Application Note

Concentration and colocalization analysis of fluorescent EVs using the NanoQNT

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Introduction

Extracellular vesicles (EVs) including exosomes (also known as small EVs), microvesicles, and apoptotic bodies (collectively called large EVs) have been identified in many biological fluids, including blood, saliva, and urine. Being produced by many organisms and cell types, EVs display different lipids, proteins, and nucleic acids, which play a vital role in cell-to-cell communication (Figure 1). EVs have demonstrated tremendous potential in the fields of regenerative medicine, therapy, and the diagnosis of diseases.

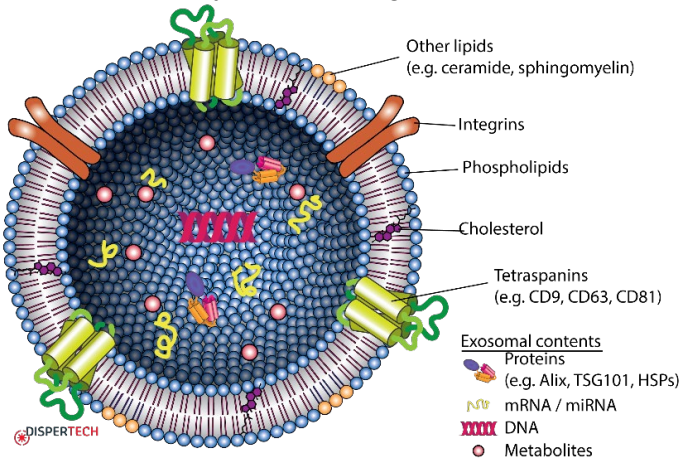

Figure 1. Schematic representation of an extracellular vesicle and its lipids, proteins, and nucleic acids components.

Colocalization of biomarkers with the NanoQNT

This application note demonstrates the potential of the NanoQNT, a device developed by Dispertech that utilizes light sheet fluorescence microscopy to rapidly determine EV particle concentrations. The measurements make use of user-defined sectioned image analysis of fluorescent EVs immobilized in a hydrogel (Figure 2a). As the light sheet is moved through the hydrogel with nanometer precision, an accurate sample volume can be reconstructed from the sectioned images (Figure 2b). Subsequently, the Z-stack of images is analyzed from which the concentration of EVs can be determined (Figure 2c).

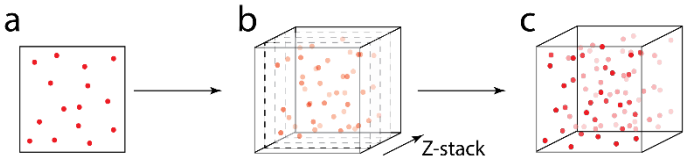

Figure 2. (a) The light-sheet illuminated sections in the hydrogel are imaged. (b) A Z-stack is reconstructed from which the measurement volume can be determined. (c) The analysis is performed on the Z-stacks from which particle concentration can be determined.

The hydrogel immobilization process also ensures that the fluorescent EVs remain in the field of view, allowing fluorescent biomarker concentration analysis and colocalization using up to 4 excitation wavelengths in rapid succession.

We utilized the FluoEVs, produced by HansaBioMed Life Sciences. FluoEVs are fluorescent EVs stably mono-labeled expressing EGFP or mCherry as a fusion protein with CD9 or CD63, or dual labeled expressing both fusion proteins CD9-mCherry/CD63-EGFP. In this application note we demonstrate the potential of our NanoQNT to accurately determine EV particle concentrations and perform colocalization of dual-labeled EVs within minutes.

Material and methods

FluoEVs were analyzed using nanoparticle tracking analysis (NTA) in fluorescence mode (Zetaview analyzer, Particle Metrix). Each sample of lyophilized FluoEVs was reconstituted in water according to the manufacturer's specifications to achieve a concentration of 10^8 particles/mL and subsequently diluted 10-fold with phosphate buffered saline (PBS) to achieve a particle concentration of 10^7 particles/mL.

The NanoQNT (Dispertech, Amsterdam, The Netherlands) was utilized to rapidly determine the concentration of the FluoEVs based on fluorescent EGFP or mCherry-labeled tetraspanins CD9 and CD63. The FluoEV sample was immobilized in an acrylamide / bis-acrylamide–based hydrogel created using a 96-square well plate.

Two fluorescence channels were employed in the NanoQNT to obtain the excitation of EGFP at 488 nm, mCherry at 561 nm, or both EGFP and mCherry at 488 and 561 nm, respectively. The resulting stacks of images where fluorescent EVs are revealed as spots were analyzed by custom-made NanoQNT analysis software.

