From Biogenesis to Uptake: Endogenous Labelling of Extracellular Vesicles with Aco-Dyes™

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Introduction

Extracellular vesicles (EVs) are lipid bilayer-enclosed particles released by cells through either the endosomal pathway or direct budding from the plasma membrane[1,2]. As key mediators of intercellular communication, EVs have attracted growing interest as both biomarkers and therapeutic agents. Given their membrane-bound nature, fluorescent probes that selectively target lipid bilayers are valuable tools for EV detection and characterisation.

Conjugated oligoelectrolytes (COEs), commercialised as Acoerela™ Dyes (Aco-Dyes™), are fluorescent probes that intercalate into lipid bilayers. This unique mechanism enables robust and consistent labelling of both cells and EVs, facilitating their visualisation and characterisation across a range of fluorescence-based analytical platforms.

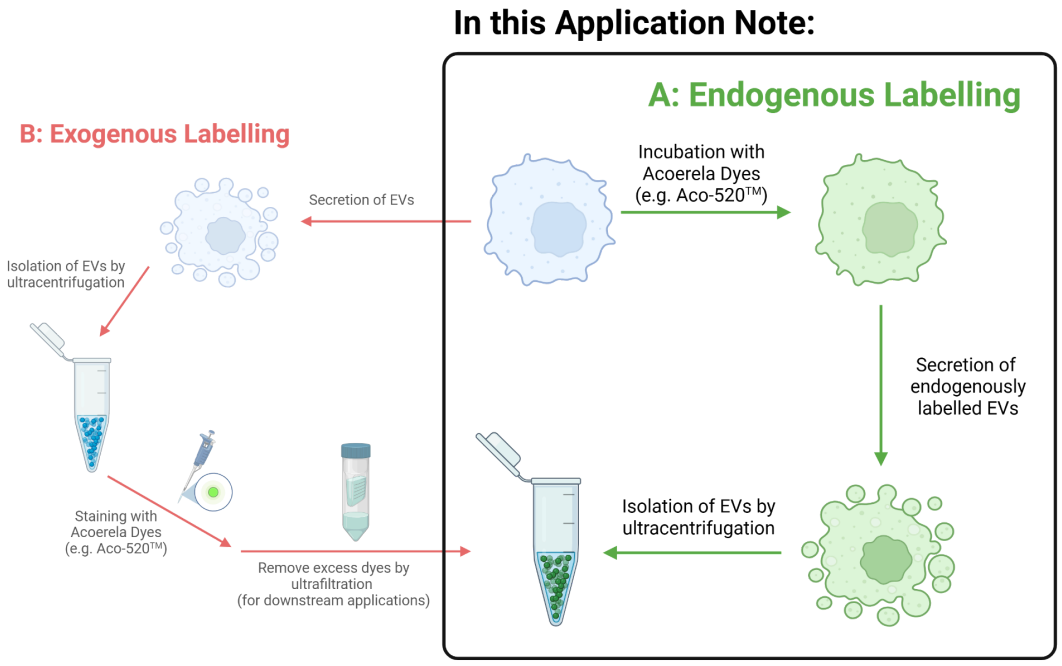

Figure 1: Schematic workflow for (A) endogenous labelling of EVs (green arrows) and (B) exogenous labelling of EVs (red arrows). This figure was created using BioRender.

In this application note, we examine the use of Aco-Dyes for the production of endogenously labelled EVs[3] (Figure 1). By incubating cells with Aco-Dyes, the probes are incorporated into cellular membranes and subsequently into EVs during biogenesis, enabling the direct isolation of fluorescent EVs for downstream analysis. This approach provides clear evidence of a lipid bilayer, a defining feature of EVs, while preserving structural integrity. Notably, endogenous labelling eliminates the need for post-isolation staining and additional purification steps, helping to maintain EV yield and native characteristics. These endogenously labelled EVs can be utilised for a wide range of downstream applications, including EV uptake and biodistribution studies.

Materials and Methods

Cell Culture

A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 mM pyruvate in a 5% CO2 incubator at 37°C.

Isolation of Extracellular Vesicles

2.5×10^6 A549 cells were cultured in T75 cell culture flasks and allowed to adhere overnight. The cells were subsequently incubated with 5 µM of Aco-520™-supplemented DMEM for 24 hours. The dye-supplemented media was removed and the cells were washed thrice with phosphate buffered saline (PBS). The cells were then incubated in DMEM supplemented with 10% EV-depleted FBS for two days. The resultant cell culture media was collected for EV isolation, and centrifuged at 500×g for 10 minutes before being filtered with a 0.22 µm filter. The supernatant was then centrifuged at 120,000×g for 90 minutes to obtain the EV pellet. The EV pellet was resuspended with PBS before undergoing another round of ultracentrifugation at 120,000×g for 90 minutes. The final pellet was resuspended in PBS for further characterisation and analysis.

