Supporting Information 3: “Calibration of flow cytometers enables reproducible measurements of extracellular vesicle concentrations and reference range establishment”

MIFlowCyt-EV of study “EV reference range study”

This document aims to provide the minimum information required to reproduce the flow cytometry experiments on extracellular vesicles (EVs) performed in the study “EV reference range study”. This document is based on three published standardization frameworks and guidelines [1–3].

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1 Experiment overview

1.1 Contact details

1.1.1 Experiment leader

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1.1.2 Flow cytometry execution

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1.2 Purpose

The goal of this flow cytometry experiment was to establish reference ranges of cell-type specific EV concentrations in human plasma from 224 healthy volunteers.

1.3 Keywords

Calibration, extracellular vesicles, flow cytometry, human blood plasma, reference ranges

1.4 Experiment variables

Age and gender differ between healthy volunteers whos' blood was collected.

1.5 Experiment design and quality controls

All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate. The entire study involved 12 96-well plates that were measured within 2 months. Each well plate contained a buffer-only control, reagents in buffer controls and isotype controls. Flow rate, and light scattering calibrations were performed daily. Fluorescence calibration was performed once.

1.6 Dates

Samples were collected on 07.10.2019, 08.10.2019 and 09.10.2019 in the Amsterdam University Medical Centers, Location Academic Medical Centre. Flow cytometry experiments were performed between 11.08.2021 and 01.10.2021.

1.7 Conclusions

In this manuscript we established the first-ever reference ranges of erythrocyte-, leukocyte-, and platelet-derived EVs in human blood plasma (n=224). The reference ranges for (1) erythrocyte-derived EVs is $8.54 \cdot 10^6 - 3.88 \cdot 10^7$ EVs/mL, with a median concentration of $2.02 \cdot 10^7$ EVs/mL, (2) leukocyte-derived EVs is $7.58 \cdot 10^6 - 3.03 \cdot 10^7$ EVs/mL with a median concentration of $1.75 \cdot 10^7$ EVs/mL, and (3) platelet-derived EVs is $1.01 \cdot 10^7 - 6.57 \cdot 10^7$ EVs/mL with a median concentration of $3.39 \cdot 10^7$ EVs/mL in cell-depleted human blood plasma. These concentration ranges apply to EVs with a fluorescence intensity >228 MESF PE for erythrocyte-derived EVs, >112 MESF APC for leukocyte-derived EVs, and >77 MESF BV421 for platelet-derived EVs, and a diameter between 150 and 1,000 nm.

2 Sample details

2.1 Sample description

2.1.1 Sample source description

Plasma was prepared from blood of healthy volunteers that were screened for hepatitis B, hepatitis C, and human immunodeficiency virus (HIV).

2.1.2 Sample description

Intravenously collected blood.

2.2 Sample collection

Venous blood was collected in Ethylenediaminetetraacetic acid (EDTA) blood collection tubes (6 mL, 9203871, Becton Dickinson (BD) Biosciences) with a 21-gauge needle (368607, BD Biosciences). The first 3.5 mL of blood was discarded to avoid the activation of endothelial cells and platelets, as well as contamination

2.3 Sample storage

Within 15 minutes from blood collection, plasma was prepared. Therefore, whole blood was centrifuged at 2,500× g for 15 minutes at 20 °C, acceleration speed 9, and deceleration speed 1 using a Rotina 380 R equipped with a swing-out rotor and radius of 155 mm (Hettich Zentrifugen). Plasma was collected 10 mm (determined with a Lego brick) above the buffy coat using a plastic Pasteur pipette (86.1171.001, SARSTEDT) and transferred into a new 15-mL polypropylene centrifuge tube (62.9924272, SARSTEDT). Subsequently, the plasma was centrifuged at the same settings used for whole blood. Afterward, plasma was collected to 10 mm above the pellet to reduce platelet contamination and transferred into a new 15-mL tube. Next, 100 µL plasma was aliquoted into cryogenic vials (72.692.005, SARSTEDT) frozen in liquid nitrogen for 15 minutes, and stored at −80 °C until analysis.

