Supplementary materials: MIFlowCyt-EV

1 Flow cytometry

1.1 Experimental design

The aim of flow cytometry (A60-Micro, Apogee Flow Systems, Hemel Hempstead, UK) was to determine the concentrations of extracellular vesicles (EV) labeled with CD61 (platelet origin), CD235a (erythrocyte origin), and lactadherin (phosphatidylserine), and platelets labelled with CD61 in serum and platelet-depleted plasma (PDP) collected with blood collection tubes (BCT) containing different anticoagulant or stabilization additives and prepared following increasing blood processing intervals (BPI). We hypothesised that (1) EV concentrations (of all types) and residual platelet concentrations are affected by the choice of BCT and BPI, (2) lipoprotein particle concentrations are unaffected by the choice of BCT, (3) serum contains the highest concentration of EV, and (4) EV size distributions are unaffected by the choice of BCT.

All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate. The entire study involved seven 96-well plates that were measured between February 11th 2019 and April 3rd 2019. Each well plate contained buffer-only controls, antibody in buffer controls, isotype controls, and unstained controls. Flow rate and scatter calibrations were performed daily. Fluorescence calibrations were performed once during the entire period. To automatically determine optimal samples dilutions, apply calibrations, determine and apply gates, and generate graphical and numerical data reports, we used custom-built software (MATLAB R2018b, Mathworks, Natick, MA, USA).

1.2 Sample dilutions

As the particle concentration in serum and PDP differs between individuals, BCT and BPI, samples require different dilutions to (1) avoid swarm detection [1] and (2) achieve statistically significant counts within a clinically applicable measurement time. Although serial dilutions are recommended to find the optimal dilution, we consider serial dilutions unfeasible in a study with hundreds of samples. Therefore, we developed a procedure to estimate to optimal sample dilution (section 1.2 of https://doi.org/10.6084/m9.figshare.c.4753676). In sum, we showed that for our flow cytometer and settings used, a count rate ≤ $5.0 \cdot 10^3$ events second unlikely results in swarm detection.

To find the dilution resulting in a count rate ≤$5.0 \cdot 10^3$ events per second, we diluted each sample 200-fold and 2000-fold in Dulbecco phosphate buffered saline (DPBS) and measured the total concentration of particles for 120 seconds without staining (see Figure 1A). Based on the measured total concentration of particles and flow rate, we calculated the minimum dilution required before staining (section 1.3) to achieve a count rate ≤$5.0 \cdot 10^3$ events per second after staining (see Figure 1B). The staining procedure adds an extra dilution of 11.3-fold to the overall dilution. To simplify procedures, samples were divided into 10 categories of pre-staining dilution: 2.5-fold, 5-fold, 10-fold, 16-fold, 25-fold, 40-fold, 65-fold, 100-fold, 160-fold, 200-fold, and 320-fold.

1.3 EV staining

EV in pre-staining diluted samples were stained with antibodies and lactadherin. Prior to staining, antibodies were diluted in DPBS and centrifuged at 18,890 g for 5 min to remove aggregates. Table 1 shows an overview of the used reagents and antibody concentrations during staining. Each sample was labelled with CD61-APC (allophycocyanin) and lactadherin, and with CD235a-FITC. To stain, 20 µL of pre-staining diluted sample (section 1.2) was incubated with 2.5 µL for CD61 and lactadherin or 2.5 µL for CD235a and kept in the dark for 2 h at room temperature. Post-staining, samples were diluted by adding 200 µL of DPBS to decrease background fluorescence from unbound reagents.

1.4 Buffer-only control

Each 96-wellplate contained at least 1 well with filtered DPBS, which was measured with the same flow cytometer and acquisition settings as all other samples. The mean count rate was 8.9 events per second, which is substantially lower than the target count rate (2.5-$5.0 \cdot 10^3$ events per second) for labelled samples.

1.5 Buffer with reagents control

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. CD61-APC, CD235a-FITC, and lactadherin in buffer resulted on average in 2, 114, and 98 fluorescence positive events, respectively. For comparison, CD61-APC, CD235a-FITC, and lactadherin resulted on average in 1,818, 1,073, and 3,094 counts in the labelled samples, respectively.

