Supporting information

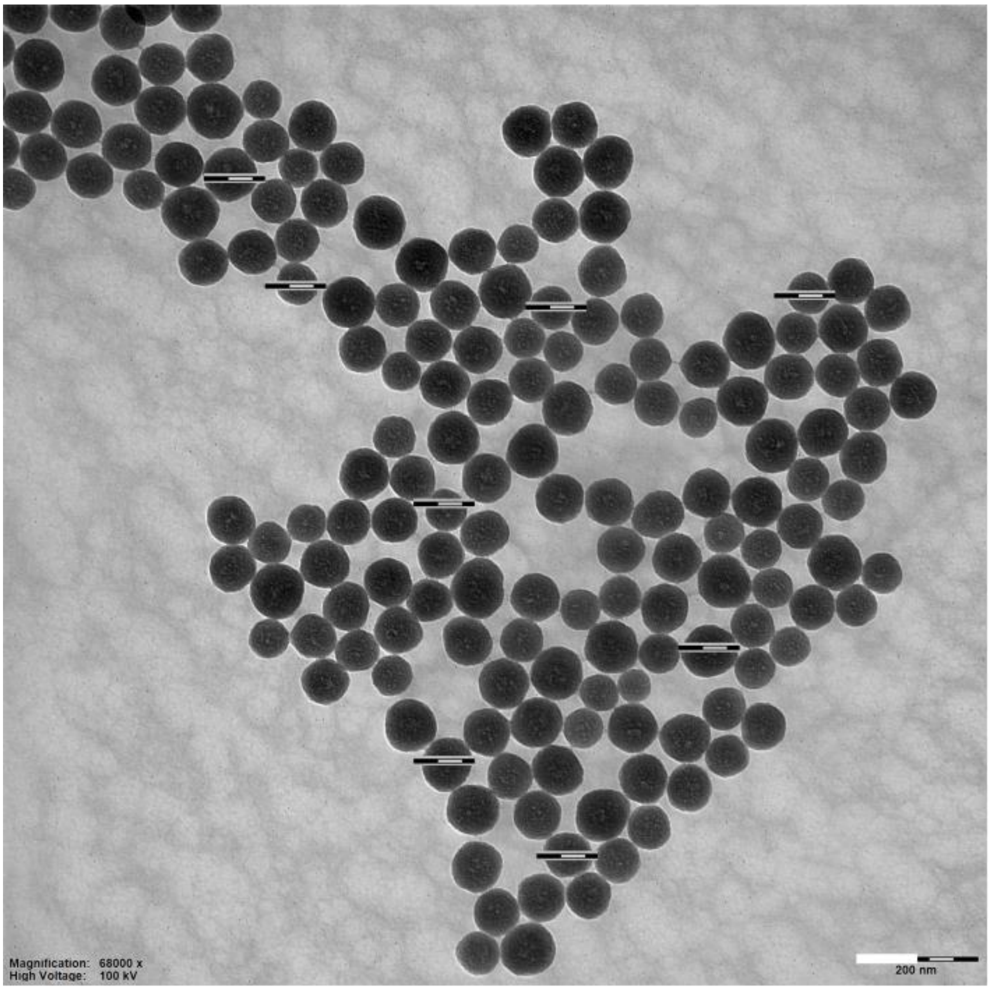

Figure S1: Transmission electron microscopy (TEM) image of SI-0.1 beads (batch GK1231543-02) purchased from Kisker Biotech GmbH, Steinfurt, Germany. The mean diameter as specified by Kisker is 100 nm. Evaluation of 46 TEM images from the actual bead sample revealed a mean diameter of 75 nm with a standard deviation of 7.7 nm. Black scale bars represent 100 nm.