2.4 Sample characteristics

Frozen human plasma samples are expected to contain the following particles: erythrocyte ghosts, EVs, lipoproteins, platelets, precipitated salt crystals, proteins, and complexes of the aforementioned particles.

2.5 Sample dilution

As the concentration of particles in plasma differs >10^2-fold between donors, samples require different dilutions to (1) avoid swarm detection and (2) detect a statistically significant number of events within a measurement time of a few minutes. The optimal dilution factor is the minimum dilution factor that is required to prevent swarm detection. For the flow cytometer and settings used, the optimal dilution factor for plasma is ≥$1.1\cdot10^2$ -fold and should result in a count rate <$1.1\cdot10^4$ events·s^{-1} [4].

To find the dilution resulting in a count rate <$1.1\cdot10^4$ events·s^{-1}, we diluted each plasma sample 200-fold and 2,000-fold in Dulbecco's phosphate buffered saline (DPBS, 21-031-CVR, Corning) and measured the total concentration of particles for 30 s without staining. Figure 1A shows a distribution of the measured total particle concentrations of all samples in the study. Taking into account the measured concentration and flow rate, we calculated the minimum dilution factor required before staining (section 2.6) to achieve a count rate $<1.1 \cdot 10^4$ events$\cdot s^{-1}$ after staining. The staining procedure adds an extra dilution factor of 11.1-fold to the overall dilution. To simplify the pipetting procedures, samples were divided into 12 categories of pre-staining dilution factors: 2-fold, 3-fold, 5-fold, 7-fold, 10-fold, 16-fold, 25-fold, 40-fold, 65-fold, 100-fold, 160-fold, and 260-fold. Figure 1B shows a distribution of the applied dilution factors of all samples in the study.

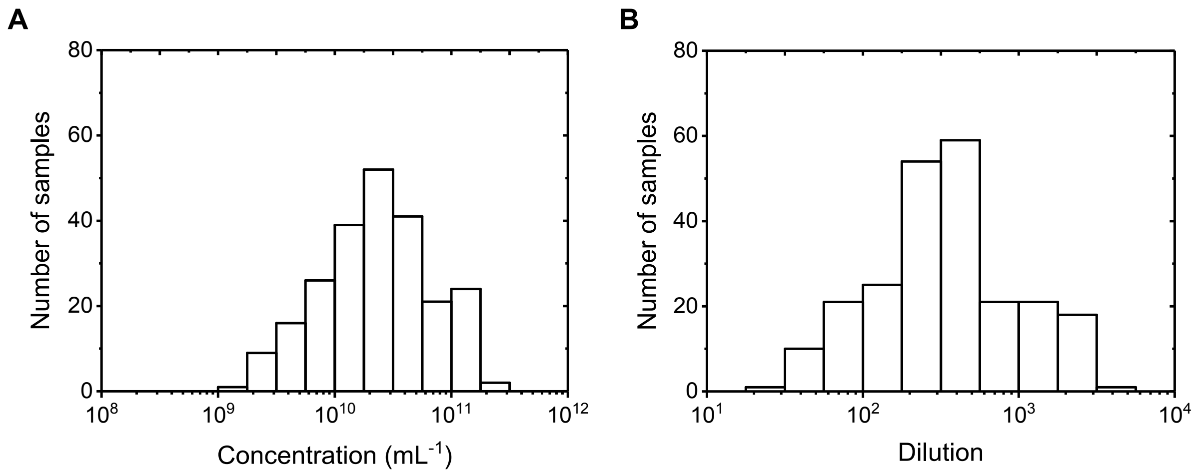

Figure 1: (A) Distribution of the total concentration of particles exceeding the trigger threshold for all samples in the study. (B) Distribution of the applied dilution factors of all samples in the study.

2.6 Sample staining

Table 1 shows an overview of the antibodies that were used to stain EVs in plasma. Prior to staining, the antibodies were diluted in DPBS. For each antibody the optimal dilution factor was determined by titration (Table 1). To remove aggregates, diluted antibodies were centrifuged at 18,890 g for 5 min at 20 °C. The supernatant minus 10 µL of the starting volume was collected and used for staining. Each sample was stained with an antibody cocktail of CD45-allophycocyanin (APC),CD61-brilliant violet 421 (BV421), and CD235-phycoerythrin (PE), or Immunoglobulin G (IgG)-APC, IgG-BV421, and IgG-PE. For the staining, 20 µL of pre-staining diluted (Figure 1B) plasma was incubated with 2.5 µL of each antibody or isotype control and kept in the dark for 2 hours at room temperature. After the incubation, samples were diluted in 200 µL DPBS to decrease background fluorescence from unbound reagents.

