TECHNICAL GUIDE

EV Profiler 2

EV Profiler 2, AutoEV and the Nanoimager provide a fast, user-friendly platform for visualizing, sizing, and phenotyping EVs. EV Profiler 2 combines functionalized surface chemistry, biochemical capture, and detection molecules and is compatible with the Nanoimager and ONI's cloud-based analysis platform, Collaborative Discovery software (CODI), to streamline sample preparation, imaging, and analysis. This Technical Guide will review critical considerations for adapting the assay to your EVs and biomarkers of interest.

EV compatibility and purification methods

EV Profiler 2 is compatible with EVs purified from biofluids or cell culture media. EV samples should be purified before being deposited onto the Assay Chip. EV Profiler 2 allows you to analyze EVs in purified samples irrespective of the purification method, but you should use a gentle purification method that maintains their morphology and cargo and does not induce aggregation in order to characterize the phenotype, size, and shape of the native EVs. We recommend size exclusion chromatography, tangential flow filtration, or differential centrifugation of EVs. The EV Profiler 2 assay was verified and validated using samples prepared with size exclusion chromatography (IZON, $qEV_{Original}$ 70nm, Product # ICO-70).

Please see our webinar on EV preparation if assistance is needed regarding suggested EV purification for dSTORM imaging.

Guidance for EV capture strategy

There are five validated EV capture options for EV Profiler 2. Four are antibody-based capture methods: anti-CD81, anti-CD63 or anti-CD9, and anti-Tetraspanin Trio (a mix of the three separate tetraspanin antibodies). The fifth, phosphatidylserine (PS) Capture, utilizes a protein that efficiently captures EVs based on binding to phosphatidylserines. If the EV source is known to be enriched with an individual tetraspanin, isolating with that tetraspanin for capture can help isolate an EV sub-population. Otherwise, combined tetraspanin capture can isolate a broad range of EVs.

PS Capture may be used with EVs enriched in phosphatidylserine (e.g., cancer-derived EVs). PS Capture is valuable when you do not want to enrich for a protein biomarker. If PS content is unknown, we suggest comparing PS Capture to anti-CD81, anti-CD63, anti-CD9, or anti-Tetraspanin Trio Capture.

EV Profiler 2 requires an input volume of 10 µL of EV sample per lane. The concentration of the input EVs depends on the selected capture molecule and on the expression and accessibility of the target molecule on the EVs. As a starting point, we recommend 10^9 EVs per mL.

Guidance for user-provided capture molecules

The EV Profiler 2 Assay Chips can be used with any biotinylated capture molecule. Isolating EV subpopulations using an alternative biotinylated capture molecule is possible, although it is not provided in the kit. Biotinylated antibodies are readily available from commercial suppliers.

Optimization of the user-defined alternative capture molecules should include the following steps:

I. **Assess capture molecule specificity:** To determine specificity, stain tissue culture cells that express the target and a negative control (e.g., knockout or peptide blocking) with a fluorescently labeled version of the capture molecule. Ideally, the negative controls should include less than 10% of the density of the positive control samples; however, the user should predetermine this threshold.

II. **Assess capture molecule compatibility with detection antibodies:** When using a new biotinylated capture molecule, ensure that at least one lane with the capture molecule does not contain EVs and that less than 100 clusters are detected in the EV-negative lane when stained similarly.

III. **Reduce background:** If the no-EV control lanes with the new capture molecule result in high background, 0.5%-2.5% BSA can be included in the biotinylated capture molecule dilution.

IV. **Optimize capture efficiency and reproducibility:** Start with 70 nM of biotinylated capture molecule for the most efficient capture and test 20% higher and lower concentrations for ideal capture efficiency. A 75-minute incubation time is recommended, but 20% shorter and longer incubation times can also be tested for ideal capture efficiency and reproducibility.

Guidance on labeling for EV and biomarker detection

The assay contains two protocols for detection: Tetraspanin Detection (three colors) or user-defined protein, Pan-EV, and Tetraspanin Trio Detection.

I. User-defined protein, Pan-EV, and Tetraspanin Trio Detection

This protocol enables the identification of the EV population, EV sizing, visualization of EVs, tetraspanin positivity (CD81, CD63, and CD9 presence (in 1-color)), and surface or luminal user-defined protein detection. It can be used with Tetraspanin Capture, PS Capture, or user-defined capture.

The user provides the primary antibody for user-defined protein detection and a matched isotype control fluorescently labeled with Alexa Fluor™ 647 (AF647) or other far-red dSTORM-compatible fluorophore as a control.

Guidance for using alternative detection molecule

The Pan-EV (488) and Tetraspanin Trio (561) workflow can also probe surface or luminal user-defined molecules in the 647 channel.

Optimization of user-defined detection molecules should include the following steps:

I. **Assess detection molecule specificity:** To determine specificity, stain tissue culture cells that express the target and a negative control (e.g., knockout or peptide blocking) with a fluorescently labeled version of the detection molecule. Ideally, the negative controls should include less than 10% of the density of the positive control samples; however, the user should predetermine this threshold.

