Supplemental Data

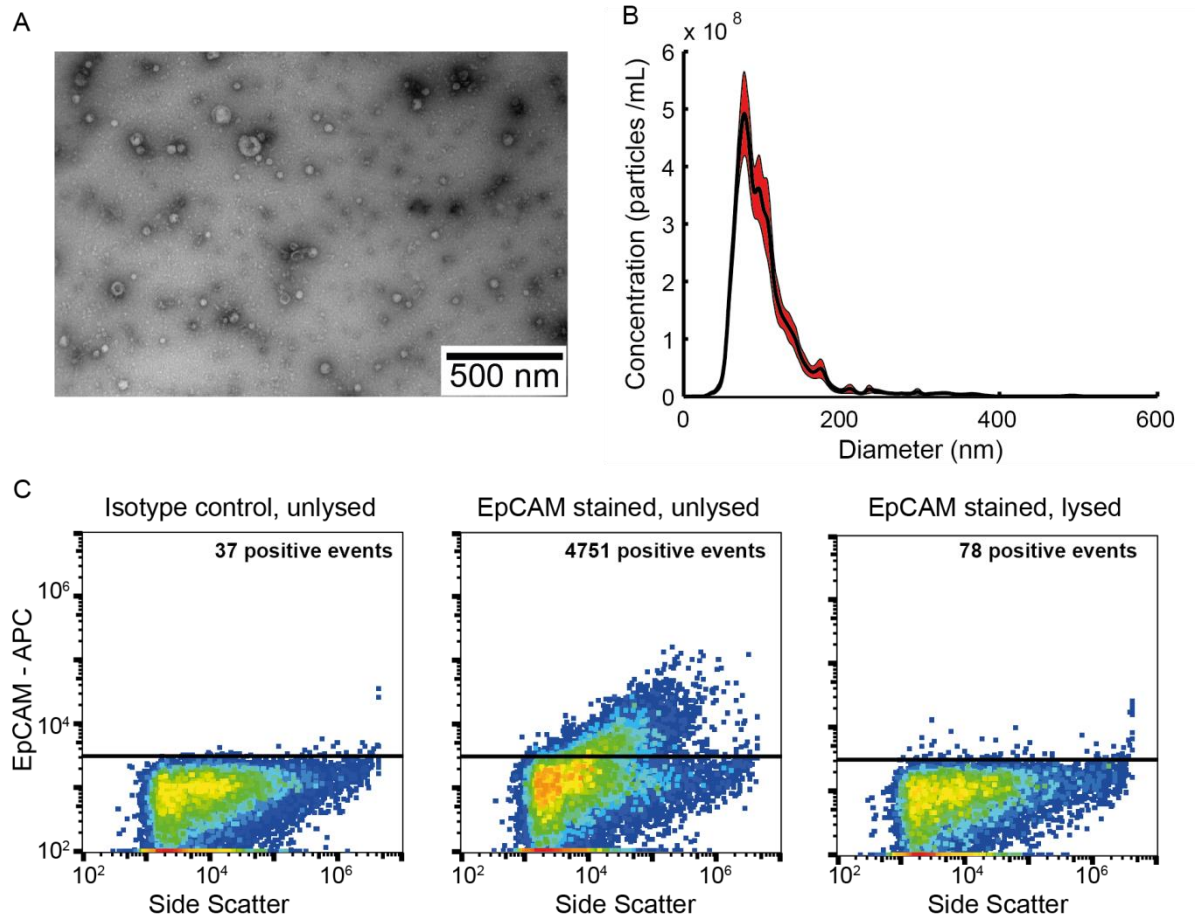


Fig. 1: Characterization of MCF7-EV sample. A) Transmission electron microscopy image of MCF7-EVs, after centrifugation at 154,000g for 60 minutes. No cellular membrane debris is visible on this or any of the other 5 images taken. Scale bar represents 500 nm. B) Size distribution of the MCF7-EVs, as measured by Nanoparticle Tracking Analysis. Black lines indicate the average size distribution, red areas indicate the standard error of the mean. C) Detergent sensitivity of $EpCAM^{+}$ particles. Addition of 4-nonylphenyl-polyethylene glycol (NP-40, Sigma-Aldrich) in a final concentration of 1% v/v, lysed >98% of EpCAM+ particles in the MCF7-EVs sample.

