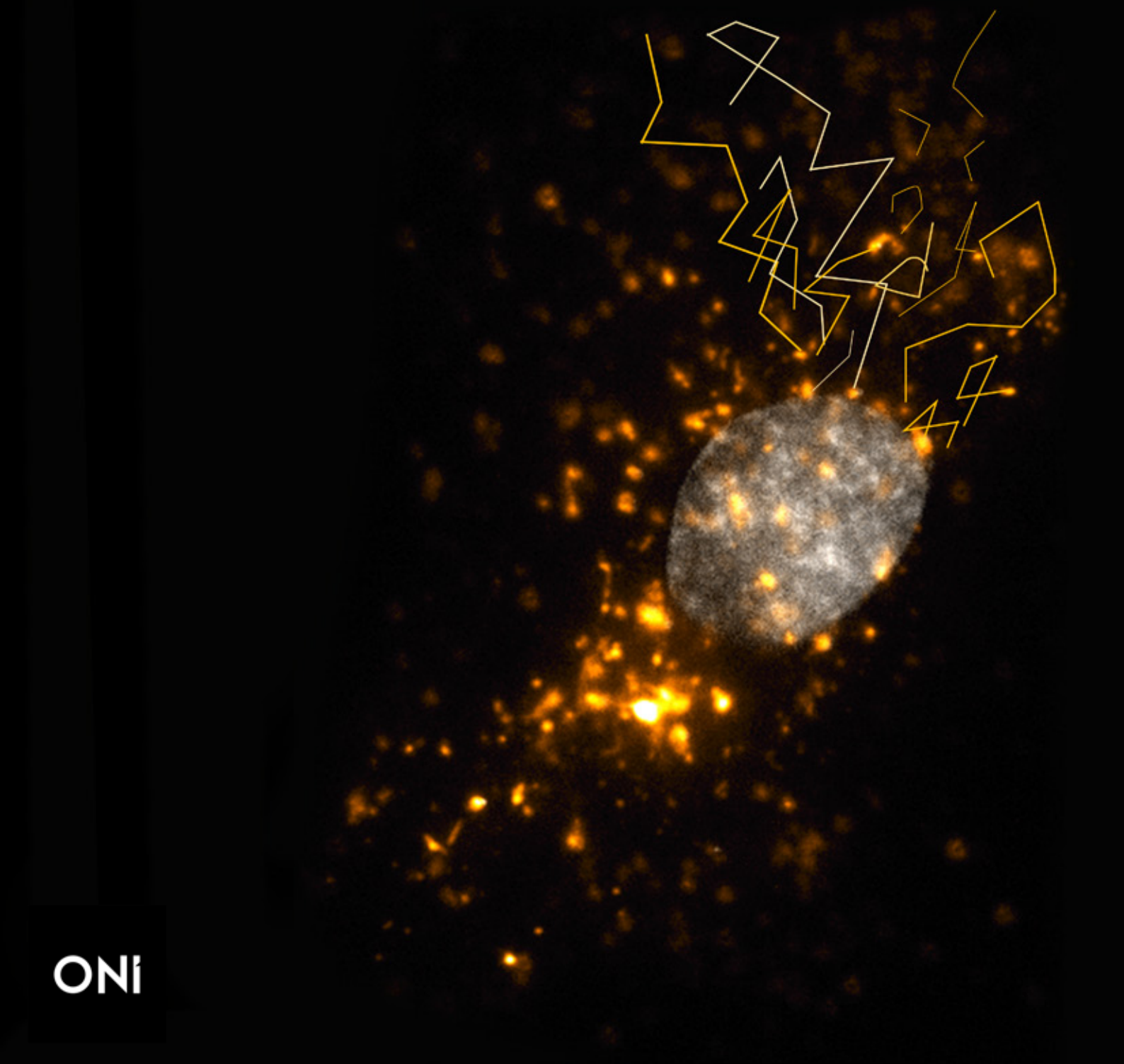
A guide to imaging EVs with super-resolution microscopy



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Extracellular vesicles and super-resolution microscopy

Extracellular vesicles (EVs) play key roles in cell-to-cell communication. EVs can cross biological barriers (such as the blood-brain barrier) and get internalized into the cell with a high degree of specificity. Thus, they are an ideal candidate for novel drug delivery methods and disease diagnostics. This guide focuses on recent progress in fluorescent super-resolution imaging and characterization of extracellular vesicles.

