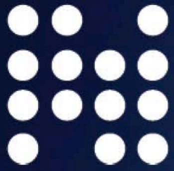

NanoView
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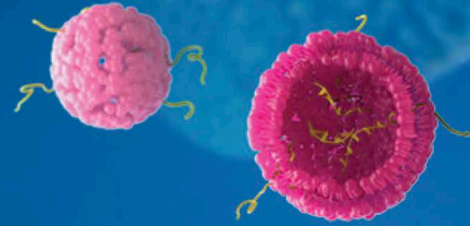
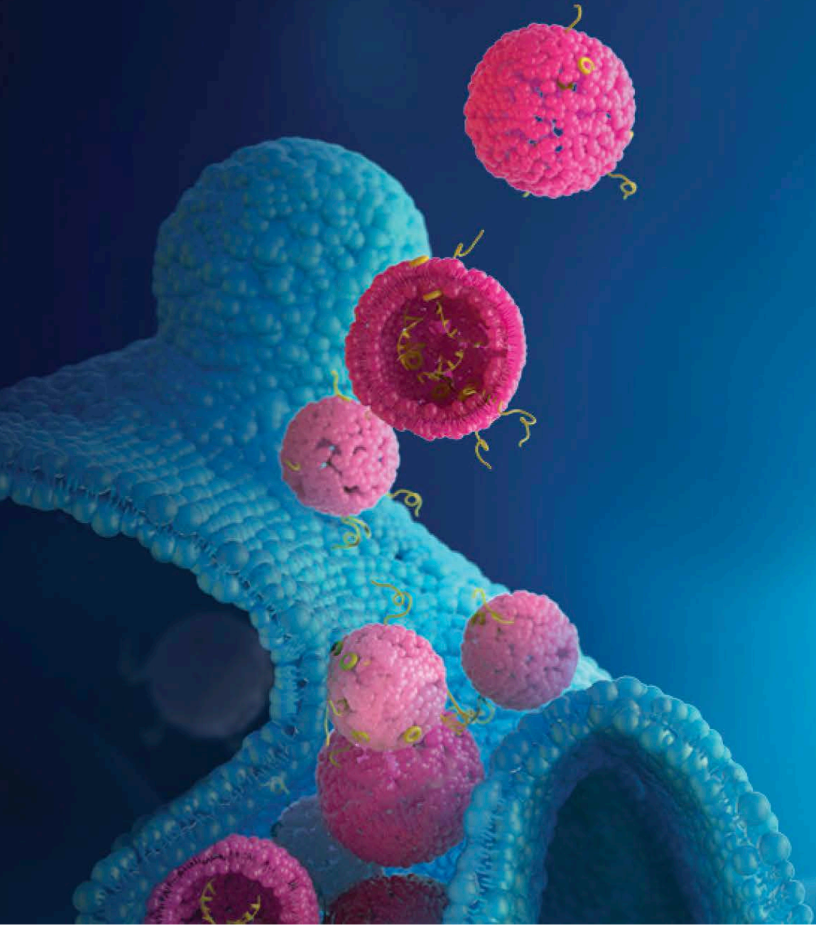
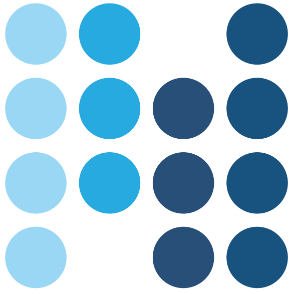




A Critical Comparison of Nanoparticle Tracking Analysis (NTA) and ExoView®



WHITE PAPER THEMES:

- Comparison of NTA and ExoView® When Measuring Small EVs
- Understanding the Limitations of NTA for EV Sizing
- Evaluating NTA User Variability
- Phenotyping of EVs with NTA and ExoView®

NTA vs ExoView®

Nanoparticle Tracking Analysis (NTA) has established itself as the de facto “Go To” method for the sizing and counting of extracellular vesicles (EVs) . It is considered by reviewers to be a standard methodology and discussed at length in current MISEV guidelines [1] and is generally required when submitting scientific papers in the field of EV research.

