ONI

Extracellular Vesicles

TECHNICAL GUIDE

EV sample preparation for super-resolution imaging

Super-resolution imaging is a powerful tool to characterize heterogeneous samples of Extracellular Vesicles (EVs). Careful sample preparation will give the best results in terms of precise and clear images, and reproducible data. Here are some considerations and guidelines for optimal EV sample preparation for successful imaging.

1. Sample purity and contaminant reduction

EV source

Consider the animal species, the culturing medium/biofluid, the presence of added serum.

Serum-free media is preferred

If serum is required for optimal cell growth, EV-depleted serum can be purchased or prepared in-house.

Avoid introducing exogenous EVs

It often occurs via culture media components. For example, fetal bovine serum naturally contains bovine EVs and human serum albumin naturally contains human EVs.

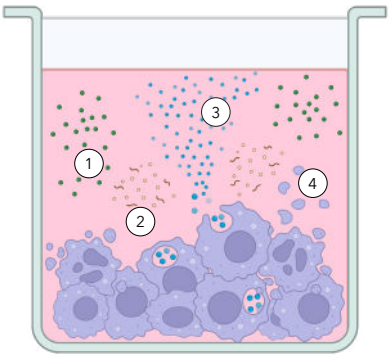

Figure 1. EV production in cell culture. Purification and concentration steps are needed in order to isolate EVs for super-resolution imaging. 1. Bovine EVs from FBS, 2. Cell debris, protein aggregates, 3. Target EVs, 4. Apoptotic bodies

2. EV collection and storage

Extracellular vesicles can be isolated from various sources, including cell culture media and biofluids including milk, urine, and blood. Perform low-speed centrifugation to remove debris, and store the EV-containing supernatant at 4 °C for up to a week. Fresh EVs are preferable for imaging; short term storage at 4 °C is recommended, but if long-term storage is necessary, minimize freeze-thaw cycles to maintain EV integrity. Even a single freeze-thaw cycle can reduce EV numbers and increase particle size.[1]

Cell culture-derived EVs

Ensure appropriate culture conditions and collect media at regular intervals to maximize EV yield.

Biofluid-derived EVs

Process fresh samples, store at 4 °C for less than 24 hours, and separate vesicle-containing plasma or supernatant from other components through centrifugation (1200 to 2500 x g for 10-15 min).

3. EV purification and particle concentration methods

The choice of an extracellular vesicle (EV) isolation and purification method depends largely on the type of EV, source, and specific application, such as super-resolution imaging. This guide outlines some, but not all, common methods, each with unique benefits and limitations.

One method may not suit all scenarios and adjustments may be necessary based on your specific requirements and sample characteristics.

Concentration

A concentration of 10^{10}-10^{12} particles/mL is recommended for EV super-resolution imaging using ONI's Nanoimager. Raw EV sources, such as cell culture media and biofluids, often have lower concentrations than desired.

Methods

Ultrafiltration: Ultrafiltration enables buffer exchange and particle concentration by size, retaining particles larger than the membrane's pore. Utilize an ultrafiltration device or centrifugal filter unit with a 100 kDa molecular weight cutoff to concentrate EVs and reduce volume before and/or after purification.[2, 3]

Differential low-speed centrifugation: EV-containing fluid is spun at low speed (300 xg), removing cells, debris, and membrane fragments. The supernatant is subsequently spun at higher speeds (2000 xg) to pellet larger classes of EVs. While cost- and time-effective, smaller EV species may be excluded.

