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.

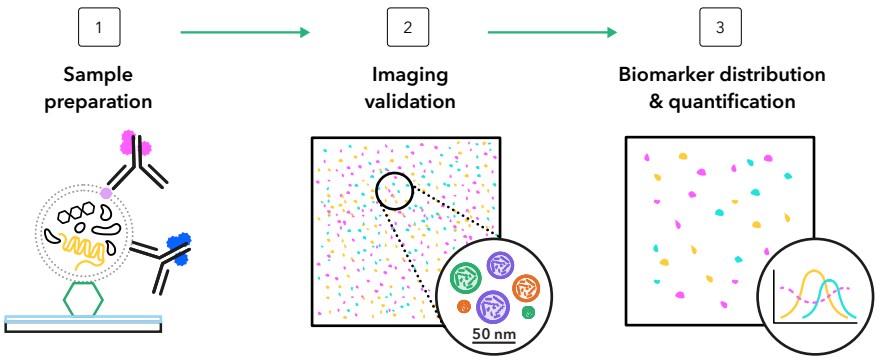
This type of research supports the:

- 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.

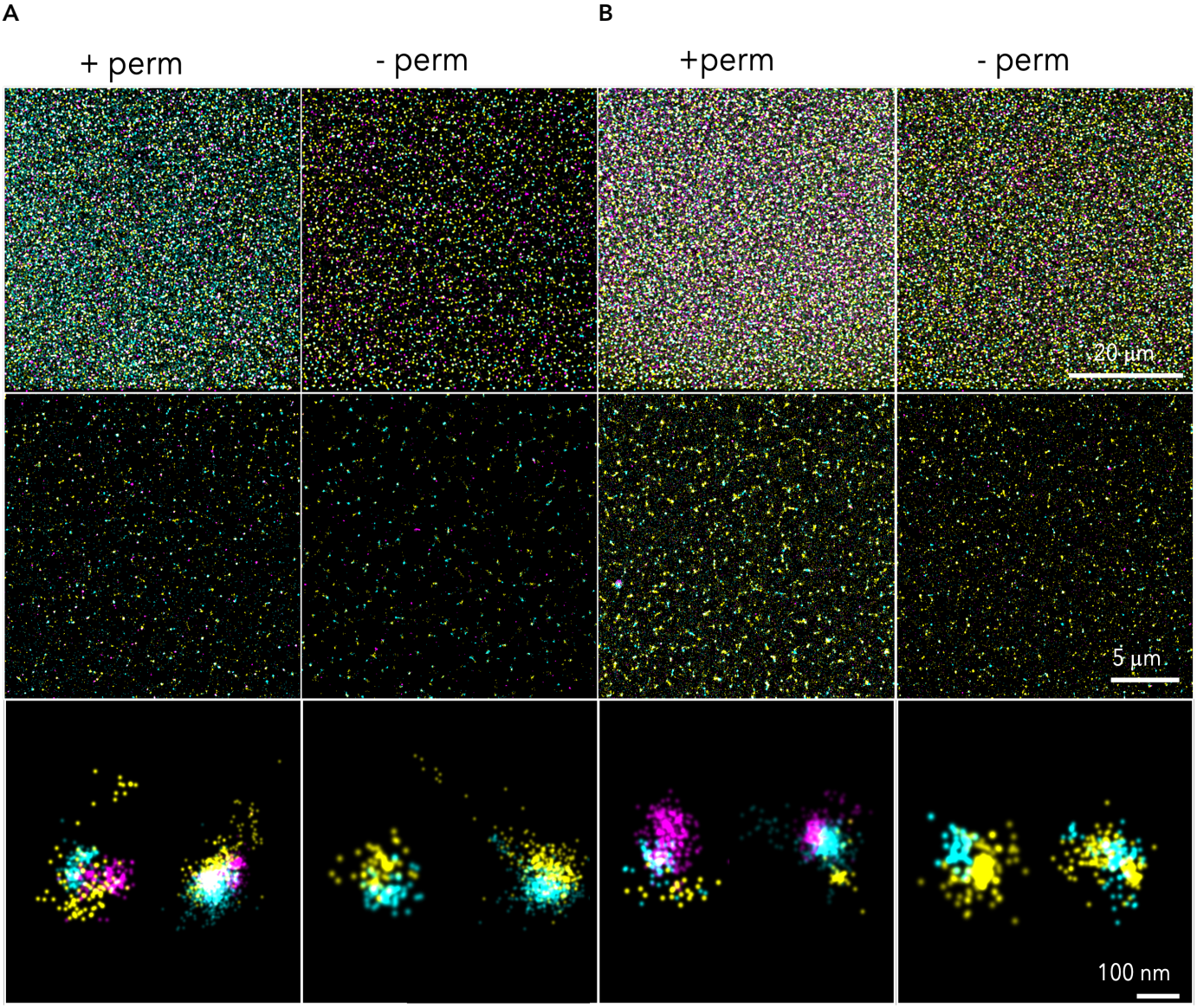

Figure 1 | A) Detection of Alix and (B) Syntenin-1 within Extracellular vesicles. EV samples were permeabilized (left column) to detect Alix or Syntenin-1 cargo within single-EVs and compared with non permeabilized samples (right column). Magnifications show a large field of view going