Chapter 5

Protocol for Measuring Concentrations of Extracellular Vesicles in Human Blood Plasma with Flow Cytometry

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Abstract

Extracellular vesicles (EVs) are lipid membrane enclosed particles that are released from cells into body fluids, such as blood. EVs offer potential new biomarkers of diseases, because the cellular origin, composition, concentration, and function of EVs change in health and disease. The concentration of EVs from specific cell types in blood can be determined with flow cytometry. A flow cytometer measures fluorescence and light scattering signals from single EVs, but only if these signals are sufficiently bright to be detected. Measured concentrations of EVs are therefore only reproducible and comparable if the detection ranges are known and reported in standard units, such as molecules of equivalent soluble fluorophore (MESF) for fluorescence signals and the diameter in nm for scatter signals. The goal of this protocol is to discuss all steps needed to derive the concentration of cell-type specific EVs within a known diameter range and fluorescence range. More specifically, this protocol describes how to determine the concentration of CD61+ (Integrin beta-3, platelet marker), CD235a+ (Glycophorin A, erythrocyte marker), and CD45+ (leukocyte common antigen) EVs in human blood plasma with an Apogee A60-Micro flow cytometer using scatter-based triggering. The principles behind this protocol could lay a firm basis for the design of a protocol suitable for other flow cytometers and body fluids.

Key words Calibration, Extracellular vesicles, Flow cytometry, Fluorescent antibody labeling, Human blood plasma, Number concentration, Standardization

1 Introduction

Extracellular vesicles (EVs) are lipid membrane enclosed particles that are released from cells into body fluids, such as blood [1]. Blood contains different types of EVs originating from a variety of cells, including erythrocytes, leukocytes, and platelets [2]. EVs offer potential new biomarkers of diseases, because the cellular origin, composition, concentration, and function of EVs change in health and disease [2].

The concentration of EVs in blood can be determined with flow cytometry [3]. A flow cytometer measures fluorescence and light scattering signals from single EVs, but only if the signals

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originating from an EV are sufficiently bright to be detected [4]. Measured concentrations of EVs are therefore only reproducible and comparable if the detection ranges are known and reported in standard units [5]. Fluorescence signals are used to detect the presence of labeled transmembrane proteins at the surface of EVs, thereby revealing the cellular origin of EVs. The standard units of choice for fluorescence are molecules of equivalent soluble fluorophore (MESF). Light scattering signals can be used to determine the diameter of EVs in units of nm [6]. The goal of this protocol is to guide the reader through all steps needed to derive the concentration of cell-type specific EVs within a known diameter range and fluorescence range, e.g.: "We measured a concentration of $8.3 \cdot 10^4$ CD45+ EVs $\cdot \mu L^{-1}$ with a diameter between 160 and 1000 nm and an allophycocyanin (APC) fluorescence intensity exceeding 90 MESF."

Figure 1 shows a schematic of the protocol. In short, the protocol describes how to collect blood, prepare plasma, store plasma, and stain plasma specifically for flow cytometry analysis. To enable the generation of reproducible data, the protocol describes how to use reference materials to calibrate the fluorescence and light scattering signals. Here, calibration means to relate the arbitrary units of a flow cytometry measurement to standard units. In addition, to verify whether the flow cytometer is stable during a study, the protocol describes how to run and analyze daily quality controls. The last sections of the protocol describe how to verify that signals are originating from EVs and how to determine the concentration of cell-type specific EVs within known detection ranges.

More specifically, this protocol describes how to determine the concentration of CD61+ (Integrin beta-3, platelet marker), CD235a+ (Glycophorin A, erythrocyte marker), and CD45+ (leukocyte common antigen) EVs in human blood plasma with an Apogee A60-Micro flow cytometer. The protocol is designed for scatter-based triggering. The principles behind this protocol could lay a firm basis for the design of a protocol suitable for other flow cytometers and body fluids.

2 Materials

All reagents are prepared and stored at room temperature unless indicated otherwise.

2.1 Blood Collection and Storage

1. 21-gauge Needle.
2. 6-mL Plastic blood collection tubes (at atmospheric pressure) containing the anticoagulant ethylenediaminetetraacetic acid (EDTA; *see* **Note 1**).
3. Storage vials with a screw lid and rubber ring (*see* **Note 2**).