Visualize extracellular vesicles – see them in cells or in solution

A substantial and growing body of evidence highlights extracellular vesicles as critical components in cell-to-cell communication pathways. The visualization of EVs is fundamental to our understanding of the role of EVs in all aspects of cellular transmission; from the packaging of signaling molecules and nucleic acids during vesicle biogenesis, to tracking of their uptake and fate after internalization within selected target cells or tissues.

CHALLENGES IN VISUALIZING EXTRACELLULAR VESICLES

Extracellular vesicles originating from different cells or tissues are destined for specific target sites where they carry out particular functions. As EVs vary greatly in both size and protein content, it is believed that the presence of specific biomarkers (including tetraspanins, integrins and other proteins) on different sub-groups of EVs may be the root cause of this selectivity (Lyden et al 2015). However, characterizing these biomarkers on individual EVs poses a significant challenge to researchers in the field.

While electron microscopy techniques have the capability to resolve individual EVs, they do not easily allow detection of multiple markers at the same time, and are limited to fixed cells. With conventional light microscopy techniques, a number of proteins can be labeled but the small size of EVs means that the majority fall well below the resolution limit of light microscopy, restricting the usefulness of these techniques in identifying different sub-populations of vesicles.

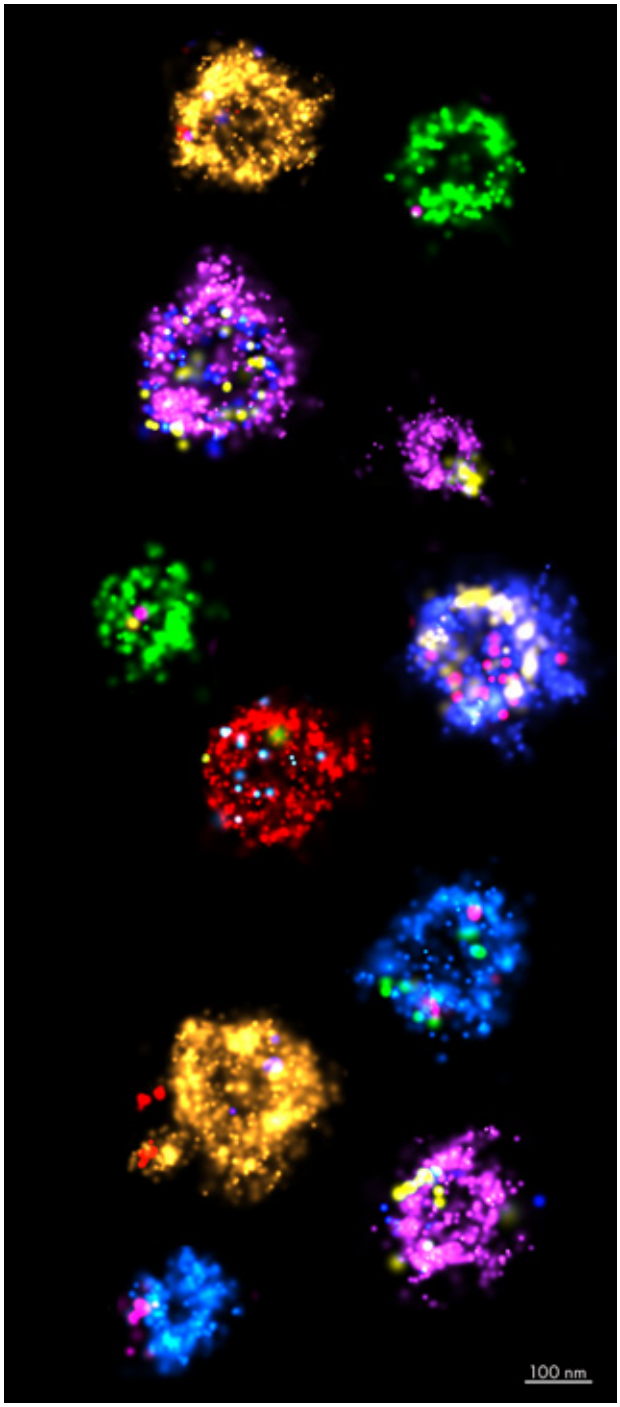


FIGURE 1

dSTORM images of EVs with labeling of tetraspanins cocktail (small clusters and inner rings) and the vesicle membrane surface (WGA, outer rings), colored randomly. For more information click on the hyperlinked image.

