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🌐 FCMPASS - Acquisition and gating of fluorescence reference materials

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We use this protocol and it's working

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This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization. By using the FCMPASS software you agree to the following terms and conditions.

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Abstract

This protocol outlines the steps required to acquire fluorescence reference material data for use with the FCMPASS software. This is one of a number of protocols in the pipeline for performing small particle calibration using the fcmpass software package.

Materials

MATERIALS

⊗ Flow Cytometer

⊗ Vortex

⊗ Rainbow calibration beads **SpheroTech Catalog** #RCP-30-5A

⊗ DPBS **Invitrogen - Thermo Fisher Catalog** #14190

⊗ Falcon® 5 mL Round Bottom Polystyrene Test Tube with Snap Cap Sterile Individually Wrapped **Corning Catalog** #352003

⊗ Fluorescence Calibration Reference Material

MESF Bead Calibration

1   Vortex each fluorescence reference bead bottle before use.

2   Add 1 drop (~50 µL) of each bead population to separate FACS tubes containing 250 µL of DPBS.

2.1   Due to the high autofluorescence of the 'Blank' beads, their use is not recommended to use as 0 MESF.

2.2   Many commercially available fluorescence calibration beads are bright and will require extrapolation instead of interpolation to obtain the dim fluorescence values. The accuracy of the extrapolation will therefore be influenced by a number of factors including the gating of the populations. While less ergonomic, it is preferable to analyze 1 bead population at a time. This allows for gating on scatter parameters, rather than fluorescence parameters, making the statistics less biased by the gating strategy. Analyzing one bead population at a time will also minimize the subjectivity when gating fluorescence populations that overlap, sometimes causing small peaks.
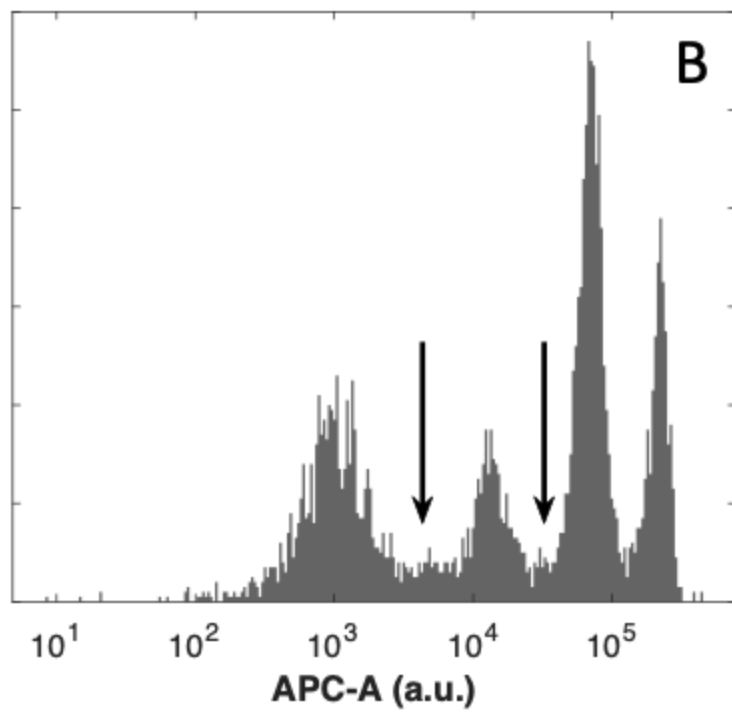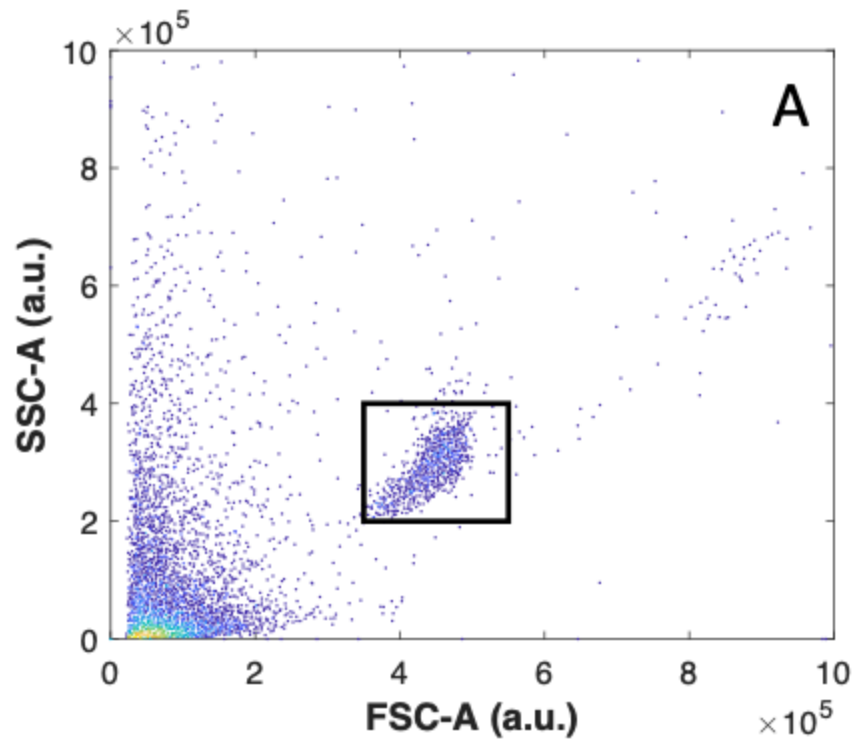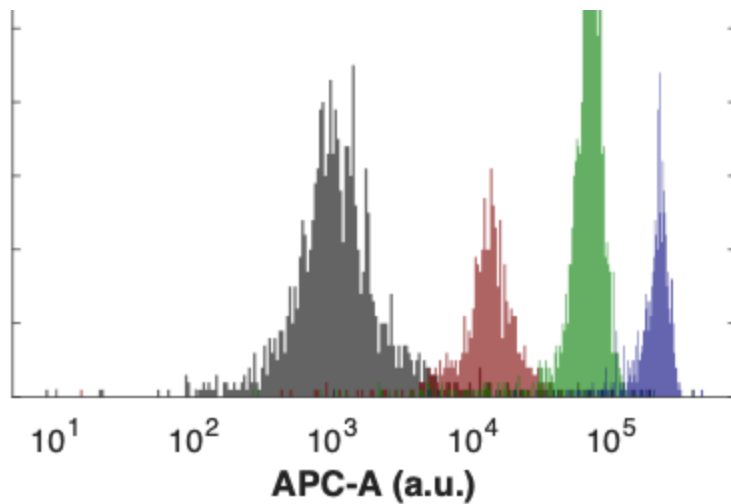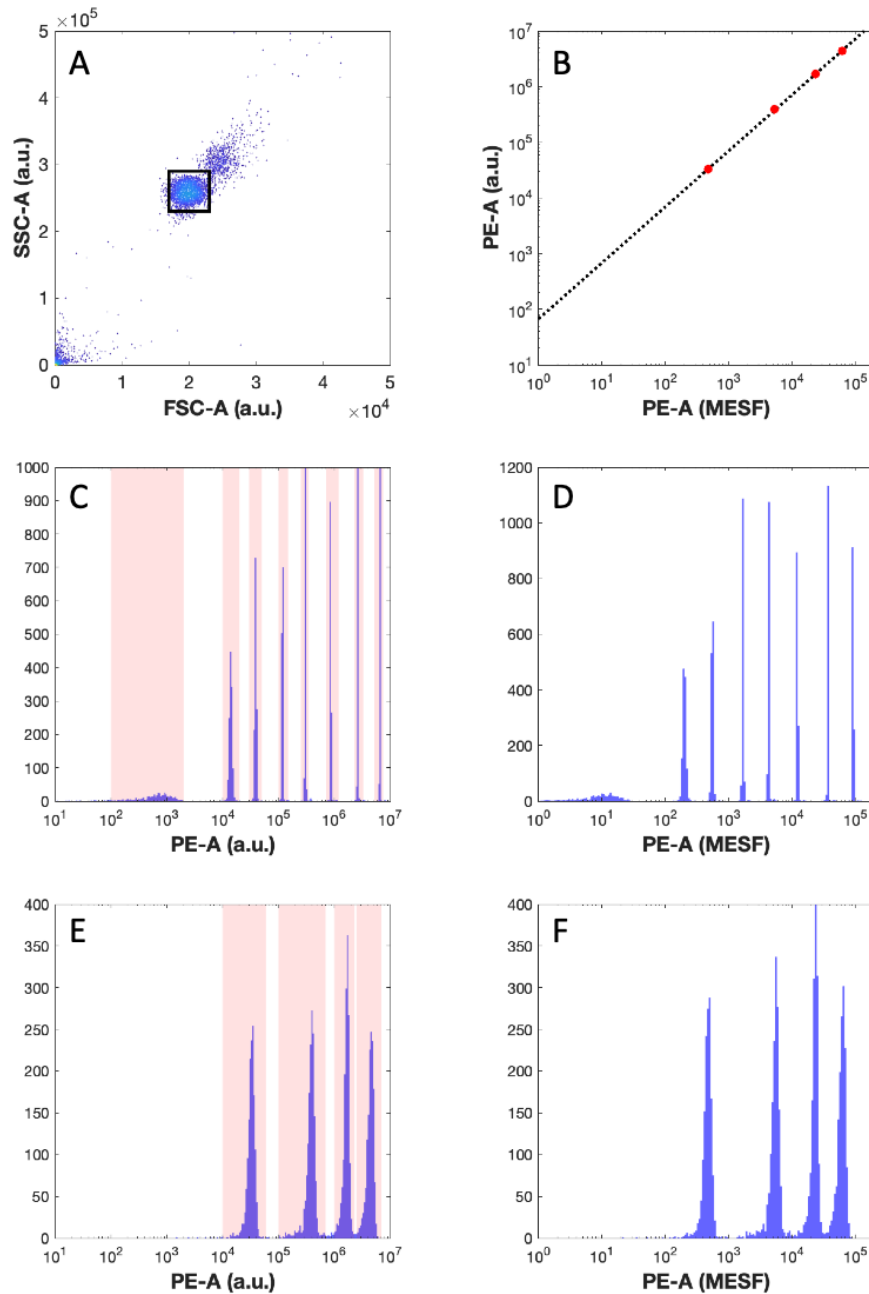
Gating fluorescence reference beads.*A) Gating of bead population using FSC-A and SSC-A. B) Histogram of all four APC MESF bead population in a cumulative distribution. Arrows highlight areas of overlap between beads that may lead to subjectivity on where to manually draw gates. C) Histogram of individual APC MESF bead populations.*

