Comparison of Generic Fluorescent Markers for Detection of Extracellular Vesicles by Flow Cytometry

Leonie de Rond,[1,2,3]* Edwin van der Pol,[1,2,3] Chi M. Hau,[2,3] Zoltan Varga,[4,5] Auguste Sturk,[2,3] Ton G. van Leeuwen,[1,2] Rienk Nieuwland,[2,3] and Frank A.W. Coumans[1,2,3]

BACKGROUND: Extracellular vesicles (EVs) in biofluids are potential biomarkers of disease. To explore the clinical relevance of EVs, a specific generic EV marker would be useful, one that does not require antibodies and binds to all EVs. Here we evaluated 5 commonly used generic markers for flow cytometry.

METHODS: Flow cytometry (A60-Micro, Apogee) was used to evaluate the ability of the generic EV markers calcein acetoxymethyl ester, calcein acetoxymethyl ester violet, carboxyfluorescein succinimidyl ester (CFSE), 4-(2-[6-(dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium (di-8-ANEPPS), and lactadherin to stain EVs from MCF7 human breast adenocarcinoma cell line-conditioned culture medium [epithelial cell adhesion molecule positive (EpCAM$^+$)] or platelet EVs from human plasma [integrin $\beta3$ positive (CD61$^+$)]. Side scatter triggering was applied as a reference, and the influence of non-EV components (proteins and lipoproteins) was evaluated.

RESULTS: Di-8-ANEPPS, lactadherin, and side scatter detected 100% of EpCAM$^+$ MCF7 EVs. Lactadherin and side scatter detected 33% and 61% of CD61$^+$ EVs, respectively. Di-8-ANEPPS detected platelet EVs only if soluble protein was first removed. Because all generic markers stained proteins, at best 33% of platelet EVs in plasma were detected. The calcein markers and CFSE were either insensitive to EVs in both samples or associated with swarm detection.

CONCLUSIONS: None of the generic markers detected all and only EVs in plasma. Side scatter triggering detected the highest concentration of plasma EVs on our A60-Micro, followed by lactadherin. The choice between scatter or lactadherin primarily depends on the analytical sensitivity of the flow cytometer used.

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Extracellular vesicles (EVs)[6] are cell-derived particles (50–1000 nm) with a phospholipid membrane. EV populations in biofluids are potential biomarkers of disease, and such EVs can be identified by immunophenotype (1–6). The immunophenotype of single EVs above the detection threshold can be determined at high throughput and analytical sensitivity using flow cytometry (7). In flow cytometry, particles are detected based on light scattering or fluorescence. However, because EVs are weak light scatterers and common flow cytometers have a high optical background and insensitive scatter detectors, detection of EVs based on light scattering is difficult. A generic marker, defined here as any form of nonimmunoaffinity labeling, may have advantages over scatter triggering, including better separation from noise (8), well-established calibration methods (7, 9), and lower susceptibility to swarm detection (10). Ideally, a generic EV marker (a) stains all EVs, i.e., 100% sensitivity, (b) stains only EVs, i.e., 100% specificity, (c) is compatible with fluorescent immunostaining, (d) can be directly used in biofluids, and (e) has a staining protocol <8 h for clinical applicability. See the Statistics paragraph of the Methods section for the definition of sensitivity and specificity used in this study.

We considered including calcein acetoxymethyl ester (calcein AM), calcein acetoxymethyl ester violet (calcein violet), carboxyfluorescein succinimidyl ester (CFDA-SE, here and commonly referred to as CFSE), 4-(2-[6-(dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium (di-8-ANEPPS), annexin V,

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lactadherin, and Paul Karl Horan 67 (PKH67) (8, 9, 11–15). Table 1 shows an overview of the reported properties and results for these markers, except calcein violet and lactadherin, which have not been used as generic markers previously. Calcein AM (495/515 nm absorption/emission) and calcein violet (400/452 nm) are both membrane permeable and nonfluorescent, requiring hydrolysis to be converted into a membrane-impermeable fluorescent analog (11, 16). Because intravesicular esterases catalyze the hydrolysis, both markers label intact EVs only (11). Furthermore, calcein violet has limited spectral overlap with commonly applied fluorophores, which simplifies combining calcein violet with immunofluorescent labeling. Like calcein AM, CFDA-SE is also nonfluorescent and requires hydrolysis to be converted to CFSE (492/517 nm), which then binds covalently to free amines (17). Di-8-ANEPPS (467/631 nm) is nonfluorescent in solution and becomes fluorescent when bound to phospholipid membranes and possibly proteins (9, 18). Annexin V binds to membrane phosphatidylserine in the presence of calcium. Because calcium may trigger clotting of plasma or blood, we used lactadherin since (a) both annexin V and lactadherin bind to phosphatidylserine, (b) the affinity of lactadherin for phosphatidylserine is higher than that of annexin V (19), and (c) binding of lactadherin to phosphatidylserine is calcium independent. Both annexin V and lactadherin, however, are not true generic markers because phosphatidylserine is not exposed on all EVs (20). This also holds true for calcein AM, calcein violet, and CFSE, as it is uncertain whether all EVs efficiently hydrolyze the markers. Therefore, for convenience, all markers tested are termed "generic markers." Finally, PKH67 was not evaluated because the labeling protocol is longer than 8 h and not clinically applicable (8, 21).

