Received: 19 January 2023 | Revised: 26 March 2023 | Accepted: 16 April 2023

https://doi.org/10.1016/j.jtha.2023.04.042

REVIEW ARTICLE

Standardization of extracellular vesicle concentration measurements by flow cytometry: the past, present, and future

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Funding information
B.B. acknowledges funding by METVES II. This project (METVES II) has received funding from the EMPIR program cofinanced by the Participating States and from the European Union's Horizon 2020 research and innovation program. E.v.d.P. acknowledges funding from the Netherlands Organisation for Scientific Research—Domain Applied and Engineering Sciences (NWO-TTW), research programs VENI 15924 and VIDI 19724. Z.V. was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

Abstract

Concentrations of extracellular vesicles (EVs) in body fluids are being explored as disease biomarkers. Most laboratories use flow cytometry to characterize single EVs at high throughput. A flow cytometer (FCM) detects light scattering and fluorescence intensities of EVs. However, detection of EVs by flow cytometry is complicated for 2 reasons. First, EVs are small and have weak light scattering and fluorescence signals compared to cells and are, therefore, hard to detect. Second, FCMs differ in sensitivity and provide data in arbitrary units, which complicates data interpretation. Due to the mentioned challenges, the measured concentration of EVs by flow cytometry is cumbersome to compare between FCMs and institutes. To improve comparability, standardization and development of traceable reference materials to calibrate all aspects of an FCM are needed, as are interlaboratory comparison studies. Within this article, we will provide an overview of the standardization of EV concentration measurements, including the current effort to introduce robust calibration of FCMs, thereby enabling comparable concentration measurements of EVs, which in turn can be used to establish clinically relevant reference ranges of EV concentrations in blood plasma and other body fluids.

KEYWORDS
calibration, extracellular vesicles, flow cytometry, interlaboratory comparison study, standardization

Manuscript handled by: Patricia Liaw

Final decision: Patricia Liaw, 17 April 2023

1 | INTRODUCTION

Extracellular vesicles (EVs) are cell-derived, membrane-enclosed particles present in body fluids. As the composition and concentration of EVs are disease-dependent, cell-type–specific EV concentrations are being explored as potential biomarkers of disease, including cancer and cardiovascular disease [1–4]. However, before EV concentrations can be utilized as biomarkers, standardization of concentration measurements is essential to obtain comparable data. Due to the heterogeneous nature of EVs, in particular their broad size distribution and biochemical composition, standardization of EV concentration measurements is challenging.

Flow cytometry is a popular technique to measure the concentration of EVs due to its ability to detect and characterize single particles at a throughput of thousands of particles per second. Flow cytometers (FCMs) were originally designed to detect light scattering and fluorescence signals of single cells in a hydrodynamically focused fluid stream. Given that all cells exceed the detection threshold and assuming that the flow rate is known, the concentration of cells can be determined. However, sample and hardware-related issues complicate EV concentration measurements, as illustrated in Figure 1.

EVs are so heterogeneous and small that most FCMs are unable to detect the smallest EVs in a population, neither by light scattering nor by fluorescence [6,7]. The red line in Figure 1A shows an indicative size distribution of EVs in human blood plasma. The shape of the size distribution is based on published cryoelectron microscopy measurements of EVs in human plasma [5], whereas the concentration at the vertical axis is added by fitting the size distribution onto flow cytometry data, which are shown as black dots. Figure 1A shows that EVs have a broad size distribution and that small EVs outnumber large EVs.

Furthermore, only the fraction of EVs right from the vertical dashed line is efficiently detected with this FCM. The vertical dashed line indicates the lower limit of detection (LoD) of our FCM, which we defined as the diameter at which the size distribution peaks. In reality, however, the LoD of an FCM is not a straight line but a complex function that requires a formal definition in the future. Please note that LoDs are FCM and detector dependent and therefore differ between instruments.

To explain why EVs are difficult to detect, Figure 1B shows an indication of the relationship between light scattering (blue line) and diameter and fluorescence intensity (green line) and diameter measured by an FCM. Most strikingly is not only the strong decrease of particularly light scattering signals but also fluorescence signals for decreasing diameters of EVs. Please note that the vertical scales are logarithmic. By combining the data from Figure 1A, B, we obtain the light scattering and fluorescence signal distributions of EVs, shown in Figure 1C, D, respectively. The solid lines predict how EVs with the size distribution shown in Figure 1A would scatter light and emit fluorescence, whereas the data points show the actual light scattering and fluorescence distributions measured with our FCM.

Figure 1C, D show that our FCM, and this holds true for most FCMs, is unable to detect all EVs by light scatter or fluorescence. Furthermore, Figure 1C, D show that optical detection of all EVs in plasma is demanding, because scatter and fluorescence detectors require to cover a 10 000 000-fold and 10 000-fold difference in signal levels, respectively. Due to the LoD of the FCM, most FCMs only detect the fraction of EVs exceeding the LoD. On the other hand, specialized instruments with the sensitivity to detect the dimmest EVs will miss the largest EVs due to an insufficient detection range [8]. Thus, due to differences in detection ranges, FCMs measure different concentrations of EVs in a given sample. Consequently, standardization requires that EV concentrations are reported within the same signal ranges, which is difficult because flow cytometry data have arbitrary units [6,8]. In sum, the small size and heterogeneity of EVs, together with differences between detection ranges of FCMs and data representation in arbitrary units, complicate data interpretation and comparison of EV measurement results among different FCMs [6,9].

Due to the aforementioned challenges, the measured concentrations of EVs by flow cytometry are hitherto incomparable between instruments and institutes [10]. In this article, we provide an overview of the history of EV flow cytometry standardization and currently available reference materials (RMs) to calibrate FCMs for EV measurements. We will explain what the metrological meaning of calibration, RMs, and quality control samples are and how calibration of flow rate, light scattering, and fluorescence intensity of FCMs is the only way to achieve comparable EV concentration measurements.

2 | THE PAST

2.1 | Early detection of EVs

The first attempt to standardize EV flow cytometry measurements was based on comparing light scattering signals between cells and EVs. In a landmark study dating back to 1989, 3 populations of particles exposing platelet-specific proteins were identified in diluted whole blood by flow cytometry [11]. To discuss the earlier interpretation of these results, we repeated the experiment (Figure 2). Figure 2B shows the side scattered light intensity (SSC) vs forward scattered light intensity (FSC) of 3 different particle populations in whole blood exposing the platelet-specific protein CD61. The brightest population scattered more amount of light than platelets and therefore "*appears to represent platelets associated with the larger white blood cells*" [11] (Figure 2A). Figure 2B–D shows that the dimmest population scattered less light than platelets and was thought to represent platelet-derived EVs having an average diameter of 100 nm. By adding calcium ionophore to platelet-rich plasma, which stimulates the platelets to release EVs, Nieuwland et al. [12] confirmed that the dimmest population are indeed platelet-derived EVs (Figure 2D).

