Detection and Sorting of Extracellular Vesicles and Viruses Using nanoFACS

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Extracellular vesicles (EVs) are sub-micron-sized membranous spheres secreted by cells. EVs play a functional role as intercellular communicators and are associated with a number of diseases. Research into EVs is an area of growing interest due their many potential uses as therapeutic agents, as diagnostic and theranostic biomarkers, and as regulators of cellular biology. Flow cytometry is a popular method for enumerating and phenotyping EVs, even though the majority of EVs are below the detection sensitivity of most commercially available flow cytometers. Here, we present optimized protocols for EV labeling that increase the signal-to-noise ratio of EVs by removing residual antibody. Protocols for alignment of high-resolution jet-in-air flow cytometers are also provided. Published 2020. U.S. Government.

Basic Protocol 1: Bulk EV staining with CFSE protein binding dye
Basic Protocol 2: Antigen-specific staining of EV markers with fluorochrome-conjugated antibodies
Basic Protocol 3: Astrios EQ instrument setup and sample acquisition
Basic Protocol 4: Counting particles and EVs on Astrios EQ with spike-in reference beads

Keywords: EV sorting • EVs • extracellular vesicles • flow cytometry • flow virometry • nano flow cytometry • nanoFACS • virus sorting

How to cite this article:
Morales-Kastresana, A., Welsh, J. A., & Jones, J. C. (2020). Detection and sorting of extracellular vesicles and viruses using nanoFACS. *Current Protocols in Cytometry*, *95,* e81. doi: 10.1002/cpcy.81

INTRODUCTION

Recent advances in high-resolution flow cytometry (HRFC), which show improvements in both light scatter and fluorescence sensitivity over earlier instruments, have resulted in the development of techniques to better isolate, stain, and analyze single EVs (Boing et al., 2014; Groot Kormelink et al., 2016; Morales-Kastresana et al., 2019; Morales-Kastresana et al., 2017; Stoner et al., 2016; van der Vlist, Nolte-'t Hoen, Stoorvogel, Arkesteijn, & Wauben, 2012). Below, we describe protocols to fluorescently label EVs using CFDA-SE (hereinafter called CFSE), as well as antibodies targeted at specific EV surface proteins. We also provide guidelines for removal of residual dye and antibody, appropriate data acquisition by HRFC, and EV counting by HRFC. Figure 1 summarizes these protocols.

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols in Cytometry e81, Volume 95
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpcy.81

