Tech Tip: Fluorescent Detection of EVs by Flow Cytometry

Written by Alexis Madrid, PhD (Assistant Director of Bioscience at Biotium) and Alesha Cox-Harris (Research Associate II at Biotium)

Introduction to EV Staining

As developers of fluorescent dyes, we are frequently asked about dyes for labeling extracellular vesicles (EVs), including exosomes, so we have been working hard to develop good solutions for our customers. Because of their small size (~30-200 nm in diameter, similar to most viruses), the isolation and detection of EVs can be extremely challenging. New methods and tools are constantly being developed but it can be difficult to know which to use.

In this tech tip we will share the expertise we have acquired for optimal fluorescent staining and detection of EVs. To learn about EV and exosome biology, see our blog post on the subject.

Isolation: Getting a Clean EV Prep

When attempting to detect EVs 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 EV prep.

Sample Source

The abundance and quality of the EVs 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 or urine) for diagnostics. For researchers purifying EVs 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 EV-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 48-72 hours before collecting the conditioned medium for EV isolation (Fig. 1).

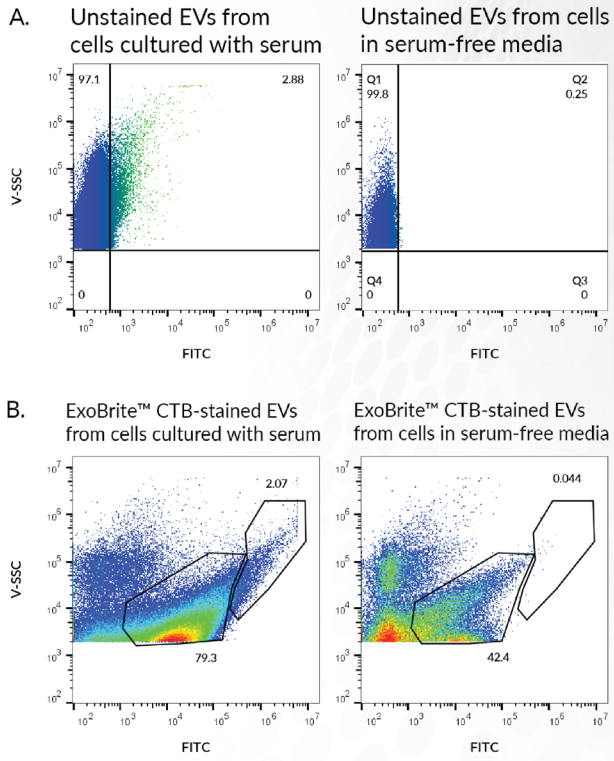

Figure 1. A. EVs were enriched using PEG precipitation from MCF-7 conditioned medium, and then analyzed by flow cytometry without any staining. EVs from cells that were grown in complete medium containing FBS show more particles with false-positive signal in the FITC channel compared to EVs from cells 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 CTB EV 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.

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Isolation methods

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

- Differential ultracentrifugation (UC) was an early popular method; it uses low-speed spins to remove large vesicles and particles, and high-speed ultra-centrifugation to pellet EVs.
- Size exclusion chromatography (SEC) uses a column of porous 70 nm resin that allows separation of particles based on size.
- Polyethylene glycol (PEG) is a polymer that 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 EV markers.
- Spin column-based kits use various types of resins to enrich EVs.

We provide our assessment of EV 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). See our Tech Tip: EV Isolation and Staining Protocols for more notes and protocols related to EV isolation.

Technical Tip:

Prior to EV isolation, it is a good idea to do a low-speed spin (i.e., 2000 x g for 10 minutes) to remove cells and large debris. It is optional to perform a filtration step through a 0.22 um cellulose acetate filter.