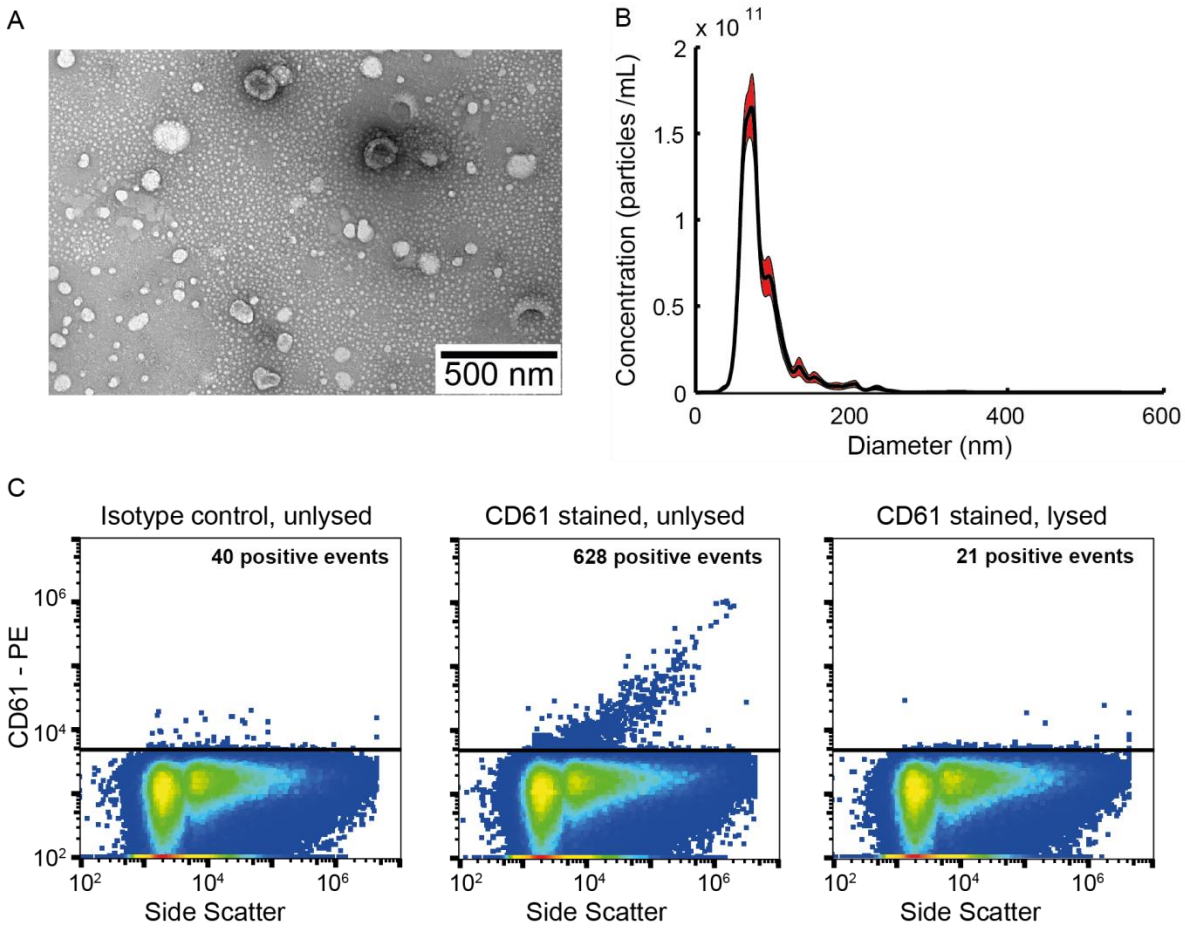


Fig. 2: Characterization of the plasma sample. A) Transmission electron microscopy image of plasma after size exclusion chromatography and centrifugation at 150,000g for 60 minutes. No cellular membrane debris is visible on this or any of the other 5 images taken. Scale bar represents 500 nm. B) Size distribution of the plasma sample, as measured by Nanoparticle Tracking Analysis. Black lines indicate the average size distribution, red areas indicate the standard error of the mean. C) Detergent sensitivity of $CD61^+$ particles. Addition of 4-nonylphenyl-polyethylene glycol (NP-40, Sigma-Aldrich) in a final concentration of 1% v/v, lysed >96% of $CD61^+$ particles in plasma.