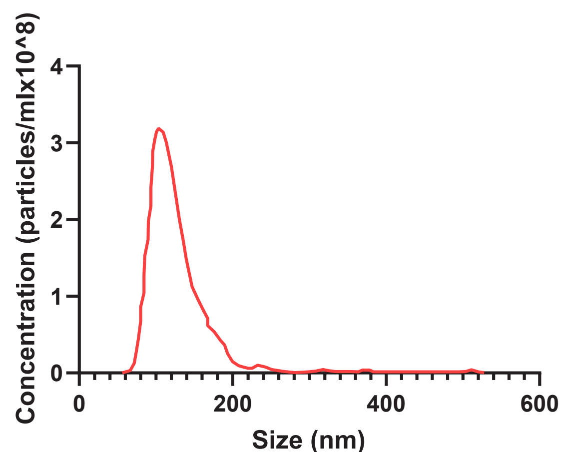
Given its status as a go-to methodology, have we lost sight of critically assessing the quality and validity of data produced by NTA, especially considering the emergence of perhaps more suitable technologies that provide a more detailed understanding of EV samples?
In this review article we will highlight some of the critical areas in which NTA data should be carefully considered and present arguments for alternative approaches that may overcome some of these analytical challenges.

NanoView is one of a number of emerging technologies that offer alternative methods to more established technologies for measuring EVs. NanoView’s technology (ExoView) is able to size, quantitate and phenotype individual EVs. EVs can be phenotyped with up to 4 surface or cargo proteins and sub-populations of EVs measured by their size, count or protein expression.

Sizing of Extracellular Vesicles

Size distributions are consistently reported in the literature to provide orthogonal validation that EV-sized events are measured. In general, however, there are relatively few articles that validate that the sizes generated are measurements of actual EVs through phenotypic information. In general, this information is inferred through sample purification meaning that the quality of the pre-purification is a critically important variable in subsequent size distribution analysis. Removal of confounding contaminants such as lipoproteins and other biological nanoparticles is critically important if phenotypic information is not provided in combination with size measurement.

FIGURE 1 A recreation of a literature-typical size distribution generated by NTA for purified EVs with modela size of 104nm

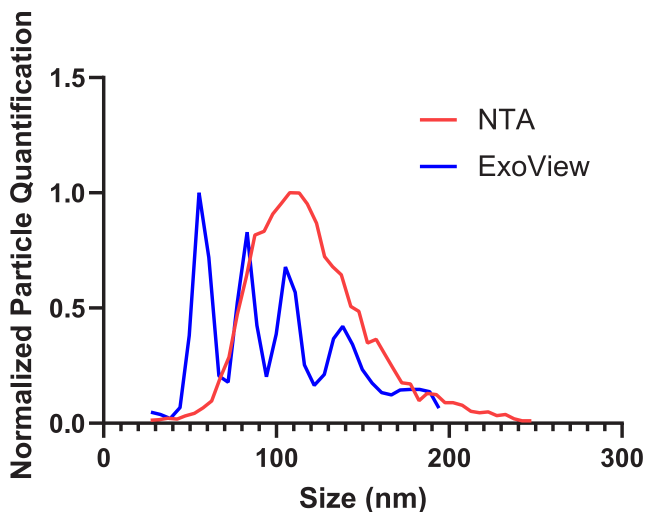


[2] Coughlan et al

Challenges Measuring Heterogenous Samples with NTA

In a publication by Arab et al [3], four orthogonal technologies were challenged with a mixture of silica beads. Silica beads were chosen due to their relatively uniform size and similar refractive index to EVs. In the study, a mixture of 68 nm, 91 nm, 113 nm and 151 nm were measured and a selection of the data is recreated in Figure 2 showing size distributions from NTA and ExoView for this heterogenous sample preparation. As can be seen, ExoVIew is able to resolve four distinct populations of beads, whereas NTA produced a single and unresolved distribution for the mixture (it must be noted that other techniques based on flow cytometry and coulter sizing also produced a more accurate size distribution when compared to NTA).

FIGURE 2 A recreation of sizing data from Arab et al publication. Data shows comparison of NTA and ExoView for a silica bead mix of 68nm, 91nm, 113nm and 151nm beads.