Table 1: Comparison of isolation methods

Isolation method	Pros	Cons
Differential ultracentrifugation	• The traditional isolation method • Sequential steps allow removal of larger vesicles	• Does not remove particles smaller than EVs • Requires a high-speed ultracentrifuge • May result in damaged EVs
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	• Additional concentration steps may be needed
PEG precipitation	• Inexpensive method • Utilized by several commercial kits • No special equipment required	• Does not select for EVs by size • Co-precipitates many non-specific particles • Requires an overnight incubation • Resulting EV prep contains residual PEG
Immuno-capture beads	• Selecting for tetraspanins should enrich for EVs • Capture enables washing before or after staining, difficult with other methods • Bead-bound EVs can be stained with antibodies for detection • Bead-bound EVs detected as bright, dense population	• Capturing based on one tetraspanin may bias analysis • Cannot analyze individual EVs • Hydrophobic dyes will stick directly to beads
Spin column kits (e.g., ExoQuick, Exo-spin™)	• Sold by several companies • Simple procedures • No special equipment required	• Poor purity, many non-EV contaminants

Technical Tip:

We have found size exclusion chromatography (SEC) using 70 nm to be an easy-to-use method that yields enriched EVs at concentrations useful for downstream analysis. It is a readily accessible option for most researchers, because it doesn't require an ultracentrifuge. See our example protocol.

Fluorescence Labeling and Detection of EVs

The small size of EVs makes them difficult to differentiate from particles or debris in a sample and means fewer binding sites for an antibody or dye. Therefore, EV 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 EVs, and our favorite methods of solving them.

Challenges of fluorescent EV 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 EVs** below).
- *Difficulty differentiating EVs from debris and aggregates*
  - **Tip:** Choose an isolation method that does a good job of separating EVs from other particles (see **Isolation methods** on page 2).
  - **Tip:** Use an antibody or dye at the lowest useful concentration, to decrease background.
  - **Tip:** Choose a stain that is not prone to aggregation (see **Using EV surface stains** on page 5).
- *Low signal over background*
  - **Tip:** Choose a bright and validated probe (see recommendations for fluorescent antibodies and surface stains in the sections below).
  - **Tip:** Titrate the probe to find the concentration that gives the best signal-to-noise.

Best practices for flow cytometry detection of EVs

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

- Move the side scatter (SSC) filter to the violet laser (i.e., V-SSC), for improved small particle sensitivity (Reference 4).
- Use fluorescent sizing beads to determine the limit of size detection for your instrument, set the V-SSC threshold accordingly.
- Dilute all samples in 0.2 um-filtered PBS, or the buffer recommended for running on the instrument.
- Use bright, validated antibody conjugates or dyes (see **Tips for staining EVs with fluorescent antibodies** on page 4 and **Tips for staining EVs with fluorescent surface stains** on page 5).
- Use the lowest antibody or stain concentration that gives a bright signal, to reduce background from fluorescent aggregates.
- Run the samples at a slow rate, adjusting the flow and sample concentrations to achieve low abort rate (see Reference 3).
- Always run the same stain or antibody in buffer alone to check for aggregation.

Technical Tip:

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

Fluorescence Labeling and Detection of EVs with Antibody Conjugates

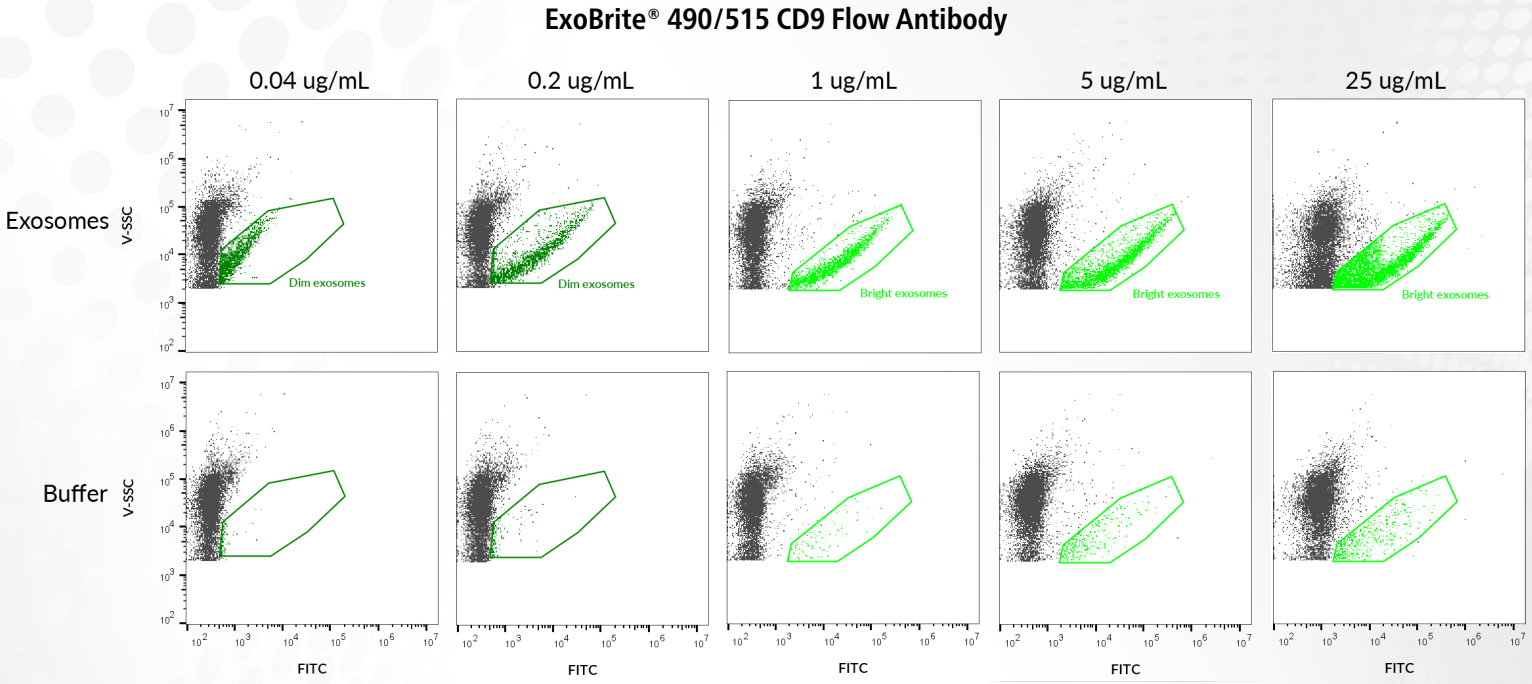

Figure 2: MCF-7-derived SEC-purified EVs were stained with a 5-fold dilution series of ExoBrite® 490/515 CD9 Flow Antibody. The two lowest antibody concentrations gave dim EV staining (dark green dim gate), while the other concentrations gave bright EV staining (light green gate). However, increasing 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.

Using fluorescently-labeled antibodies in flow cytometry

The most well-established EV markers are the tetraspanin proteins CD9, CD63, and CD81 found in the membranes of EVs. Using fluorescently-labeled antibodies against one or more of these proteins can be an effective way to label EVs for detection by flow cytometry or other methods.

Tips for staining EVs with fluorescent antibodies

- Use clones that have been validated for EV detection – We have observed that not all clones that stain cells also stain EVs so it is important to use clones that have been validated for EV detection such as the ExoBrite™ line of antibodies.
- Choose bright fluorophores for the antibody conjugates. Compared to cells, EVs have many fewer copies of each target protein, so for an EV to be detectable, each labeled antibody should be as bright as possible. We have selected and validated bright fluorophores in our ExoBrite™ line, or customers can contact our Technical Support for other suggestions.
- Titrate the antibody to determine the lowest amount needed for good staining. EVs have very few target proteins, and the sample may be dilute, so we find that we can typically use a lower concentration of antibody to stain EVs than to stain cells. 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 EVs, and ~ 10 ug/mL for bead-bound EVs.