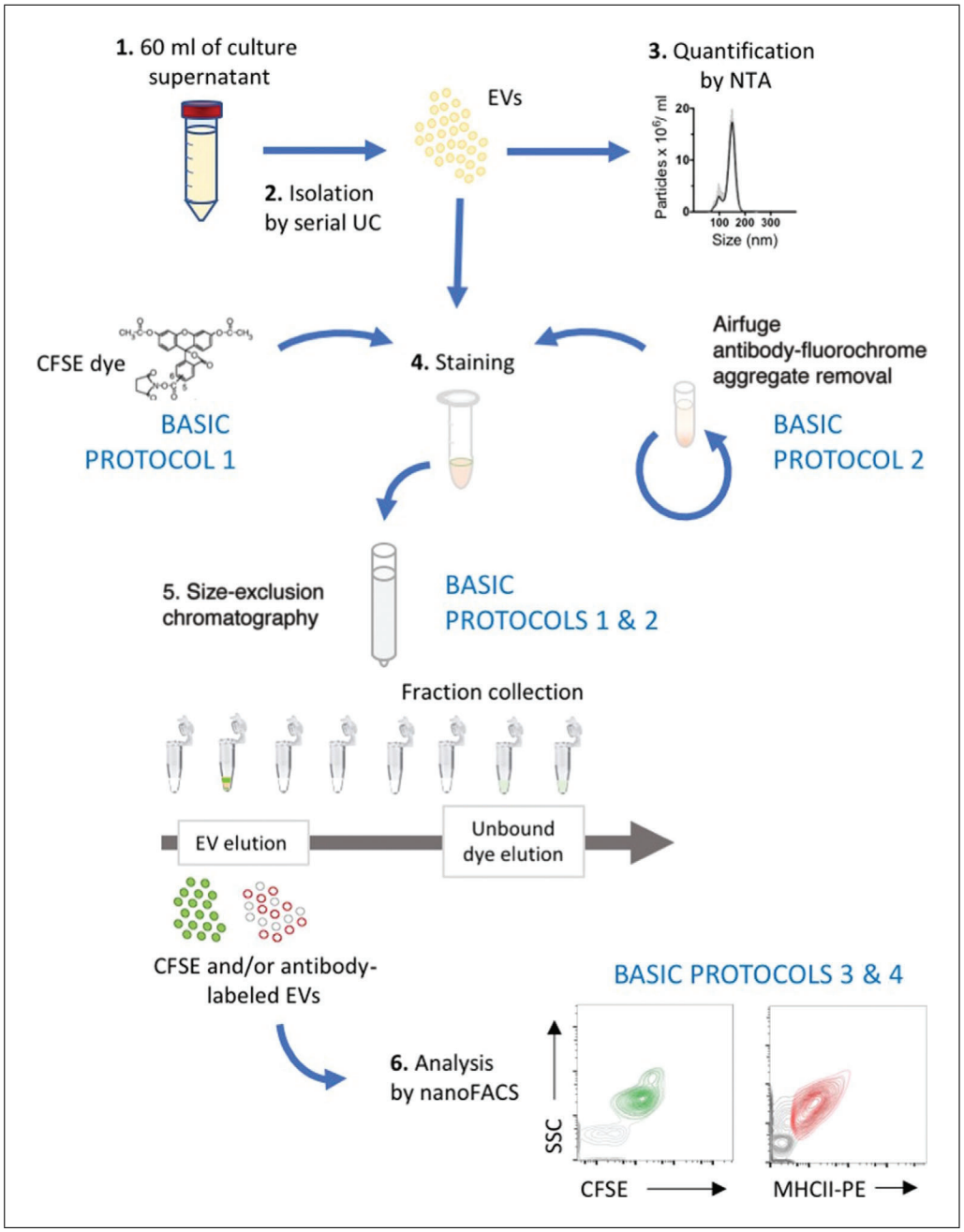

Figure 1 Diagram of the workflows described in this article for staining EVs in bulk with CFSE and for specific antigens with fluorochrome-conjugated antibodies.

The EVs used in this protocol are derived from the DC2.4 cell line and from bone-marrow-derived dendritic cells (BMDCs). DC2.4 cells are immature dendritic cells (DCs) with very low surface expression of typical DC markers (Hargadon, Forrest, & Reddy, 2012; Shen, Reznikoff, Dranoff, & Rock, 1997) that release a morphologically homogeneous population of EVs (~130 nm in diameter). DC2.4 EVs are used to demonstrate CFSE staining in Basic Protocol 1 and as a negative control for antibody-based staining methods in Basic Protocol 2. EVs derived from bone marrow dendritic cells (BMDCs) are more heterogeneous in diameter (100-200 nm) and composition (Morales-Kastresana et al., 2019) and express DC markers such as major histocompatibility class II (MHCII) molecules. BMDC EVs are used to demonstrate antigen-specific staining with fluorochrome-conjugated antibodies in Basic Protocol 2. DC2.4- and BMDC-derived EVs are isolated by serial ultracentrifugation and their concentration and diameter

distribution are characterized by nanoparticle tracking analysis (NTA: Morales-Kastresana et al., 2019; Morales-Kastresana et al., 2017).

BULK EV STAINING WITH CFSE PROTEIN BINDING DYE

BASIC PROTOCOL 1

Carboxyfluorescein diacetate succinimidyl ether (CFDA-SE) is a small molecule that has been used extensively for labeling and tracking cells by flow cytometry, along with other imaging techniques (Lyons, Blake, & Doherty, 2013; Perfetto, Ambrozak, Roederer, & Koup, 2004). CFSE irreversibly binds to positively charged functional groups, such as lysine and arginine groups in proteins, through a covalent bond.

The two diacetic groups in CFDA-SE's structure render the molecule highly permeable and nonfluorescent (Breeuwer et al., 1995). Therefore, it was originally believed that esterase, the main enzyme catalyzing the hydrolysis of the diacetic groups, was required to convert CFDA-SE to fluorescent CFSE (Wang, Duan, Liu, Fang, & Tan, 2005). In fact, however, the presence of esterases merely accelerates the hydrolysis of CFDA-SE, in which they act as catalysts; contrary to the common misconception, esterases are not required for this. Furthermore, other factors, such as high CFSE concentration, long incubation times, and high temperatures, can also promote the hydrolysis of diacetic groups (Bergsdorf, Beyer, Umansky, Werr, & Sapp, 2003; Hoefel, Grooby, Monis, Andrews, & Saint, 2003). Therefore, the approach used in this protocol has been optimized for staining cell-culture-derived EVs with a fluorescent dye, regardless of the concentration of esterases in the EVs (Morales-Kastresana et al., 2017).

The following protocol was developed to bulk-stain EV populations (Thery et al., 2001; Thery, Ostrowski, & Segura, 2009). The protocol was optimized for achieving good signal-to-noise ratios while avoiding excessive loss of EVs (Morales-Kastresana et al., 2017). To maximize the fluorescent intensity of the EVs, the authors recommend using CFDA-SE aliquots prepared in DMSO rather than aqueous solutions, as this minimizes fluorescein quenching with light and loss of diacetic groups through hydrolysis, which will render the molecule less permeant (Banks et al., 2013). This protocol can be performed in nonsterile conditions, although appropriate handling of reagents to ensure the absence of contaminating particulates is highly recommended. Though this method is very useful for purified EVs, it is not appropriate for use in the setting of highly heterogeneous samples, such as plasma or serum, where extracellular proteins (including lipoproteins and ribonucleoproteins) not associated with EVs would be labeled along with the EV-associated proteins.

Materials

Ca^{2+}-, Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS; Thermo Fisher Scientific, cat. no. 14190250)
EV sample in DPBS at a known concentration (particles per milliliter)
10 mM CFDA-SE (Thermo Fisher Scientific, cat. no. V12883) in DMSO (store under anhydrous conditions once prepared)

1.7-ml microcentrifuge tubes (Avanti)
0.5-ml-bed-volume size-exclusion chromatography (SEC) columns (Sephadex G-25 or, equivalently, NAP-5 or PD-10 columns, GE Healthcare)
Rack or similar to hold the chromatography columns
Micropipettor and tips
Incubator, 37°C

1. Prepare 15 µl of DPBS containing between $\sim 1 \times 10^8$ and $\sim 2.5 \times 10^9$ DC2.4-derived EVs in a 1.7-ml microcentrifuge tube.

