MIFlowCyt list

Requirement	Please Include Requested Information
1.1. Purpose	The purpose of this study is to find the most efficient trigger strategy for extracellular vesicle (EV) detection. Hereto, we compare 6 trigger strategies, e.g. triggering on five different generic EV markers or side scatter, for their ability to detect EVs.
1.2. Keywords	exosome, extracellular vesicles, flow cytometry, fluorescent dyes, microparticle.
1.3. Experiment variables	Trigger strategy, generic marker staining, EV sample
1.4. Organization name and address	Academic Medical Centre Meibergdreef 9 1105 AZ Amsterdam The Netherlands
1.5. Primary contact name and email address	Leonie de Rond, l.derond@amc.nl
1.6. Date or time period of experiment	August 2016 – January 2017
1.7. 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 flow cytometer, followed by lactadherin. The choice between scatter or lactadherin primarily depends on the analytical sensitivity of the flow cytometer used.
1.8. Quality control measures	Mean fluorescent intensity (MFI) was converted to molecules of equivalent soluble fluorochrome (MESF) for phycoerythin (PE), fluorescein isothiocyanate (FITC) and 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 (Chase and Hoffman 1998) 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 at al. 2016) as:

	$$R = \frac{4\sqrt{Q \cdot B} + 1}{Q}$$ Scatter sensitivity was characterized by reporting the smallest polystyrene bead that is distinguishable from the noise.
1.9 Other relevant experiment information	
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	MCF-7 cell culture supernatant and human plasma
2.1.1.2. Biological sample source description	Breast cancer cell line MCF-7 (ATCC HTB-22)
2.1.1.3. Biological sample source organism description	10 healthy non-fasting volunteers, 5 male 5 female
2.1.2.2. Environmental sample location	
2.2 Sample characteristics	MCF-7 cell culture supernatant is expected to contain EVs and possibly some small cell fragments or remaining fetal calf serum molecules. Platelet free plasma (PFP) is expected to contain EVs, lipoproteins and proteins.
2.3. Sample treatment description	MCF7 breast cancer cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 10 units/mL penicillin and 10 µg/mL streptomycin (all ThermoFischer Scientific). Cells were cultured in a T75 culture flask (Corning) at 37 ^{o}C, 5% CO_2. At 80-90% confluence, cells were washed with phosphate buffered saline (PBS, Fresenious Kabi) and cultured in FCS-free medium before harvesting EVs. After 48 hours, the conditioned culture medium was centrifuged at 1,000g for 30 minutes (Rotina 46 RS, Hettich) to remove cells, and the resulting supernatant is called MCF7-EVs throughout the text. To prepare platelet-free plasma (PFP), citrate-anticoagulated blood (0.32% final concentration) was collected as described elsewhere (22) from 10 healthy donors. Informed consent and approval from the Ethics Committee was obtained. The blood was pooled and plasma was prepared by centrifuging the blood twice at 1,560g, 20 ^{o}C for 20 minutes. Both MCF7-EVs and the plasma sample were snap-frozen in liquid nitrogen, stored at -80 ^{o}C and thawed in a 37 ^{o}C water bath before use. See

	Supplemental Data, Fig. S1, S2 for characterization of the MCF7-EVs and plasma sample.
2.4. Fluorescence reagent(s) description	Calcein AM (eBioscience, Waltham, USA), Calcein AM Violet (eBioscience), carboxyfluoresceinsuccinimidyl ester (CFDE-SE, here and commonly referred to as CFSE; ThermoFisher Scientific, Waltham, USA), 4-(2-[6-(Dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium (Di-8-ANEPPS; Sigma-Aldrich, St. Louis, USA) and lactadherin-FITC (Haematologic Technologies, Essex Junction, USA) were used as generic EV markers. MCF-7 EV were identified by staining with anti-EpCAM-APC (mouse IgG1κ, clone HEA-125, MiltenyiBiotec, BergischGladbach, Germany). Platelet EV were identified by staining with ant-CD61-APC (mouse IgG1κ, clone Y2/51, Dako, Glostrup, Denmark).
3.1. Instrument manufacturer	Apogee, Hertfordshire, UK
3.2. Instrument model	A60-Micro
3.3. Instrument configuration and settings	Samples were analyzed at a flow rate of 3.01 µL/min on an A60-Micro, equipped with a 405 nm laser (200 mW), 488 nm laser (100 mW) and 638 nm laser (75mW). Samples were measured for 4 minutes when triggering on EpCAM-APC, CD61-APC or generic marker fluorescence and 1 minute when triggering on side scatter (405 nm laser). Fluorescence triggered measurements were longer because we expected lower detection rates and wanted to ensure a statistically relevant number of detected particles. Trigger thresholds for both scatter and fluorescence were set to a value resulting in 10-20 counts/s in buffer. Triggering on calcein AM, CFSE and lactadherin fluorescence occurred in the 488-green channel (525/50 nm band pass filter), calcein violet on the 405-blue channel (445/50 nm band pass filter) and di-8-ANEPPS on the 488-red channel (680/35 nm band pass filter). APC signals were collected in the 638-D Red channel (long pass 652 nm filter). PMT voltages were set to 380 V for SALS, 375 V for LALS, 520 V for 488-green, 580 V for 488-red, 500 V for 405-blue and 510 V for 638-D Red. Trigger thresholds for both scatter and fluorescence were set to a value resulting in

	10-20 counts/s in buffer. This was 14 for LALS, 41 for 488-green (197 MESF), 28 for 488-red,75 for 405-blue and 62 for 638-D Red (0.12 MESF).
4.1. List-mode data files	May be requested by emailing l.derond@amc.nl
4.2. Compensation description	No compensation was required since no fluorophore combinations were used that have overlapping emission spectra.
4.3. Data transformation details	No data transforms were applied.
4.4.1. Gate description	Gates were based on fluorescence of a blanco sample (e.g. not stained with a generic marker), labeled with IgG1-APC (See Supplemental Data, Fig. S6 for scatter plots). For both mAb and generic marker, positive ($^{+}$) is defined as a fluorescent signal higher than the gate.
4.4.2. Gate statistics	The number of positive events was corrected for flow rate, measurement time and dilutions performed during sample preparation. The resulting concentration of positive events was corrected for background in acquisition time matched IgG1 labelled samples and reported in the Venn diagrams of the manuscript. Percentages of events within each gate of the scatter plot represent percentage of the total population.
4.4.3. Gate boundaries	Images of the gates can be seen in the scatter plots of the accompanying manuscript and Supplemental Data, Fig. S6.