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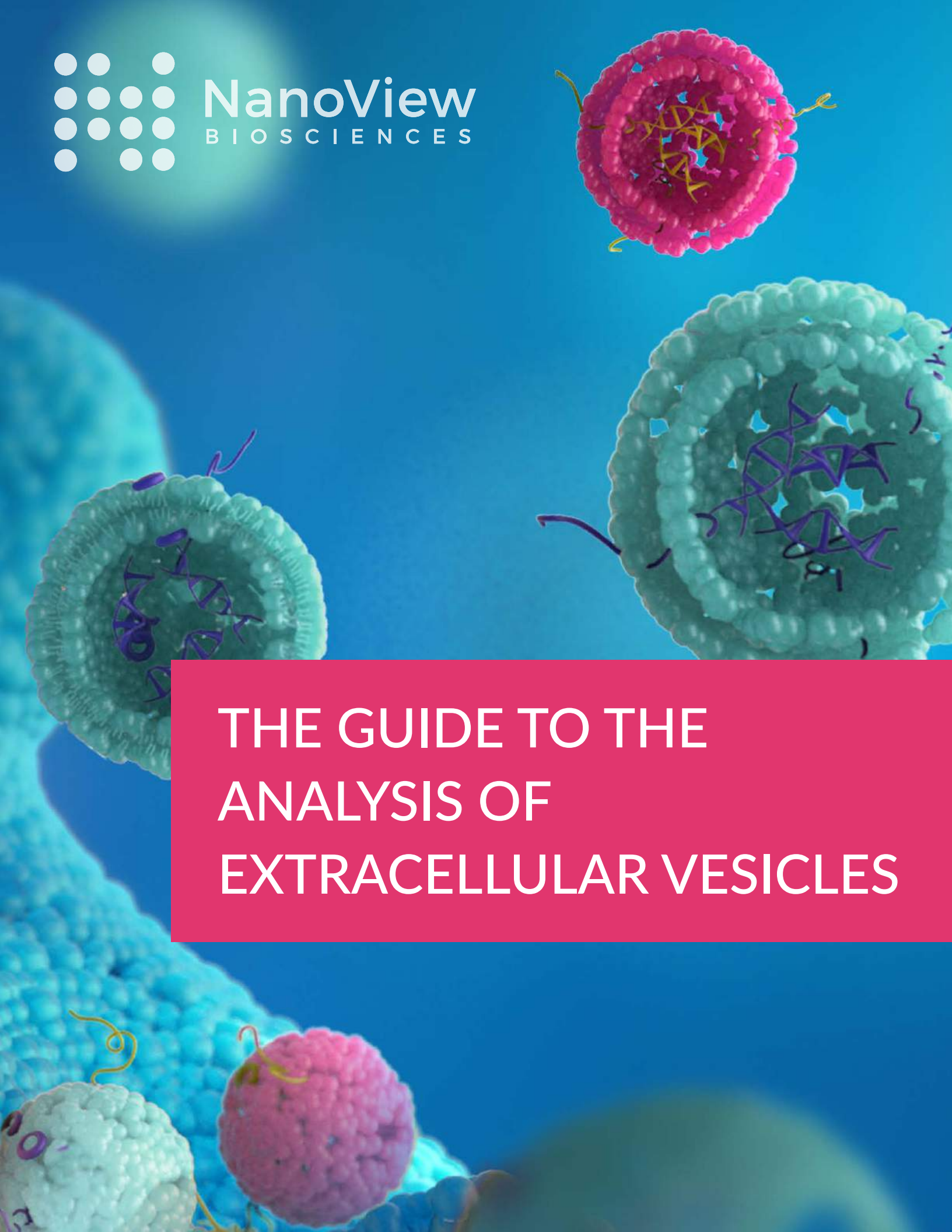

THE GUIDE TO THE ANALYSIS OF EXTRACELLULAR VESICLES

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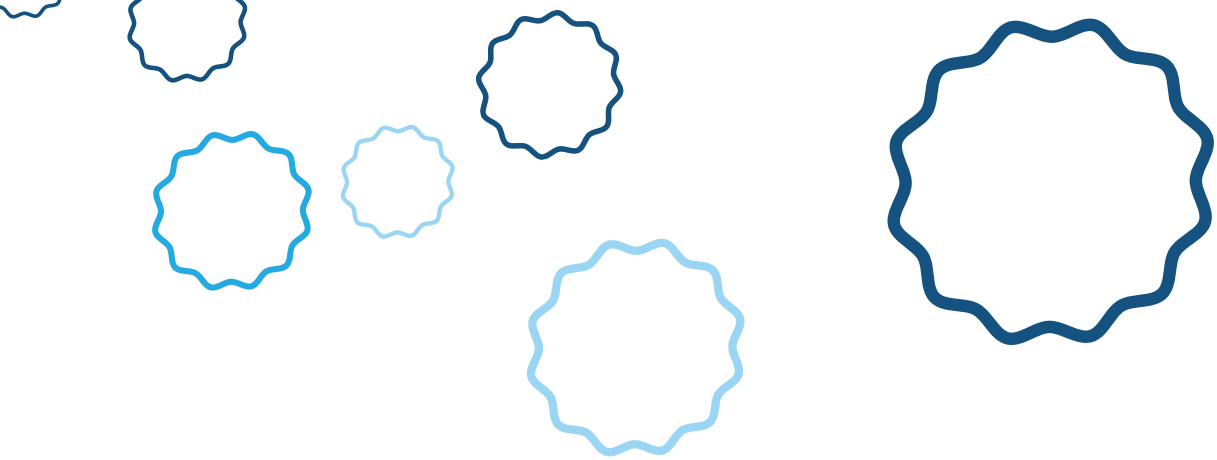
MULTIPARAMETRIC ANALYSIS OF EXTRACELLULAR VESICLES



Introduction

Extracellular vesicles (EVs) are a broad range of vesicular bodies smaller than one micron in diameter containing lipids, proteins, and nucleic acid, and released by cells into the extracellular milieu. Interest in these vesicles has exploded in recent years as they have been implicated as key mediators of non-contact intercellular communication and have been identified as candidates for early-stage diagnostics as well as therapeutic delivery vectors. Of the 59,141 publications on extracellular vesicles, 43,114 were published in the past decade and span a variety of scientific fields.[1] The activity of exosomes, a distinct class of small EVs, is reported in a diverse range of disease states, including cancer[2,3,4,5] and neurodegenerative[6,7,8] and cardiovascular diseases.[9,10] Additionally, exosomes have been shown to drive phenotypic changes in recipient cells through nucleic acid transfer.[11,12,13]

While the exact function of EVs is not yet known, investigations to further our understanding are underway. Today, we know that EVs can be taken up from circulation by recipient cells and modulate protein expression.[14] There are several distinct classes of EVs defined by biogenesis, and their release into the extracellular milieu is a carefully coordinated signaling act.[15]

The development of EV research is, however, constrained by the capabilities of the available analytical tools. Vesicle complexity also contributes to the challenges involved in EV characterization. Specifically, EVs are small in size and often express low copy numbers of specific proteins, which makes them hard to detect. They are present in complex sample matrices and may express a variety of protein markers of interest, some of which may only be present in the lumen

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of the vesicle. The 2018 edition of the International Society of Extracellular Vesicles opinion paper titled "Minimum Information Supporting Extracellular Vesicle Research," or MISEV, thoroughly describes isolation and analysis protocols and experimental designs necessary to ascribe functional capabilities to the EVs of interest.[16]

Here we demonstrate how ExoView® from NanoView Biosciences addresses these challenges delivering multidimensional data sets and enabling an advanced understanding of complex EV populations.

EV Analysis with ExoView

The ExoView platform provides a turn-key solution for the multiparametric characterization of single EVs. ExoView is designed to address the current challenges of EV research and enables researchers to meet many of the criteria highlighted in MISEV, which describes state-of-the-art guidelines for EV analysis.[14] With ExoView, single-vesicle analysis can be performed to determine the size and protein profile of individual EVs. The technology behind the powerful capabilities of ExoView has been described previously.[17,18] At the core of this technology is antigen-specific capture of EVs (Figure 1). An ExoView chip specifically captures exosomes via a microarray chip functionalized with antibodies. EVs that carry specific proteins bind to the corresponding antibodies on the chip. ExoView analyzes EVs using visible light interference for size measurements and fluorescence for protein profiling. Figure 2 shows an example image.

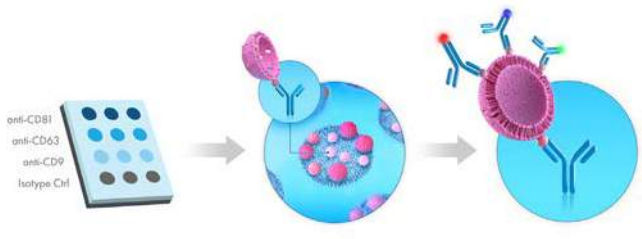

Figure 1: Principle of EV detection by ExoView. Capture antibodies specific to EV proteins immobilize EVs on the chip. EVs are subsequently stained with three fluorescent antibodies. High-resolution measurements of specific EVs are acquired.

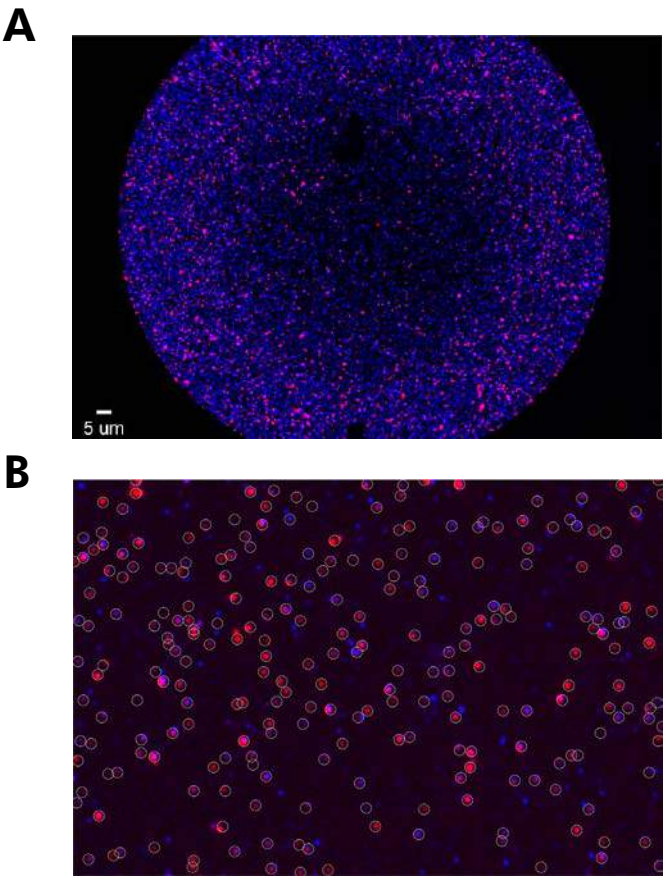

Figure 2: ExoView fluorescence imaging data. Each fluorescent spot corresponds to an EV. CD9 (blue), CD63 (red). (**A**) The image of an entire antibody capture spot. (**B**) Close-up frame. Circles indicate EVs identified by the ExoViewer software in the red channel.