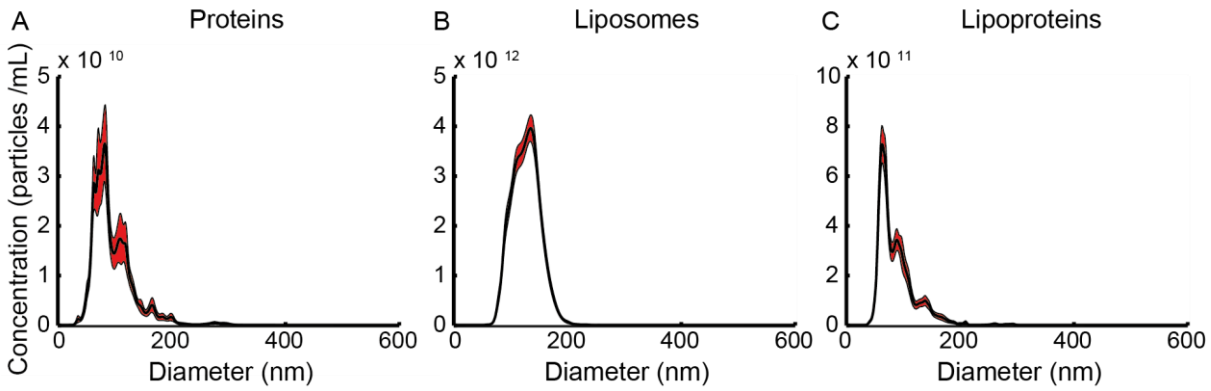


Fig. 3: Size distribution of the protein (A), liposome (B) and lipoprotein sample (C), as measured by Nanoparticle Tracking Analysis. Black lines indicate the average size distribution, red areas indicate the standard error of the mean.