1.6 Unstained controls

Unstained controls were measured but not used in our analysis.

1.7 Isotype controls

Table 1 shows an overview of the used isotype controls. For particles with a diameter >200 nm and a refractive index <1.42, as reported in this study, the median APC+ events were 3 for IgG-APC and the median FITC+ events were 29 for IgG-FITC during 120 seconds. For comparison, CD61-APC and CD235a-FITC resulted on average in 1,818, and 1,073 counts in the labelled samples, respectively.

1.8 Trigger channel and threshold

Based on the buffer-only control (8.9 events s^{-1}), the acquisition software was set up to trigger at 14 arbitrary units SSC, which is equivalent to a side scattering cross section of 10 nm^2 (Rosetta Calibration, v1.23, Exometry, Amsterdam, The Netherlands).

1.9 Flow rate quantification

The A60-Micro is equipped with a syringe pump with volumetric control, which we checked on a daily basis using the green marker bead in the Rosetta Calibration mixture. We obtained a mean flow rate of 2.95 µL/min with a standard deviation of 0.19 µL/min. Therefore, we assumed that the flow rate is equal to the adjusted flow rate of 3.01 µL/min for all measurements.

1.10 Fluorescence calibration

Calibration of the fluorescence detectors from arbitrary units (a.u.) to molecules of equivalent soluble fluorochrome (MESF) was accomplished using 2 µm Q-APC beads (2321-175, BD) and FITC Quantum (13734, Bangs Laboratories Inc.).

For each measurement, we added fluorescent intensities in MESF to the flow cytometry data files by custom-build software (MATLAB R2018a) 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 figures 2A-B, respectively.

1.11 Light scatter calibration

We used Rosetta Calibration to relate scatter measured by FSC or SSC to the scattering cross section and diameter of EV. Figure 3 shows print screens of the scatter calibrations. We modelled EV 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. For each measurement, we added the FSC and SSC scattering cross sections and EV diameters to the flow cytometry datafiles by custom-build software (MATLAB R2018a). The SSC trigger threshold corresponds to a side scattering cross section of 10 nm^2.

1.12 EV diameter and refractive index approximation

Flow-SR was applied to determine the size and refractive index of particles and improve specificity by enabling label-free differentiation between EV and lipoprotein particles (presumably chylomicrons) [3,4]. Flow-SR was performed as previously described [3,4]. Lookup tables were calculated for diameters ranging from 10 to 1000 nm, with step sizes of 1 nm, and refractive indices from 1.35 to 1.80 with step sizes of 0.001. The diameter and refractive index of each particle was added to the .fcs file by custom-build software (MATLAB R2018a).

As Flow-SR requires accurate measurements of both FSC and SSC, we applied Flow-SR only to particles with diameters >200 nm and fulfilling the condition:

$\mathrm{SSC}(\mathrm{nm}^2) > -0.7 \cdot \mathrm{FSC}(\mathrm{nm}^2) + 3.0$	Equation 2

Size distributions of EV (RI < 1.42, see section 1.14) were obtained from the diameters obtained by Flow-SR. Size distributions had a bin width of 10 nm. Right from the peak, size distributions were fitted with an exponential decaying function. The decay constants of the exponential fits were reported in the manuscript.

1.13 MIFlowCyt checklist

The MIFlowCyt checklist is added to Table 2.

1.14 EV number concentration

The concentrations of EV reported in the manuscript describe the number of particles (1) that exceeded the SSC threshold, corresponding to a side scattering cross section of 10 nm^2, (2) that were collected during time intervals, for which the count rate was within 25% from the median count rate,

(3) with a diameter >200 nm as determined by Flow-SR [3], (4) fulfilling the condition of equation 2, (5) having a refractive index <1.42 to omit false positively labeled lipoprotein particles, and (6) that are positive at the corresponding fluorescence detector (CD61-APC > 150 MESF & lactadherin-FITC > 1000 MESF; CD61-APC < 150 MESF & lactadherin-FITC > 1000 MESF; CD235a-FITC > 825 MESF), per mL of serum or PDP.

