Considerations for MESF-bead based assignment of absolute fluorescence values to nanoparticles and extracellular vesicles by flow cytometry

Estefanía Lozano-Andrés^{1, #}, Tina Van Den Broeck^{2, #}, Lili Wang³, Majid Mehrpouyan⁴, Ye Tian⁵, Xiaomei Yan⁵, Marca H.M. Wauben^{*1, #}, Ger J.A. Arkesteijn^{*1}

¹ Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

² BD Biosciences, Erembodegem, Belgium

³ Biosystems and Biomaterials Division, National Institutes of Standards and Technology (NIST), Gaithersburg, MD 20899, USA

⁴ BD Biosciences, San Jose, CA 95131, USA

⁵ Department of Chemical Biology, MOE Key Laboratory of Spectrochemical Analysis & Instrumentation, Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, People's Republic of China

[#] TRAIN-EV Marie Skłodowska-Curie Action-Innovative Training Network, train-ev.eu

^{*}Both authors contributed equally

Corresponding author:

Dr. G.J.A. Arkesteijn

Department of Biomolecular Health Sciences

Faculty of Veterinary Sciences

Utrecht University

Yalelaan 2, 3584 CM

Utrecht, The Netherlands

T: +31 30 2535451/2535833

E: G.J.A.Arkesteijn@uu.nl

Abstract

Flow cytometry is a promising technique to characterize nanoparticles (NPs) and extracellular vesicles (EVs). However, the majority of reported experiments, use arbitrary units to indicate fluorescence intensity. This hampers comparison of results from different laboratories and different platforms. We investigated the advised use of calibrated molecules of equivalent soluble fluorophores (MESF)-beads for assignment of absolute fluorescence to NPs and EVs. Firstly, we evaluated the use of two different FITC MESF bead sets as calibrators on three different flow cytometry platforms (BD Influx™, CytoFLEX LX™ and SORP BD FACSCelesta™). Secondly, NPs and biological 4T1 mammary carcinoma-EVs were analyzed using the BD Influx and their fluorescence signals calibrated by using different sets of FITC and PE MESF beads. Although fluorescence calibration, using bright calibrators designed for cellular flow cytometry, makes inter-platform comparison possible for fluorescently labeled cells and brightly labeled particles, but the uncertainty of the currently available calibrators, which are far out of the fluorescence range of the sub-micron particles, hampers a reliable assignment of absolute MESF numbers based on

extrapolation into the dim fluorescence range. Our results illustrate the need for calibration materials specifically designed for NPs and EVs to enable a reliable assignment of absolute fluorescence values in the lower fluorescent ranges.

Key words: flow cytometry, fluorescence calibration, standardization, MESF, extracellular vesicles, nanoparticles

Introduction

Flow cytometry has attracted the attention of many researchers working in the field of cell-derived extracellular vesicles (EVs) as a valuable tool for high-throughput single particle-based analysis [1]. It allows for quantitative multiparametric characterization of subsets of EVs within the heterogeneous EV-population based on the detection of light scatter signals and fluorescence. Although flow cytometry is one of the methods of choice for EV analysis, several requirements need to be met and data need to be reported in a proper way to improve rigor and reproducibility of flow cytometric EV analysis [2]. In spite of the advancements made in the past years, the vast majority of EV flow cytometric data is still published in arbitrary units. While expressing percentages of subpopulations or median fluorescence intensity (MFI) relative to the experimental controls is a common practice for cell measurements by flow cytometry, this approach is less desirable for the reliable analysis of the much smaller and dimmer EVs whereby positively-stained events cannot be discriminated from negative counterparts as a clearly separate population. Especially for these small and dim particles, the variability between the measured outcome caused by the diversity in fluidics, optics and electronics between different platforms inevitably will be reflected in the results reported as arbitrary units of fluorescence. Calibration could make clear whether a specific instrument can fully resolve or partly detect a certain EV or NP population.

Altogether, the use of standardized units for EV flow cytometric analysis will strongly contribute to rigor and reproducibility and makes inter-platform and inter-laboratory analysis possible. Therefore, the three-society EV flow cytometry working group, i.e. ISEV-ISAC-ISTH EV flow workgroup, has recommended in the recently published MIFlowCyt-EV framework for standardized reporting of EV flow cytometry experiments, the use of fluorescence calibration [2]. Fluorescence calibration is successfully applied for cell flow cytometry in which arbitrary units are translated to standardized units of fluorescence that are established by National Institute of Standards and Technology (NIST), such as molecules of equivalent soluble fluorophores (MESF) or equivalent number of reference fluorophore (ERF) [3-6]. The currently available calibrator materials are designed for the calibration of fluorescence of labeled cells and hence are much brighter in fluorescence when compared to the fluorescence from stained EVs and other NPs. We here investigated whether regression analysis could be used to assign absolute units of fluorescence to stained EVs that are far out of the range of these calibrator intensity levels. We evaluated the performance of the different calibrator beads sets and their inter-platform variation on three different flow cytometers capable of single EV analysis. Finally, we studied how the use of different bead sets would affect the calibration of fluorescence signals from synthetic NPs and biological fluorescently stained and antibody labeled EVs.

