Tech Tip

BioTechniques

Tips and Best Practices to Optimize Your Exosome Labeling

Written by Alexis Madrid, PhD (Principal Scientist at Biotium) and Alesha Cox-Harris (Research Associate at Biotium)

INTRODUCTION TO EXOSOME STAINING

Extracellular vesicles (EVs) are small, membrane-bound particles secreted from cells and thought to function as cellular messengers, carrying cargo from one cell to another. In biomedical research, exosomes and their cargo are used as diagnostic biomarkers for cancer and other diseases. However, the isolation and detection of exosomes can be extremely challenging due to their small size (~30-200 nm in diameter, similar to most viruses). New methods and tools are constantly being developed but it can be difficult to know which to use.

In this tech tip, Biotium scientists share their expertise for optimal fluorescent staining and detection of exosomes by flow cytometry. To learn about EV and exosome biology, see Biotium's recent blog post on the subject.

ISOLATION: GETTING A CLEAN EXOSOME PREP

When attempting to detect exosomes stained with fluorescent dyes or antibodies, one of the biggest challenges is that the dye or antibody may bind non-specifically to contaminants in the preparation, such as aggregated proteins and membranous cellular debris. The tips below are our suggestions for reducing these contaminants in an exosome prep.

Sample source

The abundance and quality of the exosomes in your prep will depend a lot on the source material. Some researchers won't be able to change the source material much, for example those who are using a specific biological fluid (e.g., blood, urine) for diagnostics. For researchers purifying exosomes from cultured cells, choice of growth media is important. Bovine serum commonly used for cell culture contains bovine EVs, as well as aggregated protein components like fibrinogen that will confound downstream detection. We recommend growing cells using either exosome-depleted FBS, or in serum-free medium, depending on the needs of your cells of interest.

Technical Tip: For immortalized cell lines that can tolerate serum-free conditions, a simple solution we have found is to grow cells to the desired confluency in serum-containing medium, then switch to serum-free medium for 48 hours before collecting the conditioned medium for exosome isolation (Fig. 1).

Isolation methods

There are many different methods that are used to isolate exosomes:

- Differential centrifugation has been called the gold standard of exosome isolation due to its early adoption; it uses low-speed spins to remove large vesicles and particles, and high-speed ultra-centrifugation to pellet exosomes.
- Size exclusion chromatography (SEC) uses a column of porous resin which allows separation of particles based on size.

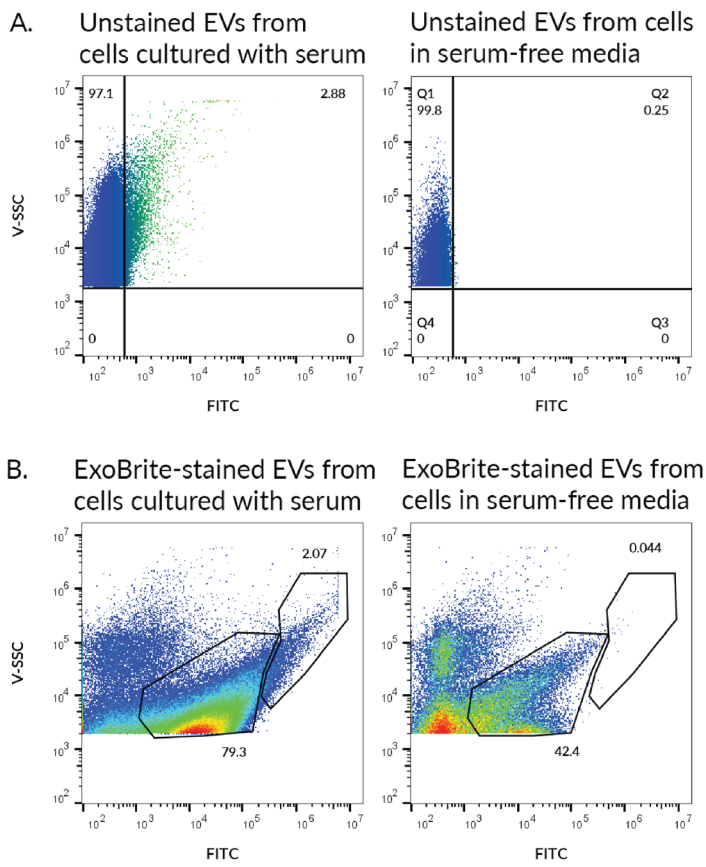

Figure 1: A. EVs were enriched using PEG precipitation from MCF-7 conditioned medium, and then analyzed by flow cytometry without any staining. EVs from cells that were grown in complete medium containing FBS show more particles with false-positive signal in the FITC channel compared to EVs from cells grown for 2 days in serum-free medium. B. EVs were enriched using PEG precipitation from MCF-7 conditioned medium, stained with Biotium's ExoBrite™ 490/515 EV Membrane Stain, and then analyzed by flow cytometry. EVs from cells grown for 2 days in serum-free medium are a more homogenous population, with fewer high molecular weight species, compared to EVs from cells grown in medium with FBS.

