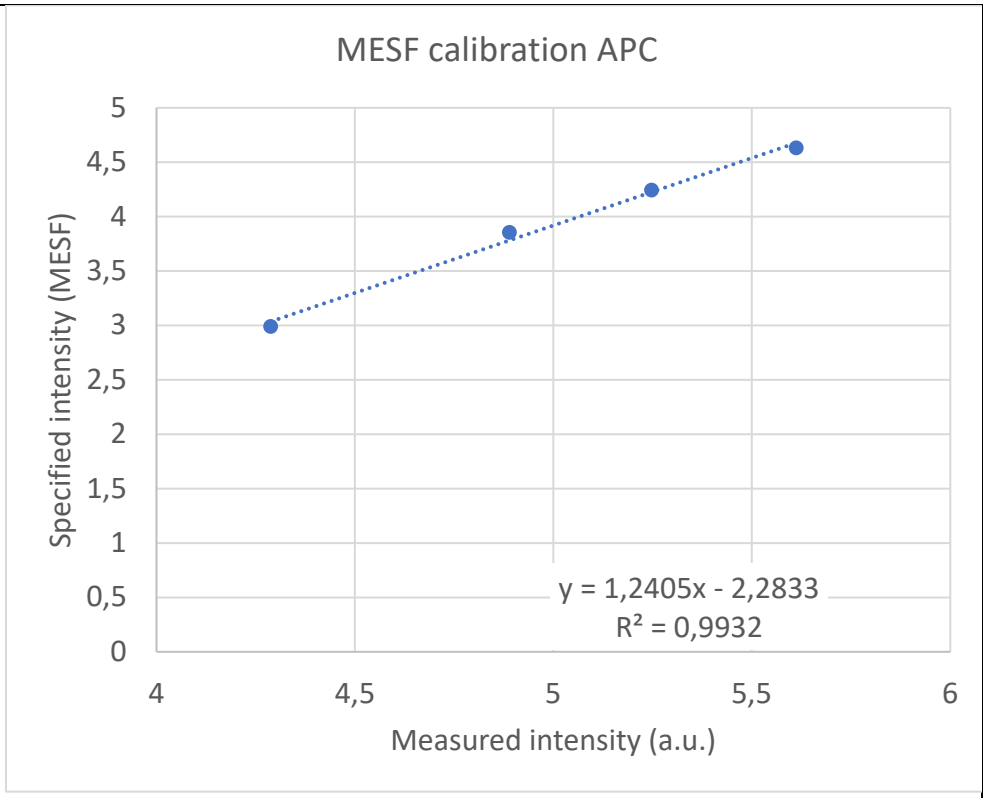
Supporting information 1: MIFlowCyt checklist of Study B "Removal of platelets from blood plasma to improve the quality of extracellular vesicle research"