There are 2 reasons why platelet-derived EVs were thought to have an average diameter of 100 nm. First, Sims et al. [13] showed in 1988 that platelet-derived EVs and 100 nm fluorescent reference particles had similar FSC. As Sims et al. [13] triggered on fluorescence, they probably did detect 100-nm reference particles and possibly a fraction of the largest EVs. However, the used FSC detectors were

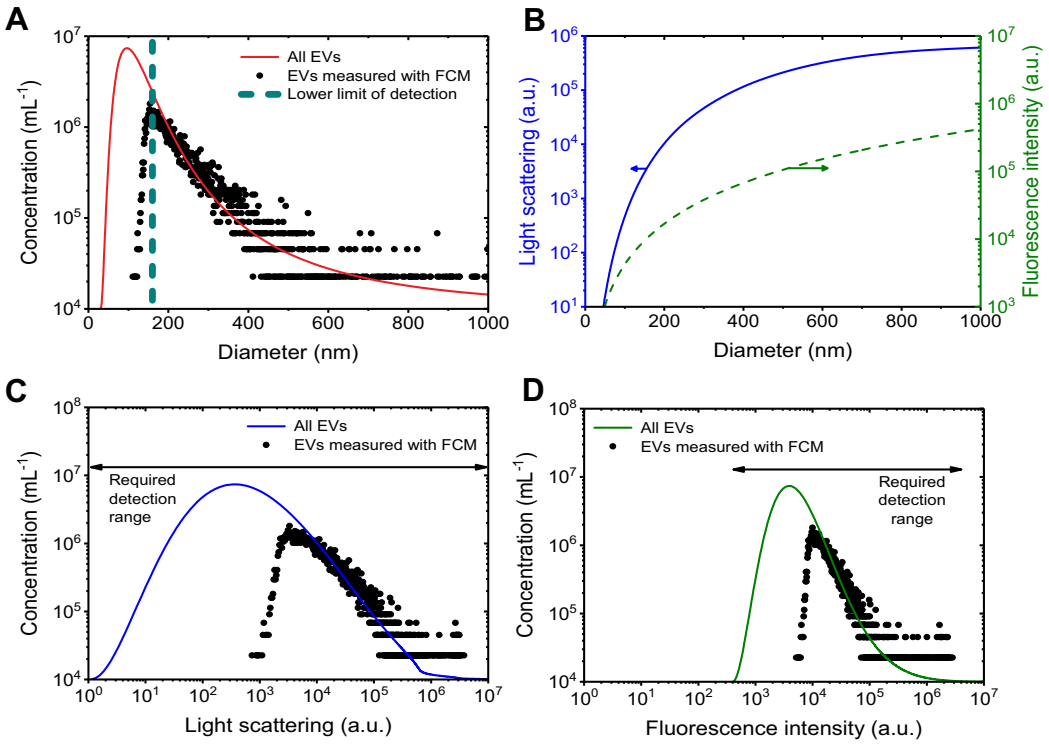

FIGURE 1 (A) Indicative size distribution of extracellular vesicles (EVs) in human blood plasma measured with cryo-EM (red line) [5] and measured with flow cytometry (Apogee A60-Micro) following lactadherin staining (symbols). The vertical scale is logarithmic. For flow cytometry measurements, diameters were determined by Rosetta Calibration assuming a shell thickness of 6 nm, a shell refractive index of 1.48, and a core refractive index of 1.38. The lower limit of detection (cyan dashed line) was defined as the diameter at which the size distribution peaks. Symbols represent only lactadherin+ EVs. (B) Theoretical light scattering- (blue line) and fluorescence-to-diameter (green line) relationship for EVs. Fluorescence scales quadratically with diameter, whereas scattered light is even stronger diameter dependent. (C) Concentration of all EVs (blue line) and EVs measured with our flow cytometer (FCM) following lactadherin staining (symbols) in blood plasma vs light scattering signals in arbitrary units (a.u.). The horizontal arrows indicate the detection range required to detect all EVs. (D) Concentration of all EVs (green line) and EVs measured with our FCM following lactadherin staining (symbols) in blood plasma vs fluorescence intensity in a.u. The horizontal arrows indicate the detection range required to detect all EVs. For details, please see Supplementary Information S1.

incapable of detecting submicrometer EVs by flow cytometry [6,14] and likely measured background noise. Second, Sims et al. [15] assumed that the "platelet-derived EVs" measured by flow cytometry were similar to the 100-nm microparticles observed in electron micrographs of C5b-9–treated platelets. However, neither did they provide evidence that these membrane blebs were released as EVs nor would these membrane blebs be detectable, even not with most state-of-the-art FCMs [6]. Altogether, it is unlikely, if not impossible that Sims et al. [15] measured 100-nm, platelet-derived EVs at the time.

Given the limited sensitivity of FCMs that were used in premillennial studies, at best, the relatively large spherical EVs were being studied, although it may well be that filopodia and/or empty erythrocytes or platelets were being detected [5]. The interpretation of these early days flow cytometry studies on EVs is cumbersome due to the incomplete description of preanalytical variables, the use of antibodies with unknown specificity, the use of insensitive FCMs, and the lack of calibration. These shortcomings have led to many single-center publications reporting orders of magnitude for different EV concentrations [10].

2.2 | Standardization using polystyrene bead gates on light scattering

Between 2000 and 2010, awareness grew that clinical applications of EV concentration measurements require comparable and reproducible data, which could only be achieved by standardization. Throughout this article, the term "standardization" refers to measuring a comparable concentration of cell-type–specific EVs in a body fluid.

The first standardization studies aimed to standardize EV concentration measurements in human plasma. The approach was based on measuring FSC signals of 500 nm and 900 nm fluorescent polystyrene beads, which were used to define an "MP gate", where MP referred to "microparticles," which is an older term for EVs [16]. Based on this approach, a standardization study was initiated by the *Scientific Standardization Committee on Vascular Biology of the International Society on Thrombosis and Haemostasis* in 2010 [17]. To explain the approach, Figure 3 shows FSC vs SSC of an "MP gate" and particles in human plasma. The lower and upper limits of the "MP gate" are defined by the FSC signals of 500 nm and 900 nm polystyrene beads, respectively. These studies showed that standardization of particle