The authors of the paper question the importance of peak-to-peak resolving capabilities as it relates to biological material, stating that biological samples are unlikely to exhibit such distributions and hence the inadequacies of NTA sizing may not be a limiting consideration. While this is true, one highly relevant consequence of NTA's inability to effectively size mixtures of beads is that the resultant quantification of the sample will also be detrimentaly influenced.

Given that many users are interested in quantification of EVs, the inability to size effectively in a heterogenous population will limit the accuracy of the quantification due to certain populations of the distribution being ineffectively characterized. As can be seen in Figure 2, NTA measures low concentrations of 68 nm beads when compared with NanoView that demonstrates that the 68 nm beads are in fact the dominant population by number of events. As such, any subsequent quantification of concentration via NTA will be flawed by the inability to measure the small events especially in the presence of sample heterogeneity.

Why is NTA unable to resolve distinct particle peaks and measure the smaller particles within the mixed sample?

Table 1 shows how the important variables that define sizing capabilities, vary in both techniques across a range of sizes. The ability to measure smaller particles is related to absolute sensitivity of the technique, as well as, the ability to visualize smaller particles in the presence of larger and brighter events.

Light scattering in NTA varies according to radius^6 whereas in NanoView (interferometry) it varies according to radius^3. As can be seen in Table 1, this has a drastic influence on the brightness of EVs as they get larger. In comparison to a 50 nm particle, a 400 nm particle will be x262,144 brighter in NTA whereas the brightness in NanoView will only vary by x512 across the same size range. Put in another way, the signal from larger particles will drown out the signal from smaller particles

Challenges Measuring Small EVs with NTA

TABLE 1 Comparison of the variables that define peak-to-peak resolving capabilities and the ability to image small EVs simulataneously with larger particles. Data shows comparative values versus a 50nm bead.

	50 nm	100 nm	200 nm	400 nm
	Particle Brightness			
NanoView	1	x8	x64	x512
NTA	1	x64	x4,096	x262,144
	Sizing Measurement			
NanoView	1	x8 (brighter)	x64 (brighter)	x512(brighter)
NTA	1	x2 (slower)	x4 (slower)	x8 (slower)

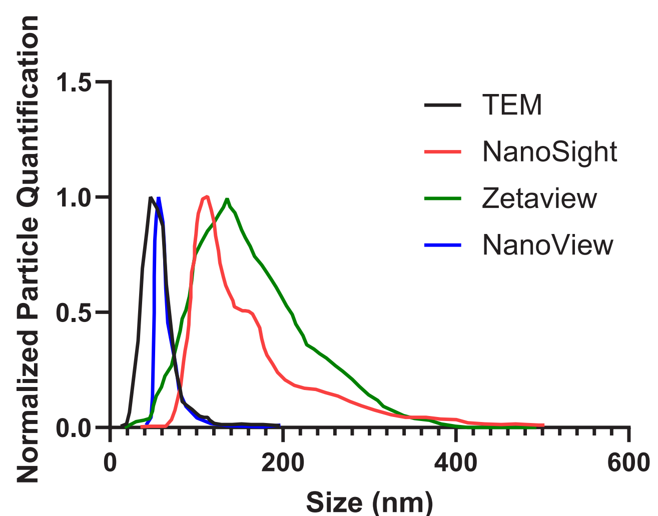
in a much more severe way in NTA making smaller particles much harder to detect in a heterogenous population.

So smaller particles are harder to detect in NTA but the manner in which size is calculated, also reduces resolving capability. NTA uses Brownian Motion to calculate particle size, whereas NanoView uses particle brightness to calculate size. Table 1 shows the effect of this.

A 400 nm particle will diffuse x8 more slowly than a 50 nm particles (linear relationship between size and motion), whereas NanoView benefits from a x512 difference in measurable signal when calculating size. The bigger the difference in the measurable signal between two different EVs, the better the ability to resolve them in terms of their size differential.