Challenges in the analysis of extracellular vesicles

Sample purification is at the center of EV analysis and presents significant challenges in the subsequent characterization of EVs. Lipoproteins, protein aggregates, bovine EVs, and numerous other contaminants commonly co-purify with EVs. MISEV 2018 guidelines highlight that complete purification of EVs is not possible. This poses a challenge for the quantification of EVs with commonly used particle characterization tools like NTA and TRPS. These technologies do not specifically measure EVs and therefore measure co-isolated contaminants and EVs indiscriminately. Therefore, an antigen-specific EV characterization technique is required to differentiate between EVs and co-isolated contaminants.

The consequence of contaminants is most apparent when characterizing EVs from complex biofluids like plasma or serum, which are known to have an abundance of lipid-based particles that are not EVs. As Sódar *et al.* describe, the majority of particles purified from blood serum in the size range of EVs did not express EV markers and were found to

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be lipoproteins (predominantly LDLs).[19] The study found that lipoproteins co-purify with EVs in all purification techniques tested. Therefore, particle characterization techniques like NTA and TRPS do not reflect the actual concentration of EVs.

The impact of this finding can be demonstrated with a theoretical exercise. Previous studies suggest that EVs constitute at most, 10% of particles in the EV size range. Of this population, disease-specific EVs may represent only 1% (Figure 3). Therefore, of all the events measured by particle characterization techniques, only 0.1% may be disease-specific EVs.

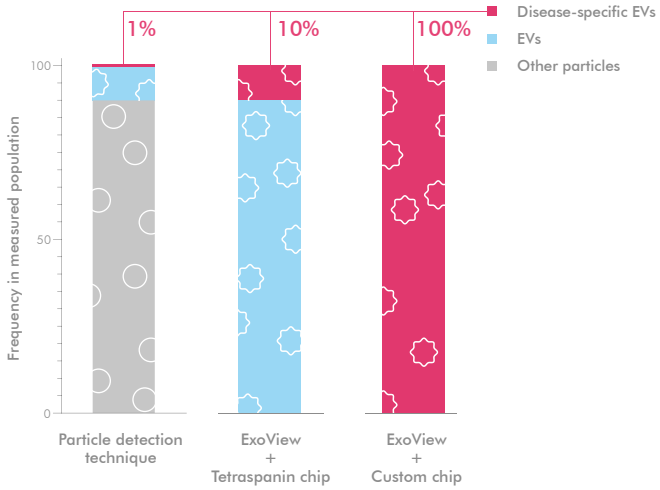

Figure 3: Relative abundance of EVs in analyses using particle detection techniques and in analysis using ExoView. Of the particles within the appropriate size range, EVs constitute up to 10%, and only ~1% of all EVs are disease-specific. With ExoView, all analyzed particles are EVs. Custom chips enable enrichment for EVs of interest in the analyzed population.

Applying this to the measurement of 10,000 particles:

- 10,000 events measured, of which 9000 are lipoproteins, 1000 are EVs, and 10 are disease-specific EVs.

With ExoView which specifically measures EVs:

- 10,000 events are measured, 10,000 of which are EVs, and 100 are disease-specific EVs
- ExoView can also positively select for disease-specific EVs through customization of capture antibodies. In this instance, 100% of the EVs captured will be disease-specific (assuming appropriate selection of capture antibodies).

Given that conventional particle characterization techniques are likely to measure significantly fewer than 10,000 events, the number of disease-specific events measured may be less than 10.

Furthermore, inherent variability associated with particle detection techniques (such as NTA and TRPS) is well documented in the literature.[20,21,22,23,24] Experimental variability may further obscure the presence of disease-specific EVs requiring even larger changes in the count of disease-specific EVs to gain meaningful data. This variability and low sensitivity make particle detection techniques less practical in the analysis of serum samples. At the same time, the high sensitivity and specificity of antigen-specific techniques like ExoView position them to provide insight in diagnostic applications.

ExoView also has the potential to overcome many of the challenges associated with EV purification. With ExoView, multidimensional data sets are obtained from every experiment, enabling sizing, quantification, protein quantification, protein colocalization, and cargo detection. Unprocessed samples can be directly analyzed, as can purified samples from a variety of techniques. EVs from cell culture supernatant, plasma, serum, urine, and other matrices can be measured directly. The diversity of the data and sample types has made ExoView the instrument of choice for researchers who are deepening the understanding of EV biology, who are studying cancer and neurodegenerative diseases, and who are developing EV-based therapeutics.

Measuring EV heterogeneity

The ExoView Tetraspanin Kit is a protein microarray chip with antibodies against commonly expressed EV Tetraspanin proteins CD9, CD63, and CD81. The ExoView Tetraspanin Plasma Kit, in addition to the three Tetraspanins, includes CD41a capture antibodies for platelet-derived EVs. All chips also include appropriate isotype negative controls to test for non-specific binding. The ExoView Tetraspanin Kit comes with human or with mouse antibodies.

Customization of Exoview kits allows for specific proteins to be studied. Customization can be achieved through modifying the fluorescent antibodies in the assay. In this way, custom proteins can be detected on EVs expressing one of the four standard capture antibodies on an ExoView chip (CD9, CD63, CD41a, or CD81). At times, a research project demands focus on a subset of EVs positive for a specific marker. In this case, a custom assay can be designed in which EVs are captured using an antibody to the specific marker. Custom chips can be designed with streptavidin capture for flexible customization. Rather than offering an analysis of all EVs, a custom kit can specifically capture the subpopulation of interest.

Conclusion

Extracellular vesicles are some of the smallest cellular compartments, yet they fulfill diverse biological roles. EV research presents a great opportunity to uncover biological phenomena, to develop diagnostics, and to engineer therapeutics. The ExoView platform enables high-resolution multidimensional characterization of EVs. ExoView combines size, count, and protein expression measurements at the single-vesicle level, making it a uniquely powerful technology.

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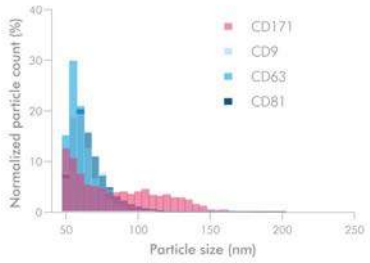
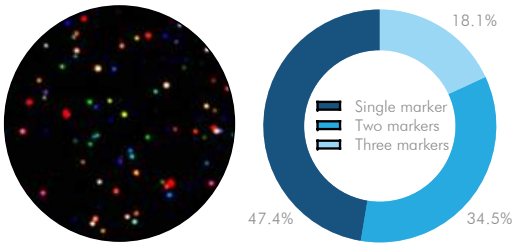
Sizing and Counting EVs



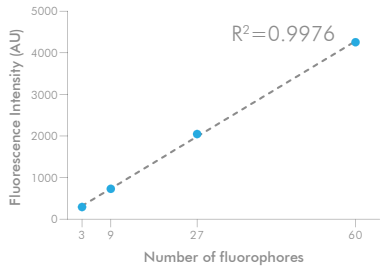
ExoView offers high-resolution size distributions and quantification of EV subpopulations. Here we compared EVs from CD171-OE HEK 293 cultures. EVs expressing CD81, CD63, and CD9 (blue) exhibit typical distributions with a peak at 55nm, vesicles carrying the overexpressed protein CD171 (pink) are distinct in their size distribution. To learn about how ExoView sizes EVs and why you can trust ExoView data, refer to Technical Note: Sizing.

Protein Profiling and Colocalization



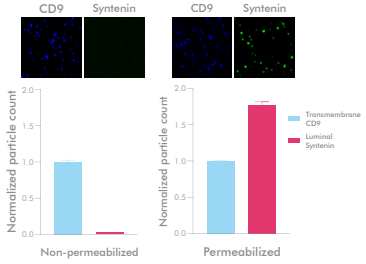
To study colocalization of proteins (for example, to identify disease- and tissue-specificity), ExoView offers detection of up to 4 proteins on an individual EV. The combination of specific markers expressed by individual EVs can be measured across all EVs imaged. EVs from HEK 293T cultures were stained with three fluorescent antibodies and distinct subpopulations of EVs were quantified according to their relative protein expression. To learn about other types of protein profiling data and fluorescent measurements using ExoView, please see Technical Note: Precision phenotyping with single-vesicle analysis.

Detecting Rare Events



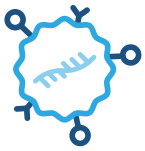
Highly sensitive detection is critical for the analysis of rare EV populations, such as disease-specific EVs. We demonstrate the sensitivity of ExoView EV detection by measuring fluorescent standards. ExoView reliably detects standards carrying three fluorescent molecules. The antibodies used in ExoView assays typically carry at least three fluorescent molecules per antibody, thus, ExoView can detect EVs carrying a single copy of the protein of interest. To learn more about the sensitivity as well as the specificity of ExoView measurements, refer to Technical Note: Precision phenotyping with single-vesicle analysis.

Measuring Cargo



EV cargo is involved in intercellular communication and fulfills a variety of functions. In this experiment, we used the ExoView Cargo Kit to visualize luminal syntenin. This figure shows EVs captured on a CD63 spot and labeled with antibodies against Syntenin and CD9. The non-permeabilized sample shows CD9 counterstaining only. Permeablized samples show counterstaining for both CD9 and Syntenin with more vesicles stained for Syntenin, demonstrating that some of the vesicles captured on the CD63 spot do not express CD9. For further information on cargo detection via ExoView, please refer to Technical Note: EV cargo detection.

Technology key features



EV CARGO

Probe for luminal EV proteins and cargo in single EVs

FLUORESCENCE

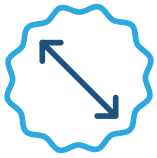
Detect lowest-abundance proteins with single-binding-event sensitivity across 3 color channels

BIOMARKER COLOCALIZATION

Quantify relative protein expression of up to 4 proteins per EV

PURIFICATION NOT REQUIRED

Detect the changes in your sample, not the biases from your purification technique



EV SIZE

Acquire high-resolution measurements of the size of individual antigen-positive EVs

EV COUNT

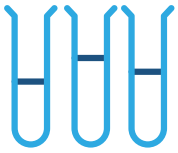
Count the number of antigen-positive EVs directly from sample, no purification required

MULTIPLEXED SAMPLE ANALYSIS

Characterize multiple populations of EVs from a single sample using up to 6 surface markers

STREAMLINED WORKFLOW

Reduce hands-on time and increase sample throughput with automated measurement and parallel sample preparation



VALIDATED ASSAYS

Innovate with custom assays and study specific proteins with custom-designed chips

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PHENOTYPING

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TECHNICAL NOTE:
PRECISION PHENOTYPING WITH SINGLE-VESICLE ANALYSIS

- Protein expression levels (up to 3)
- Protein colocalization
- Subpopulation characterization
- Direct-from-sample characterization

What the reader can expect to learn from this Technical Note:

- The analytical challenges in measuring small EVs due to size and protein expression.
- How ExoView® addresses these challenges through single binding event sensitivity fluorescence measurements.
- How ExoView measures events with three fluorescent molecules and how fluorescence intensity measurements scale according to the number of fluorescent molecules.
- How ExoView fluorescence measurements can be used to estimate protein expression on single EVs.
- How four proteins can be measured on a single EV using ExoView.
- How protein colocalization can be measured and how this relates to protein profiling on single EVs in diagnostic applications.