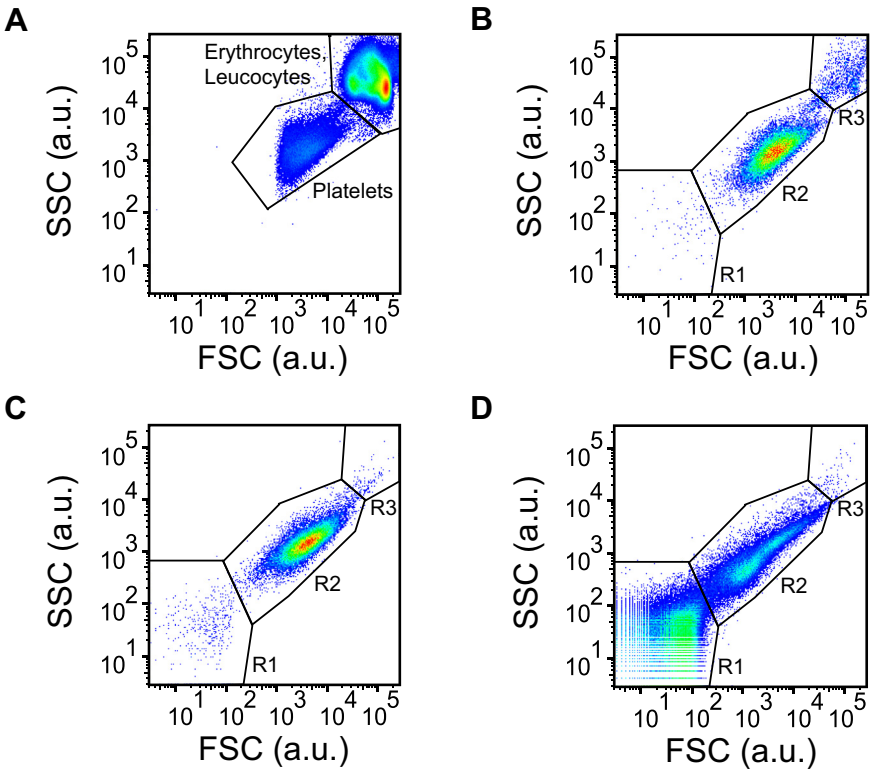

FIGURE 2 (A) Representative dot plots of light scattering signals in arbitrary units (a.u.) of erythrocytes, leucocytes, and platelets (gates) in diluted whole blood measured by flow cytometry without antibodies. Cells such as erythrocytes, leucocytes and platelets can be identified based on their scattering patterns. (B) Side scattered light intensity (SSC, a.u.) versus forward scattered light intensity (FSC, a.u.) in diluted whole blood when using an anti-platelet antibody. R1 represents platelet-derived EVs, R2 platelets, and R3 platelet-leucocyte complexes. (C) SSC (a.u.) versus FSC (a.u.) in diluted blood plasma when using an anti-platelet antibody. R1 represents platelet-derived EVs, R2 platelets, and R3 platelet-leucocyte complexes. (D) SSC (a.u.) versus FSC (a.u.) in diluted blood plasma with activated platelets when using an anti-platelet antibody. R1 represents platelet-derived EVs, R2 platelets, and R3 platelet-leucocyte complexes. The data was generated based on a repeated experiment based on a publication from Nieuwland et al. [12]. For details, please see Supplementary Information S2.

concentration measurements in cell-depleted plasma is feasible but only among FCMs of the same type.

2.3 | Standardization based on light scattering theory

The "MP" gate assumed that the diameter and shape are the dominant properties of particles affecting light scattering. However, also the refractive index (RI) contrast between a particle and the medium determines how efficiently a particle scatters light. As most EVs are spherical [5], light scattering detected by an FCM can be described by Mie theory [18]. In 2011, Chandler et al. [19] realized the importance of the RI of EVs in light scattering measurements and developed a light scattering model based on the Mie theory to calculate the relation between FSC and the diameter of polystyrene beads (RI, 1.59), silica beads (RI, 1.463), and EVs (RI, 1.39). As experimental data on the RI of EVs was lacking in 2011, the RI was based on literature values of biological particles with a structure similar to EVs. The authors experimentally confirmed that the "MP gate" [19] selects platelets rather than platelet-derived EVs. Moreover, they confirmed that the "MP gate" selects different particle size ranges at different FCMs,

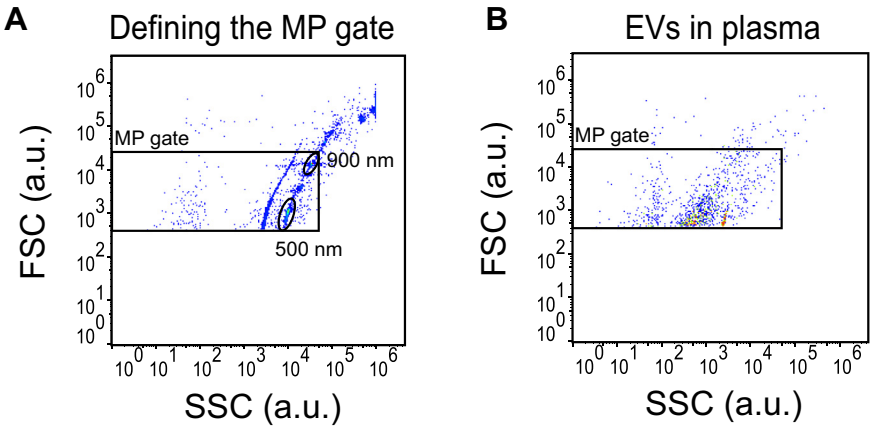

FIGURE 3 (A) Definition of the "microparticle (MP) gate." Representative dot plot of forward scattered light intensity (FSC) in arbitrary units (a.u.) versus side scattered light intensity (SSC, a.u.) of the Megamix SSC beads. The black gate shows the construction of the "MP gate" based on a publication from Robert et al. [16]. (B) Extracellular vesicles (EVs) in plasma. Dot plot of the "MP gate" on a plasma sample measured by flow cytometry. For details, please see Supplementary Information S3.

indicating that gates based on polystyrene beads cannot be used to standardize concentration measurements of EVs within the same size ranges among different types of FCMs.

In 2012, van der Pol et al. [14] designed a model based on Mie theory to relate light scattering of spherical particles to their diameter and RI. In contrast to the model of Chandler, this model also takes the optical configuration of the FCM into account. The theory describes the light scattering data of polystyrene and silica beads well (R^2 = 0.99) and was used to establish a scatter-to-diameter relation of EVs, again assuming an RI based on literature values of biological particles with a structure comparable to EVs. For the first time, the scatter signal of an FCM was related to the diameter of EVs in nm.

2.4 | Swarm detection

Due to the insights from Mie theory and the development of new particle sizing technologies, van der Pol et al. [14] discovered that during EV measurements by flow cytometry, multiple (hundreds or more) particles that are present at or below the detection limit may be simultaneously and continuously illuminated, and together be erroneously registered as single events. This effect was called "swarm detection," which is a special form of coincidence detection. Similar to coincidence detection, swarm detection causes an erroneous estimate of EV concentrations measured by flow cytometry and can be reduced by dilution [14,20,21].

2.5 | Refractive index measurements of EVs

Calibration with Mie theory was based on the assumption that EVs have a lower RI than silica and polystyrene. In 2012, Konokhova et al. [22] published the first RI measurements of EVs. With a mode RI just below 1.40, Konokhova et al. [22] experimentally confirmed the previously assumed RI values of EVs. In 2014, the low RI of EVs was further confirmed by measurements with nanoparticle tracking analysis [23,24] and a method called *flow cytometry scatter ratio* [25].

The RI measurements of EVs were important to the field because they (i) confirmed that "MP gates" based on polystyrene beads select larger particles than EVs, such as platelets, (ii) are essential input to Mie theory models, and (iii) substantiate the theory underlying swarm detection. Mie theory models allow to relate measured light scattering signals to the diameter of particles under the assumption that the RI of a particle is known. Thus, the more accurate the RI of EVs is known, the more accurate the diameter of EVs can be determined. Consequently, the better EV concentrations can be compared between FCMs within the same size range.

2.6 | Relevance of EV size distributions to EV flow cytometry standardization

The reason why accurate diameter determination of EVs is relevant to the standardization of EV concentration measurements became clear in 2014, when 2 studies published the size distribution of EVs in human body fluids [5,26]. The studies showed that within the detection range of most FCMs, the concentration of EVs strongly decreases with increasing diameter, as shown in Figure 1A. Hence, measured EV concentrations are highly dependent on particularly the LoD of the FCM. Therefore, the reported EV concentrations should always be accompanied by the detection limit in comparable units.