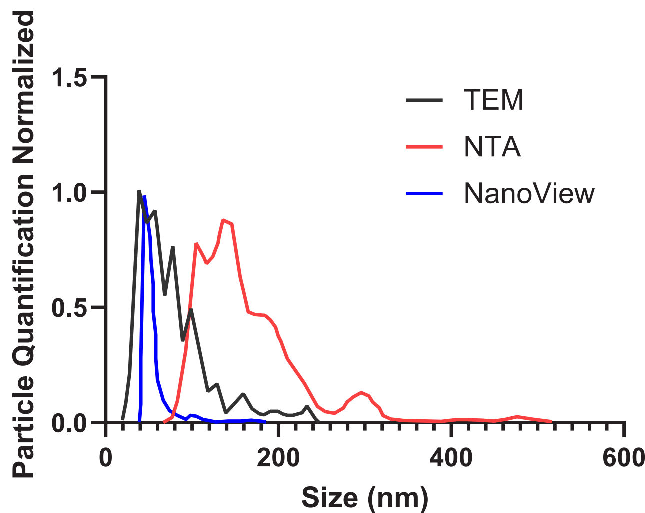
In a paper by Bachurski et al [4] these phenomena are yet further evidenced in the measurement of EV samples. In the study, L-540 derived EVs were prepared with 100k ultracentrifugation and measured using NanoView, TEM and two different manufacturers of NTA. A recreation of a selection of data is shown in Figure 3. As can be seen, the size measured by NanoView and TEM on this sample were highly comparable with a modal particle size in 60 nm size range. Both manufacturers of NTA produced size distributions highly biased towards the larger events within the sample with literature-typical size distributions of 100 to 200 nm for both manufacturers. Both NanoView and TEM detect relatively few events in 100-200 nm size range in this sample further demonstrating the potential disconnect between sizing and quantification of NTA and the smaller and likely significantly more prevalent events at the smaller size ranges.

FIGURE 3 A recreation of sizing data from Bachurski et al publication. Comparison of NTA (NanoSight and ZetaView), TEM and NanoView (ExoView) sizing on L-540 derived EVs



Mizenko et al [5] show similar results to the Bachurski paper in their analysis of SK-OV-3 derived EVs by TEM, NanoView and NTA with NTA demonstrating significantly higher sizes than TEM and NanoView which largely agree in their size determination.

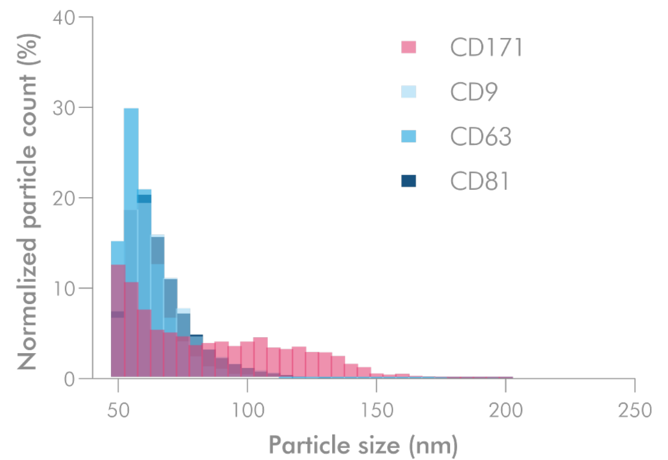
FIGURE 4 A recreation of sizing data from Mizenko et al publication. Comparison of NTA NanoSight, TEM and NanoView sizing on SK-OV-3 EVs



Size Vs EV Phenotype Via NanoView

As demonstrated, NanoView sizing more closely matches gold-standard TEM size measurements for the examples presented. In addition, size distributions can be generated on a per antibody basis. As Figure 5 demonstrates, different EV phenotypes can exhibit different sizes. EVs expressing CD171 demonstrate a larger and broader size distribution than CD9/CD81/CD63 positive EVs.

FIGURE 5 Size distributino comparison of EVs dervied from CD171-over expression HEK 293 cultures. CD171 positive EVs demonstrating a larger particle size than CD9/CD63/CD81 positive EVs



NTA size distributions suffer from the inability to accurately measure the smaller constituents in a heterogeneous population. Small EVs are obscured by larger EVs which influences both size and concentration measurements detrimentally.

ExoView produces size distributions that more accurately match known sizes in mixtures of silica beads and match more closely with TEM size distributions in EV preparations. ExoView also provides the ability to discriminate size distributions of specific sub-populations of EVs expressing specific protein biomarkers.

