ONI

Extracellular Vesicles

Nanoimager

The first complete solution for single-molecule imaging of EVs

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Extracellular Vesicles
Application Notes

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Extracellular Vesicles

EV characterization: introducing super-resolution microscopy for single-EV analysis

Introduction

Extracellular vesicles (EVs) are membrane-bound vesicles secreted by all cells that facilitate intercellular communication. EVs are enriched in surface proteins (such as ALIX, flotillin, and tetraspanins) and contain luminal content (including DNA, RNA, and siRNA Figure 1). Importantly, these small nanosized vesicles that transfer active molecules and genetic materials can be isolated from complex biofluids such as blood, urine, and saliva, which makes them ideal candidates for disease diagnostics and therapeutic applications.

Since EVs resemble the composition of their parental origin, secreted vesicles are becoming a promising source for biomarker discovery, as the specific types of surface proteins and cargo may have profound pathophysiological significance. The ability to identify and measure the presence or abundance of particular components could represent particular "disease signatures". Therefore, characterizing molecular profiles of EVs may yield powerful diagnostic capabilities that more accurately determine the onset of distinct cancers[1], neurodegeneration[2], and cardiovascular diseases[3]. In this application note, we will discuss how super-resolution microscopy can be used as a powerful tool for EV characterization and imaging.

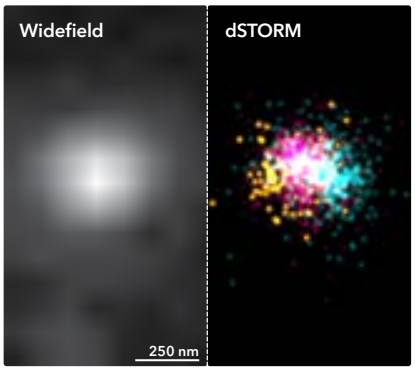

Figure 1 | 3-color widefield vs dSTORM image of CD9-ATTO488, CD63-Alexa Fluor®-647 and CD81-Alexa Fluor®-555 EVs.

Open questions

Owing to their diverse biological heterogeneity, the term EV itself encompasses a number of subpopulations referred to as exosomes, microvesicles, outer membrane vesicles (OMVs) and ectosomes. The myriad of names mentioned throughout the literature[4,5] (Table 1), stems from the cellular origin, separation and isolation abilities as well as molecular profiles of the different EVs. To unify and establish a set of universal guidelines for EV characterization, a list of minimal information for studies of extracellular vesicles (MISEV) was published in 2014 and updated in 2018 by leaders within the field to outline an official set of characterization requirements. This in turn, has greatly contributed to the development and advancement of our understanding of EVs.

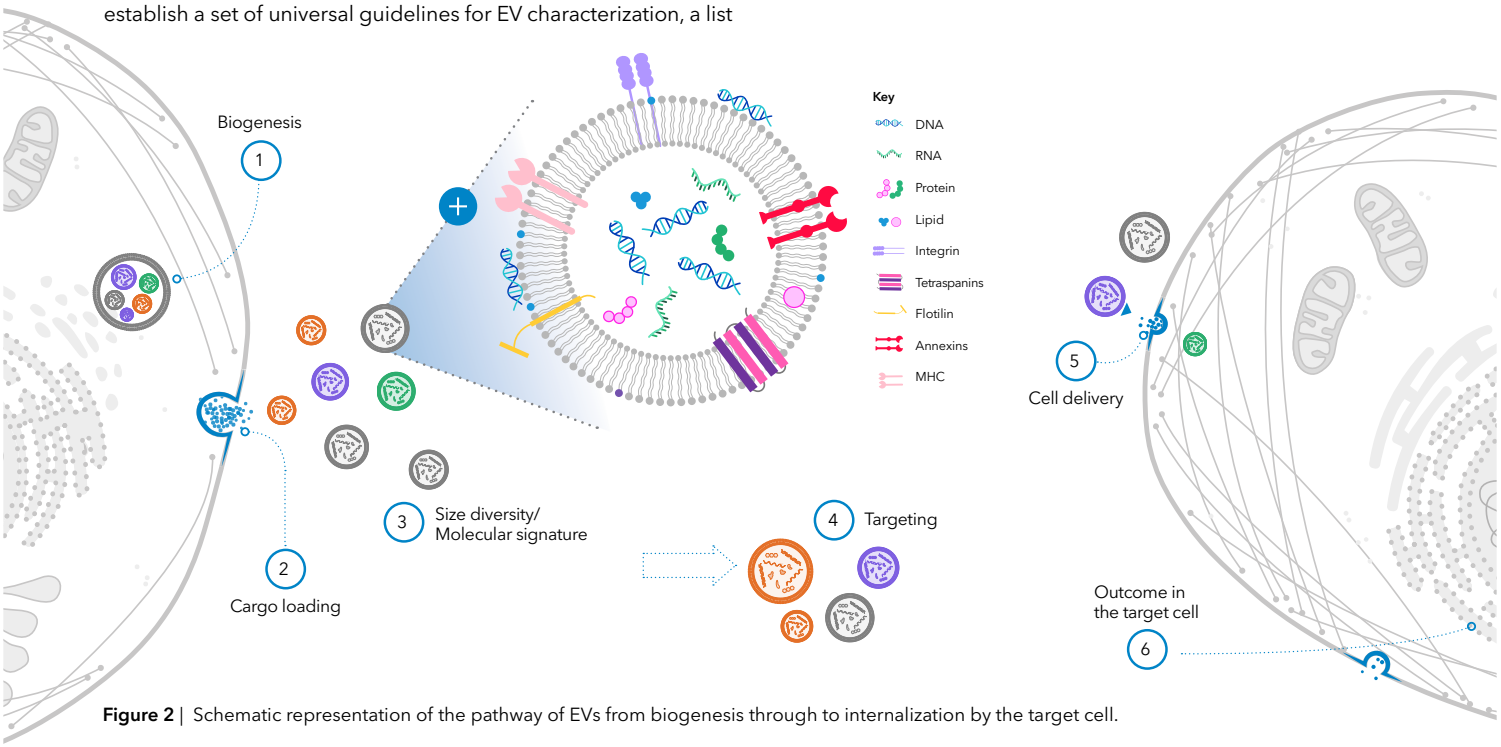

Figure 2 | Schematic representation of the pathway of EVs from biogenesis through to internalization by the target cell.

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However, there are still many fundamental unanswered questions surrounding the journey EVs take from biogenesis through to internalization by target cells (Figure 2). For instance, whilst it is understood that EVs selectively capture predefined proteins and nucleic acids, the mechanics that regulate such sorting of materials and cargo loading into the EVs is not. Another open question surrounding the EV journey relates to their distinct targeting capabilities, with studies investigating how differences in cargo and surface proteins influence their respective targets. Interestingly, the amount of cargo transported within an EV has been shown to be very low[6], which formulates the hypothesis that EV targeting needs to be highly efficient and specific, as the precision may be less important when a large number of EVs are released. Delivery is postulated to depend on the very specific surface molecules that function as 'barcodes', recognized by a receptor or "reader" situated on the cell membrane, but how this relates to an EVs target and internalization remains elusive. To answer such questions requires a complete analysis of EVs at a single-molecule level to determine individual molecular profiles of associated components, so we can ultimately harness their properties for diagnostic and therapeutic purposes.

Table 1 | Classification of EV subpopulations. Adapted from Borges et al (2013)[7].

