Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	The purpose of this study is to systematically evaluate adaptations to the optical configuration and fluidics of a common flow cytometer (FACSCanto, Becton Dickinson (BD), Franklin Lakes, NJ), and show the resulting effects on the forward (FSC), side (SSC) scatter and phycoerythrin (PE) fluorescence sensitivity. The aim is to enable detection of scatter signals from 100 nm EVs on both the FSC and SSC detector.
1.2. Keywords	exosomes, extracellular vesicles, flow cytometry, light scattering, microparticle
1.3. Experiment variables	Blocker bar shape, laser power, pinhole diameter, detector type, sample stream width, sample flow velocity, flow channel dimensions and the pore size of the sheath filter
1.4. Organization name and address	Amsterdam UMC University of Amsterdam Dept. Biomedical Engineering and Physics Cancer Center Amsterdam Amsterdam Cardiovascular Sciences Meibergdreef 9 1105AZ, Amsterdam The Netherlands
1.5. Primary contact name and email address	Leonie de Rond, l.derond@amsterdamumc.nl
1.6. Date or time period of experiment	October 2018 – June 2019
1.7. Conclusions	The SNR improved a total of $3.8 \cdot 10^4$-fold on FSC and 30-fold on SSC. As a result, the estimated detection limits for EVs (assuming refractive index 1.40) went from 1,220 nm on FSC and 180 nm on SSC, to 250 nm on FSC and 90 nm on SSC. Another ~50 fold improvement on FSC is still necessary to detect 100 nm EVs.
1.8. Quality control measures	
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	A mixture of green fluorescent polystyrene (PS) beads of 100, 300, 500 and 900 nm (Megamix-plus FSC, Stago BNL, Leiden, the Netherlands) was used to evaluate flow cytometer sensitivity with every adaptation. The sensitivity of the final optimal system was demonstrated using non-fluorescent 100, 125, 147, 203, 402 nm NIST traceable PS beads (Thermo Fischer Scientific, Rockford, IL), 75 nm silica bead (Kisker Biotech GmbH, Steinfurt, Germany) and 189 and 374 nm hollow silica beads [21] and a urine EV sample.
2.1.1.2. Biological sample source description	Urine
2.1.1.3. Biological sample source organism description	Five overnight fasting healthy male donors
2.1.2.2. Environmental sample location	N/A
2.3. Sample treatment description	Urine from five overnight fasting healthy male donors was collected and processed as described earlier [21]. Informed consent and approval from the ethics committee was obtained. Briefly, urine was pooled and centrifuged for 10 min at 180 g, 4 °C, followed by 20 min at 1,560 g, 4 °C to remove cells. 1 mL aliquots of the resulting cell-free urine

	were snap-frozen in liquid nitrogen and stored at -80 °C. Before use, 12 aliquots of cell-free urine were thawed at 37 °C, pooled and centrifuged for 10 min at 1,560 g, 4 °C to remove salt precipitation. The resulting cell- and salt-free supernatant was diluted in phosphate buffered saline (PBS, Corning, Corning, NY) before analysis.
2.4. Fluorescence reagent(s) description	N/A
3.1. Instrument manufacturer	Becton Dickinson, Franklin Lakes, NJ
3.2. Instrument model	FACSCanto A
3.3. Instrument configuration and settings	See accompanying manuscript for a description of the applied adaptations to the optical configuration and fluidics. Megamix-plus FSC beads were diluted in 10-fold concentrated PBS (BE17-525F, Lonza, Basel, Switzerland) to obtain Megamix beads in 1-fold concentrated PBS. The Megamix beads were measured using FITC triggering to ensure detection of all beads throughout the adaptations. A minimum of 1,000 events per bead population were acquired. Noise levels were estimated by measuring PBS using a FITC trigger with low threshold (200 arbitrary units (a.u.)) and high voltage (600 V). This results in randomly triggered events and thus enables an approximation of FSC and SSC noise. To enable SNR determination, and thereby monitor the sensitivity, both noise and bead signal need to be within the dynamic range of every detector. This can be done by changing the PMT voltage. A standardized protocol was used to set the PMT voltages after every adaptation. The voltage on the FITC channel was configured to attain a rate of 1000 (noise) events/s in PBS using FITC triggering. Next, the SSC voltage was set such that the 300 nm bead had an intensity of 10^5 a.u., and the PE voltage such that the 500 nm bead had an intensity of 10^4 a.u.. Since only the 900 nm bead was detectable on FSC in the standard configuration, the FSC PMT voltage was instead configured to center the noise in the FSC channel around 10^2 a.u.. After the illumination and detection adaptations, the noise on SSC was no longer within the dynamic range using the 300 nm bead setting approach, so for the fluidics experiments the noise on SSC was also centered around 10^2 a.u. with the PMT voltage.
4.1. List-mode data files	All data has been uploaded to flowrepository.org (FR-FCM-Z25X).
4.2. Compensation description	N/A
4.3. Data transformation details	Matlab (Matlab R2018b (Mathworks, Natick, MA) was used to transform the data.
4.4.1. Gate description	Bead populations were gated using Matlab. First, the noise/background peak was gated out using a FITC-H histogram. An SSC-H histogram was created of the remaining events, using which the bead populations were gated and identified. In case some of the bead populations were off scale on the SSC-H histogram, an FSC-H histogram

	was used to gate and identify the remaining bead populations.
4.4.2. Gate statistics	The SI and robust coefficient of variation (rCV) for each bead population were calculated as follows: $$SI = \frac{median_{bead} - median_{noise}}{2 \cdot rSD_{noise}} \quad (1)$$ $$rCV = \frac{rSD_{bead}}{median_{bead}} \cdot 100\% \quad (2)$$ with $median_{bead}$ the median intensity of the bead population, $median_{noise}$ the median intensity of the noise and rSD_{noise} and rSD_{bead} the robust standard deviation of the noise and bead, respectively, defined as: $$rSD = \frac{1}{2}(percentile_{84.13} - percentile_{15.87}) \quad (3)$$ with $percentile_{84.13}$ and $percentile_{15.87}$ the intensity of the noise or bead population at those percentiles.
4.4.3. Gate boundaries	N/A

Notes

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