The analysis of Extracellular Vesicles by Flow Cytometry (RFCC 2020)

Note: The term "extracellular vesicles" is the term endorsed by the International Society of Extracellular Vesicles as the name for particles released from a cell that contain a lipid bilayer and do not contain a functional nucleus [1].

The structure of this protocol is based on an outline recently published in the Journal of Extracellular Vesicles [2]. The table attached at the end of this protocol is the recommended framework for information that should be provided for all publications in which extracellular vesicles are analyzed by flow cytometry. This protocol will primarily address the steps for preparation of EV samples for analysis by flow cytometry after isolation: instrument preparation and setup, measurement of instrument background, determining limits of detection, appropriate controls, and fluorescence calibration. Companies and products mentioned in the protocol are not a promotion by this facility, but rather a suggestion of resources for improving your analysis strategy.

For guidelines addressing the isolation of EVs, the following references offer guidelines:

The method for isolating EVs is dependent upon the source of the EVs and the downstream application.

- Witwer KW et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles. 2013 May 27; 2:20360
- Cossarizza A et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol. 2019 Oct; 49(10): 1457-1793
- Thery C et al. Minimal Information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018 Nov 23; 7(1): 1535750
- Thery C et al. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Current Protoc Cell Biology. 2006 Apr; 3: 3.22

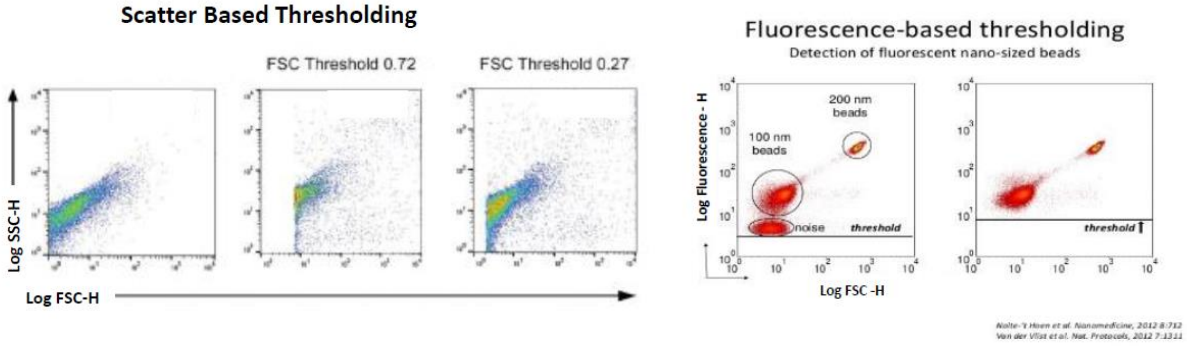
You may visit the International Society of Extracellular Vesicles (ISEV) (https://www.isev.org), the International Society for the Advancement of Cytometry (ISAC) (https://isac-net.org) or the Extracellular Vesicle Flow Cytometry Working Group (www.evflowcytometry.org) for additional resources.

Important information to obtain prior to flow cytometric analysis of EVs:

EV size distribution and concentration should be evaluated prior to analyzing the samples by flow cytometry. Use an independent and accurate method, such as the NanoTracking Analysis (NTA) or the Tunable Resistance Pulse Sensing (TRPS) technologies. Flow cytometers are limited in their ability to detect particles below a certain size, and it is important to understand if you are detecting the majority of EVs in your sample by flow cytometry or just a small subset. You can calculate the concentration of EVs analyzed and compare that number to the concentration of EVs prior to analysis to determine what percentage of EVs you are actually capturing.