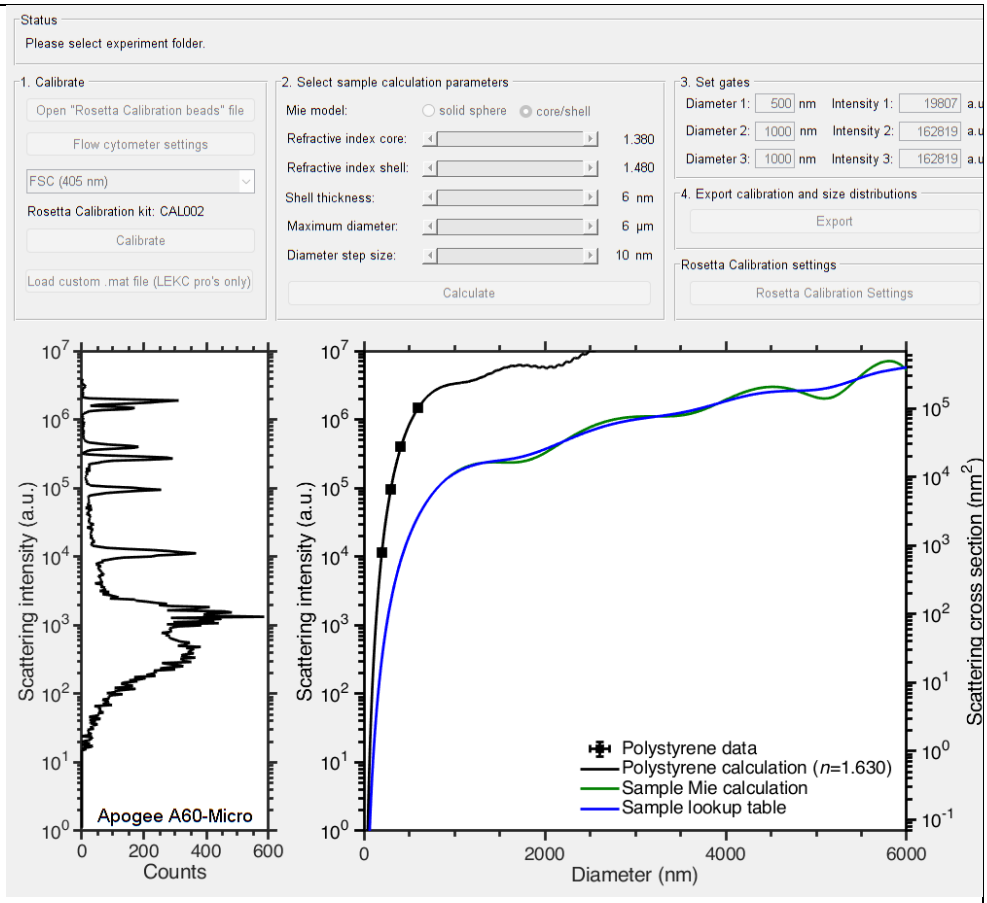
Requirement	Please Include Requested Information
1.1. Purpose	The aim of the flow cytometry experiment was to determine the concentration of platelets stained with CD61-APC in double centrifuged plasma of 10 healthy individuals after freeze-thawing. We hypothesized that the platelet concentration ranges between 10^5 and 10^7 mL^{-1}.
1.2. Keywords	Platelets, plasma
1.3. Experiment variables	Blood is collected from 10 healthy donors and anticoagulated with citrate, dipotassium ethylenediaminetetraacetic acid (K2 EDTA; spray), K2EDTA (gel plug), or ACD-A.
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	February 11th, February 14th, February 18th to 20th, April 2nd and 3rd, 2019
1.7. Conclusions	The concentration of platelets ranged from $1.1 \cdot 10^5$ mL^{-1} to $2.3 \cdot 10^6$ mL^{-1}, with a mean concentration of $9.6 \cdot 10^5$ mL^{-1} and a standard deviation of $7.1 \cdot 10^5$ mL^{-1}.
1.8. Quality control measures	The adjusted flow rate was 3.0 µL min^{-1} and validated with Rosetta Calibration beads (Exometry, Amsterdam, The Netherlands). The measured flow rate was 2.95 ± 0.19 µL min^{-1} (mean ± standard deviation). To calculate platelet concentrations, we assumed a flow rate of 3.0 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 in seven days.
2.1.1.1. Sample description	Double-centrifuged plasma (section 2.1.1.2) from 10 healthy volunteers (section 2.1.1.3) collected with 4 different, commonly used anticoagulants (citrate, K2 EDTA [spray], K2EDTA [gel plug], or ACD-A), snap frozen in liquid nitrogen, stored at -80 °C and thawed at 37 °C.