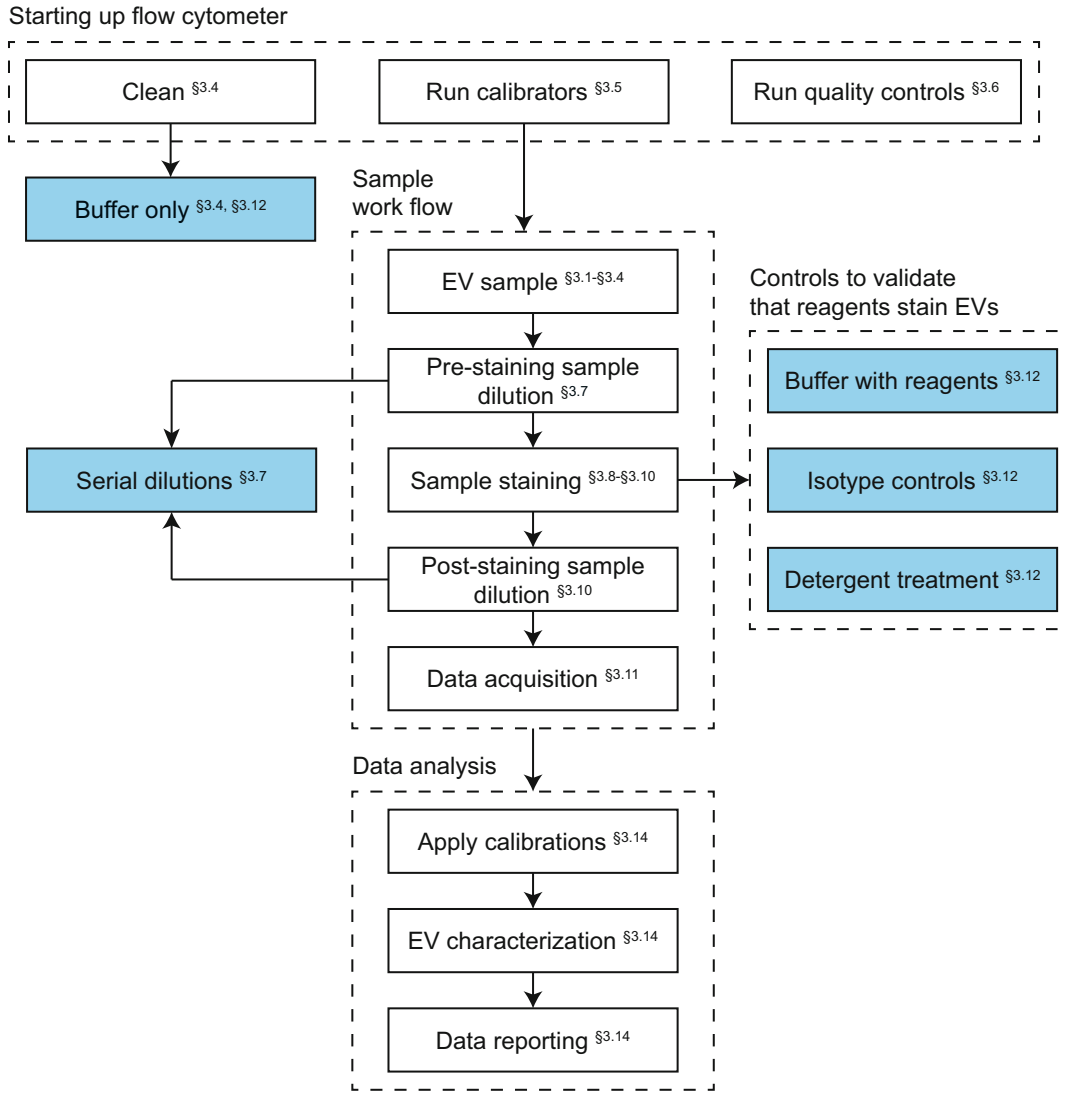

Fig. 1 Diagram of the protocol for measuring concentrations of extracellular vesicles (EVs) from human blood plasma with flow cytometry. § Subheading

2.2 Reference Materials for Calibrating Fluorescence and Light Scattering Detectors

1. APC calibrant: 2 μm APC Quantitative (Q-APC) Beads (custom-order, BD, USA). Reconstitute the dried pellet by adding 500 μL of Dulbecco's phosphate-buffered saline (DPBS) and vortex for 10 s. Use the same day.
2. Brilliant Violet 421 (BV421) calibrant: Dried 3-micron BV421 Quantitative Beads (custom-order, BD, USA). Reconstitute the dried pellet by adding 500 μL of DPBS and vortex for 10 s. Use the same day.
3. Phycoerythrin (PE) calibrant: PE Easy Calibration Kit, 5 peaks (Spherotech, USA). Manually tilt PE Easy Calibration Kit

ten-fold before use. Add one droplet from each dropper bottle (5 bottles in total) to 300 μL of DPBS and vortex for 10 s. Use the same day (*see* **Note 3**).

4. Light scattering detector calibrant: Rosetta Calibration beads (Exometry, The Netherlands). Manually tilt Rosetta Calibration beads ten-fold before use. Add one droplet of Rosetta Calibration beads to 160 μL of Milli-Q water and vortex for 10 s. Use the same day.

2.3 Quality Controls

1. QC sample for fluorescence detectors: SPHERO™ Rainbow Calibration particles, 8 peaks (Spherotech, USA). Manually tilt SPHERO™ Rainbow Calibration particles ten-fold before use. Add 2 droplets to 1000 μL of Milli-Q water, vortex 10 s, and label the tube with the date. Add 200 μL from the prepared solution to a new tube to be measured. Store the remaining solution at 4 °C for a maximum period of 5 days.
2. QC sample for light scattering detectors and sample volume: ApogeeMix (Apogee Flow Systems, UK). Manually tilt ApogeeMix ten-fold before use. Add 100 μL of ApogeeMix to 100 μL of Milli-Q water and vortex for 10 s. Use the same day.

2.4 Reagents for Staining and Lysing Extracellular Vesicles

1. CD45-APC: APC mouse anti-human CD45 antibody (BioLegend, USA), clone HI30, isotype mouse IgG_1.
2. CD61-BV421: BV421 mouse anti-human CD61 antibody (BD, USA), clone VI-PL2, isotype mouse IgG_1.
3. CD235a-PE: PE mouse anti-human CD235a antibody (Agilent Dako, USA), clone JC159, isotype mouse IgG_1.
4. APC mouse IgG_1 (BD, USA).
5. BV421 mouse IgG_1 (BD, USA).
6. PE mouse IgG_1 (BD, USA).
7. 96-Well microplate (flat well bottom).
8. X-Pierce™ film (Sigma-Aldrich, USA).
9. Detergent treatment reagent: prepare a solution of 10% Nonidet™ P 40 Substitute (Sigma-Aldrich, USA) by adding 200 μL of Nonidet™ P 40 Substitute to 1800 μL of DPBS. Filter the solution with a 50-nm Nuclepore Hydrophilic Membrane filter (Whatman plc, UK).

2.5 Solutions for Cleaning the Flow Cytometer Fluidics

1. BD FACSRinse Solution (BD, USA).
2. 1.5% CITRANOX® acid detergent: dilute CITRANOX® acid detergent (Sigma-Aldrich, USA) stock solution 66.7-fold in Milli-Q water and vortex for 10 s.
3. COULTER CLENZ® Cleaning Agent (Beckman Coulter, USA).
4. ProClin™ 300 (Sigma-Aldrich, USA).

2.6 Equipment

1. Table centrifuge with a minimum acceleration force of 2500 × *g* and an adjustable break setting.
2. Microcentrifuge with a minimum acceleration force of 18,890 × *g* and an adjustable break setting.
3. Flow cytometer: A60-Micro (Apogee Flow Systems, UK) equipped with an autosampler and a 405-nm, 488-nm, and 638-nm laser (*see* **Note 4**).
4. Vortex mixer with a minimum number of 2500 rotations · min^{-1}.
5. Water bath.

3 Methods

All procedures are performed at room temperature unless specified otherwise.

3.1 Blood Collection

1. Collect blood from overnight fasting individuals using a 21-gauge needle [7–9].
2. Avoid prolonged use of a tourniquet [10].
3. Discard the first 2 mL of collected blood [11, 12].
4. Collect blood in plastic collection tubes containing the anticoagulant EDTA (*see* **Note 1**).
5. Fill the tubes to get the appropriate EDTA to blood ratio and mix gently [13].
6. Transport the blood collection tubes vertically.
7. Minimize, measure, and report the time interval between blood collection and plasma preparation [14–17].
8. Report all pre-analytical details [1, 7].