SUPER-RESOLUTION FOR EXTRACELLULAR VESICLE CHARACTERIZATION

Super-resolution microscopy surpasses the resolution barrier of conventional light microscopes and enables detection and quantification of single proteins and nucleic acids at the sub-vesicular level. Furthermore, the structural composition of vesicle membranes can be reconstructed with single-molecule localization microscopy (SMLM) reaching 20 nm resolution and can be used to identify the specific biomolecules involved in EV signalling and targeting.

The application of super-resolution imaging techniques has high value in identifying and visualizing sub-populations of EVs and may additionally provide important information into the role EVs play in disease progression.

TRACKING EXTRACELLULAR VESICLES MOVEMENTS IN LIVE CELLS

When extracellular vesicles reach their target cells, they bind to the plasma membrane where they are either internalized or release their contents into the cytoplasm via membrane fusion, a process that is poorly understood.

Advanced fluorescence microscopy allows detection of single EVs in real-time, tracking their interaction and uptake within living cells, as well as visualizing their movements post-internalization. Read our Case study for more information.

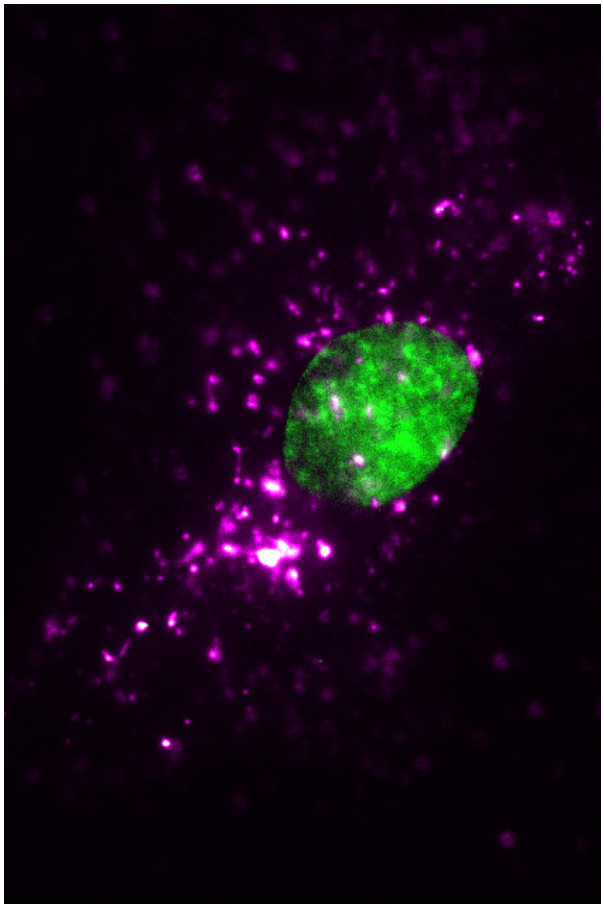
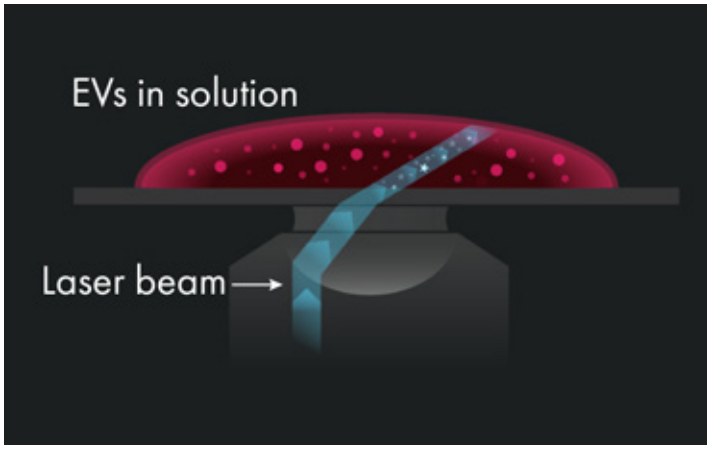


FIGURE 2

Human umbilical vein endothelial cell with the nucleus labelled with Hoechst (green) and EVs labelled with AF647 (magenta). Samples provided by Ms. Maria Panagopoulou and Dr. Margaret Paterson from the lab of Prof. Christopher Gregory, University of Edinburgh, for a video click on the hyperlinked image.