For the samples stained with CD61-APC and lactadherin-FITC, two extra gates were applied between aforementioned steps 2 and 3. To omit residual platelets, only events with a side scattering cross section <300 nm^2 and an APC intensity <7000 MESF were included. To omit other residual cells, only events with a side scattering cross section <300 nm^2 and a FITC intensity <50,000 MESF were included.

The total EV concentration was determined by applying aforementioned gates 1 to 5. The lipoprotein particle (presumably chylomicron) concentration was determined by applying aforementioned gates 1 to 5, but with gate 5 selecting particles with a refractive index >1.45 instead of <1.42.

The residual platelet concentration was determined by applying gates 1 to 2, followed by selecting particles with a side scattering cross section >300 nm^2 and CD61-APC >7000 MESF.

1.15 Data sharing

Raw data, data with standard units, and a summary of all flow cytometry scatter plots and gates applied will be shared upon request.

2 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. *J Thromb Haemost* 2012; **10**: 919–30.

[2] Gasecka A, Nieuwland R, Budnik M, Dignat-George F, Eyileten C, Harrison P, Lacroix R, Leroyer A, Opolski G, Pluta K, van der Pol E, Postuła M, Siljander P, Siller-Matula JM, Filipiak KJ, others. Ticagrelor attenuates the increase of extracellular vesicle concentrations in plasma after acute myocardial infarction compared to clopidogrel. *J Thromb Haemostaris* 2020; **18**: 609–23.

[3] van der Pol E, de Rond L, Coumans FAW, Gool EL, Böing AN, Sturk A, Nieuwland R, van Leeuwen TG. Absolute sizing and label-free identification of extracellular vesicles by flow

cytometry. *Nanomed Nanotechnol Biol Med* 2018; **14**: 801–10.

[4] de Rond L, Libregts SFWM, Rikkert LG, Hau CM, van der Pol E, Nieuwland R, van Leeuwen TG, Coumans FAW. Refractive index to evaluate staining specificity of extracellular vesicles by flow cytometry. *J Extracell Vesicles* 2019; **8**: 1643671.

Figures

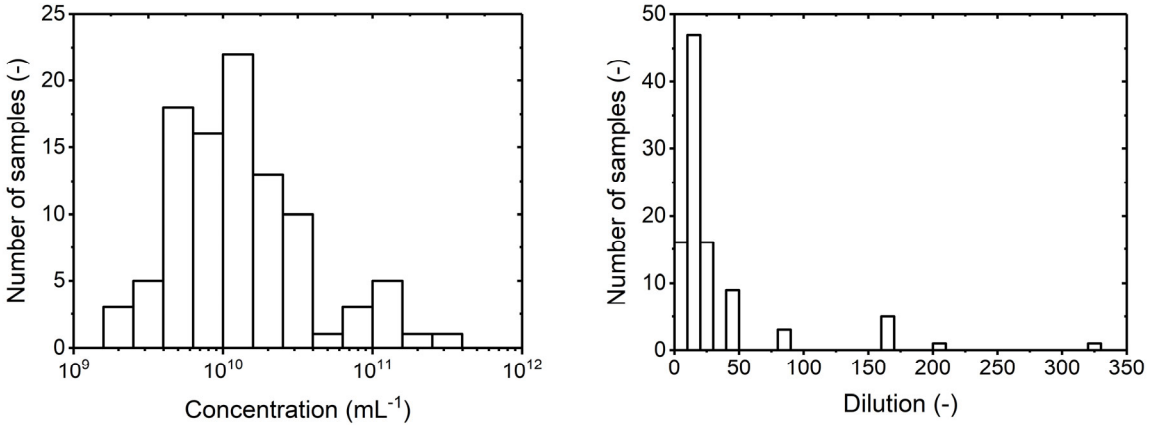
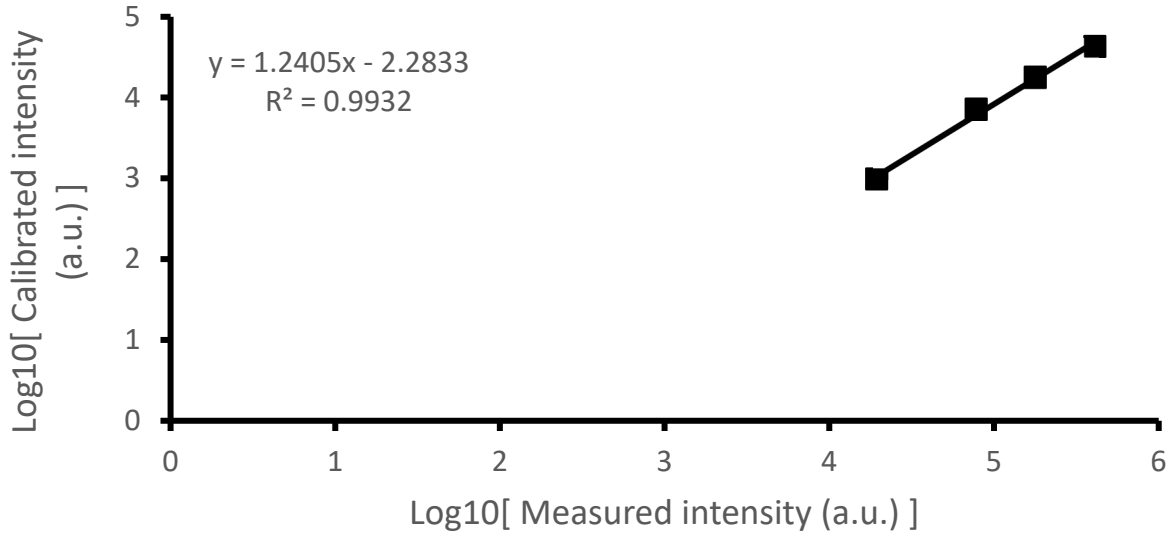