This number of EVs corresponds to ∼0.1-2.5 µl of EV stock, if prepared as described previously (Morales-Kastresana et al., 2017), starting with 60 ml of supernatant from cells cultured for 48 hr in EV-depleted medium. The reaction volume and CFSE concentrations are optimized for staining ∼1 × 10^9 EVs, and the use of a different amount of EVs may therefore result in suboptimal staining. When larger amounts of labeled EVs are required, this protocol can be scaled up with similar results.

2. In a separate 1.7-ml microcentrifuge tube, prepare 15 µl DPBS containing 80 µM CFDA-SE by adding 0.12 µl 10 mM CFDA-SE to 15 µl DPBS.

   Intermediate dilutions in DPBS may be prepared to facilitate the dilution process. We recommend storing the CFDA-SE stock reconstituted in DMSO in small aliquots at −80°C. Avoid storing CFDA-SE in the form of DPBS solution or other aqueous solutions because this favors the hydrolysis of its diacetic groups, thereby decreasing the dye's permeability and its incorporation into EVs (Banks et al., 2013; Bergsdorf et al., 2003; Hoefel et al., 2003). For the same reason, it is critical that the aliquots be stored in an anhydrous manner. If the EV number and reaction volume are scaled up in step 1, scale up the CFDA-SE quantity accordingly to achieve an 80 µM CFDA-SE solution.

3. Pipette the CFDA-SE solution on top of the EV solution. Mix by pipetting and incubate 2 hr at 37°C in the dark. The incubated CFSE concentration is now 40 µM.

   The incubation time may be extended to increase incorporation of CFDA-SE into EVs. However, the authors have observed a decrease in the EV number after long incubation periods (Morales-Kastresana et al., 2017).

4. 15 min before the end of the incubation, wash a 0.5-ml-bed-voume SEC column with 10 ml DPBS. Never allow the column to dry.

   To automate the washing process, a pump can be setup to help add DPBS onto the column.

5. Prepare collection tubes for the collection of two fractions. To facilitate the visualization of eluted sample, use a marker pen to draw a line indicating the 500-µl mark on each collection tube.

   The first fraction is the "dead volume," of buffer alone, that elutes before fractions containing material from the loaded sample. The majority of DC2.4 EVs appear in fraction 2. Some EVs may, however, elute in fraction 3. Free dye elutes in fractions ∼7-8.

6. When the 2-hr incubation period is complete, increase the volume of the CFSE-stained EV preparation to 100 µl by adding 70 µl DPBS, and mix by pipetting.

   If the staining is scaled up in step 1 to increase the number of EVs, then add sufficient DPBS to obtain a 100-µl final volume. If the total volume is >100 µl, use multiple columns to wash the sample.

7. Pipette the 100 µl CFSE-stained EVs onto the SEC column and immediately start collecting 500-µl fractions.

8. When the 100 µl of sample has completely entered the column bed, add 500 µl DPBS and continue collecting fractions. Approximately 80% of the eluted EVs will be collected in fraction 2 (Fig. 2).

   Waiting until the whole sample enters the column bed before adding more DPBS avoids dilution of the sample and improves the collection of concentrated EV fractions.

   The NTA-detectable DC2.4 EVs are ∼130 nm in diameter. For other size ranges, the elution profile might be different. In that case, the authors recommend collecting smaller fractions (250 µl each) and assessing the EV elution profile by nanoFACS or other means.

9. Further fractions can be collected if desired by continuing to add DPBS onto the column and collecting fractions as in step 8.

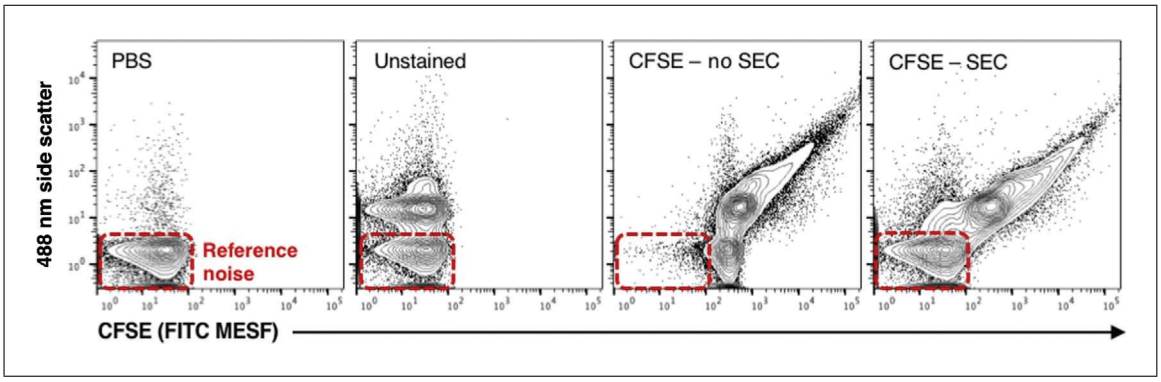

Figure 2 Representative contour plots of PBS, unstained DC2.4 EVs, and CFSE-stained DC2.4 EVs before and after SEC. The number of fluorescein molecules incorporated in the EVs is shown in the plot (the procedure for calculating the MESF is provided in Basic Protocol 4). Red boxes indicate system reference noise.