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Introduction

Interest has been growing in EVs as their importance in diagnostic and therapeutic applications becomes apparent. Research has exploded in the fundamental principles of EV biogenesis, in the use of EVs as predictive and prognostic diagnostics, and in stem-cell-derived and engineered therapeutic vesicles.[1,2] As these fields develop, a need has emerged for sensitive and reliable EV characterization methods.[3]

Several factors make EV characterization challenging:

- Sensitivity – the ability to detect rare events or poorly expressed proteins on single EVs;
- Specificity – the ability to distinguish between the target protein and other molecules;
- Signal linearity with EV concentration – the ability to compare EV counts between samples;
- Signal interpretation – the ability to distinguish between high protein copy number on a few EVs and low protein copy number on a large number of vesicles;
- Signal colocalization – the ability to determine if proteins detected are located on the same vesicle or different vesicles;
- EV association – the ability to associate proteins with single EVs rather than bulk measurements.

ExoView measures the physical properties of EVs alongside protein expression at the single-EV level with single-binding-event sensitivity and excellent specificity. The ExoView signal is linear with the number of EVs and with protein copy number on individual EVs. Finally, ExoView offers a unique capability to detect colocalization of up to four protein markers on a single EV. These proteins can be customized to measure both surface and luminal proteins on individual EVs.

Figure 1 summarizes the principle of operation of ExoView fluorescence mode. After the sample is incubated on the chip and EVs have bound to capture antibodies, EVs can be stained with fluorescent antibodies for up to three additional proteins (Figure 1). By measuring both light interference and fluorescence, ExoView probes single EVs for up to four proteins, thus enabling characterization of unique EV subpopulations based on protein expression levels.

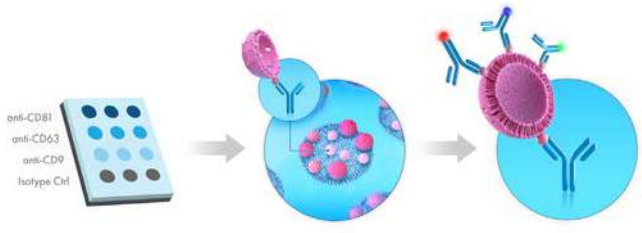

Figure 1: ExoView detects up to 3 fluorescent antibodies on a single EV. Capture antibodies specific to EV proteins immobilize EVs to the chip. EVs can be subsequently stained with three fluorescent antibodies.

Detecting single-molecule events

Exosomes are characterized as small EVs in the range of 30-200nm. At this size range, EVs often express a limited number of proteins, making their detection challenging. Highly sensitive analytical equipment is, therefore, required to detect their presence. The ExoView platform has single-binding-event sensitivity, making it the instrument of choice for EV characterization. The sensitivity of ExoView was determined by measuring Gattaquant DNA origami standards. These standards have tightly controlled fluorophore distributions, are small in size, and are highly homogeneous, thus serving as excellent fluorescence sensitivity standards.

ExoView was used to measure standards that contained 3, 9, 27, and 60 Alexa fluorophore molecules, and results are shown in Figure 2. The result demonstrates that ExoView can routinely measure three fluorophores and that the fluorescence intensity signal was linear between 3 and 60 fluorescent molecules.

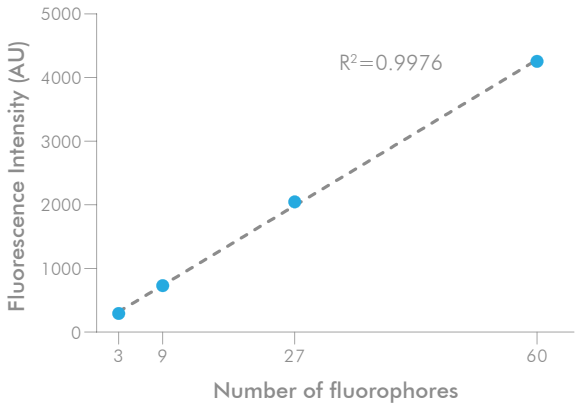

Figure 2: Fluorescence signal measured by ExoView is linearly proportional to the number of fluorophores. Gattaquant DNA origami nanostructures with 3, 9, 27, or 60 fluorophores were measured by ExoView.

Typical ExoView fluorescent antibodies are conjugated to an average of three fluorescent molecules. This experiment demonstrates that ExoView has single-binding-event sensitivity and can be used to relate fluorescence intensity to the number of binding events, with appropriate experimental calibration. The sensitivity and linearity of fluorescence intensity measurements position ExoView for analyzing any EV, including small exosomes with low protein expression.

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Specificity of protein detection on EVs

In EV analysis, validation via orthogonal techniques is often challenging, and therefore ensuring specificity of the measured signal is paramount. To demonstrate the specificity of ExoView, wild type (WT) and knock-out (KO) HEK 293 cell lines were tested. The ExoView Tetraspanin Kit enables capture of EVs based on the expression of ubiquitous tetraspanin markers: CD81, CD63, and CD9. The captured EVs can be subsequently stained for other proteins of interest.

Figure 3 shows the analysis of EVs captured using anti-CD81 and subsequently stained using fluorescent antibodies against CD81, CD63, and CD9. EVs from CD81 KO samples show no binding as would be expected. EVs from CD63 and CD9 KO cultures, while captured by CD81 capture antibodies, show a drop in signal for CD63 and CD9 surface staining, respectively.

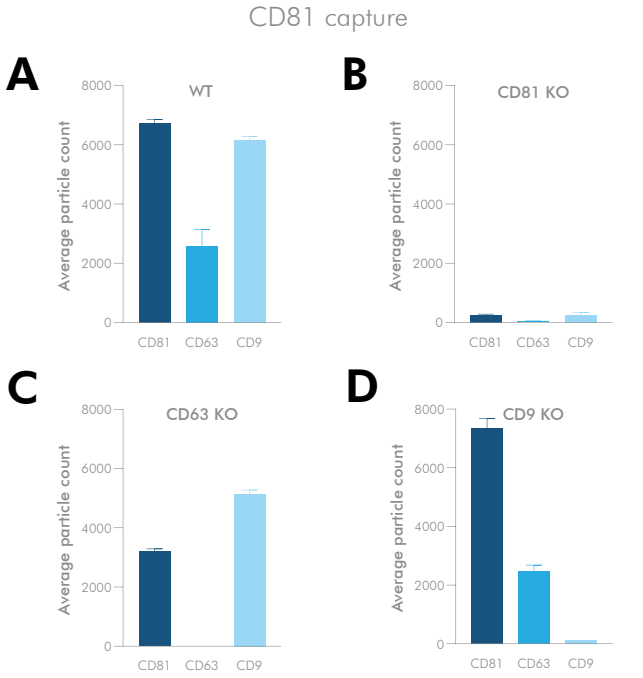

Figure 3: Specificity of ExoView signal is verified in knock-out experiments. EVs from HEK 293 cell lines are analyzed using ExoView and Tetraspanin Kit. EVs were captured using anti-CD81 antibody and stained using anti-CD81, anti-CD63, and anti-CD9 fluorescent antibodies. (**A**) Staining for all three tetraspanins was observed in EVs from WT cells. (**B**) No EVs were captured in the CD81 KO samples. (**C**, **D**) CD81-positive EVs were captured in CD63 and CD9 KO cultures, with no staining for CD63 and CD9, respectively.

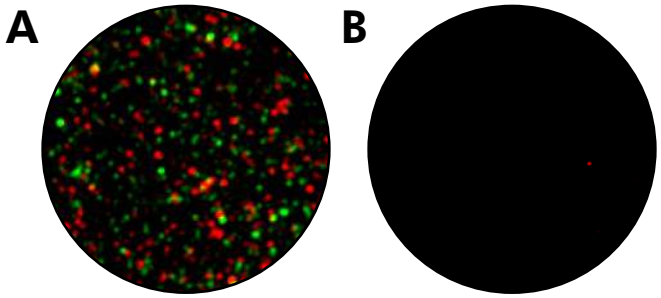

Figure 4: The ExoView Isotype Control is a built-in check of each assay. EVs are incubated on a chip with anti-CD81 capture antibody and stained with fluorescent anti-CD81 (green), anti-CD63 (red), and anti-CD9 (blue) antibodies. (**A**) CD81 capture spot showing bound vesicles counterstained with fluorescent antibodies and (**B**) Isotype Control from same chip showing very limited non-specific response.

Isotype controls serve as a negative control to identify any non-specific binding in both interference and fluorescence measurements. Figure 4 displays a typical image with abundant fluorescence signal on the experimental spot (A) and a minimal singnal on the built-in negative control spot present on all ExoView chips (B).

Measuring protein expression levels on EVs

Several perturbations lead to a change in protein expression levels on EVs, including: drug treatment, gene knock-down, gene knock-out, gene overexpression, inhibitors, artificial transcription factors, and others. Measuring protein expression at the single-vesicle level overcomes issues associated with bulk analysis, like Western blot. Bulk analysis obscures whether high signal is due to a few vesicles with high protein expression or a lot of vesicles with low protein expression. In addition, bulk analysis does not distinguish between proteins associated with EVs and proteins in solution. Single-EV data enables the researcher to deconvolve signal and draw definitive conclusions about the location and the level of expression of a given protein.

To illustrate the resolution ExoView data offers, we analyzed expression levels in a knock-down (KD) system. To compare protein expression levels of individual EVs between the KD and control samples conditioned cell culture media (CCM), we generated a scatter plot of fluorescence intensity against particle diameter (Figure 5A). EVs in both the KD and the control sample demonstrated a range of expression levels, but the KD levels were clearly reduced. We further analyzed the EV fluorescence intensity data and performed an unpaired two-tailed t-test to compare the two distributions (Figure 5B). The linearity of the ExoView fluorescence data enabled quantitative comparison of protein expression on single EVs.

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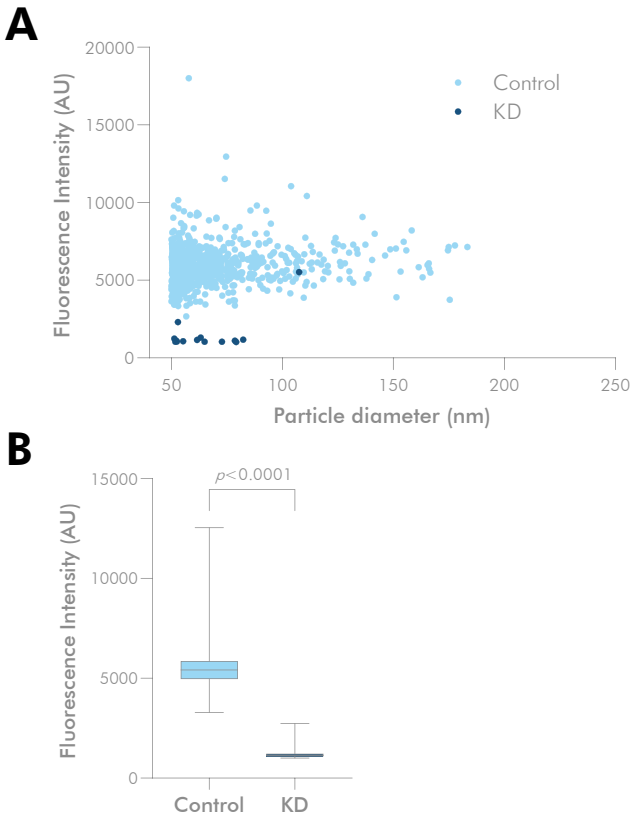

Figure 5: Protein expression levels can be measured via fluorescence signal. EVs from CCM of Control (light blue) and knock-down (dark blue) cultures were captured and stained for the protein of interest. (**A**) Protein expression level was measured at the single-EV level and was deterined to be lower in the KD than in Control EVs. (**B**) Fluorescence intensity was quantified, and the EVs in the KD condition were found to have a 10-fold decrease in expression compared to Control.