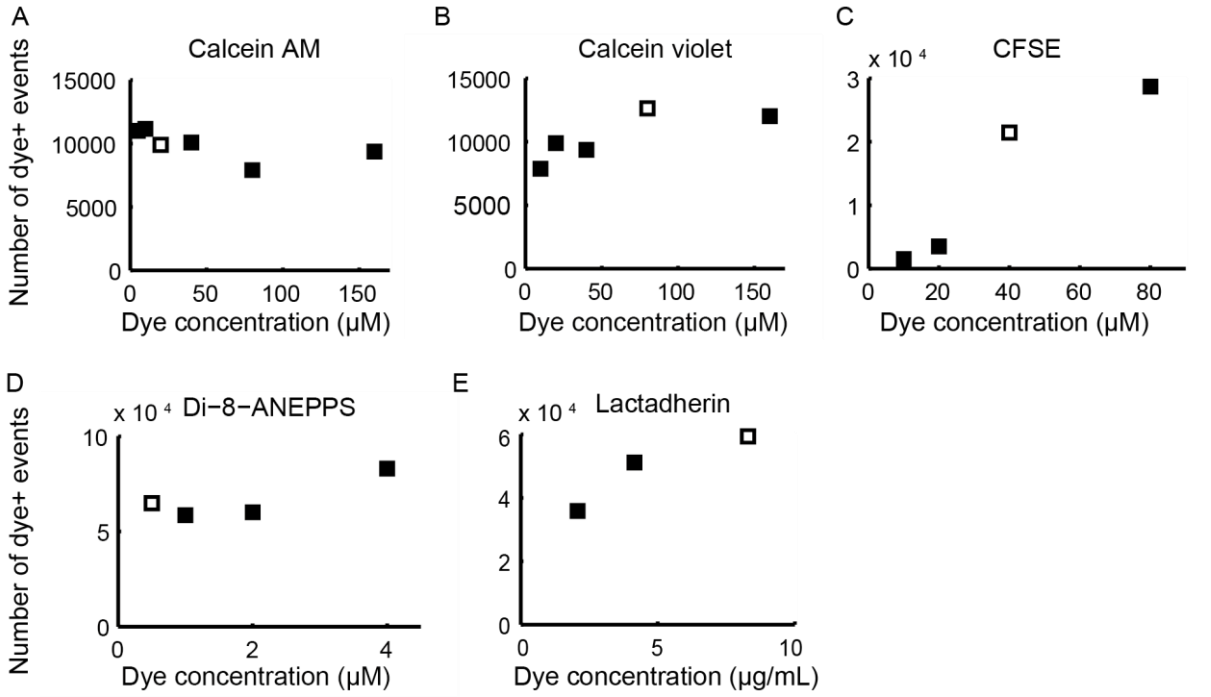


Fig. 4: Titration curves of the generic markers on MCF7-EVs. Open symbols indicate the concentration at which the generic marker was used in the present study. MCF7-EVs were used for generic marker titration because this is a less complex sample than plasma.

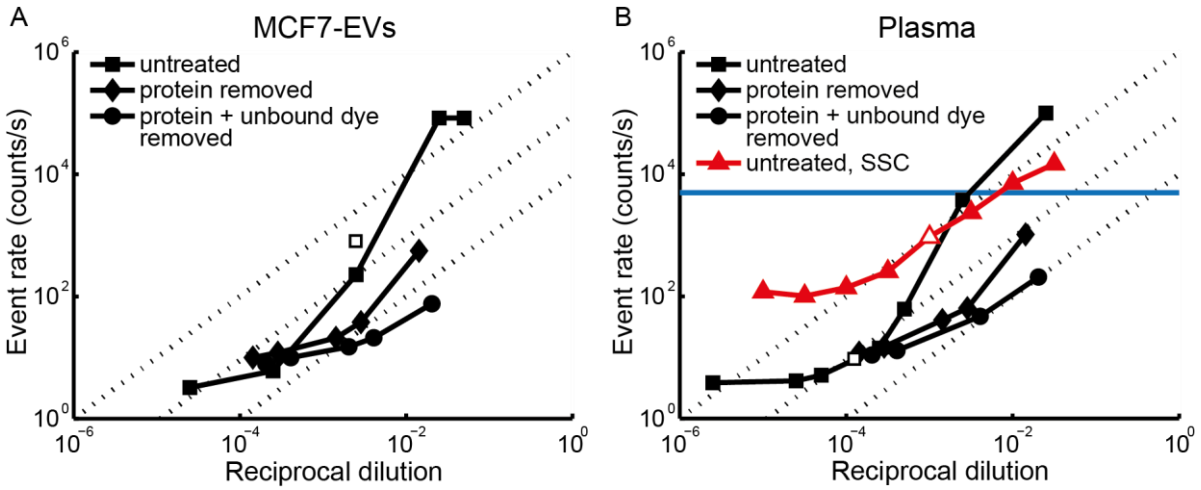


Fig. 5: Dilution curves of MCF7-EVs (A) and plasma (B), triggered on CFSE (black) or side scatter (SSC, red). With regard to the CFSE triggered samples (black), samples were directly stained with CFSE and measured (squares). Alternatively, protein was removed by size exclusion chromatography (SEC) before staining (diamonds), or SEC was applied before and after staining, before staining to remove protein and after staining to remove unbound CFSE (circles). Shown is the event rate per second versus the reciprocal dilution.
When no swarm is present, a relation exists in which an increase by factor *k* in the reciprocal dilution causes the event rate to increase by a factor *k* as well. The dotted lines represent such a relation. The SSC triggered data in B) shows a region in which the dilution curve is aligned with the dotted lines, indicating that at these dilutions no swarm is present. On our flow cytometer this coincides with an event rate below 5,000 counts/s (blue horizontal line) for these samples. For the CFSE triggered data (black), the dilution curves never aligns with the dotted lines, indicating that swarm is present at all sample dilutions for all samples. Data shown in Fig. 1 and Fig. 2 are measured at dilutions indicated by the open symbols.