3.2 Plasma Preparation

1. Remove cells by centrifugation at 2500 × *g* for 15 min [18]. Turn off the break or set the lowest deceleration on the centrifuge.
2. Collect the plasma to 10 mm above the buffy coat and pipette the plasma into a clean plastic tube.
3. Remove residual cells by centrifugation at 2500 × *g* for 15 min [18]. Turn off the break or set the lowest deceleration on the centrifuge.
4. Collect the plasma to 10 mm above the bottom of the tube and pipette the plasma into a clean plastic tube.
5. Confirm the absence of residual platelets and hemolysis in plasma using clinical routine laboratory tests (*see* **Note 5**).

6. Report all pre-analytical details [1, 7], including the centrifugation settings, the type of centrifuge and tubes used, the volume of fluid in the tubes during centrifugation, and the volume of collected supernatant [19].

3.3 Plasma Storage

1. Pipette aliquots of 100 μL into the storage vials.
2. Snap-freeze aliquots in liquid nitrogen [20].
3. Store aliquots at or below −80 °C until further use [21, 22].
4. Report all pre-analytical details [1, 7], including the type of storage vials used.

3.4 Starting Up the Flow Cytometer

1. Turn on the flow cytometer and start the data acquisition software.
2. Verify whether there is sufficient sheath fluid to run all samples (*see* **Note 6**). When required, fill the sheath tank(s) with Milli-Q water (*see* **Note 7**).
3. Apply the function "flow cell soaking" once every 2 weeks (*see* **Note 7**).
4. Apply the function "flow cell clean" daily (*see* **Note 7**).
5. Apply the function "remove air from syringe" daily (*see* **Note 7**).
6. Load the data acquisition settings optimized for EV characterization (Table 1).
7. Run Milli-Q water to verify whether the flow cytometer is clean and whether the trigger threshold is set above the background noise level. The flow cytometer is clean when the count rate is $\leq$50 events $\cdot$ s^{-1} (*see* **Note 7**).
8. Repeat **step 7** until the flow cytometer is clean. If the flow cytometer does not become clean, apply **steps 1** and **2** of Subheading 3.13 and repeat **steps 3–5**, and/or replace the sheath fluid filter, and/or increase the trigger threshold, and/or request maintenance.

3.5 Run Reference Materials to Calibrate Fluorescence and Light Scattering Detectors

1. Run the APC calibrant, the BV421 calibrant, and the PE calibrant with settings optimized for fluorescent beads (Table 1).
2. Verify whether $\geq$3 distinct bead populations exceed the background fluorescence level of the corresponding fluorescence detector and whether each population contains $\geq$500 events (Fig. 2a, *see* **Note 8**).
3. For each bead population, plot the logarithm of the specified MESF value versus the logarithm of the measured median fluorescence intensity in arbitrary units. Fit the data points with a linear regression (Fig. 2b). Verify whether the coefficient

Table 1
Data acquisition settings of the A60-Micro to measure EVs and fluorescent beads

Setting	EV characterization	Fluorescent beads
Aspirated sample volume (μL)	140	140
Data storage buffer (events)	5,000,000	(default) 500,000
Flow rate ($\mu L \cdot min^{-1}$)	3.0	4.5
Measurement time (min)	2	5
Number of flush cycles	2	2
PMT voltages fluorescence detectors	Optimal	Optimal
PMT voltages FSC + SSC	Optimal	Beads should not saturate detector
Sheath pressure (mbar)	130	130
Trigger threshold detector	SSC	SSC

PMT photomultiplier tube, *FSC* forward light scattering, also named small angle light scattering (SALS), *SSC* side light scattering, also named large angle light scattering (LALS)

of determination (R^2) exceeds 0.99. Save the intersect and slope of the linear regression for **step 1** in Subheading 3.14 (*see* **Note 9**).

4. Run the light scattering detector calibrant with settings optimized for EV characterization (Table 1).
5. Verify whether ≥3 distinct populations exceed the background level of the side scattered light (SSC) detector, whether at least 1 population exceeds the background level of the fluorescein (FITC) detector, and whether each population contains ≥1000 events (Fig. 2c, *see* **Note 8**).

3.6 Daily Quality Controls

1. Run the QC sample for fluorescence detectors with settings optimized for fluorescent beads (Table 1).
2. Verify whether ≥3 distinct peaks exceed the background fluorescence level of the APC, BV421, and PE detectors, and whether each population contains ≥1000 events (Fig. 3a).
3. Verify whether the fluorescence detectors are stable during all measurement days. Therefore, plot the median APC, BV421, and PE fluorescence intensities of a dim and a bright bead versus time (Fig. 3b). The fluorescence detectors are considered stable if the coefficient of variation (CV) of the median fluorescence intensities is <10%, more preferably <5% (*see* **Note 10**).
4. Run the QC sample for light scattering detectors and sample volume with settings optimized for EV characterization (Table 1).

