Supporting information for: Absolute sizing and label-free identification of extracellular vesicles by flow cytometry

Edwin van der Pol[1,2,3],*, Frank A.W. Coumans[1,2,3], Leonie de Rond[1,2,3], Elmar L. Gool[1,2,3], Anita N. Böing[2,3], Auguste Sturk[2,3], Rienk Nieuwland[2,3], and Ton G. van Leeuwen[1,3]

[1]Biomedical Engineering & Physics; [2]Laboratory Experimental Clinical Chemistry; [3]Vesicle Observation Center, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Manufacturer	Catalog number	Material	Reference diameter (nm)
Thermo Fisher Scientific	3100A	PS	100 ± 8
	3150A		147 ± 4
	3200A		203 ± 5
	3300A		296 ± 5
	3400A		400 ± 7
	3600A		600 ± 10
	3800A		799 ± 5
	4010A		994 ± 10
Kisker Biotech GmbH	PSi-0.2	Silica	206 ± 18
	PSi-0.4		391 ± 20
	PSi-0.6		577 ± 21
	PSi-0.8		772 ± 10
	PSi-1.0		918 ± 14

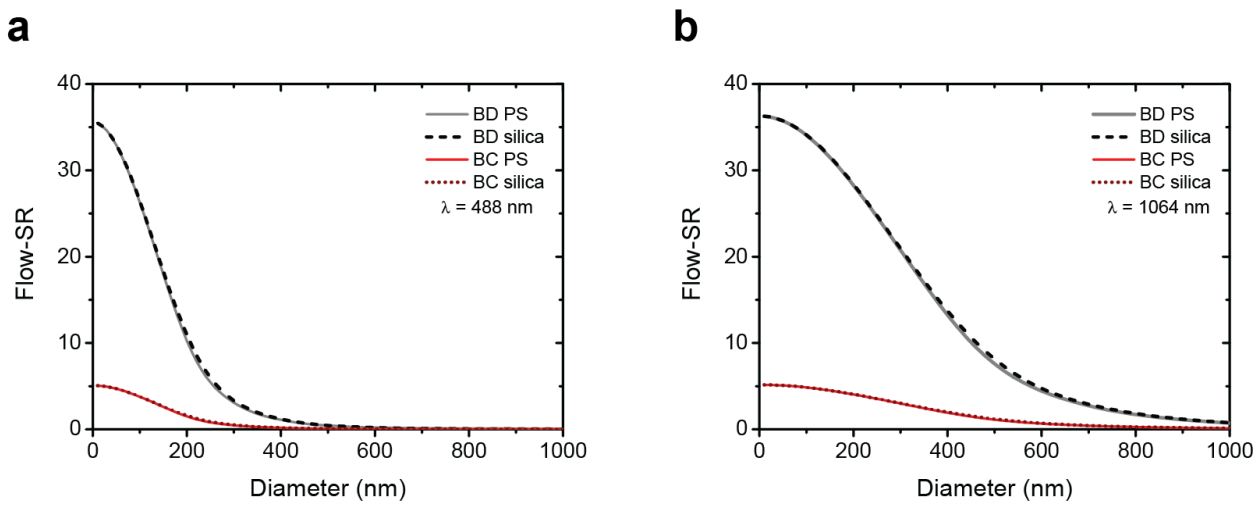
Supplementary Table 1: Manufacturer, catalog number, material, and reference diameter of polystyrene (PS) and silica beads used to relate forward and side scattered light to the diameter and refractive index of nanoparticles.

