

## RECOMMENDATIONS AND GUIDELINES

# Minimum information to report about a flow cytometry experiment on extracellular vesicles: Communication from the ISTH SSC subcommittee on vascular biology

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### Abstract

The Extracellular Vesicle Flow Cytometry Working Group (<http://www.evflowcytometry.org>) is formed by members of the International Society for Extracellular Vesicles (ISEV), the International Society for Advancement of Cytometry (ISAC), and the International Society on Thrombosis and Haemostasis (ISTH). This working group of flow cytometry experts develops guidelines for best practices regarding flow cytometry detection of extracellular vesicles. To improve rigor and standardization, this working group published a framework outlining the minimal information to report about a flow cytometry experiment on extracellular vesicles (MIFlowCyt-EV) in the *Journal of Extracellular Vesicles*, the ISEV journal, in 2020. In parallel, an article explaining MIFlowCyt-EV was published in *Cytometry Part A*, one of the ISAC journals, and now will be introduced to the ISTH as an SSC Communication in the *Journal of Thrombosis and Haemostasis*. The goal of this SSC Communication is to explain why flow cytometry is becoming the instrument of choice to characterize single extracellular vesicles, the obstacles that have been identified and (mostly) overcome by developing procedures to calibrate flow cytometers, and the relevance of reporting minimal information to improve reliability and reproducibility of experiments in which flow cytometers are used for characterization of extracellular vesicles.

### KEYWORDS

calibration, extracellular vesicles, flow cytometry, reproducibility of results, research design

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# 1 | INTRODUCTION

At present, more than 5000 articles are published annually on extracellular vesicles (EVs), a term encompassing particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate,<sup>1</sup> such as exosomes and microparticles or microvesicles. EVs are an intrinsic component of all body fluids and are being widely explored as potential biomarkers of disease, particularly because the function and properties of EVs change with disease. However, biomedical literature has a general reproducibility crisis that without doubt also affects EV research. Without improving rigor and standardization, future clinical applications of EVs are unreproducible and therefore unreliable.

## 1.1 | MIFlowCyt-EV

To start improving rigor and standardization, the Extracellular Vesicle Flow Cytometry Working Group (<http://www.evflowcytometry.org>) published a framework about the minimal information to report about a flow cytometry experiment on extracellular vesicles (MIFlowCyt-EV) in 2020.<sup>2</sup> This working group, founded in 2015, has members from the International Society for Extracellular Vesicles (ISEV), the International Society for Advancement of Cytometry (ISAC), and the International Society on Thrombosis and Haemostasis (ISTH), and develops guidelines for best practices regarding flow cytometry detection of EVs. The goal of this communication is to bring the MIFlowCyt-EV framework to the attention of the readership of the *Journal of Thrombosis and Haemostasis*, which is particularly relevant because (1) the ISTH was the first international organization to realize the importance of standardizing EV concentration measurements,<sup>3</sup> and (2) the results of four interlaboratory comparison studies to standardize EV measurements were published in the *Journal of Thrombosis and Haemostasis*.<sup>4-7</sup>

## 1.2 | Why flow cytometry to detect extracellular vesicles?

Flow cytometry is a technique that measures fluorescence and light scattering signals of single particles in a hydrodynamically focused flow. The working principle of a flow cytometer is well described in the literature.<sup>8-10</sup> To measure the concentration of cell-type-specific EVs in body fluids, flow cytometry has four advantages over other single particle detection techniques. To substantiate this statement, Table 1 shows the particle count rate during analysis, the error of the measured concentration, the lower limit of detection (LoD) in terms of EV diameter, the error and precision of the measured diameter, and the possibility to phenotype EVs for flow cytometry and four other single particle detection techniques.

First, flow cytometry is capable of characterizing particles with a count rate that is 2–4 orders of magnitude higher than the other techniques in Table 1. Due to this high throughput, flow cytometry can

**TABLE 1** Particle count rate during analysis, error of the measured concentration, lower limit of detection (LoD) in terms of the diameter of extracellular vesicles (EVs), error of the measured diameter of polystyrene reference particles exceeding the LoD, precision of the measured diameter of polystyrene reference particles exceeding the LoD, the possibility to phenotype EVs for flow cytometry, and references substantiating the numbers for hybrid interferometric reflectance imaging–fluorescence microscopy, single particle tracking, resistive pulse sensing, and transmission electron microscopy