Material and Methods

MESF calibration beads

For calibration of the fluorescence axis in the fluorescein isothiocyanate (FITC) channel we used two different sets of FITC MESF beads (custom-made, 6 μ m lot MM2307 #131-10; #131-8; #130-6; #130-5 and 2 μ m lot MM2307#156; #159.1; #159.2; #122.3, BD Biosciences, San Jose, CA).

For calibration of the fluorescence axis in the PE channel, we used two different sets of PE MESF beads (6 μm , commercial QuantiBrite, Catalog. No. 340495 lot 62981 and 2 μm , custom made, lot MM2327#153.1; #153.2; #153.4; #153.5; #153.6, BD Biosciences, San Jose, CA).

The 6 μm FITC MESF beads were prepared by reacting various concentration of FITC with PMMA Beads (Bangs Labs) in borate buffer at pH 9.2. The 2 μm FITC beads were prepared by reacting various concentrations of FITC-BSA (with a FITC/BSA molar ratio of 2) with 2 μm carboxylic beads (Bangs Labs) using EDC/NHS chemistry. The 2 μm PE beads were made as described above except various concentrations of PE were used with 2 μm carboxylic beads in EDC/NHS chemistry. The beads were analyzed on LSR Fortessa™ (BD Biosciences) and their MESF values were assigned using commercially available MESF beads (Flow Cytometry Standards Corp.) as calibrators. The FITC ERF values were assigned to both 2 μm and 6 μm beads using a specific lot of FITC-FC Bead (BD Biosciences) as a calibrator with known ERF value, which has been assigned by NIST.

Flow cytometer platforms

In this study three flow cytometers were used. A jet in air-based BD Influx (BD Biosciences, San Jose, CA), a BC CytoFLEX LX (Beckman Coulter, Brea, CA) with a cuvette-based system and a cuvette-based SORP BD FACSCelesta™ (BD Biosciences, San Jose, CA) equipped with a small particle side scatter (SSC) module.

The BD Influx flow cytometer was modified and optimized for detection of submicron-sized particles [7]. In brief, FITC was excited with a 488 nm laser (Sapphire, Coherent 200 mW) and fluorescence was collected through a 530/40 bandpass filter. PE was excited with a 562 nm laser (Jive, Cobolt 150 mW) and fluorescence was collected through a 585/42 bandpass filter. Optical configuration of the forward scatter detector was adapted by mounting a smaller pinhole and an

enlarged obscuration bar in order to reduce optical background. This reduced wide-angle FSC (rwFSC) allowed detection of sub-micron particles above the background based on forward scatter [7, 8].

Upon acquisition, all scatter and fluorescence parameters were set to a logarithmic scale. To minimize day to day variations, the BD Influx was standardized at the beginning of each experiment by running 100 and 200 nm yellow-green (505/515) FluoSphere beads (Invitrogen, F8803 and F8848). The instrument was aligned until predefined MFI and scatter intensities were reached with the smallest possible coefficient of variation (CV) for rw-FSC, SSC and fluorescence. After optimal alignment, PMT settings required no or minimal day to day adjustment and ensured that each measurement was comparable. MESF beads and NPs were measured with a FSC threshold set at 1.0 while for biological EVs a fluorescence threshold was set at 0.67 by allowing an event rate of 10-20 events/second while acquiring a clean PBS sample.

When performing quantitative and qualitative analysis of synthetic NP and biological EV samples, preparations were diluted in PBS as indicated. Upon loading on the Influx, the sample was boosted into the flow cytometer until events appeared, after which the system was allowed to stabilize for 30 seconds. Measurements were performed by a fixed 30 seconds time or by setting a gate around the spike-in beads and allowing to record a defined number of events in the gate (80 000 events) using BD FACS Software 1.01.654 (BD Biosciences).

The CytoFLEX LX was used without any tailor-made modifications in the configuration. Before measurements, the manufacturer recommended startup and QC procedure were run first. All scatter and fluorescence parameters were set to a logarithmic scale. FITC was measured with a 50 mW 488 nm laser and fluorescence was measured through a 525/40 band pass filter at gain 1.0. FITC MESF beads were recorded with a FSC threshold at 1000. Measurements were performed using CytExpert 2.1 (Beckman Coulter).

