Enhanced quantification and cell tracking using dual-fluorescent labeled extracellular vesicles

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Extracellular vesicles (EVs) are membrane-limited lipid bilayer vesicles released by many cell types, including both prokaryotic and eukaryotic organisms. One of the main functions of EVs that makes them so interesting, is their key role in cell-to-cell communication. [1], [2]. According to the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2023 guidelines, EVs subtypes are classified as a function of different parameters, i.e., size, biogenesis, and composition [3], [4]. Accordingly, EVs have been divided into three classes: small-sized extracellular vesicles (also known as exosomes), microvesicles (MVs) and apoptotic cell-derived vesicles [5]. Nevertheless, EVs subtypes show overlapping dimensions, share some biomarkers and are similar in shape and density. Consequently, it remains difficult to cleanly isolate and distinguish between them. For this reason, sometimes it is difficult to refer to one specific type and, thus, they are referred to as small EVs (sEVs), including the group whose size comprises 30-150 nm [6].

Due to their natural tropism, there is an increasing interest in using sEVs, for diagnostic and therapeutical applications [7]. Regarding this last application, sEVs have certain advantages that make them a preferred choice for new therapeutic developments. sEVs display a more defined composition and tend to be more homogenous in terms of size compared to other types. and sEVs show an increased specificity and efficiency in targeting or delivering cargo to recipient cells [8]. In addition, since they exhibit unique biocompatibility, low immunogenicity, and reduced toxicity, so they can travel safely throughout extracellular fluids, sEVs have been explored as natural drug nanodelivery systems [9].

Despite the well-defined properties of EVs, their clinical studies and applications present technical and biological limitations that make it still marginal [10]. Distinguishing specific subpopulations of EVs from heterogeneous EVs remains a challenging task due to their heterogeneity and the limitations of current detection methods and the development of new strategies is needed since it allows a proper control of targeted EVs. These limitations, together with the lack of exclusive biomarkers, cost-effective quantification methods, and high-resolution visualization techniques, complicate the development of reliable and efficient techniques for purification, characterization, quantification and tracking studies of sEVs [11]. For this reason, is essential the development of advanced techniques that allow the distinction and quantification of sEVs within any type of sample.

An interesting approach to better distinguish sEVs from the background, consists in their selective labelling. The development of fluorescence-based monitoring tools has become of great interest for developing new techniques that ease the research in sEVs and their applications, including, cargo loading, recipient cells uptake and biodistribution [12], [13]. One promising method is the production of fluorescently labelled EVs by generating genetically engineered donor cells lines expressing fluorescent proteins fused to other proteins that are enriched in EVs, with minimal disturbance of their physicochemical and biological properties.

A single labelling of a tetraspanin (EV transmembrane protein) is not enough to identify a specific type of EVs population. In order to overcome this drawback, in this study, we aimed to develop a dual fluorescence reporter system using a tricistronic vector, which enables stable co-expression of two transgenes along with antibiotic resistance for mammal cell cultures selection [14], [15]. For this, eGFP and mCherry were fused to the N-terminal domain of CD63 and CD9, respectively, which are tetraspanins specific or enriched in sEVs.

Double fluorescently labelled sEVs were produced in a stable transfected HEK293SF-3F6 cell line. Fluorescently labelled sEVs were characterized using a variety of techniques (**Figure 1**), including, Flow cytometry (FC), Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), Western Blot (WB) and Confocal Spectral Fluorescent Microscopy (CSFM). The results about protein expression showed that the fused proteins were efficiently incorporated into sEVs. The size distribution and concentration of modified sEVs were compared with controls, indicating that the genetic engineering did not affect the structure and morphology of sEVs. The functionality of fluorescently labelled sEVs was assessed by recipient cells uptake assay. Results showed that sEVs functionality persists post-purification (**Figure 2A and B**). Moreover, fluorescently labelled sEVs were quantified by nanoflow cytometry, allowing to distinguish sEVs from other EVs or particles, and these values were compared to direct fluorescence measurements, obtaining a linear correlation, statistically validated, enabling a sEVs quantification method. In conclusion, this study describes a feasible, cheap, and fast methodology for EVs characterization, trafficking, and intercellular communication, as well as for sEVs quantification.

In conclusion, the production of dual-fluorescent labelled extracellular vesicles, specifically for small-sized EVs subpopulation, enhances the precision of sEVs tracking and enables a robust, cost-effective, and rapid quantification and characterization for sEVs of interest. This method also improves control over dosage-dependent studies involving sEVs. Likewise, modified sEVs preserved their attributes and their functionality, as demonstrated by executing the current MISEV guidelines. This strategy is a valuable tool for therapeutic sEVs development and drug delivery.

References

[1] A. Bobrie and C. Thery, "Exosomes and communication between tumours and the immune system: Are all exosomes equal?," in Biochemical Society Transactions, Feb. 2013, pp. 263–267. doi: 10.1042/BST20120245.

[2] M. Simons and G. Raposo, "Exosomes - vesicular carriers for intercellular communication," Aug. 2009. doi: 10.1016/j.ceb.2009.03.007.

[3] J. A. Welsh et al., "Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches," J Extracell Vesicles, vol. 13, no. 2, Feb. 2024, doi: 10.1002/jev2.12404.

[4] M. Yáñez-Mó et al., "Biological properties of extracellular vesicles and their physiological functions," 2015, Co-Action Publishing. doi: 10.3402/jev.v4.27066.

