NOV 02, 2023

DOI:
dx.doi.org/10.17504/protocols.io.261ge324ol47/v1

Protocol Citation: Sean M Cook, Vera A. Tang, Joanne Lannigan, Jennifer Jones, Joshua A Welsh 2023. Resource 6: rEV Fluorescent Detector Setting Incrementation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261ge324ol47/v1

Protocol status: Working

Created: Jan 30, 2023

Resource 6: rEV Fluorescent Detector Setting Incrementation

In 1 collection

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Translational Nanobiology Section

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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.

Last Modified: Nov 02, 2023

PROTOCOL integer ID: 76106

Funders Acknowledgement:
NIH
Grant ID: ZIA BC011502
NIH
Grant ID: ZIA BC011503
NIH
Grant ID: 4UH3TR002881-03

MATERIALS

Exosome standards fluorescent **Millipore Sigma Catalog #SAE0193**, referred to as 'rEVs'

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

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

Protein LoBind Tubes (5 mL) **Thermo Fisher Scientific Catalog #0030122356**, referred to as 'low-binding Eppendorf tubes'

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

100 nm polystyrene NIST bead **Thermo Fisher Scientific Catalog #3100A**, referred to as '100 nm NIST bead'

Sample Preprepation

5m

1 Briefly centrifuge rEVs at 100 x g, 4°C, 00:05:00 before opening 5m

2 Add 100 µL of 4 °C deionized water to rEV vial. Pipette up and down to mix well.

> **Note**
>
> Do not vortex

3 Create 5 mL of a 1:2000 dilution of rEVs by pipetting 1 µL rEVs into 1999 µL DPBS in a low-binding Eppendorf tube. This gives a 5E6 p/mL solution.

4 Label a FACS tube as 'DPBS'. Pipette 500 µL DPBS into the tube.

5 Label as FACS tube as 'QbSure', and add 500 µL DPBS. Vortex the QbSure beads for 5 sec and add 3 drops QbSure beads into FACS tube.

Cytometer Setup

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

7 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'.

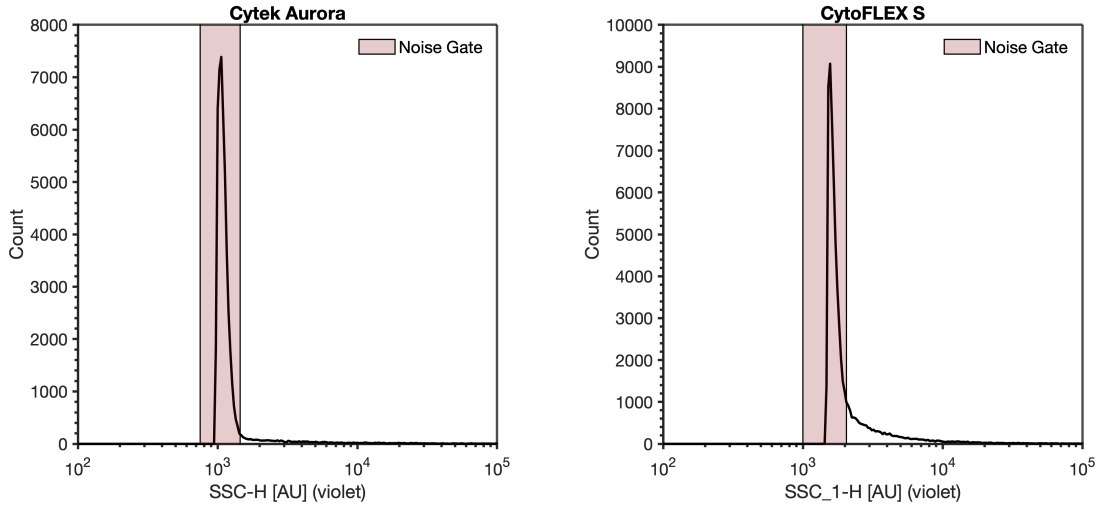
8 Create a histogram plot with the FITC parameter (CytoFLEX: FL1 | B-525-40, Aurora: B2 | B-524.5-17) on the X-Axis and make sure it is plotted on a log-scale.

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

10 Set the cytometer triggering threshold to the violet SSC parameter at the same settings used to acquire the bead fluorescent voltration. **All samples should be acquired with the lowest flow rate, typically ~10-15 µL min^{-1}.**

11 Acquire the 'DPBS' tube while viewing the SSC histogram plot from go to step #9. 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.