Sensitivity of rare event detection

In diagnostic applications, the number of disease specific EVs can be very small relative to the total pool of EVs in circulatory blood. As such, the detection of rare EVs in a specific and reliable manner is of paramount importance. Moreover, when comparing differences between samples, it is critical to establish that the signal is linear and both statistically and biologically relevant.

The linearity of ExoView fluorescent EV counts and thus the capability of ExoView to reliably quantify EVs at a range of concentrations was tested. HEK 293 supernatant was measured on ExoView (Figure 6) across a series of 1:3 dilutions spanning five orders of magnitude. A linear function was fitted to the data (R^2=0.9931), indicating that the signal from the dilutions is in fact linear with EV concentration over three orders of magnitude. Thus, ExoView measurement enables direct comparison of EV counts between samples within a range of three orders of magnitude.

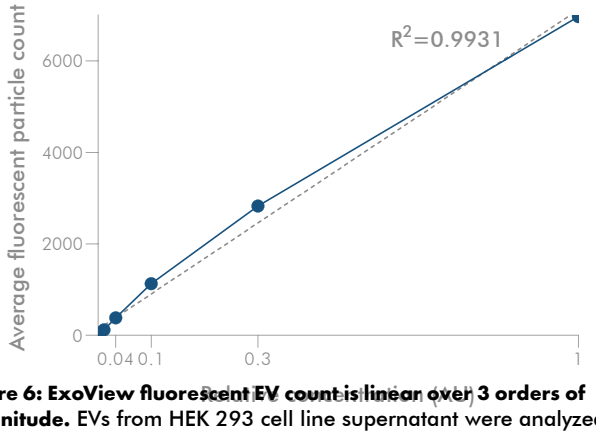

Figure 6: ExoView fluorescent EV count is linear over 3 orders of magnitude. EVs from HEK 293 cell line supernatant were analyzed using ExoView. EVs were captured using anti-CD63 antibody and stained with anti-CD81 antibody. Linear fit to the data was added (R^2=0.9931).

Quantifying protein colocalization on single EVs

For research questions that require an in-depth understanding of an EV subpopulation, single-vesicle protein colocalization analysis can help uncover fundamental insights, such as the tissue of origin of an EV carrying a cancer marker. To demonstrate the unique capability of ExoView to determine protein colocalization on single EVs, tetraspanin colocalization was assessed. EVs were purified from HEK 293 cell line supernatant, incubated with anti-CD81 capture chip and stained with anti-CD81 (green), anti-CD63 (red), and anti-CD9 (blue) fluorescent antibodies. Fluorescence images of EVs captured on the CD81 antibody were performed using ExoView (Figure 8A). ExoView takes images in red, green, and blue channels and reconstructs a single image where R/G/B images are overlaid. Table 1 shows how protein expression is represented in a 3-color overlay.

Table 1: Signal color in fluorescence mode imaging indicates protein colocalization.

Signal color	Protein profile
Green	CD81
Red	CD63
Blue	CD9
Yellow	CD81, CD63
Cyan	CD81, CD9
Purple	CD63, CD9
White	CD81, CD63, CD9

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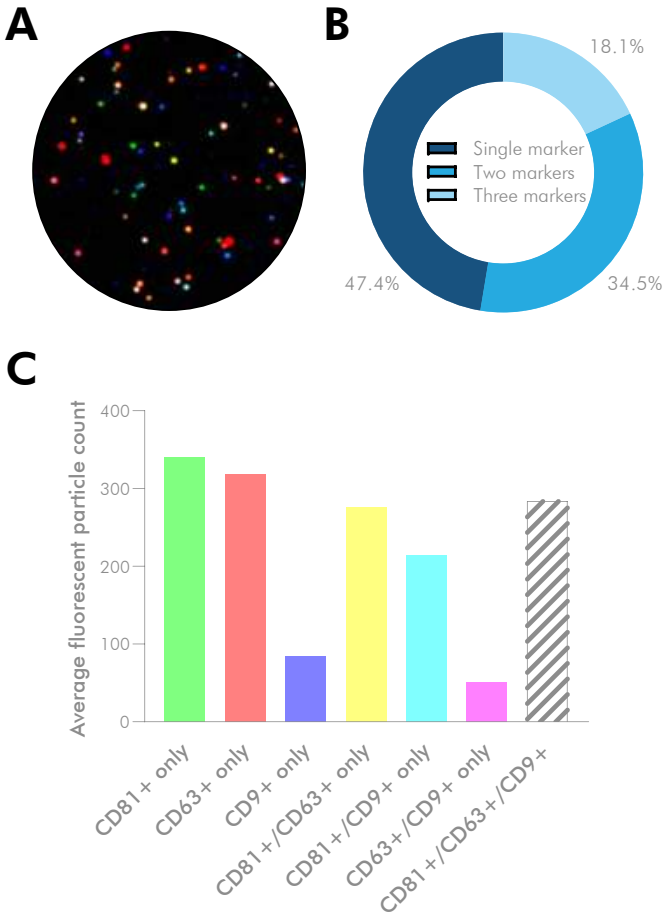

Figure 7: Single-EV protein expression data enables protein colocalization analysis. EVs in the CCM of HEK 293 cells were captured using anti-CD81 capture antibody and stained for CD81 (green), CD63 (red), and CD9 (blue). (**A**) The EV population was imaged using ExoView fluorescence mode. (**B**) Each observed EV was classified as carrying one, two, or three markers, and EVs within these categories were quantified. (**C**) Each observed EV was classified based on the specific combination of tetraspanins that it carried. EVs were quantified within these subpopulations.

The ExoView images can be quantified to determine the number of vesicles that express a specific protein. Data is represented in terms of percentages of the total fluorescent counts that correspond to each color (and therefore each protein expression combination). 47.4% of the population was found to carry a single marker (CD81, CD63, or CD9), 34.5% to carry two markers, and 18.1% carried all three proteins. This protein profile analysis of EV subpopulations enables assessment of changes in subpopulations across samples.

The tetraspanin marker colocalization is only an example of the data that can be acquired, and the choice of fluorescent secondary antibody is readily customizable to offer insight into different biological systems. Antibodies conjugated to fluorescent molecules that excite and emit at wavelengths that match the R/G/B filter sets in ExoView R100 are fully compatible with the platform. Understanding colocalization of disease-specific markers with tissue-of-origin markers is one obvious application for this capability.

Conclusion

As the understanding of the complexity of EVs deepens, research questions demand a high-resolution analysis of EVs. Parameters such as presence of a protein, protein expression level, and protein colocalization constitutes core aspects of EV research. Applying a straightforward protocol, a variety of samples were labeled and analyzed for protein expression levels, protein colocalization, and fraction of population positive for one, two, or three markers. To learn more about how the multidimensional ExoView data can give you insights into the EV subpopulations you are studying, visit www.nanoviewbio.com/characterize.

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TECHNICAL NOTE:
EV CARGO DETECTION

- Detection and quantification of cargo proteins
- Colocalization of cargo and surface markers
- Subpopulation characterization
- Direct-from-sample characterization

What the reader can expect to learn from this Technical Note:

- An appreciation for the importance of Extracellular Vesicle (EV) cargo detection.
- How ExoView® measures EV cargo at a single-vesicle level, providing valuable insights into EV based diagnostics and therapeutics.
- How the ExoView platform measures EVs without purification and enables simultaneous EV surface and luminal protein analysis.
- How ExoView offers analyses that enable adherence to the best practices in EV measurement by closely following MISEV guifelines.

Introduction

As studies into the role of EVs in disease advance, understanding the cargo that EVs use for cell-to-cell communication has become critically important. EV cargo molecules have been identified as possible biomarkers for Alzheimer's disease, inflammation, mortality, and others.[1,2,3,4] In cancer cells, EV cargo has been shown to regulate tumorigenesis by promoting the growth of tumor vasculature, recruiting cancer-associated fibroblasts, increasing metastatic potential, and evading immune detection.[5,6,7]

In therapeutic applications, EVs are considered ideal candidates for drug delivery to targeted sites, especially as they readily cross the blood brain barrier. Understanding how efficiently small or large molecule therapeutics are loaded into EVs and how efficiently these vectors are purified is a crucial requirement.

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The Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines recommend intracellular cytosolic protein detection as part of standard protocols for EV characterization. The MISEV guidelines aim to demonstrate the presence of a lipid bilayer enclosing intracellular material to reject the possibility that cellular fragments are erroneously measured. For this purpose, measurement of intracellular cytosolic proteins, including ALIX and Syntenin, is recommended in conjunction with external transmembrane or lipid-bound proteins.

The ExoView platform provides the ability to measure both internal and external proteins at the single-vesicle level and to colocalize their presence on single EVs, thus validating that the particles studied are indeed EVs. EV subpopulation fractions can be quantified in terms of the number of vesicles containing specific cargo proteins. Lastly, simultaneous measurement of EV size and count of EV subpopulations, together with single-antibody sensitivity and purification-free analysis, make the ExoView platform a robust and widely applicable research tool.

Cargo detection through permeabilization

EVs are immobilized on ExoView chips by affinity capture against EV transmembrane proteins (CD81, CD63, and CD9). Once affinity captured, EVs are fixed and permeabilized with the ExoView Cargo Kit to enable access of antibodies to EV cargo. EVs are incubated with up to three fluorescent antibodies or dyes, which can be targeted against surface and luminal proteins, and labeled EVs on chips are read out using the ExoView R100 instrument.

HEK293 cell culture supernatant was tested with and without EV permeabilization (Figure 2). Surface tetraspanin CD9 and luminal Syntenin were probed using fluorescent antibodies. As expected, permeabilization enabled detection of luminal Syntenin, which is otherwise undetectable. Permeabilization was achieved through the use of the ExoView Cargo Kit.

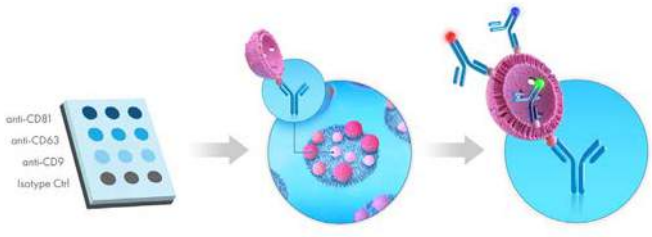

Figure 1: ExoView detects up to 4 proteins per EV, one capture probe plus three fluorescent probes. EVs are affinity captured against ubiquitous EV transmembrane proteins. EVs are subsequently fixed and permeabilized and stained with up to three fluorescent antibodies targeted to surface or luminal proteins.