2.1.1.2. Biological sample source description	Venous blood was collected from 10 healthy individuals who denied having a disease or using drugs and/or medication. Collection of biological samples was according to the Ethical Committee of Ghent University Hospital approval EC/2015/0260 and in accordance to the guidelines and regulations of the Helsinki Declaration. Participants had given written informed consent.
2.1.1.3. Biological sample source organism description	Healthy human volunteers.
2.2 Sample characteristics	Plasma is expected to contain detectable extracellular vesicles, lipoproteins proteins, and platelets.
2.3. Sample treatment description	A 21-Gauge straight needle was used for venipuncture of an antecubital vein after applying a light tourniquet. The first few milliliters of blood were collected in a serum tube and discarded. Subsequently, blood was collected in 4 blood collection tubes, each containing different anticoagulants and/or preservatives: citrate (Vacuette sodium citrate 3.2%, volume 9 ml, Greiner Bio-One), EDTA spray (Vacutainer K2EDTA, volume 10 ml, BD Biosciences), EDTA gel (Vacuette K2EDTA with separator, volume 8 ml, Greiner Bio-One), and ACD-A (Vacutainer ACD Solution A, volume 8.5 ml, BD Biosciences). The drawing sequence of collection tubes was randomized. Time between start and finish of blood collection was not more than 5 minutes. To mix anticoagulants with blood, each blood collection tube was inverted by turning the tubes vertically for 180° and back to the starting position ten times immediately after collection, as per manufacturer's instructions. The blood collection tubes were held in a rack in an upright position at room temperature (20° C). Venipuncture was performed within the same laboratory as sample preparation and samples were not transported. Centrifugation was performed exactly 60 min after collection of the last tube. The ISTH protocol for preparation of platelet depleted plasma was applied. Platelet depleted plasma was prepared by two serial centrifugations at 2,500·g for 15 min at 20 °C using an Eppendorf 5810 R (Eppendorf, Hamburg, Germany) benchtop centrifuge. No brake was applied. After each centrifugation step, plasma was transferred to a clean 5-mL polypropylene centrifuge tube (Eppendorf). At least 5 mm of plasma was left in the tube to avoid contamination with cells. The concentration of residual platelets was measured by a hematology analyzer (XP-300, Sysmex), which has a detection limit of 10^7 platelets/mL. If residual platelets were detected, one additional centrifugation step was performed. Platelet depleted plasma was aliquoted, snap frozen in liquid nitrogen, and stored at -80 °C until analysis.
2.4. Fluorescence reagent(s) description	Table S1.1 contains all details about the staining reagent. Prior to staining, samples were diluted 2.5-fold to 160-fold in DPBS (21-031-CV, Corning, USA) to achieve a count rate <5,000 events/s upon measuring the stained samples, thereby preventing swarm detection for our assay and instrument. To remove antibody aggregates, anti-human CD61-APC antibody (17-

