Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	To demonstrate how to perform light scatter calibration (conversion of arbitrary units into standard units) for Imaging Flow Cytometers (IFCM), and to demonstrate the value of light scatter-to-diameter calibration for the reproducibility of Extracellular Vesicle detection.
1.2. Keywords	Imaging Flow Cytometry, Extracellular Vesicles, Calibration, Standardization, Reproducibility
1.3. Experiment variables	Polystyrene (PS) and Hollow Organosilica Beads (HOBs) of various submicron sizes. PE and APC MESF quantitation beads. Plasma EV Test Sample (PEVTES) pre-stained with CD235a-PE and CD61-APC to identify erythrocyte- and platelet-derived EVs, respectively.
1.4. Organization name and address	Erasmus Medical Center, University Medical Center Rotterdam, The Netherlands. Wytemaweg 80, 3015 CN, Rotterdam
1.5. Primary contact name and email address	Wouter W. Woud, w.woud@erasmusmc.nl
1.6. Date or time period of experiment	2022-2023
1.7. Conclusions	Combined calibration of light scattering and fluorescent signals for IFCM enhances 1) the reproducibility of EV detection between different IFCMs, and 2) allows comparison of EV-data with other flow cytometric platforms with different optical configurations and settings.
1.8. Quality control measures	The instrument calibration tool ASSIST® was used upon each startup to optimize performance and consistency between experiments.
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	Different sizes of PS beads (81, 92, 100, 122, and 152-nm) were used to calibrate light scatter signals. PS beads (Catalogue numbers 3080A, 3090A, 3100A, 3125A, and 3150A, Nanosphere™ Size Standards NIST Traceable PS Beads, Thermo Fisher) were diluted in 0.1 µm filtered MilliQ down to a working concentration of ~E^8 particles/mL before acquisition with IFCM. Different sizes of HOBs (216, 280, 351, and 396-nm) were used to validate the light scatter calibration [23]. HOBs were diluted down to working concentrations of ~$1E^8$/mL in MilliQ: 216-nm (~$3.3E^{10}$/mL) and 351-nm (~$2.9E^{10}$/mL) HOBs were diluted 300-fold, while 280-nm HOB (~$3.8E^{10}$/mL) was diluted 400-fold, and 396-nm ($2.2E^9$/mL) HOB 20-fold. Dried 2-µm PE, and APC Quantitation beads (Custom order, 2364-87, 2364-89, Becton Dickinson (BD) Biosciences) with known MESF values were used to calibrate the fluorescent detection channels.
2.1.1.2. Biological sample source description	The PEVTES was prepared as described previously [28]. PEVTES prepared for this article were stained with CD235a-PE (R7078, JC159, f.c. 25 µg/mL; Dako) to identify erythrocyte-derived and CD61-APC (17-0619-42, VI-PL2,