Size and Count with Particle Tracking

In addition to imaging EV dynamics within live cells, single-molecule fluorescence microscopy can be used to follow the trajectories of fluorescently labeled vesicles in solution through a technique commonly known as nanoparticle tracking analysis (NTA); a tracking based analysis of the brownian motion of single particles. NTA calculates the rate of particle movement, known as the diffusion coefficient, and from here estimates the size of the nanoparticles. As NTA analysis detects single particles, it is also useful in measuring the concentration of particles in a solution. These features of NTA provide quantitative information on the properties of EVs in solution, namely size and concentration, which explains the wide adoption of NTA in the Extracellular Vesicle research community.



ADVANTAGES OF SINGLE-MOLECULE FLUORESCENCE FOR NTA-STYLE CHARACTERIZATION

The detection of single extracellular vesicles for NTA analysis can be achieved either by light scattering or fluorescence signal coming from a single particle. Fluorescence imaging can provide significant advantages over the light scattering-based NTA analysis. For example, light scattering leads to non-specific detection of particles while antibody or membrane staining-based fluorescence imaging provides highly specific detection, so one only quantifies the particles they are interested in.

Additionally, a single EV can be stained with two biomarkers, each with a different fluorescence signature. Imaging fluorescence from labeled EVs in two fluorescent channels at the same time and combining this with two-color NTA adds another layer of valuable information:

- Characterization of extracellular vesicles and their subpopulations: by using one generic marker of EVs (like a membrane dye) in one color and a specific marker for EVs (such as CD9) in another, it is possible to study the fraction of EVs with a particular marker in a single experiment.
- Highly specific labelling of Extracellular Vesicle populations: EVs contain multiple markers and studying EV populations with highly specific markers (like CD9 or specific RNA molecules) using two color imaging can be a very useful way to confidently identify your EV of interest. It allows following only dual-labeled EVs and exclude the unwanted population during analysis.
- Size distribution of specific populations: two color NTA allows the detection of a specific EV population and measurements of its size distribution within a wider population.

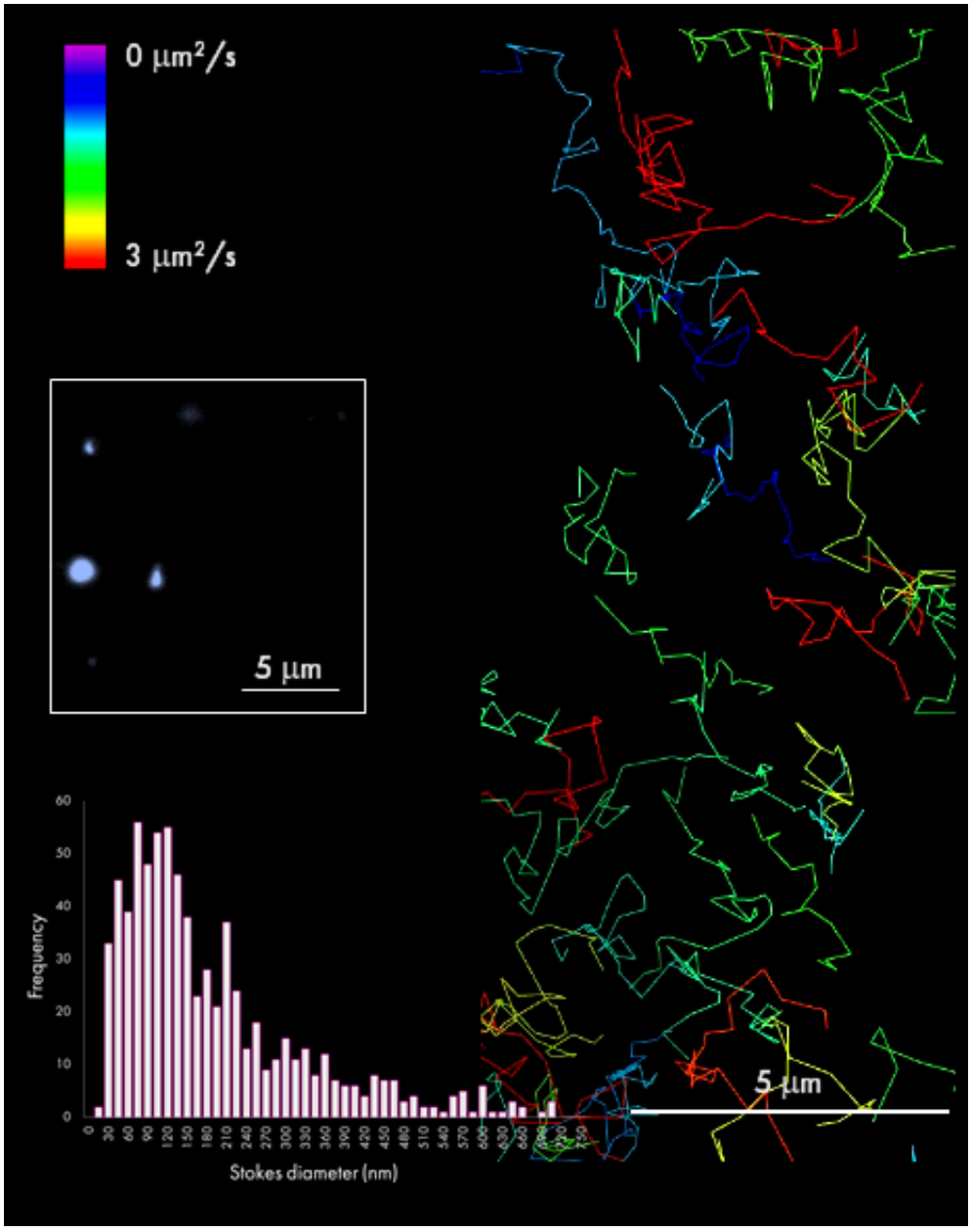
Figure 3 presents an example of tracking using labeled extracellular vesicles in solution. Purified EVs were labeled with PHK67 (membrane stain) and imaged at 37 C. EVs displayed a range of diffusion coefficient as indicated with the color-coded tracks and shown in the histogram. Inset shows an example frame of wide-field with EVs (in light blue). Obtained information was used to determine the distribution of EV size, which turned out to be a relatively wide range peaking at around 100nm.