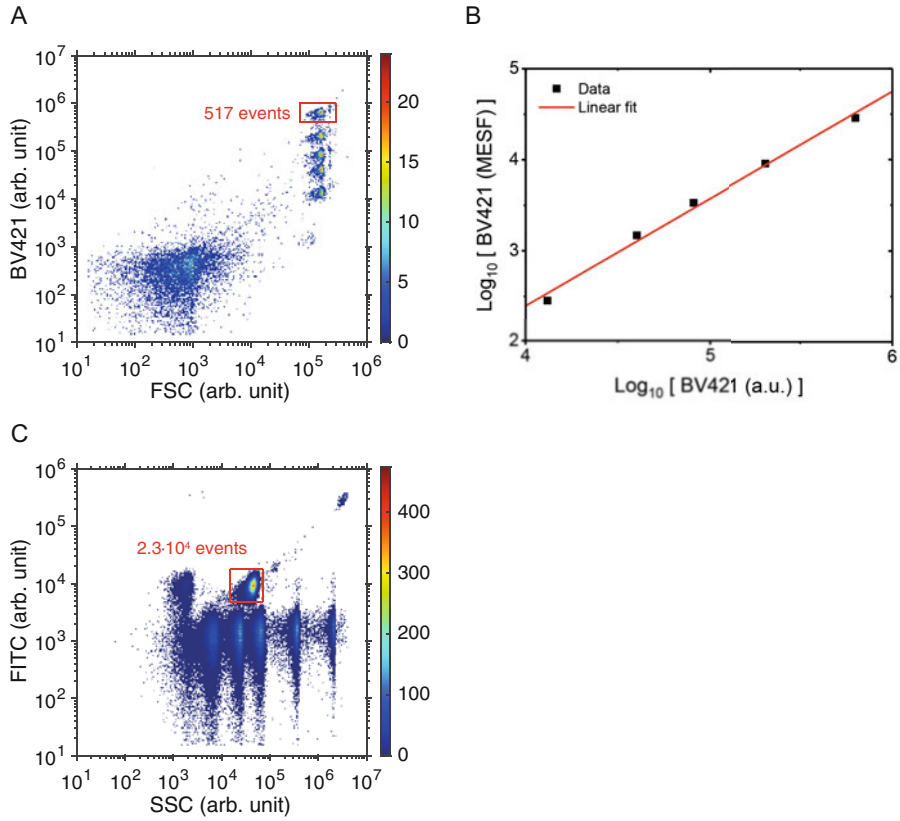

Fig. 2 Reference materials to calibrate fluorescence and light scattering detectors measured with an A60-Micro flow cytometer. (**a**) Brilliant Violet 421 (BV421) fluorescence intensity versus forward scattered light (FSC) intensity in arbitrary units (arb. unit) of the BV421 calibrant. Five populations can be distinguished from the background noise and each population contains ≥500 events. (**b**) Logarithm of the specified BV421 molecules of equivalent soluble fluorophore (MESF) value versus the logarithm of the measured median BV421 fluorescence intensity in arbitrary units (symbols). The slope of the fit (line) is 1.178, the intercept is −2.316, and the coefficient of determination (R^2) is 0.992. (**c**) Fluorescein (FITC) fluorescence intensity versus side scattered light (SSC) intensity in arbitrary units (arb. unit) of the light scattering detector calibrant. Five distinct populations exceed the background level of the side scattered light (SSC) detector, and at least one population exceeds the background level of the FITC. Each population contains ≥1000 events

5. Verify whether the 110-nm FITC beads exceed the background level of both the FITC detector and the SSC detector (Fig. 3c).
6. Verify whether the LALS detector is stable. Therefore, plot the median scattering intensities of the 110-nm FITC beads versus time (Fig. 3d). The LALS detector is considered stable if the CV of the median scattering intensities is <10%, more preferably <5%.
7. Verify whether the measured sample volume is within 5% of the adjusted sample volume during all measurement days. Therefore, plot the measured sample volume versus time (Fig. 3e)

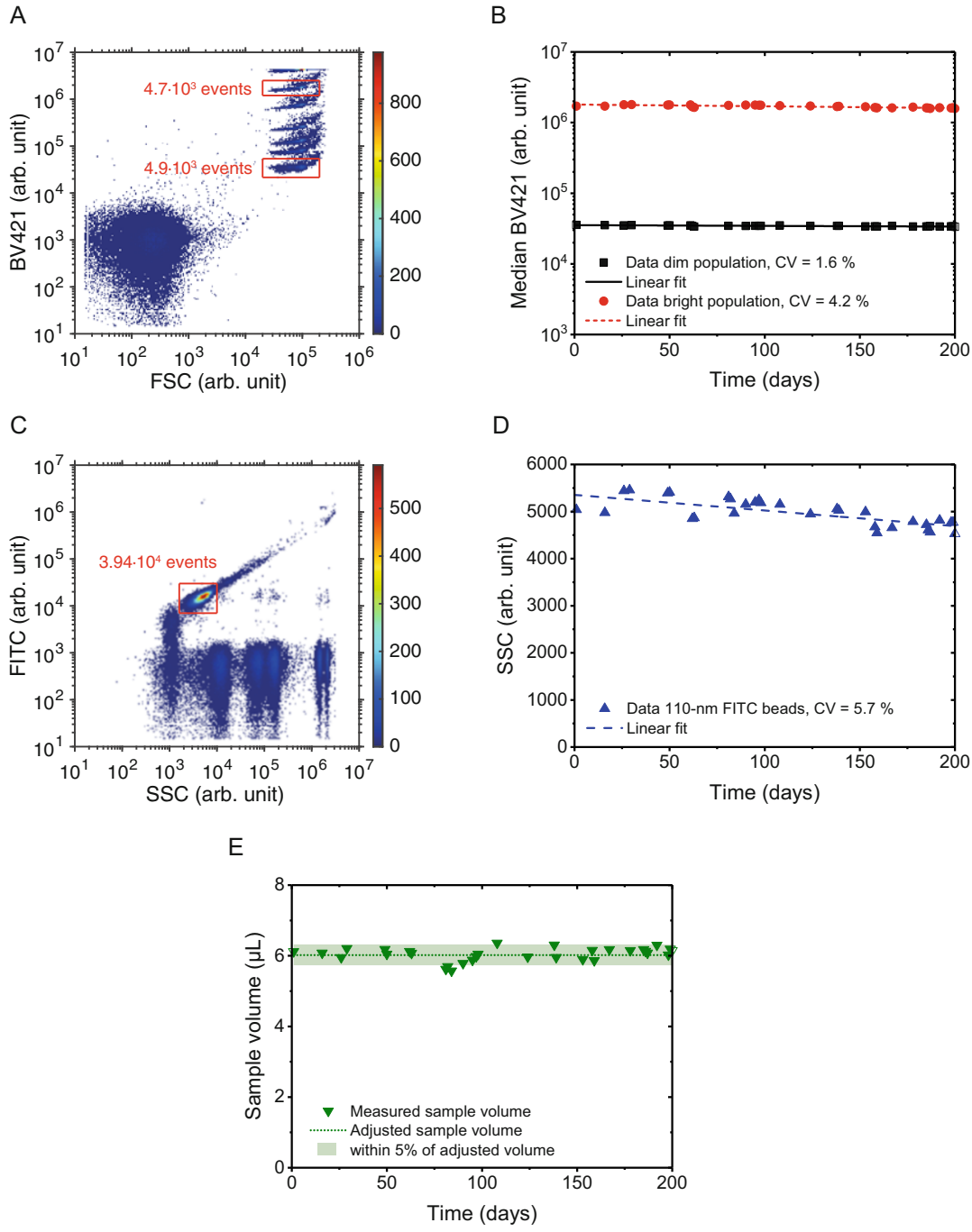

Fig. 3 Daily quality controls. (**a**) Brilliant Violet 421 (BV421) fluorescence intensity versus forward scattered light (FSC) intensity of the quality control (QC) sample for fluorescence detectors. Seven populations can be distinguished from the background noise. The brightest population saturates the BV421 detector. Each population contains $\geq$1000 events. (**b**) Median BV421 fluorescence intensity versus time in days for the dim (squares) and bright (circles) populations indicated in panel A. The coefficient of variation (CV) is <5%, which indicates that the BV421 detector was stable during these 200 days. (**c**) Fluoresceine (FITC)

and compare the results to the adjusted sample volume. The measured sample volume V_m [μL] is given by:

$$V_m = \frac{n}{C_s} \tag{1}$$

where n is the number of detected 110-nm FITC beads and C_s is the specified concentration [μL^{-1}] of the 110-nm FITC beads. The brackets indicate the units of the physical properties. The adjusted sample volume V_a [μL] is given by

$$V_a = Q \cdot t \tag{2}$$

where Q is the flow rate [μL/min] and t is the measurement time [min]. In this protocol, $Q = 3.0\ \mu L \cdot min^{-1}$, $t = 2$ min (Table 1), and therefore $V_a = 6.0$ μL per measurement.