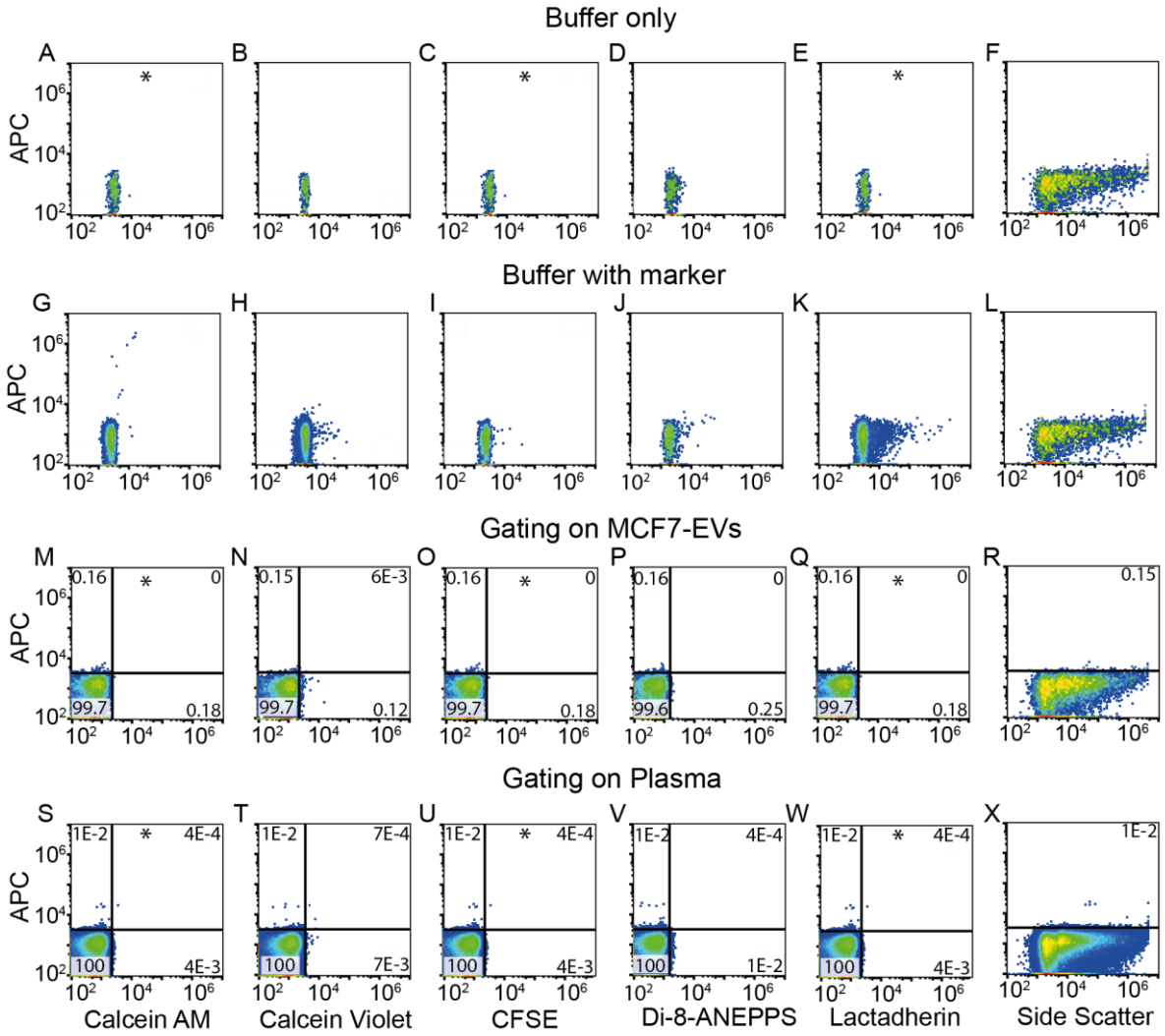
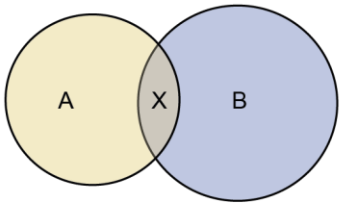