Single Particle Analysis using Flow Cytometry

EVs were analysed using the NanoFCM Flow NanoAnalyzer (NanoFCM) and CytoFLEX LX (Beckman Coulter) flow cytometers. Antibody staining was performed to characterise the EVs using the protocol described in our co-labelling application note[4]. The samples were stained with Alexa Fluor® 647 (AF647) anti-human CD63 antibody (BioLegend, Catalogue No.: 353016) (4 µg/mL) for 30 minutes at room temperature and subsequently analysed using flow cytometry with an appropriate dilution.

Cellular Uptake of Endogenously Labelled EVs

5×10^4 A549 cells/mL were plated on an 8-well chamber slide. The cells were subsequently incubated with 5×10^9 EVs/mL for 6 and 24 hours respectively. The cells were then washed with PBS thrice before imaging on the FV3000 confocal microscope (Evident Scientific).

Results

EVs isolated from Aco-520-labelled A549 cells were found to be endogenously labelled with Aco-520 (Figure 2). Single particle analysis demonstrated a predominantly Aco-520-positive EV population, indicating the successful incorporation of the dye into the EV membrane.

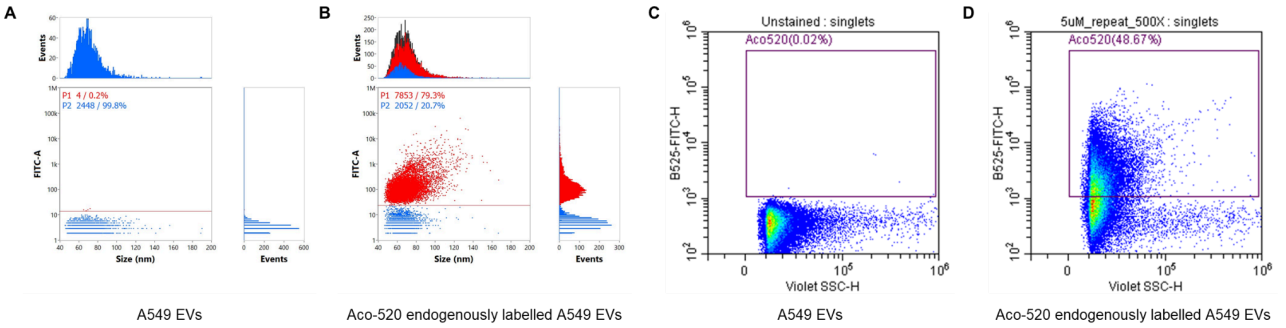

Figure 2: Single particle analysis of A549 EVs. (A) Unlabelled A549 EVs and (B) A549 EVs endogenously labelled with Aco-520 analysed on the NanoFCM Flow NanoAnalyzer. Corresponding analyses of (C) unlabelled A549 EVs and (D) A549 EVs endogenously labelled with Aco-520 on the CytoFLEX LX.

To further characterise the EV populations, the expression of tetraspanin marker CD63 was assessed using a dual-staining approach (Figure 3). With this approach, Aco-520 verified the presence of a lipid bilayer, while the AF647-conjugated anti-CD63 antibody identified a specific CD63-positive EV subpopulation. Notably, the inclusion of Aco-520 provides an additional layer of confidence in EV identification by distinguishing bona fide membrane-bound vesicles from non-vesicular events, such as protein or antibody aggregates, which may otherwise contribute to false-positive signals[5].