The aforementioned generic markers for labeling EVs were evaluated on various samples and flow cytometers (Table 1). Using flow cytometry to detect epithelial cell adhesion molecule positive (EpCAM$^+$) EVs from MCF7 cell culture and integrin β3 positive (CD61$^+$) EVs from human plasma, we compared the performance of the most promising generic markers, namely, calcein AM, calcein violet, CFSE, di-8-ANEPPS, and lactadherin, and used side scatter as a reference. We also evaluated the influence of common coisolates of EVs and marker micelles or aggregates on the detection of EVs with generic markers.

Materials and Methods

REAGENTS

Calcein AM and calcein violet were obtained from eBioscience. CFSE, Pluronic F-127, RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin were all from ThermoFisher Scientific. Di-8-ANEPPS

and BSA (purity ≥98%) were from Sigma-Aldrich, and lactadherin–fluorescein isothiocyanate (FITC) was from Hematologic Technologies. PBS was from Fresenious Kabi, and VLDLs were from Merck Millipore. Allophycocyanin (APC)-conjugated EpCAM (mouse IgG1κ, clone HEA-125) was from Miltenyi Biotec, CD61-APC (mouse IgG1κ, clone Y2/51) was from Dako, and IgG1 isotype control was from BD. The buffer used in all experiments, including size-exclusion chromatography (SEC), was PBS [154 mmol/L NaCl, 1.24 mmol/L Na$_2$HPO$_4$.2H$_2$O, 0.2 mmol/L NaH$_2$PO$_4$.2H$_2$O, pH 7.4; 50 nm filtered (Nuclepore, GE Healthcare)], supplemented with 0.32% trisodium citrate.

EV SAMPLES

MCF7 breast cancer cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mmol/L L-glutamine, 10 U/mL penicillin, and 10 μg/mL streptomycin. Cells were cultured in a T75 culture flask (Corning) at 37 °C, 5% CO$_2$. At 80% to 90% confluence, cells were washed with PBS and cultured in fetal bovine serum-free medium before harvesting EVs. After 48 h, the conditioned culture medium was centrifuged at 1000g for 30 min (Rotina 46 RS, Hettich) to remove cells. We call the resulting supernatant MCF7 EVs throughout the text.

To prepare platelet-free plasma, citrate-anticoagulated blood (0.32% final concentration) from 10 healthy donors was collected as described elsewhere (22). Informed consent and approval from the ethics committee was obtained. The blood was pooled, and plasma was prepared by centrifuging the blood twice at 1560g, 20 °C for 20 min.

Both MCF7 EVs and the plasma sample were snap-frozen in liquid nitrogen, stored at −80 °C, and thawed at 37 °C before use. Figs. 1 and 2 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue4 show the characterization of the MCF7 EVs and plasma sample.

PREPARATION OF NON-EV COMPONENTS

Non-EV components in plasma include proteins and lipoproteins. Because liposomes are currently being investigated as reference particles for EV measurements (9), we also studied whether generic markers stained liposomes. We found, however, that the composition of our liposomes was unsuitable for evaluation of generic markers (see Table 1 of the online Data Supplement) and, therefore, excluded the results from any further discussion.

Non-EV components studied here include marker in buffer, a protein solution containing 40 g/L BSA and lipoproteins. Fig. 3 of the online Data Supplement provides the size and concentration information of the non-EV samples.

Table 1. Published uses of generic markers for fluorescence triggering of extracellular vesicles.

Marker	Mechanism	EV sample	Flow cytometer	Fluorescence resolution limit, MESF	Smallest bead measured[a], nm	Conclusion[b]	Reference number
Calcein AM	Fluoresces upon hydrolysis	Cell culture, PFP,[c] erythrocyte EVs	BD LSRII	–	760	Labels only intact EVs	(11)
CFSE	Fluoresces upon hydrolysis and then binds to amines	Cell culture, malignant ascites	Apogee A50	–	100	Labels more EVs than annexin V, protocol <12 h	(12)
Di-8-ANEPPS	Voltage-sensitive membrane marker	PRP	Customized FACSCanto	FITC 236 PE 71	530	Allows EV sizing	(9)
Annexin V	Binds to phosphatidylserine	PFP	BC Gallios	Cy5 1263	300	Fluorescence triggering detects 77x more platelet EVs than scatter triggering	(13)
PKH67	Intercalates in membrane	Cell culture, seminal fluid	Customized BD influx	–	200	Fluorescence triggering distinguishes EVs from noise	(8)

[a] Smallest polystyrene bead diameter distinguished in indicated reference, 180-nm refractive index 1.42 bead in reference 12 converted to polystyrene bead equivalent using scatter model (28).
[b] Conclusion as drawn by the authors in reference.
[c] PFP, platelet-free plasma; PRP, platelet-rich plasma; Cy5, cyanine 5.