2.7 | Standardization using different polystyrene bead gates for forward scattered light and side scattered light

The earlier introduced "MP gate," based on polystyrene beads, did not lead to comparable EV concentrations among different FCMs because the "MP gate" selects different EV sizes on FCMs with different light collection angles. To mitigate this problem, a study initiated by the *International Society on Thrombosis and Haemostasis Scientific Standardization Committee on Vascular Biology* proposed to use 2 sets of selected beads adapted to FSC or SSC dependent on which scatter parameter provides the best resolution to detect EVs [27]. Cointe et al. [27] applied their approach to a selection of 26 out of 52 participating FCMs and achieved coefficient of variations of 28% and 37% for the measured platelet-derived microparticle concentrations in 2 different samples. Although the approach resulted in comparable EV concentrations and did not require modeling with Mie theory, there are 3 caveats. First, the optical configuration, such as the illumination wavelength and collection angles differ between FCMs. As the optical configuration affects the scatter-to-diameter relation, a given gate based on the light scattering signals of polystyrene beads will lead to a selection of different EV sizes on the different FCMs and hence result in measuring different concentrations of EVs [6]. Second, based on current knowledge from Mie theory, the gates defined by Cointe et al. [27] do include particles >1000 nm, such as platelets. Third, a gating strategy based on 2 polystyrene bead sizes limits the flexibility of users to define gates matching the detection range of their FCM.

2.8 | Standardization by EV diameter estimation using Mie theory

An interlaboratory comparison study was initiated involving 46 FCMs to standardize EV concentration measurements within predefined EV diameter ranges. With their most sensitive light scattering detector, 6 FCMs detected EVs down to 300 nm, 22 FCMs detected EVs down to 600 nm, and 32 FCMs detected EVs down to 1200 nm. Although all study participants had a track record in EV detection by FCM, 14 FCMs were unable to detect 400 nm polystyrene beads and therefore have none or limited utility for EV research with the settings used. The used reference particles and Mie model, which considers the differences between optical configuration of FCMs and the RI of EVs [6],

became commercially available under the name Rosetta Calibration (Exometry B.V.).

The study further revealed that flow rate calibration is essential to standardization because the actual flow rate differed up to 2-fold from the set flow rate. Within the 1200- to 3000-nm gate, the coefficient of variation of the measured EV concentration was 81% using the Mie theory model compared with 139% using a gate based on polystyrene beads. In sum, Mie theory modeling provides insight into the size of detected EVs and improves the interlaboratory variability. However, the Mie theory modeling relies on the assumed RI of EVs, which is much lower than the RI of polystyrene beads and requires extrapolation. In turn, the approach by van der Pol et al. did not include calibration of the fluorescence detectors.

2.9 | Beads with a similar refractive index as EVs

Whereas "MP gates" based on polystyrene beads select particles of unknown size that differ between FCMs, Mie theory requires an extrapolation, as polystyrene beads have a higher RI than EVs. In 2018, Varga et al. [28] proposed an alternative to polystyrene beads, called hollow organosilica beads (HOBs), to set EV size gates and potentially calibrate FCMs. HOBs have an approximately 10-nm thick organosilica shell with an RI of 1.46 and an aqueous core with an RI of 1.34. Consequently, HOBs have a similar RI distribution as EVs, thereby mimicking the light scattering behavior of EVs.

2.10 | Standardization by fluorescence calibration

Fluorescence calibration for FCMs was already developed in the 1980s [29,30], with molecules of equivalent soluble fluorochrome (MESF) being the standardized unit. If a particle has a fluorescence intensity of given MESF, this means that the particle emits the same fluorescence intensity as an equivalent number of molecules of the fluorochrome dissolved in solution [31]. Other units of fluorescence intensity, like antibody binding capacity (ABC) and equivalent number of reference fluorophores (ERF), followed in the 1990s and 2000s, respectively [32,33].

Despite the long-term existence of fluorescence calibration procedures [34], it was first used in the EV field by Mobarrez et al. [35] in 2009 and explicitly elucidated by Stoner et al. [36] and Arraud et al. [37] in a special issue on "*Measurements of EVs and other submicron size particles by FCM*" that appeared in *Cytometry A* in 2016. Similar to light scattering, fluorescence signals of stained EVs are close to and below the LoD of most FCMs [38], as shown in Figure 1D. Therefore, the measured concentration of stained EVs depends on the applied fluorescence gate, which should therefore be reported in standard units.

2.11 | Calibrating light scattering and fluorescence

In 2020, Welsh et al. [39] measured comparable concentrations of fluorescently stained viruses by calibrating both light scattering and fluorescence signals of 2 different FCMs. The study by Welsh et al. [39] is an important milestone to EV flow cytometry standardization because it confirmed that seemingly different results become comparable after calibration. The authors noted that *"fluorescence and light scatter calibration are not widely adopted by the small particle community as methods to standardize flow cytometry (FCM) data,"* thus *"further support in the form of education"* is required. In addition, calibrations of both scatter and fluorescence detectors could assist in standardizing procedures to optimize acquisition settings [7,36,37].

2.12 | Standardized reporting

Data comparison between studies does not only require solid standardization strategies, but also standardized reporting. In 2020 the EV flow cytometry working group (www.evflowcytometry.org) published a framework (MIFlowCyt-EV) to support standardized reporting of information regarding EV flow cytometry experiments [40]. MIFlowCyt-EV provides a structure for sharing EV-FC results, included assay controls, data acquisition, instrument calibration, and sample preparation.

In sum, most problems of EV flow cytometry standardization have been tackled during the past decade. The EV flow cytometry field went from studies without calibration to standardization of both size and fluorescence. Standardization by light scattering and fluorescence calibration was implemented into the field of EV flow cytometry, allowing to express the measured EV concentration within calibrated ranges in standard units of the *International System of Units* (SI), like nanometers. Table 1 [41–44] provides an overview of the history of EV flow cytometry standardization.

3 | THE PRESENT

From the history, it became clear that calibration is the key to EV FCM standardization [45]. Although in the EV field, the word "calibration" is used in different contexts, in this article we refer to calibration in the metrological context. In the next section, we will explain what calibration means in metrology and whether the current standardization procedures in EV flow cytometry are compliant with metrology. Table 2 [47,49] provides an overview of the formal definitions and what they mean to the EV field.

3.1 | Calibration in a metrological context

In the context of flow cytometry and metrology, the term calibration means an operation to establish the relationship between the measured arbitrary units and the corresponding quantity values realized by measurement standards [45,50]. With quantity values, we mean a number and a measurement unit that together express the magnitude of a quantity, such as the diameter of an EV expressed in nm. Measurement standards are definitions of a given quantity and are preferably expressed in base units of the SI [51], so that after a calibration, the

TABLE 1 Overview of the history of extracellular vesicle flow cytometer standardization.

Year	Authors	What has been done	Multicenter study
1986	Brown et al. [30]	MESF calibration	No
1988	Sims et al. [13]	Detection of "100-nm platelet-derived microparticles"	No
1990	Abrams et al. [11]	Direct detection of "platelet-derived microparticles in humans" Forward scatter (a.u.) does not equal particle size	No
1996	Fuller et al. [41]	Fluorescence based sizing of synthetic EVs	No
1997	Nieuwland et al. [12]	Platelet-derived "microparticles" are procoagulant	No
2009	Robert et al. [16]	Standardization using polystyrene beads	Yes
2010	Mobarrez et al. [35]	MESF calibration in EV research	No
2010	Lacroix et al. [17]	Interlaboratory comparison study on platelet-derived microparticles	Yes
2011	Chandler et al. [19]	Mie theory to understand light scattering of EVs	No
2012	van der Pol et al. [14]	Mie theory and swarm detection	No
2012	Konokhova et al. [22]	EV refractive index measurements	No
2012	van der Vlist et al. [42]	EV detection by fluorescent labeling	No
2013	Nolan et al. [43]	Swarm detection, trigger artifact	No
2014	Gardiner et al. [24], van der Pol et al. [23]	EV refractive index measurements	No
2014	Arraud et al. [5], van der Pol et al. [26]	EV size distribution in human plasma and urine	No
2014	Zhu et al. [8]	Detection of 24-nm silica beads	No
2014		Start of the EV FCM working group	
2016	Stoner et al. [36]	Fluorescent-based sizing	No
2017	Cointe et al. [27]	Interlaboratory comparison study on platelet-derived microparticles	Yes
2018	de Rond et al. [7]	Optimal trigger strategy depends on FCM	No
2018	van der Pol et al. [6]	Mie theory-based standardization study	Yes
2018	Varga et al. [28]	Hollow organosilica beads	No
2018	de Rond et al. [9]	Scatter-based sizing	No
2019	Welsh et al. [40]	Standardized reporting, MIFlowCyt-EV	No
2020	Welsh et al. [39]	Simultaneous fluorescence and light scatter calibration	Yes
2022	Woud at al. [44]	Fully calibrated imaging FCM	No