	Exosomes	OMVs	Microvesicles	Ectosomes
Size	40-120 nm	100-400 nm	50-1000 nm	100-1000 nm
Cellular origin	All cell types	Gram negative bacteria	Megakaryocytes, blood platelets, monocytes, neutrophils, tumor cells and placenta	All cell types
Known contents	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Virulence factors, proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins and nucleic acids (non-coding RNAs)
Common markers	Alix, Annexins, tetraspanins (CD81, CD63, CD9), flotillin	Outer surface proteins (OmpA, OmpC, OmpX, AbompA), PAMPs (LPS)	Integrins, selectins, CD40	Cholesterol, sphingomyelin and ceramide

EVs and super-resolution microscopy

Current methods of EV characterization include flow cytometry, nanoparticle tracking analysis (NTA), epifluorescence imaging techniques and electron microscopy (EM). However, despite the commonplace of these techniques, they often fail to give the necessary resolution to accurately characterize individual EVs or, in the case of EM, struggle to visualize multiple markers in a single-experiment, limiting the ability to assess the molecular signatures of EVs.

Super-resolution imaging techniques, featured in the MISEV18 guidelines, can overcome the resolution limits associated with conventional light microscopy. Through Single-molecule localization microscopy (SMLM) techniques, such as dSTORM and PALM, EV biomarkers are labeled with a class of fluorophores of which the photochemical properties can be exploited to stochastically switch them between a dark and an emissive state. This allows small subsets of fluorophores to be detected in isolation and the localization of each fluorescent molecule to be fitted with a Gaussian function. By imaging in this way, SMLM retains the advantage associated with traditional fluorescence imaging techniques while circumventing the diffraction-limit, providing an achievable resolution exceeding 20 nm and allowing the spatial distribution of EV markers to be visualized with single-molecule sensitivity.

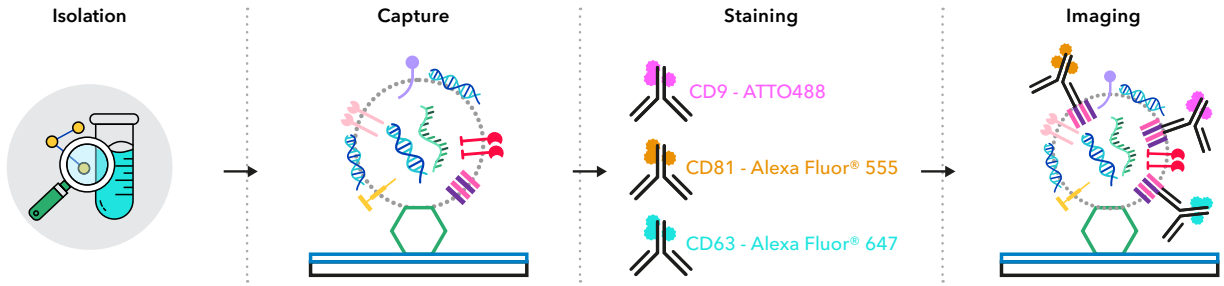

Figure 3 | Schematic representation of isolated and purified EVs, adhered to a glass slide and stained with tetraspanin markers on their surface.

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The Nanoimager is a comprehensive SMLM platform for precise and detailed molecular characterization of EVs. With a multitude of imaging modalities available, this data-driven platform facilitates exploration into composition and behavior of EVs, to provide deeper insights into vesicle subpopulation signaling pathways and intracellular interactions from both a fixed sample and live-cell perspective. To demonstrate the capabilities of SMLM on the Nanoimager in EV characterization, HCT116 EVs were isolated, purified and immunostained with commercially available antibodies against surface membrane tetraspanins, CD9, CD63 and CD81 (Figure 3) and imaged using dSTORM (Figure 4). The results demonstrated a heterogeneous population of EVs that were either single (A-C), double (D-F) or triple (G) positive for tetraspanins. Subsequent analysis would allow for the size profiles and relative abundance of each of the markers to be quantified across the EV population. This data highlights the multi-factor characterization capabilities that follow SMLM imaging, enabling researchers to assess unique protein signatures or changes in biomarker number across populations, which could have important connotations for early disease diagnostics and better understanding EV functionality.

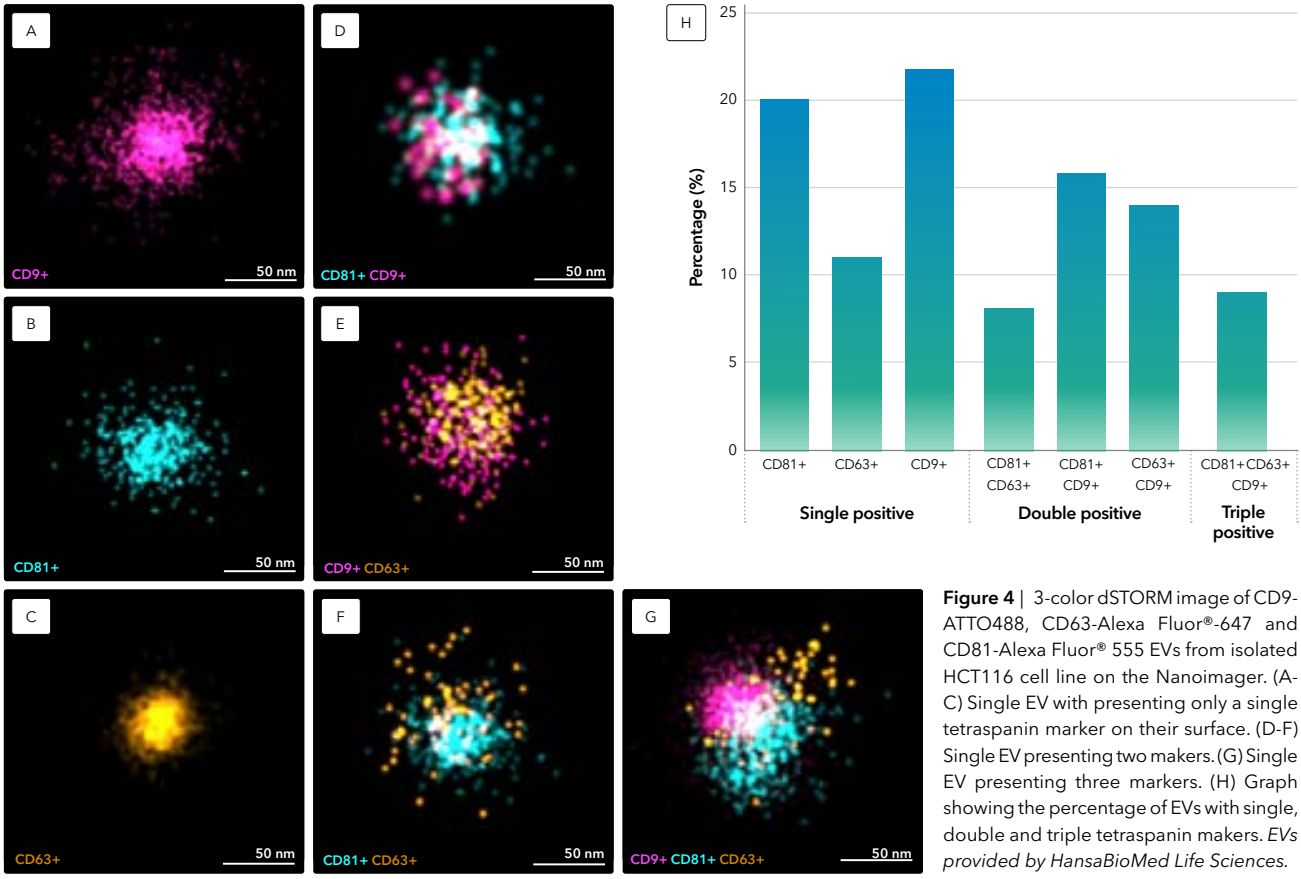

Figure 4 | 3-color dSTORM image of CD9-ATTO488, CD63-Alexa Fluor®-647 and CD81-Alexa Fluor® 555 EVs from isolated HCT116 cell line on the Nanoimager. (A-C) Single EV with presenting only a single tetraspanin marker on their surface. (D-F) Single EV presenting two makers. (G) Single EV presenting three markers. (H) Graph showing the percentage of EVs with single, double and triple tetraspanin makers. *EVs provided by HansaBioMed Life Sciences.*