Fluorescence Labeling and Detection of EVs with EV Surface Stains

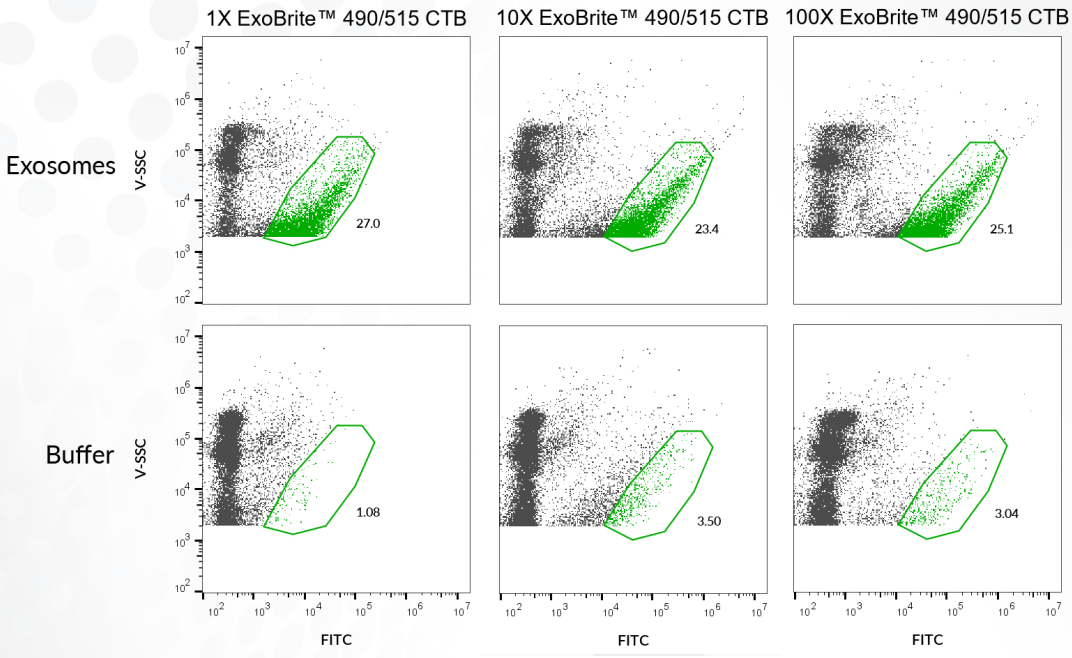

Figure 3: MCF-7-derived SEC-purified EVs were stained with 1X, 10X, or 100X ExoBrite™ 490/515 CTB EV Stain. Increasing the concentration of stain only slightly increased the brightness of the stained EVs, but also increased the frequency of non-specific aggregates (bottom row). We recommend titrating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

Technical Tip:

As a starting point, we recommend using 1X ExoBrite™ Annexin, ExoBrite™ WGA, or ExoBrite™ CTB EV Stains for purified EVs, and 10X ExoBrite™ WGA or ExoBrite™ CTB EV Stains for bead-bound EVs.

Using EV surface stains

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

Biotium has tested more than 40 different surface stains by flow cytometry and selected those that offer the best detection of EVs. This work led us to develop the ExoBrite™ line of EV stains. Our ExoBrite™ Annexin, ExoBrite™ WGA, and ExoBrite™ CTB EV Stains are based on fluorescent protein ligands for cell surface targets. We continue to develop and evaluate other types of dyes and products for EV research, including EV stains based on membrane intercalating dyes.

Tips for staining EVs with fluorescent surface 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, usually due to aggregation and/or poor coverage.
- Titrate the dye to determine the lowest amount needed for good staining (Fig. 3). EVs 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 fluorescent aggregates that give false positive signals.
- When setting up your experiment, always include a control of buffer plus stain alone (without EVs), to see whether the stain shows any aggregation (Fig. 3).

References

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