a.u., arbitrary units; EV, extracellular vesicle; FCM, flow cytometer; MESF, molecules of equivalent soluble fluorochrome; MIFlowCyt-EV, Minimum Information about a flow cytometry experiment (MIFlowCyt) standard in an EV flow cytometry-specific reporting framework.

measurement results can be compared between different instruments. An example of a measurement standard is the meter, which is the SI unit of length and defined as 30.66331899 wavelengths of radiation emitted by the unperturbed ground-state hyperfine transition of the cesium 133 atom [52]. From the example, it is clear that a measurement standard cannot be directly used to calibrate an FCM, because a measurement standard is merely a definition. To perform a calibration in practice, RMs are needed.

3.2 | Reference materials

RMs defined by the *International Organization for Standardization* guide 30:2015 is a generic term that refers to a "*material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process*" [48]. RMs can be divided into (i) quality control materials and (ii) certified RMs, which are also called calibrants [48,53]. The quality control materials and generally also calibrants can be used to perform technical replicates and to develop and test detection methods and assays [51]. However, only calibrants can be used to calibrate.

Therefore, calibrants have additional requirements such as specified quantity values that (i) cover the detection range of the instrument used and (ii) have an associated uncertainty (see Section 3.3). Metrology institutes determine the quantity value and associated uncertainty of calibrants in a traceable manner, meaning that the quantity value and associated uncertainty can be related to the measurement standards in SI units through a documented unbroken

TABLE 2 Formal definitions and what they mean (to the extracellular vesicle field).

Term	Formal definition	Reference	Meaning to the EV field	Examples
Calibration	"Operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and in a second step, uses this information to establish a relation for obtaining a measurement result from an indication."	JCGM, 2008, metrology vocabulary [45]	Calibration is a procedure to relate the arbitrary units of measured data to comparable standard units, preferably SI units	Relating arbitrary units of light scattering signals to the diameter of EVs in nm [6,46]. Relating arbitrary units of fluorescence signals to MESF [35,39,46].
Measurement accuracy	"Closeness of agreement between a measured quantity value and a true quantity value of a measurand"		Quantitative term to describe the closeness of a measured value to the "true value."	Measuring NIST traceable polystyrene beads with nanoparticle tracking analysis to determine the difference between the measured and specified mean diameter.
Measurement uncertainty	"Nonnegative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used"	JCGM 200: 2012 [45]	Uncertainty is the technical term for the level of doubt about an obtained measurement result. Uncertainty is not the same as error.	Reference value mean size and expanded uncertainty of Reference Material 8013 Gold Nanoparticles, 60 nm diameter (NIST) measured by TEM is 56.0 ± 0.5 nm [47].
Primary reference measurement procedure	"Reference measurement procedure used to obtain a measurement result without relation to a measurement standard for a quantity of the same kind."		Method of which every aspect involved in the measurement is known, including the uncertainty. Therefore, primary reference measurement procedures do not require calibration.	Specialized, well-characterized equipment in metrology institutes, such as electron microscopes, atomic force microscopes, and small-angle x-ray scattering for dimensional characterization.
Reference material	"Material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process."	ISO guide 30:2015 [48]	A sample that can be used to perform quality controls and/or calibrations	Quality control material: Recombinant EVs [49] Calibrant: NIST Traceable Particle Size Standards or MESF beads

(Continues)

TABLE 2 (Continued)

Term	Formal definition	Reference	Meaning to the EV field	Examples
Secondary measurement standard	"Measurement standard established through calibration with respect to a primary measurement standard for a quantity of the same kind."	JCGM 200:2012 [45]	An RM that has been calibrated against a primary standard.	NIST traceable polystyrene beads to calibrate size.
Traceable	"Property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty."	ISO_IEC_17025, JCGM, 2008, Metrological vocabulary, ISO/IEC guide 99 (https://www.iso.org/obp/ui/#iso:std:iso-iec:guide:99:ed-1:v2:en)	When a property of an RM is traceability characterized, this means that the value and uncertainty of the property are specified in SI units.	The diameter of NIST traceable polystyrene beads is traceably determined, eg, 100 ± 3 nm.

EV, extracellular vesicle; ISO, International Organization for Standardization; JCGM, Joint Committee for Guides in Metrology; MESF, molecules of equivalent soluble fluorochrome; NIST, National Institute of Standards and Technology; RM, reference material; SI unit, International System of Units; TEM, Transmission electron microscopy.

chain of comparisons, each contributing to the measurement uncertainty [39,54].

3.3 | Measurement uncertainty

The establishment of measurement uncertainty is an important aspect of a calibration. No matter how accurate the measurement technique, a measurement does not result in an exactly determined quantity value. The measurement uncertainty is the dispersion of the quantity values attributed to the quantity thought to be measured. As an example, reporting the mean diameter of measured EVs without an uncertainty statement has little meaning, because it means that the true mean diameter can have any value. However, reporting the mean diameter of measured EVs with an uncertainty statement means that, based on the uncertainty attributed to the measurement standards, calibration procedure, and measurement procedure, the true mean diameter lies with a known probability within the stated uncertainty range of the reported mean diameter. Thus, the attribution of a measurement uncertainty reveals the accuracy of a measurement technique and is therefore helpful for data comparison, for example, when different techniques generate different measurement results and to judge which results to trust.

3.4 | The present state of flow cytometry calibrations

EV FCM measurements are typically performed to determine the number concentration of fluorescently stained EVs. The measurement of the number concentration requires calibration of the sample volume and the assurance that all particles in the measured sample volume are measured, which is unlikely if not impossible for EV FCM measurements because part of the EVs have light scattering and fluorescence signals that fall below the LoD of the FCM. Consequently, light scattering signals and fluorescence also require calibration, such that the detection ranges wherein EVs are measured can be reported.

From a metrological perspective, an obvious unit for the power of optical signals is Watt ($kg \cdot m^2 \cdot s^{-3}$). However, the power in Watt has little utilization to standardize EV FCM measurements, because a given particle results in different optical powers when measured at different FCMs due to hardware differences. To realize comparable EV concentration measurements, ideally the physical properties of EVs, such as the diameter and the number of stained biomolecules, are derived from the measured signals, including an uncertainty statement and an estimate of the LoD of the FCM.