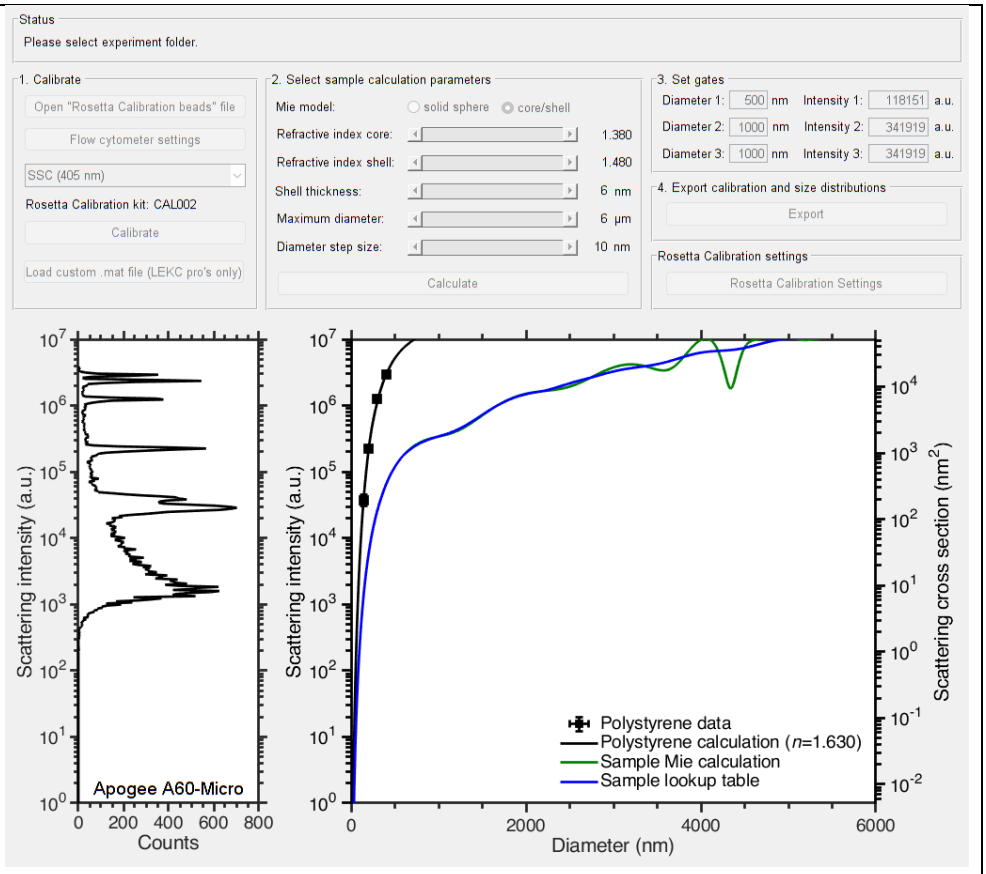
	0619-42, eBioscience; clone VI-PL2; final concentration 12.5 µg/mL) and Lactadherin-FITC (BLAC-FITC, Haematologic Technologies; final concentration 83.3 µg/mL) was diluted in DPBS and centrifuged at 18,890 · g for 5 minutes. To measure the concentration of platelets, 20 µL of diluted plasma was incubated with 2.5 µL of each antibody and kept in the dark for 120 minutes at room temperature. Next, samples were further diluted by adding 200 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 3.0 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, which corresponds to a side scattering cross section of ~10 nm^2 (Rosetta Calibration). For FSC and SSC, the PMT voltages were 380 V and 350 V, respectively. For all detectors, the peak height was analyzed. 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.19122668
4.2. Compensation description	Not applicable.
4.3. Data transformation details	Calibration of the APC fluorescence detector:



Calibration of the forward scattered light detector:

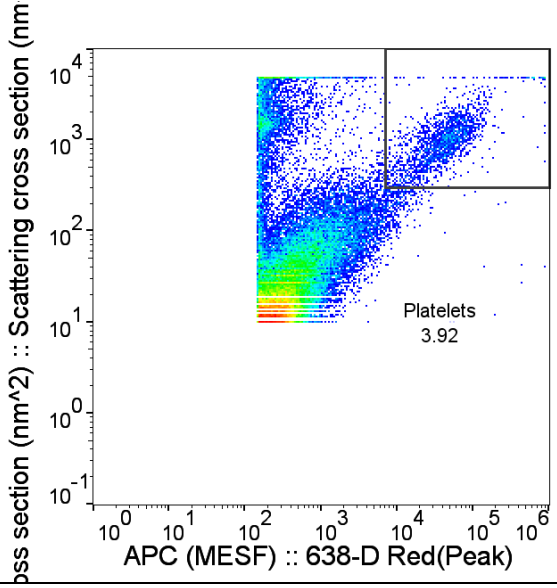


Calibration of the side scattered light detector:



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 25% of the median count rate, were included. Platelets were defined as events with a fluorescent brightness >7,000 MESF APC and a side scattering cross section exceeding 300 nm^2. The next figure shows the platelets gate applied to the concatenated data of all 40 measurements.



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.19122668

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

Table S1.1: 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
Phosphatidyl-L-Serine		Bovine Lactadherin	FITC	none	none	83.3	Haematologic Technologies	BLAC-FITC	HH0216

APC: allophycocyanin; CD: cluster of differentiation; FITC: fluorescein.