Figure 1. Distribution of the total measured particle concentrations and sample dilutions.

A. MESF calibration APC



B. MESF calibration FITC

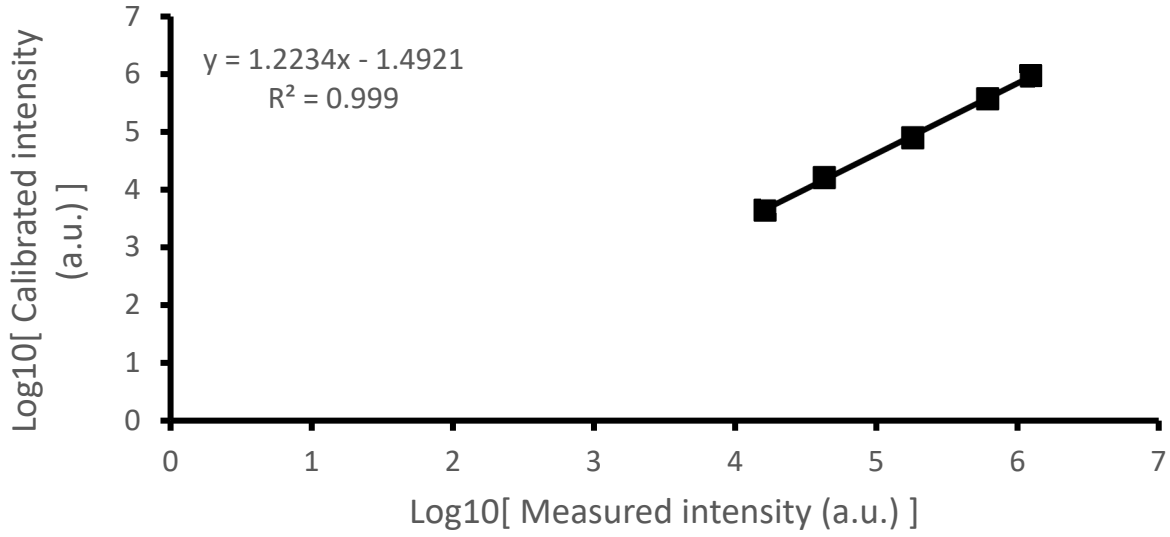


Figure 2. Calibration of the fluorescence detectors from arbitrary units (a.u.) to molecules of equivalent soluble fluorochrome (MESF). Logarithmic MESF versus logarithmic mean fluorescence intensity (MFI) for (A) allophycocyanin (APC), (B) fluorescein (FITC). Data (symbols) are fitted with a linear function (line).

