TECHNICAL NOTE

Implementing EV cargo detection using custom antibodies and single-molecule imaging

Detection and quantification of Extracellular Vesicle (EV) cargo can be challenging due to the small size of EVs, the low-abundance of some EV biomarkers, and the need to ensure EV morphology remains intact after isolation and capture.

Here, we provide a framework for implementing EV cargo marker detection using permeabilization and Pan-EV labeling, as well as controls tailored to the specific requirements of single-molecule localization microscopy (SMLM), namely dSTORM imaging. This allows researchers to gather critical data on EV size, EV biomarker distribution, and EV cargo of interest using custom antibodies while ensuring experiment reproducibility and reliability.

1. EV isolation & purification

For a comprehensive guide on how to prepare EV samples for dSTORM imaging, including EV sources, collection, storage, EV particle concentration methods and more, read our Technical Guide "Preparing EV samples for super-resolution imaging". Here, we used PANC1-derived EVs for the detection of ALIX, a well-characterized cargo protein contained in many EVs types and implicated in tetraspanin recruitment[1].

Cell culture

PANC1 cells were cultured in phenol red-free DMEM supplemented with 10% EV-depleted FBS and 1% penicillin/streptomycin (Gibco™ 21063045, A2720803 and 15140122).

EV purification

- Conditioned medium was collected after 48 hours and spun at 300 x g for 10 minutes to remove cellular debris.
- EV-containing samples were then concentrated to 400 µL using Vivaspin™ 20 100 kDa concentrators (Cytiva 28932363) at 1000 x g in 10-minute intervals.
- Concentrated medium was loaded into IZON qEVoriginal Gen 2 70 nm column (IZON ICO-70) equilibrated with ONI's Wash Buffer.
- Size Exclusion Chromatography (SEC) collection was performed manually, using gravity flow. 3 mL of void volume was discarded, then 500 µL of fractions 7-10 were collected off the qEVoriginal column.
- Fraction 7 was used for subsequent preparation using ONI's EV Profiler 2 kit.

2. EV capture

- EVs were captured using phosphatidylserine (PS) Capture. PS Capture may be used with EVs enriched in phosphatidylserine (e.g. cancer-derived EVs), and is also useful when you do not want to enrich a protein biomarker.
- If PS content is unknown, we suggest comparing PS Capture to anti-CD81, anti-CD63, anti-CD9 (individual or combined) capture.
- Combined tetraspanin capture can be used to isolate a broad range of EVs.
- EV Profiler 2 may be compatible with a user-defined capture molecule, as long as the target of interest is membrane-associated, accessible, and tested following the product user guide.

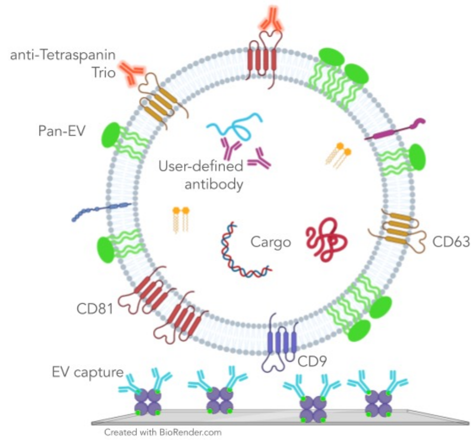
3. Target labeling: surface & cargo markes

Pan-EV stain

A generic membrane stain that binds all EVs helps ensure accurate detection, morphology assessment and sizing of EVs. Commonly used markers, such as wheat germ agglutinin (WGA) or concanavalin A (ConA), make membrane visualization dependent on glycosylation. Here, we used the EV Profiler 2 kit Pan-EV stain, which offers membrane visualization independently of protein expression or membrane glycosylation.

Tetraspanin labeling

The Pan-EV stain used together with the Tetraspanin Trio (CD9, CD63, and CD81) in the EV Profiler 2 kit allows users to confidently identify and size EVs in accordance with the latest MISEV guidelines[2], and characterization of tetraspanin-negative EVs. Additionally, this configuration allows for detection of protein targets in a third dSTORM channel/color (details below).



Custom marker detection

In this modality, users provide the primary antibody for user-defined protein detection, and a matched isotype control. These shall be fluorescently labeled with a dSTORM compatible fluorophore, such as AlexaFluor™ 647 or other far red dSTORM-compatible fluorophore.