The SORP BD FACSCelesta™ was equipped with a small particle SSC module for improved scatter detection. Before measurement, the standard setup and QC procedures were performed and the recommended CS&T performance check was run to monitor performance on a daily basis and to optimize laser delay. All scatter and fluorescence parameters were set to a logarithmic scale. 100 nm yellow-green (505/515) FluoSphere beads (Invitrogen, F8803) were acquired and used to set optimal small particle-SSC and fluorescence (FITC detector) PMT-V values. FITC was measured with a 100 mW 488 nm laser through a 530/30 band pass filter. FITC MESF beads were recorded with a SSC threshold at 200. Measurements were performed using BD FACSDiva™ Software v8.0.3 (BD Biosciences).

Further descriptions of each instrument and methods are provided in Data S1 (MiFlowCyt checklist) and Data S2 (MiFlowCyt-EV framework).

Preparation of FITC-doped silica nanoparticles

Synthetic silica nanoparticles (SiNPs) of 550 nm diameter with six different FITC fluorescence intensities were produced by using a modified method of literature reports [9-12]. Briefly, the amine reactive FITC molecules were covalently linked to the silane coupling agent, (3-aminopropyl)-triethoxysilane (APTES) in anhydrous ethanol. Monodisperse silica seeds of ~90 nm prepared by using amino acid as the base catalyst [9, 10] were suspended in a solvent mixture containing ethanol, water and ammonia. Then tetraethyl orthosilicate (TEOS) and different volumes of APTES–FITC solutions were added for growing FITC-doped SiNPs by a modified Stöber method [11, 12]. Upon washing three times with anhydrous ethanol, the FITC-doped SiNPs were reacted with TEOS in the solvent mixture to allow growth of a silica layer. The synthesized SiNPs were washed three times with anhydrous ethanol and stocked in anhydrous ethanol. The diameters of SiNPs were measured by transmission electron microscopy.

Isolation and fluorescent staining of biological Extracellular Vesicles for flow cytometric analysis

EV-containing samples were obtained from 4T1 mouse mammary carcinoma cell culture supernatants (ATCC, Manassas, VA) as previously described [8, 13, 14]. EVs were stained with 5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, hereinafter referred as CFSE) (ThermoFisher, catalog number C1157) and separated as described previously [7]. Briefly, 2 μ l of the isolated 4T1 EVs (corresponding to a concentration of 1.44 E12 particles/mL as determined by nanoparticle tracking analysis) were mixed with 18 μ l PBS/0.1% aggregate-depleted (ad)BSA. For antibody labeling, samples were first resuspended in 15.5 μ l PBS/0.1% adBSA and incubated with 0.5 μ g of rat anti-mouse CD9-PE (Clone: KMC8, IgG2a, κ , lot 7268877, BD Biosciences) or matched isotype antibodies (Rat IgG2a, κ , PE-conjugated, lot 8096525, BD Biosciences) for 1h at RT while protected from light. EVs were then stained with 40 μ M CFSE in a final volume of 40 μ l. The sealed tube was incubated for 2h at 37°C while protected from light. Next, staining was stopped by adding 260 μ l PBS/0.1% adBSA. After fluorescent staining, EVs were separated from protein aggregates and free reagents by bottom-up density gradient centrifugation in sucrose for 17.30 h at 192,000 g and 4°C using a SW40 rotor (k-factor 144.5; Beckman Coulter, Fullerton, California, USA). Twelve fractions of 1 mL were then collected from the top of the gradient and respective densities were determined by refractometry using an Atago Illuminator (Japan). For analysis by flow cytometry, EV samples corresponding to a 1.14 g/mL density were diluted 1:20 in PBS prior measurement. MIFlowCyt-EV guidelines [2] were followed whenever applicable (Data S2).

Concentration determination by using spike-in beads

EV concentration was normalized using a spiked-in external standard containing 200 nm orange (540/560) fluorescent beads (Invitrogen, F8809). The concentration of the beads was determined by Flow NanoAnalyzer N30 (NanoFCM, Xiamen, China) and stocked at 5.7×10^{10} particles/mL. Beads were diluted $1:10^4$ in PBS and added to the EV samples, mixed and measured on the flow cytometer. Bead count was used to calculate the EV concentration.

Data analysis

For fluorescence calibration each bead peak population was gated using FlowJo Version 10.5.0 and MFI were obtained for further least square linear regression analysis. Data was handled in Microsoft Excel and figures were prepared using GraphPad Prism version 8.0 (GraphPad Software Inc). The software FCM PASS Version v2.17 was used to generate files with calibrated axis units in the histograms and dot plots shown [15] (Software is available on <http://go.cancer.gov/a/y4ZeFtA>).