[5] Q. Cai et al., "Message in a Bubble: Shuttling Small RNAs and Proteins between Cells and Interacting Organisms Using Extracellular Vesicles," Jun. 17, 2021, Annual Reviews Inc. doi: 10.1146/annurev-arplant-081720-010616.

[6] H. Aheget, L. Mazini, F. Martin, B. Belqat, J. A. Marchal, and K. Benabdellah, "Exosomes: Their role in pathogenesis, diagnosis and treatment of diseases," Cancers (Basel), vol. 13, no. 1, pp. 1–45, Jan. 2021, doi: 10.3390/cancers13010084.

[7] Y. S. Kim, J. S. Ahn, S. Kim, H. J. Kim, S. H. Kim, and J. S. Kang, "The potential theragnostic (diagnostic+therapeutic) application of exosomes in diverse biomedical fields," Mar. 01, 2018, Korean Physiological Soc. and Korean Soc. of Pharmacology. doi: 10.4196/kjpp.2018.22.2.113.

[8] J. P. Nederveen, G. Warnier, A. Di Carlo, M. I. Nilsson, and M. A. Tarnopolsky, "Extracellular Vesicles and Exosomes: Insights From Exercise Science," Feb. 01, 2021, Frontiers Media S.A. doi: 10.3389/fphys.2020.604274.

[9] S. M. Van Dommelen et al., "Microvesicles and exosomes: Opportunities for cell-derived membrane vesicles in drug delivery," Jul. 20, 2012. doi: 10.1016/j.jconrel.2011.11.021.

[10] L. Bahmani and M. Ullah, "Different Sourced Extracellular Vesicles and Their Potential Applications in Clinical Treatments," Jul. 01, 2022, MDPI. doi: 10.3390/cells11131989.

[11] X. Li et al., "Challenges and opportunities in exosome research-Perspectives from biology, engineering, and cancer therapy," Mar. 01, 2019, American Institute of Physics Inc. doi: 10.1063/1.5087122.

[12] G. Corso et al., "Systematic characterization of extracellular vesicles sorting domains and quantification at the single molecule–single vesicle level by fluorescence correlation spectroscopy and single particle imaging," J Extracell Vesicles, vol. 8, no. 1, Dec. 2019, doi: 10.1080/20013078.2019.1663043.

[13] K. Dooley et al., "A versatile platform for generating engineered extracellular vesicles with defined therapeutic properties," Molecular Therapy, vol. 29, no. 5, pp. 1729–1743, May 2021, doi: 10.1016/j.ymthe.2021.01.020.

[14] B. Carrillo Sanchez, M. Hinchliffe, and D. G. Bracewell, "GFP-tagging of extracellular vesicles for rapid process development," Biotechnol J, vol. 17, no. 6, Jun. 2022, doi: 10.1002/biot.202100583.

[15] K. Lee, S. Y. Kim, Y. Seo, H. Kwon, Y. J. Kwon, and H. Lee, "Multicistronic IVT mRNA for simultaneous expression of multiple fluorescent proteins," Journal of Industrial and Engineering Chemistry, vol. 80, pp. 770–777, Dec. 2019, doi: 10.1016/j.jiec.2019.06.042.

Figures

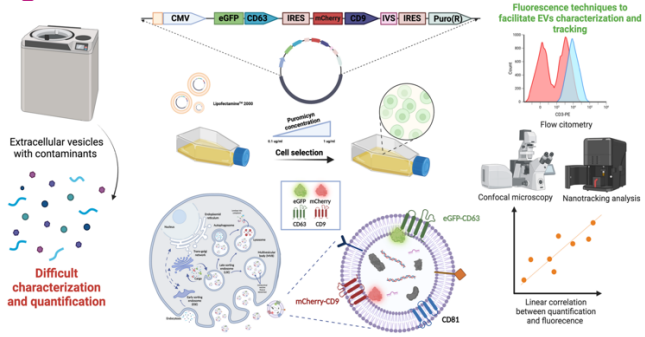

Figure 1. Existing problematic and followed methodology for obtaining double labeled stable sEVs and subsequent characterization.

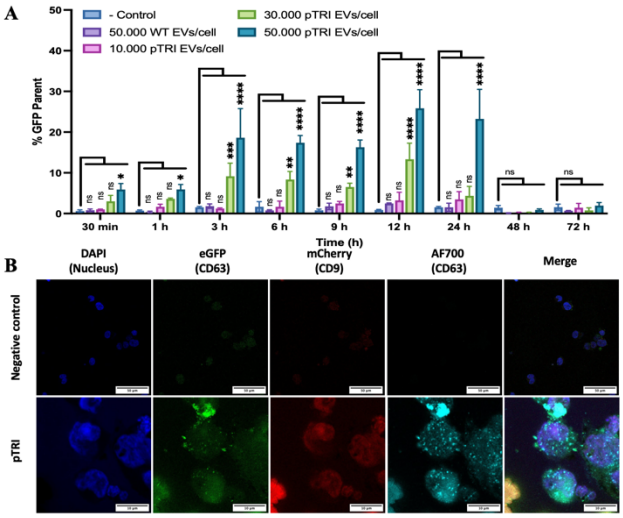

Figure 2. HEK293SF-3F6 double labelled EVs uptake study. **A.** Fluorescent eGFP positive percentage quantification assay with three different EVs concentrations. **B.** Qualitative evaluation of the uptake levels of 50.000 part/cell after 4 h and localization, upper negative control with non-modified EVs