10. If CFSE-labeled DC2.4 EVs are to be used on the same day for further experiments, store at 4°C and avoid exposure to direct light. If the CFSE-labeled EVs are to be used on a different day, prepare aliquots if necessary and store at −80°C protected from light (e.g., wrapped in foil; Morales-Kastresana et al., 2017).

ANTIGEN-SPECIFIC STAINING OF EV MARKERS WITH FLUOROCHROME-CONJUGATED ANTIBODIES

BASIC PROTOCOL 2

The use of antibodies for EV staining and analysis via flow cytometry poses challenges due to the relatively large sizes of antibodies and fluorochrome conjugates in comparison to the majority of EVs. A conventional IgG measures ~3 × 11 nm, not including any conjugated fluorochrome(s) (Reth, 2013). Antibodies conjugated to fluorescent proteins such as phycoerythrin (PE) and allophycocyanin (APC; 250 and 105 kDa, respectively) are considerably bigger. Furthermore, commercial antibody preparations contain some aggregates that can be misidentified as positive events if not removed (Gagnon & Beam, 2009). Therefore, it is crucial to choose a method that efficiently eliminates antibody aggregates and discriminates labeled EVs from unbound antibody conjugates.– Some groups have reported successful removal of unbound antibodies after gradient ultracentrifugation in sucrose or iodixanol or repetitive ultracentrifugation (Groot Kormelink et al., 2016; Higginbotham et al., 2016; van der Vlist et al., 2012). In this protocol, we use high-speed centrifugation to remove antibody aggregates and size-exclusion chromatography (SEC) to wash the unbound antibody after staining.

Materials

Ca^{2+}-, Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS, Thermo Fisher Scientific)
Fluorochrome-conjugated antibody: anti-MHCII-PE antibody (Biolegend, cat. no. 107608)
Fc Block (anti-CD32) (BD Pharmingen, cat. no. 553142)
EV sample in DPBS with a measured concentration (particles per milliliter)
20% (v/v) ethanol

1.7-ml microcentrifuge tubes
Rack or equivalent to hold the chromatography columns
Micropipettor and clean tips
Airfuge (Beckman Coulter, cat. no. 347854)
A100/18 rotor (Beckman Coulter, cat. no. 347593)
Plastic rotor lids for A100/18 rotor (Beckman Coulter, cat. no. 339643)

Thin-walled polypropylene tubes for A100/18 rotor (Beckman Coulter, cat. no. 342630)
Gentle mixer
Sharp tweezers
10 ml qEV-Original (or Sepharose-2B) size-exclusion chromatography (SEC) columns (Izon Bioscience)

1. Wash one qEV column per EV preparation with 20 ml DPBS. Never allow the columns to become dry.
2. Pipette 1×10^9 EVs in a 10-µl volume of DPBS and add 2 µg Fc Block reagent to block Fc receptors. Incubate with no agitation for 10 min at room temperature.

   The presence of Fc receptors on EVs is not well documented. However, adding Fc Block will not only block putative Fc receptors, but also serve as a source of protein to block other nonspecific binding sites of fluorescent antibodies.
3. Into a 1.7-ml microcentrifuge tube, pipet 1.5 µg of fluorochrome-conjugated antibody, and add DPBS to 120 µl final per sample. Mix by pipetting up and down. If multiple samples are to be stained with the same antibody, prepare a master mix.

   This antibody quantity is a reference starting point when testing a new antibody. Antibody titration is recommended to achieve optimal staining and avoid the use of unnecessary material. Many anti-human antibodies are provided in a test volume format (microliters per test) rather than a concentration format (micrograms per milliliter).
4. Transfer the 120 µl of antibody solution to an Airfuge tube, and mark one side of the tube with a waterproof marker. Place the tube with a corresponding balance into an A100/18 rotor, with the mark facing up. Place a lid on the rotor.

   The mark is a reference for the location of the antibody aggregates after Airfuge centrifugation. Using the rotor cover can reduce sample evaporation during centrifugation.
5. Place the rotor into the Airfuge and close the Airfuge lid tightly. Open the air source to the centrifuge until the gauge reads 22 psi (~130,000 RCF) and leave for 5 min.

   To avoid extreme heat during centrifugation, this step can be performed in a cold room. Alternatively, the authors cool down the rotor before using it.
6. When the Airfuge step is complete, use sharp tweezers to remove the tubes from the rotor and place them on the corresponding rack.
7. Gently pipet off the top 70 µl of solution and add it on top of the EV solution.

   Antibody aggregate pellets cannot always be observed. For that reason, leaving a reasonable volume in the bottom of the tube and using the top part of the solution is recommended.
8. Incubate the EVs with antibody for 15-30 min in the dark at room temperature with gentle agitation.

   As with CFSE, time is a parameter that can be modulated to increase the labeling with antibodies. The authors have observed slight improvements of staining with certain epitopes upon increasing the staining period up to 1 hr.
9. Prepare collection tubes for 12 fractions. To facilitate the visualization of eluted sample, use a marker to draw a line indicating the volume of each fraction (500 µl) on the side of the collection tubes.
10. Add DPBS to the EV prep to 500 µl final volume and proceed to remove unbound antibody by SEC using qEV columns.

