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Resource 4: rEV Serial Dilution

Forked from rEV Acquisition

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.

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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'

Low Protein Binding Collection Tubes (1.5 mL) **Thermo Fisher Scientific Catalog #90410**, 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'

rEV Reconstitution

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

2 Add 100 µL of 4 °C deionized water.

3 Pipet up and down to mix.

Note

Do not vortex

4 Aliquot 20 µL into low-binding Eppendorf tubes.

Equipment

Low Protein Binding Microcentrifuge Tubes	NAME
Microcentrifuge Tubes	TYPE
Thermo Scientific	BRAND
90410	SKU
https://www.thermofisher.com/order/catalog/product/90410	LINK

5 Store at 2-4 °C for up to one day (up to 2 weeks at -80 °C).

rEV Dilutions

6 Label 10 low-binding Eppendorf tubes:

	A	B
	Label	Dilution
	1	50
	2	100
	3	200
	4	400
	5	800

	A	B
	6	1600
	7	3200
	8	6400
	9	12800
	10	25600

7 Add 980 µL of DPBS (preferably 0.1 µm filtered) to the tube 1 from go to step #6 .

8 Add 500 µL of DPBS (preferably 0.1 µm filtered) to the remaining tubes.

9 Add 20 µL of the stock reconstituted rEVs to the tube labeled 1 (go to step #6), pipet up and down to mix.

> **Note**
>
> Do not vortex

10 Pipette 500 µL of the 1:50 dilution in tube 1 into the 1:100 dilution in tube 2, pipet up and down to mix.

> **Note**
>
> Do not vortex

11 Repeat the above step for each of the remaining dilution tubes until the serial dilution has been completed for all dilutions.

12 Keep on ice during acquisition.

Flow Cytometer Acquisition

13 Use the instrument settings that were established from the previous gain incrementation and scatter calibration resources. On the Aurora, set the window extension to 0. On the CytoFLEX, use the high acquisition mode. Collect for at least 1 minute at a low flow rate with a 30 second recording delay if using plate 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'.

13.1 Set your instrument to trigger on the most sensitive side scatter channel at the FCM_{PASS} optimal gain output as determined by the FluoSpheres scatter voltration. Set the threshold in the noise such that the event rate is ~1000 events/sec when running DPBS.

> **Protocol**
>
> NAME
> **Resource 1: Scatter Detector Setting Incrementation for FCMPASS**
>
> CREATED BY
> **Jennifer Jones**
>
> PREVIEW

13.2 Set the FITC parameter to the FCM_{PASS} optimal gain output as determined by the QbSure fluorescent voltration.

Protocol

NAME

Resource 2: Fluorescence Detector Setting Incrementation for FCMPASS

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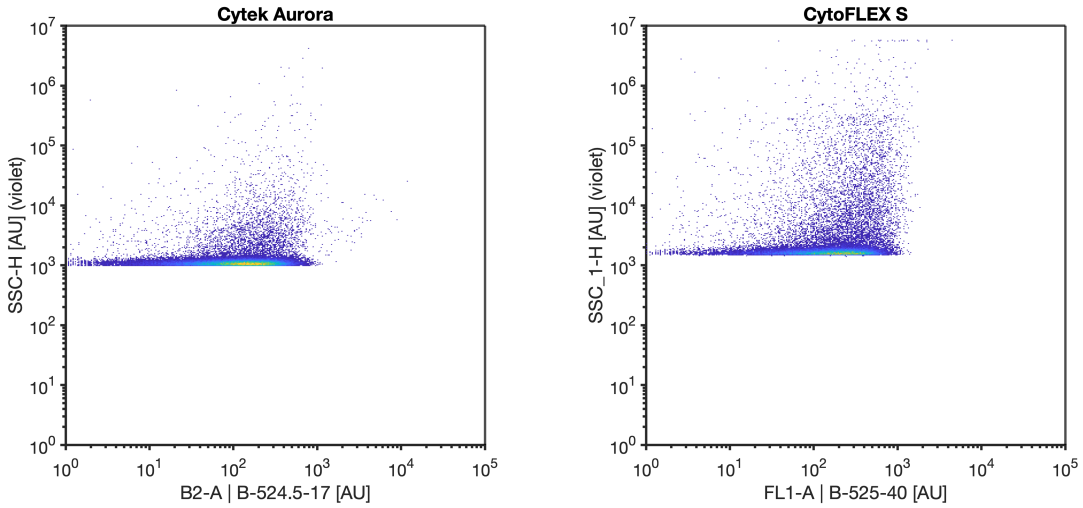
Jennifer Jones