Analysis of mono-labeled EVs

The results were obtained using the NanoQNT analysis software, which algorithmically determines the particle concentration based on image analysis, where each fluorescent EV appeared as a bright spot on the image taken by a camera.

Table 1. Expected concentrations based on NTA versus determined concentrations using the NanoQNT.

EV sample	Expected conc. (particles/mL)	NanoQNT conc. (particles/mL)
CD9-EGFP	1×10^8	1.9×10^8
	1×10^7	1.5×10^7
CD63-EGFP	1×10^8	2.0×10^8
	1×10^7	2.9×10^7
CD63-mCherry	1×10^8	6.0×10^7
	1×10^7	5.6×10^6

As presented in Table 1, the EV particle concentrations of the EGFP or mCherry-labeled FluoEVs were within the expected concentration range as determined by NTA.

Analysis and colocalization of dual-labeled EVs

Analysis of the stack of images derived from the dual-labeled EVs consisting of CD9-mCherry and CD63-EGFP was also performed. Figure 2 shows a predominant overlap between fluorescent spots in the EGFP channel (Figure 2a) and in the mCherry channel (Figure 2b). However, not all spots exhibit colocalization , suggesting that a subset of EVs only has one fluorescently labeled tetraspanin.

The asymmetry of the scattering plot shown in Figure 2c, confirms the difference in fluorescence intensity between the two channels. Further analysis of the EVs demonstrates the concentration differences between the two channels and particles with dual labeling (Table 2).

Table 2. Analysis of the dual-labeled (CD9-mCherry/CD63-EGFP) EVs using the NanoQNT.[a]

Measured dual labeled conc. (particles/mL)	Measured EGFP conc. (particles/mL)	Measured mCherry conc. (particles/mL)
8.75×10^7	1.75×10^8	1.15×10^8

[a] Total expected particle conc.: 10^8 particles/mL.

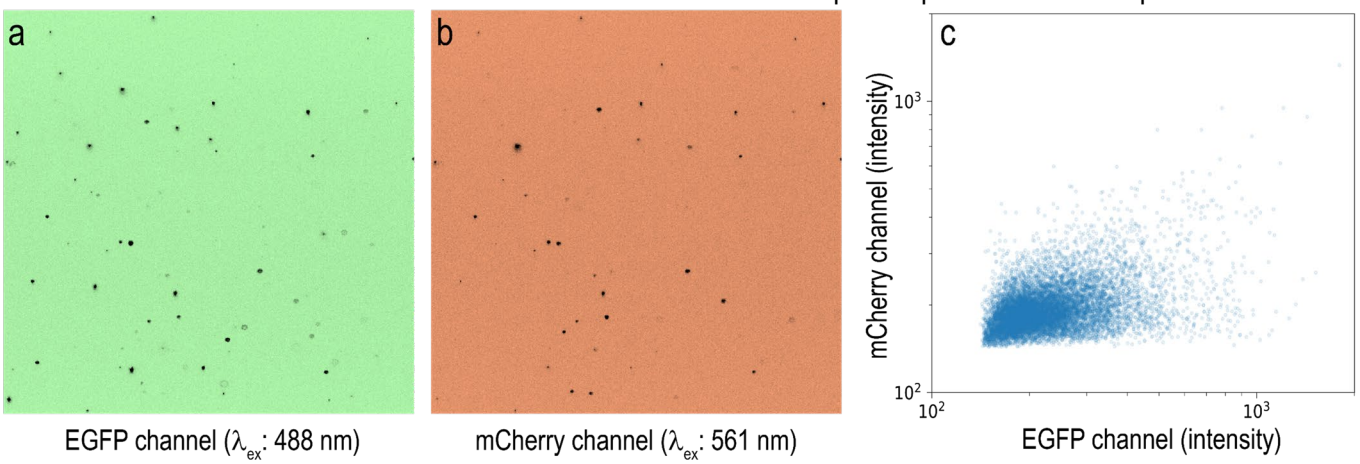

Figure 2. Colocalization analysis of CD9-mCherry/CD63-EGFP dual labeled EVs showing (**a**) the EGFP channel using a laser operating at 488 nm and (**b**) the mCherry channel using a laser operating at 561 nm. (**c**) A scattering plot showing the fluorescence intensity from the EGFP and mCherry channels.

Conclusions

- Accurate analysis of EV particle concentrations within minutes.
- Colocalization analysis of dual-labeled EVs.
- Population analysis and quantitation of dual-labeled EVs.

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