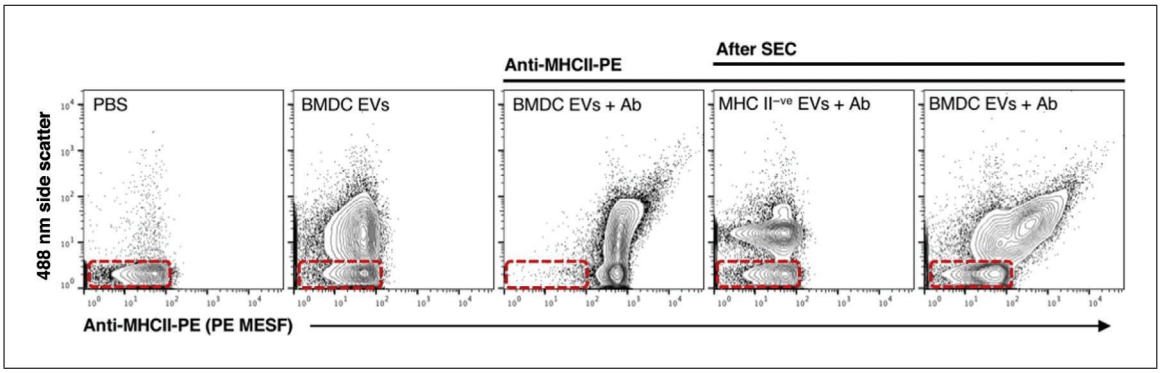

Figure 3 Representative contour plots showing PBS, unstained BDMC EVs, and MHCII-stained BMDC EVs (before and after SEC) and control DC2.4 EVs that lack MHCII on their surface. Red boxes indicate system reference noise.

Samples that will not be immediately loaded on columns can be stored at in the dark at 4°C overnight (up to 18 hr).

11. Wait until all of the DPBS used to prewash the column has entered the column bed. Immediately load 500 µl of the sample and simultaneously start collecting 500-µl fractions.
12. Keep adding DPBS (500 µl each time) while collecting fractions. Stained EVs will start eluting in fraction 7, with the majority in fractions 8-9. For maximum recovery, harvest fraction 10 too (Fig. 3).
13. Store EVs at 4°C in the dark until ready to perform flow cytometric analysis.
14. qEV columns can be stored at 4°C and reused with extensive washing. The authors recommend washing with a minimum of 50 ml DPBS, to elute as much remaining antibody as possible, followed by 10 ml 20% ethanol diluted in DPBS to keep the columns aseptic during storage. Before reusing a column, wash with 40 ml DPBS to make sure that any traces of ethanol are removed.

ASTRIOS EQ INSTRUMENT SETUP AND SAMPLE ACQUISITION

BASIC PROTOCOL 3

This protocol presents the general steps to follow to achieve optimal laser-stream-detector alignment and sample acquisition on the Astrios EQ flow cytometer (Beckman Coulter). The general concept, however, can be applied to other jet-in-air instruments.

Materials

FACSRinse
Ca^{2+}-, Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS, Gibco)
200-nm Fluosphere fluorescent beads: red (Life Technologies, cat. no. F8794, excitation/emission 580/605) and yellow-green (Life Technologies, cat. no. F8811, excitation/emission 505/515)
FACSClean

Astrios EQ flow cytometer
Polystyrene FACS tubes

1. Turn on the Astrios EQ instrument at least 1 h before running the samples. Let the pressure and stream stabilize, and prime the fluidics system to remove bubbles in the circuit. Turn lasers on and allow them to warm up while shuttered.

2. Wash the sample line with FACSRinse solution for ∼20 min at a high differential pressure (1 psi over the sheath pressure). Repeat same procedure with clean DPBS for another 20 min.

3. Adjust the vertical alignment.

   To test whether the stream is vertical, raise the nozzle while looking at the relative position of the stream to the pinholes. If the position does not change, the stream is vertically well aligned; if it does change, then the verticality must be tweaked. A good vertical alignment maximizes the detection of parameters in all laser paths.

4. Set the triggering threshold to the 561-SSC channel. Adjust the triggering threshold channel and voltage to allow the visualization of the noise population, to ∼10,000 events per second (which is approximately a noise event rate of 20% of the total triggered event rate when samples are running, and will be proportionately lower on most other flow cytometers).

   The noise is a random representation of the diffusely scattered photons from the laser beam and stream intercept. Because of the high event rate of these low-level signals, on some instruments inclusion of these events is only feasible in a very limited way due to limitations in the baseline restoration algorithms and other signal-processing attributes. Background noise is informative because (i) it serves as a window into the population of EVs that fall under the triggering threshold, (ii) it makes it possible to determine when there is too much free dye in the interrogation point, and (iii) it helps identify when EV samples are being analyzed at too high a concentration and are therefore at risk of coincident detection (Morales-Kastresana et al., 2017; van der Pol, van Gemert, Sturk, Nieuwland, & van Leeuwen, 2012). For these reasons, the authors refer to the noise as "background reference noise."

5. Load a sample containing a mixture of 200 nm yellow-green and red Fluosphere beads at 1×10^7 beads/ml (an $\sim 1 \times 10^6$-fold dilution of original stock) to fine-tune the stream-laser alignment.

   Any combination of beads that are excited by different lasers can be used. The goal is to have two populations whose fluorescence will be collected in different pinholes, in such a way that the stream is aligned according to two pinholes. This ensures correct vertical alignment.