NTA User-Variability

Another common issue cited in the literature is the user dependence of NTA, particularly versus software features such as detection threshold and camera level (Maas et al [6], Vestad et al [7]). Gardiner et al [8] highlight the extensive sources of variability within NTA measurements namely; inaccurate temperature measurements, incorrect viscosity estimation, external instrument vibration, type of camera used, laser wavelength, depth of laser beam, cleanliness of optical elements over which sample are suspended, duration of measurements and operator proficiency.

Gross [9] et al provide a well-articulated summary of the issues associated with user parameters in NTA (NanoSight). The papers explains that for mono-disperse samples, NTA can produce precise and reproducible sizing measurements when predefined and constant user parameters are used. However, the paper goes on to explain that for heterogeneous sample populations, that the results are highly dependent upon user settings.

The paper challenged NTA with a mixture of 50 nm, 400 nm and 600 nm beads at a ratio of 9:2:1, the summary of the results is as follows:

- With all user settings, the total concentration measured in the heterogenous sample was incorrect versus calculated concentrations.
- Changing the detection threshold and camera level resulted in the appearance and disappearance of various size peaks.
- With all user settings, the concentration of 50 nm particles was drastically under-reported due to the smaller particles being obscured by the presence of the larger brighter particles.
- Camera Level (CL) and Detection Threshold (DT) (NanoSight-specific user variables) were found to have the biggest influence on measurements.
- Increasing the CL increases the reported concentration because in part it increases the detection of noise or false centers, but conversely is required for the detection of smaller particles in a heterogenous sample. Changes in the DT work antagonistically to CL but with the same influence. The selection or bias of these user settings, define how many particles are included in the analysis and of which size - thus having a drastic influence on reported concentration and size. Similar findings were demonstrated by Tian et al [11] using heterogenous bead samples.

Phenotyping EVs

To assess the performance of NTA as it relates to phenotyping EVs, a Google Scholar search was performed and the results shown below:

- exosome* AND (“NTA”OR “nanoparticle tracking analysis”) = 13,600 publications
- exosome* AND (“fNTA” OR “fluorescence nanoparticle tracking analysis” OR “fluorescent nanoparticle tracking analysis”) = 257 publications

As can be seen, there is a paucity of published articles that include search terminology pertaining to the use of NTA in fluorescence mode. In fact, only 1.9% of all NTA-EV related publications include terminology that relates to the use of fNTA or fluorescence-based terminology.

Why is this the case? In a field in which the function of EVs is inextricably linked to its phenotype, then surely more publications would cite fNTA if it were something that could be readily achieved?

Out of the publications that do cite fNTA, the majority utilize membrane stains rather than fluorescent antibodies in an attempt to discriminate EVs in general from contaminant particles. To date, very few published articles cite fNTA with antibody mediated fluorescence for EV phenotyping.

The main reasons for the lack of antibody mediated fluorescence based detection with NTA are two-fold:

1. Lack of absolute sensitivity required to detect fluorescently stained EVs and
2. Sample photobleaching that results from the use of:

- High-power density laser excitations sources.
- Due to their size, few fluorescent antibodies can attach to a single EV.
- The requirement to measure EVs for a period of time for it to be successfully tracked via NTA making photobleaching problematic to tracking

Whether the goal is to characterize EV sub-populations as it relates to their biological function, or alternatively discriminate lipoproteins from EVs in general, it would seem that fNTA is a challenging technique that has been succesfully implemented rarely in the literature.

There are numerous papers that cite that lipoproteins are present in the blood at concentrations significantly higher than EVs [11] and that when EVs are purified from blood (by all purification techniques tested), that lipoproteins constitute the vast majority of all events due to the fact that their density and size span those of EVs.

NTA suffers from a drastic reduction in the ability to visualize small particles in the presence of large particles due to relative particle brightness. It also suffers from a limited ability to discriminate EVs of different sizes due to use of Brownian Motion to calculate EV size. When combined, these phenomena result in flawed size distributions and quantification inaccuracies when measuring heterogeneous EV preparations. It is becoming more widely accepted that EVs have log-normal distribution down to the smaller sizes, meaning that the majority of EV events, reside at the smaller sizes. Missing this part of the fraction can result in large inaccuracies in EV quantification via NTA.

The lack of fNTA data within the literature highlights the limitations in capabilites in EV phenotyping via NTA meaning that overcoming the aforementioned limitation via fNTA is a chellenging endeavour.