SEC

To investigate the impact of protein on staining of EVs by the generic markers, protein was removed using SEC before generic marker staining and compared with untreated samples. SEC columns (qEVsingle, Izon Science; 3.5 mL Sepharose CL-2B) were washed once with 6 mL of buffer before loading 100 μL of plasma, followed by buffer elution. The first 1 mL of eluate was discarded, after which the 0.5-mL fraction containing EVs was collected (23).

STAINING PROTOCOLS

Sample incubation conditions were based on literature (9, 11, 12): 20 min at 37 °C for calcein AM and calcein violet, 60 min at 37 °C for CFSE, and 60 min at room temperature for di-8-ANEPPS and lactadherin. Generic markers were titrated on MCF7 EVs (see Fig. 4 of the online Data Supplement). A 10-μL sample containing EVs or non-EV components was incubated with either 10 μL of calcein AM (final concentration, 20 μmol/L), calcein violet (80 μmol/L), CFSE (40 μmol/L), di-8-ANEPPS (0.5 μmol/L) with 0.05% Pluronic F-127, lactadherin (8.3 μg/mL), or buffer.

MCF7 EVs were identified by exposure of EpCAM (24); platelet EVs, by CD61 (25). APC-conjugated antibodies were used for immunostaining because of negligible spectral overlap with the evaluated generic markers. Next, 2.5 μL of EpCAM-APC (22 μg/mL), CD61-APC (25 μg/mL), or IgG1 isotype control at a matching concentration was added to 10 μL of generic marker-stained sample and incubated for 2 h at room temperature in the dark. To remove aggregates, antibodies and generic markers were centrifuged at 19000g for 5 min before use.

After incubation, samples were diluted in buffer to event rates below approximately 5000/s to prevent swarm when triggering on side scatter (see Table 2 and Fig. 5 of the online Data Supplement). A separate control experiment demonstrated that >96% of antibody positive (mAb$^+$) particles in the EV samples lysed upon detergent treatment, indicating that most mAb$^+$ particles were probably EVs (see Figs. 1 and 2 of the online Data Supplement).

FLOW CYTOMETRY

All samples were analyzed on an A60-Micro (Apogee) at a flow rate of 3.0 μL/min. Samples were measured for 4 min when triggering on EpCAM-APC, CD61-APC, or generic marker fluorescence and for 1 min when triggering on side scatter (405-nm laser). Fluorescence-triggered measurements were longer to ensure a statistically relevant number of detected particles. Trigger thresholds were set to a value resulting in 10 to 20 counts/s in buffer. Gates were based on isotype controls without generic marker. For both mAb and generic marker, positive ($^+$) is defined as a fluorescent signal exceeding the gate. Particle

concentrations were calculated by compensating the number of detected particles for flow rate, measurement time, and sample dilution. All concentrations were corrected using acquisition time-matched plain buffer or IgG1 controls to estimate the number of sample-specific events. A full description of the flow cytometer configuration, operating conditions, and data analysis is found in the MIFlowCyt list of the online Data Supplement.

For each generic marker, 6 repeated measurements were performed for both EV samples and 2 measurements for the non-EV samples. In all, 115 of 124 EV sample measurements were successful, with a minimum of 3 successful measurements per generic marker. Reported results are the mean of individually stained samples.