Instrument preparation and set up:

1. The cytometer must be very clean in order to detect EVs above background signals. Background signals include electronic noise (events detected when a sample is not running), contaminants in the sheath fluid, and contaminants in the cytometer fluidic system. The sheath fluid should be filtered through a 0.22um filter at the minimum. A lengthy instrument clean may be necessary to achieve acceptable background levels. The flow cytometry core oversees lengthy instrument cleaning. Thorough instrument, SIP and sample line cleaning are vital and involve the use of detergent, bleach, and DI water [3]. Running Coulter Clenz or 5% Contrad for 10 minutes on HIGH, followed by 10% bleach for 10 minutes, and 0.1um filtered DI water for 10 minutes should sufficiently clean the SIP and sample line. If background events are not at an acceptable level (see line 9), speak to the flow core staff about the possibility of scheduling additional instrument cleaning. Carryover from a previously run sample into the next sample should be negligible; however, it may be desirable to run a fresh tube of filtered buffer in between your EV samples, to reduce the possibility of carryover.
2. Display FSC and SSC parameters on a log scale as opposed to a linear scale [3,4].
3. Display FSC, SSC, and fluorescence parameters on a height log scale as opposed to an area log scale [3,4].
4. Establish a trigger channel threshold that best resolves EVs from instrument noise (e.g. electronic noise from the PMT). This will be instrument specific as flow cytometers vary in design and detection sensitivity. 405 SSC is commonly used as trigger, since a shorter wavelength allows for better detection of smaller particles; however, 488 SSC, 561 SSC, 488 FCS (if the cytometer has a FSC PMT) have been used. Since flow cytometry is based on the collection of large amounts of light, whether scatter or fluorescence, the detector chosen should allow for the largest angle of light collection, have the highest sensitivity, and the lowest noise. If you are considering using a fluorescence signal for triggering, you must use something that labels all EVs (typically a membrane dye).



5. Run samples at low pressure (low flow rate) especially if EV concentration is going to be calculated [2,3,4].
6. Record sample acquisition for a set period of time instead of recording a set number of events [2,4]. Using time as the parameter for stopping data collection allows for comparisons between controls and test samples. It also allows for data comparison between laboratories. If your instrument of choice allows for stopping data acquisition on volume, it can be used as another option to compare control and test samples. If you choose to use a volumetric stop, the volumetric

measurement must be validated [5]. Since many instruments do not have volumetric measurements, choosing this option may not allow for interlaboratory comparisons.

7. It may be beneficial to include time as a parameter in the EV analysis and create a plot of time vs SSC or time vs fluorescence. Monitoring the events over time will assist in identifying a problem that occurred during data acquisition. This gate may later be utilized during post acquisition analysis to exclude questionable data due to a possible instrument problem.
8. There is much debate on the correct way to establish an EV population gating strategy. Scatter, fluorescence, or a combination of these may be used. The upper and lower limits of the EV gate must be defined. Historically, beads have been used for the purpose of establishing an EV size gate. Unfortunately, polystyrene beads do not have the same refractive index as EVs, and the size gates established by this method were overestimated. Beads may be utilized for establishing a preliminary gating strategy and later used as a reference for checking the gate. The current strategy under discussion for establishing an EV size gate utilizes one of several MATLAB based software programs in development for calibrating light scatter, and converting the light scatter of beads of known composition and size to EV diameter. This EV diameter may then be used to establish an EV size scatter gate (e.g. FCM_{PASS} or Rosetta Calibration) [6,7]. The calculations created by the programs are approximations, as they make certain assumptions regarding EV membrane thickness, uniformity of EV spherical shape, and uniformity of EV refractive index. The gates must be verified with biological references. The recently established company, Cellarcus Biosciences, advertises calibration standards more appropriate for EVs and fluorescent liposomes of varying sizes to help establish and verify EV gating strategies. Additionally, fluorescently labeled retroviruses (ViroFlow Technologies) have been proposed as controls for assisting in EV identification and enumeration; however, they cannot be used for establishing EV size [3].
9. Establish an acceptable background level using 0.22um filtered (preferably 0.1um filtered) PBS or DI water. This background level should be established at the same flow rate the samples will be run. A low flow rate is recommended [4]. Background levels will vary by instrument and detector chosen for triggering. Acceptable background levels should be expressed as events per second (e.g. 5 events per second [4]). For example, if triggering on fluorescence background should be <10 events/sec, if triggering off scatter <100 events/sec (Joanne Lannigan, personal communication).
10. Fluorescence calibration: Instrument fluorescence calibration helps determine the instrument's fluorescence sensitivity and converts data (median fluorescence intensity) to standard measurement units such as molecules of equivalent soluble fluorochrome (MESF) or equivalent number of reference fluorophores (ERF) [8]. MESF calculation of unstained EVs is the best way to determine positive and negative cutoffs for fluorescence. Once you know the MESF or ERF of the unlabeled EVs, you can than describe the positive population as having a fluorescence value greater than that of the negative population, plus or minus one or two standard deviations. Fluorescence calibration allows for comparison between laboratories and methodologies, and establishes a uniform standard of measurement for reporting results. MESF beads are commercially available surface dyed microspheres. Unfortunately, MESF beads are not available for all fluorophores. Commercially available alternatives are microspheres coated with increasing amounts of antibody. Each coated population binds a specific number of monoclonal antibodies of the noted species, which is equal to its Antibody Binding Capacity (ABC) value. If you are using a membrane dye (which will not bind to an ABC bead), hard dyed ERF beads can be used. It should be noted the ERF beads are less sensitive as they have a broad emission spectrum and do not