Data availability

All EV data of our experiments have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210047)[16]. All flow cytometric data files have been deposited at the Flow Repository (FR-FCM-Z3FJ).

Results

Cross-platform assessment of MESF-bead based calibration of fluorescent signals using different bead sets

Two differently-sized FITC MESF bead sets of $6 \mu\text{m}$ and $2 \mu\text{m}$, each containing 4 bead populations per set and respectively representing an overall brighter ($6 \mu\text{m}$) and an overall dimmer ($2 \mu\text{m}$)

beads set, were measured and their use for fluorescence calibration of FITC fluorescent signals was evaluated using three different flow cytometer platforms, namely a BD Influx, a BC CytoFLEX and a SORP BD FACSCelesta. Every bead population measured on each of these three platforms was gated and respective singlet gated populations are displayed as overlays in histograms (Figure 1a) showing their FITC fluorescence. The 4 peaks in the histograms of both the 6 μm and 2 μm beads are indicated from dim to bright fluorescent as p1, p2, p3 and p4 (Figure 1b). The bigger sized 6 μm FITC MESF beads contained overall brighter fluorescent intensities, whose assigned values range from 25,910 to 715,225 FITC MESF. The smaller sized 2 μm FITC MESF beads covered a dimmer part of the fluorescence intensity range with assigned values ranging from 3,634 to 103,706 FITC MESF. As expected, Figure 1b shows that MFI values alone expressed in arbitrary units do not allow the comparison of measurements across the three instruments. Therefore the FITC MFI of every bead population was next correlated to the FITC MESF values provided for each bead population using least square linear regression. We found a different slope in the two lines generated by either the 6 μm or the 2 μm MESF beads, consistent within each platform and indicating that this is an inherent quality of these specific bead sets (Figure 1c). To evaluate the precision and accuracy of FITC MESF assignments we used specific bead populations from one set, referred to as ‘unknown’ in Figure 1, to calculate the FITC MESF by extrapolation of the regression line based on the other bead set. The ‘unknown’ samples used where: 1) p1 and p4 of the 2 μm bead set for which the FITC MESF values were calculated using the regression line of the 6 μm bead set and 2) p1 and p2 of the 6 μm bead set for which the FITC MESF values were calculated using the regression line of the 2 μm bead set (Figure 1c). Using this approach, the extrapolated FITC MESF values of the ‘unknown’ p4 from the 2 μm beads and the ‘unknown’ p2 from the 6 μm beads could successfully be compared between platforms, proving the precision of

the fluorescence calibration and facilitating the interpretation of data acquired on the three different platforms (Figure 1d-e and Figure 1h).

To quantify accuracy we decided to set a range of 20% difference as acceptable (10% above or 10% below actual values, as indicated by the green band in Figure 1d-e) for proper assignment to MESF values. We found less than 20% difference from the expected value for all calculated FITC MESF values of the ‘unknown’ signal from the p4 of the 2 μ m beads on the 6 μ m bead-set regression line and the p2 of the 6 μ m beads on the 2 μ m bead-set regression line. This observation was consistent for all platforms and independent of the bead set that was used. Thus, further demonstrating not only precision but also the accuracy of fluorescence calibration (Figure 1d-e and Figure 1h).

However, when extrapolating the ‘unknown’ bead signal from p1 of the 2 μ m beads on the 6 μ m bead-set regression line and from p1 of the 6 μ m beads on the 2 μ m bead-set regression line (Figure 1f-g) we found that although calculated FITC MESF values were again comparable between platforms and hence calibration proved to be precise, the accuracy was uncertain. Comparison of the calculated MESF values of the ‘unknown’ beads to the expected MESF values in these cases revealed more than a 20% difference, leading to either an underestimation or an overestimation of the absolute number of FITC MESF (Figure 1f-h).

This can be explained by the fact that slight differences in the slope of the calibration lines of the different bead sets become more prominent when the extrapolation is extended into the dimmer or brighter range of the fluorescence intensities of the calibration beads, so out of the range of the calibrators. Importantly, the same trend was observed in all three platforms evaluated. It is unlikely that differences in slopes are caused by instrument non-linearity since it is a recurrent pattern on all three platforms (Figure 1) and linear plotted values do not show non-linearity either (Figure S1a-c). Also, variability between separate measurements could be excluded (Figure S2). Finally,

the same phenomenon occurred when 6 μ m and 2 μ m calibrator bead sets for PE MESF were used. In this case the opposite situation occurred; the dimmer 2 μ m beads displayed a steeper slope than the brighter 6 μ m beads (Figure S1d-f). Hence, our observations are pointing towards an uncertainty in the assignment of the both FITC and PE beads.

