Removing blood platelets from human plasma to reveal extracellular vesicle-associated miRNA profiles

Supplementary File 3. MIFlowCyt-EV checklist

1. Flow cytometry

1.1. Experimental design

The purpose of this flow cytometry (A60-Micro, Apogee Flow Systems, Hemel Hempstead, UK) experiment was to quantify the concentration of extracellular vesicles (EVs) derived from platelets (CD61+), erythrocytes (CD235a+) and leukocytes (CD45+) in plasma- and extracellular vesicle (EV) samples, before and after filtration with a polycarbonate membrane filter having a 0.8-µm pore diameter. We hypothesized that filtration would remove remaining cells, including (activated) platelets and erythrocyte-ghosts (ery-ghosts), without affecting EV concentrations. Downstream analysis was performed to decipher how the removal of remaining cells affects the quantity of miRNAs that are thought to be associated with EVs (EV-miRNAs).

Pre-analytical variables, such as blood collection and plasma preparation by two different protocols, have been reported in the manuscript. Double centrifuged plasma samples were (1) filtered with a 0.8 µm pore-size polycarbonate membrane filter (IsoporeTM, Merck Millipore, Darmstadt), or (2) used as starting material to isolate EVs with size-exclusion chromatography CL-2B (SEC2B). The EV-enriched fractions (F8-10), obtained using SEC2B were pooled and used for further analyses. One mL of the pooled EV fraction was filtered using the polycarbonate membrane filters. Platelet-, activated platelet- and ery-ghost concentrations were determined using a FACS Canto II. Details can be found in the MIFlowCyt documents added to the supplements.

To determine EV concentrations, all samples were measured with an Apogee A60-micro in a 96-well plate (one plate per experiment), using an autosampler. Experiments were performed in triplicate, and each experiment contained antibody in buffer controls, corresponding to the antibodies included in this experiment, and a buffer-only control. Scatter calibration and flow rate calibration were performed on the day of the experiments. Fluorescence calibration was performed two to three months before the experiments. To automatically process data,

determine optimal samples dilutions, apply calibrations, determine and apply gates, generate reports with scatter plots and generate data summaries, MATLAB R2018b software (Mathworks, Natick, MA) was used.

1.2. Sample dilutions

As the particle concentration in plasma differs between individuals, samples require different dilutions to avoid swarm detection[1,2] and to achieve statistically significant counts within a reasonable measurement time. Although serial dilutions are recommended to find the optimal dilution, we consider serial dilutions unfeasible in a study with 48 samples. Therefore, we developed a procedure to estimate the optimal sample dilution[2]. In sum, we showed that for our flow cytometer and the used settings, a count rate $\leq 5.0 \cdot 10^3$ events/second unlikely results in swarm detection.

To find the dilution resulting in a count rate $\leq 5.0 \cdot 10^3$ events per second, we measured the total concentration of particles for 30 seconds without staining and calculated the minimum dilution required. Samples having a count rate $>5.0 \cdot 10^3$ were diluted in Dulbecco's Phosphate-buffered saline (DPBS, Corning, Corning, NY) and re-measured. The staining procedure adds an extra dilution of 11.3-fold to the overall dilution.

1.3. EV staining

EVs in plasma and pooled EV-enriched fractions were stained using anti-CD61-FITC (Dako Amstelveen, The Netherlands), anti-CD45-APC (Biolegend, San Diego, CA, USA), and anti-CD235a-FITC (Dako, Amstelveen, The Netherlands). Prior to staining, antibodies were pre-diluted in DPBS, as described in **Table S2.1**, and centrifuged at 18,890 x g for 5 minutes to remove aggregates. Two-and-a-half µl of each antibody was incubated with 20 µl pre-stained diluted sample for 2 hours at room temperature in the dark. CD61-FITC and CD45-APC were used for double staining, by adding a total of 5 µl antibody to each sample. Post-staining, samples were diluted by adding 200 µl DPBS to each sample.

1.4. Buffer-only control

Each 96-well plate contained 1 well with DPBS, which was measured with the same flow cytometer and acquisition settings as all other samples. The mean count rate was 38 events per second, which is lower than the target count rate ($2.5\text{-}5.0 \cdot 10^3$ events per second) for plasma

and EV samples. The minimum count rate of buffer-only control was 23 events per second, the maximum count rate measured was 61 events per second.

