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Resource 2: Fluorescence Detector Setting Incrementation for FCMPASS

Forked from Flow Cytometer Fluorescence Voltration for FCMPASS

In 1 collection

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DISCLAIMER

This protocol summarizes key steps for a specific type of method, which is one of a collection of methods and 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.

ABSTRACT

Flow cytometry (FCM) is a common extracellular particles (EPs), including viruses and extracellular vesicles (EVs), characterization method. Frameworks such as MIFlowCyt-EV exist to provide reporting guidelines for metadata, controls, and data reporting. However, tools to optimize FCM for EP analysis in a systematic and quantitative way are lacking. Here, we demonstrate a cohesive set of methods and software tools that optimize FCM settings and facilitate cross-platform comparisons for EP studies. We introduce an automated small particle optimization (SPOT) pipeline to optimize FCM fluorescence and light scatter detector settings for EP analysis and leverage quantitative FCM (qFCM) as a tool to further enable FCM optimization of fluorophore panel selection, laser power, pulse statistics, and window extensions. Finally, we demonstrate the value of qFCM to facilitate standardized cross-platform comparisons, irrespective of instrument configuration, settings, and sensitivity in a cross-platform standardization study utilizing a commercially available EV reference material.

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MATERIALS

QbSure **Cytek Catalog #B7-10005**, referred to as 'QbSure'

DPBS **Thermo Fisher Scientific Catalog #14190144**, referred to as 'DPBS'

5 mL Round-bottom tube **Corning Catalog #352052**, referred to as 'FACS tube'

FITC-5 MESF Beads **Bangs Laboratories Catalog #555B**, referred to as 'FITC MESF beads'

Sample preparation

10s

1 Vortex QbSure bottle on a high setting for 00:00:05. 5s

> **Note**
>
> This protocol in principle is compatible with all multi-peak rainbow beads. Validation has only been tested on QbSure and Spheretech 8-peak beads. QbSure beads have been highlighted due to having consistently cleaner negative populations in our testing.

2 Pipette 500 µL of DPBS to two FACS tubes. Label one tube 'DPBS" and the second tube 'Beads'.

> **Note**
>
> An observation from our protocol development is that it is important not to use a low protein binding tube for this step, as it can result in excess unbound fluor from the beads creating background noise increases leading to excessive event rate.

3 Add 3 drops of QbSure beads to the 'Beads' tube and vortex for 00:00:05. 5s

Cytometer Setup

4 Ensure cytometer is clean and that -Height and -Area statistics are set to be collected on all parameters and that all parameters are on.

5 On the Cytek Aurora, set window extension to 0. On the CytoFLEX platform turn on 'High Acquisition Mode'.

Note

To set the window extension to 0 on the Cytek Aurora, navigate to the 'Lasers' tab under the sample acquisition settings and set the window extension to 0.

To change the acquisition mode on the CytoFLEX S, click on the 'Advanced' menu on the top-of-the-screen ribbon. Next click on 'Event Rate Settings' menu, change the acquisition mode to 'High', and press 'Ok'.

6 Create a pseudocolor plot with FSC-H on the X-Axis and (488 nm) B-SSC-H on the Y-Axis and make sure both parameters are being plotted on a linear-scale.

7 Create a histogram plot with (405 nm) V-SSC-H on the X-Axis and make sure it is plotted on a log-scale.

8 Set the cytometer triggering threshold to (405 nm) V-SSC-H. **All samples should be acquired with the lowest flow rate, typically ~10-15 µL min^{-1}.**

Note

Cytometer Voltage/Gain and threshold settings are subjective due to their dependency on alignment, and the scatter filters in place, amongst other variables. The following are **guide** values to start with and may need adjustment for optimal acquisition.