2.7 Fluorescence reagents

Characteristic measured	Analyte	Analyte detector	Reporter	Isotype	Clone	Concentration (µg mL^{-1})	Manufacturer	Catalog number	Lot number
Leukocyte common antigen	CD45	Anti-human CD45 antibody	APC	IgG1	HI30	4.5	Biolegend	340910	B272158
Integrin	Human CD61	Anti-human CD61 antibody	BV421	IgG1	VI-PL2	3.125	Beckman Dickinson Biosciences	744381	191784
Glyco-protein	CD235a	Anti-human CD235a antibody	PE	IgG1	JC159	12.5	Dako	R7078	20079786
Affinity for Fc receptor	Fc receptor	IgG1	APC	n.a.	MPOC21	4.5	Beckman Dickinson Biosciences	554681	8261691
	Fc receptor	IgG1	BV421	n.a.	X40	3.125	Beckman Dickinson Biosciences	659453	8242928
	Fc receptor	IgG1	PE	n.a.	X40	12.5	Beckman Dickinson Biosciences	345816	035605

Table 1. Overview of staining reagents. Characteristics being measured, analyte, analyte detector, reporter, isotype, clone, concentration during staining, manufacturer, catalog number and lot number of used staining reagents. The concentration of staining reagents during measurements was 11.1-fold lower than the concentration during staining. APC: allophycocyanin; BV: brilliant violet; CD: cluster of differentiation; IgG: immunoglobulin G; PE: phycoerythrin.

3 Flow cytometer

3.1 Model and manufacturer

A60-Micro, Apogee Flow Systems, Hemel Hempstead, UK. The flow cytometer has not been altered. All components are original and came with the flow cytometer.

3.2 Configuration and settings

3.2.1 Flow rate and acquisition time

The flow cytometer is equipped with a syringe pump with volumetric control. Samples were analysed for 120 s at a flow rate of 3.01 µL/min.

3.2.2 Light sources

The flow cytometer has three lasers that illuminate a fixed-alignment cuvette flow cell. The adjusted powers were 100 mW, 100 mW and 100 mW for the 405-nm, 488-nm, and 638-nm laser, respectively.

3.2.3 Detectors

Table 2 shows an overview of the detectors used in this study.

Detector name	Detected property	Voltage (V)	Spectral filter bandwidth (nm)
405-SALS	Forward scattered light	470	
405-LALS	Side scattered light	375	
405-Blue	Brilliant violet fluorescence	460	445/50
488-Orange	PE fluorescence	450	575/30
638-D Red	APC fluorescence	480	>650

Table 2. Detector name, detected property, voltage and spectral filter bandwidth of the detectors used in this study. APC: allophycocyanin; CD: cluster of differentiation; PE: phycoerythrin.

3.2.4 Trigger detector and threshold

Based on the buffer-only control, a trigger threshold of 24 arbitrary units was applied to the side scattering detector. As (1) the A60-Micro applies the trigger threshold analogically, thus before digitalization of the signal, and (2) the arbitrary unit channel numbers differ between the data acquisition software and the flow cytometry datafiles, the trigger threshold was expressed in standard units by taking the modes of the effective side scattering cross section distribution and optical diameter distribution (section 5.3.3.1) of a plasma sample. Plasma samples have a size distribution with a peak far below the detection limit of the A60-Micro. The modes of the effective side scattering cross section distribution and optical diameter distribution therefore represent the trigger threshold. The trigger threshold is equivalent to an effective side scattering cross section of 8 nm^2 and an optical diameter of 154 nm for EVs[1].