So the question arises, unless experiments and samples are carefully selected and the conclusions drawn from those experiments taken at very general levels, what level of accuracy and insight do NTA analyses really provide in the field of EV research?

What Alternatives Are Available?

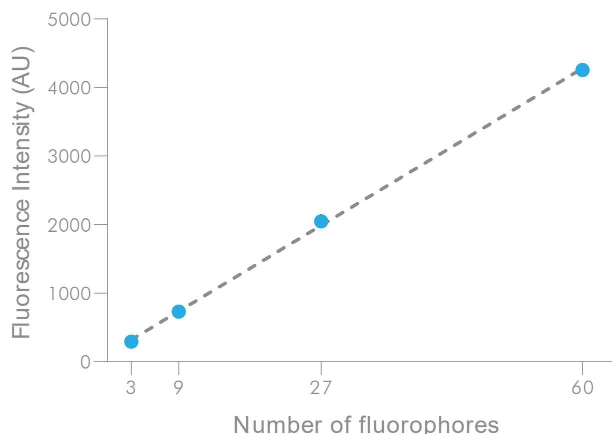
Data comparing the sizing capabilities of NanoView versus NTA have been presented, however how else can NanoView's technology overcome the challenges presented in this review? NanoView's technology is designed to routinely operate in fluorescence mode with sensitivities that enable the measurement of even the smallest EVs that express specific proteins at low concentrations.

NanoView's technology works by binding EVs to the surface of a chip via common EV markers (CD9/CD63/CD81/CD41a), once bound to the chip EVs can be subsequently fluorescently labeled with up to 3 fluorescent antibodies. Figure 6 shows the basic assay design with a standard or custom antibody binding an EV to an ExoView chip that can be subsequently labeled with 3 standard or custom fluorescent antibodies. The surface can be functionalized with up to 6 different antibodies types therefore EV analysis can be highly plexed to study various phenotypes of EV. Analysis requires no purification and size, count and phenotyping information can be gathered from the technology. Analysis requires no purification and size, count and phenotyping information can be gathered from the technology.

Given the discussion in this article about and importance of measuring small EVs versus NTA's inability to do so, the sensitivity of any alternative methods is of paramount importance. As such, to validate the sensitivity of NanoView's technology, DNA origami structures were made with highly controlled numbers of fluorescent molecules. Samples with 3, 9 27 and 60 fluorescent molecules were measured, and results plotted in Figure 7. Figure 7 demonstrates that not only is the ExoView sensitive down to at least three fluorescent molecules, that the fluorescent intensity is quantifiable and linear across the range of samples. This means that ExoView platform is easily sensitive enough to detect and measure very small EVs with poor protein expression overcoming the challenges associated with NTA and fNTA.

FIGURE 6 Basic assay design for ExoView showing capture antibody and 3 fluorescent antibodies allowing four proteins to be colocalized on a single EV

FIGURE 7 Fluorescent sensitivty of ExoView showing ability to measure 3 fluorescent molecules.



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How Does ExoView Help Your Research?

FIGURE 8 Three color fluorescent images of single vesicles

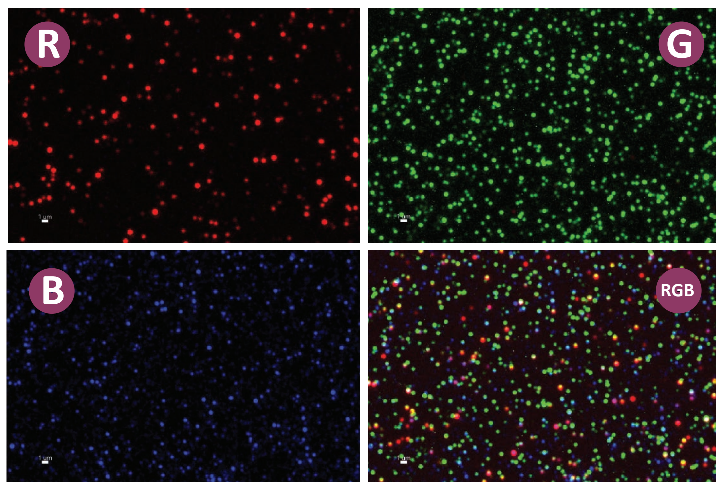


FIGURE 9 Purification-free analysis: Normalized EV counts from unpurified conditioned culture media versus UC purified.