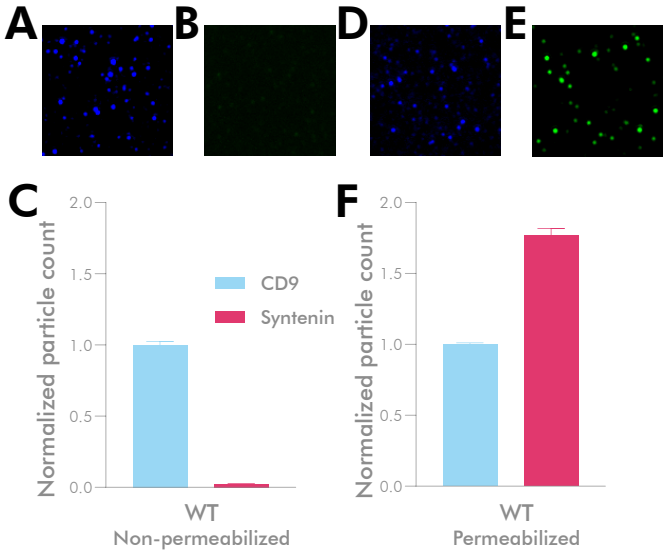

Figure 2: ExoView Cargo Kit enables detection of luminal Syntenin. EVs from WT HEK293 cultures were probed for the transmembrane CD9 and for luminal Syntenin under non-permeabilized (A-C) and permeabilized (D-F) conditions. (**A**, **D**): CD9-positive EVs. (**B**, **E**): Syntenin-positive EVs. (**C**): Quantification of imaging data in A, and B. (**F**): Quantification of imaging data in D and E. More permeabilized vesicles stained for Syntenin, demonstrating that some of the vesicles captured on the CD63 spot do not express CD9.

Testing specificity of ExoView cargo detection

Syntenin knock-out (KO) HEK 293 samples were tested to demonstrate the specificity of the ExoView Cargo Kit. EVs from KO HEK 293 cultures were permeabilized and probed using fluorescent anti-syntenin antibodies as per the ExoView Cargo Kit protocol. The absence of Syntenin signal in the KO samples indicates the specificity of Syntenin labeling in the ExoView Cargo Kit.

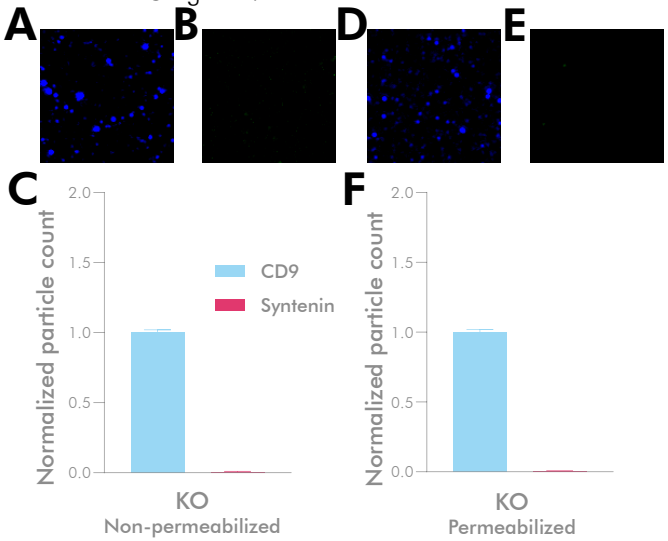

Figure 3: Syntenin detection is specific in ExoView Cargo Kit. EVs from Syntenin KO HEK293 cultures were treated and probed as in Figure 4.

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Colocalization and quantification of surface and cargo proteins

As discussed, colocalization of EV surface proteins and cargo markers on single EVs is critical in classifying EVs, especially in the context of diagnostic and therapeutic applications. The ExoView platform provides protein measurements and determines surface and cargo protein colocalization on a single EV. Figure 4 shows a scatter plot with EVs that express the transmembrane tetraspanin CD63 and luminal Syntenin. This data offers insight both into protein expression level (Syntenin level is high in permeabilized sample, pink; low in non-permeabilized sample, grey) and into colocalization of CD63 and Syntenin. The utility of the measurement of a surface and a cargo protein on the same EV is easy to imagine in a diagnostic application, for example, where a cancer and a tissue marker colocalizing on a single EV would provide diagnostic insight. When working on an EV therapeutic, this data can help determine the efficiency of cargo loading into vesicles. Learn more about the types of analyses ExoView fluorescence data offers in Technology Note: Precision phenotyping with single-vesicle analysis.

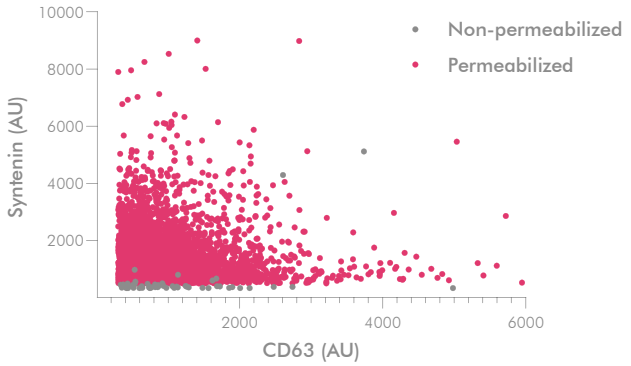

Figure 4: Colocalization of surface CD63 and Syntenin cargo on individual EVs. EVs from HEK 293 cultures were analyzed for CD63 and Syntenin with and without permeabilization. Each dot represents a single EV

Conclusion

The ExoView platform is the first commercially available tool that enables the measurement of luminal EV proteins and surface EV proteins. Measuring both at a single-vesicle resolution helps researchers comply with MISEV guidelines to address the specific analysis of EVs. The application of the ExoView permeabilization kit allows the measurement of specific cargo proteins that may be important in diagnostic applications. Staining of nucleic acid cargo may also be possible through the use of either sequence-specific probes or general nucleic acid stains (NanoView has validated neither to date).

Lastly, in therapeutic applications, fluorescent small-molecule cargo or large molecules fluorescently labeled (via antibodies or fluorescent tags or proteins) can be detected and quantified at a single-EV level. This can allow for measurement of the loading capacity of drug delivery vectors in therapeutic process optimization.

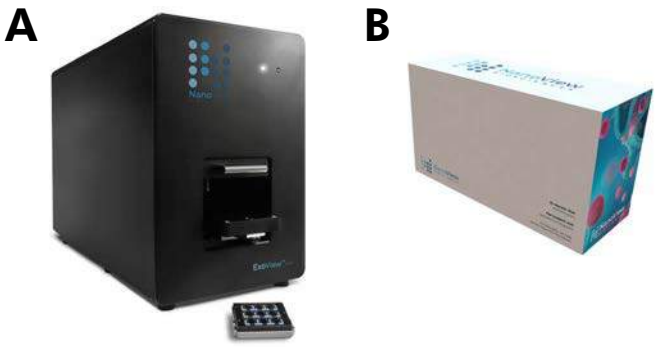

Figure 5: ExoView Cargo Kit for luminal protein detection. **(A)** ExoView R100 instrument for reading out EVs on microarray chips. **(B)** ExoView Cargo Kit with capture-antibody-functionalized microarray chips and fluorescent antibodies for cargo labeling.

To learn about how ExoView data can transform your EV cargo analysis, visit www.nanoviewbio.com/characterize.

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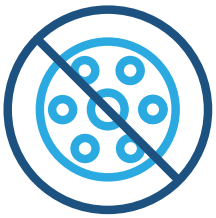

TECHNICAL NOTE:
DIRECT-FROM-SAMPLE EV ANALYSIS

- Direct-from-sample analysis
- Simple, bias-free workflow
- Sizing and quantification
- Detection of up to 4 markers

What the reader can expect to learn from this Technical Note:

- MISEV guidelines and challenges associated with EV purification.
- The challenges associated with particle sizing techniques when detecting disease-specific EVs from complex biological solutions.
- How specific measurement of EVs using ExoView® provides the ability to measure complex samples like plasma or serum without the need for sample purification.
- How ExoView offers a way to monitor downstream purification of EV-based therapeutic vectors.
- Examples of cell culture supernatant and urine samples characterized with and without purification
- How costs can be saved by removing the need for purification and expensive EV-depleted growth media.

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Introduction

The Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines state that "absolute purification or complete isolation of EVs from other entities is an unrealistic goal."[1] It is widely considered a challenge to get high purity and high EV recovery in tandem. High recovery is often associated with loss of specificity and vice versa (Figure 1).

Depending on the mode of operation, purification techniques are likely to add technique-specific biases. The time and cost of sample purification add additional challenges to the field, especially when considering large volume purification, as may be the case in therapeutic vector production or large sample numbers that may be associated with diagnostic applications. These technique-specific biases introduce contaminants that carry forward into downstream sample analysis and may confound or bias data generated using downstream analytical tools.

Common contaminants co-isolated with EVs are High- and Low-Density Lipoproteins (HDL, LDL), viruses, and protein aggregates as well as bovine EVs from media supplements. To combat bovine EVs being mischaracterized as EVs of interest, many users turn to expensive EV-depleted cell culture media, which add significant costs to sample workflows. A summary of co-purifying contaminants adapted from MISEV guidelines is shown in Table 1. The guidelines also state that the choice of purification method is the single most critical determinant of the results of subsequent downstream analysis.

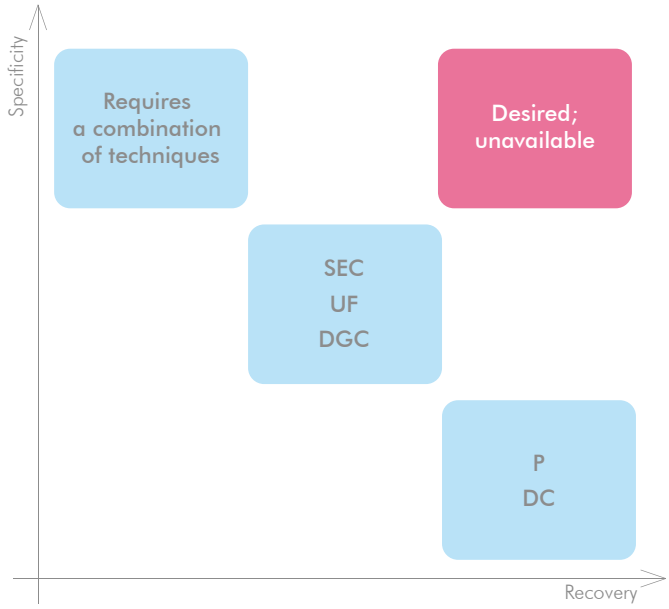

Figure 1: Tradeoffs between recovery and specificity during purification. SEC: Size exclusion chromatography; UF: Ultrafiltration; DGC: Density gradient centrifugation; P: Precipitation; DC: Differential centrifugation.

Purification and analysis specificity

As Sódar *et al.* describe, the majority of particles purified from blood serum in the same size range as EVs did not express EV markers and were found to be lipoproteins (predominantly LDL).[2] The study found that lipoproteins co-purify with EVs in all purification techniques tested. Particle characterization techniques, such as NTA and TRPS, are therefore prone to biases from these particles in subsequent analyses. This highlights the need for antigen-specific particle characterization, especially for complex samples.

Table 1: Comparison of different purification methods. Adapted from MISEV guidelines.