4 Assay controls

Assay controls recommended by the MIFlowCyt-EV framework were performed to confirm that signals originate from EVs. Fluorescence-minus-one and single-stained controls were not performed due to thorough experience with the used antibody panels and because the emission spectra of the used fluorophores do not have spectral overlap. Procedural controls were not performed because no methods to isolate EVs were applied after staining. Serial dilution control were performed on the A60-Micro for six representative plasma samples and results were published [4]. Section 2.5 explains how swarm detection was prevented.

4.1 Buffer-only controls

Each 96-well plate contained 2 wells with DPBS, which were measured with the same flow cytometer and acquisition settings as all other samples. The median count rate for all DPBS measurements was 16 events s^{-1}, which is lower than the target count rate (2.5-5.0·10^3 events s^{-1}) for events in stained plasma samples.

4.2 Buffer with reagents controls

Each 96-wellplate contained a buffer with reagent control for each reagent (Table 1), which was measured with the same flow cytometer and acquisition settings as all other samples. Table 3 shows a summary of the results of the buffer with reagents controls.

Reagent	Mean number of fluorescence positive events in buffer (120 s^{-1})	Mean number of fluorescence positive events in stained samples (120 s^{-1})	Mean number of fluorescence positive events in buffer / stained samples (-)
CD45-APC	10	539	0.019
CD61-BV421	108	980	0.110
CD235a-PE	42	716	0.059

Table 3. Results of the buffer with reagents controls. APC: allophycocyanin; BV: brilliant violet; CD: cluster of differentiation; PE: phycoerythrin.

[1] EVs are modelled as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm.

4.3 Unstained controls

Unstained controls were measured with a 200-fold and 2,000 -fold dilution factor, which differs from the dilution factor with which the stained plasma samples were measured. Unstained controls were not considered in this analysis.

4.4 Isotype controls

Each sample was stained with IgG1, which was measured with the same flow cytometer and acquisition settings as all other samples. For each sample the ratio of the number of fluorescence positive events in isotype control and the number of fluorescence positive events in the stained sample was determined. When the ratio between the IgG1 control and stained sample was >0.15, the sample was excluded from further analysis.

4.5 Detergent treatment controls

Detergent treatment control experiments were performed prior to measuring the healthy volunteer samples. For the detergent treatment control NP-40 was added to a pooled plasma sample, which contains blood plasma from 8 healthy volunteers. The sample was measured with the same flow cytometer and acquisition settings as all other samples. Table 4 shows a summary of the results of the detergent treatment controls.

Reagent	Number of fluorescence positive events after detergent treatment ($120\ s^{-1}$)	Number of fluorescence positive events in stained sample ($120\ s^{-1}$)	Mean number of fluorescence positive events after detergent treatment / stained samples (-)
CD45-APC	51	613	0.083
CD61-BV421	472	5216	0.090
CD235a-PE	53	1656	0.032

Table 4. Results of the detergent treatment controls. APC: allophycocyanin; BV: brilliant violet; CD: cluster of differentiation; PE: phycoerythrin.

5 Data analyses

To automatically determine optimal samples dilutions, apply calibrations, determine and apply gates, generate reports with scatter plots and generate data summaries, we developed and applied custom-build software (MATLAB R2020b, Mathworks, USA).

5.1 Data sharing

Data is available via: https://figshare.com/s/60590e618d96cc1374f8

5.2 Compensation details

No compensation was applied because no fluorophore combinations were used that have overlapping emission spectra.

5.3 Calibrations

5.3.1 Flow rate

At the start of each measurement day, we applied the automated quality control system ApoCal (#1524, Apogee Flow Systems), which checks whether the flow rate is within 20% of the adjusted flow rate of 3.01 $\mu L \cdot min^{-1}$. For all days, the flow cytometer passed this quality control check.