Figure 1. Evaluation of two different FITC MESF bead sets for the calibration of fluorescent intensities across three flow cytometer platforms. (a) Histogram overlays (axis in arbitrary units) of FITC fluorescent intensity peaks derived from the 6 μm or the 2 μm FITC MESF beads (b) Table showing the median fluorescence intensity (MFI) statistic derived from each of the fluorescent intensity peaks from dimmer to brighter being expressed in arbitrary units. (c) Least square linear regression analysis of 6 μm (black circles) and 2 μm (grey triangles) FITC MESF beads. Provided FITC MESF and measured FITC MFI values were transformed to log and plotted in a log-log fashion for the three platforms. (d-g) Bar graphs showing the expected absolute FITC MESF and the calculated FITC MESF values for different unknowns as indicated in the figure. The absolute number of FITC MESF was calculated for the indicated fluorescent intensity peak population and compared to the expected value on the three flow cytometers. For comparison, we included a 20% variation from the expected FITC MESF of the population as a reference point (green). (h) Table indicating the expected and calculated FITC MESF values for each sample used in the analysis.

MESF assignments of FITC fluorescence intensities from synthetic silica nanoparticles depend on the MESF-bead calibrator set used

So far we have demonstrated that accuracy of assignment is negatively influenced when signal strength of the unknown particle is further away from the average fluorescence intensities of the calibrator beads. We next investigated how calibration with the FITC MESF bead-sets impact absolute fluorescent assignment when nanoparticles with even dimmer fluorescence were measured. For this purpose we selected one platform (BD Influx) to measure and analyze 550 nm silica NPs, containing 6 populations with different FITC fluorescence intensities. Singlets were gated (Figure S3) and an overlay histogram showing their 6 different FITC fluorescence intensities was generated (Figure 2a, left). We next performed fluorescence calibration and generated two additional histograms showing calibrated FITC MESF units based on either the 6 μm (Figure 2a, middle) or the 2 μm FITC MESF calibrator bead set (Figure 2a, right). We obtained the MFI and

CV values for each population and further calculated their FITC MESF values based on the two different FITC MESF calibrator bead sets (Figure 2b). Our data show that when using brighter 6 μ m calibration beads, the calculated FITC MESF values were always lower as compared to the use of the 2 μ m bead-set regression line (Figure 2b). In addition, the multi-intensity peak analysis of the silica NPs in one measurement revealed that the difference in the absolute FITC MESF values of the synthetic silica NPs obtained by the two calibrator bead sets increased in the dimmer range of the fluorescence, with a 27.3% variation for the highest fluorescent peak (p6) and 76.5% variation for the dimmest population of these NPs (Figure 2b). To further visualize this, we used the calculated FITC MESF values (Figure 2b) to generate an overlay bar graph showing the differences in calculated FITC MESF from the six FITC fluorescent populations within the 550 nm NPs (Figure 2c). Our results demonstrate that the variation between using the 6 μ m (dark green) or 2 μ m (light green) FITC MESF bead set progressively increased from brightest (p6) to dimmest (p1) NP populations (Figure 2c). As indicated before, this can be ascribed to the differences in the slopes of the regression lines of the two different bead sets and the extrapolation into the dim area. To confirm that this phenomenon was not limited to the use of FITC MESF beads we additionally analysed 6 μ m and 2 μ m PE MESF calibrator bead sets on the BD Influx (Figure S1d-f). By measuring a sample containing 200 nm broad spectrum fluorescent polystyrene NPs we could determine its fluorescent intensity in terms of ERF (Figure S4a). The variation in the absolute PE ERF value obtained by the two different calibrator sets used to assign the absolute calibrated ERF value was in this case 41.3% (Figure S4b).

Figure 2. MESF bead-based calibration of fluorescence signals from synthetic silica NPs. (a)

Histogram overlay showing FITC fluorescence in arbitrary units (a.u.) from each population next to FITC

MESF calibrated axis based on the 6 μm or 2 μm FITC MESF beads from the six differently FITC-labeled 550 nm NP gated populations. **(b)** Table showing the MFI and CV of each population and the calculated FITC MESF values for each of the unknown populations with the percentage of variation between the two calculated reference values. **(c)** Bar graph showing the calibrated FITC MESF values for each of the six differently FITC-labeled populations based on the 6 μm (dark green, generally lower numbers) and 2 μm (light green, generally higher numbers). Bars from 6 μm bead values and bars from 2 μm bead values are presented as overlays.