Antibody-dye conjugation

Molecules (antibodies, proteins etc.) can be purchased fluorescently labeled or can be labeled by the user with a dSTORM-compatible dye. The number of fluorophores per protein can be modulated by increasing the molar ratio of dye to protein during the conjugation.

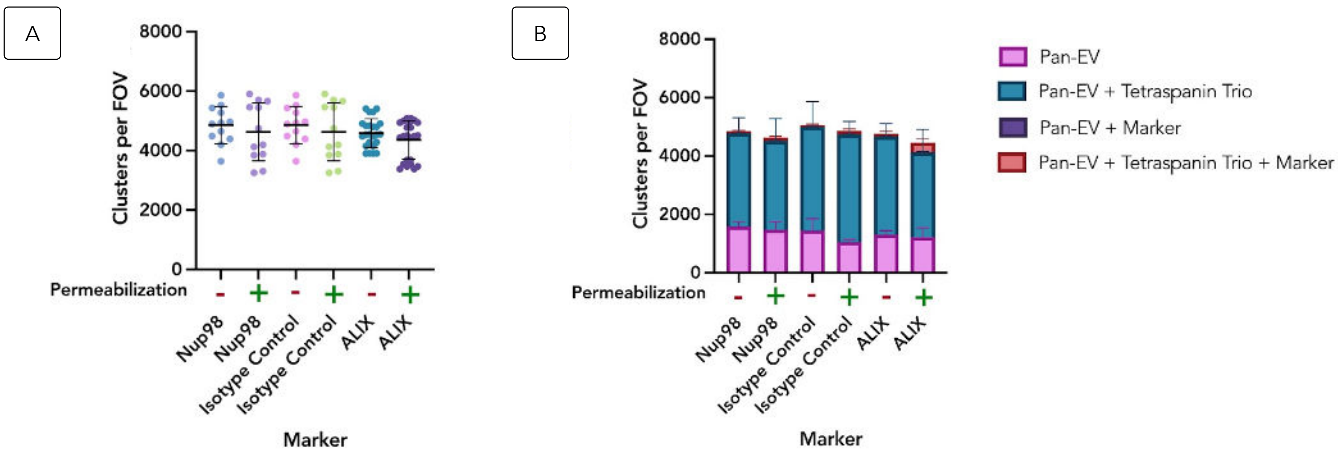
Cargo detection using permeabilization

Here, ALIX was detected using a recombinant rabbit monoclonal antibody (Abcam ab275387) from EV Profiler 2. Staining Solution from ONI's dSTORM Training Kit was used as the rabbit anti-Nup98 control. Rabbit isotype control was purchased (Thermo Fisher 02-6102). All cargo antibodies (incl. controls) were directly conjugated to AlexaFluor™ 647. Permeabilization was performed using the EV Profiler 2 kit Permeabilization Buffer, and incubating for 10 min prior to antibody detection, as per the product user guide.

4. Controls to assess permeabilization effects

No permeabilization control

- It allows users to assess EV integrity after permeabilization and detect non-permeabilized EVs.
- If EV size distribution or number of EVs captured changes substantially, over-permeabilization may be occurring, and some EVs are lost as a result.
- It also allows users to assess non-specific binding of antibodies targeting internal cargo molecules. Any signal in the non-permeabilized samples suggests that non-specific antibody binding is occurring.
- The abundance of the cargo molecule in the permeabilized sample should be compared to the level detected in the non-permeabilized samples.
- We recommend that the difference between cargo detected in permeabilized and non-permeabilized samples be statistically significant. Changes of approximately 1% would not be significant enough to demonstrate cargo detection,



