NTA goes colocalization: Detection and measurement of CD9 and CD41 on double-stained human platelet derived MSC-EV preparations

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Abstract

Over the last decade, Nanoparticle Tracking Analysis (NTA) has become a well-established technique for the characterization of extracellular vesicles (EVs). Initially, NTA was used for the un-targeted measurement of EV size distribution and concentration, while operated in standard scattering mode. More recent innovations reached a milestone when low-bleach fluorescence-based NTA (F-NTA) became available, providing a much-improved detection efficiency and more targeted analysis, as compared to unmodified F-NTA. With the newly released Particle Metrix ZetaView® system, model PMX-230 TWIN (a two laser NTA design), it is now possible to detect two independent fluorochromes on the same particle. Due to ultra-fast switching times between the two excitation lasers and their respective fluorescence emission filters, it is possible to overlay and colocalize the corresponding fluorescent signals. In this report, we describe the quantification of colocalization ratios on double-stained MSC-derived EVs using fluorochrome conjugated antibodies against the cell surface antigens CD9 and CD41, which also serve as EV marker proteins. Furthermore, we compare the NTA colocalization results with the results of imaging flow cytometry and elucidate the comparability of both orthogonal methodologies.

Keywords: colocalization, Nanoparticle Tracking Analysis (NTA), extracellular vesicles, CD41, CD9, antibody, fluorescence, membrane

Introduction

Exosomes are derivatives of the cellular endosomal system. When using NTA, together with other small EVs, exosomes appear as nanoparticles with diameters between 70 – 150 nm [1, 2]. Small EVs are involved in many cellular processes such as (i) antigen-specific T-cell response, (ii) cell-cell communication, (iii) inflammatory processes, (iv) metastasis of tumors and (v) many other patho-physiological activities [3-7].

Small EVs can be prepared from various liquid biopsies including blood, urine and various cell culture supernatants, e.g. from mesenchymal stem/stromal cells (MSCs) conditioned media [8-10]. Many important cell-specific surface molecules like integrins, cell adhesion molecules and tetraspanins are associated with small EVs [11-13]. Because of their pivotal therapeutic potential, EVs from selected cell types are intensively studied in many laboratories around the world. In addition to classical biochemical and molecular biological techniques, NTA emerged as a key method for the characterization of EVs [14].

The traditional NTA method measures the total concentration of particles from a range of 10 - 1000 nm; however, targeted examination of biological particles is not feasible with only light scattering detection.

With the development of fluorescence-NTA, fluorescently labelled sub-populations, marked by lipid dyes or labelled antibodies, can be analysed. In addition, newly developed colocalization NTA (C-NTA) measurements provide more specific biological information by detecting fluorescence signals from distinct particle phenotypes within a single sample, all in one single and very fast measurement.

We describe the quick & easy antibody staining of human platelet lysate-derived mesenchymal stem cell EVs (MSC-EVs) using combined antibodies, anti-CD9 & anti-CD41. We show successful colocalization measurements on double-positive EVs, using C-NTA and we confirm these results with orthogonal data from imaging flow cytometry.

Methods

Cell culture and purification of MSC-EVs

EVs were derived from MSC conditioned media grown in the presence of human platelet-lysate via a PEG precipitation procedure [15, 16].

Fluorescence labelling

For antibody labelling, a cocktail of (i) 1 µl of MSC-EVs with a measured concentration of $3.7x10^{11}$ EV/ml, were mixed with (ii) 7.5 µl of a 1:7.5 dilution of anti-Hu CD9, an APC-conjugated antibody (clone MEM-61), (iii) 10 µl of a 1:10 dilution of anti-Hu CD41-AF488 antibody (clone MEM-06), and (iv) 1.5 µl particle-free PBS buffer (Gibco, Fisher Scientific Paisley, UK). Both pre-labeled antibodies were obtained from EXBIO, Vestec (Czech Republic). The cocktail was incubated for 2 hours in the dark at room temperature. After incubation, the mixture was supplemented with PBS buffer to a final volume of 2 ml and used for subsequent NTA and IFCM analyses (200 µl).