	Count rate (min <sup>-1</sup> )	Concentration measurement error (%)	Diameter measurement error (%)	Diameter measurement precision (CV %)	Diameter lower LoD (nm)	Phenotyping	References
Flow cytometry	10 <sup>5</sup> –10 <sup>6</sup>	15–18	0–5	1–4	91–190	Fluorescent labels	12,13
Hybrid interferometric reflectance imaging–fluorescence microscopy	10 <sup>2</sup> –10 <sup>3</sup>	X	1–22		~50	Fluorescent labels	11
Single particle tracking	10 <sup>2</sup> –10 <sup>3</sup>	1–150	2–9	4–23	70–90	Detection of fluorescent labels hampered by bleaching	13
Resistive pulse sensing	10 <sup>2</sup> –10 <sup>3</sup>	0–24	2–5	5–10	70–100	X	13
Transmission electron microscopy	10 <sup>2</sup> –10 <sup>3</sup>	X	~–12		<30	Immunogold labelling	13

Note: Flow cytometry refers to flow cytometers capable of characterizing thousands of particles per second. Abbreviations: CV, coefficient of variation; empty cell, data unavailable; X, not applicable.

provide statistically significant information to differentiate populations of EVs present in, for example, human blood plasma, such as EVs from platelets, EVs from erythrocytes, and EVs from endothelial cells.<sup>8</sup>

Second, flow cytometry determines the concentration and particle diameter more accurately and more precisely than the other techniques in Table 1 for which data on the accuracy and precision are available.<sup>11-14</sup> The high accuracy and precision of flow cytometry enable reporting of the concentration of the measured EVs within a well-defined fluorescence and size range, which is a prerequisite for generating reproducible data.<sup>2,15,16</sup>

Third, flow cytometry can be used to phenotype fluorescently labeled EVs. Hybrid interferometric reflectance imaging–fluorescence microscopy also support fluorescence detection and with transmission electron microscopy immunogold labels can be used to phenotype EVs. Detection of fluorescently labeled EVs with single particle tracking is difficult in practice because common fluorophores bleach during particle tracking.

Fourth, flow cytometry is commonly available in clinical laboratories. The main advantage of the wide availability of flow cytometry is the presence of an infrastructure required to perform flow cytometry experiments on EVs. In practice, however, not all flow cytometers have the sensitivity required for EV research. For example, a recent international comparison study showed that 24% of the evaluated flow cytometers had limited utility for EV research. On the other hand, modern flow cytometers have an LoD, expressed in terms of the smallest detectable EV diameter, ranging from 91 nm to 190 nm using light scattering (Table 1). With highly specialized instruments it is even feasible to detect 50 nm EVs, albeit at the expense of throughput.<sup>17</sup> Such specialized instruments are unsuitable for the characterization of cells and are therefore not true flow cytometers but rather “flow nanometers.”

### 1.3 | Problems with extracellular vesicles flow cytometry

Most flow cytometers are developed to detect single cells rather than single EVs. Consequently, standardization of EV flow cytometry experiments faces problems caused by (1) the optical properties of EVs, (2) the complexity of EV-containing samples, (3) differences between flow cytometers.<sup>6,13,18-20</sup>

Regarding the optical properties of EVs, EVs produce both dim fluorescence signals and dim light scattering signals. Consequently, signals from EVs are partly at or below the LoD, which hampers the detection of EVs by flow cytometry. Here, the LoD refers to the lowest signal level that can be differentiated from background noise. The light scatter properties of EVs in question are the diameter and refractive index, which together determine how efficiently a particle scatters light. In 2012, Konokhova et al.<sup>21</sup> showed that EVs with a diameter of 500 nm and larger have a lower refractive index than commonly used reference particles of the same diameter, such as polystyrene beads or silica beads. Therefore, EVs scatter light less efficiently than reference particles of the same diameter. Two years

later the low refractive index of EVs was also confirmed for EVs down to 100 nm.<sup>22,23</sup> A low refractive index together with a sub-micrometer size result in dim light scattering signals. Furthermore, the submicrometer size of EVs restricts the available binding places for antibodies and therefore also results in dim fluorescence signals.

With regard to the complexity of EV-containing samples, the first property that comes to mind is the complex relation between the EV concentration and diameter. In 2014, it was shown that the most abundant EVs present in normal human plasma have a diameter of less than 200 nm.<sup>24</sup> Typically, the size distribution of EVs is non-normal, with a peak somewhere below 200 nm and a long tail pointing toward larger diameters.<sup>13</sup> Figure 1C shows an example of a size distribution of EVs. To the right of the peak, the concentration of EVs rapidly decreases with increasing diameter. In other words, there is a high concentration of small EVs and a low concentration of large EVs, but the relationship between concentration and diameter is not normally distributed. Therefore, the LoD of a flow cytometer strongly affects the measured concentration of EVs. Moreover, the size distribution implies that the concentration of EVs right below the LoD exceeds the concentration of EVs above the LoD. At physiological concentrations, EVs below the LoD may be simultaneously present in the (relatively large) illumination volume of a flow cytometer and together generate a detectable signal that is recorded as a “single event.” This special case of coincidence is of course an artifact and was named “swarm detection” in 2012.<sup>18</sup>

The second property regarding the complexity of EV-containing samples is the presence of non-EV particles. For example, plasma often still contains platelets and platelet fragments, whereas EVs in plasma and serum are outnumbered by lipoprotein particles. Due to the low signal intensities of EVs that often overlap with signals generated by non-EV particles present in the sample, buffer, and reagents, non-EVs may be misidentified as EVs.