excite or emit the same as the fluorophore used to stain cells [8]. The manufacturers will provide a table or calibration program for converting median fluorescence into MESF or ERF. The beads are much larger than EVs, have a higher antibody binding capacity than EVs (and therefore the fluorescence may be much brighter than EVs) and have a different refractive index. They may not provide an accurate lower limit of fluorescence detection. Cellarcus Biosciences advertises nanoscale fluorescence calibration standards that should improve detector calibration for weaker fluorescence signals, i.e. the lower limit of fluorescence detection.

Reagent preparation:

As the limit of detection on most standard flow cytometers is 200nm (0.2um), all buffers should be filtered through a 0.22um filter, preferably a 0.1um filter. As previously stated, the sheath fluid should be filtered through a 0.22um filter at the minimum.

Antibody aggregates may mimic EV signals, so it is recommended that antibodies be ultra-centrifuged or filtered before using [4]. Centrifugation of EVs is very difficult (and may introduce unintended artifacts), so samples are diluted after antibody labeling instead of washing them. Antibody titration is important. High antibody concentrations could increase signals that mimic EVs, so it is important to look at background signals at the various antibody concentrations. Maximum fluorescence detection should be calculated for each fluorophore by calculating the staining index for each antibody at different detector voltages [3,9]. Specially engineered retroviruses (engineered with antigens commonly found on EVs) are being investigated for possible use as positive controls or for antibody titration [3].

Assay controls:

You will need to bring these controls with you every time you analyze EV samples. The rationale behind these controls is to determine whether the detected events are actually EVs rather than antibody aggregates, buffer contaminates, etc. Reference [2] goes into greater detail behind the rationale for these controls. These controls should be run at the same instrument settings at which you will be running your EV samples (same flow rate, same data acquisition stop, etc).

1. Buffer only.
2. Buffer plus reagents (e.g. antibodies); same concentration of reagents used with EVs.
3. Unstained control.
4. Isotype controls.
   This control may assess non-specific binging to Fc receptors. Some protocols do not recommend the use of Fc block as it may introduce increased background signals. If you use an Fc block, remember to bring a control of buffer plus Fc block to assess background levels.
5. Single stained controls.
6. Procedural controls.
   These controls assess the possible introduction of artifacts that could be mistaken for EVs. For example, processing a portion of the buffer only control in the same manner EV samples are processed. If you centrifuge your EV samples after antibody staining, the buffer control and the buffer plus reagent controls should be centrifuged at the same speed and for the same length of time as your EV samples.