Different MESF-bead sets assign different absolute numbers of ERF and MESF to fluorescently CFSE stained and CD9 labeled extracellular vesicles

Next, we investigated the impact of this phenomenon on the assignment of absolute FITC-ERF units and PE MESF units to a biological EV sample measured on the same platform by using the four different calibration bead sets, i.e. 6 and 2 μm FITC MESF and the 6 and 2 μm PE MESF beads. Since EVs scatter less light than beads and NPs, we used a fluorescence threshold to measure the biological EVs [7]. To this end, we used CFSE as a luminal dye to fluorescently stain EVs and a tetraspanin CD9 antibody, since this tetraspanin transmembrane protein is known to be enriched in the surface of EVs and is considered as EV-marker. Next to the CFSE stained and CD9-PE labeled EVs, we measured an unstained control containing EVs but without CFSE or antibody and a matching isotype control (Figure 3a). Since 200 nm spike-in beads were added to the samples for EV concentration measurement we defined an EV-gate and a spike-in bead gate in these samples and relevant additional controls (Figure S5a). Our data revealed a 76.6% variation in the MFI expressed in absolute CFSE ERF units on CFSE stained EV and a 156.9% variation in the MFI expressed in absolute PE MESF units on CD9-PE labeled EVs when the two different calibrator bead sets were used on the same platform (Figure 3a-b). The fluorescent threshold value of 0.67

used on the BD Influx corresponds to an equivalent of 150 FITC MESF based on the 6 μ m beads or 300 FITC MESF based on the 2 μ m beads (Figure 3a) and indicates the level of detection using this fluorescence threshold. Therefore, especially when a fluorescence threshold is used to measure EVs, accurate calibrators are required to be able to report the fluorescence threshold in calibrated units.

Lastly, in the overlay histograms it is shown how the fluorescence intensities of the calibrators relate to the dimmer signals generated by CFSE stained and CD9-PE labeled EVs (Figure 3c-d).

Figure 3. MESF bead-based calibration of fluorescence signals from biological EV samples. (a) Analysis of three biological EV samples by using a fluorescence threshold. Unstained EVs control, CFSE + isotype-PE EVs next to CFSE + CD9-PE stained EVs (from left to right plots) with dot plots showing CFSE fluorescence vs PE fluorescence in arbitrary units (a.u.) (upper row) or CFSE ERF vs PE MESF calibrated axis based on either the 6 μ m (middle row) or 2 μ m beads (lower row). The discontinuous line in each dot plot indicates the fluorescence threshold value used, which is 0.67 and on this BD Influx corresponds to an equivalent of 150 FITC MESF based on the 6 μ m beads or 300 FITC MESF based on the

2 μ m beads. Number of events for each sample within the EV gate and MFI values for either CFSE or PE fluorescence (a.u.) are indicated in the top row. Once calibrated dot plots were generated the median fluorescence intensity in either CFSE ERF or PE MESF standardized units of fluorescence were obtained based on the 6 μ m (middle row) or 2 μ m beads (lower row) and are indicated for each dot plot. **(b)** Table showing the ERF or MESF values obtained after calibration for the CFSE + CD9-PE EVs and the percentage of variation between the use of the 6 μ m or 2 μ m bead sets. **(c)** Histogram overlays displaying fluorescence in arbitrary units from CFSE stained EVs (blue) next to the 6 μ m or 2 μ m FITC MESF bead set (green). **(d)** Histogram overlays displaying fluorescence in arbitrary units from CD9-PE labeled EVs (purple) next to the 6 μ m or 2 μ m PE MESF bead set (red).

Discussion

The calibration of fluorescence measurements obtained by flow cytometry in terms of MESF or ERF is essential for proper and robust comparison of data between different instruments, different labs and different time points. EV flow cytometry is pursued as a tool in the rapidly expanding EV research field and the definition of what and how much can be detected is only starting to evolve. Besides the inter-comparability of EV data, the use of calibrated values also allows to create consensus about the range of fluorescence that can be expected by EV flow cytometry in case of generic staining and epitope density, thereby facilitating the further identification of EV in flow cytometry data. Therefore, it is desirable to express flow cytometry data from EVs in calibrated units as well [2]. Our results clearly illustrate why it is important to perform fluorescence calibration, as previously reported in other studies [17, 18], since MFI values in arbitrary units cannot be directly compared when samples are measured on different flow cytometers.