Lysis controls were prepared with non-ionic detergent NP-40 Alternative (herein named NP-40), from Calbiochem, Merck, Darmstadt (Germany) and used to rupture the membrane of EVs. Following incubation, 20 µl of 0.5% NP-40 solution was added to the labeled EV cocktail, resulting in a final concentration of 0.25% NP-40, and then incubated an additional 30 minutes at room temperature in the dark. The lysed cocktail was also diluted into 2 ml of PBS for NTA and IFCM analyses (200 µl).

NTA and Colocalization measurements

NTA measurements of size and concentration were carried out using a ZetaView® PMX-420 QUATT equipped with the software version 8.05.14 SP7 as well as a PMX-230 TWIN running the Particle Metrix Software Suite and the ZetaNavigator software version 1.0 (SOPs in Tables 1 & 2). The TWIN model was used to obtain colocalization data. As described previously [17], colocalization experiments with a ZetaView® instrument comprise an automated sequence of steps. Short video sequences are recorded from the red (ex640/F660) and green (ex488/F500) fluorescence channel, followed by an overlay of both channels. A colocalization event occurs when a particle containing both red and green fluorescent tags yields detection in both channels, also within a certain Brownian range of motion. Colocalization ratios are calculated by dividing the number of colocalization events by the detections of the channels ex640/F660 and ex488/F500, respectively.

Table 1: Measurement parameters on a PMX-420 QUATT

Measurement parameters	Scatter Mode (λ=488 nm)	Fluorescence Mode (λ=488F500 nm)	Fluorescence Mode (λ=640F660 nm)
Positions	11	11	11
#Cycles	2	2	2
Number of frames	30 (Med.)	30 (Med.)	30 (Med.)
Sensitivity	80	95	94
Shutter	100	200	200
MinBrightness	30	30	30
MaxArea	1000	1000	1000
MinArea	10	10	10
Tracelength	15	15	15
Framerate	30 fps	30 fps	30 fps
Tracking Radius	100	100	100

Table 2: Additional parameters used for colocalization measurements on a PMX-230 TWIN

Measurement parameters	Scatter Mode (λ=488 nm)	Fluorescence Mode (λ=488F500 nm)
Positions	3	3
#Cycles	1	1
Video length	35	35
Sensitivity	93	95
Shutter	300	300
Min Size / nm	1	1
Max Size / nm	1000	1000
MinArea	1	1
MaxArea	1000	1000
Framerate	30	30
Measurement parameters	Additional colocalization settings	
Trace Length	3	
Link Radius	10	
Switch Frame	6	
Laser off frames	5	

A unique algorithm is required to measure colocalization data with the PMX-230 TWIN, resulting in different camera settings from other NTA analyses with the PMX-420 QUATT.

Imaging flow cytometry (IFCM)

IFCM measurements were carried out by using an Amnis® ImageStream®X Mark II (Luminex Corporation, Seattle, WA, USA) with the parameters shown in Table 3.

Table 3: Measurement parameters used for IFCM

Laser λ [nm]	Used Laser Power	Max. Laser Power	Filter [nm]
375	70	70	-
488	100	100	FITC (Ch02) 488-560
561	200	200	-
648	150	150	APC (Ch11) 642-745
785 (SSC)	70	70	SSC (Ch06) 756-780

Results & Conclusions

Size and concentration measurements

Standard NTA measurements in scatter mode with the ZetaView® system resulted in a median diameter (X50) of 127 nm and a concentration of $3.68x10^{11}$ particles per ml.

To specifically detect EV phenotypes in our samples, the particles were dual-labelled with an anti-CD9 APC antibody, which is reported as a typical EV-marker [18], and a human platelet lysate-specific anti-CD41 antibody conjugated with AF488. Subsequent F-NTA measurements with both markers yielded the desired results as all positive detections are derived from only CD9 positive or CD41 positive vesicles, respectively. All F-NTA measurements were performed in 5 replicates.

