Supplemental materials 1: pulse height versus pulse area

In the manuscript we analyze the pulse height of fluorescence signals. However, the relation between the measured number of photoelectrons and the registered signal differs for the pulse height and pulse area. In contrast to the pulse area, the pulse height is biased towards positive fluctuations of the noise. Consequently, the pulse area is expected to scale linearly with the measured number of photoelectrons whereas close to the background noise level the pulse width shows an offset. To evaluate whether this offset affects our results, we have simulated the registered signal versus signal level for the pulse height and pulse area. As input parameters for the simulation, we determined the detection efficiency Q and background B of the phycoerythrin (PE) detector [1].

We related the arbitrary units of fluorescence to units of molecules of equivalent soluble fluorophore (MESF), as shown in Figure S2.1D. Next, we determined Q and B of the PE detector of the Apogee A60-Micro using Sphero Rainbow Calibration Particles (RCP-30-5A, Spherotech, USA). Thereto, we (1) determined the coefficient of variation (CV) and the calibrated brightness of each bead population, (2) calculated the standard deviation squared of each bead population by subtracting the CV of the brightest population, and (3) plotted the corrected standard deviation squared versus brightness, as shown in Figure S1.1. The inverse of the slope is equal to Q, which is 0.138 photoelectrons/MESF, and the intercept divided by the slope is equal to B, which gives 57 MESF or 8 photoelectrons per acquisition time.

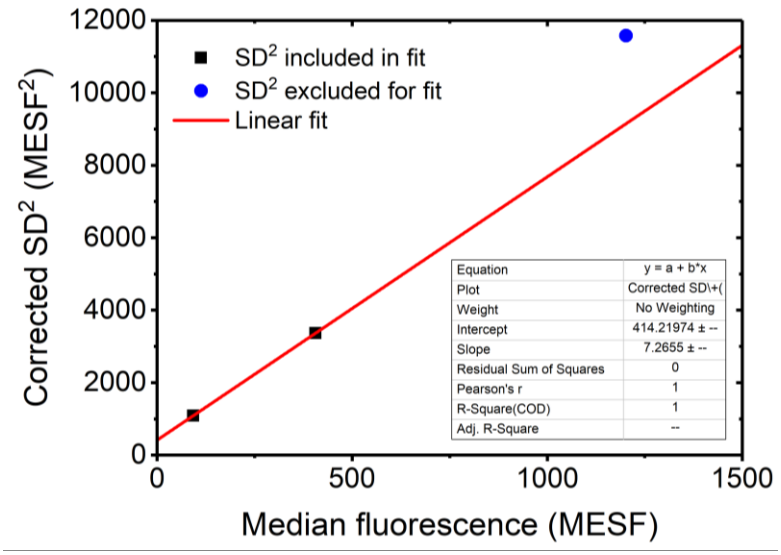

Figure S1.1. Corrected standard deviation (SD) squared versus the median fluorescence in units of molecules of equivalent soluble fluorophore (MESF) measured (symbols) with rainbow particles on an Apogee A60-Micro flow cytometer and fitted (line) with a linear function (slope = 7.266, intercept = 414.2). The two dimmest rainbow particles were used for fitting to guarantee that the corrected SD^2 is dominated by shot noise.

Next, we simulated the registered signal for the pulse height and pulse area for signal levels of 1 to 100 photoelectrons using a step size of 1 photoelectron. For each signal level we simulated 1,000 Gaussian pulses at a count rate of 1 kHz. The full width at half maximum of each Gaussian pulse was 1.2 µs, the sampling frequency was 10 MHz (personal communication with Apogee Flow Systems), and the mean background level was 8 photoelectrons per acquisition time (previous paragraph). To take into account the random arrival of photoelectrons, we added Poisson noise to both the signal and background levels. We determined the background level by taking the median signal level during 1 ms and subtracted the background level from the pulses. For each pulse, the height and area were calculated and plotted as a function of the signal levels, as shown in Figure S1.2.