- Ultrafiltration vials are centrifugation devices with a membrane of a certain pore size (e.g., 100 kD); particles smaller than the molecular weight cutoff pass through, while larger particles are retained and concentrated on top.
- Polyethylene glycol (PEG) is a polymer which can be used to facilitate the precipitation of particles in a sample.
- Immuno-capture beads can be used to facilitate both purification and detection by flow cytometry. This method involves magnetic beads coated with antibodies against one of the three tetraspanin proteins (CD9, CD63, or CD81) that are commonly used as exosome markers.
- Several companies now offer spin column-based kits for exosome isolation.

We provide our assessment of exosome isolation methods in Table 1, based on our own testing of several of these methods here at Biotium, as well as recently published review articles (References 1,2).

Technical Tip: Prior to exosome isolation, it is a good idea to filter the conditioned cell medium or biological fluid through a 0.22 um filter (cellulose acetate or PES). We have found better yield with cellulose acetate.

Technical Tip: We have found size exclusion chromatography (SEC) to be an easy-to-use method that yields a relatively pure population of exosomes. It is a readily accessible option for most researchers, because it doesn't require an ultracentrifuge.

FLUORESCENCE LABELING AND DETECTION OF EXOSOMES

The small size of exosomes makes them difficult to differentiate from particles or debris in a sample and means fewer binding sites for an antibody or dye. Therefore, exosome probes need to have bright fluorescence to reach the limit of detection, and at the same time little to no aggregation to reduce nonspecific particles. In this section, we will delve into the main challenges of fluorescence staining and detection of exosomes, and our favorite methods of solving them.

Challenges of fluorescent exosome detection by flow cytometry

- Low sensitivity and high noise for cytometric detection of small particles.
  - **Tip:** Determine the limit of size detection of your instrument using sizing beads, and follow best practices for small particle detection by flow (see **Best practices for flow cytometry detection of exosomes** on page 3).
- Difficulty differentiating exosomes from debris and aggregates.
  - **Tip:** Choose an isolation method that does a good job of separating exosomes from other particles (see **Isolation methods** on page 1).
  - **Tip:** Use an antibody or dye at the lowest useful concentration, to decrease background.
  - **Tip:** Determine the limit of size detection of your instrument, and follow best practices for small particle detection by flow (see **Best practices for flow cytometry detection of exosomes** on page 3).
- Low signal over background.
  - **Tip:** Choose a bright and validated probe (see recommendations for fluorescent antibodies and membrane dyes in the following sections).
  - **Tip:** Titrate the probe to find the concentration that gives the best signal:noise.

Using fluorescently-labeled antibodies in flow cytometry

The most well-established exosome markers are the tetraspanin proteins CD9, CD63, and CD81 found in the membranes of exosomes. Using fluorescently-labeled

Table 1: Comparison of isolation methods

Isolation method	Pros	Cons
Differential centrifugation	• The traditional isolation method • Sequential steps allow removal of larger vesicles	• Does not remove particles smaller than exosomes • Requires a high-speed ultracentrifuge • May result in damaged exosomes
Size exclusion chromatography (SEC)	• Columns select particles by size • Large and small contaminants are excluded • No special equipment required • May use wide range of sample volumes if concentrated first	• Final sample may be dilute • May need to concentrate sample by ultrafiltration first
Ultrafiltration vials (e.g., 100 kDa cut-off)	• Removes small particles • Results in concentrated sample • No special equipment required	• Does not remove particles larger than EVs
PEG precipitation	• Inexpensive method • Utilized by several commercial kits • No special equipment required	• Does not select for exosomes by size • Co-precipitates many non-specific particles
Immuno-capture beads	• Selecting for tetraspanins should enrich for exosomes/EVs • Capture enables washing before or after staining, difficult with other methods • Bead-bound exosomes can be stained with antibodies for detection • Bead-bound exosomes detected as bright, dense population	• Capturing based on one tetraspanin may bias analysis • Cannot analyze individual exosomes • Many dyes are hydrophobic and stick to beads
Spin column kits (e.g., ExoQuick, Exo-spin™)	• Sold by several companies • Simple procedures • No special equipment required	• Poor purity, many non-exosome contaminants

antibodies against one of these proteins is an effective way to label exosomes for detection by flow cytometry or other methods.

