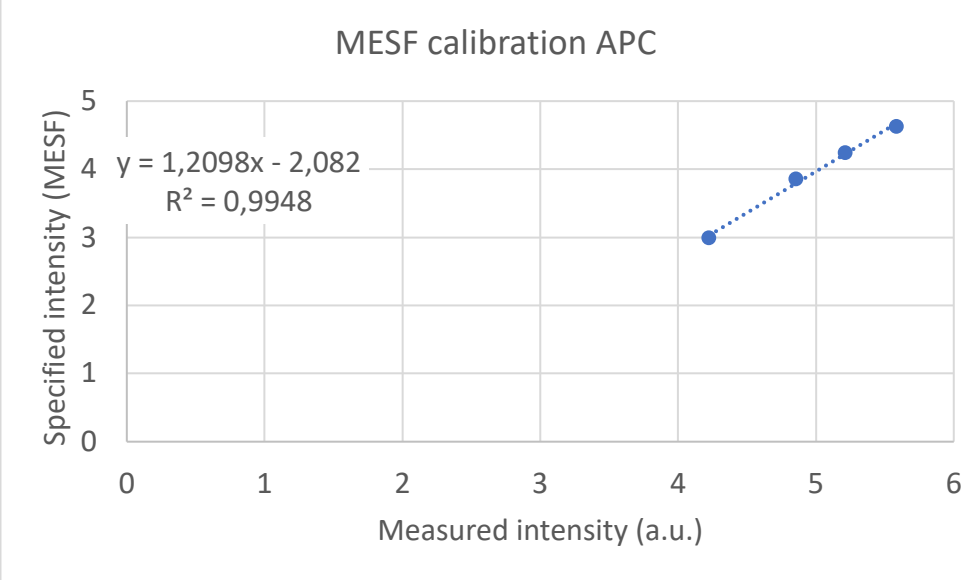
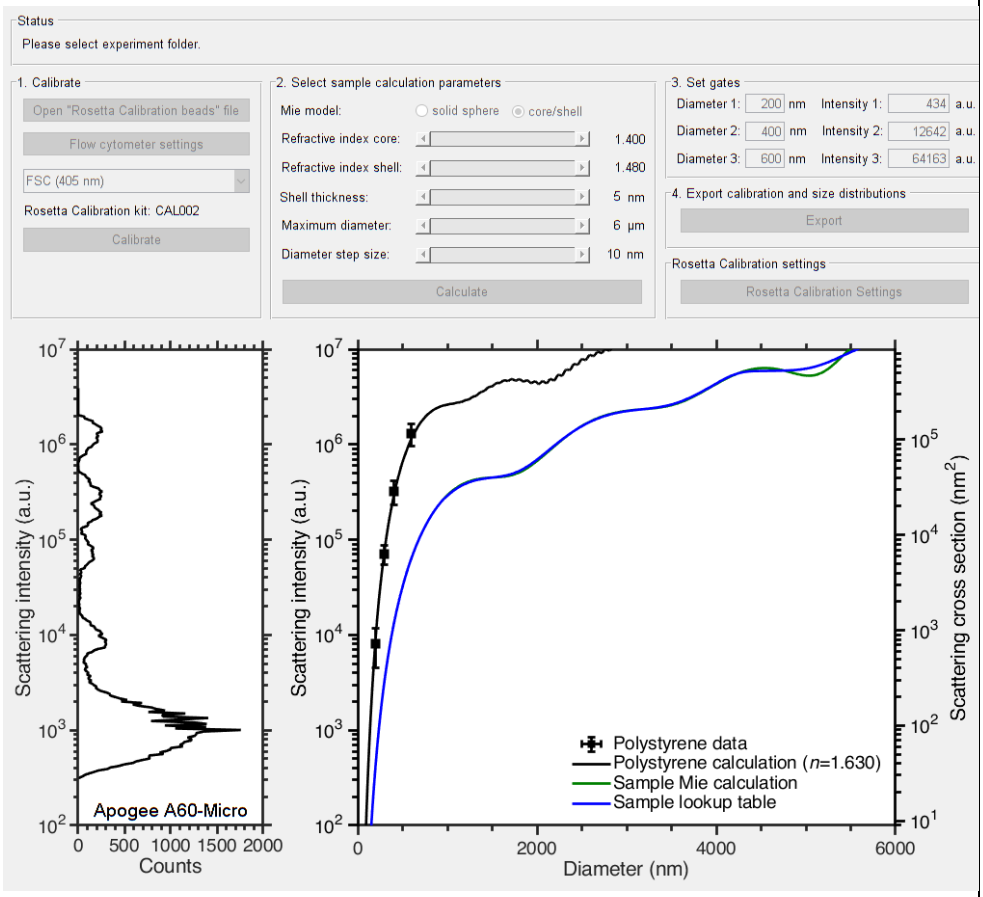
Supporting information 2: MIFlowCyt checklist of "Removal of platelets from blood plasma to improve the quality of extracellular vesicle research"