Ultracentrifugation: Spins as fast as 100,000 x g can be used to pellet small particles such as EVs. Ultracentrifugation yields concentrated EV samples, though it is nonspecific with regard to particle type and can result in aggregation of EVs and other particles.[4]

Immunoaffinity isolation: Magnetic beads with attached antibodies capture target EVs. Provides good purity and moderate concentration, though reagent costs may accumulate over time.[2]

Size exclusion chromatography: Separates particles by size classes. Offers good purity at a relatively low cost and quick process. Results in a more dilute vesicle population.[5]

Density gradient centrifugation and polymeric precipitation: Inexpensive and simple methods to concentrate EVs, though may result in low specificity samples and aggregation of EVs and non-EV material. Resulting contaminants can obscure the true fluorescence signal of interest and affect the quality of the data obtained.[6]

Tangential flow filtration (TFF): TFF can be used to exchange buffers, separate particles by size, as well as concentrate the particle species of interest. Handles large volumes of EV-containing fluids and results in good purity, but requires specialized equipment.[7]

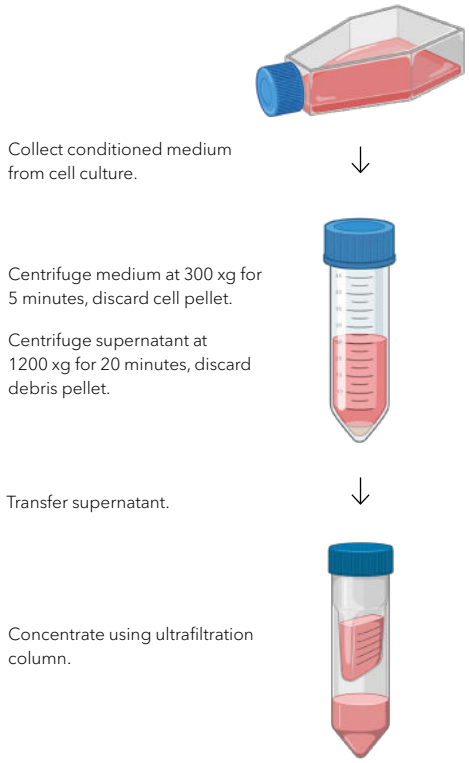

Figure 2. Flow chart of example EV purification and concentration method.

4. EV capture onto imaging surface

Surface capture and immobilization of biological samples is crucial for achieving high-quality imaging with TIRF microscopy and single molecule localization microscopy (SMLM) techniques like dSTORM. Titration of the capture molecule for surface coating is recommended for optimal capture and subsequent imaging.

A relatively high density of target particles must be captured onto the imaging surface for successful super-resolution imaging. Good density for EV analysis is 1000 or higher per field of view (50 x 80 µm).

EV-specific capture

Phosphatidylserine (PS)-based capture targets EVs enriched in surface-expressed PS, which is a classical uptake signal for target cells and is often highly expressed on EV surfaces.[8] Advantages of PS-based capture include its high capture efficiency for EVs produced by many commonly used cancer cell lines, as well as the fact that it is not species-specific. Drawbacks include that it can miss low-PS EVs, such as those produced by some primary cells or in biofluids from healthy tissue.[9] PS-mediate capture can be calcium-dependent.

EDTA in your sample buffers will chelate calcium and interfere with the PS capture. Adding additional calcium can help compensate for any EDTA present.

Target-specific capture
Antibody capture against EV-associated tetraspanin(s) or other surface markers of interest allows for specific capture of target-expressing EVs. Assess whether the target marker is also expressed on the cell membrane, as cell membrane debris can then be captured along with EVs.

Non-specific capture
Poly-lysine can result in the detection of a wide range of particles, but it may also lead to high levels of background noise. This non-specificity can be advantageous or disadvantageous depending on the imaging goals of your EV experiment.

5. Fixation

While not strictly necessary for dSTORM, fixation helps maintain the structural integrity and stability of extracellular vesicles during sample preparation and image acquisition, enabling high spatial resolution and analysis of specific structures and molecules making up the vesicles.

Over-crosslinking
Use gentle-to moderate fixation conditions to avoid over-crosslinking, which can occur from prolonged or excessive fixation and cause masking of epitopes, reducing antibody-target binding.[10]

Fluorophore-fixative compatibility
Ensure your fluorophores are fixation-compatible. Some fluorophores, such as pH-sensitive or tandem dyes, can be affected by fixation.[11]

6. Fluorescent labeling EV imaging

Fluorophores
Choose fluorophores compatible with single molecule localization microscopy. Popular choices for a three-color dSTORM experiment include Alexa Fluor 647,CF 568, and Atto 488. See our guides: Popular fluorophores for dSTORM imaging and Popular fluorophores for PALM imaging.