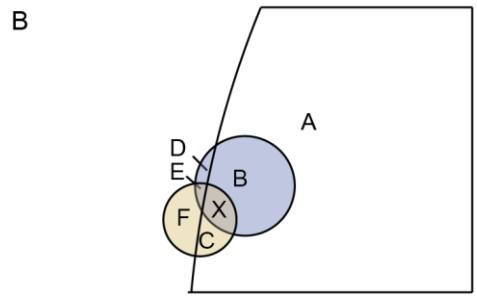
Fig. 6: A-F) Buffer only controls. G-L) Generic marker in buffer controls, note: for scattering no marker is needed so data shown in L is the same as that in F. M-R) Isotype controls of MCF7-EVs. MCF7-EVs were stained with IgG1-APC and triggered on scatter, fluorescence in the channels of the markers was evaluated to set the gates used in this study. The same method was used to set the gates for plasma (S-X). Data of one representative minute are shown. Numbers in quadrants indicate percentage of total population. Within each row, data marked with asterisk is the same data, since Calcein AM, CFSE and lactadherin fluorescence are all detected in the same channel. All axes are in arbitrary units.

A



X: [mAb+marker+] when triggered on marker
A: [mAb+] when triggered on mAb - X
B: [marker+] when triggered on dye - X

B



X: [mAb+marker+scat+] when triggered on scat
A: [mAb-marker-scat+] when triggered on scat
B: [mAb-marker+scat+] when triggered on scat
C: [mAb+marker-scat+] when triggered on scat
D: [mAb-marker+] when triggered on marker - B
E: [mAb+marker+] when triggered on marker - X
F: [mAb+] when triggered on mAb - X - C - E

Fig. 7: Schematic representation on how the Venn diagrams are calculated. A) Calculation for the Venn diagrams in Fig. 1 and 2. Marker can be replaced by scatter in case of Fig. 1L and Fig. 2L, R. B) Calculation for the Venn diagrams in Fig. 3.

Cause of low liposome staining with generic markers

In contrast to an earlier study, our liposomes did not stain for di-8-ANEPPS (1). The main difference is the composition of the used liposomes. Stoner used phospholipids that have a transition temperature below 0 ^{o}C. The membranes of such phospholipids are thus in the "liquid crystalline phase" at room temperature, and therefore allow marker incorporation. The liposomes in our study are in the "ordered gel phase" at room temperature, which will complicate incorporation of the marker. When we labeled our liposomes at a temperature above the transition temperature of our phospholipids (60 ^{o}C), the concentration of di-8-ANEPPS positive liposomes increased 27-fold (Table 1, PCPGlip-200 are the liposomes used in this study, Egg-PC liposomes are similar to the liposomes used in Stoner 2016). In addition, our liposomes have a relatively high concentration of cholesterol, which causes a blue-shift in the spectrum of di-8-ANEPPS (2). This blue-shift decreases the intensity of di-8-ANEPPS in the channel we used to detect its fluorescence.
Furthermore, there is a difference in detection filters used in the two studies. Stoner applied an optical long pass filter that transmits approximately 5-fold more di-8-ANEPPS light than our narrowband filter (based on Fluorescence Spectraviewer, ThermoFischer).
Together, these factors explain the difference between the earlier results of Stoner and our present findings. The composition of liposomes is thus of key importance when using liposomes as a reference material for EVs.

Table 1: Detected concentrations of di-8-ANEPPS positive liposomes

Liposome	Room temperature		60 ^{o}C	
	red positive [a] (10^9 /mL)	orange positive [b] (10^9 /mL)	red positive [a] (10^9 /mL)	orange positive [b] (10^9 /mL)
PCPGlip-200	10	70	60	1,900
Egg-PC	1,500	200	-	-

[a] 488-red positive, 680/35 nm band pass filter
[b] 488-orange positive, 575/30 nm band pass filter
All concentrations are corrected for marker in buffer controls. -: not determined.

PCPGlip-200 liposomes: 13.2 mM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, NOF Corp.), 3.3 mM 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt (DPPG, NOF Corp.), 16.5 mM cholesterol (Sigma-Aldrich).
Concentration and size as determined by NTA $2.54 \cdot 10^{14}$ /mL, mean diameter 127 ± 25 nm (See Fig. S3). Liposomes were prepared by the Research Centre for Natural Sciences (Budapest, Hungary) using 200 nm pore filters (3).
Egg-PC liposomes: 27 mM L-α-phosphatidylcholine (Egg-PC, Avanti Polar Lipids).
Concentration and size as determined by NTA $3.77 \cdot 10^{14}$ /mL, mean diameter 78 ± 26 nm.

