EV Analysis using Flow Cytometry

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Most flow cytometers were developed for analyzing cells. Since extracellular vesicles (EVs) are smaller than cells and since no method will isolate EVs only, this document contains important guidelines for analyzing EVs using flow cytometry.

Before you start your experiment, we highly recommend that you read reference 1-3.

- It is extremely important to be consistent in all steps of your procedure between samples.
- Use 0.1 µm filtered buffer and sheath (e.g. Millipore 0.1 µm vacuum filtration system).
- Include reference material e.g. standardized beads (4,5), liposomes or virus particles (6).
- Spin down antibody aggregates before you add antibody to your samples (10 min. at 17.000 x g / 5 min. at 20.000 x g, 4°C if possible)
- Include the following controls (all controls should be handled exactly like the samples)
    - Buffer alone
    - Buffer + antibody - one for each antibody included in the experiment
    - Unstained EV control
    - Single stained EV controls
    - Procedural controls - a control sample for each step in your protocol after staining to make sure the procedure does not introduce artifacts
    - Serial dilution – to confirm single EV detection - your MFI should be stable and the count rate should decrease with decreasing concentration (Figure 1).
      If MFI decreases, you do not have single events!
    - Detergent treatment - to be able to differentiate between membrane-enclosed vesicles (which will be disrupted by detergent) and protein complexes. Small vesicles are harder to dissolve and a harder detergent may be needed (7).

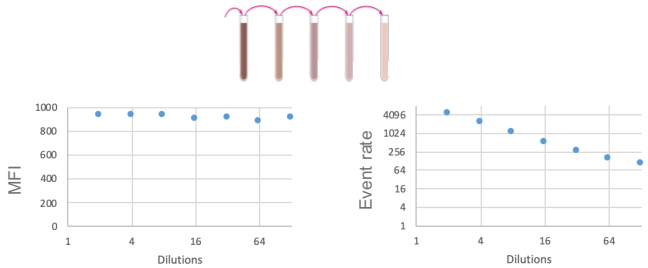

Figure 1: Make serial dilutions of your sample and determine MFI for your bacteria population. I you are detecting single events, your MFI will remain stable through your dilutions, and the eventrate will decrease. *Data from Denis Selnihhin & Anja Bille Bohn*

- Run your samples at the lowest possible speed, perform a flow rate calibration (using counting beads or by weighing) and collect the same volume for all of your samples. Report the flowrate (µl/ml) in your paper.
- Trigger on fluorescence (if not possible, trigger on SSC-H).

- In FSC vs SSC plots; use height (not area) and use a logarithmic scale.
- Use light scatter calibration using e.g. FCM pass (8).
- Use fluorescence calibration – Molecules of Equivalent Soluble Fluorochrome (MESF) using e.g. FCM pass (8) or FlowJo (9).
- Define positive and negative gate in terms of MESF (e.g. APC intensity between 5.0 x 10^2 and 11.7 x 10^3 MESF)
- The flow cytometer may not be able to detect all EVs in your sample. Therefore, report your result as e.g. 4.2 x 10^8 $CD61^+$ EVs/ml with MESF value above 5.0 x 10^2.

References

1. MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments, *Journal of Extracellular Vesicles*, 2020, 9:1, 1713526, DOI: 10.1080/20013078.2020.1713526
2. 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, *Journal of Extracellular Vesicles*, 2018, 7:1, 1535750, DOI: 10.1080/20013078.2018.1535750
3. Analysis of individual Extracellular Vesicles by flow Cytometry, J P Nolan and E Duggan, *Flow Cytometry Protocols, Methods in molecular biology vol 1678, DOI 10.1007/987-1-4939-7346-0_5*
4. MESF beads, Bangs Laboratories https://www.bangslabs.com/products/flow-cytometry/fluorescence-quantitation
5. vCal antibody capture nanobeads or vCal MESF calibrator nanobeads, Cellarcus Biosciences https://www.cellarcus.com/product-category/standards/
6. Engineered Retroviruses as Fluorescent Biological Reference Particles for Small Particle Flow Cytometry, V Tang et al. bioRxviv, 2019 doi.org/10.1101/614461
7. Differential detergent sensitivity of extracellular vesicle subpopulations, Xabier Osteikoetxea et al., Org. Biomol. Chem., 2015, **13**, 9775-9782
8. FCMPASS Software Aids Extracellular Vesicle Light Scatter Standardization, Joshua Welsh et al., *Cytometry part A*, 2019 DOI: 10.1002/cyto.a.23782
9. FlowJo software

You can follow guideline developments from the International Society for Extracellular Vesicles (ISEV), International Society for Advancement of Cytometry (ISAC) and the International Society on Thrombosis and Haemostasis (ISTH) here:
http://www.evflowcytometry.org/