	Differential Centrifugation (DC)	Density Gradient Centrifugation (DGC)	Size Exclusion Chromatography (SEC)	Ultrafiltration (UF)	Immunocapture (IC)	Precipitation (P)
Contaminants	Lipoproteins, protein aggregates, viruses	Lipoproteins (HDL)	Lipoproteins, proteins, protein aggregates, viruses	Same-size particles	Soluble proteins	Proteins
Major Artefacts	EV-particle aggregates			EV-particle aggregates		Protein complexes, EV-particle aggregates
EV recovery (%)	2-80	10	40-90	10-80		90
Assay time (h)	3-9	16-90	0.3	0.5	4-20	0.3-12
Sample volume	mL-L	μL-mL	μL-mL		μL-mL	μL-mL

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To demonstrate the significance of contaminants, consider the sensitivity of measurement to a change in the population of disease-specific EVs. As Sodar *et al.* demonstrated, EVs constitute at most 10% of the population of particles detected by a non-specific particle detection technique.[2] If disease-specific EVs constitute at most 1% of EVs, there will be a single disease-specific EV per 1000 particles of similar size. A researcher who wants to measure the impact of a disease (such as cancer) on the release of EVs and who is using a particle characterization technique to measure EVs will observe a doubling of the signal when the number of disease-specific EVs has increased 1000-fold (Figure 2A). By comparison, the same researcher, when using an antigen-specific technique, will observe a doubling of the signal when the number of disease-specific EVs doubles (Figure 2B). This is true even if the actual particle count is extremely low, making antigen-specific techniques well-suited for rare event detection.

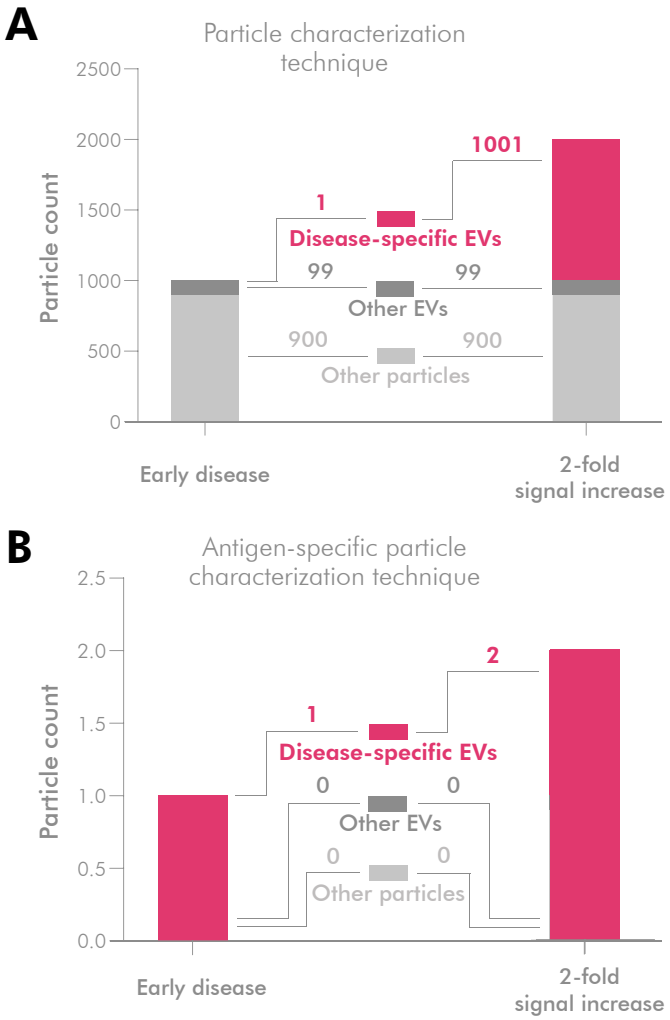

Figure 2: Signal increases with change in disease-specific EVs. (**A**) Given the frequency of disease-specific EVs, a doubling in signal occurs when disease-specific EVs have increased 1000-fold. (**B**) When measuring EVs using an antigen-specific technique, the signal doubles when disease EVs have doubled.

Detecting and characterizing EVs directly from sample using ExoView

ExoView is a technology that addresses purification limitations by analyzing unprocessed samples from serum, plasma, cell culture supernatant, urine, and other biological sources without the need for pre-purification. 35µl of sample is required for analysis; however, in most cases, samples are diluted before measurement. Affinity capture of EVs from solution on a multiplexed microarray chip enables direct analysis without sample purification. The single-use chip is functionalized with an array of antibody spots (Figure 3), with EV specific antibodies (CD81, CD63, and CD9) present on the chip. Each antibody spot serves to capture EVs that present the corresponding EV protein. This allows for positive selection and analysis of antigen-positive EV subpopulations from a complex mixture without any labeling.

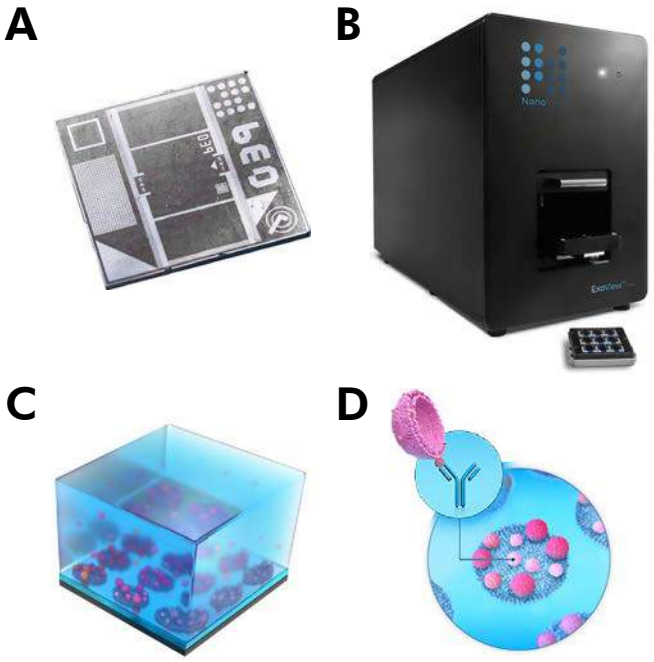

Figure 3: The ExoView microarray chip works through EV affinity. (**A**) Photograph of the microarray chip with specific affinity antibody spots. (**B**) Photograph of the ExoView instrument, in which the microarray chips are read out. (**C**) Sample incubated on ExoView microarray chip. (**D**) EVs that carry corresponding antigens bind to the antibodies on the microarray chip and are ready for analysis.

Interferometric detection of exosomes in the ExoView R100 platform has been previously discussed.[3,4,5] Light is emitted from a visible light source onto the surface of the ExoView chip where EVs are captured. The light is scattered based on the size of the captured EV and reflected from the chip surface. Interference of the reflected light results in a high-resolution size distribution of the antigen-positive EVs. To learn more about measuring size distributions of EV populations, please refer to Technical Note: Sizing.

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In fluorescence mode, the same antigen-captured EVs can be labeled using up to three different fluorescent antibodies. With the addition of fluorescence, up to four proteins can be measured on single EVs. Either surface or luminal proteins are measured, colocalized, and relative expression levels estimated on each individual EV captured. To learn more about fluorescent data collected by ExoView, please refer to Technical Note: Precision phenotyping with single-vesicle analysis and Technical Note: EV cargo detection.

Conditioned media analyzed with and without purification

Conditioned cell culture media (CCM) was measured on the ExoView platform with and without purification. Purification of the conditioned media was performed using industry standard Ultracentrifugation (UC) protocols and compared with untreated conditioned media from the same sample. A standard ExoView chip that captures EVs expressing CD81, CD63, and CD9 was used. EV counts on all three capture antibodies were very similar when comparing purified and unpurified samples (Figure 3). This suggests that ExoView measures EVs specifically disregarding contaminants in the sample.

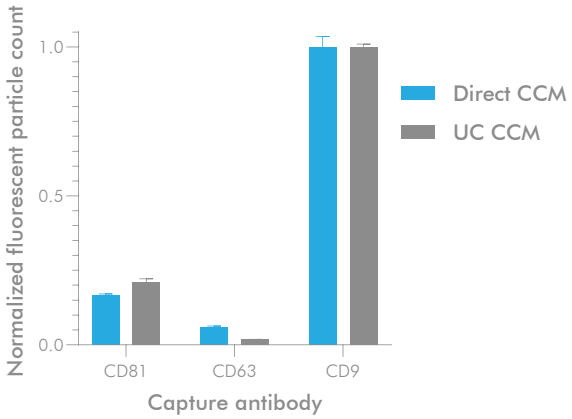
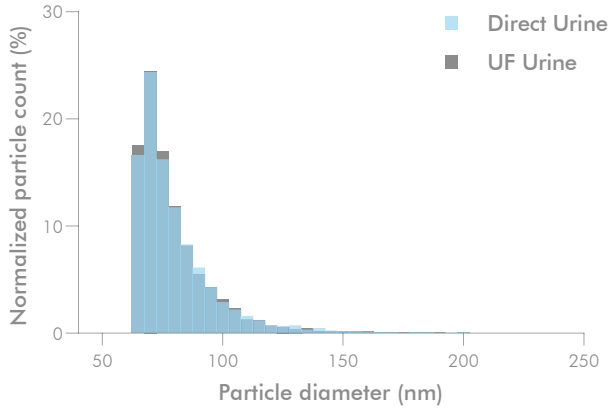


Figure 3: Direct-from-sample and purified sample analyses. EVs from conditioned culture media (CCM) were either analyzed directly (Direct CCM) or after purification via ultracentrifugation (UC CCM). EVs were captured using anti-CD81, anti-CD63, and anti-CD9 capture antibodies and stained for CD81, CD63, and CD9. Total fluorescent EV counts were acquired and normalized to CD9 in both samples. Direct and UC samples show the same trend.

Sizing of EVs from urine with and without purification

Urine samples were measured on the ExoView platform with and without purification. EVs were captured on standard chips with antibodies against CD81, CD63, and CD9, and size measurements performed using interferometric light measurements on the EVs captured on each antibody spot. Urine samples were measured with and without ultrafiltration. Sizing and counting data for CD9-positive vesicles is shown in Figure 4A. EVs positive for CD81 and CD63 showed a similar trend. Quantification of vesicles positive for CD81, CD63, and CD9 is shown in Figure 4B. The observed distribution of the vesicles measured in purified and non-purified samples was similar. When EVs are measured specifically, similar results can be measured directly-from-sample and post-purification. This illustrates how ExoView can specifically and accurately measure EV populations with and without sample purification.

A



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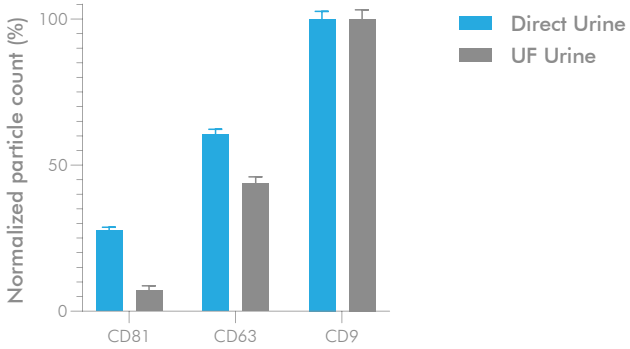

Figure 4: Direct-from-sample urine analysis shows a similar size distribution and protein makeup as ultrafiltration-purified urine. (**A**): Size distribution of CD9-positive vesicles in an unpurified (Direct Urine) and a purified (UF Urine) urine sample. (**B**) Particle counts of particles pre and post purification on CD81, CD63, and CD9 capture antibodies.