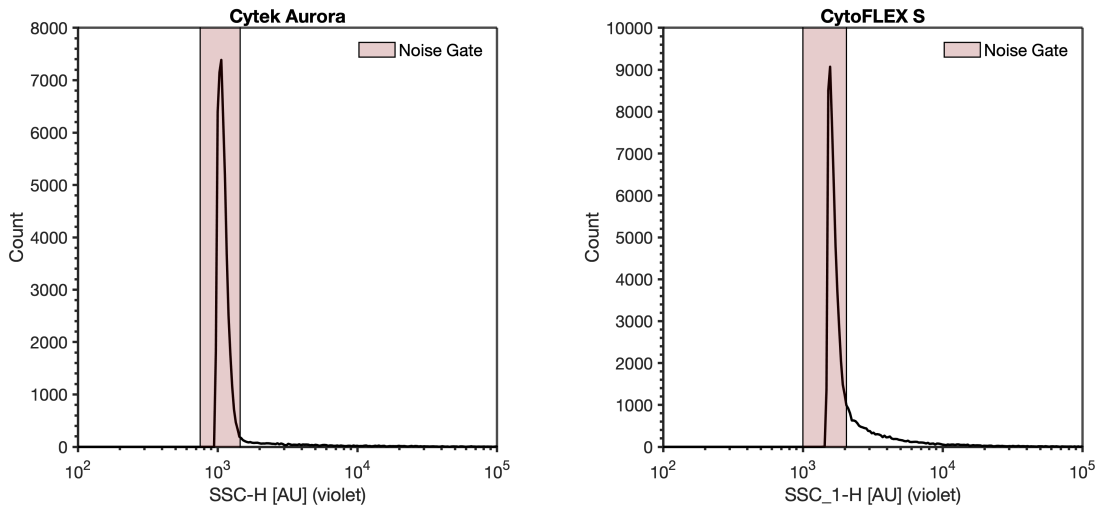
Beckman Coulter, CytoFLEX [405 nm OD0 filter, 488 nm OD2 filter]

- Threshold V-SSC-H = 1000;
- V-SSC Gain = 200
- FSC Gain = 100
- B-SSC Gain = 100

Cytek Bioscience, Aurora [405 nm OD0 filter, 488 nm OD2 filter]

- Threshold V-SSC-H = 1000;
- V-SSC Gain = 2500
- FSC Gain = 150
- B-SSC Gain = 40

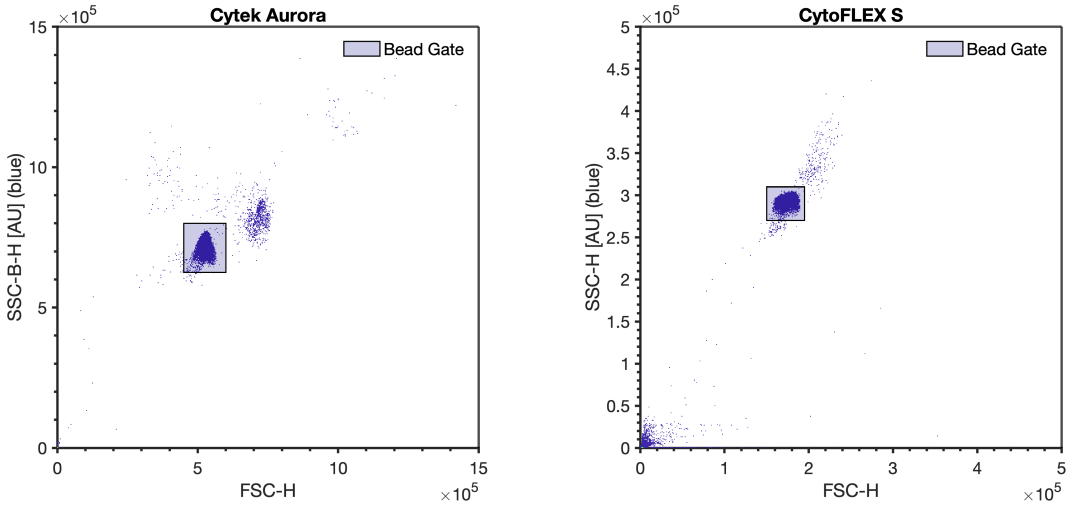
9 Acquire the DPBS tube while viewing the histogram plot from go to step #7. Adjust the detector gain or trigger threshold until the instrument noise is being **acquired at ~1000 events/sec.** The instrument noise floor is distinct from detected background events in sheath as it has a sharp increase. In a system with debris there may be a tail that elongates out of this this sharp peak.



Example of threshold on the instrument noise floor on the Cytek Aurora and CytoFLEX S. Cytek Aurora triggered on SSC-H channel at gain 2500 and threshold 975. CytoFLEX S triggered on SSC_1-H channel at gain 250 and threshold 1200.

