Resource 7: rEV immunophenotyping

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.

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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 **Merck MilliporeSigma (Sigma-Aldrich) 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'

Low Protein Binding Microcentrifuge **Thermo Fisher Scientific Catalog #88379**

96-well V bottom plate **Contributed by users**
PE anti-human CD81 (TAPA-1) Antibody **BioLegend Catalog #349505**, referred to as 'anti-CD81-PE'
APC anti-human CD81 (TAPA-1) Antibody **BioLegend Catalog #349509**,
referred to as 'anti-CD81-APC'
Pacific Blue™ anti-human CD81 (TAPA-1) Antibody **BioLegend Catalog #349515**
, referred to as anti-CD81-PB'
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 Preparation

35m

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

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

Note

Do not vortex

3 Dilute the reconstituted rEVs 1 in 5 in PBS for staining* Take 50 µL of the rEV stock and add 200 µL of PBS.

Note

The sample concentration chosen for staining should take into consideration the need to dilute the sample further after staining to reduce the concentration of free antibodies, since the samples will be acquired without a washing step to remove these antibodies as in the case of cells.

4 Prepare the antibody dilutions for a 2x staining concentration of each of the antibodies to be used in the antibody titration. See the example below for sample calculations prepared for a staining concentration of 2 µg/ml.

	A	B	C	D	E	F
		AB stock concentration (µg/ml)	2x staining conc (µg/ml)	**Stock volume for 2x staining conc (µL)**	Total volume for 2x staining conc (µL)	**Volume of PBS to be added (uL)**
	anti-Human CD81-PE	120	4	**2**	60	**58**
	anti-Human CD81-APC	200	4	**2**	100	**98**
	anti-Human CD81-PB	300	4	**2**	150	**148**

Sample calculations prepared for a staining concentration of 2 µg/ml. All Antibodies clones are 5A6.

5 Samples will now be prepared using the following plate map.

	A	B	C	D	E	F	G	H
		CD81 PE	**CD81 APC**	**CD81 Pacific Blue**	**CD81 PE**	**CD81 APC**	**CD81 Pacific Blue**	
		rEV	rEV	rEV	PBS	PBS	PBS	
	0 µg/mL	A1	A2	A3	A4	A5	A6	
	0.0625 µg/mL	B1	B2	B3	B4	B5	B6	

	A	B	C	D	E	F	G	H
	0.125 µg/mL	C1	C2	C3	C4	C5	C6	
	0.25 µg/mL	D1	D2	D3	D4	D5	D6	
	0.5 µg/mL	E1	E2	E3	E4	E5	E6	
	1 µg/mL	F1	F2	F3	F4	F5	F6	
	2 µg/mL	G1	G2	G3	G4	G5	G6	**QbSure Beads**
		PBS	PBS	PBS	PBS	PBS	PBS	**100 nm PS NIST-Traceable Beads**

6 In a 96-well V-bottom plate, add 10 µL 1:5 rEV solution to wells A1-A3

7 Add 10 µL DPBS to wells A1-A3 and reverse pipet to mix. These wells will serve as the rEV controls

8 Add 20 µL DPBS to wells A4-A6. These wells will serve as buffer only controls

9 In the same 96-well V-bottom plate from go to step #6, add 10 µL 1:5 rEV solution to wells B1-G3. Add 10 µL DPBS to wells A1-G1. Add 10 µL from the PE working solution tubes to wells B1-G1, add 10 µL from the APC working solution tubes to wells B2-G2, add 10 µL from the PB working solution tubes to wells B3-G3.

10 In the same 96-well V-bottom plate from go to step #6, add 10 µL from the corresponding PE working solutions tube to wells B4-G4. Add 10 µL from the corresponding APC working solutions tube to wells B5-G5. Add 10 µL from the corresponding PB working solutions tube to wells B6-G6. These wells will serve as the DBPS+AB control wells.

11 Cover and incubate this plate for 00:30:00 at RT. 30m

12 Using a separate 96-well V-bottom plate, add 199 µL DPBS into wells A1-G6. Pipet 1 µL from the incubation plate into the new plate in the same wells and reverse pipette to mix.

> **Note**
>
> Steps 11 and 12 will be repeated for the ON incubations as well to bring rEVs down to running concentration that doesn't swarm the instrument detectors.

13 Add 200 µL DPBS into wells A7-G7. These wells will help reduce sample carryover after each rEV+AB combination is acquired.

14 Re-cover incubation plate and let incubate ON at RT to repeat measurements next day as directed in the following steps.

15 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

16 Setup cytometer to acquire at FCM_{PASS} output gains for both light scatter and fluorescent detectors as determined by the FCM_{PASS} detector optimization module outputs. For the Aurora, an optimal gain template is returned to import into the Spectraflo software.

17 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.

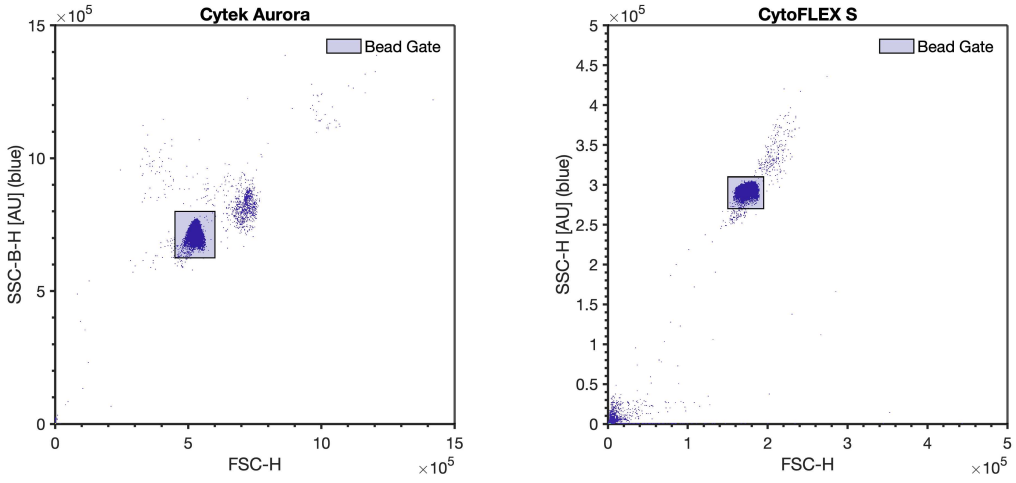
To change the acquisition mode on the CytoFLEX S, click on the 'Advanced' menu on the top-of-the-screen ribbon. Next click on 'Event Rate Settings' menu, change the acquisition mode to 'High', and press 'Ok'.

18 Set the cytometer triggering threshold to the violet SSC parameter and run a DPBS control first to ensure the cytometer is clean and thresholds/event rates have remained unchanged. The background event rate should be ~1000 events/sec. **All samples should be acquired with the lowest flow rate, typically ~10-15 µL min^{-1}.**

19 Acquire all wells for at least 60 sec.

20 In an open well, 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.

21 In an open well, add 200 µL from the QbSure FACS tube and collect 10,000 bead events at the same settings as the rEVs.



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.

22 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

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PREVIEW

23 Don't forget to repeat the acquisition for the samples incubating ON.