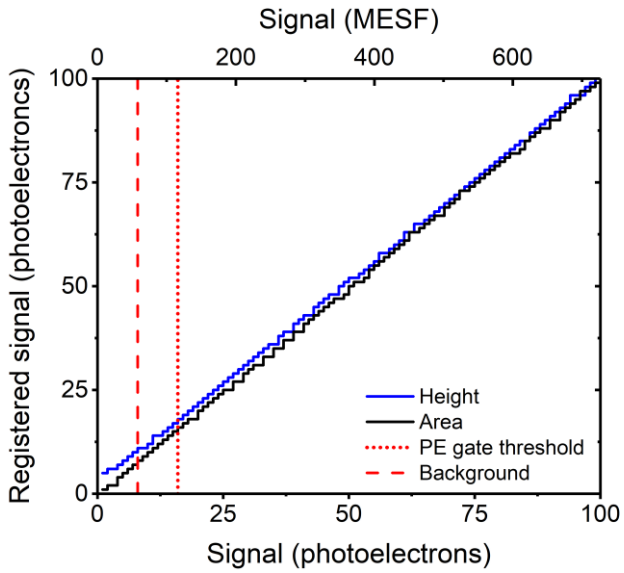

Figure S1.2. Registered signal for the pulse height (blue, upper line) and pulse area (black, lower line) versus signal level, showing an offset of the pulse height. The vertical lines indicate the background level (dashed line) and the applied threshold (dotted line) of the phycoerythrin (PE) gate.

Figure S1.2 shows that pulse height analysis introduces a 13% overestimation of the registered signal at the PE gate threshold. This negligible overestimation can be explained by three factors. First, with 8 photoelectrons per acquisition time, the background level is low. Consequently, the background noise level, which contributes to the overestimation of the pulse height, is low. Second, the PE threshold is set at 2-fold the background level. Third, due to the low sampling frequency relative to the pulse width, each pulse is sampled only 11 times. Consequently, for 80% of the pulses, the maximum of the Gaussian pulse is located at the center bin of the Gaussian pulse and hence, the positive bias of the noise is limited. We assume that other fluorescence detectors of our flow cytometer show similar behavior.

Our main motivation to analyze pulse height instead of pulse area is that for our flow cytometer, the pulse height results in substantially lower (robust) CVs then pulse area, as shown in Table S1.1.

Table S1.1. Robust coefficient of variation (rCV) of the pulse area and pulse height of the dimmest rainbow bead measured by the allophycocyanin (APC), Brilliant Violet, fluorescein (FITC), and phycoerythrin (PE) detector.

	APC	Brilliant Violet	FITC	PE
rCV area (%)	7.6	20.0	41.1	98.4
rCV height (%)	6.7	8.3	28.1	36.5

References

1 Hoffman RA, Wood JCS. Characterization of flow cytometer instrument sensitivity. *Curr Protoc Cytom* Wiley Online Library; 2007; **40**: 1–20.

Table S1.2. Overview of staining reagents. All of the four fluorophores are coupled to an anti-human CD61 antibody, detect human CD61 (integrin beta 3), and have IgG1 as an isotype control. Shown are the four different fluorophores, clones of the antibodies, final antibody concentration during staining, manufacturer, catalog - and lot number of used staining reagents.

Fluorophore	Clone	Final antibody concentration (µg mL^{-1})	Manufacturer	Catalog number	Lot number
APC	VI-PL2	8.33	eBioscience	17-0619-42	2026494
BV421	VI-PL2	6.3	Becton Dickinson	744381	0191784
BV421	RUU-PL7F12	6.3	Becton Dickinson	744767	9297433
FITC	VI-PL2	1.6	Becton Dickinson	557291	0184775
FITC	Y2/51	12.5	Dako	F0803	20027302
PE	VI-PL2	3.0	eBioscience	12-0619-42	1934552

APC: allophycocyanin; BV421: Brilliant Violet 421; FITC: fluorescein isothiocyanate; PE: phycoerythrin