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

rEV Acquisition

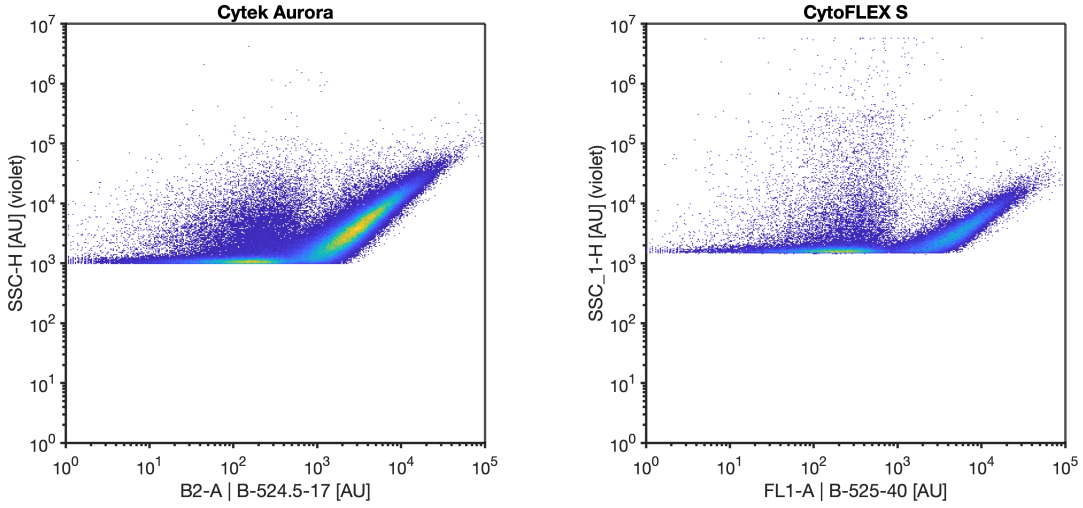
12 Validation of FCM_{PASS} outputs can now be performed by acquiring the rEVs at the same voltration gains used when acquiring the QbSure fluorescence voltration.

Note

For flow cytometers with avalanche photodiodes its is recommended that the 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. 50
2. 100
3. 200
4. 300
5. 400
6. 500
7. 750
8. 1000
9. 1250
10. 1500
11. 2000
12. 2500
13. 3000



Example of rEVs acquired on the Cytek Aurora and CytoFLEX S. Cytek Aurora triggered on violet SSC-H at gain 2500 and threshold 975. CytoFLEX S triggered on violet SSC_1-H at gain 250 and threshold 1200.

Note

The total event rate when acquiring the rEVs should not exceed 6000 events/sec. If the event rate is higher than 6000 events/sec, further dilution of the rEVs is necessary prior to acquisition.

13 Pipette 200 µL from the 5E6 rEV solution from go to step #3 into a FACS tube. Acquire the FACS tube for 60 s on the lowest flow rate at one of the fluorescence gains. Repeat this for each gain.

Note

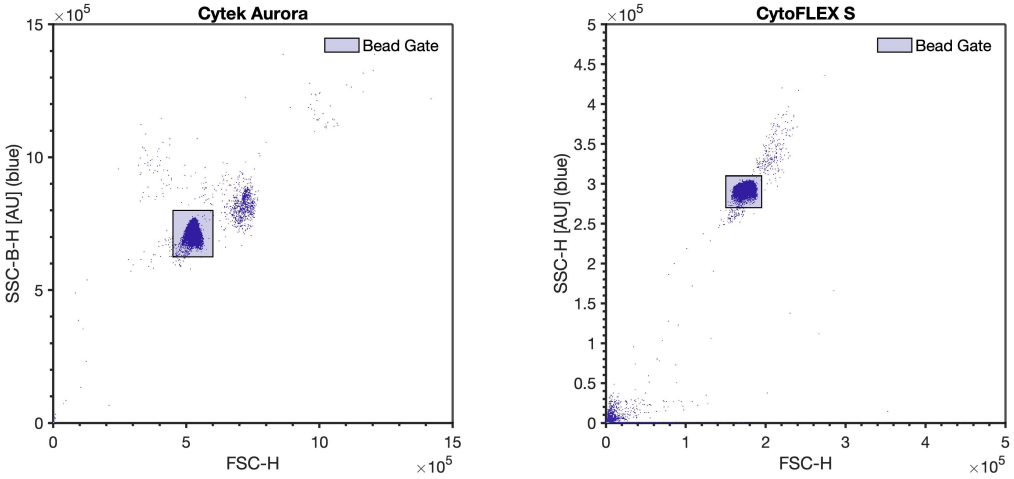
In order to prevent the crashing out of rEVs over the time course of the experiment, only pipette from 5E6 rEV solution into a FACS tube immediately prior to acquisition. Reverse pipette to mix in the FACS tube, do not vortex.

14 Run the 100 nm polystyrene NIST beads at the same scatter settings as the rEVs (diluted in DPBS to a concentration of 5E6 p/mL) until at least 10000 bead events have been acquired. The 100 nm bead only needs to be acquired once at the scatter settings used in the experiment.

Note

Acquiring the 100 nm polystyrene NIST bead allows for the calibration of the rEV side scatter data into calibrated units if the collection half-angle of the instrument has been determined in previous protocols.

15 Run QbSure beads on low at the same fluorescent settings and collect 10,000 bead events for each gain.



QbSure beads gated on blue SSC-H and FSC-H on the Cytek Aurora and CytoFLEX S. The violet SSC settings remain unchanged. In order to limit .fcs file size, a FSC scatter trigger may be used until 10000 QbSure bead events are acquired.

Note

You may need to adjust the B-SSC and FSC detector settings for the bead acquisition. Do not adjust fluorescence detector settings or high sensitivity SSC detector settings. Assuming the QbSure beads were cross calibrated in the previous protocols, this allows for the calibration of the rEV fluorescent data into calibrated units.

16 If calibration of data into standard units is desired, the 100 nm polystyrene NIST bead and QbSure beads can be used to calibrate data by following the FCM_{PASS} experiment calibration protocols.

Protocol

NAME

FCMPASS Protocol Collection

CREATED BY

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PREVIEW