Intra-EV staining
For internal biomarkers of interest, permeabilization is usually needed to stain EV cargo. If the spatial location of your biomarker is unknown, testing permeabilization vs intact conditions can distinguish between internal and external target location. Because common permeabilizing detergents such as Tween-20 and Triton-X-100 can be harsh, consider titrating to the minimal effective concentration for your experiment to preserve as much of the original EV structure as possible.

Fluorophore labeling methods
Consider your labeling method with regards to spatial constraints. Nanobodies are smaller than antibodies and labeled primary antibodies are smaller structures than primary + secondary systems. See our guide on dSTORM preparation essentials.

7. Controls[12]

Antibody controls
When using antibodies to detect EV epitopes, include an isotype control to evaluate background signals and verify that the antibody specifically recognizes the intended target epitope.

Nucleic acid controls
Treat samples with RNase or DNase to confirm probe specificity when detecting nucleic acids within or associated with EVs.

EV detection controls
Assess specificity and sensitivity by including a negative control (no EVs) and a lysed EV control to examine detection efficiency and distinguish intact from disrupted vesicles.

Permeabilization controls
When imaging internal EV cargo, compare results from permeabilized and non-permeabilized samples to determine if detected molecules are inside the EVs or associated with their surface.

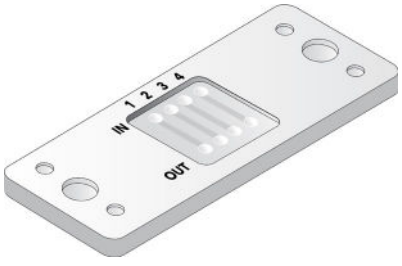

Figure 3. Schematic of the 4-lane EV chip from the ONI EV Profiler kit.

8. EV imaging

Optimized and simplified workflow with the EV Profiler Kit

Consider using the EV Profiler kit offered by ONI. This comprehensive kit provides all the necessary components for capturing, fixing, staining, and imaging EVs, ensuring a streamlined workflow for quality super-resolution images.

Imaging surface

Capture the EVs on a 1.5H coverslip (not a glass slide) in a sealed format which allows buffer application. This ensures compatibility with the imaging system for optimal image quality.

STORM buffer

Utilize an appropriate imaging buffer to create the optimal imaging environment for dSTORM. Low-oxygen and reducing conditions allow for fluorophore blinking and contribute to high-quality super-resolution images. Do not use hard mounting media.

Illumination angle

To minimize background fluorescence, employ total internal reflection fluorescence (TIRF) microscopy. TIRF selectively excites fluorophores within a thin layer in the Z direction, enhancing axial resolution and decreasing interference from out-of-focus fluorophores. Adjust the laser illumination angle to achieve TIRF conditions.

Laser power

Laser power directly impacts the blinking behavior of fluorophores, which is essential for high-resolution dSTORM imaging. While higher laser power can enhance blinking, it also increases the risk of premature photobleaching, leading to signal loss. Therefore, it's crucial to optimize laser power for each fluorophore and imaging experiment, balancing high resolution with appropriate rates of photobleaching.

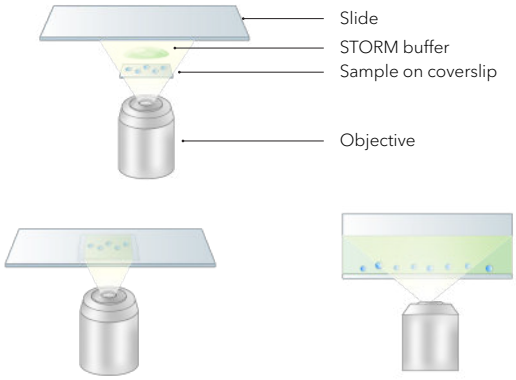

Figure 4. Diagram of optimal EV imaging setup using a coverglass and slide.

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