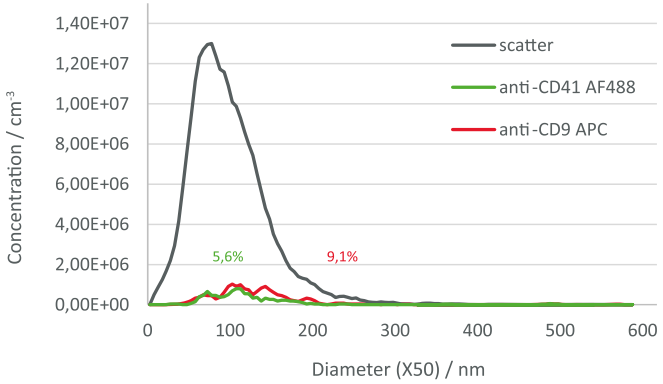

Figure 1: Size distribution of an MSC-EV preparation in scatter (grey) and fluorescence mode (green and red), simultaneously stained with anti-CD41 and anti-CD9 antibodies.

CD41-positive EVs resulted in a median diameter (X50) of 110.6 nm and a measured concentration of $2,06x10^{10}$ EV/ml. In contrast, the mean diameter of the CD9-positive EVs was 126.2 nm, which is comparable to the overall result of all particles measured in scatter mode. The measured concentration of CD9-positive EVs was $3.38x10^{10}$ EV/ml.

The percentage of CD9- and CD41-positive EVs detected in the red and green fluorescence channel were found to be 5.6% and 9.1%, respectively. This result was expected given that the sample was not purified, and contained many other different nanoparticles.

Colocalization studies

Measurement of colocalization generally refers to the simultaneous detection of at least two specific targets in close vicinity to each other within a biological specimen, where each target molecule is labelled with a specific fluorescent probe. Several key prerequisites for the accurate quantification of colocalization ratios using NTA are: i) illumination of identical measuring volumes by both lasers, ii) short switching times between laser channels, to minimize the loss of particles via diffusion out of the field of view, and iii) setting the search radius (i.e., the Brownian jump distance) to reduce false positive events [17]. A representative result of colocalization data for double-stained MSC-EVs is shown in Figure 2.

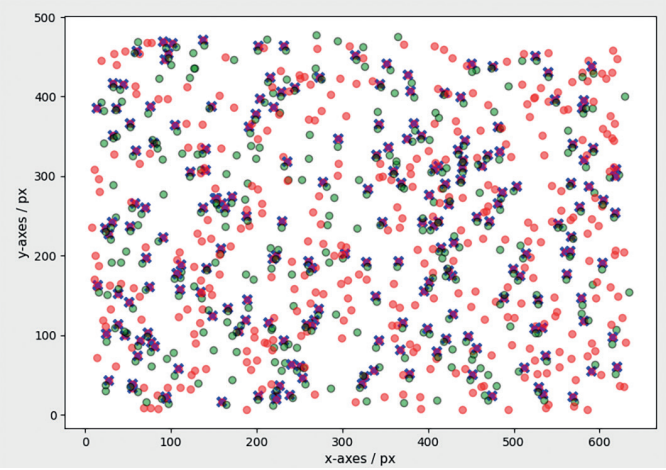

Figure 2: Representation of the x-y centroid coordinates of the scattering events of MSC-EVs double-stained with anti-CD9 APC and anti-CD41 AF488. Each green dot represents an EV positive for CD41 while each red dot indicates a CD9-positive particle. Double positive EVs are highlighted by a purple cross.

Colocalization measurements revealed an average ratio of 58.5% (+/-5.5), which means that 58% of the EVs positive for CD41 did carry the CD9 marker as well. This result is confirmed by imaging flow cytometry, (Fig. 3) which also gave 58% for double-positive EVs.