   The dimmer the fluorescent beads used, the better the alignment will be for EVs.

6. Open a dot plot depicting yellow (∼515 nm) and red (∼605 nm) fluorescent channels in each axis (or correspondent fluorescent axis for the chosen beads). Tweak the alignment until the fluorescence signal is optimized for both bead sets. While doing this, try to keep the total event rate, including the noise, at ∼10,000-20,000.

   Alignment will be optimal when the distance between the noise and bead populations is the greatest in terms of fluorescence, while the bead population remains as tight as possible. Also, the event rate should not increase significantly with respect to DPBS alone, as the contribution of the beads to the overall rate is insignificant.

   It is very useful to monitor time versus any parameter (fluorescence and scatter) to determine how the distance between noise and beads varies with alignment adjustments (Fig. 4).

   If the bead populations and the reference noise population appear as "split" populations, there is probably drop-drive noise (especially with the 70-μm nozzle), which can be eliminated by dropping the break-off point by lowering the amplitude or frequency, to the extent possible while maintaining stable Intelli-Sort settings and a stable breakoff point.

7. Once the instrument is aligned, acquire and save a representative sample of the beads used for the alignment.

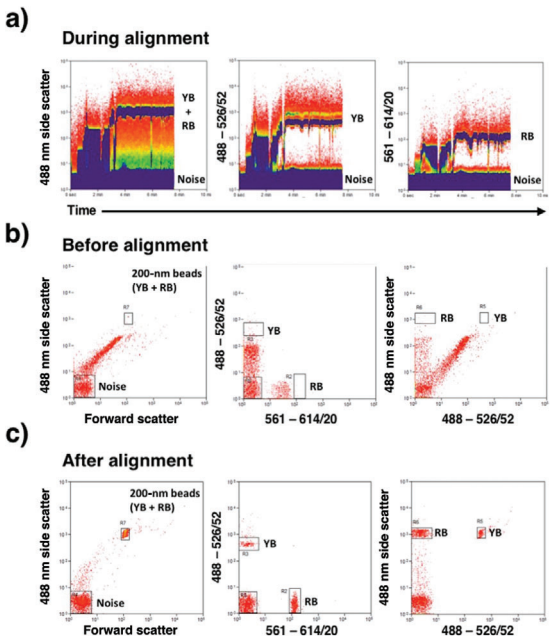

Figure 4 Plots generated before, during, and after alignment. (**A**) Plots with the time parameter on the *x* axis and scatter or fluorescence on the *y* axis during the alignment process to monitor the relative separation between the beads and noise. (**B** and **C**) Scatter and fluorescence parameters before (**B**) and after (**C**) optimization of the alignment. YB, 200-nm yellow fluorescent beads; RB, 200-nm red fluorescent beads.

This file will serve as a reference for future alignment, allowing comparisons of the alignment between experiments.

8. Wash the sample line with DPBS for 5 min at 1 psi.

   As the sample lines clear, monitor the disappearance of the 200-nm beads from the alignment steps over time by referring to the time versus scatter or fluorescence plots. If alignment beads do not disappear completely, increase the pressure up to a differential pressure of 1.4. If beads are still observed, there may be contaminating beads in the sample path (in either the lines, the junction points between fittings, or the nozzle reservoir), and you will need to clear these residual beads before continuing. It can be useful to wash the line with rinse solution followed by DPBS for 5 min each. Once washing is done, change rinse and DPBS tubes for future uses, as these solutions may contain contaminating beads from the sample injection tube.

9. Save an acquisition of 30 s of clean DPBS.

10. Load the EV sample, increase pressure up to 1 psi to accelerate the sample loading, and then decrease the differential pressure to 0.3 psi.

    A 0.3-psi differential pressure is offered here as a reference for what commonly works on Astrios EQ sorters that we have worked with. A differential pressure that permits stable detection of single EVs, avoiding coincidence, should be selected. The authors recommend keeping the differential pressure the same for all samples to be analyzed during an experiment, because event rate can be a valuable source of information.

11. Wait until the event rate is stable (use the time vs. scatter plot). Then stop the acquisition in the software (not the machine) and start acquiring again for 30 s. Save the acquisition.

    The authors recommend saving acquisitions of the same duration, in order to compare event numbers among samples.

12. Wash the sample line with DPBS for 5 min at 1 psi.
13. Run samples, save acquisitions, and wash with DPBS, as described above. *IMPORTANT*: Keep samples at 4°C and in the dark.
14. Wash the sample line consecutively with FACSClean, FACSRinse, and DPBS for 10 min each at 2 psi. Turn off the lasers and fluidics. Rinse the nozzle and place a FACS tube cap containing some clean water under the nozzle tip.

BASIC PROTOCOL 4

COUNTING PARTICLES AND EVs ON ASTRIOS EQ WITH SPIKE IN REFERENCE BEADS

There are different methods to quantify the particle count in a solution. Common counting methods are resistive pulse sensing (RPS) and nanoparticle tracking analysis (NTA). RPS uses the Coulter principle to derive size from the measurable changes in electrical impedance produced by nonconductive particles suspended in an electrolyte. NTA tracks the movement of particles by Brownian motion, allowing their diameter to be calculated using the Stokes-Einstein equation. Concentration can also be calculated based on the number of particles tracked per detection window volume. This technique requires the use of a specific instrument that may not be available to the broad scientific community on a regular basis. Here, we describe an alternative method for counting particles in a solution, in which spherical polystyrene fluorescent beads of known concentration are used as spike-in reference materials. This method, however, depends upon the accuracy of the bead stock concentration.