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Extracellular Vesicles

EV cargo: visualizing and quantifying molecular DNA in single-EVs

Introduction

Extracellular Vesicles (EVs) are membranous particles that enable cell-cell communication via their surface components and cargo which includes proteins, lipids and genetic material such as DNA, mRNA and miRNA. While most of the transported material is carried within the lumen of EVs, a number of publications have identified a significant amount of genetic material coating the outer surface of vesicles (Figure 1). It is believed that the luminal contents of EVs are directly linked to their functional effect at recipient cells however, less is known about the role of DNA on the EV surface.

Studies have demonstrated that the presence of surface DNA modified EV adhesion properties[1], contributed to the overall net negative charge of EVs[2] and mediated horizontal DNA gene transfer[3]. Furthermore, on EVs derived from cancer cells, surface DNA contained oncogenic mutations that may imply a role of EV DNA in modulating the tumour microenvironment[4]. Therefore, spatial visualization of DNA and association with vesicles of discrete sizes or molecular signatures may contribute to a better understanding of the mechanisms and distinct functions of luminal and surface DNA in disease.

Challenge

A persistent challenge with imaging EVs is their small size (the smallest sub-category measuring between 40-200 nm in diameter) which falls below the resolution limits of conventional light microscopy. These diffraction-limited techniques restrict the user's ability to accurately size EVs, detect and quantify biomarkers or cargo, or to distinguish the fluorescent signal of an intact EV from that of fragments or isolated proteins. With super-resolution imaging techniques such as dSTORM, we can overcome this resolution limit and visualize EVs with single-molecule sensitivity.

Summary

The Nanoimager enables visualization and detection of multiple proteins and genetic material simultaneously both within the lumen and on the surface of individual EVs with single-molecule sensitivity.

This type of research supports the:

- Characterization of sub-populations of EVs possessing one or more biomarkers
- Distinction of molecules within the lumen or on the surface of EVs
- Association of unique molecular signatures to EVs of a specific size
- Investigation of the function of DNA on the EV surface
- Understanding the unique role of EV subtypes in cancer and other diseases

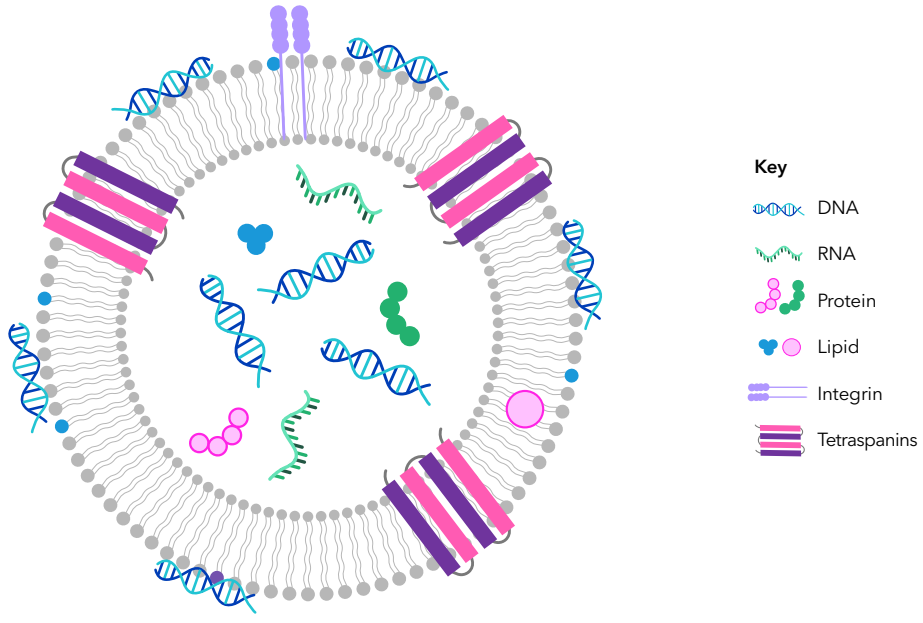

Figure 1 | Schematic representation of an EV showing the presence of DNA on both the EV surface and within the vesicle lumen.

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Results

Through multi-color dSTORM imaging on the Nanoimager, it is possible to fluorescently label and visualize DNA molecules on the surface of EVs and within the lumen, and quantify the association of the genetic material with vesicles of varying molecular signatures. Here, in collaboration with Dr. Franz Ricklefs from UMC Hamburg, EVs were purified from primary glioblastoma cells from patients. Prior to fluorescent labeling of CD63 and CD81 with Alexa Fluor® 555 and 647 respectively, and DNA molecules with the cell-impermeable SYTOX™ Green dye, EVs were subjected to one or a combination of the following conditions (Table 1 and Figure 2):

Table 1 | Conditions EVs were subjected to.

Sample	1. DNase treatment	2. Permeabilization	3. DNA labeling	Outcome
A	✓	✗	✗	Negative control
B	✓	✓	✓	Luminal DNA alone
C	✗	✗	✓	Surface DNA alone
D	✗	✓	✓	Luminal and Surface DNA

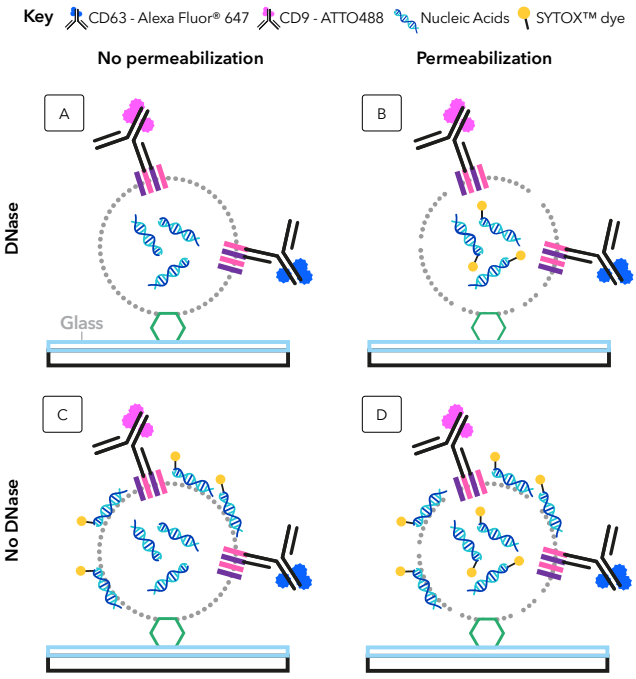

Figure 2 | Schematic representation of corresponding experiemental outcome of samples A-D.