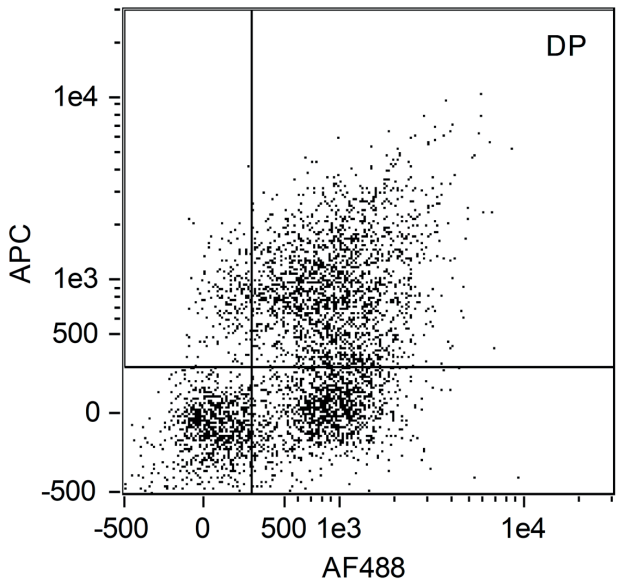

Figure 3: Scatter plot of the result of IFCM performed with double stained MSC-EVs. 58% of the EVs are considered as double-positive (DP) for CD41 and CD9.

To ensure that the fluorescent signals are derived from membranous particles and not from artefacts, lysis controls with NP-40 were carried out. Treatment of membrane containing particles with this non-ionic detergent will destroy the lipid layer and therefore diminishes the F-NTA signals.

NP-40 lysis controls resulted in very few detectable particles and a colocalization ratio average of only 4% (+/-4) via the TWIN system.

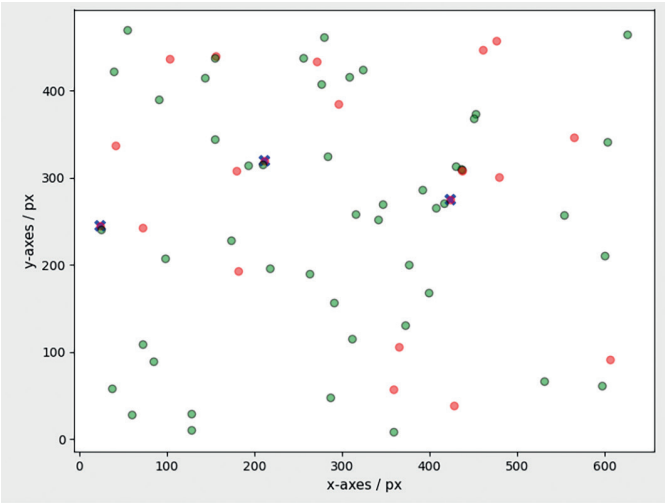

Figure 4: Representative of x-y centroid coordinates of the scattering events of double-labelled MSC-EVs incubated with 0.5% NP-40. 4% of the detected particles are double positive for both markers.

A drastic decrease in particle number as well as significant reduction in the colocalization ratio, from NP-40 controls, indicate that EV particles detected by F-NTA are indeed surrounded by a membrane. Further, our findings are confirmed by using IFCM with a value of 5% (per Figure 5).

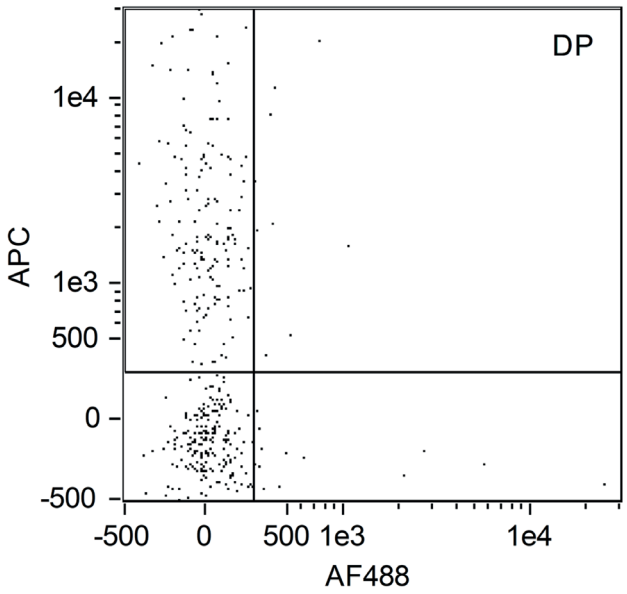

Figure 5: IFCM revealed significant reduction of colocalization to a value of 5% double-positive (DP) in the NP-40 lysis control.