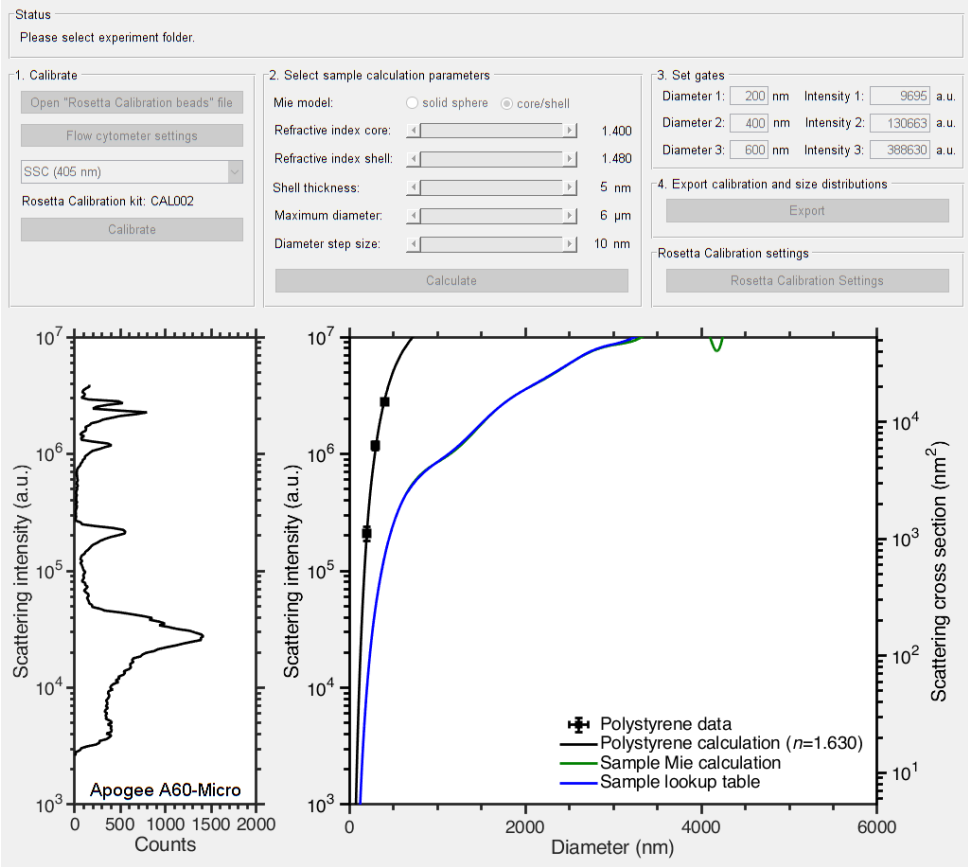
Requirement	Please Include Requested Information
1.1. Purpose	The aim of the flow cytometry experiment was to determine the concentration of platelets stained with CD61-APC in fresh double centrifuged plasma of 224 healthy individuals. We hypothesized that the platelet concentration ranges between 10^5 and 10^7 mL^{-1}.
1.2. Keywords	Platelets, plasma
1.3. Experiment variables	Blood is collected from 224 different donors
1.4. Organization name and address	Amsterdam University Medical Centers Location Academic Medical Centre Meibergdreef 9 1105 AZ Amsterdam The Netherlands
1.5. Primary contact name and email address	Edwin van der Pol, e.vanderpol@amsterdamumc.nl
1.6. Date or time period of experiment	October 7th, 8th and 9th 2019
1.7. Conclusions	The concentration of platelets ranged from $6.0 \cdot 10^4$ mL^{-1} to $9.8 \cdot 10^6$ mL^{-1}, with a mean concentration of $5.1 \cdot 10^5$ mL^{-1} and a standard deviation of $7.0 \cdot 10^5$ mL^{-1}.
1.8. Quality control measures	The adjusted flow rate was 75 µL min^{-1} and validated with Rosetta Calibration beads (Exometry, Amsterdam, The Netherlands). The measured flow rates were between 69 and 88 µL min^{-1}. To calculate platelet concentrations, we assumed a flow rate of 75 µL min^{-1}, because the A60-Micro is equipped with a syringe pump with volumetric control. The APC detector was calibrated with 2 µm Q-APC beads (2321-175, BD). The FSC and SSC detectors were calibrated with Rosetta Calibration beads and software v1.11 (Exometry).
1.9. Other relevant experiment information	The experiment was conducted within three days. Three samples were omitted from the analyses, because the flow cytometer failed to start the measurement and collect data.
2.1.1.1. Sample description	Freshly prepared double-centrifuged plasma (section 2.1.1.2) from 224 healthy volunteers (section 2.1.1.3).
2.1.1.2. Biological	Venous blood was collected from 224 healthy individuals who denied having a disease or using drugs and/or medication.