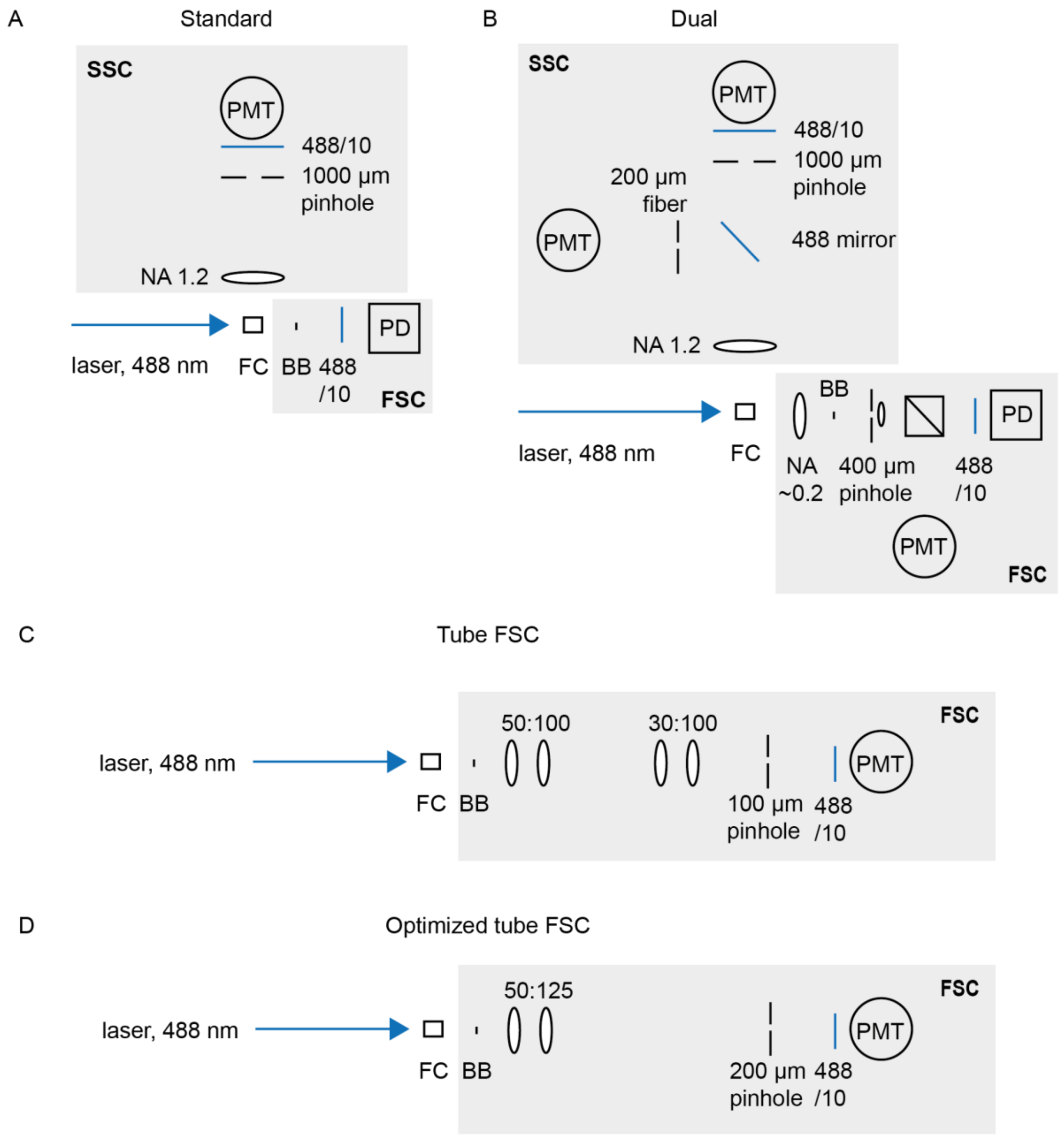

Figure S2: Schematic representation of the different forward (FSC) and side scatter (SSC) detection modules. A) Standard configuration of the FACSCanto. B) Dual FSC and SSC modules including a high resolution detection module in addition to the standard configuration. C) Tube FSC module. D) Optimized tube FSC module. 488/10: bandpass filter, 488 mirror: NFD01-488 (Semrock), BB: blocker bar, FC: flow cell, NA: numerical aperture, PD: photodiode, PMT: photomultiplier tube.