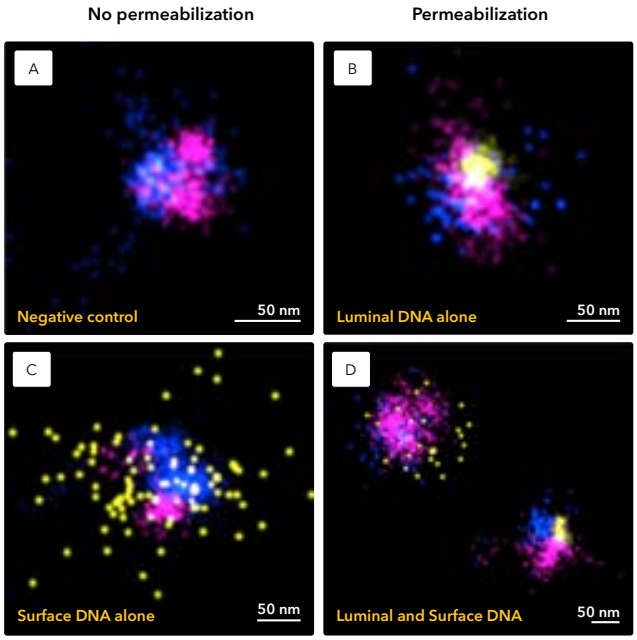

Figure 3 | Detection of DNA on the surface and within the lumen of EV samples (A-D) isolated from primary glioblastoma cells using dSTORM. DNA (yellow), CD63 (blue) and CD81 (magenta). *Dr. Franz Ricklefs, UMC Hamburg*.

Clusters of tetraspanin proteins and DNA on individual EVs were visualized by super-resolution dSTORM imaging (Figure 3) and subsequently analyzed through cluster analysis. The results illustrate that DNA molecules are present both within and on the surface of glioblastoma-derived EVs and the number of localizations of SYTOX™ signal differs greatly depending on treatment conditions (Figure 4). The use of additional techniques to further characterize the EV-associated DNA may provide insight into the role of these EVs in tumor progression or strengthen them as biomarkers for the identification and classification of tumors in clinical samples.

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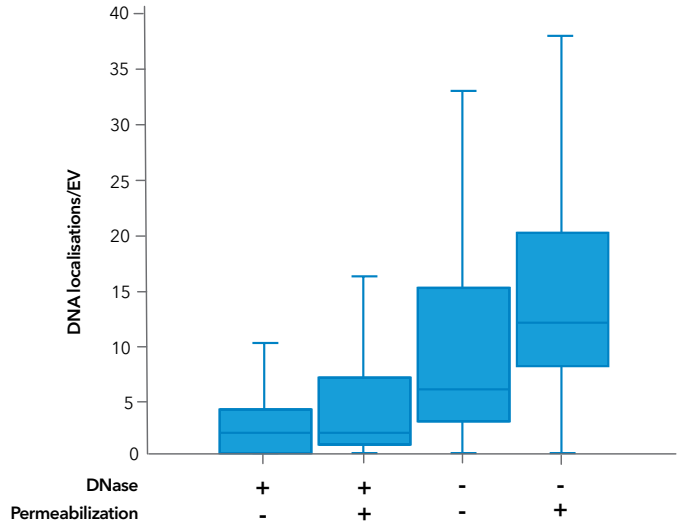

Figure 4 | Quantification of mean number of DNA localizations per EV pre-treated with either DNase I, permeabilization, neither or both, showing significant differences in localization number across conditions.

Solution with the Nanoimager

Understanding the unique molecular signatures of sub-populations of EVs and their association with specific cargo can enhance our understanding of how EVs function in cell signalling pathways and give deeper insights into the phenotypic consequences they enact at their target sites. The Nanoimager provides the complete solution for multi-color imaging of EV biomarkers including proteins, lipids and genetic material with up to 20 nm resolution for complete characterization and sizing of vesicle populations.

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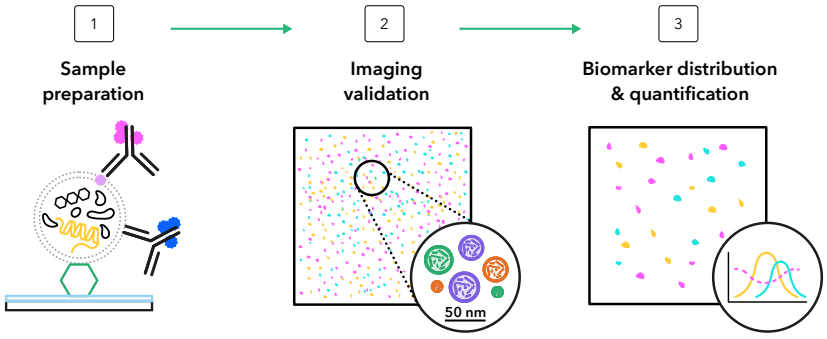

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Extracellular Vesicles

Advanced EV characterization made simple

Welcome to **CODI**. A collaborative data analysis platform for EV research.

Introduction

Super-resolution microscopy is a powerful tool for EV imaging and characterization by allowing researchers to directly visualize single-EVs, their surface biomarkers and therapeutic cargo. However, being able to extract quantitative data from these images through clustering analysis will enable researchers to unlock the secrets of EVs which until now, hasn't been fully explored. Clustering analysis allows the user to characterize the heterogeneity of an EV sample, to determine the percentage of positive markers on the surface of the EVs, and to measure DNA cargo. In this application note, we discuss how ONI's new analysis platform provides researchers with a methodical analysis pipeline that standardizes EV characterization.

Data analysis of super-resolution microscopy: CODI

To assist EV researchers in extracting quantitative information from their super-resolution images, ONI has developed a novel analysis software termed CODI. This platform hosts a wide range of advanced image analysis tools that have been designed to aid the study of EVs and address the challenges associated with their characterization. This allows for multi-factor characterization that enables users to count thousands of EVs from a single super-resolution image, assess their morphology with 20 nm resolution and visualize multiple biomarkers with single-molecule sensitivity. EV researchers can now unveil previously invisible insights from their sample through this simple, reliable and easy to use platform. The workflow for single-EV analysis on the Nanoimager starts with the preparation of EVs and finishes with clustering analysis to extract quantitative information about the EV sample (Figure 1).

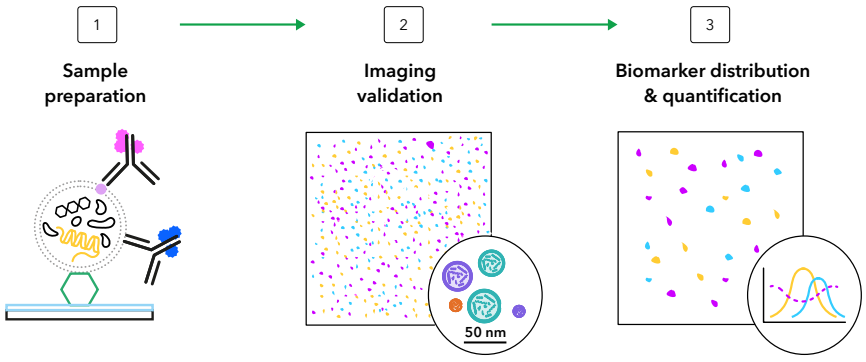

Figure 1 | Schematic representation of the EV workflow showing how EVs are first stained and adhered to the glass surface, before being imaged on the Nanoimager and analyzed using CODI.