5.3.2 Fluorescence calibration

Calibration of the fluorescence detectors from arbitrary units (a.u.) to molecules of equivalent soluble fluorochrome (MESF) was accomplished using 2 µm APC quantification beads (lot 2364-175, custom-order, Becton Dickinson Biosciences), 3 µm BV421 quantification beads (lot MM2337-25, custom-order, Becton Dickinson Biosciences), and SPHERO™ Calibration Particles PE (catalogue number ECFP-F2-5K, lot AK01, Spherotech Inc). Figure 2A, C, and D shows the 10-base logarithm of the specified MESF intensities versus the 10-base logarithm of the measured median fluorescence intensity of each bead population for the APC, BV421, and PE calibration, respectively. The data are fitted with a linear function. These fluorescence calibrations were used to assign MESF values for APC, BV421, and PE to rainbow beads (SPHERO™ Rainbow calibration particles, 8 peaks, 3.0-3.4 µm, lot EAM02, Spherotech Inc). In turn, the rainbow beads, which are hard dyed beads with long-term stability, were used to apply fluorescence calibrations. Figure 2 B, D, and F show the 10-base logarithm of the assigned MESF intensities for rainbow beads versus the 10-base logarithm of the measured median fluorescence intensity of each bead population. The data are fitted with a linear function. For each measured plasma sample, we added fluorescent intensities in MESF units to the flow cytometry data files using following equation:

$\mathrm{I(MESF)} = 10^{a \cdot \log_{10} \mathrm{I(a.u.)} + b}$	Equation 1

where I is the fluorescence intensity, and *a* and *b* are the slope and the intercept of the linear fits in Figure 2.

