Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	The purpose of this study is to investigate the possibility of deriving Q and B to quantify scatter sensitivity, using the scattering cross section (σ_s) in nm^2 as a standardized unit.
1.2. Keywords	background light, exosomes, extracellular vesicles, flow cytometry, light scattering, nanoparticles, optical efficiency, viruses, sensitivity, standardization
1.3. Experiment variables	Illumination power, bead size
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	July 26th 2019
1.7. Conclusions	As a proof of principle, we derived Q, B, and R for a photon limited light scatter detector of a flow cytometer. The approach is a step towards the comparison and interpretation of scatter data from different flow cytometers and can in turn be used to compare the sensitivity of different scatter detector designs.
1.8. Quality control measures	
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	A bead mixture containing non-fluorescent NIST-traceable polystyrene bead populations with mean diameters of 100, 125, 147, 203, 296, 400, 600, 799 and 994 nm (all 3000 Series Nanosphere Size Standards, Thermo Fisher Scientific, Waltham, MA), and two green fluorescent bead populations of 140 and 380 nm respectively (G140, G400, Thermo Fisher Scientific, Waltham, MA) was prepared in distilled water. The concentration of each bead population in the mixture was ~10^7 /ml.
2.1.1.2. Biological sample source description	N/A
2.1.1.3. Biological sample source organism description	N/A
2.1.2.2. Environmental sample location	N/A
2.3. Sample	The bead mixture was diluted 10-fold in phosphate buffered saline (PBS, 21-031-CVR, Corning, Corning,

treatment description	NY) before measuring.
2.4. Fluorescence reagent(s) description	N/A
3.1. Instrument manufacturer	Becton Dickinson, Franklin Lakes, NJ
3.2. Instrument model	Customized FACSCanto A
3.3. Instrument configuration and settings	See [1] for a full description of the customized flow cytometer configuration. SSC collected on the customized FACSCanto is split by a 488-nm mirror and simultaneously detected using a standard SSC detection module and a high-resolution SSC module. Data shown in the manuscript is measured using the standard SSC detection module. The diluted bead mixture was measured at ~40 µl/min using a trigger on the high-resolution SSC (threshold of 200 @ 267 V) to allow detection of all beads. Per bead population, a minimum of 1,000 events was acquired. The background signal on the standard SSC channel was measured while triggering particles with a light scattering intensity ranging from 200 to 400 a.u., which is an order of magnitude below the detection limit of the standard SSC detection module, on the high-resolution SSC module. We used this strategy to assure that the width of the sampling window, which affect the magnitude of the area parameter, is equal for beads and background signals. The used voltage on SSC was 670 V.
4.1. List-mode data files	All data has been uploaded to flowrepository.org (FR-FCM-Z292) and can be accessed by the reviewers using the link below: flowrepository.org/id/RvFrWZ1MFVUAEG7Q2tAenxRlXW6uPUI5NPt89NcySWTjbNaOQICYGlv3ixvWxZ4F
4.2. Compensation description	N/A
4.3. Data transformation details	Matlab (Matlab R2018b (Mathworks, Natick, MA) was used to analyze the data.
4.4.1. Gate description	Bead populations were gated using Matlab. First, the 380 nm green fluorescent bead was gated out using a FITC-H histogram. A histogram based on the hiResSSC-H parameter was created of the remaining events, using which the bead populations were gated and identified.
4.4.2. Gate statistics	Median, robust standard deviation (SD) and robust coefficient of variation (CV) of the side scatter height parameter were determined for each bead population. These parameters were preferred over the mean, and the normal standard deviation and coefficient of variation because they are less influenced by outliers and therefore a more reproducible measure [2]. To determine the median and SD of the background distribution, all measured events were included.
4.4.3. Gate boundaries	N/A

References

1 de Rond L, van der Pol E, Bloemen PR, Van Den Broeck T, Monheim L, Nieuwland R, van Leeuwen TG, Coumans FAW. A systematic approach to improve scatter sensitivity of a flow cytometer for detection of extracellular vesicles. Cytometry A. 2019; submitted.

2 Hoffman RA, Wood JCS. Characterization of Flow Cytometer Instrument Sensitivity. Curr Protoc Cytom. 2007; 40: 1.20.1-1..18.