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Firstly, EV surface proteins, membrane structures or internal cargo are labeled with a dSTORM compatible fluorescent dye and then specifically captured onto a glass surface. High resolution, dSTORM imaging is carried out, allowing for thousands of captured EVs to be directly visualized with 20 nm resolution. Lastly, the super-resolution images are subjected to clustering methods and co-localization analysis in which quantitative information detailing the different subpopulations, sizes and biomarker distributions can be obtained rapidly and automatically (Figure 2).

Summary

ONI's analysis platform provides advanced tools which have been designed to aid the characterization of extracellular vesicles using streamline workflows, that allows researchers to:

- Directly visualize single-EV allowing sample validation
- Assess and count hundred to thousands of surface bound EVs
- Quantify multiple biomarkers and assess their distribution of the EV surface
- Evaluate EV morphology with 20 nm resolution
- Measure and quantify EV cargo

Figure 2 | Screenshots of CODI, demonstrating dSTORM imaging of EVs (A) and clustering analysis (B).

Clustering analysis works by identifying the localizations from the dSTORM image which corresponds to each of the tetraspanin proteins labeled on the EV surface. All localizations within a defined radius are grouped into a dense circular cluster representing a single EV. Once these clusters have been identified, they can be constrained on parameters such as size, shape, length and circularity.

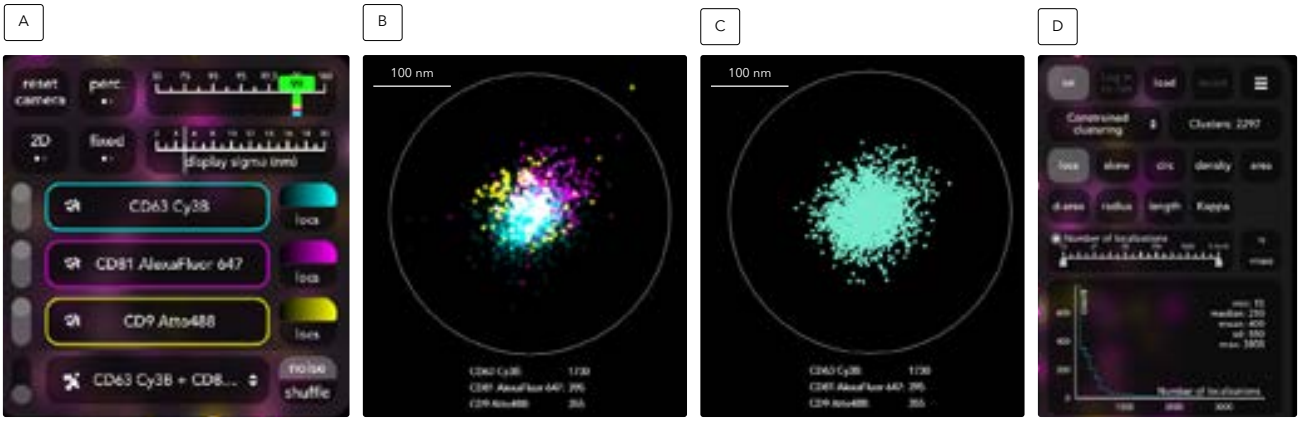

Figure 3 | Tool bar within CODI (A) showcasing how tetraspanin proteins labeled on the EV surface (B) are grouped together into one dense circular cluster, corresponding to a single EV (C). Clusters can be constrained based on parameters relating to known characteristics (D).

This ensures that EV clusters can be distinguished from any background or aggregates in the field of view in the field of view and removes them from the final results, allowing the user to have additional confidence that the clusters being analysed are in fact EVs (Figure 3).

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These constraints can be saved, exported and applied to other datasets to facilitate reproducible analysis when comparing between different datasets. Once these clusters are defined, information on specific characteristics of the EV population can be extracted. For example, the size distributions can be calculated for the global population of EVs in the sample (Figure 4).

This allows users to assess the homogeneity of their EVs, and identify specific sub-types of EVs within the sample. Additionally, other features of an EV can be quantified such as density, shape and morphology, features which are key in allowing us to understand more about the EV sub-populations.

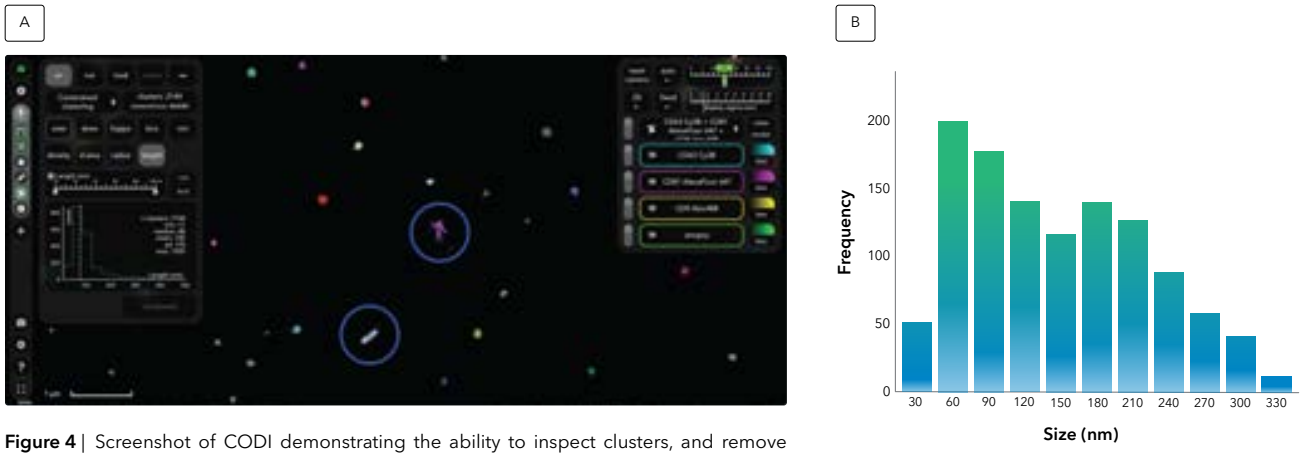

Figure 4 | Screenshot of CODI demonstrating the ability to inspect clusters, and remove localizations that do not correspond to set parameters (blue circles) (A). Histogram representing the sub-populations of EVs within a sample based on size analysis (B).

These clustering tools can be used to quantify surface biomarkers on single-EVs and assess the heterogeneity within the entire sample. This enables the percentage of single, double and triple positive EVs to be distinguished and counted for an enhanced characterization.

Additionally the amount of DNA present on the surface and within EVs can be visualized and measured, thus allowing these clustering tools to enable researchers to rapidly attain an understanding of the population distributions in samples and evaluate their therapeutic cargo.