CHARACTERIZATION OF FLOW CYTOMETER ANALYTICAL SENSITIVITY

The resolution limit of the Apogee A60-Micro was 74 phycoerythrin (PE) molecules of equivalent soluble fluorochrome (MESF), 304 FITC MESF, and 16 APC MESF. We considered the resolution limit for APC unreliable (details provided in the online Data Supplement). Scatter analytical sensitivity was characterized by reporting the smallest polystyrene bead distinguishable from noise, which was 100-nm polystyrene on our flow cytometer.
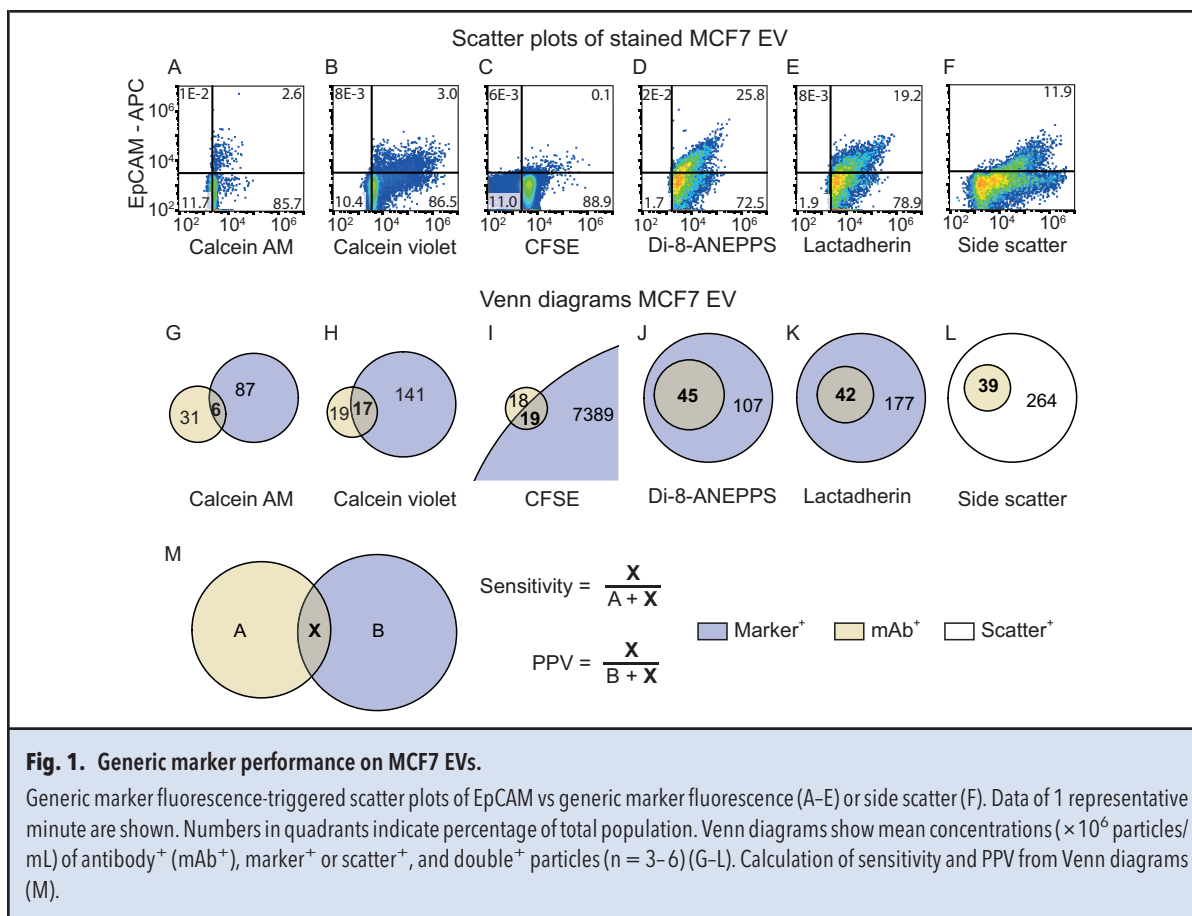
STATISTICS

Throughout this study, the performance of the generic markers was expressed in terms of sensitivity and positive predictive value (PPV) for EV detection. Unless stated otherwise, sensitivity is defined as the percentage of mAb$^+$ EVs detectably stained by the generic marker out of the total mAb$^+$ EV population. This definition hereby differs from those used for clinical and analytical sensitivity. The PPV is defined here as the percentage of mAb$^+$ EVs detectably stained by the generic marker out of the total marker$^+$ population. Sensitivity and PPV were calculated as:

$$\text{sensitivity} = \frac{[\text{mAb}^+\text{marker}^+]}{[\text{mAb}^+]} \cdot 100\% \qquad (1)$$

$$\text{PPV} = \frac{[\text{mAb}^+\text{marker}^+]}{[\text{marker}^+]} \cdot 100\% \qquad (2)$$

with [mAb$^+$marker$^+$] the detected concentration of mAb$^+$ particles while triggering on the generic marker, [mAb$^+$] the detected concentration of mAb$^+$ particles while triggering on the antibody, and [marker$^+$] the detected concentration of marker$^+$ particles while triggering on the generic marker. It was not possible to calculate the specificity of the generic markers because marker$^-$ particles (i.e., true negatives) were not detected when

Fig. 1. Generic marker performance on MCF7 EVs.

Generic marker fluorescence-triggered scatter plots of EpCAM vs generic marker fluorescence (A–E) or side scatter (F). Data of 1 representative minute are shown. Numbers in quadrants indicate percentage of total population. Venn diagrams show mean concentrations ($\times 10^6$ particles/mL) of antibody$^+$ (mAb$^+$), marker$^+$ or scatter$^+$, and double$^+$ particles (n = 3–6) (G–L). Calculation of sensitivity and PPV from Venn diagrams (M).

triggering on fluorescence. Instead, the PPV was calculated and used as a measure for specificity.

To evaluate the significance of differences between plain buffer (n = 10) and non-EV components (n = 2), a 1-sided (because buffer has the fewest particles), 1-sample *t*-test was applied to determine *P*.