A. Forward scatter calibration B. Side scatter calibration

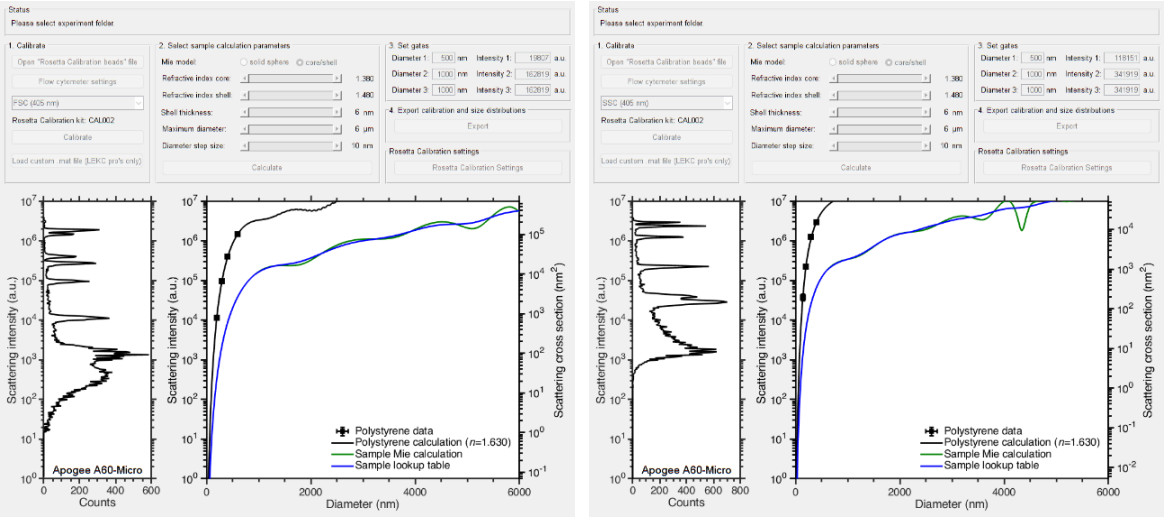


Figure 3. Forward scatter and side scatter calibration of the A60-Micro by Rosetta Calibration. To relate scatter to the diameter of EV, we modelled EV as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm.

Table 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 9-fold (CD61 + lactadherin) or 10-fold (CD235a) 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	VI-PL2	50	eBioscience	17-0619-42	2026494
	Human CD235a	Anti-human CD235a antibody	FITC	IgG1	JC159	100	Dako	F0870	20043299
	Bovine Lactadherin		FITC	n.a.	n.a.	83.3	Hemaetologic Technologies	BLAC-FITC	HH0430 and HH0216
Affinity for Fc receptor	Fc receptor	IgG1	APC	n.a.	MPOC-21	200	Beckman Dickinson	554681	7075605
	Fc receptor	IgG1	FITC	n.a.	X40	50	Beckman Dickinson	345815	7010851

APC: allophycocyanin; FITC: fluorescein isothiocyanate; CD: cluster of differentiation; IgG: immunoglobulin G;

Table 2. MIFlowCyt checklist.

Requirement	Please Include Requested Information
1.1. Purpose	See section 1.1.
1.2. Keywords	Biomarkers; Proteomics; RNA sequencing; Extracellular Vesicles; Exosomes; Serum; Plasma; Anticoagulants; Preservatives; Cancer
1.3. Experiment variables	Blood collection tubes and blood processing intervals
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 2019 and April 3rd 2019.
1.7. Conclusions	Total EV ($p<0.001$), activated platelet ($p<0.001$) and non-platelet EV ($p<0.001$), erythrocyte EV ($p=0.012$) and residual platelet concentrations ($p<0.001$) were all significantly affected by BCT type, while lipoprotein particle concentrations were not ($p=0.25$). Serum contained the highest number of total EV (5.80E+08 ± 1.60E+08 mL^{-1}), with 3.87-fold ($p<0.001$) higher concentrations compared to citrate PDP (1.50E+08 ± 1.30E+08 mL^{-1}). Similarly, serum contained the highest number of activated platelet EV (3.70E+08 ± 9.60E+07 mL^{-1}), representing 68 ± 9.1 % of total EV count in serum. Activated platelet EV concentrations were 77.1-fold ($p<0.001$) higher compared to citrate PDP (4.80E+06 ± 1.50E+06 mL^{-1}), in which 5.2 ± 3.6 % of total EV were represented by activated platelet EV. In addition, PDP prepared from Paxgene ccfDNA and Streck BCT RNA contained 1.40- ($p=0.004$) and 3.13-fold ($p=0.003$) higher activated platelet EV concentrations compared to citrate PDP. EDTA PDP contained significantly lower numbers of activated platelet EV ($p=0.003$). The highest numbers of non-platelet EV were measured in serum and Roche cfDNA, with 1.59- ($p=0.043$) and 1.79-fold ($p=0.006$) higher concentrations compared to citrate PDP. Residual platelets were still present in serum and PDP prepared from all BCT in concentrations under