Label	Manufacturer	Catalog number	Material	Reference diameter (nm)	Measured diameter (nm)
A	Thermo Fisher Scientific	3125A	PS	125 ± 5	
B	Microparticles GmbH	M-PS-F-0.25	PS	240 ± 7	246 ± 13
C	Microparticles GmbH	PS-F-0.3	PS	315 ± 5	316 ± 10
D	Thermo Fisher Scientific		PS	380 ± 7	383 ± 11
E	Microparticles GmbH	SiO2-F-0.25	Silica	255 ± 10	269 ± 18
F	Kisker Biotech GmbH	PSi-0.4	Silica	391 ± 20	420 ± 33

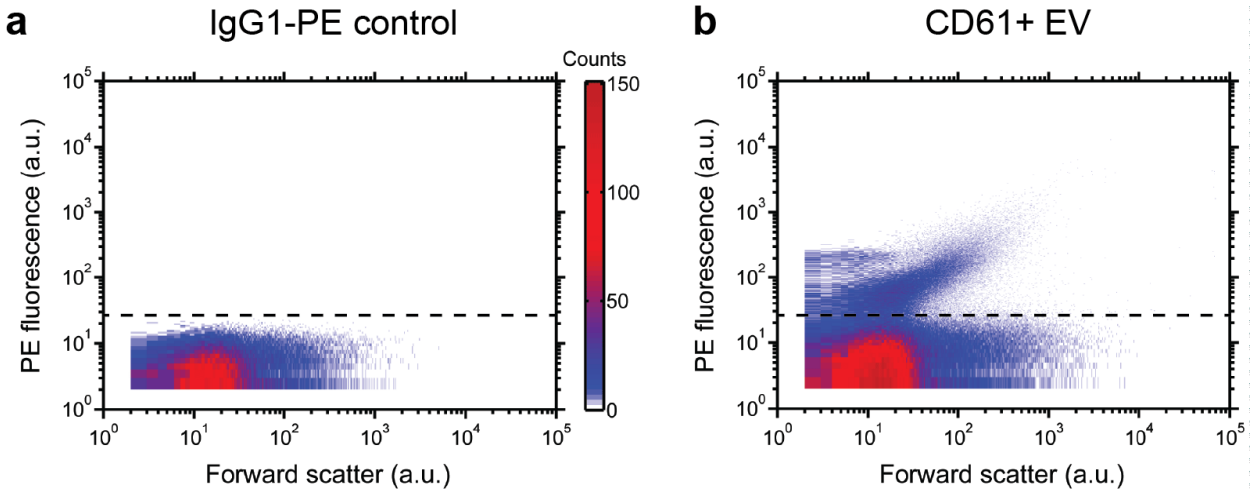
Supplementary Table 2: Label, manufacturer, catalog number, material, reference diameter, and diameter as measured by Flow-SR of polystyrene (PS) and silica beads used to validate size and refractive index determination by flow cytometry.

Sample	FSC voltage (V)	FSC threshold (a.u.)	SSC voltage (V)	SSC threshold (a.u.)	PE voltage (V)
Bead mixture, oil emulsions, Intralipid	360	30	340	20	480
200 nm gold nanoparticles	360	0	280	5	600
Platelet-depleted plasma	360	40	340	20	480
Breast milk and infant formula	345	30	340	20	480

Supplementary Table 3: Forward scattered light (FSC) and side scattered light (SSC) detector voltage and threshold, and phycoerythrin (PE) detector voltage for each sample.



Supplementary Figure 1: Calculated flow cytometry scatter ratio (Flow-SR; side scatter / forward scatter) versus diameter for polystyrene (PS) and silica beads for (a) typical Becton Dickinson (BD; dashed line) and Beckman Coulter (BC; dotted line) flow cytometers with an illumination wavelength of 488 nm, and (b) similar flow cytometers with an illumination wavelength of 1064 nm. The polar angle of the forward scatter detector was integrated from 1° to 7° for BD instruments and 1° to 19° for BC instruments. The side scatter detector was assumed to be a lens with a numerical aperture of 1.2. For these instruments and wavelengths, theory predicts that Flow-SR versus diameter is independent of the RI and provides a unique solution for particles with a diameter below the illumination wavelength.



Supplementary Figure 2: Scatter plot of phycoerythrin (PE) fluorescence versus forward scatter of platelet-depleted supernatant of a platelet concentrate labeled with (a) 1.5 µg/mL IgG1-PE and (b) PE-conjugated CD61. Based on the isotype control, a fluorescence threshold of 25 a.u. was selected.