7. Serial dilutions.
   The concentration of EVs in your sample should be established. Serial dilutions of your EV samples will establish whether the EV signals are single particles or multiple particles, termed the swarm effect [10]. The fluorescence intensity of the particles should remain the same while only the frequency of the events should decrease.
8. Detergent treated EV samples.
   Intentional detergent lysis (e.g. 0.05% TritonX-100 [3] or 0.5% NP-40 [4]) will establish the lability of the EVs. EV membranes will be disrupted by the detergent while artifacts such as antibody aggregates will not. The detergent and concentration may differ for EVs depending on their source (purified EVs from a cell culture or EVs in plasma) and will depend on EV concentration.
9. Reference standards.
   Beads may be used as references to check instrument settings. For example, you may use a 400nm bead as a reference [7] for light scatter to determine if the voltages need to be adjusted to keep your EVs in your light scatter gate (if you use one). Commercially available fluorescently engineered retroviruses or fluorescently labeled liposomes that may be used as reference standards.
10. Fluorescence calibration standards.
    MESF, ABC, or ERF beads.
11. FMO (Fluorescence Minus One) controls.
    This control may help determine if FRET or quenching is occurring from the use of multiple fluorescent antibodies.

Additional extracellular vesicle characterization:

Additional characterization of the EV population may be requested by a reviewer. Information requested may include: EV diameter, refractive index, concentration, brightness, and epitope number.

Approximate EV diameter and refractive index may be calculated using of light scatter calibration and Mie theory [11]. These protocols require extensive knowledge of the flow cell design, instrument light collection angles, etc. There are a few MATLAB based software programs in development for calibrating light scatter and converting the light scatter of beads to EV diameter and EV refractive index. FCM_{PASS} and Rosetta Calibration are two of the programs available [6,7]. The programs require the analysis of different beads of known composition and size, and uses scatter measurements to calculate scatter cross sectional area and calculate a scatter ratio. The programs then use the information to create plots from which EV diameter and refractive index may be extrapolated. As stated earlier, the calculations created by the programs are approximations. In order to make the calculations possible, the programs makes certain assumptions regarding flow cell design and angles of light collected, as well as certain assumptions regarding EV membrane thickness, uniformity of EV spherical shape, and uniformity of EV refractive index. Beads must be analyzed on the flow cytometer before analyzing the EVs. The information from the beads may then be used to set up an EV scatter gate for analysis, or, if not using a scatter gate, the scatter information may be collected and used post analysis to calculate EV diameter and refractive index.

It is important to report detected EVs as a concentration rather than percentage [2,7,9]. Concentration of the EVs analyzed may be assessed with the aid of counting beads or, if available in the instrument software, volumetric measurement. Counting beads are commercially available from several

manufacturers (BD, Beckman Coulter, Bangs Labs, Sperotech). The manufacturer will provide you with the bead count per volume to be used in the calculation of sample concentration. Some instruments have a volume stop as opposed to a time stop. If that is not the case for your instrument of choice, the volume may be calculated based on sample weight before and after analysis, or volume measurement before and after analysis [5,7]. In order to perform these calculations, the sample flow rate must be known. Volume measurements are difficult on instruments like the LSR and Fortessa due to the fact that these instruments are continually aspirating sample (even when loading the sample tube) so some of the sample volume is lost prior to data collection, so the use of counting beads is the better choice.

Epitope estimation on the EV population may be requested. The use of Antibody Binding Capacity beads may be helpful. Unfortunately, ABC beads are much larger than EVs, and may not be accurate for low numbers of epitopes. Cellarcus Biosciences has recently introduced a line of nanosized ABC beads that may prove helpful.

All .fcs files should be shared through a data repository. Web resources include: https://flowrepository.org/, https://www.cytobank.org/, and http://evtrack.org/. FlowRepository.org is a public flow cytometry data repository for reviewing data sets referenced in published literature [12]. It provides a score based on MIFlowCyt recommendations to show the degree of MIFlowCyt compliance, and tracks provided, partially provided, or missing data. Cytobank.org is a commercial site for data analysis and a data repository. EV-TRACK is a data repository specifically for EV data [13]. It provides a metric, based on MISEV guidelines, to assess whether or not the information provided is sufficient for interpreting and reproducing the experiment.