3.7 Determine Optimal Sample Dilution Factor

1. Thaw the (selection of) sample aliquot(s) in a water bath at 37 °C for 60 s.
2. Vortex for 10 s.
3. Dilute the sample 1000-fold in DPBS (*see* **Note 7**). Therefore, add 5 μL of sample to 95 μL of DPBS (*see* **Note 11**), vortex for 10 s, and add 4 μL of the 20-fold diluted sample to 196 μL of DPBS. Discard or keep the remaining 95 μL of sample for Subheading 3.10.
4. Run the 1000-fold diluted, unstained samples with settings optimized for EV characterization (Table 1).
5. If the count rate is not between 200 and 5000 events $\cdot\ s^{-1}$, increase or decrease the dilution factor to achieve a count rate between 200 and 5000 events $\cdot\ s^{-1}$ (*see* **Notes 7** and **12**).
6. Per sample, calculate the measured total particle concentration C_m [μL^{-1}], which is given by:

$$C_m = \frac{D_u \cdot n}{Q \cdot t} \tag{3}$$

where D_u is the dilution factor of the unstained sample (presumably 1000-fold).

Fig. 3 (continued) fluorescence intensity versus side scattered light (SSC) intensity of the QC sample for light scattering detectors and sample volume. Two populations can be distinguished from the background noise. The brightest population saturates the FITC and SSC detectors. Each population contains ≥1000 events. (**d**) Median SSC intensity versus time in days for the 110-nm FITC populations (triangles) indicated in panel C. The CV is <5.7%, which indicates that the SSC detector was stable during these 200 days. (**e**) Adjusted (dotted line) and measured (triangles) sample volume versus time in days. The measured sample volume is based on the specified concentration of 110-nm FITC beads in the QC sample for light scattering detectors and sample volume. For all but 4 days, the measured sample volume is within 5% (marked area) of the adjusted sample volume

7. Per sample, calculate the optimal sample dilution factor D_o, which is given by:

$$D_o = \frac{C_m \cdot Q}{60 \cdot J_m} \tag{4}$$

where $J_m = 5000$ events $\cdot$ s^{-1} is the maximum count rate to minimize the risk of swarm detection [23] (*see* **Notes 7** and **13**).

8. Per sample, calculate the pre-staining dilution factor D_p, which is given by:

$$D_p = \frac{D_o}{D_s} \tag{5}$$

where $D_s = 11.4$ is the dilution factor that is introduced by staining the sample in Subheading 3.10 (*see* **Notes 14** and **15**). Save the sample-specific pre-staining dilution factor D_p for use in **step 2** in Subheading 3.10.

3.8 Antibody Titration

1. The steps below apply to each antibody (CD45-APC, CD61-BV421, and CD235a-PE). Dilute the antibody one-fold (undiluted), two-fold, four-fold, eight-fold, 16-fold, and 32-fold in DPBS. Use higher dilutions if required. For each dilution, the volume after dilution should be 32 μL.
2. Centrifuge 32 μL of the diluted antibodies for 5 min at 18,890 × *g* using a microcentrifuge to remove aggregates. Turn off the break or set the lowest deceleration on the centrifuge.
3. Pipette the supernatant into a new tube and leave 10 μL of the reagent and the pellet in the tube. Avoid disturbing the pellet.
4. Pipette 2.5 μL of the prepared antibody dilutions into 2 new tubes.
5. Add 20 μL of diluted (D_p) reference plasma sample, such as plasma pooled from multiple healthy individuals in a study, to the prepared antibody dilutions in the first tubes of **step 4** and gently stir with the pipette tip (*see* **Note 16**).
6. Add 20 μL of DPBS to the prepared antibody dilutions in the second tubes of **step 4** and gently stir with the pipette tip (*see* **Note 16**).
7. Incubate 120 min in the dark.
8. Add 200 μL of DPBS to dilute the antibodies before measuring and gently stir with the pipette tip (*see* **Note 16**).
9. Follow the steps in Subheading 3.11 to measure the DPBS and reference plasma sample stained with the prepared antibody dilutions.

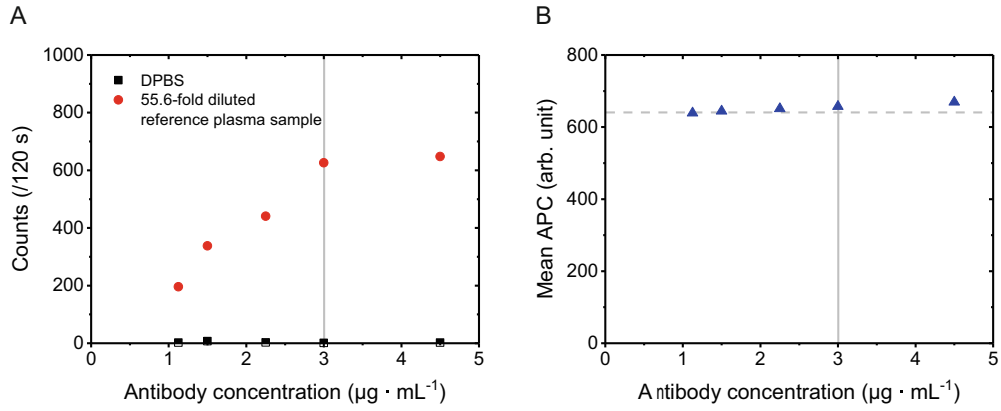

Fig. 4 Antibody titration. (**a**) Measured counts per 120 s versus concentration of CD45-allophycocyanin (APC) antibody in Dulbecco's phosphate-buffered saline (DPBS; squares) and a 55.6-fold diluted (D_0) reference plasma sample (circles). The measured counts in plasma increase with antibody concentration and reach a plateau at 3 μg · mL^{-1}, which is the optimal antibody concentration (vertical line). The measured counts in DPBS are negligible. (**b**) Median APC intensity versus concentration of CD45-APC antibody (triangles). The median APC intensity gradually increases with antibody concentration. The dashed line is a guide to the eye and the vertical line indicates the optimal antibody concentration