1.5. Buffer with reagents control

Each 96-well plate contained a buffer with reagent control for each reagent (**Table S2.1**), which was measured with the same flow cytometer and acquisition settings as all samples. For CD61-FITC combined with CD45-APC in buffer and CD235a-FITC in buffer, an average of 87 and 118 events per second were measured, respectively. This is higher than in the buffer-only control (38 events per second). To investigate whether the relatively high background counts caused by CD61-FITC/CD45-APC and CD235a-FITC affected the reported results, we applied the same calibrations and gates to CD61-FITC/CD45-APC and CD235a-FITC in buffer as to the plasma and EV samples stained with the corresponding antibody. On average, we obtained 29 CD61-FITC+ and 35 CD45-APC+ events in buffer, while the mean of CD61-FITC+ and CD45-APC+ events in plasma and EV fractions were 1551 and 583, respectively. Therefore, the number of positive background events is acceptable in these samples. For CD235a-FITC, we obtained an average of 413 CD235a-FITC+ events in buffer and an average of 782 CD235a+ events in plasma and EV samples. The number of false positive CD235-FITC events is high relative to the number of CD235a+ EVs in samples.

1.6. Unstained controls

Unstained controls were measured with the same dilution and settings as the stained samples. Unstained plasma samples prepared according to the ISTH protocol had a minimum count rate of 280 events per second and a maximum count rate of 2577 events per second. For unstained plasma samples prepared using the non-ISTH protocol, a minimum count rate of 165 events per second and a maximum count rate of 2856 events per second were measured. Low count rates (< 500 events per second) of unstained controls (and samples) in one of the experiments was unexpected, since the most optimal pre-dilution factors prior to staining were established between 2067 and 4374 events per second, which was not comparable with the count rates during measurements.

1.7. Isotype controls

No isotype controls were included in this experiment, since the used antibodies did not show any non-specific binding in previous experiments.

1.8. Trigger channel and threshold

Based on the buffer-only control (38 events per second), the acquisition software was set up to trigger at 24 arbitrary units SSC, which is equivalent to a side scattering cross section of 6 nm^2 (Rosetta Calibration v1.28, Exometry, Amsterdam, The Netherlands).

1.9. Flow rate quantification

On the measurement days, we used 110-nm FITC beads with a specified concentration (Apogee calibration beads, Apogee Flow Systems, Hemel Hempstead, UK) to validate the flow rate of the A60-Micro. As the A60-Micro is equipped with a syringe pump with volumetric control, and we assumed a 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 Quantum™ MESF Kits (13734, Bangs Laboratories Inc., Fishers, IN, USA). Calibrations of the APC and FITC detectors were performed on 2021-07-15. Apocal was used to correct for variations in the APC detector. For each measurement, we added fluorescent intensities in MESF to the flow cytometry data files (MATLAB R2018a) using the following equation:

$\mathrm{I(MESF)} = 10^{a \cdot \log_{10} \mathrm{I(a.u.)} + b}$	Equation S1

where I, is the fluorescence intensity, and *a* and *b* are the slope and the intercept of the linear fits respectively, see **Table S2.2**.

1.11. Light scatter calibration

We used Rosetta Calibration (v1.28, Exometry) to relate scatter measured by forward scattering (FSC) and side scattering (SSC) to the effective scattering cross section and diameter of EVs. **Figure S2.1** shows print screens of the scatter calibrations. We modelled EVs 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 cross sections and EV diameters to the cytometry data files. The SSC trigger threshold corresponds to a side scattering cross section of 6 nm^2.

1.12. MIFlowCyt checklist

The MIFlowCyt checklist can be found in the MIFlowCyt documents added to the supplements.

1.13. EV number concentration

The concentrations reported in the manuscript describe the number of particles (1) that exceeded the SSC threshold, corresponding to a side scattering cross section of 6 nm^2, (2) that were collected during time intervals, for which the count rate was within 750 counts/second above and below the mean count rate, (3) with a diameter < 1,000 nm as measured by SSC after light scatter calibration (section 1.11) and (4) that were positive for APC, or FITC, per mL of plasma.

1.14. Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.c.6126768.v1

1.15. References

1. van der Pol E, van Gemert MJ, Sturk A, Nieuwland R, van Leeuwen TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. *J Thromb Haemost.* 2012;10(5):919-930.
2. Buntsma NC, Gasecka A, Roos Y, van Leeuwen TG, van der Pol E, Nieuwland R. EDTA stabilizes the concentration of platelet-derived extracellular vesicles during blood collection and handling. *Platelets.* 2022;33(5):764-771.

Figures and tables

Figure S2.1: Rosetta Calibration to relate scatter to the diameter of EVs.