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Monitoring downstream purification of EVs

We have demonstrated that the ExoView platform is equally well-suited for measuring purified and complex biological samples containing EVs. This has clear implications when considering development and production of EV therapeutic vectors. When purifying a vector for therapeutic use, it is essential to measure the efficiency of purification from one step to the next and to test the vector against QC standards. ExoView provides an ideal tool for monitoring these processes. ExoView is the only tool that can routinely measure samples without sample purification and, therefore, can be used throughout the vector purification process from cell culture supernatant to final product.

Conclusion

In this Technical Note, we discussed the challenges associated with the purification of EVs. We showed that ExoView makes specific measurements of EVs in purified and complex samples. ExoView is an effective solution that can eliminate purification steps required for sample analysis and therefore improve time and investment costs that are associated with typical EV workflows. When purification is necessary to generate a final product (as would be the case in therapeutic vector development), ExoView provides a tool capable of monitoring the efficiency of purification from start-to-finish of the purification process.

The ability to measure EVs in an antigen-specific way is critically important for fundamental EV research as well as for rare event detection in dianostic and therapeutic applications. Disease-specific EVs may be present at low concentrations in plasma relative to other circulating EVs and contaminants. The ability to measure specific EVs without purification or bias from other EV populations provides the means to more effectively study these rare events.

To learn what analyses the multidimensional ExoView data enables you to perform, visit www.nanoviewbio.com/characterize.

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NanoView BIOSCIENCES

TECHNICAL NOTE: SIZING

- Direct-from-sample characterization
- Sizing of heterogeneous samples
- Subpopulation characterization: sizing and protein profiling

Introduction

The 2018 edition of the International Society of Extracellular Vesicles opinion paper titled "Minimum Information Supporting Extracellular Vesicle Research," or MISEV, describes isolation and analysis protocols as well as experimental design necessary to ascribe origin and functional capabilities to the EVs of interest.[1] Specifically, every EV sample needs to be analyzed along the following parameters:

- Quantification
- Sizing
- Biophysical features
- Presence of membrane-bound cytosolic compartment
- Purity control
- Compartment of origin
- Manner of association of functional proteins.

ExoView® delivers rich multidimensional data that addresses a number of these parameters. For additional materials on how to use ExoView to meet MISEV requirements, see www.nanoviewbio.com/characterize. Here we focus on analyzing the size of extracellular vesicles.

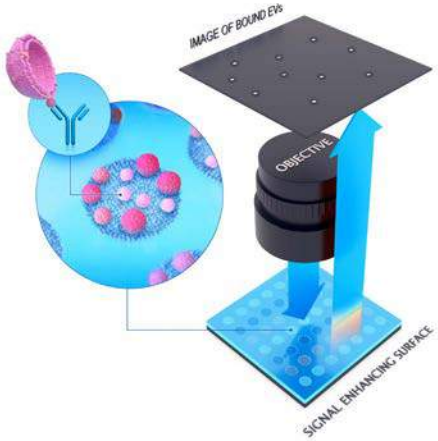

Figure 1: ExoView detects captured EVs using interferometry. Spots on a chip are coated with antibodies specific to a protein on the surface of an EV. During sample incubation, EVs are captured by the antibodies and become bound to the chip. During analysis, light emitted from a source onto the chip is reflected by both the signal-enhancing silicon dioxide chip surface and the captured EVs. The interferometer measures the interference of this reflected light to determine the size of the captured EV.

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The ExoView platform provides a turn-key solution for comprehensive characterization of EVs. It analyzes single EVs and delivers the size distribution for a defined EV population in the sample. The analyzed populations are defined by the capture antibody on the surface of the ExoView chip. Multiple antibodies can be immobilized on the chip to allow characterization of several populations at a time. Samples can be analyzed directly from cell culture supernatant, plasma, serum, urine, or another sample matrix without the need for purification.

The chip is functionalized with an array of antibody spots. Each spot serves as a capture area for EVs that present the corresponding antigen. This allows for positive selection and specific analysis of antigen-positive EVs from a complex mixture without any labeling. The interferometric detection of exosomes used in the ExoView platform has been previously discussed.[2,3,4] Briefly, light is emitted from a visible LED source onto the silicon dioxide surface of the ExoView chip. Light reflected from the chip surface interferes with light scattered by captured EVs in a size-dependent manner. By measuring light interference, ExoView delivers a high-resolution size distribution of antigen-captured particles.

Detecting heterogeneous populations

To demonstrate the ability of ExoView to discriminate between particles of different sizes, we immobilized EVs to the chip and measured a mix of NIST-traceable polystyrene nanospheres of 50, 100, 150, and 200 nm. While polystyrene is not a perfect surrogate for EVs in terms of their refractive index, these nanospheres have tight size distributions and here serve to demonstrate the peak-to-peak resolving capability of ExoView. Figure 2A shows the label-free image of the polystyrene nanospheres acquired through interferometric imaging. Figure 2B demonstrates the close alignment of the observed (solid blue line) size distribution and the modeled (dashed grey line) peaks for the polystyrene nanospheres as provided by the manufacturer. In this multimodal particle population of sizes relevant to EV research, ExoView detects and correctly determines particle sizes with the same acquisition settings across all sample types.

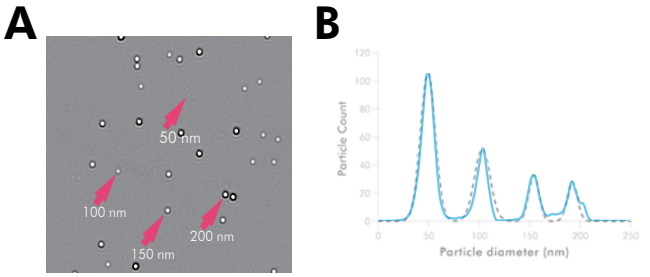

Figure 2: ExoView can detect a multimodal population of particles sized between 50 and 200 nm. A mix of polystyrene nanoparticles of sizes 50 nm, 100 nm, 150 nm, and 200 nm was analyzed using ExoView. (**A**) Representative high-magnification image of an area on a single capture spot. Blue circles encapsulate algorithmically identified particles. (**B**) Average particle count (y-axis) was plotted against particle diameter (x-axis). Observed and modeled size distribution in solid blue and dashed grey, respectively.

Size consistency with high-resolution imaging

In a recent study, Bachurski *et al* compared sizing data from ExoView and two commercially available Nanoparticle Tracking Analysis (NTA) technologies against Transmission Electron Microscopy (TEM) sizing data for a serum sample and media from L-540 cells that were purified by ultracentrifugation (10,000x*g* and 100,000x*g* pellets).[5] In all cases, the ExoView platform produced mean sizes of ~50nm which were consistent with the mean sizes derived from TEM. At the same time, both NTA instruments produced mean sizes of over 100nm across the samples.

To confirm that the observed small particles were, in fact, EVs, we performed an immunogold staining against CD63, a protein commonly associated with EVs. EVs from BxPC3 cell culture were stained for TEM with 1% Uranyl acetate. Figure 3A shows a TEM image of the EVs (size labels in

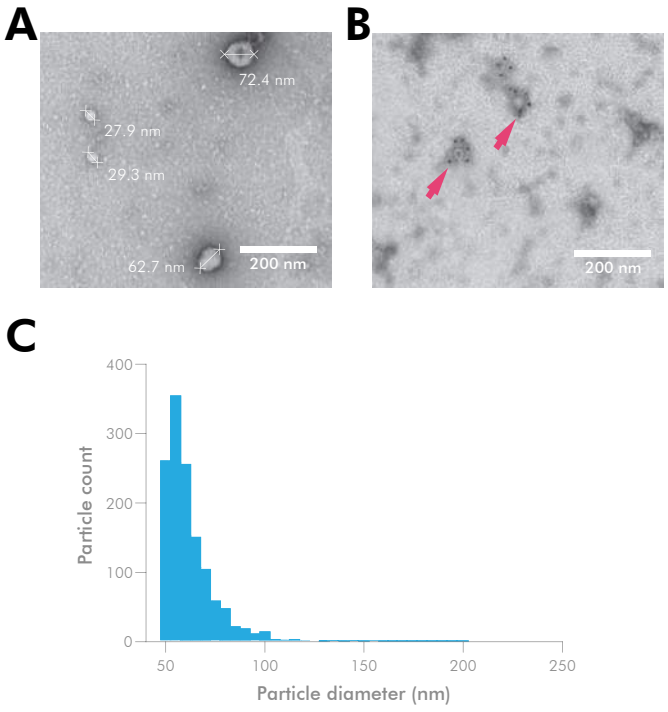

Figure 3: ExoView size distribution is consistent with Transmission Electron Microscopy (TEM). EVs from BxPC3 cell line were purified using Size Exclusion Chromatography (SEC) and imaged with TEM. (**A**) EVs were sized (sizes annotated). (**B**) EVs were immunostained against CD63 using gold nanoparticles and imaged using TEM. Arrows indicate examples of CD63-positive EVs. (**C**) EV size distribution measured with ExoView. EVs in the conditioned media from BxPC3 cell line were captured using CD63 antibodies. Sizes were calculated using interferometry measurements in ExoView. The average particle count (y-axis) from 3 antibody spots was plotted against vesicle diameter (x-axis). The population exhibited a mode of 55 nm and a mean of 70.8 nm.

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white), while Figure 3B is a TEM image of the immunogold anti-CD63 staining. Red arrows indicate examples of small vesicles positive for CD63. The size distribution of the population in Figure 3C has a mode of 55 nm, reflecting the inclusion of small EVs observed in TEM.

Specific Sizing of EVs and EV subpopulations

Many existing technologies do not specifically measure the size of EVs, but rather all particles in a solution within a certain size range in the sample. Given the challenges in purification, this often leads to indiscriminate co-analysis of mixed pools of EVs as well as contaminants (lipoproteins, LDL, HDL, protein aggregates, cell debris, etc). ExoView only analyzes EVs that carry a specific protein signature enabling selective analysis of the population of interest and confidence in the specificity of the measured size distribution. With ExoView, protein signatures can be translated into custom panels to enable side-by-side comparisons of EV subpopulations of interest.

Sizing from raw and purified samples

With purification procedures lengthy and costly over sample cohorts, the ability to measure particle size in the sample directly is advantageous. As discussed above, ExoView is uniquely able to perform such measurements due to the specificity of EV capture technology.

We measured EV size distribution directly from conditioned media as well as after performing density centrifugation using OptiPrep density gradient medium. Figure 4 shows size distribution as measured directly from conditioned media (Figure 4A) and after OptiPrep density centrifugation (Figure 4B). The two samples exhibit a mode of 55 nm and 50 nm and a mean of 60.6 nm and 59.2 nm, respectively. While the absolute counts differ, likely due to purification process and dilution, the mode and the mean of the two populations are comparable.

To adjust for different total counts, we generated normalized size distributions by dividing the number of EVs in a size bin by the total number of EVs detected (Figure 4C). The resulting size distributions exhibit the same features. Although the purified sample slightly favors smaller vesicles, the majority of the vesicles in both samples are 55 nm or smaller. Importantly, characterization of conditioned media and of density-purified EVs converge. The results suggest that ExoView can be used for direct-from-sample analysis of heterogeneous EV populations and deliver measurements reflective of the true EV population composition and unaffected by contaminants.