Materials

200-nm Fluosphere fluorescent beads: e.g., red (Thermo Fisher Scientific, cat. no. F8794) or yellow-green (Thermo Fisher Scientific, cat. no. F8811)
Ca^{2+}-, Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS, Gibco)
EVs in DPBS

Polystyrene FACS tubes
Orthogonal EV particle counter: e.g., resistive-pulse sensing device or nanoparticle tracking analysis device

1. Prepare a stock of 200-nm polystyrene reference beads by diluting 50 µl Fluosphere beads in 50 ml DPBS (1000-fold dilution) and keep this solution as a "Big Stock." Determine the particle concentration of the beads in the "Big Stock" using NTA or any other method.

   Companies provide an estimated concentration of beads in solution, based on the size and weight. This tends to be a rough estimation; we therefore recommend preparing a 10,000× dilution of "Big Stock" beads in DPBS to perform quantification of particles per milliliter. Polystyrene beads are stable in DPBS, with no significant loss of particles or fluorescence over time, and can therefore be quantified once and used over the next couple of years. As a reference, the authors' "Big Stock" of 200 nm yellow-green beads is ∼1.8 × 10^{10} particles/ml, as measured by NTA. It is worth noting that every time an aliquot is taken from the "Big Stock," its concentration is likely to decrease from the previous time. It is therefore recommended that several smaller "Big Stock" aliquots be made to ensure better consistency over long periods of time.

2. Vortex the bead stock solution for 5 s.
3. Prepare a working stock of beads by diluting them 100-fold in DPBS (∼1.8 × 10^8 particles/ml). Keep this working stock for the whole experiment.

4. Prepare EVs in a known volume of DPBS and annotate the dilution, if any

   If EVs are derived from Basic Protocols 1 and 2, expect some EV loss during the protocols and dilute accordingly.

5. Vortex the working stock of beads for 5 s.

6. Spike the beads by diluting them 100-fold in the EV prep. The final concentration of reference beads, if following previous example, will be 1.8×10^6 particles/ml. Mix the sample.

7. Run the EV sample containing the spiked reference beads as described in Basic Protocol 4.

   200-nm polystyrene beads may overlap in scatter with some EVs. The authors therefore use 200-nm yellow-green beads if EVs are stained with PE or APC, or 200-nm red beads if EVs are stained with CFSE or fluorescein isothiocyanate (FITC). This allows easier differentiation of the EV and bead populations and provides a more reliable count.

8. Record the EV and bead event count as shown in Figure 4.

   The detectable concentration is dependent upon the limit of detection of the instrument and on how the gating strategy was defined. In accordance with the MIFlowCyt-EV standardized reporting framework (http://www.evflowcytometry.org/), the parameters being used to gate EVs and the threshold of the instrument should be calibrated into standard units to allow the detectable concentration to be defined in standard units that can be validated (Welsh et al., 2020). See Welsh & Jones (2020) for information on how to calibrate fluorescence and light scatter parameters into standard units.

9. Multiply the EV count by the bead concentration before dividing by the bead count, as shown in Equation 1.

$$\text{detectable EV concentration} = \frac{\text{EV gate count} \times \text{bead concentration}}{\text{bead gate count}}$$

Equation 1

If there is any noise or background source in the EV gate, these background counts must be subtracted to obtain approximate EV counts. A clear example of this background would be any events that are observed when running clean PBS. In that case. use Equation 2:

$$\text{detectable EV concentration} \frac{(\text{EV gate count} - \text{background}) \times \text{bead concentration}}{\text{bead gate count}}$$

Equation 2

COMMENTARY

Background Information

Extracellular vesicles (EVs) mediate intercellular communication both within multicellular organisms and among unicellular organisms (Colombo, Raposo, & Thery, 2014; Kaparakis-Liaskos & Ferrero, 2015; Schwechheimer & Kuehn, 2015; Valadi et al., 2007). EVs are a heterogeneous group of vesicles that include exosomes, microvesicles, apoptotic bodies, outer membrane vesicles, etc. carrying a variety of lipids, proteins, and nucleic acids (Bobrie, Colombo, Krumeich, Raposo, & Thery, 2012; Lai et al., 2016; Raposo & Stoorvogel, 2013; Thery et al., 2001; Yanez-Mo et al., 2015). In humans, EVs have been shown to be key components in multiple physiological processes in health and disease, with their origin, cargo, and destination specific to their derivation (Hoshino et al., 2015; Kaparakis-Liaskos & Ferrero, 2015; Melo et al., 2015; Robbins & Morelli, 2014; Segura et al., 2005; Thery et al., 2001; Thery et al., 2009). The complexity of EV composition is hard to study due to their small size (Colombo et al., 2014), and such studies have traditionally been limited to methods that assess the qualitative characteristics of a bulk EV population (Thery, Amigorena, Raposo, &