This analysis has the potential of uncovering relative abundance of different population subtypes based on their diffusion and size.

FIGURE 3

Tracking of extracellular vesicles in solution.

For a video click on the hyperlinked image.



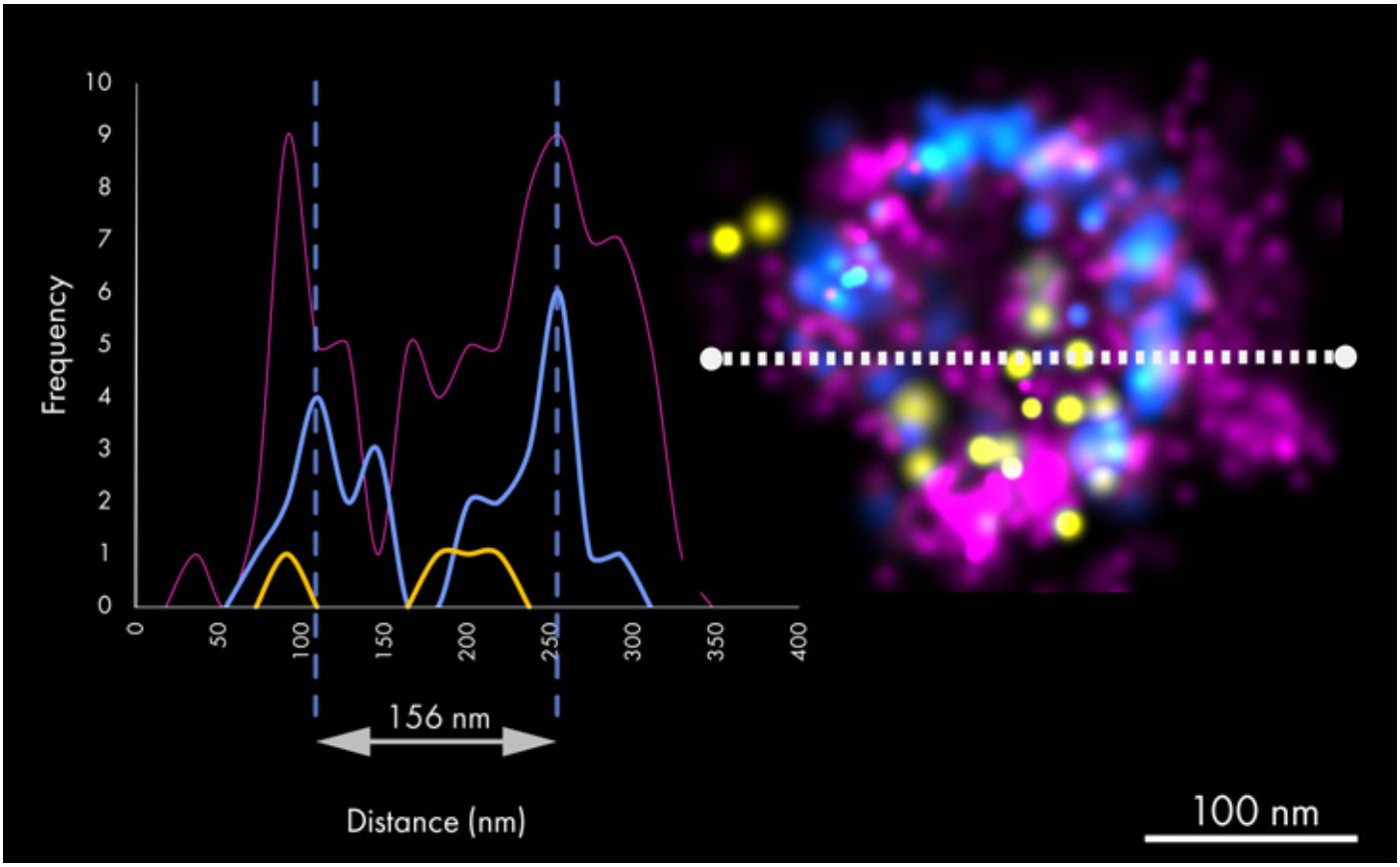


FIGURE 4

EVs isolated from human keratinocyte culture media, acquired by dSTORM. The image reveals the spatial organization of the most commonly known tetraspanins, CD63 (blue) and CD81 (yellow) at the molecular level relative to the vesicle membrane surface (magenta). For more information click on the hyperlinked image.

Size, count, image... understand

One of the most challenging aspects of studying EVs, and perhaps one of the most important, is combining multiple complementary characterization techniques. The array of tools available to define EV size and number rarely seem to agree, and seeing EVs at the single vesicle level remains difficult. What if one tool could provide independent sizing measurements and allow you to directly compare size and number with in vivo data at single-vesicle resolution?

MEASURE AND FOLLOW

Single-molecule fluorescence microscopy offers the most sensitive fluorescence measurements available. When configured in the correct way, this technique can be used to follow the trajectories of fluorescently labeled vesicles in solution. Based on the trajectories and the corresponding diffusion measurements, the user can quickly estimate the size distribution and concentration of an EV population as described in the previous chapter. Importantly, using a single-molecule fluorescence microscope allows us to extract this information with greater sensitivity than with any other tracking-based fluorescence instrument.

A special advantage of using a single-molecule super-resolution microscope is that it provides tracking-based size and concentration measurements, and also offers exceptional resolution for visualizing the same population of EVs in cells. After categorizing the vesicles with tracking data, you can add them to cells and investigate their biological function by visualizing their interaction