down to single-EVs, 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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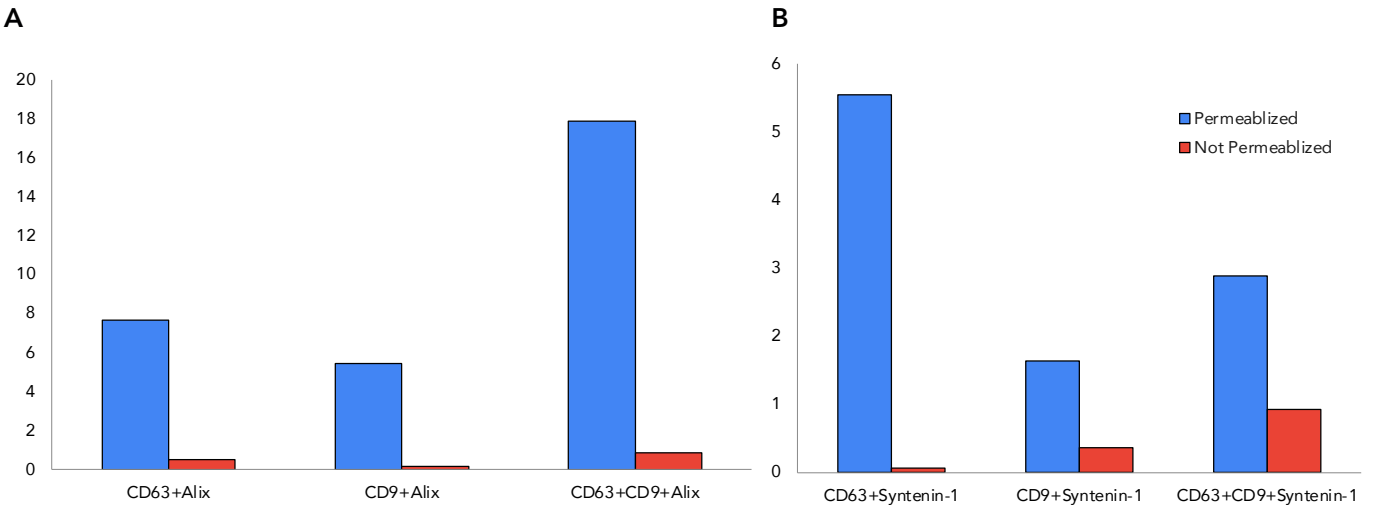

Figure 2 | Stacked histogram plots showing the percentage of EVs containing Alix (A) or Syntenin-1 (B) cargo. Each graph contains a comparison of permeabilized vs non-permeabilized samples. Only EVs containing protein cargo which co-localized with the surface biomarkers were analyzed, following MISEV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