3   Ensure cytometer fluorescence settings are those used for small particle analysis.

4   If the beads are >1 µm in diameter the use of a forward-scatter trigger threshold will likely yield optimal detection and reduced background.

5   Analyze each bead sample at the same acquisition settings until >5000 bead events are recorded.

6   Gate each bead population on FSC-A vs. SSC-A and obtain the median area statistic for the fluorescence parameter being calibrated to move on to the 'FCMPASS Fluorescence Calibration' protocol.

6.1   By default, flow cytometers trigger the acquisition of an event using the pulse height parameter. In cases where a trigger threshold is being defined e.g. SSC. It is recommended that the pulse-height is used so that the limit of detection can be defined in calibrated units. There is no consensus within the small particle community over the use of pulse height vs. area. We recommend that, in general, if the parameter being calibrated was not used as a trigger channel the pulse area statistic should be used due to it tending to be linear at low signal intensities and therefore a more reliable method for extrapolation.

Multipeak Rainbow Bead Cross-Calibration

7   Vortex the rainbow multi-peak reference bead and MESF bead bottles before use.

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8   Add 1 drop (~50 μL) of each MESF bead population to separate FACS tubes containing 250 μL of DPBS.

9   Add 1 drop (~50 μL) of the 8-peak bead population to separate FACS tubes containing 250 μL of DPBS.

10   Ensure cytometer fluorescence settings are those used for small particle analysis

11   If the beads are >1 μm in diameter the use of a forward-scatter trigger threshold will likely yield optimal detection and reduced background.

12   Analyze each bead sample at the same acquisition settings until >2000 bead events are recorded. For the 8-peak beads this will be >16,000 events.

13   Gate each MESF bead population on FSC-A vs. SSC-A and obtain the median statistic for the parameter and perform calibration of the MESF and 8-peak bead files.

14   Once the 8-peak rainbow beads are calibrated in PE MESF units, gate the population on FSC-A vs. SSC-A to obtain singlets. Using the singlet population gate each of the 8-peak populations.

14.1   The gating the individual fluorescent bead populations can be done in the parameter which best separates each population. This may be a different fluorescence detector than the calibrated parameter. While the gating of each population does not have to be on the MESF parameter itself, the MESF parameter should be checked to ensure all populations are on scale. In some 3rd party software the scale limits (minimum and maximum value) will influence the outputted statistic due to how the data is binned.

15   Once each of the 8-peak populations has been gated, obtain the median MESF value for each of the populations. These values are now the cross-calibrated values for these beads and can be used on the same instrument at different gains.

Gating and cross-calibration of fluorescence reference beads.*A) Gating of 8-peak bead population using FSC-A and SSC-A. B) Regression of PE MESF bead reference values vs. acquired arbitrary statistics for each population. C) Histogram of gated (Panel A) 8-peak reference beads and gating of each population (red). D) Histogram of gated (Panel A) 8-peak reference beads converted to PE MESF units using regression (Panel B). E) Histogram of gated PE MESF beads and gating of each population (red). F) Histogram of gated PE MESF beads converted to PE MESF units using regression (Panel B).*

15.1   If an instrument is re-aligned or filters are changed these values will no longer be valid and will require cross-calibration to be performed again. In general, it is good practice to regularly cross-calibrate 8-peak bead reference values, e.g. once a month.