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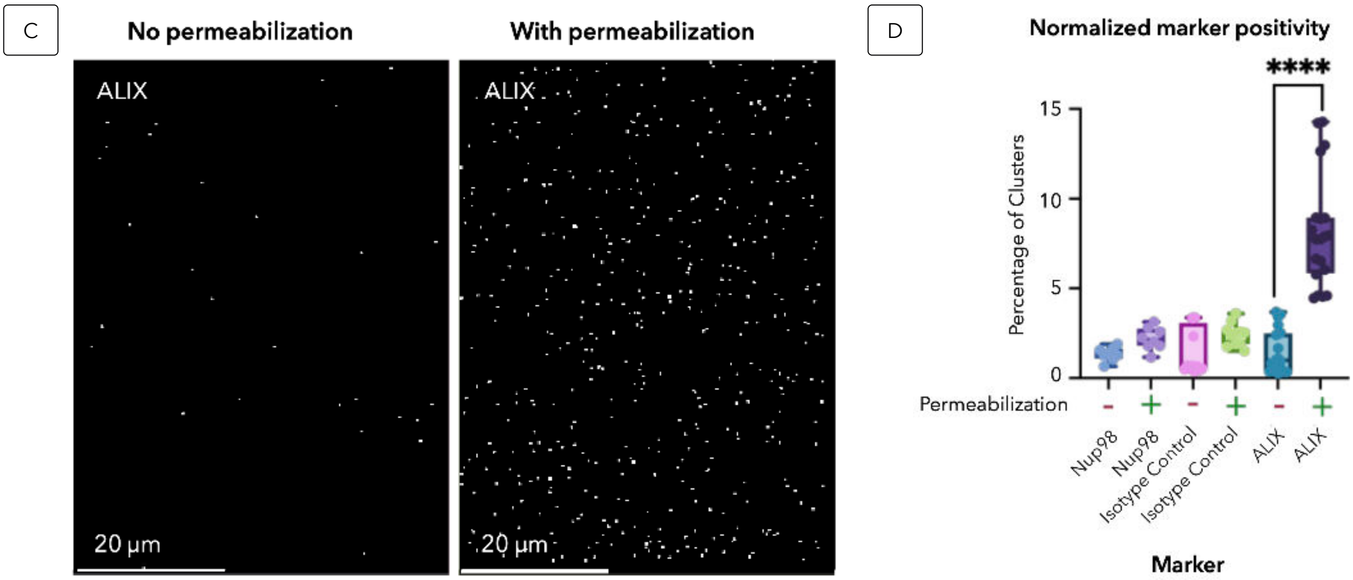

Figure 1 | Significant increase in ALIX signal and similar number of EVs detected after permeabilization. A) Quantification of detected clusters for each condition, each point represents a FOV, the mean ± SD is plotted. B) Quantification of clusters detected for each experimental condition, plotted with biomarker distribution. All FOVs are represented within each bar; each biomarker bar represents the mean, with SD shown for all FOVs from that condition. C) Representative images of a whole FOV, with only the ALIX signal shown. Left image is a non-permeabilized sample; the right image is a permeabilized sample. D) Percentage of clusters positive for the marker labeled with AlexaFluor™ 647. Each FOV was normalized to total clusters in the FOV; each point represents an FOV. **** = p<0.000001, unpaired t-test.

Lyzed EVs control

- In cases where the results between the no permeabilization and permeabilization conditions are inconclusive, one may also perform a lyzed EV control.
- This is done using a high concentration of a common detergent (e.g. 1% saponin or Triton X-100) on the EV sample instead of the permeabilization step, resulting in captured EVs being lyzed, and a very sparse Pan-EV signal.
- If the lyzed EV results look similar to regular permeabilization results, EVs may be over-permeabilized. This indicates that a gentler detergent step may be required.

5. Controls to verify biomarker detection

EV cargo molecules are often low-abundance molecules and must be detected with high sensitivity. To ensure that your antibody is detecting your cargo molecule of interest, we recommend the following controls when validating a cargo marker for the first time.

Isotype control or detection of antigen not found in EVs

- Isotype controls serve to check non-specific antibody binding.
- Ideally, a sample stained with a species- and isotype-matched irrelevant antibody control should result in little staining; and the amount of staining should be similar between permeabilized and non-permeabilized samples.
- If the amount of EVs positive for the isotype control is similar to that of your cargo molecule of interest, it may indicate that your cargo is not present at detectable levels or that your antibody lacks specificity.
- If desired, an antibody targeting a protein not found in EVs (such as Nup98, a nuclear pore complex component) may be used instead of a specifically-raised isotype control.

Cell staining

To determine antibody targeting specificity, staining should be performed in tissue culture cells expressing the target alongside a negative control (e.g. knockout line, knockdown treated or peptide blocking) with a fluorescent-labeled version of the detection molecule. Ideally, the negative control should include less than 10% of the density of the positive control samples, however, this threshold should be predetermined by the user.

Concentration titration

For most efficient detection, start with 5 µg/mL of labeled antibody. The antibody or the detection molecule can be titrated around 1-10 µg/mL. Use the negative control lanes (no permeabilization for internal cargo, isotype controls for surface cargo) to guide detection molecule concentration.