	the detection limit of a standard hematology analyzer. Citrate (6.64E+05 ± 5.36E+05 mL^{-1}) and ACD-A plasma (4.95E+05 ± 3.22E+05 mL^{-1}) contained the lowest numbers of residual platelets. Conversely, PDP prepared from Paxgene ccfDNA and Streck BCT RNA contained 4.07- ($p<0.001$) and 4.97-fold ($p=0.001$) higher residual platelet concentrations compared to citrate plasma, explaining the release of higher numbers of activated platelet EV in PDP prepared from these BCT. PDP prepared from the remaining BCT, except for EDTA gel, also contained significantly higher numbers of residual platelets compared to citrate plasma, but this did not lead to significantly elevated activated platelet EV levels. Roche cfDNA PDP contained the highest number of erythrocyte EV (3.80E+07 ± 9.40E+06 mL^{-1}), with 1.46-fold ($p=0.036$) higher concentrations compared to citrate plasma (2.60E+07 ± 2.40E+07 mL^{-1}), corresponding to the significant hemolysis observed in these BCT. Conversely, ACD-A plasma contained 1.86-fold ($p=0.042$) lower numbers of erythrocyte EV (1.40E+07 ± 7.50E+06 mL^{-1}). Finally, flow cytometry data were also used to calculate EV size distributions (RI < 1.42) in serum and PDP across the selected BCT. The decay constant of exponential fits of the size distributions were unaffected by the type of BCT, confirming the observations from the (f)NTA and TEM experiments on spiked rEV and EV separated from plasma. Total EV, activated platelet and non-platelet EV, erythrocyte EV and residual platelet concentrations were all unaffected by short-term storage (1 h, 8 h), regardless of BCT. After long-term storage (72 h), the number of total EV was least affected in ACD-A and Streck RNA complete BCT, with fold changes of 5.63 ($p=0.043$) and 6.33 ($p=0.009$), respectively, compared to baseline. With mean fold changes ranging from 14.96 to 21.96, total EV counts in the remaining BCT were significantly higher ($p<0.001$). Activated platelet and non-platelet EV counts at T3 were least affected in Streck RNA complete BCT, with fold changes of 20.01 (p=NS) and 3.58 (p=NS), while erythrocyte EV counts were least affected in ACD-A (p=NS). Conversely, the concentration of EV subtypes after long-term storage (72h) was the most significantly

	affected in EDTA and Streck BCT DNA, with fold changes of 186.11 and 79.27 for activated platelet EV, 21.03 and 24.30 for non-platelet EV, and 43.93 and 127.03 for erythrocyte EV, respectively ($p<0.001$). After long-term storage (72 h), a large share of the total EV count was represented by activated platelet EV and erythrocyte EV in all BCT. Despite applying an additional 2500 g centrifugation step for non-platelet depleted plasma samples, residual platelets remained abundantly present in PDP prepared from EDTA ($p<0.001$) and Streck BCT DNA ($p=0.001$).
1.8. Quality control measures	All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate (section 1.9). Each well plate contained buffer-only controls (section 1.4), antibody in buffer controls (section 1.5), unstained controls (section 1.6) and isotype controls (section 1.7). The flow rate was checked with Rosetta Calibration beads. All used detectors were calibrated (section 1.10 and 1.11).
1.9 Other relevant experiment information	The entire study involved seven 96-well plates that were measured within 2 months.
2.1.1.1. Sample description	For flow cytometry experiments, venous blood samples from healthy volunteers were collected into blood collection tubes containing different anticoagulants and/or preservatives. All donors were non-smokers. Healthy donors did not take any medication during the previous ten days, nor did they suffer from any chronic or acute disease at the time of venipuncture. Cancer patients were not on anticoagulant therapy at the time of sample collection. The erythrocyte count, hemoglobin, mean cellular volume, mean cellular hemoglobin, mean cellular hemoglobin concentration, platelet count and leukocyte count were in the normal range for all included donors. Venipuncture was performed while the donors were fasting. 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 11 BCT each containing different anticoagulants and/or preservatives: Serum (Vacuette Z serum with separator and clot activator, volume 9 ml, Greiner Bio-One, Frickenhausen, Germany);