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

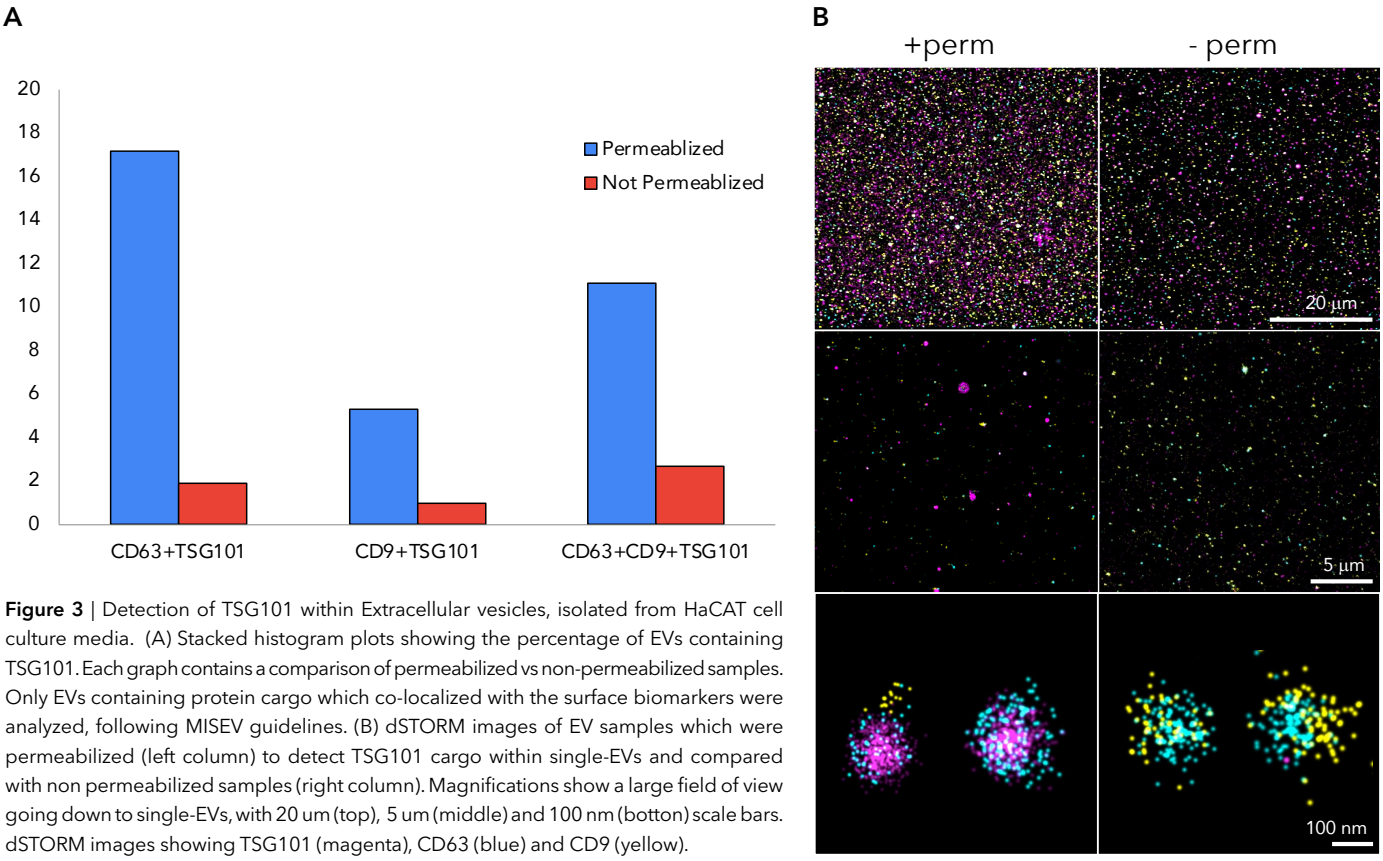

Figure 3 | Detection of TSG101 within Extracellular vesicles, isolated from HaCAT cell culture media. (A) Stacked histogram plots showing the percentage of EVs containing TSG101. Each graph contains a comparison of permeabilized vs non-permeabilized samples. Only EVs containing protein cargo which co-localized 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.

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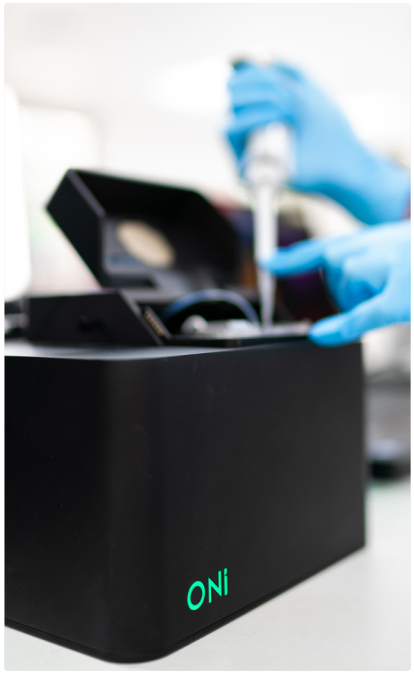
Here, the dSTORM images show an increase in TSG101 signal (magenta) in the permeabilized samples in comparison to the non-permeabilized samples which is to be expected (Figure 3, B). The high-resolution images show the detection of larger sized vesicles, since the media was not subjected to further purification, 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).

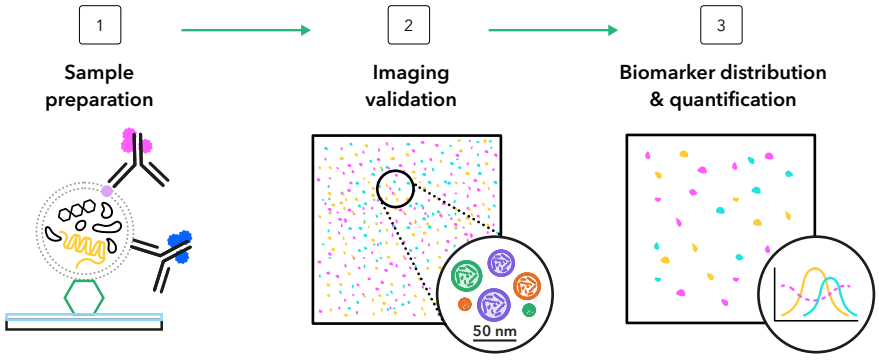
Being able to visualize extracellular vesicles using super-resolution techniques is extremely important, especially for characterizing protein cargo within EVs, to ensure analysis is being done on true EVs populations over any cell fragments. This confidence can only be achieved by using single-molecule 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**



References

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