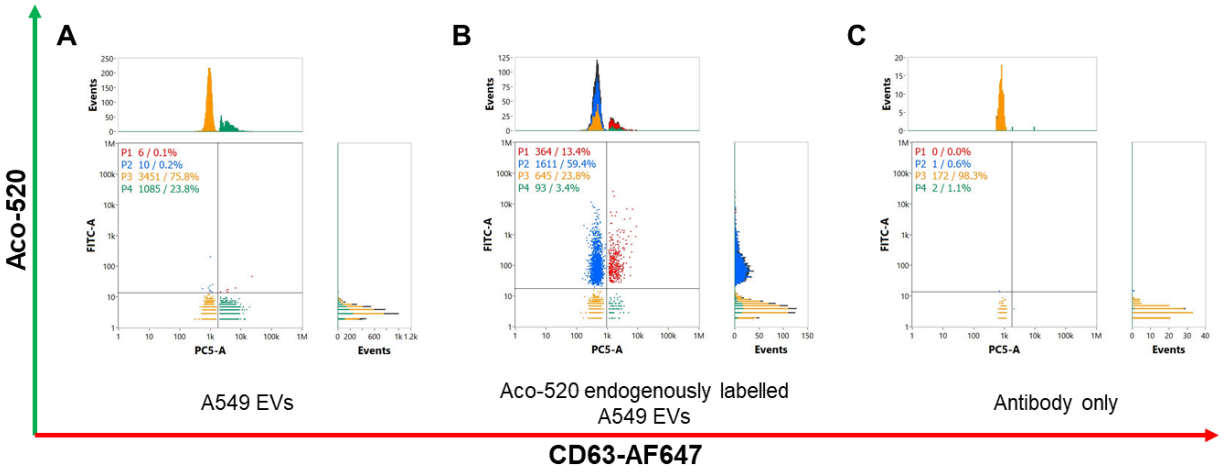

Figure 3: NanoFCM Flow NanoAnalyzer analysis of protein marker CD63 on (A) unlabelled A549 EVs and (B) A549 EVs endogenously labelled with Aco-520. (C) Free antibody included as a negative control.

The Aco-520 endogenously labelled EVs were subsequently evaluated in an in vitro cellular uptake study. Following incubation with recipient cells, Aco-520 remained readily detectable for up to 24 hours, enabling the visualisation and tracking of EV uptake over time (Figure 4). These results demonstrate the stability of the endogenous Aco-520 label.

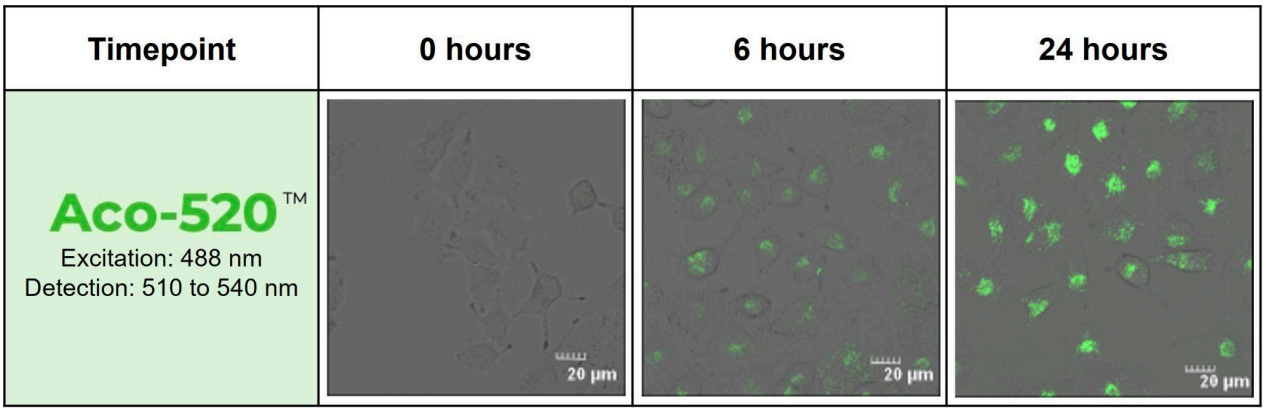

Figure 4: Confocal imaging of A549 cells incubated with Aco-520 endogenously labelled EVs for 0, 6 and 24 hours respectively.

Conclusion

Aco-520 demonstrated successful endogenous labelling of EVs, producing fluorescently labelled vesicles that are readily compatible with downstream analytical workflows. In this study, the resulting endogenously labelled EVs were utilised for cellular uptake studies and visualised by confocal microscopy, providing a practical approach for investigating EV uptake and intracellular tracking.

The ability to generate fluorescently labelled EVs during biogenesis offers several advantages over conventional post-isolation staining approaches. As the label is incorporated directly into EV membranes during vesicle formation, no additional staining or purification steps are required following EV isolation. This minimises sample handling and avoids downstream processing steps, such as ultracentrifugation or ultrafiltration, which have been reported to reduce EV recovery and alter EV characteristics[6].

As other Aco-Dyes share similar membrane-intercalating and fluorogenic properties, this endogenous labelling strategy may be extended across the broader Aco-Dyes portfolio. Endogenously labelled EVs can support a wide range of applications, including EV characterisation, investigation of intra- and intercellular communication, and biodistribution tracking. The utility of COEs for endogenous labelling extends beyond cultured cells. Using a similar strategy, Aco-650™ has been employed in organoid models to visualise endosomal membrane transfer between organoids, highlighting the versatility of COEs for studying vesicle-mediated communication in complex biological systems[7]. By enabling direct labelling

during EV biogenesis while preserving EV yield and native vesicle characteristics, Aco-Dyes provide researchers with a streamlined workflow for studying EV biology and exploring the therapeutic potential of EVs.

Guidelines for Using Aco-Dyes

1. Acoerela dyes vary in intensity and staining efficiency. It is recommended to optimise the method for each dye, cell type and study condition.
2. Conduct a titration study to determine the optimal concentration and duration to label the cells. Suggested concentration range: 1 to 20 µM. Suggested duration range: 24 to 48 hours.

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