10. Plot the counts in DPBS versus the antibody concentration (Fig. 4a).
11. Plot the counts in the reference plasma sample versus the antibody concentration (Fig. 4a).
12. Plot the mean fluorescence of the relevant fluorescence detector (APC, BV421, or PE) versus the antibody concentration (Fig. 4b).
13. Select the optimal antibody concentration. The optimal antibody concentration is the concentration that leads to (1) negligible counts in DPBS, (2) maximum counts in the reference plasma sample, and (3) a minimum background fluorescence level (*see* **Note 17**).
14. Report the selected optimal antibody concentration in comparable units, such as μg · μL^{-1} or mole · L^{-1} (*see* **Note 18**).

3.9 Antibody Mixture Preparation

1. Per antibody (CD45-APC, CD61-BV421, and CD235a-PE), calculate the required volume of antibody from the stock solution, V_{ab}·[μL], to stain all samples:

$$V_{ab} = \frac{3.5 \cdot N \cdot C_{\text{optimal}}}{C_{\text{stock}}} + 10 \qquad (6)$$

where N is the number of samples, C_{stock} is the stock concentration of the antibodies, and C_{optimal} is the optimal antibody concentration obtained from **step 13** in Subheading 3.8.

2. Per antibody, dilute a volume V_{ab} of the stock in DPBS to obtain the optimal antibody concentration obtained from **step 13** in Subheading 3.8 and gently stir with the pipette tip (*see* **Note 16**).
3. Per antibody, centrifuge the diluted antibodies for 5 min at 18,890 × *g* using a microcentrifuge to remove aggregates. Turn off the break or set the lowest deceleration on the centrifuge.
4. Per antibody, pipette the supernatant into a new tube and leave 10 μL of the reagent and the pellet in the tube. Avoid disturbing the pellet.
5. Pool the different aggregate-depleted antibodies to obtain the antibody mixture for staining plasma samples and gently stir with the pipette tip (*see* **Note 16**).

3.10 Sample Staining

1. Thaw the (selection of) sample aliquot(s) in a water bath at 37 °C for 60 s or continue using the thawed samples from **step 1** in Subheading 3.7.
2. Dilute the samples in DPBS using the sample-dependent pre-staining dilution factor D_p calculated in **step 8** in Subheading 3.7.
3. Pipette 7.5 μL of the antibody mixture prepared in Subheading 3.9 into a new tube.
4. Add 20 μL of diluted (D_p) sample to the antibody mixture (**step 3**) and gently stir with the pipette tip (*see* **Note 16**).
5. Incubate 120 min in the dark.
6. Add 200 μL of DPBS to dilute the antibodies before measuring and gently stir with the pipette tip (*see* **Note 16**).
7. Transfer 200 μL of the stained samples to a 96-well plate.
8. Cover the 96-well plate with X-Pierce™ film.

3.11 Data Acquisition

1. Create a worklist with descriptive sample names and positions on the 96-well plate (*see* **Note 19**).
2. Measure all samples on the 96-well plate with settings optimized for EV characterization (Table 1). Make sure to use suck and spit and to rinse the needle and flow cell twice between different samples (*see* **Note 20**).

3.12 Assay Controls

1. Apply the buffer-only control. To verify whether the DPBS and the flow cytometer are clean, run DPBS with settings optimized for EV characterization. DPBS is clean and suitable as a dilution buffer when the total concentration is $\leq 1 \cdot 10^3$ particles $\cdot$ μL^{-1}, preferably $\leq 5 \cdot 10^2$ particles $\cdot$ μL^{-1} (Fig. 5a; *see* **Notes 7** and **21**).