sample source description	
2.1.1.3. Biological sample source organism description	Healthy human volunteer.
2.2 Sample characteristics	Plasma is expected to contain detectable extracellular vesicles, lipoproteins proteins, and platelets.
2.3. Sample treatment description	Venous blood was collected from 224 healthy individuals who denied having a disease or using drugs and/or medication. Venous blood was collected using a 21-Gauge needle, and the first 3.5 mL of blood was discarded. One tube with 6 mL of EDTA blood (BD Biosciences) was collected, mixed gently, and processed within 15 minutes. To prepare plasma, the blood collection tube was double centrifuged using a Rotina 380 R equipped with a swing-out rotor and radius of 155 mm (Hettich Zentrifugen, Tuttlingen, Germany). Whole blood was double centrifuged at 2,500 g, 15 minutes, 20°C, acceleration speed 9, deceleration speed 1. Plasma was collected 10 mm above the buffy coat with a plastic Pasteur pipette (VWR, Radnor, PA) and transferred into 15-mL polypropylene centrifuge tubes (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). Subsequently, plasma was centrifuged at 2,500 g, 15 minutes, 20°C, acceleration speed 9, deceleration speed 1. Plasma was collected to 10 mm above the pellet, transferred into a new 15-mL polypropylene centrifuge tube (Greiner Bio-One B.V.), mixed by pipetting, and further transferred to 0.5-mL low protein binding tubes (Sarstedt, Nümbrecht, Germany). The concentration of platelets was measured in diluted, stained fresh plasma.
2.4. Fluorescence reagent(s) description	Table S2.1 contains all details about the staining reagent. Prior to staining, samples were diluted 5-fold in DPBS (21-031-CV, Corning, USA). To remove antibody aggregates, anti-human CD61-APC antibody (17-0619-42, eBioscience; clone VI-PL2; final concentration 8.33 µg/mL) was diluted in DPBS and centrifuged at 18,890 · g for 5 minutes. To measure the concentration of platelets, 40 µL of 5-fold diluted plasma was incubated with 5 µL of antibodies and kept in the dark for 30 minutes at room temperature. Next, samples were further diluted by adding 400 µL of DPBS and measured by flow cytometry.
3.1. Instrument manufacturer	Apogee Flow Systems (Hemel Hempstead, UK)
3.2. Instrument model	A60-Micro
3.3. Instrument configuration and settings	Stained samples were analysed for 120 seconds at a flow rate of 75 µL min^{-1} on an A60-Micro, equipped with a 405 nm laser (100 mW), 488 nm laser (100 mW) and 638 nm laser (75 mW). The trigger threshold was set at 14 arbitrary units SSC and 100 arbitrary units APC fluorescence, which corresponds to ~350 MESF. For FSC and SSC, the PMT voltages were 380 V and 350 V, respectively. For all detectors, the peak height was

	analysed. APC signals were collected with the 638-D Red (Peak) detector (long pass 652 nm filter, PMT voltage 510 V).
4.1. List-mode data files	The .fcs files will become available via https://doi.org/10.6084/m9.figshare.19130219
4.2. Compensation description	Not applicable.
4.3. Data transformation details	Calibration of the APC fluorescence detector: 

Calibration of the forward scattered light detector:



	Calibration of the side scattered light detector: 
4.4.1. Gate description	To automatically apply gates, generate pdf reports with scatter plots, and summarize the data in a table, custom-build software (MATLAB R2018b) was used. Please find below a description of the applied gating strategy. First, only events that were collected during time intervals, for which the count rate was within 50% of the median count rate, were included. Second, events with a fluorescent brightness >12,000 MESF APC were included to select platelets.
4.4.2. Gate statistics	The number of positive events was corrected for flow rate, measurement time and dilutions performed during sample preparation.
4.4.3. Gate boundaries	An overview of all gates can be found in the compressed data summary files https://doi.org/10.6084/m9.figshare.19130219

APC: allophycocyanin; CD: cluster of differentiation; DPBS: Dulbecco's phosphate-buffered saline; EDTA: thylenediaminetetraacetic acid; FSC: forward scattering; MESF: molecules of equivalent soluble fluorophores; PMT: photomultiplier tube; SSC: side scattering.

Table S2.1: Overview of staining reagents. Characteristics being measured, analyte, analyte detector, reporter, isotype, clone, concentration, manufacturer, catalog number and lot number of used staining reagents. The antibody concentration during measurements was 11.3-fold lower than the antibody concentration during staining

Characteristic measured	**Analyte**	**Analyte detector**	**Reporter**	**Isotype**	**Clone**	**Concentration during staining (µg mL^{-1})**	**Manufacturer**	**Catalog number**	**Lot number**
Integrin	Human CD61	Anti-human CD61 antibody	APC	IgG1	YI-PL2	8.33	Invitrogen	17-0619-42	2026494

APC: allophycocyanin; CD: cluster of differentiation.