Results

GENERIC MARKER PERFORMANCE ON CELL CULTURE EVs

First, the performance of the generic markers was evaluated on EVs from MCF7 cells. EVs were identified by EpCAM-APC, and we assumed that all EpCAM binding (mAb$^+$) particles were true EVs. Fig. 1, A–E, shows representative scatter plots of MCF7 EVs triggered by fluorescence of the 5 generic markers. Side scatter triggered measurements are included for reference (Fig. 1F). Fig. 6 in the online Data Supplement shows the results for buffer only, marker in buffer, and isotype controls.

Fig. 1, G–L, shows Venn diagrams that were calculated using the concentrations of particles in the upper and lower right quadrants of the scatter plots, combined with the concentration of total EpCAM$^+$ particles from

EpCAM-APC triggered measurements (Table 4 of the online Data Supplement shows the raw numbers, and Fig. 7 of the online Data Supplement provides a calculation of the Venn diagrams). The diagrams show the concentrations of missed EVs (mAb$^+$marker$^-$), detected EVs (mAb$^+$marker$^+$), and possibly falsely detected EVs (mAb$^-$marker$^+$). The sensitivity to detect EpCAM$^+$ particles was calculated from Fig. 1, G–L, as shown in Fig. 1M and previously described in the Methods section.

Table 2 shows the sensitivity of each generic marker and side scatter to detect EVs. CFSE suffered from swarm at all dilutions, making these results unreliable (see Fig. 5 of the online Data Supplement). Ideally, a generic marker would exclusively stain EVs. Because not all marker$^+$ particles are also EpCAM$^+$ (Fig. 1, A–E), the PPV of generic markers was also calculated. For interpretation, a PPV of 29% indicates that 29% of the marker$^+$ events are also mAb$^+$, and thus EVs by our definition. Table 2 shows that all generic markers had a PPV <100%. Taken together, di-8-ANEPPS and lactadherin were the most promising generic markers for MCF7 EVs based on sensitivity and PPV. Subsequently, the ability of generic markers to detect EVs in plasma was studied because

Table 2. Summary of trigger strategies for detecting extracellular vesicles.

Trigger	Channel	Spectral overlap with	MCF7 EVs		Platelet EVs		Detects also	Platelet EVs after SEC	
			Sensitivity[a], %	PPV[b], %	Sensitivity[a], %	PPV[b], %		Sensitivity[a], %	PPV[b], %
Fluorescence									
Calcein AM	FITC	FITC	16 ± 8	3	−0.1 ± 0.3	−0.2	M, P[c]	1 ± 1	2
Calcein violet	Violet	DAPI	47 ± 8	1	9 ± 4	7	M, P	3 ± 1	1
CFSE[d]	FITC	FITC	49 ± 9	0.2	3 ± 4	0.3	M, P, LP	3 ± 1	2
Di-8-ANEPPS	PerCP	PerCP, PE, FITC	122 ± 25	29	0.3 ± 2	0.2	M, P	1 ± 1	1
Lactadherin	FITC[e]	FITC[e]	113 ± 39	19	33 ± 11	17	M, P	3 ± 1	1
Scatter	SSC	−	105 ± 30	13	61 ± 25	0.1	M, P, LP	12 ± 3	0.3

[a] Antibody+marker+ particles relative to antibody+ particles ± SD.
[b] Antibody+marker+ particles relative to marker+ particles ± SD.
[c] M, marker in buffer; P, proteins; LP, lipoproteins; PerCP, peridinin-chlorophyll-protein; SSC, side scatter channel.
[d] Results obtained with CFSE suffered from swarm (see Fig. 5 of the online Data Supplement).
[e] PE if lactadherin-PE is used.

plasma contains non-EV particles that may interfere with detection of EVs by generic markers.

GENERIC MARKER PERFORMANCE ON PLASMA EVs

Plasma contains various populations of EVs. Here we chose to study platelet EVs, assuming that detection of platelet EVs was representative of detection of all EV populations in plasma. Platelet EVs were immunostained with CD61-APC, assuming that all CD61-APC-binding (mAb+) particles were true EVs.

In analogy to MCF7 EVs (Fig. 1), we prepared Fig. 2 for platelet EVs in plasma and Table 4 in the online Data Supplement for the raw numbers. The sensitivity for detecting CD61+ particles was calculated from Fig. 2, G–L, as shown in Fig. 2S and described in the Methods section, and presented in Table 2. Again, CFSE results were unreliable because of swarm. Approximately 30% of all plasma EVs expose CD61 (20). Therefore, a PPV of 30% for CD61+ particles in plasma was considered a perfect performance, i.e., 30% of all marker+ particles would also be CD61+. Table 2 shows the PPV for each generic marker and side scatter, as calculated from the Venn diagrams (Fig. 2, G–L). In summary, lactadherin and side scatter detected CD61+ particles in plasma. The low PPVs indicated that the generic markers also stained mAb− particles, which could be EVs with mAb fluorescence below the detection limit of our flow cytometer, other EV populations, or non-EV particles. Therefore, we studied whether generic markers stained non-EV particles.