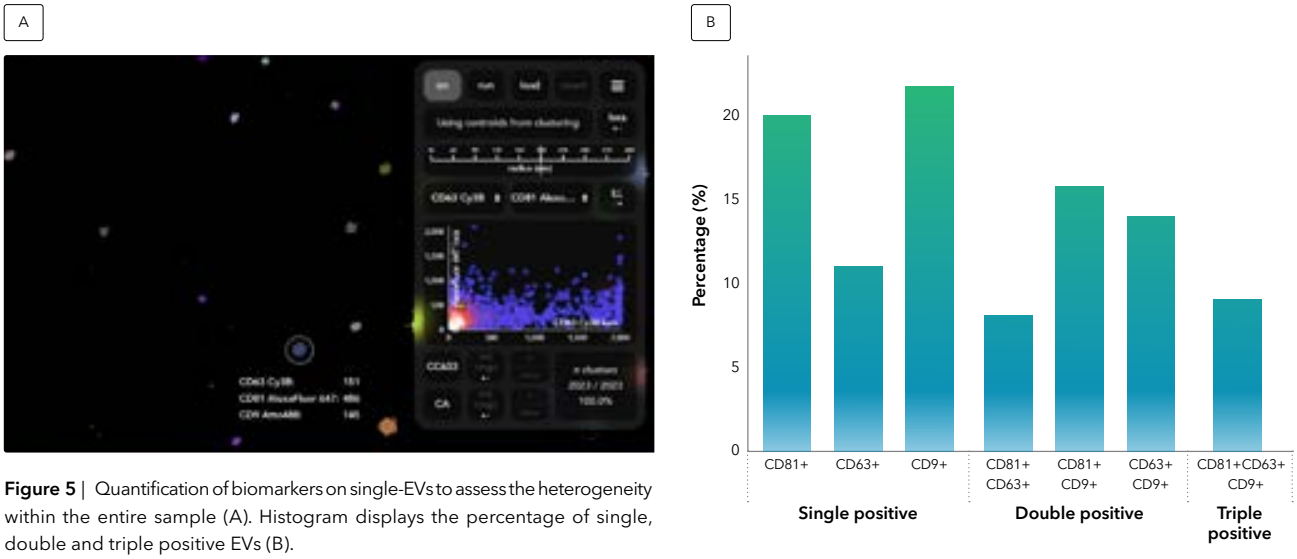

Figure 5 | Quantification of biomarkers on single-EVs to assess the heterogeneity within the entire sample (A). Histogram displays the percentage of single, double and triple positive EVs (B).

Solution with CODI

Understanding the unique molecular signatures of sub-populations of EVs and their association with specific cargo can enhance our understanding of how EVs function in cell signalling pathways and give deeper insights into the phenotypic consequences they enact at their target sites.

The Nanoimager and CODI provide a complete solution for multi-color imaging and analysis of EV biomarkers including proteins, lipids and genetic material with up to 20 nm resolution for complete characterization and sizing of vesicle populations. For more information visit **oni.bio/extracellular-vesicles**

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Extracellular Vesicles

Simultaneous visualization of EV cargo and surface markers with single-molecule imaging

Introduction

Extracellular Vesicles (EVs) are released by all cells and contain a lipid bilayer structure which encapsulates a diverse range of cargo including lipids, nucleic acids and proteins. These nanosized particles play a key role in intercellular communication through the transport of functional cargo[1]. This makes them ideal candidates for biomarker discovery, diagnostics and for therapeutic applications. In recent years, EV cargo has been widely investigated as biomarkers for neurodegenerative diseases, cancer, aging and cardiovascular diseases, among others. In addition to the naturally occurring cargo loaded into EVs, there has been increasing interest in engineered EVs loaded with therapeutic cargo for targeted therapy[2].

Challenge

Characterizing EVs by conventional light microscopy is difficult as their dimensions are below the diffraction limit of light. This hinders the ability of researchers to confidently distinguish between fluorescent signals detected from individual EVs, multiple particles or from cell debris. Without being able to bypass the diffraction limit, determination of EV morphology, accurate biomarker distributions and cargo content on a single-EV level remains a persistent challenge. Super-resolution imaging techniques can help overcome these limitations by allowing users to resolve single-EVs and their internal cargo with single-molecule sensitivity at 20 nm resolution. Furthermore, powerful clustering methods can be applied to the super-resolution image, allowing users to extract nanoscale morphological features, characterize the percentage of markers on the surface of the EVs as well as cargo proteins within single-EVs

Summary

The Nanoimager enables visualization and detection of multiple surface markers and internal protein cargo simultaneously on individual EVs with single-molecule sensitivity.

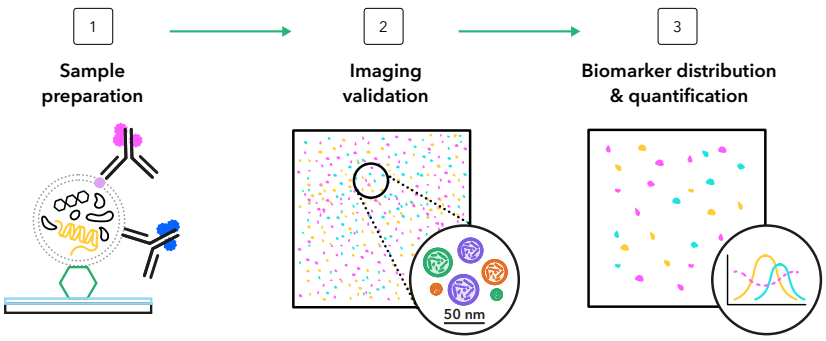
This type of research supports the:

- Use single-molecule sensitivity for better EV characterization
- Visualize single-EVs with 20 nm resolution and distinguish different biomarkers
- Detection of surface proteins alongside internal cargo proteins
- Quantify and measure the amount of cargo within a global population of EVs

Results - Characterization of protein cargo in purified EVs

The Nanoimager uses super-resolution techniques such as dSTORM to visualize and characterize surface and cargo biomarker profiles on single-EVs with increased sensitivity. For these studies simultaneous visualization of intracellular protein cargo (Alix, Syntenin-1 and TSG101, respectively) was carried out alongside tetraspanin surface markers, which follows the criteria stated in The Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines, for characterizing intracellular EV cargo[3]. All EV samples were prepared using the EVProfiler Kit and analyzed through the CODI software.

In brief, EVs isolated from human colorectal cancer cell lines were captured and immobilized onto ultracleaned passivated coverslips. EV's were then fixed and permeabilized, allowing the primary conjugated antibodies to label the internal protein cargo, whilst simultaneously labeling the surface tetraspanin markers. For a negative control, the permeabilization step was omitted thus allowing us to have a comparison of EVs that cargo could not be detected. Imaging buffer was placed into the EV chips which were then images through super-resolution dSTORM on the Nanoimager microscope.



Schematic 1 | EV characterization workflow on the Nanoimager.

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Clusters of tetraspanin markers, CD63 and CD9 can be seen on the surface of single-EVs which co-localizes with the internal protein markers Alix (Figure 1, A) and Syntenin-1 (Figure 1, B) within the permeabilized samples. This can be directly visualized from the super-resolution image, giving users additional confidence in their results . In the case where permeabilization was omitted, since the antibody cannot penetrate the EV membrane we measured low signal from each protein cargo in the non-permeabilized samples.