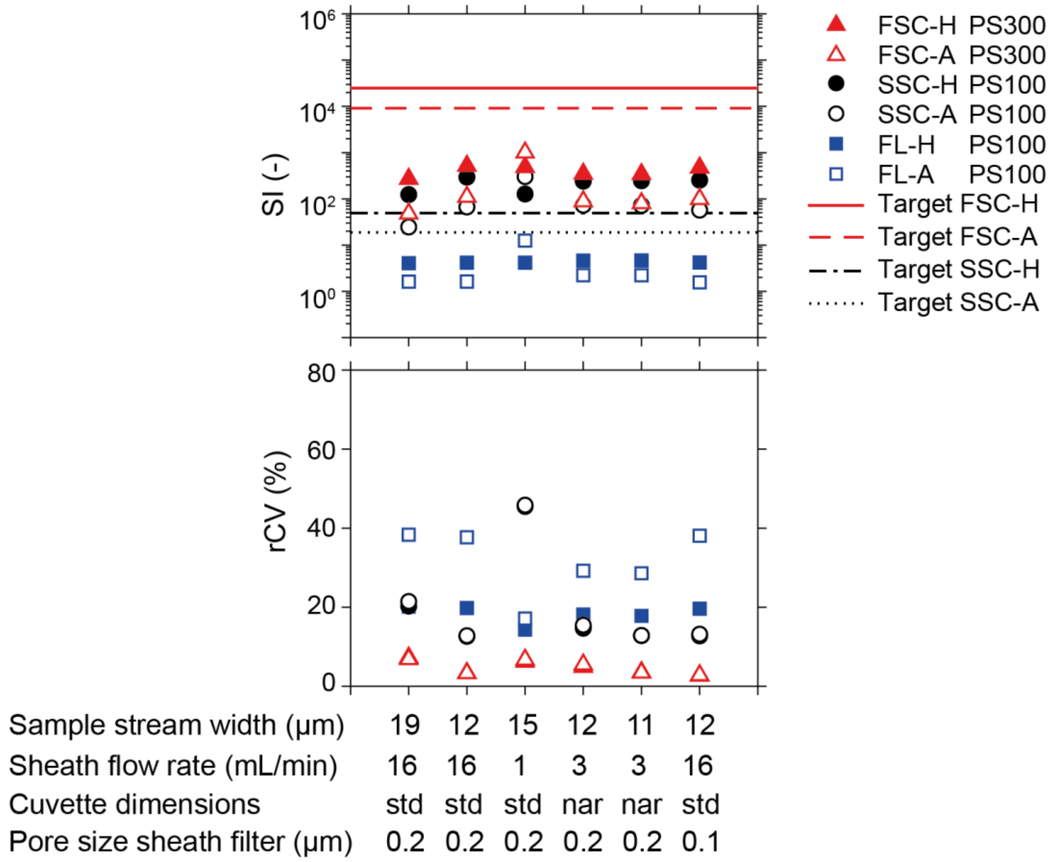

Figure S3: Influence of fluidics changes on the separation index (SI, top panel) and robust coefficient of variation (rCV, bottom panel). For forward scatter (FSC, red triangles), data of 300 nm polystyrene (PS) is shown, for side scatter (SSC, black circles) and fluorescence (FL, blue squares) data of 100 nm PS is shown. Lines represent the required SI for the depicted PS bead to allow detection of 100 nm extracellular vesicles for FSC (red lines) and SSC (black lines), as calculated using Mie theory. A: area parameter, H: height parameter, std: cuvette with a flow channel of standard dimensions, nar: cuvette with a flow channel of half the standard dimensions.