	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); ACD-A (Vacutainer ACD Solution A, volume 8.5 ml, BD Biosciences), PAXgene ccfDNA (volume 10 ml, PreAnalytiX GmbH, Hombrechtikon, Switzerland); Cell-Free DNA BCT (volume 10 ml, Streck, La Vista, NE, USA); Cell-Free RNA BCT (volume 10 ml, Streck); LBgard (volume 8.5 ml, Biomatrica, San Diego, CA, USA); Cell-Free DNA (volume 8.5 ml, Roche Sequencing Solutions, Pleasanton, CA, USA); RNA complete BCT (volume 10 ml, Streck). 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 BCT vertically for 180° and back to the starting position ten times immediately after collection. The blood collection tubes were held in a rack in an upright position at room temperature (20° C). For healthy volunteers, venipuncture was performed within the same laboratory as sample preparation and samples were not transported. For cancer patients, venipuncture was performed in the operating theatre and samples were transported on foot to the laboratory within 10 minutes. Centrifugation was performed exactly 60 min, 8h or 72h after collection of the last BCT, as indicated in the results section. Serum was prepared by centrifugation at 1200 g for 15 min at 20 °C. Platelet depleted plasma (PDP) was prepared by two serial centrifugations at 2500 g for 15 min at 20 °C (ISTH protocol). An Eppendorf 5810 R (Eppendorf, Hamburg, Germany) benchtop centrifuge was used. No brake was applied. After each centrifugation step, serum or blood plasma was transferred to a clean 5 mL polypropylene centrifuge tube (Eppendorf). At least 0.5 cm of serum or blood plasma was left in the tube to avoid contamination with cells. Serum and PDP samples were aliquoted, snap frozen in liquid nitrogen, and stored at -80 °C until analysis. Before staining (section 1.3), samples were thawed for 1 minute at 37 °C.

2.1.1.2. Biological sample source description	Venous blood samples from healthy volunteers. 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	Humans.
2.2 Sample characteristics	Serum and PDP are expected to contain erythrocyte ghosts, EV, lipoprotein particles, proteins, and residual platelets.
2.3. Sample treatment description	Please see section 1.3.
2.4. Fluorescence reagent(s) description	Please see Table 1.
3.1. Instrument manufacturer	Apogee, Hemel Hempstead, UK
3.2. Instrument model	A60-Micro
3.3. Instrument configuration and settings	Samples were analysed for 120 seconds at a flow rate of 3.01 µL/min 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 SSC 14 arbitrary units, corresponding 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. APC signals were collected with the 638-D Red(Peak) detector (long pass 652 nm filter, PMT voltage 510 V). FITC signals were collected with the 488-Green(Peak) detector (525/50 nm band pass filter, PMT voltage 520 V).
4.1. List-mode data files	Raw data, data with standard units and a summary of all flow cytometry scatter plots and gates applied are available upon request.
4.2. Compensation description	No compensation was required because no fluorophore combinations were used that have overlapping emission spectra.
4.3. Data transformation details	No data transforms were applied besides calibrations.
4.4.1. Gate description	See section 1.14
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	On visual overview of all gates is available upon request.

APC: Allophycocyanin; BCT: blood collection tube; BPI: blood processing interval; CD: cluster of differentiation; EV: extracellular vesicles; FSC: forward scattering; PDP: platelet depleted plasma; PMT: photomultiplier tube; SSC: side scattering.