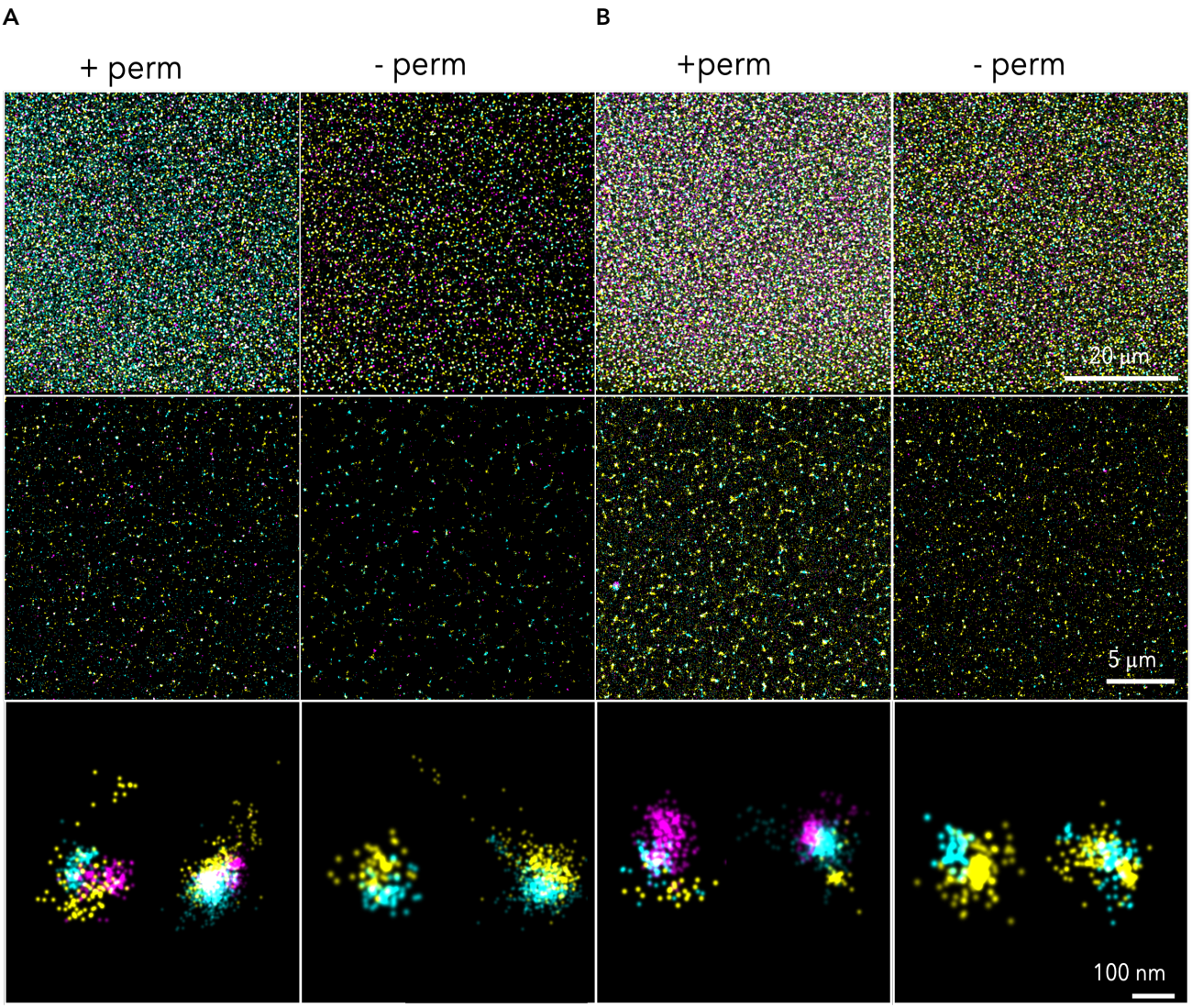

Figure 1 | A) Detection of Alix and (B) Syntenin-1 within Extracellular vesicles. EV samples were permeabilized (left column) to detect Alix or Syntenin-1 cargo within single-EVs and compared with non permeabilized samples (right column). Magnifications show a large field of view going down to single-EVs, with 20 um (top), 5 um (middle) and 100 nm (botton) scale bars. dSTORM images showing Alix or Syntenin-1, respectively (magenta), CD63 (blue) and CD9 (yellow)

Through our CODI software we can transform single-EVs into clusters, allowing users to exact quantifications on the total number of EV clusters containing protein cargo and the percentage of EVs which have protein cargo that colocalizes with the tetraspanin markers (Figure 2). For these experiments, quantifications were extracted from thousands of EVs bound to the coverslip. It was found that 29% of the EV population contained Alix cargo that co-localizes with the surface markers, which was a total of 400 out of 1044 EVs analyzed. In comparison to the non-permeabilized samples which showed a low amount of Alix detection in only 1% of the EV population, or 41 out of 843 EVs (Figure 2, A). A similar trend was observed for Syntenin-1 which showed 9% of EVs contained the protein cargo in the permeabilized samples (400 out of 726 EVs) in comparison to only 1% within the non-permeabilized samples, or 41 out of 3047 EVs (Figure 2, B).

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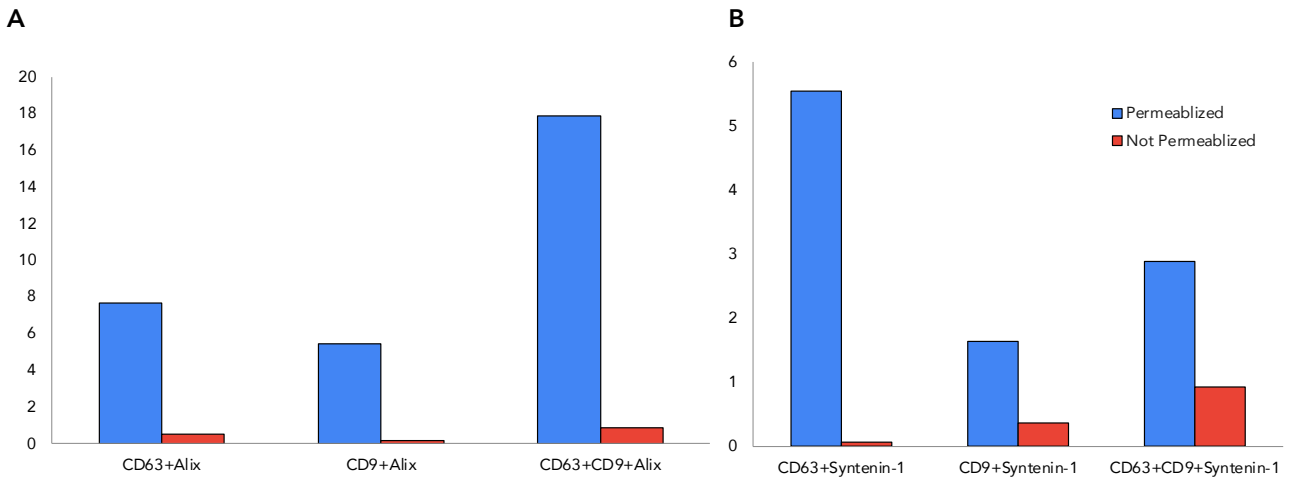


Figure 2 | Stacked histogram plots showing the percentage of EVs containing Alix (A) or Syntenin-1 (B) cargo. Each graph contains a comparison of permeabilized vs non-permeabilized samples. Only EVs containing protein cargo which co-localized with the surface biomarkers were analyzed, following MISEV guidelines.