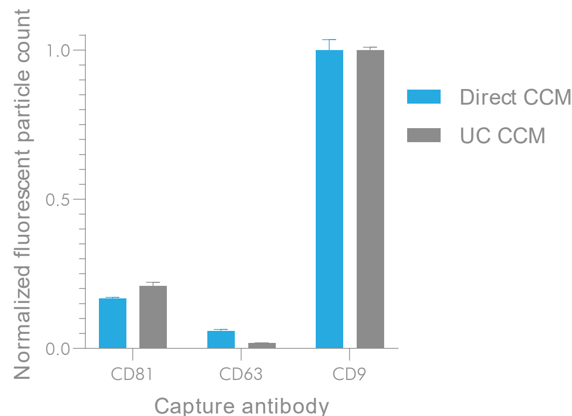


FIGURE 10 Quantification of biomarker colcalization on single EVs

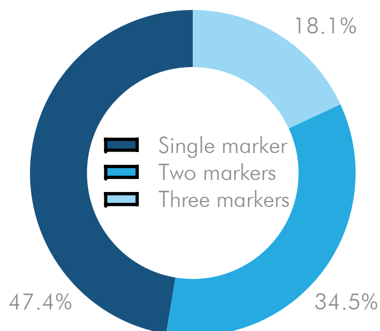


FIGURE 11 Measure specific EV populations in the presence of lipoproteins.

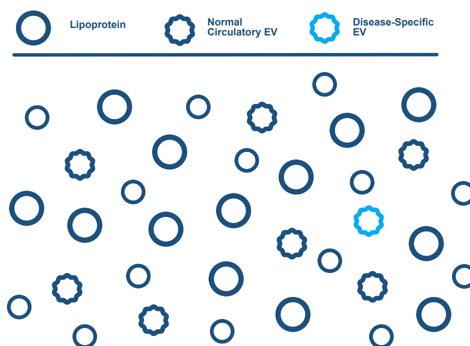


FIGURE 12 Cargo detection in individual EVs

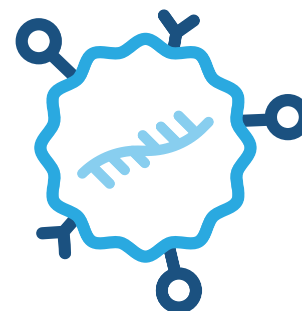
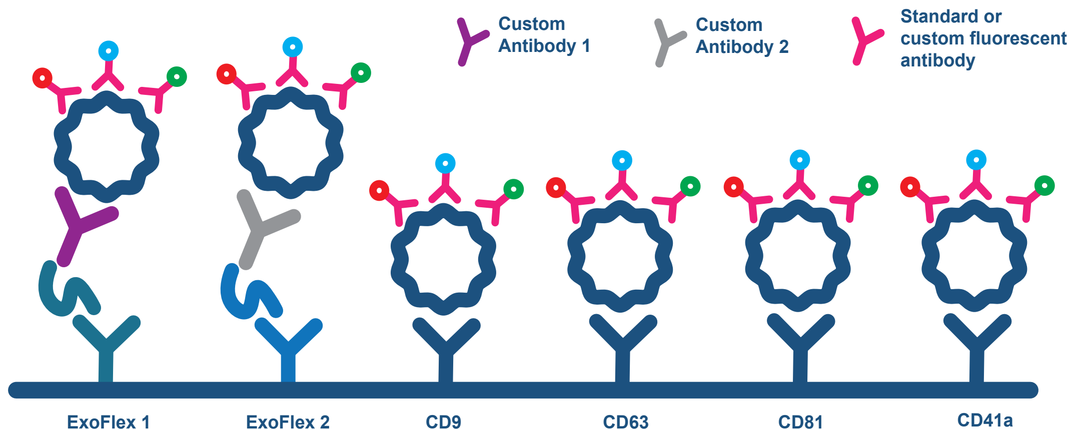


FIGURE 13 Fully user-customizable assays to capture and detect standard and custom proteins via ExoFlex™



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