INFLUENCE OF NON-EV COMPONENTS

Suspensions of EVs isolated from plasma often contain non-EV components (26). Table 3 shows the particles detected when the indicated marker was added to buffer, proteins, and lipoproteins. Because the particle concentration differed in each sample, the concentrations cannot be compared between columns. All generic markers and side scatter detected particle concentrations higher than the background (plain buffer) in the marker in buffer sample ($P < 0.001$) and in the sample containing protein ($P < 0.0001$). Only CFSE and side scatter detected particles in excess of background in the lipoprotein sample ($P = 0.002$ and $P < 0.001$, respectively). Our protein sample contained a physiological concentration of albumin (27). Therefore, binding of the generic markers to plasma proteins may explain their low sensitivity to detect EVs in plasma.

DETECTION OF PLASMA EVs BY GENERIC MARKERS AFTER PROTEIN REMOVAL

Because proteins were stained by the generic markers (Table 3), plasma proteins were removed by SEC before labeling. Fig. 2, M–R, shows the resulting Venn diagrams, from which the sensitivity and PPV for CD61+ particles were calculated (Table 2). Table 2 shows that protein removal before staining improved the sensitivity and PPV by 10- and 9-fold for calcein AM, 1- and 6-fold for CFSE, and 3- and 6-fold for di-8-ANEPPS, whereas the sensitivity and PPV reduced 0.3- and 0.2-fold for calcein violet and 0.1- and 0.7-fold for lactadherin. As a reference, for side scatter the sensitivity reduced 0.2-fold and the PPV increased 3-fold after protein removal. Thus, protein removal improved the detection of CD61+ particles in plasma for calcein AM, CFSE, and di-8-ANEPPS. Again, CFSE suffered from swarm; therefore, obtained results are unreliable.
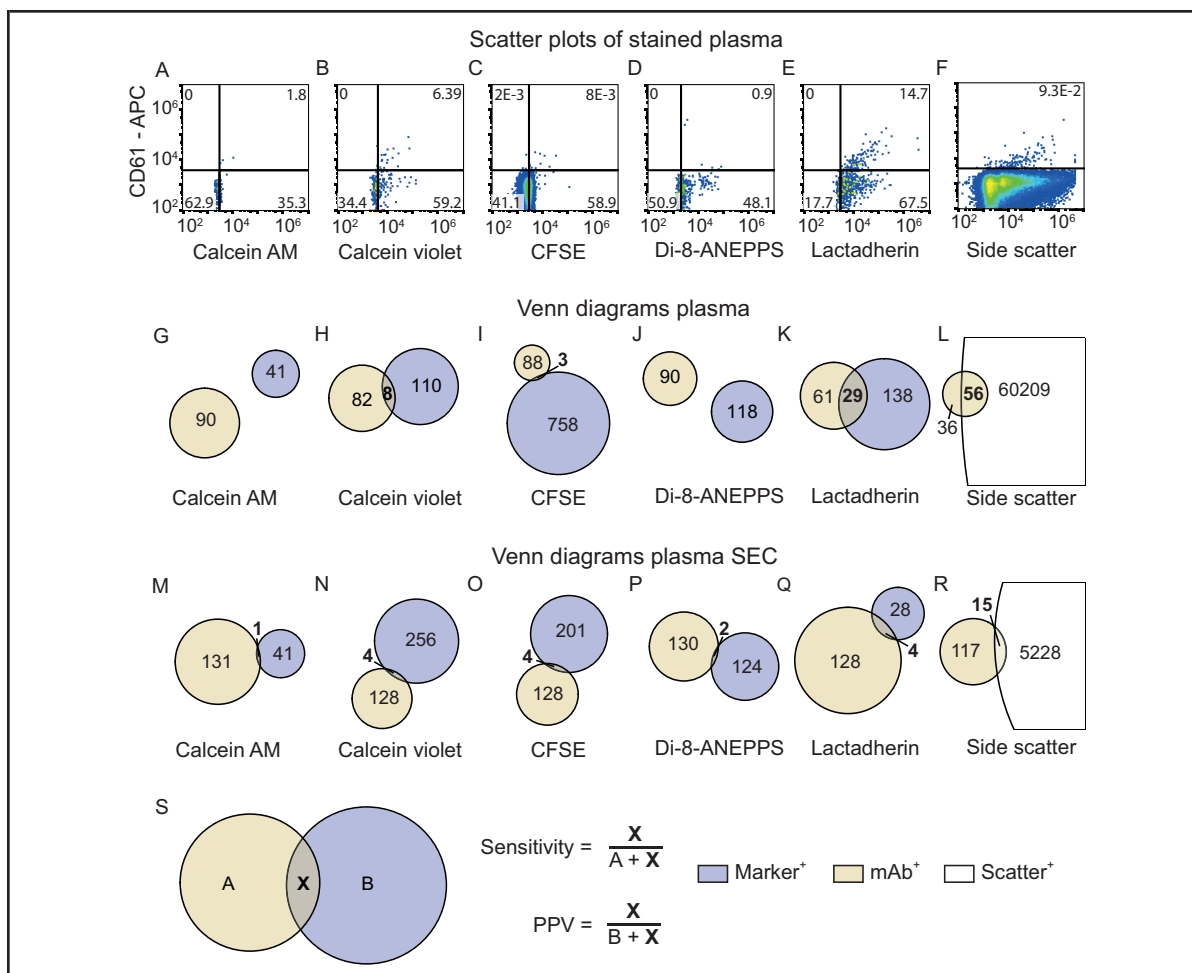
Fig. 2. Generic marker performance on plasma.

