Supporting information 4: Material and Methods for Figure 4, *"Standardization of extracellular vesicles concentration measurements by flow cytometry: the past, present and future"*

1.1. Experimental design

The aim of the flow cytometry (Apogee A60-micro, Apogee Flow Systems) experiment was to provide an example of good practice regarding flow cytometry data in form of a calibrated sample versus (vs.) arbitrary unit data. For this figure one sample of plasma was labelled with anti-human CD61-allophycocyanin (APC) to identify platelet-derived EVs with a flow cytometer (FCM). An Apogee A60-micro instrument optimized for detection of EVs was chosen for this experiment.

1.2. Blood collection and preparation of blood plasma

Blood was collected from 1 healthy individual 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 Ethylenediaminetetraacetic acid blood (9203871, 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) 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.) 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 and transferred into a new tube and pooled.

1.3. Staining platelet-derived EVs in plasma for flow cytometry

The plasma sample was 20-fold pre-diluted in Dulbecco's Buffered Saline (dPBS; 21-031-CVR, CORNING) to event rates below 5,000/s to further prevent swarm (1). EVs present in plasma were immuno-fluorescently stained with anti-human CD61-APC. Before staining,

aggregates in the antibody were removed by centrifugation at 18,890 g for 5 minutes at 20 °C. The supernatant minus 10 µL of the starting volume was collected and used for staining. Table S1 contains an overview of the staining reagents. 20 µL pre-diluted plasma were incubated for 2 hours at room temperature in the dark, with 2.5 µL of the antibody. Afterward, 200 µL dPBS were added and the sample was measured by flow cytometry.

1.4. Flow cytometry

The stained sample was measured for 120 seconds at a flow rate of 3.01 µL/min on a Apogge 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 side scattering (SSC) 14 arbitrary units, corresponding to a side scattering cross section of 10 nm^2 (Rosetta Calibration). For forward scattering (FSC) and SSC, the 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).

1.5. Software and statistics

Data analysis was performed by custom-build software (MATLAB R2020b) to automate data calibration and data processing. Graphs were made with custom-build software (MATLAB R2020b) and Adobe Illustrator (V 26.2.1).

1.6. Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.22336324.v1; https://doi.org/10.6084/m9.figshare.22336321.v1

1.7. References

1. van der POL E, van GEMERT MJC, STURK A, NIEUWLAND R, van LEEUWEN TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. Journal of Thrombosis and Haemostasis [Internet]. 2012 May 1;10(5):919–30. Available from: https://doi.org/10.1111/j.1538-7836.2012.04683.x

Table S1: Overview of staining reagents. Characteristics being measured, analyte, analyte detector, reporter, isotype, clone, concentration, manufacturer, catalog number and lot number of used staining reagents.

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	VI-PL2	25	Invitrogen	17-0619-42	2062626

APC: allophycocyanin; CD: cluster of differentiation; IgG: Immunoglobulin G.