through different imaging modes. The imaging could involve live cells: studying uptake by time-lapse SIM imaging at high resolution, or real-time tracking to study the interaction of EVs with the target cell membrane. Alternatively, it could involve fixing cells after sufficient time for the EVs to be taken up, and using 20nm-resolution dSTORM microscopy to study the interaction of EVs with molecules in the target cells (e.g. lysosomes, endocytic markers) at unprecedented resolution.

CHARACTERIZE

A robust way to characterize EVs is to apply two completely orthogonal techniques in the same instrument. dSTORM is a method for imaging fluorescently labeled molecules with 20nm resolution (figure 4). This means that dSTORM can report on the markers present on single vesicles (such as protein or RNA content), as well as their distribution on the vesicle relative to one another. Importantly, this also means that dSTORM can be used to directly infer the size of vesicles on a glass surface by imaging them, in the same way that electron microscopy has been used in the past.

In a single-molecule super-resolution microscope, the size inferred by super-resolution imaging can then be directly compared with the tracking-based measurements of size taken from the same population of vesicles diffusing in solution, as explained above.

VESICLE NANOIMAGING

The Nanoimager is one of the most sensitive microscopes available due to its unique closed-form design. It is ideal for researchers in the extracellular vesicles field as it comes with tailored analysis for sizing and concentration measurements, and it supports super-resolution microscopy as well as live-cell imaging in the most accessible format ever designed. Using this platform, EV populations can be characterized by orthogonal methods and subsequently imaged to measure their interaction and uptake in cells. Up to four molecular species can be measured in the same sample using different fluorescent markers.

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e: hi@oni.bio • t: +44 (0) 1865 951 820
ONI, Linacre House, Jordan Hill Road, Oxford, OX2 8TA, UK
WWW.ONI.BIO