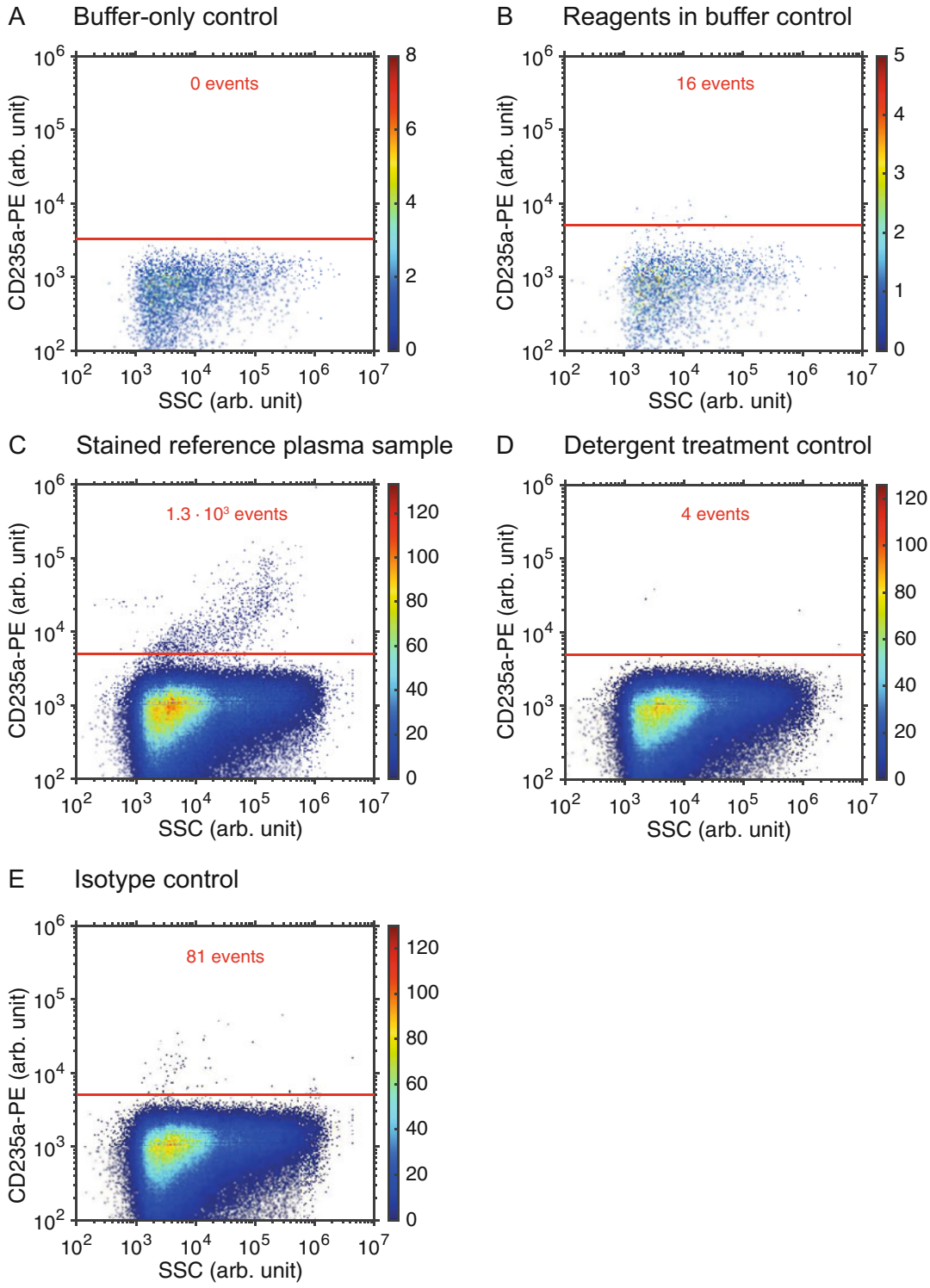

Fig. 5 Assay controls. CD235a-phycoerythrin (PE) fluorescence intensity versus side scattered light intensity of (**a**) Dulbecco's phosphate-buffered saline (DPBS), (**b**) CD45-allophycocyanin (APC), CD61-Brilliant Violet 421 (BV421), and CD235a-PE in DPBS, (**c**) a 55.6-fold diluted reference plasma sample stained with a mixture of CD45-APC, CD61-BV421, and CD235a-PE, (**d**) a 55.6-fold diluted reference plasma sample stained with a

2. Apply the reagents in buffer control. To verify whether the mixture of reagents does not contain detectable particles, stain DPBS (instead of a sample) by following **steps 3–6** in Subheading 3.10 and measure the sample as described in Subheading 3.11 (Fig. 5b; *see* **Note 21**).
3. Apply the detergent treatment control. To verify whether the stained particles lyse upon the addition of detergent, which is expected for EVs, follow the **steps 1–6** in Subheading 3.10 for a reference plasma sample. After **step 6** (Subheading 3.10), add 20 μL of detergent treatment reagent and continue with **step 2** in Subheading 3.11. Confirm the absence of fluorescence positive events after the detergent treatment (Fig. 5c and d; *see* **Note 21**).
4. Apply the isotype control. To verify whether the antibodies do not bind to Fc receptors, follow the steps in Subheadings 3.9–3.11 for a reference plasma sample, but replace the antibodies with APC mouse IgG_1, BV421 mouse IgG_1, and PE mouse IgG_1. For the isotype control, use the optimal antibody concentrations obtained from **step 13** in Subheading 3.8 (Fig. 5e; *see* **Note 21**).

3.13 Shutting Down

1. Take a 96-well plate and fill one 3 wells (200 μL per well) with BD FACSRinse Solution, 3 wells with Milli-Q water, 1 well with COULTER CLENZ® Cleaning Agent, 2 wells with 1.5% CITRANOX® acid detergent, and 3 wells with Milli-Q water.
2. Apply **step 2** in Subheading 3.11 to run the cleaning solutions and rinse the flow cytometer (*see* **Note 22**).
3. When the flow cytometer will not be used within 1 week, add 30 μL of ProClin™ 300 to each liter of sheath fluid (Milli-Q water) to preserve the sheath fluid.
4. Shut down the flow cytometer.

3.14 Data Analysis

1. For each fluorophore, use the intersect b and slope a of the linear regression of the fluorescent calibrations (*see* **step 3** in Subheading 3.5) to relate the arbitrary units of the measured fluorescence intensities I_m to the calibrated units I_c in MESF (*see* **Notes 23** and **24**):

$$I_c = 10^{a \cdot \log_{10}(I_m) + b} \tag{7}$$

Fig. 5 (continued) mixture of CD45-APC, CD61-BV421, and CD235a-PE, but after the addition of 10% Nonidet™ P 40 Substitute, and (**e**) a 55.6-fold diluted reference plasma sample stained with a mixture of APC mouse IgG_1, BV421 mouse IgG_1, PE mouse IgG_1. Antibodies and samples are used at the optimal concentration as determined by titration and measured with an A60-Micro flow cytometer. Compared to the number of CD235a stained EVs in panel **d**, the positive events in the controls are negligible

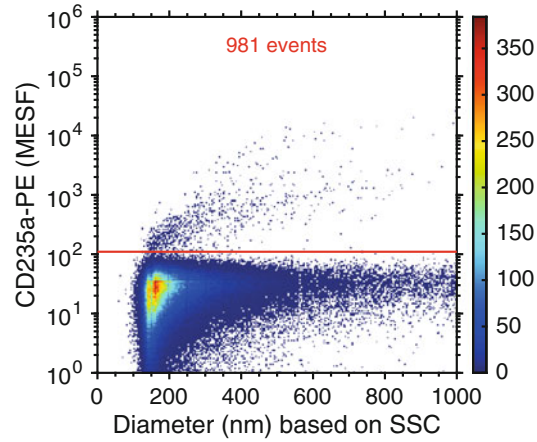

Fig. 6 Example of a calibrated plasma sample. Calibrated CD235a-phycoerythrin (PE) fluorescence intensity in units of molecules of equivalent soluble fluorophore (MESF) versus the diameter of extracellular vesicles based on the side scattered light (SSC). The plasma sample was 30-fold diluted in Dulbecco's phosphate-buffered saline prior to the staining procedure described in this protocol. Between the lower size detection limit, which is ~160 nm, and 1000 nm, 981 particles stained with CD235a-PE exceeded the fluorescent gate of 110 MESF

This step and all steps below apply to all samples.

2. Apply Rosetta Calibration software to relate the measured side scattering intensities to the diameter of EVs [6]. Follow the instructions of the manufacturer to apply the software. Use the core-shell model with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm.
3. For each fluorophore, select the positively stained EVs ranging from the lower size detection limit to 1000 nm. Therefore, plot the calibrated fluorescence intensity versus the diameter and apply a gate (Fig. 6; *see* **Note 25**).
4. For each fluorophore, relate the number of positively stained EVs, n_{EV} (previous step), to the concentration of positively stained EVs, C_{EV} [μL^{-1}], using:

$$C_{EV} = \frac{n_{EV} \cdot D_p \cdot D_s}{V_a} \tag{8}$$

5. For each fluorophore, report the measured EV concentration as well as the dynamic ranges of the used detectors. For example: "We measured a concentration of $5.6 \cdot 10^4$ CD235a+ EVs · μL^{-1} with a diameter between 160 nm and 1000 nm and a PE fluorescence intensity exceeding 110 MESF" (*see* **Note 26**).

