🌐 **Resource 5: rEV Scatter Detector Setting Incrementation**
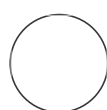
🗐 In 1 collection

NOV 02, 2023

Sean M Cook[1], Vera A. Tang[2], Joanne Lannigan[3], Jennifer Jones[1], Joshua A Welsh[1]

[1]Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health; [2]University of Ottawa; [3]Flow Cytometry Support Services

Translational Nanobiology Section

Jennifer Jones

Protocol status: Working

Created: Jan 30, 2023

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.

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 rEVs 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 FITC parameter at the same settings used to acquire the bead scatter 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 FITC 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.
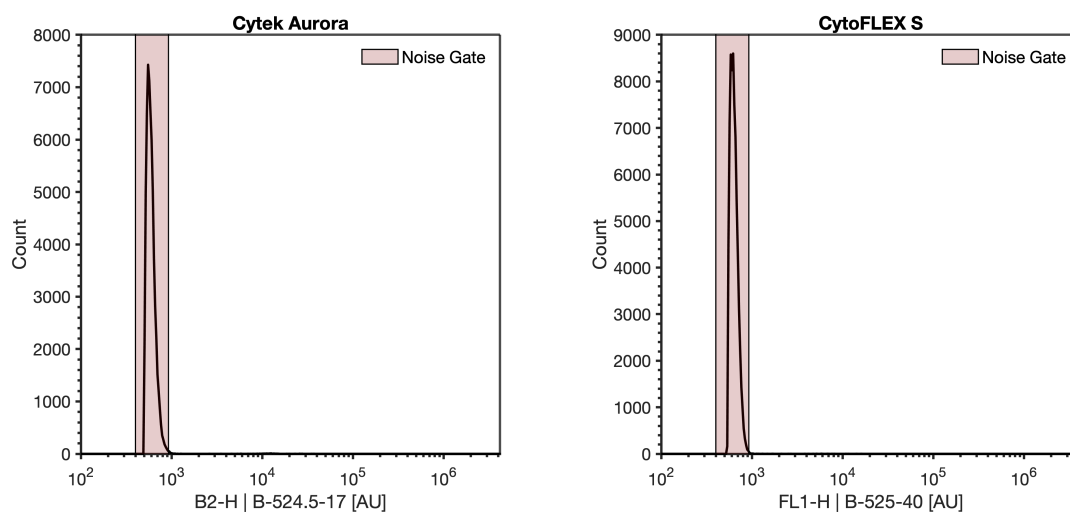
Acquisition of DPBS on the Cytek Aurora and CytoFLEX S, with the Aurora triggering on instrument noise on the B2-H channel at gain 1750 and threshold 500 and the CytoFLEX triggering on instrument noise on the FL1-H channel at gain 500 and threshold 550.

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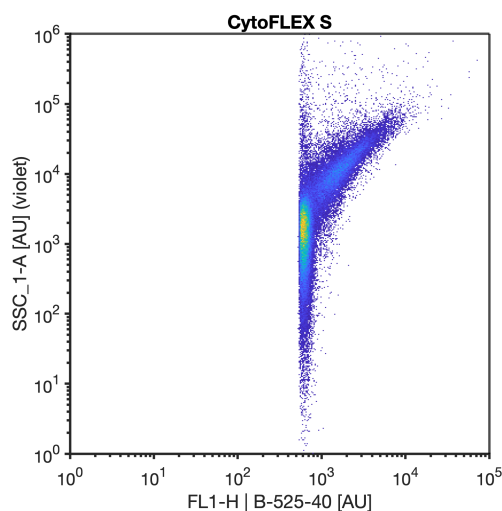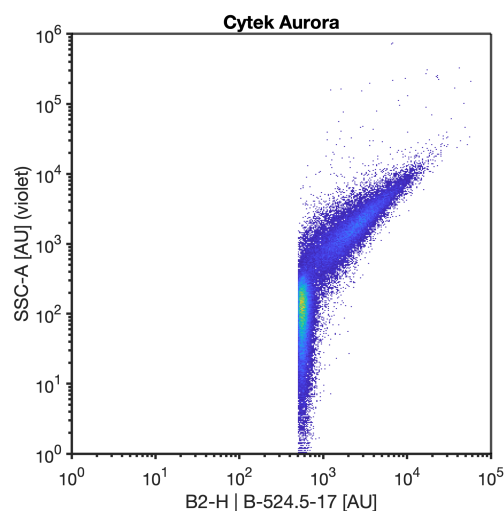
rEV Acquisition

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

Example of rEVs acquired on the Cytek Aurora and CytoFLEX S. Cytek Aurora triggered on B2-H | B-524.5-17 at gain 1750 and threshold 500. CytoFLEX S triggered on violet FL1-H | B-525-40 at gain 500 and threshold 550.

> **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 scatter gains. Repeat this for each gain.
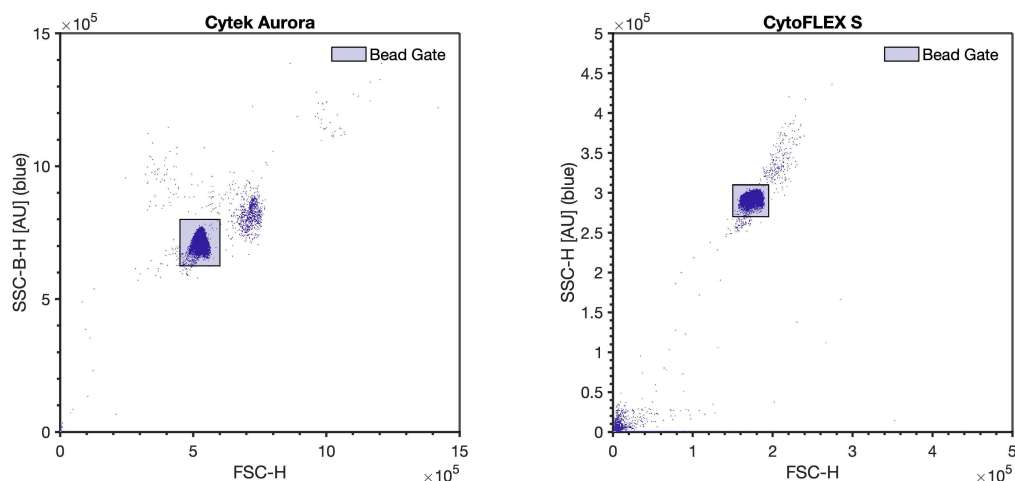
> **Note**
>
> In order to prevent the crashing out of rEVs over the time course of the experiment, only pipette from 5E6 rEV solution into a FACS tube immediately prior to acquisition. Reverse pipette to mix in the FACS tube, do not vortex.

14   Run the 100 nm polystyrene NIST beads at the same settings as the rEVs (diluted in DPBS to a concentration of 5E6 p/mL) until at least 10000 bead events have been acquired for each gain.

> **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 as the rEVs and collect 10,000 bead events. QbSure beads only need to be acquired once at the fluorescent settings used in the experiment.

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

Joshua A Welsh

PREVIEW

> **Note**
>
> Not all scatter gains will resolve the 100 nm NIST bead, but the data can still be calibrated using the 110 nm FluoSpheres that were collected in the FluoSpheres scatter voltration.