6. EV imaging

EV detection was performed using the Pan-EV (488 channel) + Tetraspanin Trio (561) + custom marker detection modality (647 channel), in this case, Alix (Figure 2A). Imaging was performed using a Nanoimager microscope. Recommended laser power from the EV Profiler 2 user guide was optimized for AF647.

If the user-defined detection molecule is not AlexaFluor™ 647, the 647 laser power needs to be titrated to ensure fluorophore blinking throughout the acquisition. Optimal laser power and number of frames for the user-defined molecule can be determined using the CODI analysis software. The optimal laser power will minimize the localization precision (increased precision leading to a decreased numerical value) and maximize the photon count while still capturing several localizations per frame over the entirety of the imaging period. This can be determined by observing the number of localizations per frame, which should have a steep decline in the first frames leading to a blinking steady state for the remainder of the imaging time.

7. Data analysis

Subsequent analysis was performed using ONI's CODI software, with preset "Pan-EV + custom marker" analysis settings. The DBSCAN algorithm was used to cluster localization data, using localizations from the Pan-EV and Tetraspanin Trio stains. Clustering did not include 647 localizations to avoid filtering bias between cargo-positive and negative EVs.

Data extracted were plotted using Graphpad Prism 10.2.2. to show similar EV diameters detected using ONI's Pan-EV stain across all samples. This confirmed that permeabilization does not significantly change EV diameter, yet ALIX-positive EVs tend to be larger. This may indicate that ALIX is preferentially packaged into larger EVs, or that the presence of ALIX results in larger EVs.