References:

[1] Clotilde Thery et al. Minimal Information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018 Nov 23; 7(1): 1535750

[2] Joshua A. Welsh et al. MIFlowCyt-EV; a framework for standardized reporting of extracellular vesicle flow cytometry experiments. J Extracell Vesicles. 2020 Feb3; 9(1): 1713526

[3] Vera A. Tang et al. Single-Particle Discrimination of Retroviruses from Extracellular Vesicles by Nanoscale Flow Cytometry. Scientific Reports. 2017 Dec 19; 7(1): 17769

[4] Heather C. Inglis et al. Techniques to Improve Detection and Analysis of Extracellular Vesicles Using Flow Cytometry. Cytometry Part A. 2015 Nov; 87(11): 1052-1063

[5] Lili Wang et al. Measurement of Microsphere Concentration Using a Flow Cytometer with Volumetric Sample Delivery. J Res Natl Inst Stand Technol. 2014 Dec; 119: 629-643

[6] Joshua A. Welsh et al. FCM_{PASS} software aids extracellular vesicle light scatter standardization. Cytometry Part A. 2020 Jun; 97(6): 569-581

[7] Edwin van der Pol et al. Standardization of extracellular vesicle measurements by flow cytometry through vesicle diameter. J Thromb Haemost. 2018 Jun; 16(6): 1236-1245

[8] Lili Wang and Robert Hoffman. Standardization, calibration, and control in flow cytometry. Curr Protoc Cytom. 2017 Jan 5; 79:1.3.1-1.3.27

[9] Andrea Cossarizza et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol. 2019 Oct; 49(10): 1457-1793

[10] Edwin van der Pol, et al. Single vs swarm detection of microparticles and exosomes by flow cytometry. J Thromb Haemost. 2012 May; 10(5): 919-930

[11] Leonie de Rond et al. Deriving extracellular vesicle size from scatter intensities measured by flow cytometry. Curr Protoc Cytom. 2018 Oct; 86(1): e43

[12] Josef Spidlen et al. Preparing a Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) Compliant Manuscript Using the International Society for Advancement of Cytometry (ISAC) FCS File Repository (FlowRepository.org). Curr Protoc Cytom. 2012 Jul; 10: 10.18.1-10.18.26

[13] Jan Van Deun et al. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. Nature Methods. 2014 Feb 28; 14(3): 228-232

Guidelines:

Framework Criteria	What to report	Completed
1.1 Preanalytical variables conforming to MISEV guidelines	Preanalytical variables relating to EV sample including source, collection, isolation, storage, and any others relevant and available in the performed study.	
1.2 Experimental design according to MIFlowCyt guidelines	EV-FC manuscripts should provide a brief description of the experimental aim, keywords, and variables for the performed FC experiment(s) using MIFlowCyt checklist criteria: 1.1, 1.2, and 1.3, respectively.	
2.1 Sample staining details	State any steps relating to the staining of samples. Along with the method used for staining, provide relevant reagent descriptions as listed in MIFlowCyt guidelines (Section 2.4 Fluorescence Reagent(s) Descriptions).	
2.2 Sample washing details	State any steps relating to the washing of samples.	
2.3 Sample dilution details	All methods and steps relating to sample dilution.	
3.1 Buffer alone controls	State whether a buffer-only control was analyzed at the same settings and during the same experiment as the samples of interest. If utilized it is recommended that all samples be recorded for a consistent set period of time e.g. 5 minutes, rather than stopping analysis at a set recorded event count e.g. 100,000 events. This allows comparisons of total particle counts between controls and samples.	
3.2 Buffer with reagent controls	State whether a buffer with reagent control was analyzed at the same settings, same concentrations, and during the same experiment as the samples of interest. If used state what the results were.	
3.3 Unstained controls	State whether unstained control samples were analyzed at the same settings and during the same experiment as stained samples. If used, state what the results were, preferably in standard units.	
3.4 Isotype controls	The use of isotype controls is applicable to immunofluorescence labelling only. State whether isotype controls were analyzed at the same settings and during the same experiment as stained samples. If utilized, state which antibody they are matched to, the concentration used, and what the results were (Section 4.2, 4.3, 4.4). Due to conjugation differences between manufacturers if should be stated if the isotype controls are from the same manufacturer as the matched antibodies.	
3.5 Single-stained controls	State whether single-stained controls were included. If used state whether the single-stained controls were recorded using the same settings, dilutions, and during the same experiment as stained samples and state what the results were, preferably in standard units (Section 4.2, 4.3, 4.4).	