4 Notes

1. This protocol is based on 6-mL plastic blood collection tubes containing the anticoagulant EDTA. Although different tube volumes could be used, please note that the volume of fluid in the tube determines the efficacy of centrifugation [19]. Plastic tubes are recommended to minimize contact activation [7]. EDTA is preferred over citrate, because EDTA blocks platelet activation, thereby stabilizing the concentration of EVs in blood samples during blood collection and transport [24].
2. The use of storage vials with a screw lid and rubber ring minimize sublimation, thereby reducing freeze-drying artifacts during storage.
3. In this protocol, a mixture of three different antibody-fluorophore conjugates (CD45-APC, CD61-BV421, CD235a-PE) is used to stain the samples. The fluorophores APC, BV421, and PE have different excitation spectra and therefore require excitation with different lasers, which prevents errors caused by spectral spillover [25, 26].
4. This protocol is based on an A60-Micro flow cytometer and assumes that the flow cytometer is well-maintained and that the optimal voltages of the photomultiplier tubes (PMTs) for EV detection are known. Examples of how to optimize PMTs voltages for flow cytometers can be found in the literature [25, 27]. Aspects in this protocol that are specific to the A60-Micro will be marked with a note. In contrast to many flow cytometers, the A60-Micro is equipped with a syringe pump that allows accurate control (measurement errors <5%) of flow rates <5 $\mu L \cdot min^{-1}$. For the A60-Micro, forward scattered light (FSC) and side scattered light (SSC) are typically called small angle light scattering (SALS) and large angle light scattering (LALS), respectively. Throughout this chapter, the terms FSC and SSC are used.
5. The detailed description of routine laboratory tests to confirm the absence of residual platelets and hemolysis is beyond the scope of this manuscript.
6. At a sheath pressure of 150 mbar, the A60-Micro uses ~1 L of sheath fluid per hour.
7. Flow cytometer specific.
8. The commercial mixtures of beads to calibrate fluorescence and light scattering signals used in this protocol may saturate the detectors of flow cytometers dedicated to the detection of EVs. Consequently, less than the specified number of bead populations may be detected.

9. The slope and intersect of the linear regression can be used to relate the measured fluorescence intensities of EVs in arbitrary units to their fluorescence intensities in standard units of MESF.
10. For experiments that span multiple days, the daily use of calibrants is laborious and expensive. Alternatively, a calibration based on MESF calibrants (*see* **steps 1–3** in Subheading 3.5 followed by **step 1** in Subheading 3.14) can be used to assign MESF values to the bead populations of the QC sample for fluorescence detectors (**step 1** in Subheading 3.6). These assigned MESF values are specific for each fluorescence detector of the flow cytometer. After the MESF assignment, the fluorescence detectors QC sample can be used for a single-step daily calibration of the fluorescence detectors.
11. To enhance the precision of pipetting viscous samples, it is recommended to pipet at least 5 μL of plasma.
12. The primary goal of running 1000-fold diluted, unstained samples is to determine the optimal dilution of each sample. Apart from the optimal dilution, the measurements of 1000-fold diluted, unstained samples are similar to the unstained controls.
13. Subheading 3.7 assumes that swarm detection is absent for a given flow cytometer and sample type when the count rate is below a given critical count rate J_m. For diluted plasma samples measured with a clean and well-maintained A60-Micro, J_m is between 5000 and 10,000 events s^{-1}. J_m can be obtained by titration and is the lowest dilution for which (1) the median light scattering intensity of all particles in the sample and (2) the measured particle concentration remain stable.
14. The optimal sample dilution factor D_o is obtained by two steps (*see* Fig. 1). First, the sample is diluted before staining with the so-called pre-staining dilution factor D_p. Second, the sample is diluted by the staining procedure. During the staining procedure, reagents are added to the samples and after incubation the reagents are diluted to minimize interference of unbound reagents with the measurement. To keep the concentration of unbound reagents constant while allowing different sample dilutions, samples are pre-diluted before staining.
15. The pre-staining dilution factors D_p calculated with Esq. (3)–(5) will have inconvenient values, which may lead to calculation and pipetting errors. Therefore, it is recommended to round up and group the sample-specific values of D_p, for example, to 2, 3, 5, 7, 10, 16, 25, 40, 65, 100, 160, 260. In this series, the dilution factors are selected such that the anticipated count rate of stained samples is between 3000 and 5000 events $\cdot s^{-1}$.

16. BV421 fluorophores form aggregates upon vortexing. To avoid aggregate formation, mix by adding the sample to the reagents and gently stir with the pipette tip.
17. Lower antibody concentrations also improve the cost-effectiveness of an assay.
18. The measurement of a reference plasma sample with a single antibody at the optimal concentration is in fact a single-stained control.
19. Stained plasma samples have different optimal dilutions. Therefore, it is recommended to keep track of the sample dilutions in filenames.
20. Running a full 96-well plate takes ~8 h under the following conditions: (1) use suck and spit before aspiring each sample, (2) the measurement time per sample is 2 min, and (3) rinse the needle and flow cell twice after measuring each sample.
21. Assay controls should be used qualitatively, for example, to confirm that signals are originating from EVs. Assay controls should not be used quantitatively, for example, to apply corrections to the number of fluorescent positive counts measured in EV samples.
22. To use the automatic shutdown function of the A60-Micro, the 12 cleaning and rinsing solutions can also be added to the final row of the 96-well plate with samples.
23. Please note that Eq. (7) is only valid when I_c is plotted versus I_m, like in Fig. 2b.
24. Flow cytometry data analysis software often have an option to parametrize signals. Alternatively, custom software could be developed to read flow cytometry data files and apply Eq. (7) to the measured fluorescence intensities.
25. According to Poisson statistics, the precision of a concentration measurement with flow cytometry in terms of CV is given by $1/\sqrt{n}$, where n is the number of counted events. The counting error of an EV concentration based on 100 counts is therefore 10%, whereas the counting error of an EV concentration based on 10 counts is 32%. Hence, the number of counted events require careful consideration.
26. Avoid expressing flow cytometry data as (%) ratios, because ratios preclude data reproducibility.

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