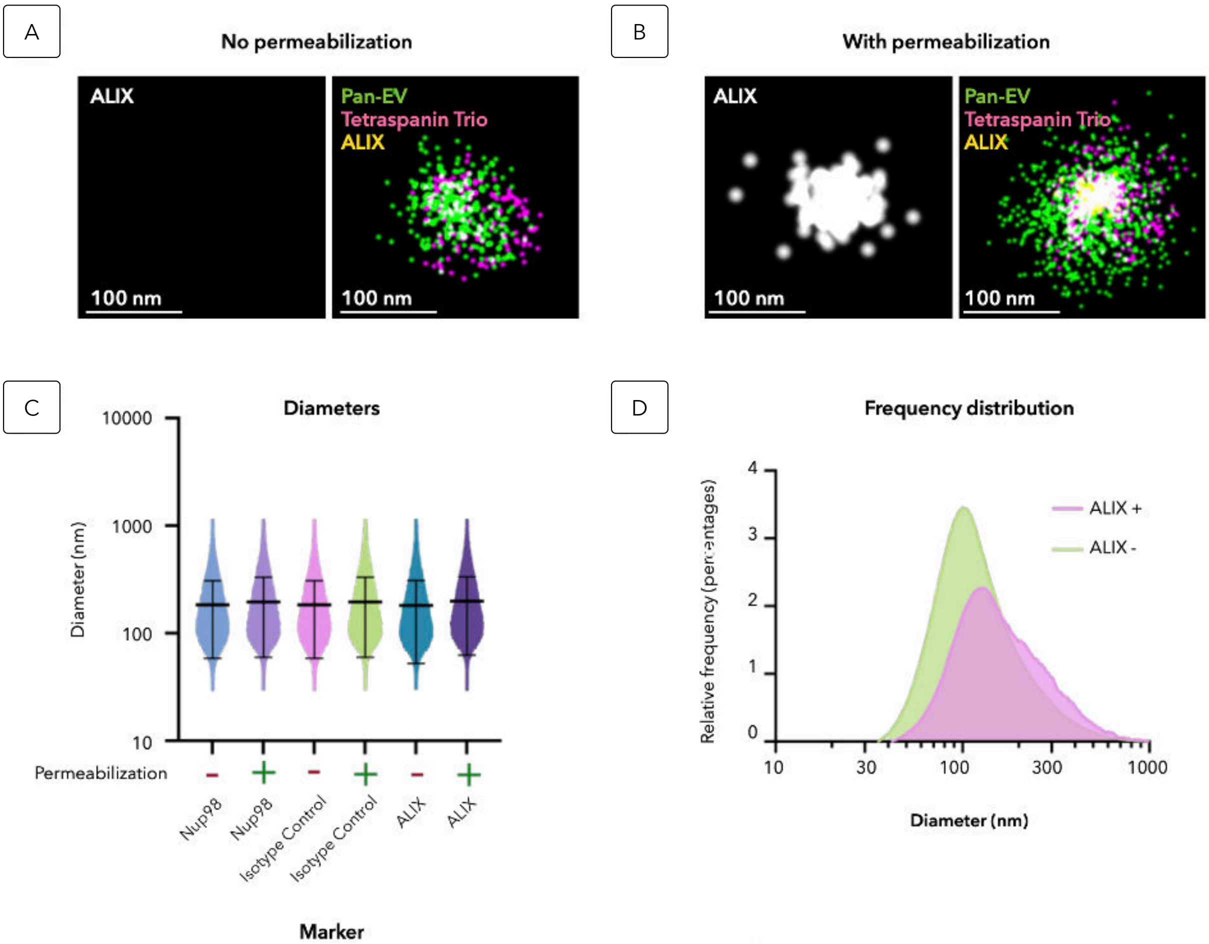

Figure 2 | Permeabilization does not significantly change EV diameter, yet ALIX+ EVs are larger. A and B) Representative images of single EVs that were non-permeabilized (A) or permeabilized (B) and stained for ALIX. C) EV diameters detected using ONI's Pan-EV stain, the mean with SD was plotted on a logarithmic scale to display the range of EV sizes. D) Histogram of the relative frequency of the diameter of ALIX positive (ALIX+) and ALIX negative (ALIX-) EVs within permeabilized samples. A bin size of 5 nm was used for the histogram, and the resultant histogram was smoothed using second-order smoothing of 9 neighbors. The X-axis is shown on a log10 scale, the Y-axis shows the relative frequency of each bin as a percentage.

When performing SMLM using ONI's EV Profiler 2 kit and the Nanoimager, users can extract the number of biomarker localizations present in a single EV. While this is valuable data, it is essential to note that one cannot correlate protein expression to localizations in a 1:1 manner (i.e., having 10 localizations of ALIX present in an EV does not mean there were 10 molecules of ALIX in that EV). However, the number of localizations generally correlates with protein content on an order of magnitude scale (i.e., an EV with 50 ALIX localizations is very likely to have more ALIX molecules than an EV with 5 ALIX localizations). Thus, we can investigate if EV size impacts the amount of ALIX present in an EV and there is a correlation between the two parameters. Interestingly, the data suggested a characteristic size at which an EV will contain the most localizations of ALIX (Figure 3). EVs with the most ALIX localizations were typically smaller than 500 nm but larger than 150 nm. This did not align with the median diameter, which is approximately 140 nm (right Y-axis, Figure 3).

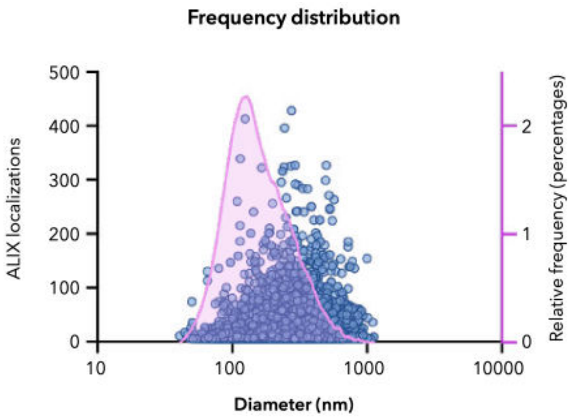

Figure 3 | ALIX+ EVs have a characteristic size. ALIX localizations (left Y-axis) vs. diameter, overlaid with relative frequency (right Y-axis, from figure 4D). A bin size of 5 nm was used for the histogram, and the resultant histogram was smoothed using second-order smoothing of 9 neighbors. The X-axis is shown on a log10 scale, the right Y-axis shows the relative frequency of each bin as a percentage.

REFERENCES

1. Larios J, Mercier V, Roux A. et al. ALIX- and ESCRT-III–dependent sorting of tetraspanins to exosomes. J Cell Biol. 219 (3) (2020). doi:10.1083/jcb.201904113

2. Welsh JA, Goberdhan DCI, O'Driscoll L., et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. J Extracell Vesicles. 13(2):e12404 (2024). doi: 10.1002/jev2.12404.

Explore EV Profiler 2