Table 1 Troubleshooting Table

Artifact	Source	What to do
False-positive signal	Drop drive noise	Reduce amplitude and/or frequency
	Nonspecific binding of antibodies	Add control EVs that do not express the target antigen
	Antibody/dye aggregates	Add control EVs that do not express the target antigen
		Add control antibody/dye alone
	Background antibody/dye fluorescence	Add control antibody/dye alone
		Noise fluorescence shifting
Low resolution of positive signal over the noise/negative population	Background antibody/dye fluorescence	Wash the excess antibody/dye
	Low antigen density	Use a more sensitive instrument, brighter fluorochrome, and/or brighter staining method
No detection of positive signal	Low antibody affinity	Increase concentration and/or incubation time
	Antibody/fluorochrome conjugate not working properly	Change antibody, clone, fluorochrome, and/or lot
Unexpected increase in event rate	Change in differential pressure of sheath tank and sample line	Time versus scatter parameter to identify these peak
	Stuck material in sample line or nozzle	Flush sample line with detergent and PBS with increased sample pressure differential (boost)
	Contaminating material from previous sample	Run filtered PBS before and after samples, to minimize the presence of contaminating particles
Coincident detection of particles	Sample is too concentrated	Dilute the sample
		Use spike-in beads to ensure an operational concentration range
Nanobubbles at the interrogation point	Nanobubbles arise by hydrodynamic de-cavitation due to depressurization when stream exits nozzle	Consider lower pressure system operation, as with 100 micron instead of 70 micron nozzle

Clayton, 2006). However, due to the heterogeneity presented in these bulk EV preparations, discrete EV subpopulations can easily be missed in bulk analyses.

Critical Parameters

• **Sterility and cleanness.** Using sterile reagents will avoid the growth of any biological contaminant that may also release EVs and prevent the deposition of aerial dust in the buffers. Because of the small size of EVs, it is recommended that a high standard of cleanliness be maintained to avoid debris in samples.

• **Protecting fluorescence.** Fluorochromes and dyes used in this protocol may have different stabilities in solution and resistance to photobleaching. HRFC analysis of small particles is challenging because the detected scatter and fluorescence signals are at the very limit of detection. Therefore, maximizing any fluorescent signal from EVs to negative or background populations is extremely

important. For this reason, any methods that improve the fluorescence stability of labeling is recommended; these include avoiding exposure to light and hydrolysis of CFSE in aqueous solutions (Banks et al., 2013; Bergsdorf et al., 2003; Hoefel et al., 2003; Quah, Warren, & Parish, 2007).

• **Stability of EVs in solution.** EVs are relatively stable in saline solutions when concentrated ($>10^{11}$ EV/ml) but tend to be unstable at lower concentrations ($<10^{9}$ EV/ml; unpublished observations). Therefore, it is recommended that labeled EV preparations either be analyzed on the day of staining or be frozen until the HRFC experiment is performed (if freezing them, prior knowledge of the effects of the freeze-thaw cycle on antibody-antigen binding and fluorochrome stability is required).

• **Controls.** Each antibody lot is unique, and therefore antibody lot represents a source of variability in HRFC experiments. It is widely recognized that many antibodies form aggregates, more so in some specific preparations of antibodies than in others, as well as having variability in their brightness of staining. For that reason, we believe that the best "negative control" for a staining is to test the same antibody on an EV sample that is known to be negative for the antigen being tested.

• **Practical considerations.** These protocols are feasible for yields that are comparable to standard flow sorting yields. Where $>10^{9}$ EVs are required (as for functional assays of interest, therapeutic purposes, or adoptive transfer studies), this method of sorting, with the requisite post-sort concentration steps, is generally impractical. Practically speaking, it should be expected that the product recovered after all steps may be only 10%-20% of the quantity of EVs that were counted as sorted. Thus, this is unlikely to be a suitable method for EV subsets.

• **Sorting fidelity.** To achieve high purity of sorted populations, it is critical to use a "Not" gate that includes the background reference noise on all relevant parameters, in order to reduce contamination of events that are present in the sub-threshold population.

Troubleshooting

A list of the most frequent artifacts observed by the authors is provided in Table 1, along with a brief explanation and the suggested controls to consider, either to identify or to avoid such artifacts.

Time Considerations

Basic Protocol 1: ∼2 hr 20 min
Basic Protocol 2: ∼ 50 min
Basic Protocol 3: ∼40 min
Basic Protocol 4: ∼10 min per sample.

Acknowledgments

A.M.K., J.A.W., and J.C.J. were supported by the Intramural Research Program of the National Institutes of Health (NIH), National Cancer Institute, and Center for Cancer Research. J.C.J. acknowledges NIH ZIA BC011502, NIH ZIA BC011503, NIH U01 HL126497, NIH UG3 TR002881, and the Prostate Cancer Foundation. J.A.W. is a 2019-2023 ISAC Marylou Ingram Scholar.

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Aizea Morales-Kastresana: Conceptualization; data curation; formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Joshua A. Welsh:** Formal analysis; investigation; methodology; software; validation; visualization; writing-review & editing. **Jennifer C. Jones:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing-review & editing.

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Internet Resources

https://doi.org/10.17504/protocols.io.bj6×krfn

Protocol collection: Due to the potential for alterations in the future, up-to-date versions of each protocol will be maintained here.

https://nano.ccr.cancer.gov

Software updates, background information, and learning resources for the software.

http://evflowcytometry.org

ISEV-ISAC-ISTH EV Flow Cytometry Working Group website, with resources related to MIFlowCyt-EV as a reporting framework along with various educational resources and materials.