9.1 Recording this noise is not necessary as this step is identifying optimal settings.

10 Acquire the 'Beads' tube from go to step #3 . Using the plot from go to step #6 adjust the FSC and B-SSC gain until the single bead population is clearly visible and can be easily gated from the doublet population to the top right of it. Use the Violet SSC trigger settings identified in go to step #9



Example of clearly resolved singlet QbSure bead population on the Cytek Aurora and CytoFLEX S. Cytek Aurora FSC and SSC-B gains set to 150 and 40 respectively. CytoFLEX S FSC and SSC gains both set to 100.

11 Creating a gate around the single bead population named 'Bead Gate';

12 Adjust the stopping criteria of the instrument to record until 10,000 events are acquired on 'Bead Gate' drawn in go to step #11 .

Performing Voltration

13 Voltration can now be performed by recording the 'Beads' tube at multiple fluorescent detector gains, leaving the trigger threshold and light scatter gains consistent. It is recommended that a recording of at least 10 fluorescent detector settings is taken. Including more increments within a voltration will result in being more confident of the subsequent optimal detector settings.

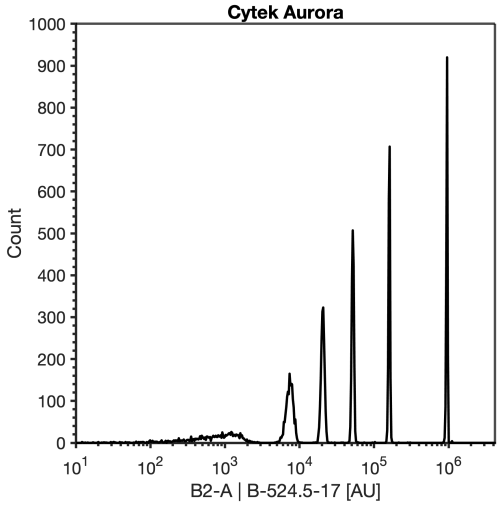
Acquisition Template.xlsx

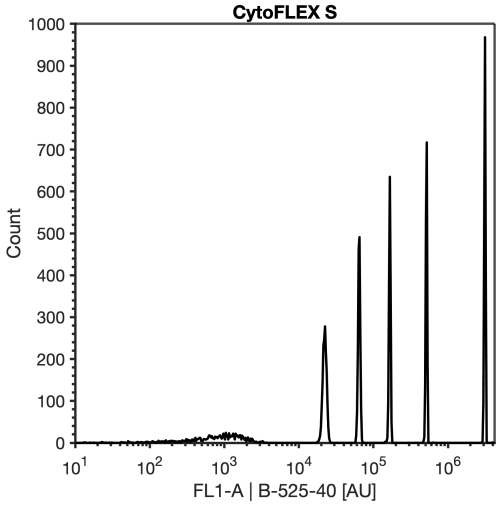
Note

For flow cytometers with avalanche photodiodes its is recommended that the fluorescent detector settings have more incrementation at lower gains than higher gains while for instruments with photomultiplier tubes they should be spaced evenly. See template for example of settings for each tube analyses

Example Gain Voltration for CytoFLEX & Aurora

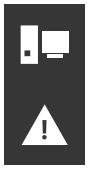
1. 100
2. 200
3. 300
4. 400
5. 500
6. 750
7. 1000
8. 1250
9. 1500
10. 2000
11. 2500
12. 3000





Example of QbSure beads acquired on Cytek Aurora and CytoFLEX S on the FITC parameter. Data has been gated to remove the noise population.

13.1 To ensure accurate data analysis, the brightest bead must be visible on at least two of the selected gains.



14 Fluorescent channels can be cross calibrated to determine lower limit of detection for the channel in calibrated units in the FCM_{PASS} software. A cross calibration between the desired MESF bead and QbSure beads should be acquired at a fluorescent detector gain where all MESF bead populations are **on-scale and fully resolved** from the noise.

Note

Cross calibration on Cytek Aurora and CytoFLEX were run with FITC MESF beads on the B2 | B-524.5-17 and FL1 | B-525-40 channels respectively. The QbSure beads were acquired at the same settings as the FITC MESF beads.