3.5 | Sample volume

Concentration is the number of particles per sample volume and thus requires calibration of the measured sample volume. FCMs exploit

various techniques to nontraceably determine the analyzed sample volume, such as a calibrated syringe pump, a flow rate sensor, and weighing. A practical procedure to determine the sample volume traceably, is the use of RMs with a traceably determined particle concentration. However, submicrometer RMs with a traceably determined particle concentration are not commercially available. Currently, laboratories, therefore, calibrate the flow rate with commercially available submicrometer counting beads that are nontraceable, such as ApogeeMix (Apogee Flow Systems), or with micrometer sized counting beads having a Conformité Européene mark for *in vitro* diagnostics, such as TruCount beads (BD Biosciences). The disadvantages of using micrometer sized counting beads are that they (i) have to be acquired with different settings than EVs and (ii) sediment, which may affect the accuracy of the calibration procedure. In sum, without an uncertainty statement, the analyzed sample volumes are still unknown from a metrological perspective.

3.6 | Light scattering intensity

Light scattering is also measured in arbitrary units. Light scattering intensity depends on the particle diameter, shape, and RI and on the RI of the surrounding medium [14]. Best current practice is to use beads (eg, polystyrene or silica) with a traceably determined diameter to calibrate scattering signals. Light scattering of beads is measured and related to their theoretical scattering cross section, which is obtained by Mie theory from the specified size and RI of the beads. However, traceably determined RI measurements of the reference particles and their surrounding medium are still missing. Furthermore, polystyrene beads (RI, 1.61 at 488 nm) and silica beads (RI, 1.46 at 488 nm) both have a higher RI than that of EVs (RI, <1.40 for EVs >∼100 nm) [22,23,25], resulting in more light scattering than that with similar-sized EVs [19,24], and therefore, misinterpretation of EV size [28].

As HOBs [28] have similar light scattering properties as EVs, they may be interesting reference particles for light scattering. However, the theoretical scattering cross section of HOBs, which is used for calibration, depends on the lumen RI, the shell thickness and RI, and the diameter, which all require traceable determination. The uncertainty of the theoretical scattering cross section is therefore expected to be higher than that of the solid beads, which only require traceable determination of the diameter and RI.

3.7 | Fluorescence intensity

Fluorescence intensities are measured in arbitrary units but can be related to standard units with ABC beads, ERF beads, or MESF beads [40]. MESF beads are most frequently used and have a specified fluorescence intensity equal to the equivalent number of molecules of the fluorochrome in a solution [32]. The MESF bead surface is stained with the same fluorochromes that are conjugated to the antibodies used for EV staining. MESF beads with fluorophores, as allophycocyanin (APC), fluorescein isothiocyanate (FITC) or phycoerythrin (PE), are commercially available. However, MESF beads have 4 limitations.

First, MESF beads and all other beads used for fluorescent calibration lack an uncertainty statement, and therefore are no metrological calibrants. Second, MESF beads have minimum MESF intensities that are order of magnitudes higher than that for EVs. Therefore, fluorescence calibration in the EV range requires extrapolation. Third, MESF beads are bigger, larger, and scatter more amount of light than EVs. This may lead to practical problems regarding triggering strategies, especially in FCMs with a narrow sample flow. Fourth, the fluorescence spectrum and intensity depend on the chemical composition and environment and can differ between MESF beads and EVs [40].

ABC beads are an alternative to MESF beads. ABC beads have a known binding capacity for immunoglobulin and capture the used conjugated antibody. Antibody capture is specific for one species and binding properties may differ per isotype and clone.

When both MESF or ABC beads cannot be used, ERF beads can be used. ERF beads have a broad emission spectrum but are less accurate than MESF and ABC beads [40]. Furthermore, they can only be used to standardize an FCM with the same spectral filters.

In sum, the fluorescent beads available for calibration lack uncertainty statements and the specified properties are not expressed in SI units. Moreover, the currently used beads are designed for cellular analysis and are bigger and brighter than EVs, requiring extrapolation for application in the EV range. In the previous section we discussed calibration and now we will continue with demonstrating how calibration improves data interpretation.

3.8 | Best practice in EV flow cytometry

To demonstrate how calibration improves data interpretation, we have applied the best current practices to calibrate light scattering and fluorescence signals of an FCM to a plasma sample stained with CD61-APC to identify platelet-derived EVs.

Figure 4A shows the fluorescence vs light scattering intensity or diameter of EVs in the stained plasma sample using arbitrary units and standard units, respectively. In Figure 4A, 2 populations exceed the fluorescence background noise, but it remains unclear which population represents the stained EVs. After calibration (Figure 4B), it becomes clear that the size and fluorescence intensity of the brightest population is typical for platelets, whereas the properties of the dimmest population are typical of EVs. Assay controls are required to confirm that the dimmest population are indeed EVs [46]. Furthermore, to achieve truly reproducible flow cytometry experiments, standardized reporting of all experimental details, including sample preparation, assay controls, calibration, and instrument settings, should be applied [40]. Examples and software developed to perform such fluorescence and light scattering calibrations can be found in the recently published "*Compendium of single extracellular vesicle flow cytometry*" [6,46,55,56].

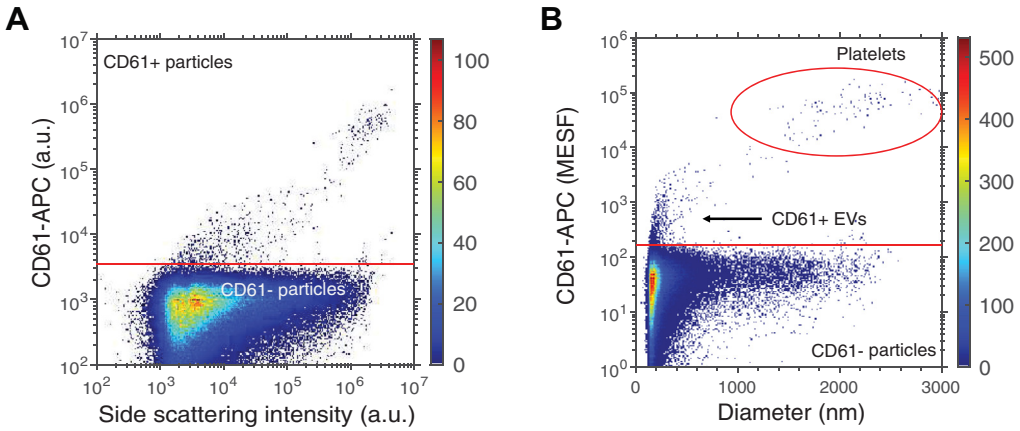

FIGURE 4 Example of the identification of extracellular vesicles (EVs) stained with anti-CD61 to identify platelet-derived EVs in arbitrary units (a.u.) and calibrated units for fluorescence in molecules of equivalent soluble fluorochrome (MESF) and diameter in nm. (A). Measured fluorescence intensity vs the side scattering intensity in a.u. of particles in plasma stained with CD61-APC (Apogee A60-Micro). The fluorescent gate (red horizontal line) differentiates stained particles (CD61+) from background noise and unstained particles (CD61-). The a.u. hinder identification of the 2 positively stained populations. (B). Measured fluorescence intensity in fluorescence standard units (MESF) vs the diameter (nm) of particles in the same sample as in panel A. Due to the calibration, the 2 populations can be identified as CD61+ EVs and platelets. For details, please see Supplementary Information S4.

4 | THE FUTURE

To enable comparison of EV concentration measurements among FCMs, standardization is needed. The key to achieve standardization is calibration. Currently, laboratories rely on polystyrene beads to calibrate light scattering and fluorescence signals. However, these beads lack uncertainty statements of the number concentration, MESF intensity, and RI.