Tips for staining exosomes with fluorescent antibodies

- Use clones that have been validated for exosome detection – we have observed that not all clones that stain cells are guaranteed to stain exosomes.
- Choose bright fluorophores for the antibody conjugates. Compared to cells, exosomes have many fewer copies of each target protein, so for an exosome to be detectable, each labeled antibody should be as bright as possible.
- Titrate the antibody to determine the lowest amount needed for good staining. Exosomes have very few target proteins and the sample may be dilute, so you may not need to use as high of a concentration as you would for cell staining. In addition, antibodies and dyes may aggregate and give false positive signals, which may be reduced by lowering the concentration (Fig. 2).

Technical Tip: As a starting point, we recommend trying ~ 1 ug/mL antibody conjugate for purified exosomes, and ~ 10 ug/mL for bead-bound exosomes.

Using membrane dyes to label exosomes

Another way to fluorescently label exosomes for detection is to stain them with a membrane dye. Being surrounded by a single membrane bilayer, exosomes and EVs should be able to bind to most membrane dyes. However, not all membrane dyes work equally well for exosome staining. Some membrane dyes, such as the classic carbocyanine dyes DiO and DiI, have poor solubility, and can thus form aggregates that can be confused with exosomes. Other membrane dyes that have been used for EV staining, such as di-8-ANEPPS, simply don't offer exosome staining that is robust or bright enough for efficient detection.

Biotium has tested more than 40 membrane stains by flow cytometry and selected those that offer the best detection of exosomes. This work led us to develop the ExoBrite™ line of exosome stains. We continue to develop and evaluate other types of dyes and products for exosome research.

Technical Tip: For fluorescent staining of exosomes, ExoBrite™ EV Membrane Stains are the best choice. They have bright signal, negligible aggregation, and have been validated with purified and bead-bound exosomes.

Tips for staining exosomes with fluorescent membrane stains

- Make sure there is data showing that the dye you select actually stains EVs. We have found that very few of the commonly used cell membrane dyes work well with EVs.
- Titrate the dye to determine the lowest amount needed for good staining (Fig. 3). Exosomes are small and the samples are often dilute, so you may not need to use as high of a concentration as you would for cell staining. In addition, lowering the concentration may help to reduce dye aggregates that give false positive signals.
- When setting up your experiment, always include a control of buffer plus dye alone (without exosomes), to see whether the dye shows any aggregation (Fig. 3).

Technical Tip: As a starting point, we recommend using 1X ExoBrite™ EV Membrane Stains for purified exosomes, and 10X for bead-bound exosomes.

Best practices for flow cytometry detection of exosomes

The ability to detect exosomes or EVs by flow cytometry is dependent upon the capabilities of the instrument itself, but there