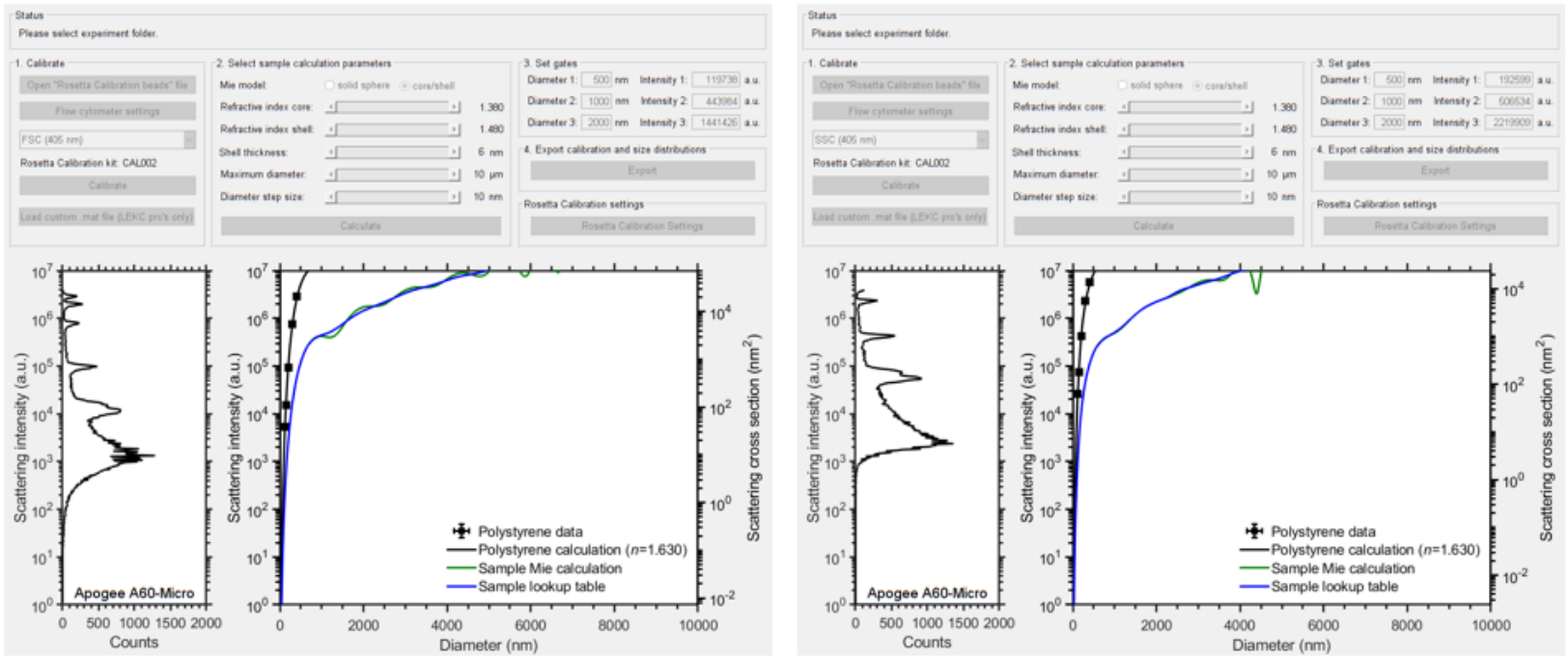

Figure S2.1. *Forward scatter and side scatter calibration of the A60-Micro by Rosetta Calibration. To relate scatter to the diameter of EVs, we modelled EVs as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm*

Table 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	FITC	IgG1	Y2/51	1.25	Dako	F0803	20027302
Glycoprotein	Human CD235a	Anti-human CD235a antibody	FITC	IgG1	JC159	5.56	Dako	F0870	20064863
Glycoprotein	Human CD45	Anti-human CD45 antibody	APC	IgG1	HI30	0.23	Biolegend	304037	B272158

APC: allophycocyanin; FITC: Fluorescein Isothiocyanate.

Table S2.2: Overview of fluorescence calibrations.

	Calibration date	Slope	Intercept	R^2
APC (2021-09-29)	2021-07-15	1.1573	-2.3959	0.9984
APC (2021-10-04)	2021-07-15	1.1573	-2.3747	0.9984
APC (2021-10-14)	2021-07-15	1.1573	-2.4100	0.9984
FITC	2021-07-15	1.3463	-3.0903	0.9932