Differences between flow cytometers affect standardization of EV measurements in two different ways. First, the LoD of a flow cytometer determines which EVs are detected and thereby determines the measured concentration of EVs. Second, the optical configuration of a flow cytometer, such as the illumination wavelength and the light collection angles, affect the relationship between the diameter and refractive index of a particle and the detected light scattering signals. Consequently, standardizing EV measurements by gating EVs based on the light scattering signals of polystyrene beads result in the selection of different size ranges of EVs at different flow cytometers. For example, a 200 nm and 400 nm polystyrene bead gate on forward scattered light selects EVs with a diameter ranging from ~300 nm to ~800 nm on a BD Influx flow cytometer, whereas the same gate on side scattered light selects EVs with a diameter ranging from ~800 nm to ~1900 nm on a BD LSR flow cytometer (based on van der Pol et al.<sup>6</sup>).

### 1.4 | Calibrating flow cytometers

All the aforementioned problems can be solved when fluorescence and light scattering signals measured by flow cytometers

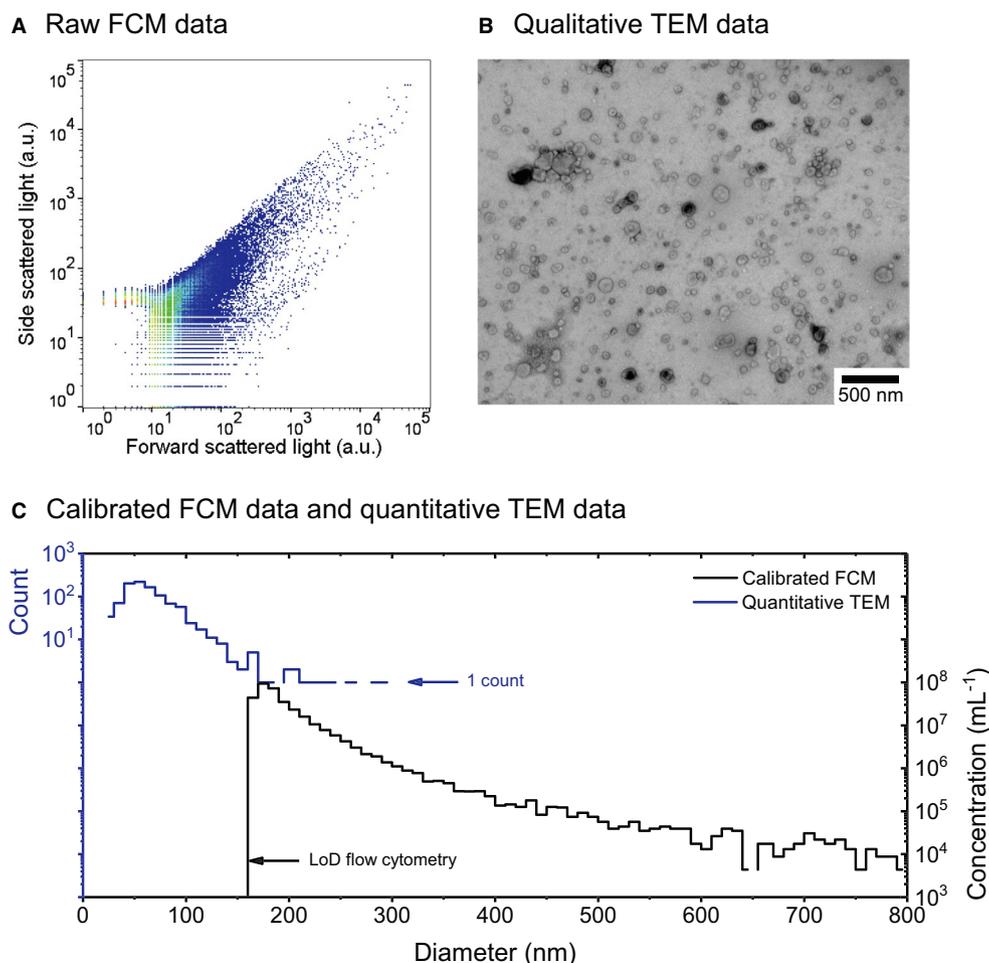
are expressed in standardized, comparable units, and not in arbitrary units. During the last ISTH interlaboratory comparison study, the light scattering signals were calibrated for the first time.<sup>7</sup> This calibration allows a conversion from arbitrary units into the International System of Units (SI) unit of length, thereby enabling comparison of the detected size ranges of EVs between flow cytometers. Procedures to convert the arbitrary units of fluorescence intensity into a standardized unit, such as the molecules of equivalent of soluble fluorochrome (MESF), are also available and improve data comparability,<sup>16</sup> but were not applied yet in interlaboratory comparison studies.