Table 2: Sample dilutions before (predilution) and after (postdilution) staining with generic markers and/or mAb.

Sample	Predilution (1:x)	Postdilution (1:x)
Marker in buffer	-	-
Proteins	-	20
Liposomes [a]	100	3,200
Lipoproteins	-	12,000
MCF7-EVs	-	40.5
Plasma	-	804
Plasma SEC	-	42.5

[a] Final sample dilution is 100 x 3,200 = 320,000

Characterization of flow cytometer analytical sensitivity

Mean fluorescent intensity (MFI) was converted to molecules of equivalent soluble fluorochrome (MESF) for phycoerythin (PE), fluorescein isothiocyanate (FITC) and allophycocyanin (APC) using the SPHERO PE Calibration kit (ECFP-F2-5K, Spherotech), Quantum FITC-5 MESF beads (555A, Bangs Laboratories) and Quantum APC MESF beads (823A, Bangs) respectively. Flow cytometer fluorescent sensitivity was characterized as described elsewhere (4) using QbSure Multipeak Cytometer Calibration beads (97-00306-01, Cytek). The resulting detection efficiency (Q) and background light (B) were used to calculate a fluorescent resolution limit (R) defined by (Stoner et al. 2016) as:

$$R = \frac{4\sqrt{Q \cdot B}+1}{Q} \quad (1)$$

Fluorescent sensitivity analysis for our Apogee A60-Micro resulted in an R of 74 PE MESF, 304 FITC MESF and 16 APC MESF. The MFI-MESF conversion assumes that the contribution of the bead autofluorescence is negligible. However, for the different APC MESF beads, the autofluorescence was 25-98% of the total MFI, see table S3. This may explain the unlikely R for APC. The R for PE and FITC were as expected.

$$MESF_{APC} = 10^{2.7938 \cdot log_{10}(MFI) - 10.98} \quad (2)$$

$$MESF_{FITC} = 10^{1.1738 \cdot log_{10}(MFI) - 1.7194} \quad (3)$$

Table 3: APC MESF bead measurements

Bead number	Specified MESF value	MFI
B (blanco)		403000
1	21008	409000
2	172008	522000
3	691111	804000
4	1969391	1780000

Table 4: Concentration of $EpCAM^+$ MCF7-EVs or $CD61^+$ EVs in plasma, detected by triggering on fluorescence of the indicated generic marker, side scatter or antibody fluorescence.

Trigger	Detected concentration of $EpCAM^+$ particles in MCF7-EV sample (10^6 /mL)	Detected concentration of $CD61^+$ particles in plasma sample (10^6 /mL)	Detected concentration of $CD61^+$ particles in plasma SEC sample (10^6 /mL)
Fluorescence			
Calcein AM	6 (3)	-0.1 (0.3)	0.9 (0.6)
Calcein violet	17 (2)	8 (2)	4 (0.5)
CFSE [a]	18 (3)	3 (3)	4 (1)
Di-8-ANEPPS	45 (7)	0.3 (1)	2 (0.7)
Lactadherin	42 (13)	30 (3)	4 (0.3)
EpCAM / CD61	37 (4)	90 (30)	132 (23)
Scatter	39 (10)	56 (12)	15 (2)

Concentrations are corrected for dilution steps and events measured in IgG1 control samples. Presented are the mean (standard deviation) of 3-6 experiments per sample. [a] Samples stained with CFSE suffered from swarm.

References

1. Stoner SA, Duggan E, Condello D, Guerrero A, Turk JR, Narayanan PK, Nolan JP. High sensitivity flow cytometry of membrane vesicles. Cytometry A 2016;89:196-206.
2. Gross E, Bedlack RS, Loew LM. Dual-wavelength ratiometric fluorescence measurement of the membrane dipole potential. Biophys J 1994;67:208-16.
3. Martin FJ, Morano JK. Liposome extrusion method. Vol.: United States Patent and Trademark Office, 1988.
4. Chase ES, Hoffman RA. Resolution of dimly fluorescent particles: A practical measure of fluorescence sensitivity. Cytometry 1998;33:267-79.