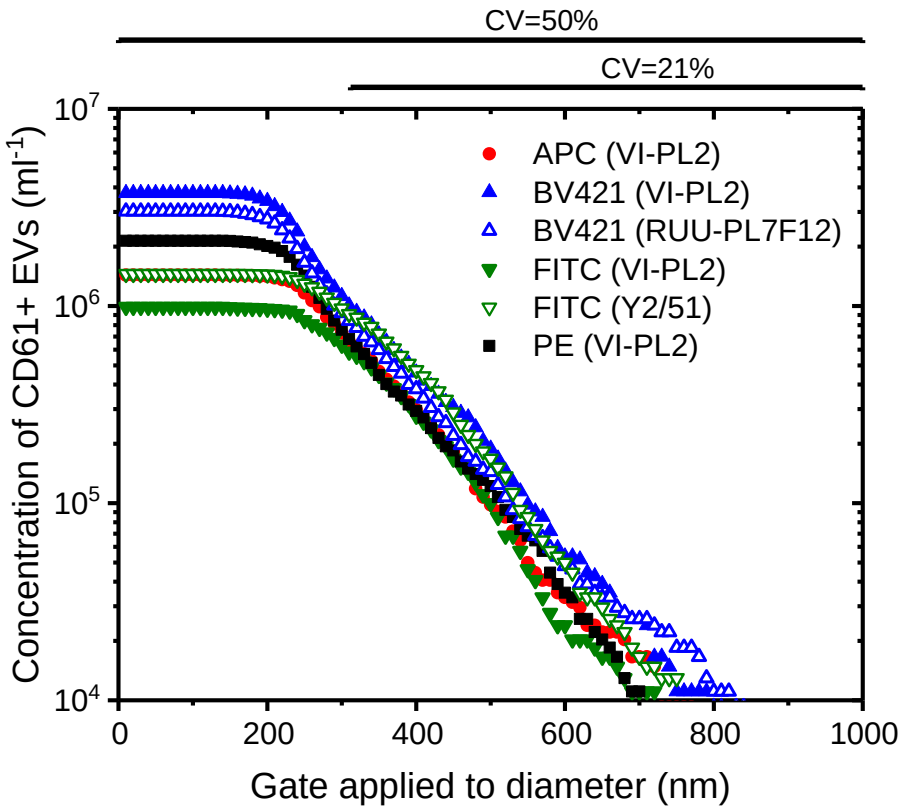

Figure S1.3. Concentration of CD61+ extracellular vesicles (EVs) measured by flow cytometry in 316-fold diluted pooled human plasma labeled with Allophycocyanin (APC, VI-PL2), Brilliant Violet-421 (BV421, VIPL2 or RUU-PL7F12), Fluorescein isothiocyanate (FITC, VI-PL2 or Y2/51), or Phycoerythrin (PE, VIPL2) versus minimum diameter of the selected EVs. In addition to a relevant fluorescence gate, a size gate was applied that ranges from the plotted minimum diameter to 1,000 nm with subsequent steps of 10 nm. A size gate of 0-1,000 nm results in up to 3.5-fold differences in the measured CD61+ EV concentrations between fluorophores and clones (CV=50%). With increasing minimum diameter, the concentrations of CD61+ EVs labeled with different fluorophores and clones converge. A minimum diameter of 310 nm provides more similar concentrations of CD61+ EVs for the different fluorophores and clones (CV=21%).

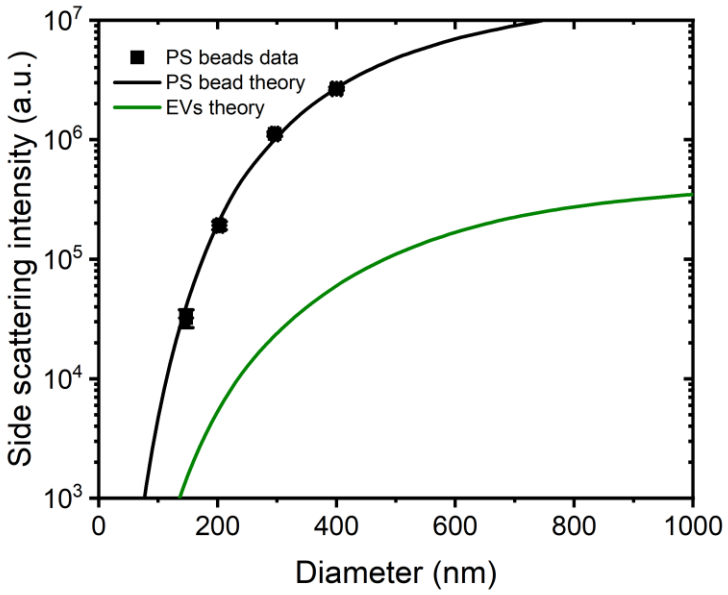

Figure S1.4. Side scattering intensity of polystyrene beads (PS; symbols) measured by flow cytometry and calculated (solid line) with Mie theory (Rosetta Calibration). The theory describes the data well (R^2=0.997). PS beads are modelled as solid particles with a refractive index (RI) of 1.633. EVs are modelled as particles with a 6 nm thick shell having an RI of 1.48 and a core having an RI 1.38.