FCM calibration requires (1) traceably characterized RMs, ie, with an uncertainty statement, so that calibrations are reliable and (2) RMs that resemble physical properties of EVs to avoid data acquisition with different settings. Sample volume calibration requires small and dim beads with a traceably determined number concentration. For light scattering calibrations the field needs traceably determined size and RI measurements. Last, but not least, the beads used for fluorescence calibration should be smaller and dimmer than the currently commercially available MESF beads, and a traceable unit for fluorescence should be established. Dedicated EV RMs will be useful to define the LoD of instruments more accurately, and to perform uncertainty evaluations of FCMs, which help to improve data reliability and comparability.

Once a robust infrastructure for EV FCM calibration is developed, this would enable data comparability and reliable reference studies of EV-type–specific concentrations in body fluids, such as blood plasma. Such reference studies open the door to multicenter studies to explore the real clinical biomarker potential and relevance of EVs.

Efforts are being undertaken to develop RMs mimicking EV characteristics. A project focusing on the development of EV-tailored RMs is 18HLT01 Metrological characterisation of microvesicles from body fluids as non- invasive diagnostic biomarkers (METVES) II (https://www.metves.eu). METVES II is a European metrology project in which metrology institutes, academia, and companies collaborate to standardize EV concentration measurements in clinical samples. The project aims to develop RMs to calibrate flow rate, light scattering and fluorescence of an FCM, and a blood plasma-based EV-containing quality control sample to validate the developed RMs. Recently, the first global interlaboratory comparison study that calibrated all aspects of 25 FCMs has been completed. The data are currently being analyzed. Preliminary results confirm that full FCM calibration improves comparability of EV concentration measurements, thus paving the road to clinically relevant multicenter studies on EVs.

ACKNOWLEDGMENTS

We thank Naomi Buntsma for providing the data for Figure 1.

AUTHOR CONTRIBUTIONS

B.B. and R.N. conceptualized the study. B.B. and E.v.d. curated the data and performed the formal analysis. B.B., R.N., and E.v.d. performed the investigation and were responsible for project administration. Z.V., R.N., and E.v.d. were responsible for the resources. B.B., R.N., and E.v.d. wrote the original draft of the manuscript. B.B., Z.V., R.N., and E.v.d. reviewed and edited the manuscript.

DECLARATION OF COMPETING INTERESTS

E.v.d.P. is cofounder and shareholder of the company Exometry B.V. (Amsterdam, the Netherlands). B.B., Z.V., and R.N. have no competing interests to disclose.

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REFERENCES

[1] Yuana Y, Sturk A, Nieuwland R. Extracellular vesicles in physiological and pathological conditions. *Blood Rev*. 2013;27:31–9.

[2] Loyer X, Vion AC, Tedgui A, Boulanger CM. Microvesicles as cell–cell messengers in cardiovascular diseases. *Circ Res*. 2014;114:345–53.

[3] Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. 2015;527:329–35.

[4] van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. 2012;64:676–705.

[5] Arraud N, Linares R, Tan S, Gounou C, Pasquet J-M, Mornet S, Brisson AR. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J Thromb Haemost*. 2014;12:614–27.

[6] van der Pol E, Sturk A, van Leeuwen T, Nieuwland R, Coumans F, ISTH-SSC-VB Working group. Standardization of extracellular vesicle measurements by flow cytometry through vesicle diameter approximation. *J Thromb Haemost*. 2018;16:1236–45.

[7] de Rond L, van der Pol E, Hau CM, Varga Z, Sturk A, van Leeuwen TG, Nieuwland R, Coumans FAW. Comparison of generic fluorescent markers for detection of extracellular vesicles by flow cytometry. *Clin Chem*. 2018;64:680–9.

[8] Zhu S, Ma L, Wang S, Chen C, Zhang W, Yang L, Hang W, Nolan JP, Wu L, Yan X. Light-scattering detection below the level of single fluorescent molecules for high-resolution characterization of functional nanoparticles. *ACS Nano*. 2014;8:10998–1006.

[9] de Rond L, Coumans FAW, Nieuwland R, van Leeuwen TG, van der Pol E. Deriving extracellular vesicle size from scatter intensities measured by flow cytometry. *Curr Protoc Cytom*. 2018;86:e43.

[10] Gasecka A, Böing AN, Filipiak KJ, Nieuwland R. Platelet extracellular vesicles as biomarkers for arterial thrombosis. *Platelets*. 2017;28:228–34.

[11] Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood*. 1990;75:128–38.

[12] Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, Maquelin KN, Roozendaal KJ, Jansen PGM, Have K ten, Eijsman L, Hack CE, Sturk A. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation*. 1997;96:3534–41.

[13] Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem*. 1988;263:18205–12.

[14] van der Pol E, van Gemert MJ, Sturk A, Nieuwland R, van Leeuwen TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. *J Thromb Haemost*. 2012;10:919–30.

[15] Sims PJ, Wiedmer T. Repolarization of the membrane potential of blood platelets after complement damage: evidence for a Ca++-dependent exocytotic elimination of C5b-9 pores. *Blood*. 1986;68:556–61.

[16] Robert S, Poncelet P, Lacroix R, Arnaud L, Giraudo L, Hauchard A, Sampol J, Dignat-George F. Standardization of platelet-derived microparticle counting using calibrated beads and a cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *J Thromb Haemost*. 2009;7:190–7.

[17] Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS, Dignat-George F, ISTH SSC Workshop. Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemost*. 2010;8:2571–4.

[18] Doornbos RM, Hoekstra AG, Deurloo KE, De Grooth BG, Sloot PM, Greve J. Lissajous-like patterns in scatter plots of calibration beads. *Cytometry*. 1994;16:236–42.

[19] Chandler WL, Yeung W, Tait JF. A new microparticle size calibration standard for use in measuring smaller microparticles using a new flow cytometer. *J Thromb Haemost*. 2011;9:1216–24.

[20] Harrison P, Gardiner C. Invisible vesicles swarm within the iceberg. *J Thromb Haemost*. 2012;10:916–8.

[21] Libregts SFWM, Arkesteijn GJA, Németh A, Nolte-'t Hoen ENM, Wauben MHM. Flow cytometric analysis of extracellular vesicle subsets in plasma: impact of swarm by particles of non-interest. *J Thromb Haemost*. 2018;16:1423–36.

[22] Konokhova AI, Yurkin MA, Moskalensky AE, Chernyshev AV, Tsvetovskaya GA, Chikova ED, Maltsev VP. Light-scattering flow cytometry for identification and characterization of blood microparticles. *J Biomed Opt*. 2012;17:057006.

[23] van der Pol E, Coumans FAW, Sturk A, Nieuwland R, van Leeuwen TG. Refractive index determination of nanoparticles in suspension using nanoparticle tracking analysis. *Nano Lett*. 2014;14:6195–201.

[24] Gardiner C, Shaw M, Hole P, Smith J, Tannetta D, Redman CW, Sargent IL. Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in extracellular vesicles. *J Extracell Vesicles*. 2014;3:25361.

[25] van der Pol E, de Rond L, Coumans FAW, Gool EL, Böing AN, Sturk A, Nieuwland R, van Leeuwen TG. Absolute sizing and label-free identification of extracellular vesicles by flow cytometry. *Nanomedicine*. 2018;14:801–10.

[26] van der Pol E, Coumans FAW, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, Sturk A, van Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J Thromb Haemost*. 2014;12:1182–92.