	final concentration (f.c.) 8.33 µg/mL; eBioscience) to identify platelet-derived EVs. To stabilize the sample a buffer of M D-(+)-Trehalose dihydrate (T0167, Sigma, f.c. 0.5 M) and 5 % Bovine Serum Albumin (A9647, Sigma Aldrich, f.c. 0.5%;) was added. [28] Bettin B, van der Pol E, Nieuwland R. Plasma extracellular vesicle test sample to standardize flow cytometry measurements. Res Pract Thromb Haemost. 2023;7(4):100181.
2.1.1.3. Biological sample source organism description	Healthy human individuals.
2.1.2.2. Environmental sample location	NA
2.3. Sample treatment description	See 2.1.1.2.
2.4. Fluorescence reagent(s) description	See 2.1.1.1 and 2.1.1.2.
3.1. Instrument manufacturer	Cytek Biosciences
3.2. Instrument model	Three different ImageStream^{X} MkII instruments, distributed over 2 labs.
3.3. Instrument configuration and settings	All samples were analyzed on three different IFCMS: 1 IFCM was located at the Rotterdam Transplant Institute at the Erasmus MC, Rotterdam, The Netherlands, and 2 IFCMs were located at the Cytek Biosciences Amnis® site in Seattle, WA, USA. The IFCM at Rotterdam was an ImageStream®^{X} (IS^{x}) Mk II instrument (Mk II 647; Cytek Biosciences, Seattle, USA) equipped with 4 lasers (405/488/642/785 nm) and a 1 CCD-camera system. Both instruments in Seattle were equiped with 6 lasers (405/488/561/592/642/785-nm) and a 2 CCD-camera system (Mk II 357 and Mk II 493). Equal settings on all instruments were used during the acquisition of both calibration and test samples. The Mk II 493 instrument is equiped with a 400-mW 488-nm laser but the samples were collected at 200-mW to match the other instruments in the study. For each instrument, SSC signals were generated with the 405-nm 120-mW laser without a notch filter before detection. Depending on the instrumental setup of the IFCM (1- or 2-camera system) SSC signals were collected in channel 1 (Ch01) or channel 7 (Ch07), which have the same spectral bandwidth when the 488-nm notch filter is used. Fluorescent signals were generated with the 488-nm and 642-nm lasers set at full power (200-mW and 150-mW, respectively) with the notch filters in. The standard 642-nm laser notch filters - allowing transmission from a wavelength of 430-nm upwards – were observed to result in a 30-40% loss of the 405-nm SSC signal. To overcome this loss of signal, we replaced these notch filters in all three instruments in this study with custom notch filters allowing 99% transmission from a wavelength of 360-nm upward. The 785-nm laser was not used in any

	of the experiments and the brightfield detection channel was turned off. All lasers (405, 488, 642-nm) were turned on for all experiments, unless specified otherwise.
4.1. List-mode data files	Representative .fcs files are available from the flow repository (flowrepository.org) under the name "Calibrated Imaging Flow Cytometry (FR-FCM-Z7UG)".
4.2. Compensation description	No compensation of fluorescent signals was performed.
4.3. Data transformation details	Scattering calibration: Light scattering signals of bead populations from PS and HOBs were fitted with Mie theory using a previously described model. The SSC detector was modeled as a detector that is placed perpendicular to the propagation direction of the laser beam. The NA of the collection lens was 0.9, and the wavelength was 405 nm. PS beads were modelled as solid spheres with a RI of 1.6328. HOBs were modelled as core-shell particles with a core RI of 1.3431, a shell RI of 1.4696, and a shell thickness of 13 nm. EVs were modelled as core-shell particles with a core RI of 1.38, a shell RI of 1.48 and a shell thickness of 6 nm. Beads were measured in water, and EVs in Phosphate Buffered Saline (PBS). Therefore, the RIs of PBS and water were assumed to be 1.3431 and 1.3451**,** respectively, at a wavelength of 405 nm. Effective scattering cross sections of the calibration beads were calculated by integrating the amplitude scattering matrix elements over 576 collection angles [18]. Data and theory were log10-transformed to scale the data onto the theory using a least-square-fit. Fluorescent calibration: Dried beads were dissolved in 100 µL BD stain buffer (554657, BD Biosciences) and vortexed briefly before acquisition. For each detection channel, a linear regression analysis was performed of the logarithmically transformed mean fluorescent intensity (MFI) against the logarithmically transformed specified MESF values. The resulting linear function was used to calibrate the log of PE and APC fluorescent intensities to the log of MESF values.
4.4.1. Gate description	Following calibration of light scatter and fluorescent signals, gating regions were set on standardized data. Events were analyzed in regions indicative of 100-1000-nm particle diameter and 3-10,000 MESF fluorescent intensity units, irrespective of the fluorophore analyzed.
4.4.2. Gate statistics	Median Fluorescent Intensity (MFI) – Objects/mL
4.4.3. Gate boundaries	See above

Notes

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