3.6 Procedural controls	State whether procedural controls were included. If used, state the procedure and if the procedural controls were acquired at the same settings and during the same experiment as stained samples.	
3.7 Serial dilutions	State whether serial dilutions were performed on samples and note the dilution range and manner of testing. The fluorescence and/or scatter signal intensity would ideally be reported in standard units (see Section 4.3, 4.4) but arbitrary units can also be used. This data is best reported by plotting the recorded number events/concentration over a set period of time at different sample dilution. The median fluorescence intensity at each of the dilutions should also ideally be plotted on the same or a separate plot.	
3.8. Detergent treated EV-samples	State whether samples were detergent treated to assess lability. If utilized, state what detergent was used, the end concentration of the detergent, and what the results were of the lysis.	
4.1 Trigger Channel(s) and Threshold(s)	The trigger channel(s) and threshold(s) used for event detection. Preferably, the fluorescence calibration (Section 4.3) and/or scatter calibration (Section 4.4) should be used in order to report the trigger channel(s) and threshold(s) in standardized units.	
4.2 Flow Rate / Volumetric quantification	State if the flow rate was quantified/validated and if so, report the result and how they were obtained.	
4.3 Fluorescence Calibration	State whether fluorescence calibration was implemented, and if so, report the materials and methods used, catalogue numbers, lot numbers, and supplied reference units for the standards. Fluorescence parameters may be reported in standardized units of MESF, ERF, or ABC beads. The type of regression used, and the resulting scatter plot of arbitrary data vs standard data for the reference particles should be supplied.	
4.4 Light Scatter Calibration	State whether and how light scatter calibration was implemented. Light scatter parameters may be reported in standardized units of nm2, along with information required to reproduce the model.	
5.1 EV diameter/surface area/volume approximation	State whether and how EV diameter, surface area, and/or volume has been calculated using FC measurements.	
5.2 EV refractive index approximation	State whether the EV refractive index has been approximated and how this was done.	
5.3 EV epitope number approximation	State whether EV epitope number has been approximated, and if so, how it was approximated.	
6.1 Completion of MIFlowCyt checklist	Complete MIFlowCyt checklist criteria 1 to 4 using the MIFlowCyt guidelines.	
6.2 Calibrated channel detection range	If fluorescence or scatter calibration has been carried out, authors should state whether the upper and lower limits of a calibrated detection channel were calculated in standardized units. This can be done by converting the arbitrary unit scale to a calibrated scaled, as discussed in Section 4.3 and 4.4, and providing the highest unit on this scale and the lowest detectable unit above the unstained population. The lowest unit at which a population is deemed 'positive' can be determined a variety of ways, including reporting the 99th percentile measurement unit of the unstained population for fluorescence. The chosen method for determining at what unit an event was deemed positive should be clearly outlined.	
6.3 EV number/concentration	State whether EV number/concentration has been reported. If calculated, it is preferable to report EV number/concentration in a standardized manner, stating the number/concentration between a set detection range.	
6.4 EV brightness	When applicable, state the method by which the brightness of EVs is reported in standardized units of scatter and/or fluorescence.	
7.1. Sharing of data to a public repository	Provide a link to the experimental data in a public data repository.	