Generic marker fluorescence triggered scatter plot of CD61 vs generic marker fluorescence (A–E) or side scatter (F). Data of 1 representative minute are shown. Numbers in quadrants indicate percentage of total population. Venn diagrams show mean concentrations (×10⁶ particles/mL) of antibody⁺ (mAb⁺), marker⁺ or scatter⁺, and double⁺ particles in plasma (G–L) and plasma SEC (M–R) (n = 3–6). Calculation of sensitivity and PPV from Venn diagrams (S).

COMPARISON OF BEST PERFORMING GENERIC MARKERS WITH SIDE SCATTER

Fig. 3 shows Venn diagrams comparing side scatter of a single sample with the best performing generic markers in this study: di-8-ANEPPS and lactadherin. For MCF7 EVs (Fig. 3, A and B), 94% of mAb⁺ particles were detected using di-8-ANEPPS, 79% using lactadherin, and 100% using side scatter. Also, Fig. 3, A and B, shows that fluorescence-based triggering resulted in 3- to 4-fold lower detection rates of mAb⁻ particles compared with side scatter. In plasma (Fig. 3C), lactadherin and side scatter detected 32% and 50% of mAb⁺ particles, respectively, with lactadherin triggering resulting in a 500-fold lower detection rate of mAb⁻ particles compared with side scatter.

Discussion

The ideal generic marker would be 100% sensitive and specific to EVs. Our results show that none of the tested generic markers fulfills this requirement in plasma. In fact, there was almost no overlap between marker⁺ and CD61⁺ populations (Fig. 2, G–L), likely because of the large number of labeled non-EV components. Our findings (Table 2) emphasize large differences in the performance of generic markers.

To summarize the results, triggering on di-8-ANEPPS, lactadherin, or side scatter on our flow cytometer resulted in comparable concentrations of EpCAM⁺ EVs in MCF7 EV samples. In contrast, CD61⁺ EVs in plasma were best detected by side scatter. Of the generic

Table 3. Detected particles in non-EV samples using different trigger strategies.

Trigger	Detected particles (10^6/mL)		
	Marker in buffer	Proteins	Lipoproteins
Fluorescence			
Calcein AM	90	400	0
Calcein violet	400	300	0
CFSE	20	14000	800
Di-8-ANEPPS	4	50	0
Lactadherin	100	200	0
Scatter		300	370000

Concentrations are means of 2 repeated measurements. All concentrations were corrected for background in buffer without generic marker. The reported concentrations in the different columns cannot be mutually compared.

markers, CD61$^+$ EVs in plasma were best detected by lactadherin. All generic markers detected particles in "stained" buffer, which could be marker aggregates/micelles and/or unbound marker that causes an increase in background fluorescence above the threshold. All PPVs were <30%, mostly owing to detection of these marker aggregates/micelles, unbound marker, and stained soluble protein. Removal of proteins from plasma increased the PPV for di-8-ANEPPS and side scatter but, surprisingly, reduced the sensitivity and PPV for lactadherin. The cause of this reduction is unknown. Side scatter triggering resulted in a detected concentration of $5.6 \pm 1.2 \cdot 10^7$ CD61$^+$ EVs/mL plasma of healthy con-

trols, comparable with the previously reported concentration of platelet EVs (20).

Calcein AM and calcein violet had a low sensitivity and PPV in both EV samples. The low sensitivity may be because of insufficient brightness of the markers or because EVs have insufficient esterase activity. A previous study reported successful staining of EVs with calcein AM (11). The scattered intensity of these EVs, however, was comparable with 1-μm polystyrene beads, which correspond to the scattered intensity of 2- to 3-μm EVs or even cells (28). Consequently, the previously detected EVs were much larger than the population of EVs detected in our present study.

Samples stained with CFSE suffered from swarm detection at low dilutions or insufficient event rates at higher dilutions in both EV samples (see Fig. 5 of the online Data Supplement), an artifact discussed elsewhere (29). Previous reports did not investigate swarm in the CFSE channel (12, 14, 15). Swarm detection may be caused by stained soluble protein or lipoprotein particles, marker micelles/aggregates, and/or unbound marker (Table 3) (14). Application of SEC to reduce soluble proteins or unbound marker did not eliminate the observed swarm (see Fig. 5 of the online Data Supplement).