II. **Optimize degree of labeling:** Molecules (antibodies, proteins, etc.) can be purchased fluorescently labeled or labeled by the user. Use a dSTORM-compatible dye that fluoresces in the 647 channel (e.g., AF647). The number of fluorophores per protein can be modulated by increasing the molar ratio of dye to protein during the conjugation. We recommend starting from a degree of labeling of 2-6, when advanced users may choose to modulate this number according to their experimental needs.

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

IV. **Optimize laser power:** The default laser power recommended in this protocol was optimized for AF647. If the user-defined detection molecule is not AF647, adjust the 647 laser power to ensure fluorophore blinking throughout the acquisition. The optimal laser power and number of frames for the user-defined molecule can be determined using the CODI analysis software.

Adjust the laser power to find a value that results in the lowest possible localization precision (less than 10 nm) and maximal photon count. ONI recommends ensuring there are at least 10 localizations per frame after filtering. 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.

II. Tetraspanin Detection

Detecting with anti-CD81 (647), anti-CD63 (561), and anti-CD9 (488) can aid in the understanding of the tissue of origin and type of EVs. Biotyping of EVs can help down-select EV capture and detection for future experiments and characterize new EV sources or EV isolation methods. The Tetraspanin Detection can be used with Tetraspanin Capture, PS Capture, or user-defined capture.

Guidance for user-provided capture molecules

Due to the small size of EVs and low abundance of many luminal cargos, permeabilization must be performed carefully and with adequate controls to ensure that the cargo signal detected is not due to assay noise or membrane fragmentation due to excessive permeabilization.

We recommend the following types of controls be used the first time a user probes for a new luminal cargo:

I. Isotype control, with and without permeabilization.
   - This control checks the specificity of your primary antibody and ensures that an aberrant signal is not detected due to permeabilization.
   - Ideally, there should be minimal signal in the no permeabilization condition and no increase in signal in the permeabilization condition.

II. User-defined antibody, with and without permeabilization.
   - This control checks that the signal in the permeabilized condition is due to the permeabilization itself rather than non-specific binding.
   - Ideally, there should be minimal signal in the no permeabilization condition and an increase in signal in the permeabilization condition.

We also recommend careful visual inspection of permeabilized EVs on CODI. The Pan-EV stain signal should still appear whole in the permeabilized EVs. Significant differences in the Pan-EV signal between non-permeabilized and permeabilized samples may indicate over-permeabilization and destruction of the EVs. Please contact ONI support if you have concerns about over-permeabilization of your sample. The data below gives an example of using appropriate controls and shows images of what intact EVs should look like following permeabilization.

Following MISEV guidelines[1], studies were performed with mild permeabilizations to detect a low-abundance cargo protein, ALIX. Isotype controls and no-permeabilization controls guided experimental design and analysis settings. In three different EV types: PC3 EVs (prostatic adenocarcinoma cells), PANC1 EVs (pancreatic duct carcinoma cells), and MSC EVs (mesenchymal stem cells), the percentage of clusters positive for ALIX was significantly higher in permeabilized EVs than in non-permeabilized EVs, as expected. These results indicate that permeabilization allows for the detection of luminal proteins with low false positive detection in non-permeabilized controls.

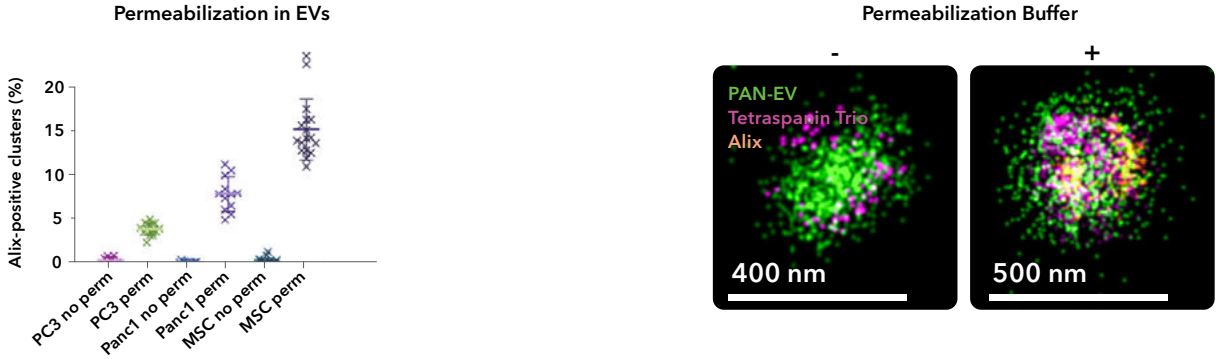

Figure 1. A) Percentage of clusters positive for a low-abundance internal cargo protein ALIX in three different EV sources with and without permeabilization. Data includes both Tetraspanin Trio Capture and PS Capture. B) Example images of non-permeabilized and permeabilized PANC1 EVs stained for ALIX.

Reference

I. Welsh, J. A., et al. Journal of Extracellular Vesicles. 13:2, e12404. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. (2024)

ONI, Linacre House, Banbury Road, Oxford, OX2 8TA, UK
ONI, ONI Inc. 11045 Roselle Street, Suite 120, San Diego, CA, 92121 USA