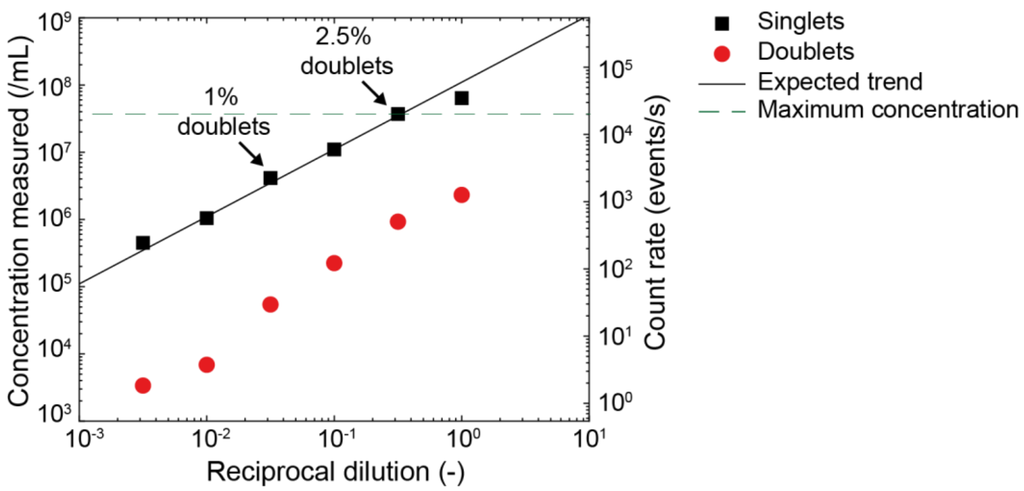

Figure S4: Measured concentration of singlets (black squares) and doublets (red circles) versus reciprocal dilution for 150 nm polystyrene beads. For concentrations ≤ $4 \cdot 10^7$ beads/mL and count rates ≤ $2 \cdot 10^4$ events/s, the singlets scale linearly with the reciprocal dilution (black line). At the maximum concentration (green dashed line), the estimated percentage of doublets is 2.5%.

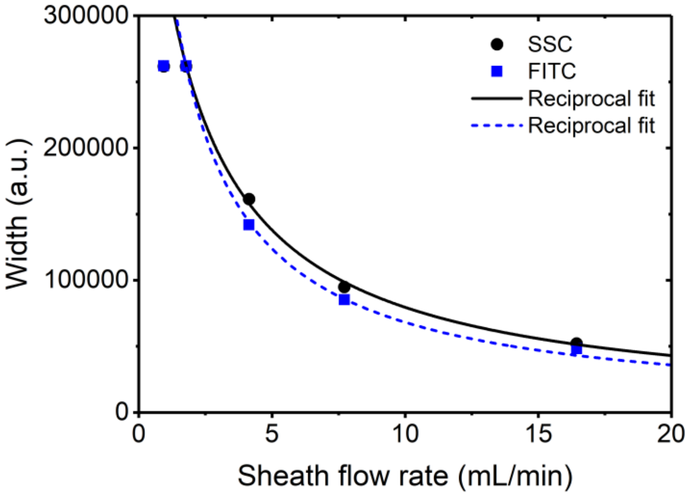

Figure S5: Median width of the 380 nm fluorescein isothiocyanate (FITC) beads of Rosetta Calibration (Exometry, Amsterdam, The Netherlands) versus the sheath flow rate. Data are fitted with the reciprocal function y=1/(a+b·x). For side scattered light (SSC), $a=1.915\cdot10^{-6}$, $b=1.066\cdot10^{-6}$, and $R^2= 1.000$. For FITC, $a=1.47627\cdot10^{-6}$, $b=1.319\cdot10^{-6}$, and $R^2=1.000$. The maximum width is 262080 arbitrary units (a.u.).