In this study, we defined EVs as all mAb$^+$ particles. This definition excluded EVs that were mAb$^-$ or mAb^{dim}, although they may be more abundant than mAb$^+$ EVs, as is the case for plasma (20). In addition, non-EV particles that are mAb$^+$ and detergent resistant would be considered EVs. Furthermore, the composition of our liposomes was unsuitable for evaluation of generic markers. First, the lipid transition temperature was above room temperature, making the membrane more imper-
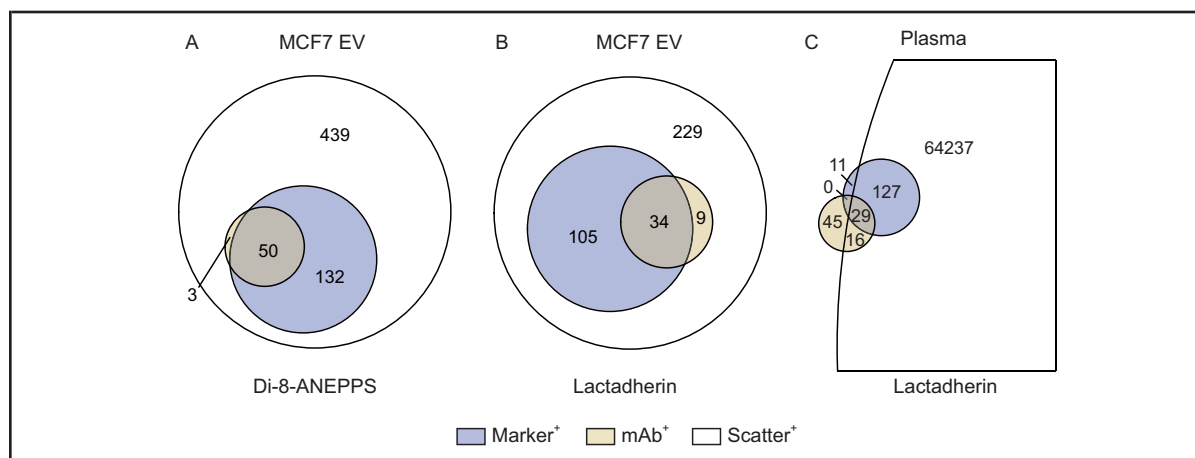

Fig. 3. Venn diagrams comparing side scatter triggering with di-8-ANEPPS and lactadherin triggering for MCF7 EVs or plasma.

Shown are mean concentrations ($\times 10^6$ particles/mL) of antibody$^+$ (mAb$^+$), marker$^+$ or scatter$^+$, double$^+$ and triple$^+$ particles in MCF7 EVs (A and B) and plasma (C).

meable to these markers, and second, at least for di-8-ANEPPS, the high concentration of cholesterol caused a blue shift in the emission spectrum (see Table 1 of the online Data Supplement) *(30)*.

None of the tested generic markers fulfilled the requirements described earlier for an ideal generic fluorescent marker. Di-8-ANEPPS and lactadherin stained 100% of EVs in MCF7 EV samples, but both markers did not specifically stain EVs. All markers were compatible with fluorescent immunostaining but posed a challenge when designing multicolor panels. Only lactadherin was directly applicable to plasma; the other markers required isolation of EVs before staining. Another way to improve marker performance would be to increase marker concentration and/or remove unbound marker after staining.

Several publications suggest that fluorescence-based triggering is superior to scatter-based triggering *(8–10, 13)*. The outcome of such comparisons, however, depends on the properties of the used flow cytometer and has little bearing on the fundamental superiority of one triggering strategy over the other. Furthermore, although in this study side scatter was most efficient to detect mAb$^+$ particles in plasma, the PPV of side scatter is, on average, 200-fold lower than fluorescence triggering on lactadherin (Fig. 3, Table 2).

Our flow cytometer has a high analytical sensitivity on scatter, a PE/FITC fluorescent sensitivity comparable with high sensitivity flow cytometers *(9)*, and is never used to measure whole plasma or blood to keep background counts low. Because most flow cytometers are either analytically sensitive on fluorescence or scatter, compromises must be made. For an informed decision, we suggest the following roadmap: (*a*) quantify the limit of detection on scatter, e.g., the smallest polystyrene bead that is distinguishable from noise, (*b*) quantify the limit of detection on fluorescence by determining Q and B, (*c*) take the sample composition into account, e.g., the presence of proteins, (*d*) compare limits of detection with reported limits of detection for scatter-sensitive and/or fluorescence-sensitive flow cytometers *(9, 14)* to select suitable trigger strategies, and (*e*) test selected trigger strategies on the sample and flow cytometer to determine which strategy detects the highest number of EVs.

Taken together, although the roadmap above can be used to select the optimal trigger strategy and marker for your application, the search for a generic EV marker continues.

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