FLuoEVs as a Robust Positive Control for EV Uptake Assays

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Introduction

The study of EV internalization is essential for understanding EV-mediated intercellular communication and therapeutic cargo delivery. Reliable interpretation of uptake experiments requires appropriate controls to distinguish true intracellular EV signal from fluorescence artefacts arising from free dye, nonspecific membrane staining, or imaging misconfiguration. The inclusion of a standardized positive control is therefore critical for reliable assay validation.

FluoEVs are highly purified EVs isolated from HEK293 cells engineered to express fluorescently tagged tetraspanins, including CD9-EGFP, CD63-EGFP, and CD81-EGFP. The genetically encoded fluorescence enables direct visualization of EVs without the need for post-isolation labelling.

In this application note, we demonstrate the use of CD63-EGFP FluoEVs as a standardized positive control in a confocal microscopy-based uptake assay using KGN cells. For contextual comparison, follicular fluid (FF)-derived EVs were fluorescently labelled using a membrane dye and analyzed under identical experimental conditions. A dye-only control was included to validate removal of unbound dye and confirm assay specificity.

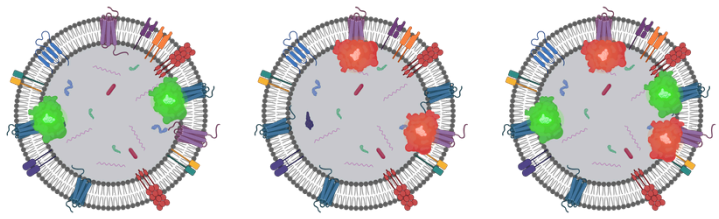

Figure 1: FLuoEVs expressing EGFP and/or mCherry in fusion with canonical EV markers, namely CD9, CD63, and CD81

Materials and Methods

FF-derived EVs were fluorescently labeled using a commercial EV Labeling kit, according to the manufacturer's instructions. To ensure efficient removal of unbound dye, labelled samples were purified using miniPURE-EVs spin columns (HansaBioMed Life Sciences). To evaluate possible dye-derived artefacts, DPBS was used as dye-only negative control. FluoEVs CD63-EGFP EVs were used directly as a positive control without additional labelling or purification steps.

The granulosa-like tumor cell line KGN was used as a cellular model for uptake experiments. A total of 70,000 cells were seeded onto 13 mm glass coverslips and cultured for 24 h prior to treatment. Cells were then incubated for 6 h with 10^8 FF EVs, 10^8 FluoEVs, or dye-treated and column-purified DPBS (negative control).

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After incubation, cells were washed 3× with DPBS and fixed with 4% paraformaldehyde for 10 min. Then, they were permeabilized with 0.5% Triton X-100 for 5 min and blocked with 5% BSA for 1 h.

To visualize cellular architecture, samples were incubated overnight at 4°C with mouse anti-tubulin antibody (cat. no 2315513, DSHB; 1:500), followed by a 1 h incubation with DyLight 405 secondary antibody (cat. no 715-475-150, Jackson ImmunoResearch; 1:300). Coverslips were mounted using ProLong Gold Antifade Mountant (Thermo Fisher Scientific).

Imaging was performed using a Zeiss LSM 900 microscope equipped with a Plan-Apochromat 63×/1.4 oil immersion objective. Serial z-stacks were acquired using bidirectional scanning with line averaging to ensure high signal-to-noise ratio. All images were captured under identical laser intensity and detector gain settings across all conditions to ensure direct comparability.

Results

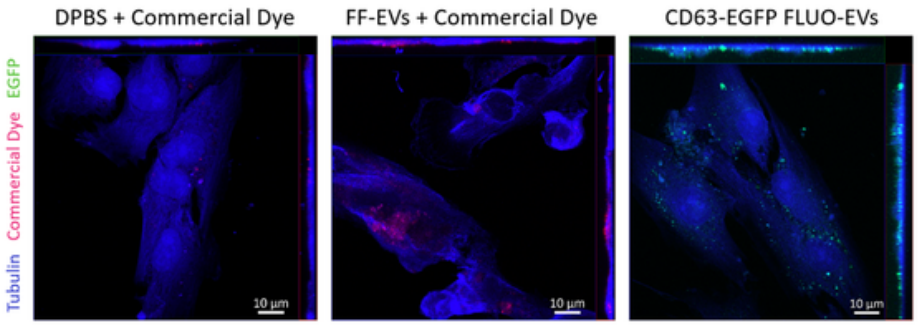

Figure 2: Maximum intensity projections of z-stack optical sections showing the uptake of EVs by KGN cells following 6 hours of incubation. Tubulin staining was used to visualize cellular structure. commercial membrane dye was used for FF EV labelling.

KGN cells treated with CD63-EGFP FluoEVs exhibited bright and well-defined intracellular fluorescent puncta distributed throughout the cytoplasmic compartment. Fluorescence was consistently detectable across multiple optical sections in z-stack acquisitions, confirming EV internalization rather than surface association.

In contrast, cells treated with membrane dye-labelled FF EVs displayed weaker and more diffuse fluorescence compared to FluoEV-treated cells.

Cells exposed to dye-treated and column-purified DPBS showed no detectable intracellular fluorescence under identical imaging settings, confirming effective removal of unbound dye and absence of dye-derived artefacts.

The strong and reproducible EGFP signal provided by FluoEVs facilitated reliable calibration of confocal imaging parameters and validation of uptake assay conditions prior to analysis of experimental EV samples.

Conclusion

- FLuoEVs provide a bright, stable and reproducible positive control for EV uptake assays, enabling reliable calibration of confocal imaging parameters and optimization of experimental conditions.
- The well-defined intracellular punctate signal generated by FluoEVs demonstrates improved clarity and consistency compared to EVs labelled with lipophilic membrane dyes.

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