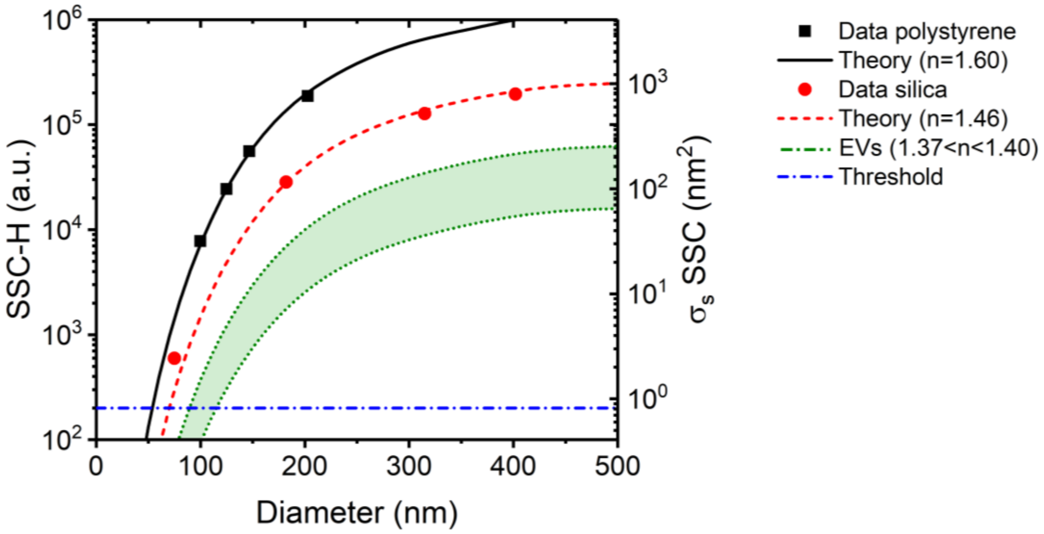

Figure S6: Measured (symbols) and calculated (lines) side scattered light (SSC-H) and side scattering cross section (σ_s) versus the diameter of polystyrene (squares) and silica beads (circles). The theory models a lens with a numerical aperture of 1.1 that is placed perpendicular to the propagation direction of a 488 nm laser beam. The theory describes the data well ($R^2=0.99$). According to this calibration, silica beads down to 70 nm exceed the SSC trigger threshold.

Determination of the target values

As defined in the data analysis section of the manuscript:

$$SI = \frac{median_{bead} - median_{noise}}{2 \cdot SD_{noise}} \quad (1)$$

For the height parameter, the ratio of the $SD_{noise}/median_{noise}$ was 0.29 in the standard configuration at 20 mW, and in the same order for the majority of other configurations. Assuming this ratio stays the same throughout the adaptations, we can thus define:

$$SD_{noise} = 0.29 \cdot median_{noise} \quad (2)$$

Substituting equation 2 into equation 1 yields for SI =1:

$$median_{bead} - median_{noise} = 2 \cdot 0.29 \cdot median_{noise} \quad (3)$$

$$median_{bead} = (2 \cdot 0.29 + 1) \cdot median_{noise} \quad (4)$$

$$median_{noise} = \frac{median_{bead}}{2 \cdot 0.29 + 1} \quad (5)$$

So by taking the median height scatter signal of a 100 nm EV (refractive index = 1.40) as modelled using Mie theory as the value for $median_{bead}$, the value for $median_{noise}$ can be calculated at which the 100 nm EV then has an SI =1. Using the resulting value for $median_{noise}$, the SD_{noise} as defined in equation 2 and the median height scatter signal as modelled using Mie theory for a polystyrene bead of chosen size as $median_{bead}$, the SI can be calculated using equation 1 and represents the SI required (i.e. the target value) to detect 100 nm EVs with an SI = 1.

For the area parameter, $median_{noise} = 0$ by definition and confirmed by measurements. So equation 1 reduces to

$$SI = \frac{median_{bead}}{2 \cdot SD_{noise}} \quad (6)$$

And for an SI =1

$$SD_{noise} = 0.5 \cdot median_{bead} \quad (7)$$

Using the median area scatter signal of a 100 nm EV (refractive index = 1.40) as modelled using Mie theory as the value for $median_{bead}$, the value for SD_{noise} can be calculated at which the 100 nm EV then has an SI =1. Similar as for the height parameter, subsequent use of the resulting value for SD_{noise} and the median area scatter signal as modelled using Mie theory for a polystyrene bead of chosen size as $median_{bead}$ allows calculation of the required SI (i.e. the target value) for the area parameter using equation 6.