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ANALYSIS OF EXTRACELLULAR VESICLES AT THE SINGLE LEVEL USING FLUORESCENCE CORRELATION SPECTROSCOPY ASSISTED BY MICROFLUIDICS *

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Abstract

We demonstrate a sensitive methodology for single-vesicle analysis of extracellular vesicles (EVs) using fluorescence correlation spectroscopy (FCS) combined with microfluidic platforms. This integrated approach enhances detection resolution, enabling quantitative characterization of EV size, concentration, and dynamics in complex biological samples.

Extracellular vesicles (EVs) are nanoscale lipid bilayer-enclosed particles secreted by virtually all cell types. They play key roles in intercellular communication, immune modulation, and tumor progression, and serve as promising biomarkers in liquid biopsies [1]. Due to their small size (30–200 nm) and heterogeneous nature, accurate characterization of EVs at the single-particle level remains a significant analytical challenge [2]. To address these challenges, we developed an integrated approach combining fluorescence correlation spectroscopy (FCS) with microfluidic control for high-sensitivity single-EV analysis. EVs were fluorescently labeled using a lipophilic dye that incorporates into the vesicle membrane, enabling reliable signal detection. Labeling efficiency and specificity were confirmed using nanoparticle tracking analysis and confocal imaging.

FCS enables high-resolution analysis of fluorescently labeled particles diffusing through a confocal volume, yielding information on particle size, concentration, and diffusion dynamics. However, conventional FCS is limited by low throughput and poor control over particle flow [3]. To overcome these drawbacks, we incorporated a precisely engineered microfluidic system that ensures stable sample flow, reduced background, and improved reproducibility. Hydrodynamic focusing was used to guide EVs through the detection region in a narrow stream, allowing consistent excitation conditions. To interpret the data under flow, we used a laminar flow-adapted diffusion model that accounts for both lateral diffusion and axial convection. This model was validated using monodisperse fluorescent beads with known physical properties.

In addition to autocorrelation analysis, we applied burst analysis to the same fluorescence time traces. This method detects and characterizes individual fluorescence bursts as single EVs transit the detection volume. Burst parameters such as intensity, duration, and count rate provide valuable information about vesicle brightness, concentration, and heterogeneity. This approach complements FCS by enabling discrete, event-based analysis and improving detection of rare or highly fluorescent particles [4]. In the EV samples derived from human plasma and conditioned cell culture media the described methods revealed in the distinct EV subpopulations with different diffusion behaviors and brightness profiles, suggesting heterogeneity in size, composition, or labeling efficiency.

Overall, the integration of FCS with microfluidics and burst analysis provides a robust and sensitive platform for high-resolution, single-vesicle characterization. This system allows for precise measurement of EV size, concentration, and heterogeneity while capturing dynamic behaviors under flow. Building on this foundation, we are now extending the method to investigate specific molecular features of individual EVs. As a first step, we aim to fluorescently label and detect key surface proteins such as CD63, CD9, and HER2, which are relevant for vesicle classification and disease-specific profiling. This direction moves us toward molecular-level characterization of exosomes in complex biological samples, with the long-term goal of enabling targeted biomarker discovery, refined diagnostics, and improved EV-based therapeutic development.

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