Characterization of TSG101 protein cargo in unpurified EVs from concentrated cell culture media

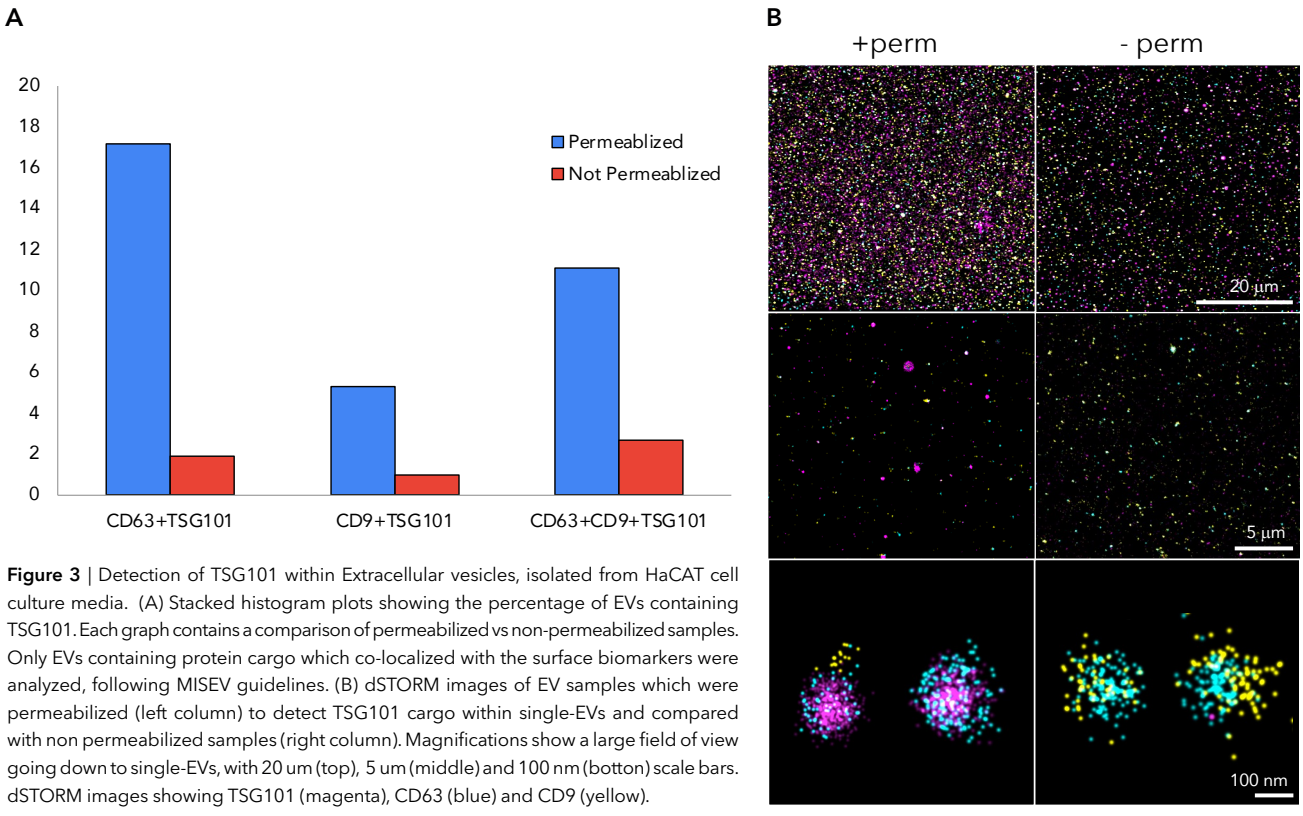
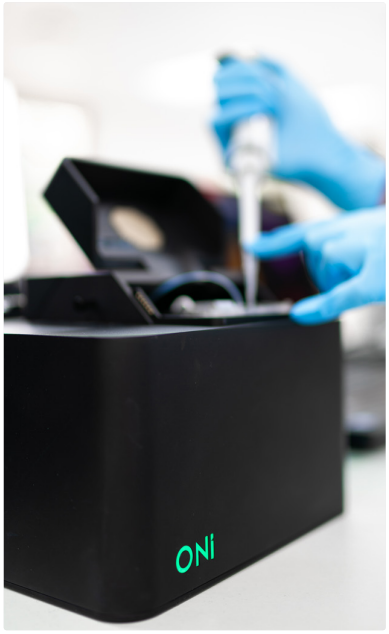


Figure 3 | Detection of TSG101 within Extracellular vesicles, isolated from HaCAT cell culture media. (A) Stacked histogram plots showing the percentage of EVs containing TSG101. Each graph contains a comparison of permeabilized vs non-permeabilized samples. Only EVs containing protein cargo which co-localized with the surface biomarkers were analyzed, following MISEV guidelines. (B) dSTORM images of EV samples which were permeabilized (left column) to detect TSG101 cargo within single-EVs and compared with non permeabilized samples (right column). Magnifications show a large field of view going down to single-EVs, with 20 um (top), 5 um (middle) and 100 nm (botton) scale bars. dSTORM images showing TSG101 (magenta), CD63 (blue) and CD9 (yellow).

According to literature TSG101 is described to be abundant in EVs from a wide range of sources however in vesicles isolated from colorectal cancer culture protein level was found to be low[4]. To assess the efficiency of TSG101 detection after permeabilization media from human keratinocyte cultures was collected, concentrated and used as a sample.

Here, the dSTORM images show an increase in TSG101 signal (magenta) in the permeabilized samples in comparison to the non-permeabilized samples which is to be expected (Figure 3, B). The high-resolution images show the detection of larger sized vesicles, since the media was not subjected to further purification, this could be a population of vesicles from non-endosomal origins being captured onto the surface of the chip. The quantifications extracted from CODI show a total of 389 out of 868 EVs contain TSG101 that co-localizes with the surface markers, which equated to 33% of the EV population. In comparison to the non-permeabilized samples which showed a low amount of TSG101 detection in only 57 out of 1023 EVs, or 5.5% of the EV population (Figure 3, A).

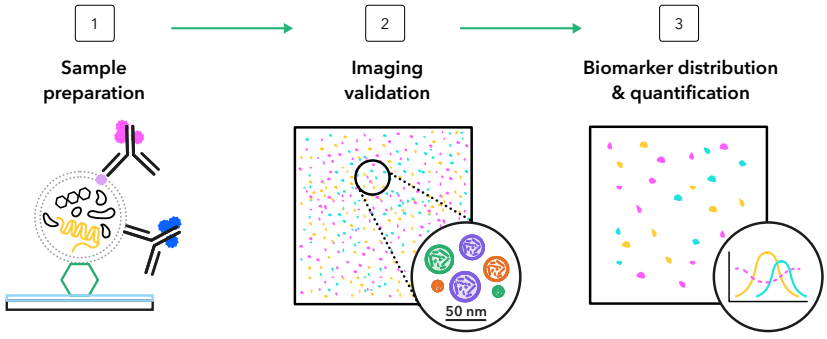
Being able to visualize extracellular vesicles using super-resolution techniques is extremely important, especially for characterizing protein cargo within EVs, to ensure analysis is being done on true EVs populations over any cell fragments. This confidence can only be achieved by using single-molecule resolution where direct visualization of protein cargo on a single-EV level, as demonstrated in this application note.



Solution with the Nanoimager

Understanding the unique molecular signatures of sub-populations of EVs and their association with specific cargo can enhance our understanding of how EVs function in cell signalling pathways and give deeper insights into the phenotypic consequences they enact at their target sites. The Nanoimager provides the complete solution for multi-color imaging of EV biomarkers including proteins, lipids and genetic material with up to 20 nm resolution for complete characterization and sizing of vesicle populations.

For more information visit **oni.bio/extracellular-vesicles**



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