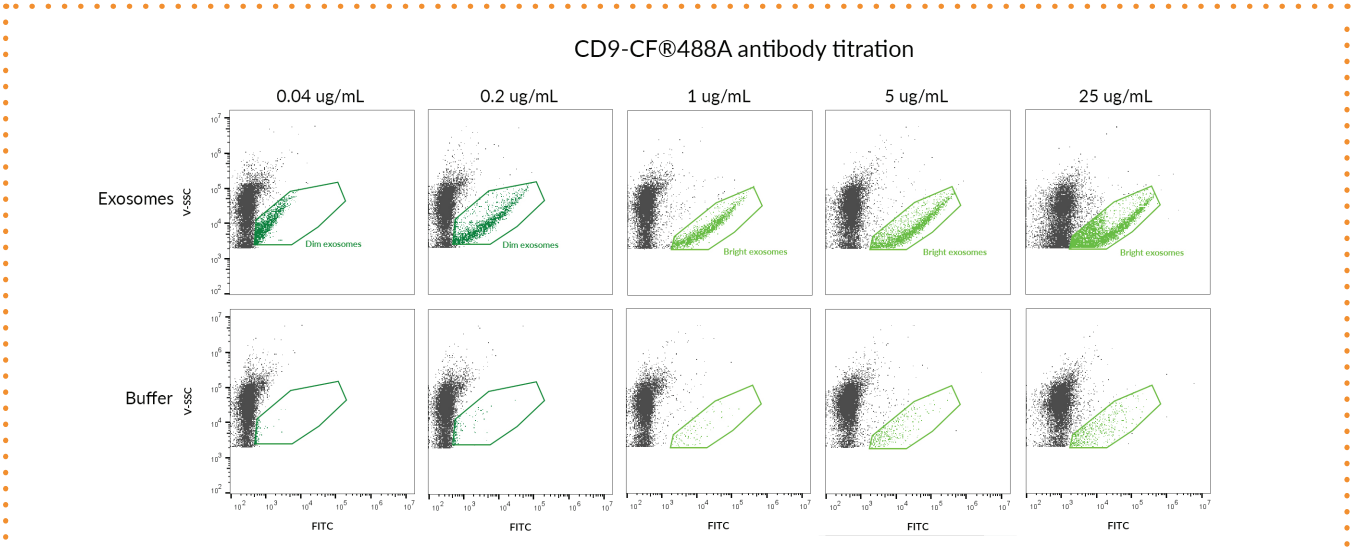

Figure 2: MCF-7-derived SEC-purified exosomes were stained with a 5-fold dilution series of CD9-CF®488A antibody. The two lowest antibody concentrations gave dim exosome staining (dark green dim gate), while the other concentrations gave bright exosome staining (light green gate). However increasing the concentration of antibody also increased the frequency of non-specific aggregates (bottom row, far right). The middle concentration, 1 ug/mL, showed both bright staining and low background. We recommend titrating antibodies to achieve good signal to noise.

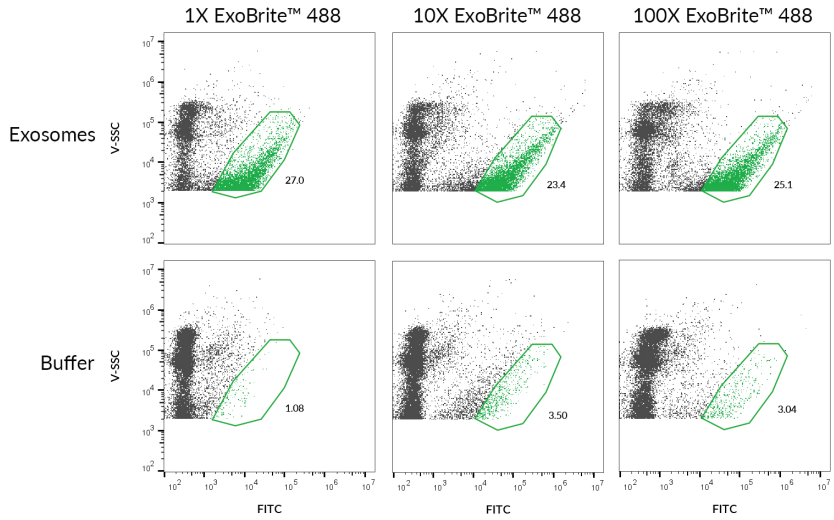

Figure 3: MCF-7-derived SEC-purified exosomes were stained with 1X, 10X, or 100X ExoBrite™ 490/515 EV Membrane Stain. Increasing the concentration of dye only slightly increased the brightness of the stained exosomes, but also increased the frequency of non-specific aggregates (bottom row). We recommend titrating stains to achieve good signal to noise.

are also experimental procedures that can help reduce background and achieve optimal detection. We have adopted the practices below for improved EV detection on the Beckman Coulter CytoFLEX flow cytometer (also see Reference 3), but the principles are the same for other instruments.

Tips for exosome detection by flow

- Move the side scatter (SSC) filter to the violet laser, for improved small particle sensitivity (Reference 4).
- Use fluorescent sizing beads to determine the limit of size detection for your instrument and set the V-SSC threshold accordingly.
- Dilute all samples in 0.2 um-filtered PBS for running on the instrument.
- Use bright, validated antibody conjugates or dyes (see **Tips for staining exosomes with fluorescent antibodies** and **Tips for staining exosomes with fluorescent membrane stains**).
- Use the lowest antibody or dye concentration that still gives a bright signal, to reduce background from dye aggregation.
- Run the samples at a slow rate, adjusting the flow and sample concentrations to achieve low abort rate and background particle counts (see Reference 3 for details).

REFERENCES

1. J. Extracell Vesicles, 4(1), 27031(2015), DOI: 10.3402/jev.v4.27031
2. Int J Mol Sci, 20(19), (2019), DOI: 10.3390/ijms20194684
3. Application Note: Set-Up of the CytoFLEX for Extracellular Vesicle Measurement. (2015). https://www.selectscience.net/application-articles/set-up-of-the-cytoflex-for-extracellular-vesicle-measurement/?artid=38228
4. J. Extracell Vesicles, 7(1), 1454776(2018), DOI: 10.1080/20013078.2018.1454776