Our findings show that extrapolating bright reference beads to dim signals is possible and calibrated values are precise irrespective the instrument used, allowing standard comparisons, which is in line

with earlier findings [18]. However, the accuracy of assigning absolute MESF or ERF values relies for a great deal on the exactness by which the calibrator beads are assigned. The effect of small variations in assignment may cause uncertainty in the slopes of the regression lines. The uncertainty in the slope affects the uncertainty for a particular point based on the spread of the points. Since the points at the ends (low intensity end) are farthest away, the uncertainty increases with the distance to the other points on average [19]. On the other hand, difference in slope between two calibrator sets per definition denotes an interception point somewhere along the line. Comparability may fully depend on the position of the interception point. In our study the interception point is close to the range where the beads reside and goes hand in hand with precision and accuracy for values in this range. However away from this point, larger uncertainties occur due to the further separation of the two regression lines. The currently available commercial MESF beads are designed to calibrate fluorescence intensity in the range of regular cell flow cytometry. However, EVs are orders of magnitude smaller than cells and their fluorescence based on generic staining or based on antibody labeling and epitope density is far dimmer than the available calibrators. To assign numbers of fluorophores to these weak signals, we rely on extrapolation of the regression line into the dim area. In this proof-of-principle study, we show that the intrinsic uncertainty of the number of assigned fluorescent molecules per calibrator bead induces a higher deviation from the expected value of MESF when the regression line is followed into the area of the dimmer particles.

It may be clear from our results with the unknown synthetic NPs and the biological EVs that the outcome of the results definitively depends on the calibrator used. Since calibration is an essential part in the identification and inter-comparability of EV data, we therefore advise that proper reporting of how fluorescent calibration is being done and adequate description of materials needs to be included to avoid confusing interpretation of calibrated data. Hence, we propose that not only

absolute fluorescence values need to be reported but also the calibration bead set and lot number used, including all MFI and MESF values. Furthermore, for future benchmarking and comparison studies, ideally the same calibrator sets should be used. Clearly, a calibrator that has a low uncertainty of assignment and has MESF values closer to the range of fluorescence intensity of the unknown particle of interest is most preferable and determination of absolute MESF/ERF values to NPs and EVs needs to be further investigated and validated before robust conclusions can be made. Moreover, novel state-of-the-art flow cytometers that are designed to measure small particles rely obligately on sub-micron beads to perform MESF calibration. These state-of-the-art platforms cannot measure the ‘standard’ 6 μm or 2 μm MESF beads [20]. Therefore, there is an urgent need for small and dim calibration beads that are especially designed for NPs and EVs to be widely used in cross-platform and inter-laboratory comparisons.

In summary, our results indicate that calibration of fluorescent signals is crucial for comparison between platforms, and that it should be utilized and properly reported. Nevertheless, accurate assignment of MESF/ERF to particles in the sub-micron size range could be hampered by the inherent uncertainty of the calibrators. Therefore, statements on the absolute number of fluorescence units, either MESF or ERF, present on NPs and EVs are related to the calibrator set used. To avoid inconsistencies, so far calibration for inter-comparison may preferably be performed using identical calibrator bead lots. For more robust assignments of fluorescence units to NPs and EVs there is a need for calibration materials that have low uncertainty of assigned fluorescent values and have a size range that is compatible for all flow cytometer platforms used for analysis of sub-micron sized particles, including both NPs and EVs.

Funding Statement

This research is supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722148 and by the National Natural Science Foundation of China (21934004 and 21627811). E.L.A. is supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722148.

Author Contributions

E.L.A. designed and performed experiments, analyzed data and wrote the manuscript. T.B. performed experiments and gave conceptual advice. L.W. gave technical and conceptual advice. M.M., Y.T. and X.Y. prepared materials and gave technical advice. G.J.A.A. and M.H.M.W. supervised the research, designed (performed) experiments and wrote the manuscript. G.J.A.A. and M.H.M.W. contributed equally as senior author. All authors critically reviewed and edited the manuscript.

Acknowledgments

The authors would like to thank Prof. An Hendrix (Laboratory of Experimental Cancer Research, Ghent University, Belgium) for the possibility to prepare and analyze M4T1 derived EV in her lab, Dr. Joshua A. Welsh (National Cancer Institute, Bethesda, MD) for helpful discussion and Ludo Monheim (BD Biosciences, Erembodegem, Belgium) for helpful technical support. FITC MESF and 2 μ m PE MESF beads were kindly provided by BD Biosciences (prepared by Dr. Majid Mehrpouyan) as part of the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722148.

Declaration of interest disclosure

Tina Van Den Broeck and Majid Mehrpouyan are both employees of BD Biosciences, a business unit of Becton, Dickinson and Company. During the course of this study, the Wauben research group, Utrecht University, Faculty of Veterinary Medicine, Department of Biomolecular Health Sciences and BD Biosciences collaborated as a co-joined partner in the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722148. Xiaomei Yan declares competing financial interests as a cofounder of NanoFCM Inc., a company committed to commercializing the nano-flow cytometry (nFCM) technology.