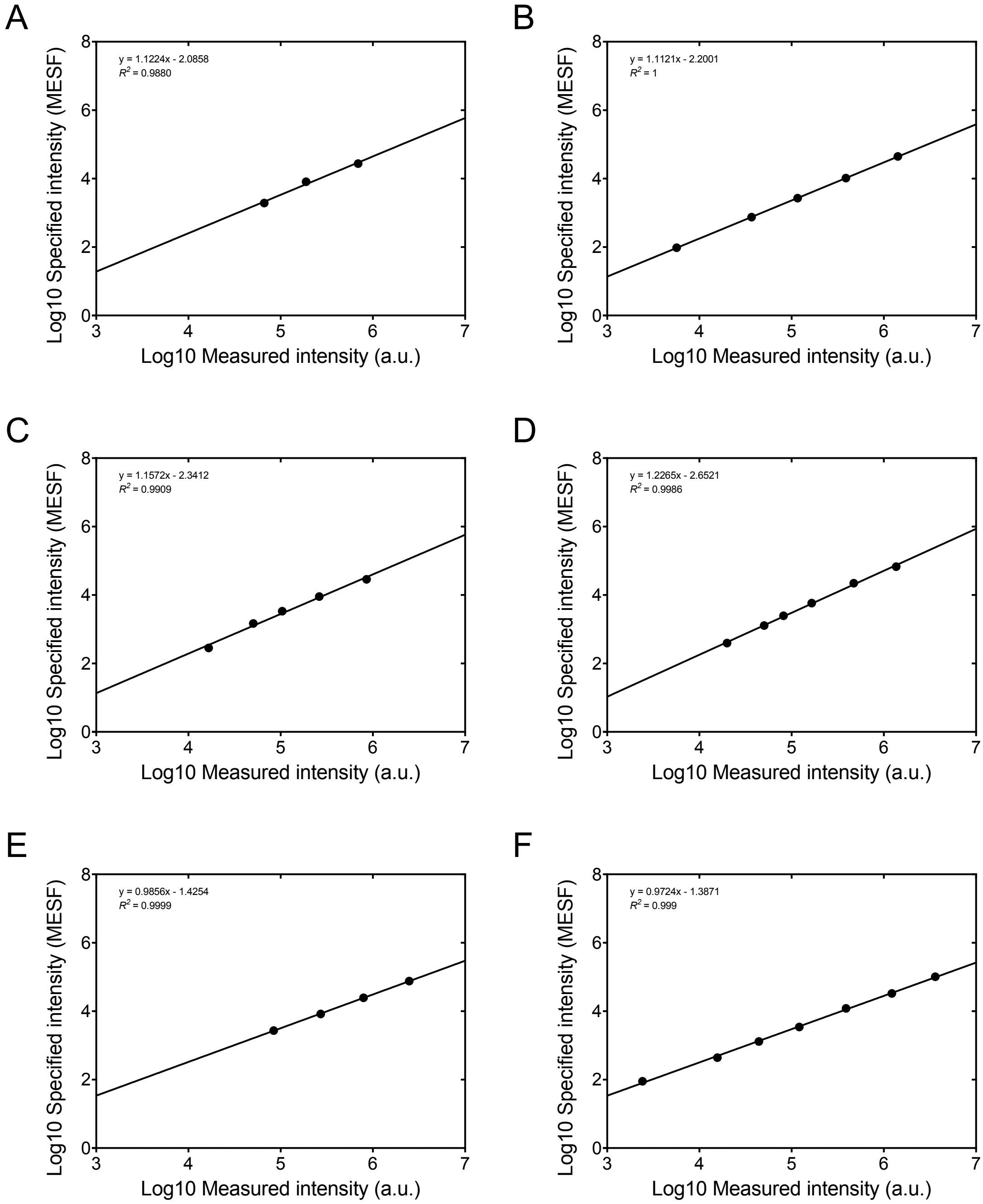

Figure 2. Calibration of the fluorescence detectors from arbitrary units (a.u.) to molecules of equivalent soluble fluorochrome (MESF). Logarithmic MESF versus logarithmic median fluorescence intensity for (A) allophycocyanin (APC), (C) brilliant violet (BV)and (E) phycoerythrin (PE). Furthermore, the 10-base logarithm of the assigned MESF intensities for rainbow beads versus 10-base logarithm of the measured median fluorescence intensity of each bead population are shown for APC (B), BV (D), and PE (F). Data (symbols) are fitted with a linear function (line).

5.3.3 Light scattering calibration

5.3.3.1 Rosetta Calibration

Rosetta Calibration (v2.05, Exometry, The Netherlands) was used to relate the forward and side scattering intensities measured at a wavelength of 405 nm to the effective scattering cross sections[2] and optical diameter[3] of EVs. EVs are modelled as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm. Figure 3 shows print screens of the light scatter calibrations.

A. Side scattering calibration

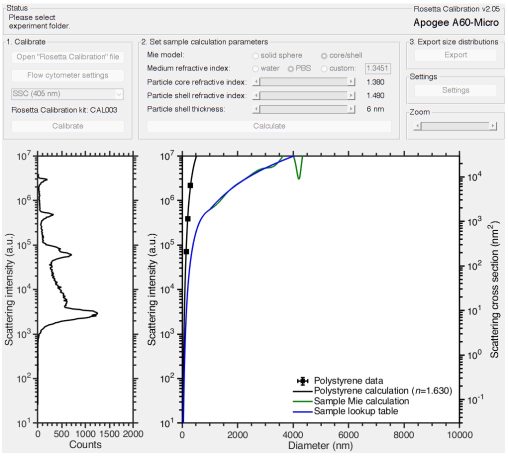

Figure 3. Side scattering calibration of the A60-Micro by Rosetta Calibration. To relate scatter to the diameter of EVs, EVs are modelled as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm.

5.4 Gate description and boundaries

The following gates has been applied to all flow cytometry data files by custom-build software (MATLAB R2020b):

1. During the second that an event was measured, the count rate was within 3.5 standard deviations from the median count rate of the entire measurement, and…
2. the condition in Equation was fulfilled to remove antibody aggregates (only applied to the CD61-BV421 antibodies), and…
3. the optical diameter is <1,000 nm, and…

    3.1.1.the CD45-APC fluorescence > 112 MESF.

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[2] The effective scattering cross section is a hypothetical area of a particle that incoming light must impinge in order to be scattered towards the lens. The calibrated effective scattering cross section axis is independent of refractive index assumptions, but depends on the illumination wavelength and collection angles of the flow cytometer [7].

[3] The optical diameter equals the physical diameter of a particle when (1) the particle is spherical and (2) the particle has the same refractive index distribution as assumed in the model.

3.1.2.the CD61-BV421 fluorescence > 77 MESF.

3.1.3.the CD235a-PE fluorescence > 228 MESF

$\log_{10} I_{BV421} > -1.1 \cdot \log_{10} \sigma_{SSC} + 1.3$	Equation 2

Here, I_{BV421} is the fluorescence intensity of the BV421 detector and σ_{SSC} is the effective side scattering cross section. PDF files with scatter plots of all applied gates are available via the online data repository (section 5.1).

6 References

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