibody clones work equally well with all EV types. It is important to test and identify the clone that works specifically for your EV sample. For instance, when PC3 EVs were labelled with CD9-FITC from clones HI9a and ALB6, only the antibody from clone HI9a produced a clear $CD9^+$ signal, while ALB6 showed minimal increase in fluorescence (Fig. 5).

Figure 5. Antibody clone selection



CD9-FITC Clone ALB6 **CD9-FITC Clone HI9a**

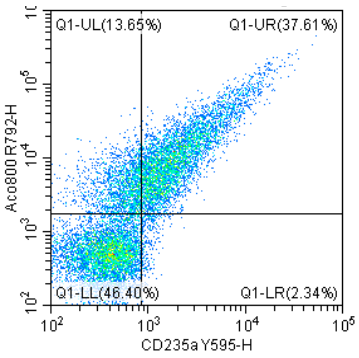
2. **Sample dilution before acquisition:** Each flow cytometer requires an optimal event rate to avoid a) swarming from overly concentrated samples, b) very weak signals from excessively diluted ones. Samples should be diluted to achieve the recommended event rate (1000-1500 events/second for the CytoFLEX nano) with a dilution factor optimised for specific sample types. The same dilution should be applied to all related samples, including controls.

3. **Co-labelling sequence:** The order of co-labelling can affect results depending on the EV type. For RBCEVs, labelling with Aco-Dye first or antibody first did not make much difference in the dual positivity (Fig. 6a). However, for pancreatic EVs, labelling with Aco-Dye before antibodies improved the dual positivity (Fig. 6b).

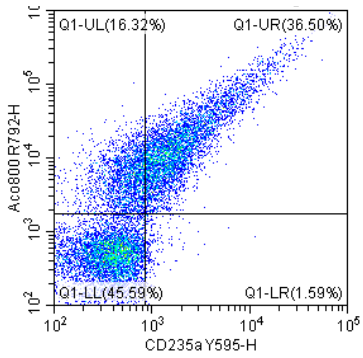
Figure 6. Co-labelling sequence

a. RBCEVs co-labelled with Aco-800 and CD235a-PE

Antibody followed by Aco-Dye

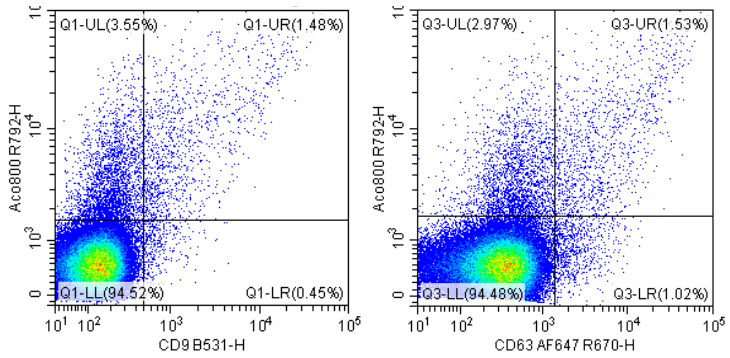


Aco-Dye followed by antibody

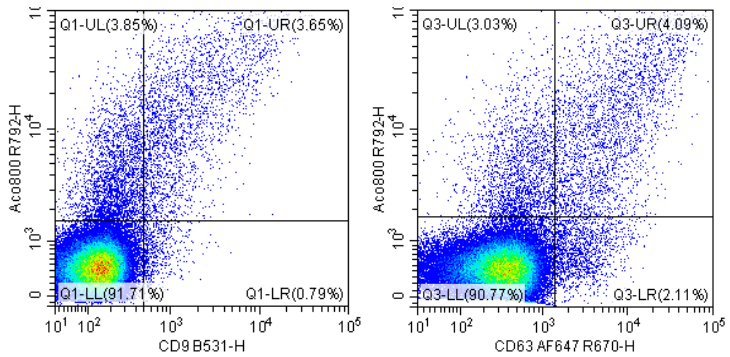


b. Pancreatic EVs co-labelled with Aco-800, CD9-FITC and CD63-Alexa Fluor® 647

Antibody followed by Aco-Dye



Aco-Dye followed by antibody



4. **Uniform settings:** Using the same instrument settings and acquisition time across all samples ensures reliable comparisons, especially between positive samples and their negative controls. It is recommended to stop acquisition based on a fixed run duration rather than a set number of events, as this allows for an accurate comparison of total particle counts between samples and controls [6].

5. **Controls:** To ensure accurate and reliable data, it is essential to include a comprehensive set of controls that help identify background signals and rule out false positives. The set should include a buffer control to assess background fluorescence, unlabelled EVs to evaluate autofluorescence, dye and antibody controls to check for non-specific binding, and a dye + antibody(ies) control to detect any interaction between the reagents in the absence of EVs. There should also be single colour controls to detect spectral overlap and enable proper compensation between fluorochromes. Additionally, for more than two colour co-labelling, a fluorescence minus one (FMO) control is useful to show the contribution of all fluorochromes except one. Including these controls improves the validity and reproducibility of the results.

Best Practices for EV Labelling with Aco-Dyes

- Sonicate the reconstituted Aco-Dye for 15 minutes at 40°C before each use.
- Each Aco-Dye has its own unique brightness and staining efficiency. Titrate and optimise the labelling concentration for each dye, EV type and study condition.
- EVs perform best when used fresh after isolation or preparation. Make a fresh 1e10 particles/mL dilution on each test day, as particle counts tend to drop over time.
- Filter all buffers through a 0.2 µm filter before use.

Acknowledgements

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