Supporting Information

Additional supporting information can be found online in the corresponding section at the end of the article.

Data S1. Author Checklist: MIFlowCyt-Compliant Items.

Data S2. MIFlowCyt-EV framework.

Figure S1. Analysis of different MESF bead sets measured on the BD Influx.

Figure S2. Evaluation of measurement variability of 2 μ m FITC MESF beads measured on the BD Influx.

Figure S3. Gating strategy for synthetic silica NPs.

Figure S4. 200 nm fluorescent polystyrene NPs.

Figure S5. Gating strategy for external spiked-in beads and biological EV samples.

ORCID

Estefanía Lozano-Andrés <https://orcid.org/0000-0002-7305-0776>

Lili Wang <https://orcid.org/0000-0003-2456-898X>

Xiaomei Yan <http://orcid.org/0000-0002-7482-6863>

Marca H.M. Wauben <https://orcid.org/0000-0003-0360-0311>

Ger. J.A. Arkesteijn <https://orcid.org/0000-0001-7739-582X>

References

1. Nolan, J.P., *Flow Cytometry of Extracellular Vesicles: Potential, Pitfalls, and Prospects*. Curr Protoc Cytom, 2015. **73**: p. 13 14 1-16.
2. Welsh, J.A., et al., *MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments*. J Extracell Vesicles, 2020. **9**(1): p. 1713526.
3. Hoffman, R.A., *Standardization, calibration, and control in flow cytometry*. Curr Protoc Cytom, 2005. **Chapter 1**: p. Unit 1 3.
4. Wang, L. and R.A. Hoffman, *Standardization, Calibration, and Control in Flow Cytometry*. Curr Protoc Cytom, 2017. **79**: p. 1 3 1-1 3 27.
5. Gaigalas, A.K., et al., *Quantitating Fluorescence Intensity From Fluorophore: Assignment of MESF Values*. J Res Natl Inst Stand Technol, 2005. **110**(2): p. 101-14.
6. Wang, L., et al., *Quantitating Fluorescence Intensity From Fluorophores: Practical Use of MESF Values*. J Res Natl Inst Stand Technol, 2002. **107**(4): p. 339-53.
7. van der Vlist, E.J., et al., *Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry*. Nat Protoc, 2012. **7**(7): p. 1311-26.
8. Arkesteijn, G.J.A., et al., *Improved Flow Cytometric Light Scatter Detection of Submicron-Sized Particles by Reduction of Optical Background Signals*. Cytometry A, 2020. **97**(6): p. 610-619.
9. Yokoi, T., et al., *Periodic arrangement of silica nanospheres assisted by amino acids*. J Am Chem Soc, 2006. **128**(42): p. 13664-5.
10. Yokoi, T., et al., *Mechanism of Formation of Uniform-Sized Silica Nanospheres Catalyzed by Basic Amino Acids*. Chemistry of Materials, 2009. **21**(15): p. 3719-3729.
11. Stöber, W., A. Fink, and E. Bohn, *Controlled growth of monodisperse silica spheres in the micron size range*. Journal of Colloid and Interface Science, 1968. **26**(1): p. 62-69.
12. Giesche, H., *Synthesis of monodispersed silica powders II. Controlled growth reaction and continuous production process*. Journal of the European Ceramic Society, 1994. **14**(3): p. 205-214.
13. Vergauwen, G., et al., *Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research*. Sci Rep, 2017. **7**(1): p. 2704.
14. Geeurickx, E., et al., *The generation and use of recombinant extracellular vesicles as biological reference material*. Nat Commun, 2019. **10**(1): p. 3288.
15. Welsh, J.A., et al., *FCMPASS Software Aids Extracellular Vesicle Light Scatter Standardization*. Cytometry A, 2019.
16. Consortium, E.-T., et al., *EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research*. Nat Methods, 2017. **14**(3): p. 228-232.
17. Nolan, J.P. and S.A. Stoner, *A trigger channel threshold artifact in nanoparticle analysis*. Cytometry A, 2013. **83**(3): p. 301-5.
18. Welsh, J.A., J.C. Jones, and V.A. Tang, *Fluorescence and Light Scatter Calibration Allow Comparisons of Small Particle Data in Standard Units across Different Flow Cytometry Platforms and Detector Settings*. Cytometry A, 2020. **97**(6): p. 592-601.
19. Draper, N.R. and H. Smith, *Applied regression analysis*. 1981, New York: Wiley.
20. Tian, Y., et al., *Protein Profiling and Sizing of Extracellular Vesicles from Colorectal Cancer Patients via Flow Cytometry*. ACS Nano, 2018. **12**(1): p. 671-680.