Negative controls prepared without MSC-EVs, but containing all other components, also show a significantly reduced number of particles compared to the positive samples (figure 6). However, the diameter of the particles remains roughly the same (figure 7). Some non-EV particles are to be expected in EV samples.

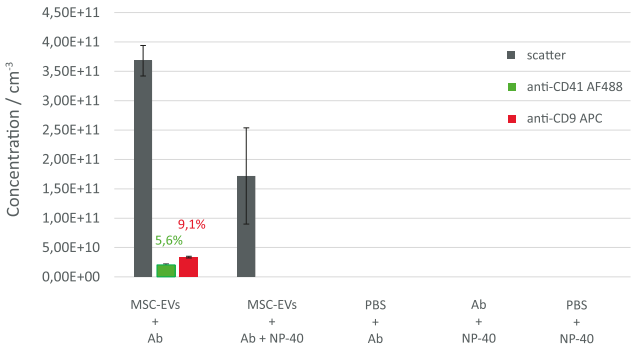

Figure 6: Negative controls analyzed in scatter and fluorescence mode analyzed by NTA. The first data set serves as comparison and represents antibody (Ab) double-labelled MSC-EVs without detergent (ref. figure 1).

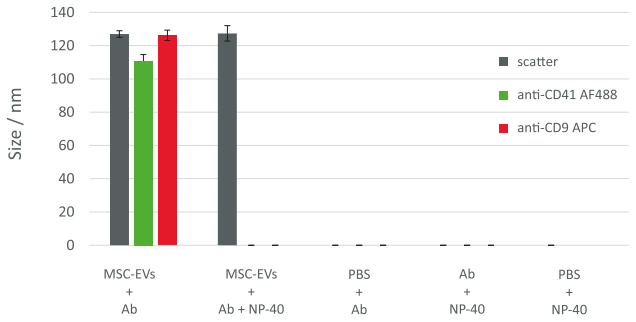

Figure 7: Comparison of particle diameter of the negative controls analyzed in scatter and fluorescence mode. The first data set serves as comparison and represents double-labelled MSC-EVs without detergent (ref. figure 1).

The NP-40 detergent control showed a particle number reduced by 54% in scatter mode and no particles in the fluorescence mode. The fact that some particles were detectable in the colocalization measurements, but not in the classic F-NTA, can be explained by the different nature of these specific measurements. While traditional F-NTA requires longer particle tracking (typically with a trace length between 10 and 30) to ensure accurate size and concentration measurement, a colocalization measurement requires a very short duty cycle in order to find the same particle within both fluorescence channels.

Summary

In this study we measured the level of EVs positive for CD41 and CD9 by comparing the number of particle detections in scatter and fluorescence mode, proving that F-NTA is an ideal method to identify human platelet-derived MSC-EVs out of a polydisperse environment of biological nanoparticles. Since the method does not require a cleaning step to eliminate unbound antibodies, it is fast and has proven to be highly reproducible.

The colocalization results obtained from C-NTA and IFCM were highly comparable, underlining that platelet derived (CD41-positive) CD9-positive EVs were present in the tested samples. The data show that targeted antibody staining can determine the level of EVs of interest via F-NTA much more specifically than with scatter-based NTA. Furthermore, C-NTA has been proven to be an accurate tool for the detection and identification of biomarkers at the single-particle level, within one single measurement, including the benefit of accurate size determination.

C-NTA is therefore complementary to single F-NTA or scatter-based NTA, offering more targeted results, thus helping to unravel the unsolved mysteries of the exciting world of extracellular vesicles.

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