PREVIEW

14 Collect a DPBS sample in a FACS tube for one minute on low.

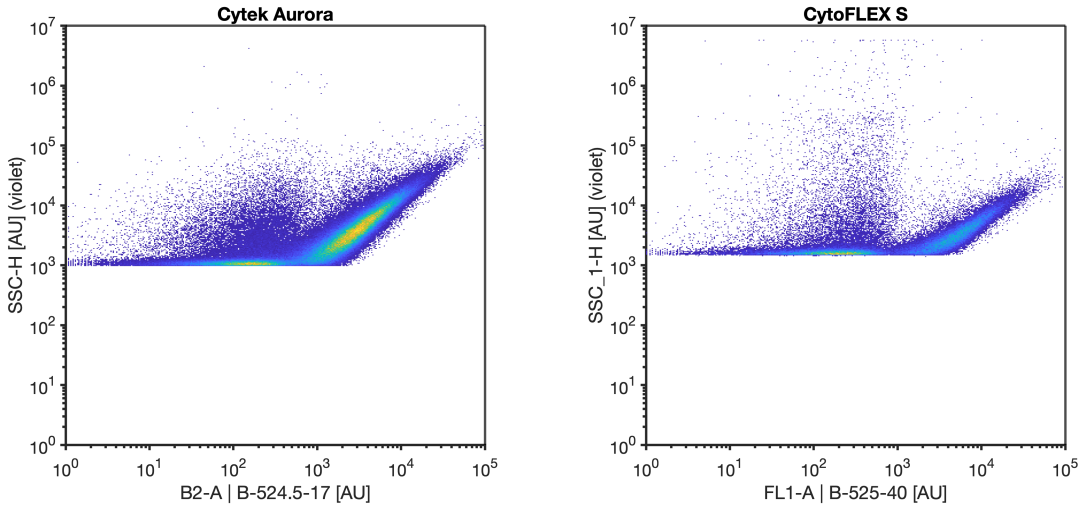
Note

This is to ensure the instrument is clean on the side scatter and fluorescent channels before acquiring the rEVs.



DPBS acquired on the Cytek Aurora and CytoFLEX S.

15 Collect all rEVs samples for one minute on low, starting with the highest dilution (least concentrated) and working down to lowest dilution (most concentrated). This will reduce the effect of carry over from high concentrations.



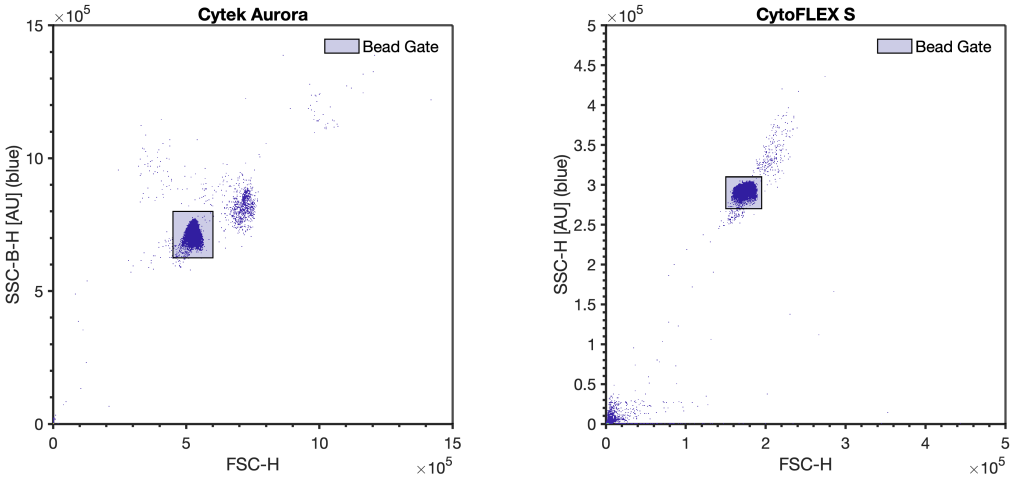
rEVs acquired on the Cytek Aurora and CytoFLEX S.

16 Run a DPBS sample in a FACS tube to make sure there are no carryover events; there should be no events in the B1 and B2 channel.

Note

If there is carry over clean instrument with bleach and flush with deionized water as appropriate.

17 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. Run QbSure beads on low and collect 10,000 bead events.



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.

18 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.

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.

19 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