Uncovering distinct populations

It is widely known that a number of cancer types lead to an increase in the overall number of EVs, which has been proposed to facilitate invasion, intravasation, circulation, extravasation, and proliferation.[6] However, the particular characteristics of this increasing population remain to be

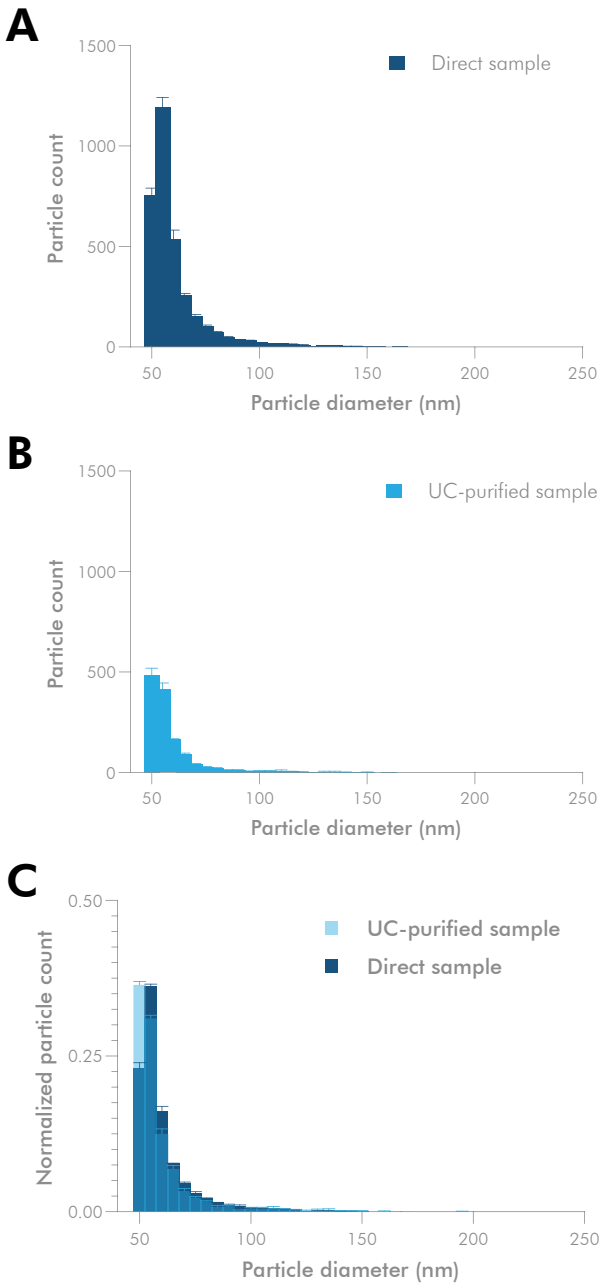

Figure 4: Size distribution measured by ExoView is unaffected by the presence of contaminants. (**A**) Size distribution of EVs direct from sample. The average particle count (y-axis) from 3 antibody spots was plotted against vesicle diameter (x-axis). Error bars display the standard error of the mean (SEM). The population exhibited a mode of 55 nm and a mean of 60.6 nm. (**B**) Size distribution of EVs after OptiPrep density centrifugation. The average particle count (y-axis) from 3 antibody spots was plotted against vesicle diameter (x-axis). Error bars display the standard error of the mean (SEM). The population exhibited a mode of 50 nm and a mean of 59.2 nm. (**C**) Overlay of relative size distributions of EVs direct from conditioned media and EVs purified from media by density centrifugation. Number of particles observed in a size bin was divided by the total number of observed EVs, and the fraction of total particles (y-axis) was plotted against particle diameter (x-axis).

elucidated. ExoView enables a detailed analysis of this population. Whereas protein expression analysis is discussed elsewhere (Technical Note: Precision phenotyping with single-vesicle analysis), here we will focus on the sizing analysis of such populations.

In a comparative study, we analyzed conditioned media from a variety of cell lines, including HEK 293, an adrenal cell-derived cell culture, and PANC 1005, a pancreatic adenocarcinoma-derived cell culture. Figure 5A shows the size distribution of EVs from HEK 293 with population average of 57.7 nm. This is typical of most cell lines and other samples. However, the EV population from PANC 1005 (Figure 5B), in addition to the typical peak around 55 nm, exhibited a peak centered around 90 nm. This distinct population consisted of larger EVs positive for CD81, CD63, and CD9. Further investigation is needed to characterize this subpopulation and identify any relevant proteins displayed on or carried by these EVs.

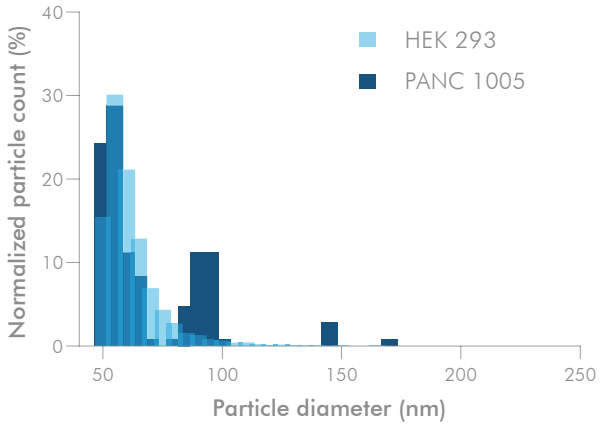

Figure 5: Size distribution of tetraspanin-positive EVs is different in two different cell types. Conditioned media from HEK 293 cell line and PANC 1005 cell line was collected, incubated on chips with CD63 capture probes, and imaged using ExoView. Relative size distribution normalized to total count of EVs derived from HEK 293 cell line (light blue) and PANC 1005 cell line (dark blue). Fraction of total particles (y-axis) was plotted against particle diameter (x-axis). A characteristic peak was observed at 55 nm in both HEK 293 and PANC 1005. In addition to the characteristic peak at 55 nm, PANC 1005 cell line exhibited a peak at 90nm suggesting a presence of a distinct, larger-sized population of EVs.

Sizing subpopulations based on surface protein

In another example, we analyzed the EV population upon introduction of an overexpression vector into HEK 293 cell line. We used OriGene CD171 overexpression (CD 171 OE) vector to transiently transfect HEK 293 cells and confirmed expression by Western Blot (Figure 6A). We then analyzed conditioned media directly using CD81, CD63, CD9, or CD171 as capture antibodies (Figure 6B and C). We estimated the size of different subpopulations by thresholding and computing the number of EVs inside the peak against the total number of observed EVs. The size

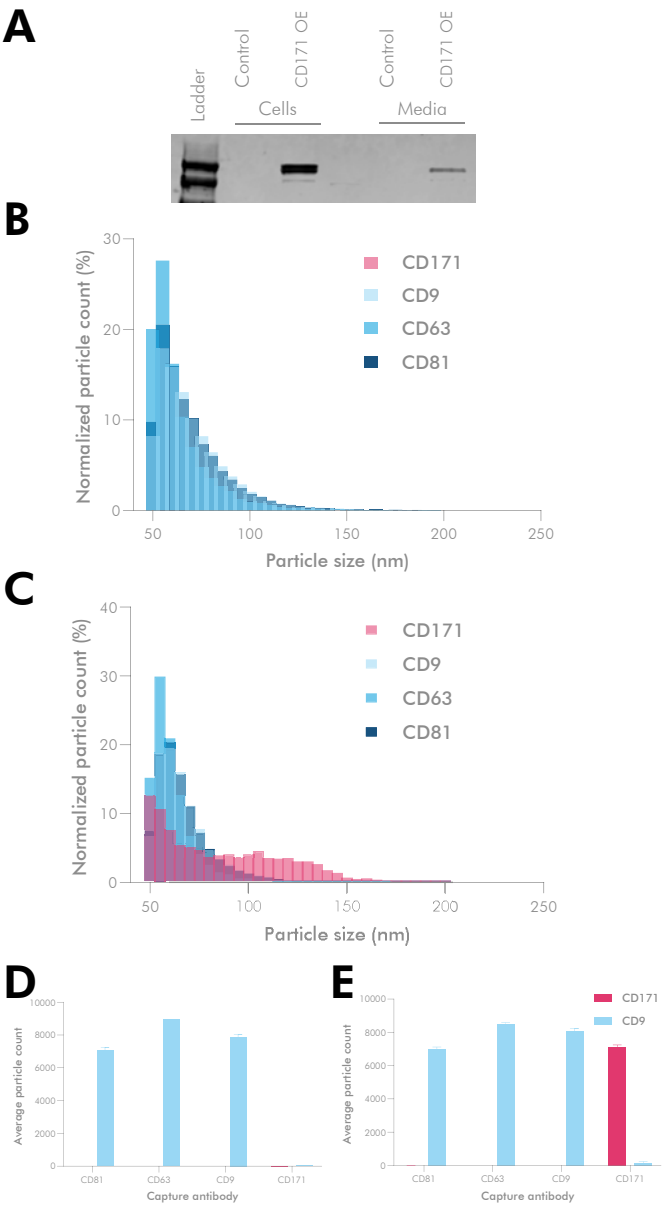

Figure 6: Size distribution of EVs in an overexpression system (CD171). (**A**) CD171 overexpression (CD171OE) was transiently induced in HEK 293 cells using OriGene vector and verified in cells and collected conditioned media via Western Blot. Conditioned media was collected from WT HEK 293 and CD171 OE HEK 293 cells, incubated on chips with CD81, CD63, CD9, or CD171 capture probes, and imaged using ExoView. Relative size distribution was normalized to the total count of EVs derived from WT HEK 293 cells (**B**) and CD171 OE HEK 293 cells (**C**) Characteristic peak was observed at 55 nm in both samples. A broad peak was observed from 80-150 nm in CD171 OE. EVs from WT HEK 293 (**D**) and CD171 OE HEK 293 (**E**) were labeled with fluorescent antibodies against CD81, CD63, and CD9 and total count was determined. EVs from the WT culture were positive for the tetraspanins and negative for CD171. Among EVs from the CD171 OE culture, those positive for the tetraspanins were negative for CD171, while those positive for CD171 were negative for the tetraspanin suggesting a distinct subpopulation of CD171-positive vesicles.

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distribution of EVs was similar for all 4 capture proteins in WT HEK 293-derived EVs, with 84% of all EVs falling within the 50-80 nm peak. However, in the population of EVs from CD171 OE HEK 293 cells, only 56% of EVs were within the 50-80 nm peak, while 38% fell into a distinct 80-150 nm broad peak.

To further analyze the broad peak subpopulation in CD171 OE sample, we used ExoView to carry out a single-vesicle surface protein profiling. Figure 6D shows the results of staining EVs using anti-CD81, anti-CD63, and anti-CD9 antibodies. The characteristic peak at 55 nm in both samples was positive for CD81, CD63, and CD9. Interestingly, however, the particles constituting the broad peak in CD171 OE sample were positive for CD171, but negative for CD81, CD63, and CD9. Further characterization may help determine the lineage of these CD171-positive particles.

Conclusion

Given the challenges with purification of EVs and the need to discriminate observations associated with EVs from those of contaminants, the ExoView platform offers a unique characterization solution to size, quantify, and perform single-vesicle direct-from-sample characterization. Here we focused on the advantages of sizing using ExoView, including detection of both small and large EVs in a heterogeneous sample and analysis of EV subpopulations specified by surface proteins. However, the most powerful analysis stems from simultaneous sizing, counting, protein profiling, and protein colocalization analysis. To learn more about how ExoView enables you to use these powerful analyses, visit www.nanoviewbio.com/characterize.

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