[27] Cointe S, Judicone C, Robert S, Mooberry MJ, Poncelet P, Wauben M, Nieuwland R, Key NS, Dignat-George F, Lacroix R. Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. *J Thromb Haemost*. 2017;15:187–93.

[28] Varga Z, van der Pol E, Pálmai M, Garcia-Diez R, Gollwitzer C, Krumrey M, Fraikin J-L, Gasecka A, Hajji N, van Leeuwen TG, Nieuwland R. Hollow organosilica beads as reference particles for optical detection of extracellular vesicles. *J Thromb Haemost*. 2018;16:1646–55.

[29] Vogt RF Jr, Cross GD, Henderson LO, Phillips DL. Model system evaluating fluorescein-labeled microbeads as internal standards to calibrate fluorescence intensity on flow cytometers. *Cytometry*. 1989;10:294–302.

[30] Brown MC, Hoffman RA, Kirchanski SJ. Controls for flow cytometers in hematology and cellular immunology. *Ann N Y Acad Sci*. 1986;468:93–103.

[31] Schwartz A, Gaigalas AK, Wang L, Marti GE, Vogt RF, Fernandez-Repollet E. Formalization of the MESF unit of fluorescence intensity. *Cytometry B Clin Cytom*. 2004;57:1–6.

[32] Schwartz A, Fernández Repollet E, Vogt R, Gratama JW. Standardizing flow cytometry: construction of a standardized fluorescence calibration plot using matching spectral calibrators. *Cytometry*. 1996;26:22–31.

[33] Wang L, Gaigalas AK, Marti G, Abbasi F, Hoffman RA. Toward quantitative fluorescence measurements with multicolor flow cytometry. *Cytometry A*. 2008;73:279–88.

[34] Wang L, Hoffman RA. Standardization, calibration, and control in flow cytometry. *Curr Protoc Cytom*. 2017;79:1.3.1–1.3.27.

[35] Mobarrez F, Antovic J, Egberg N, Hansson M, Jörneskog G, Hultenby K, Wallén H. A multicolor flow cytometric assay for

measurement of platelet-derived microparticles. *Thromb Res*. 2010;125:e110–6.
[36] Stoner SA, Duggan E, Condello D, Guerrero A, Turk JR, Narayanan PK, Nolan JP. High sensitivity flow cytometry of membrane vesicles. *Cytometry A*. 2016;89:196–206.
[37] Arraud N, Gounou C, Turpin D, Brisson AR. Fluorescence triggering: a general strategy for enumerating and phenotyping extracellular vesicles by flow cytometry. *Cytometry A*. 2016;89:184–95.
[38] Nolan JP. Flow cytometry of extracellular vesicles: potential, pitfalls, and prospects. *Curr Protoc Cytom*. 2015;73:13.14.1–13.14.16.
[39] Welsh JA, Jones JC, Tang VA. 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;97:592–601.
[40] Welsh JA, van der Pol E, Arkesteijn GJA, Bremer M, Brisson A, Coumans F, Dignat-George F, Duggan E, Ghiran I, Giebel B, Görgens A, Hendrix A, Lacroix R, Lannigan J, Libregts SFWM, Lozano-Andrés E, Morales-Kastresana A, Robert S, de Rond L, Tertel T, et al. MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments. *J Extracell Vesicles*. 2020;9:1713526.
[41] Fuller RR, Sweedler JV. Characterizing submicron vesicles with wavelength-resolved fluorescence in flow cytometry. *Cytometry*. 1996;25:144–55.
[42] van der Vlist EJ, Nolte-'t Hoen ENM, Stoorvogel W, Arkesteijn GJA, Wauben MHM. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. *Nat Protoc*. 2012;7:1311–26.
[43] Nolan JP, Stoner SA. A trigger channel threshold artifact in nanoparticle analysis. *Cytometry A*. 2013;83:301–5.
[44] Woud WW, van der Pol E, Mul E, Hoogduijn MJ, Baan CC, Boer K, Merino A. An imaging flow cytometry-based methodology for the analysis of single extracellular vesicles in unprocessed human plasma. *Commun Biol*. 2022;5:633.
[45] JCGM. JCGM 200:2008 International vocabulary of metrology-basic and general concepts and associated terms (VIM) Vocabulaire international de métrologie-Concepts fondamentaux et généraux et termes associés (VIM). 2008.
[46] Welsh JA, Arkesteijn GJA, Bremer M, Cimorelli M, Dignat-George F, Giebel B, Görgens A, Hendrix A, Kuiper M, Lacroix R, Lannigan J, van Leeuwen TG, Lozano-Andrés E, Rao S, Robert S, de Rond L, Tang VA, Tertel T, Yan X, Wauben MHM, et al. A compendium of single extracellular vesicle flow cytometry. *J Extracell Vesicles*. 2023;12:e12299.
[47] Allen AJ, Cho TJ, Grobelny J, Hackley VA, Kim DI, Namboodiri P, Becker ML, Ho DL, Karim A, Vogel BM, Watters RL. Report of Investigation Reference Material ® 8013 Gold Nanoparticles, Nominal 60 nm Diameter Office of Reference Materials Report Revision History on Last Page.
[48] ISO. ISO guide 30:2015. Reference materials—selected terms and definitions. https://www.iso.org/standard/46209.html; 2015 [accessed September 9, 2022].
[49] Geeurickx E, Tulkens J, Dhondt B, Van Deun J, Lippens L, Vergauwen G, Heyrman E, De Sutter D, Gevaert K, Impens F, Miinalainen I, Van Bockstal PJ, De Beer T, Wauben MHM, Nolte-'t-Hoen ENM, Bloch K, Swinnen JV, van der Pol E, Nieuwland R, Braems G, et al. The generation and use of recombinant extracellular vesicles as biological reference material. *Nat Commun*. 2019;10:3288.
[50] Balazs A. International vocabulary of metrology–basic and general concepts and associated terms. *Chem Int*. 2008;30:21–2.
[51] Welsh JA, van der Pol E, Bettin BA, Carter DRF, Hendrix A, Lenassi M, Langlois M-A, Llorente A, van de Nes AS, Nieuwland R, Tang V, Wang L, Witwer KW, Jones JC. Towards defining reference materials for measuring extracellular vesicle refractive index, epitope abundance, size and concentration. *J Extracell Vesicles*. 2020;9:1816641.
[52] REF 50: Consultative Committee for Length, International Committee for Weights and Measures. Mise en pratique - metre - Appendix 2 - SI Brochure. 2019.
[53] Geeurickx E, Hendrix A. Targets, pitfalls and reference materials for liquid biopsy tests in cancer diagnostics. *Mol Aspects Med*. 2020;72:100828.
[54] ISO. General requirements for the competence of testing and calibration laboratories Exigences générales concernant la compétence des laboratoires d'étalonnages et d'essais. https://www.iso.org/obp/ui/fr/#iso:std:iso-iec:17025:ed-2:v1:en; 2005 [accessed September 9, 2022].
[55] Welsh JA, Horak P, Wilkinson JS, Ford VJ, Jones JC, Smith D, Holloway JA, Englyst NA. FCM_{PASS} software aids extracellular vesicle light scatter standardization. *Cytometry A*. 2020;97:569–81.
[56] van der Pol E, Welsh JA, Nieuwland R. Minimum information to report about a flow cytometry experiment on extracellular vesicles: communication from the ISTH SSC subcommittee on vascular biology. *J Thromb Haemost*. 2022;20:245–51.

SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2023.04.042