In the performed interlaboratory comparison studies thus far, commercially available reference materials were used that were developed for measuring cells by flow cytometry. At present, however,

novel and dedicated traceable reference materials are developed to calibrate flow cytometers for the detection of EVs.<sup>19</sup> For example, reference materials developed within the 18HLT01 METVES II project ([www.metves.eu](http://www.metves.eu)) will have a known fluorescence intensity, number concentration, refractive index, and size distribution and are therefore expected to allow calibration of the full fluorescence intensity scale, light scattering intensity scale, and sample volume.

## 1.5 | The importance of calibrating

The importance of calibrating to both data interpretation and data comparison is demonstrated by Figure 1. Figure 1A shows the side scattered light versus forward scattered light of EVs from



**FIGURE 1** Calibration of flow cytometry (FCM) data improves data interpretation, enables data comparison, and thereby generates new insights. A, Side scattered light versus forward scattered light of 75 181 extracellular vesicles (EVs) from a 100-fold diluted EV-containing sample, cell-depleted urine, measured with an A50-Micro flow cytometer. The arbitrary units (a.u.) make data interpretation and comparison difficult. B, Qualitative transmission electron microscopy (TEM) image of the same cell-depleted urine, showing EVs that differ in size and morphology. C, Calibrated FCM data and quantitative TEM data of the same cell-depleted urine. The right line and vertical axis (both black) represent the number concentration versus diameter of EVs measured by FCM. The size distribution was obtained by calibrating the forward light scattering signals (Rosetta Calibration, Exometry). The left plot and vertical axis (both blue) represent the count versus diameter of 1000 EVs imaged by TEM. Both vertical axes have a logarithmic scale and the bin width is 10 nm. The flow cytometer has a lower limit of detection (LoD) of 160 nm and therefore does not detect EVs <160 nm. In contrast to FCM, TEM lacks the statistical power to depict EVs >200 nm. Owing to calibrating the FCM data, it becomes evident that under the given experimental conditions, FCM and TEM detect different EVs. FCM and TEM data are obtained from van der Pol et al.<sup>13</sup>

cell-depleted urine, measured with an A50-Micro (Apogee Flow Systems) flow cytometer.<sup>13</sup> Figure 1B shows a transmission electron microscopy (TEM) image of EVs from the same cell-depleted urine. Here, we assume that all measured particles are EVs, but in reality, these samples also contained non-EV particles, such as protein aggregates. However, for clarity we do not make this distinction. It is tempting to believe that the EVs measured by flow cytometry are attributed to the EVs depicted by TEM. However, the flow cytometry data are presented in arbitrary units, thereby making a direct comparison of the data difficult.

To compare data between flow cytometers or between a flow cytometer and another detection technique, such as TEM, calibration is required. Figure 1C therefore shows the calibrated flow cytometry data and quantified TEM data of EVs from the same cell-depleted urine. The left and right axes of Figure 1C differ because TEM measures particles on a surface (i.e., counts) whereas flow cytometry measures particles within a known volume (i.e., concentration). The right line (black) represents the number concentration versus diameter of EVs measured by flow cytometry. The size distribution was obtained by calibrating the forward scattered light signals (Rosetta Calibration, Exometry). This calibration reveals that the flow cytometer detects single EVs of 160 nm and larger. Thus, the LoD in terms of EV diameter is 160 nm, and the concentration of EVs with a diameter >160 nm decreases with increasing diameter above this detection limit.

The left line (blue) in Figure 1C represents the count versus diameter of 1000 EVs imaged by TEM. TEM can clearly detect smaller EVs than flow cytometry. Nevertheless, TEM has two limitations when introduced as a reference method for sizing EVs. First, TEM requires dehydration and fixation, which cause EVs in urine to shrink ~12%.<sup>13</sup> Second, TEM lacks the statistical power to depict EVs >200 nm within a practical time. The throughput of TEM is roughly 2–4 orders of magnitude lower than the throughput of flow cytometry, even when leaving the extensive sample preparation of TEM imaging out of consideration. Owing to calibrating the flow cytometry data, it becomes evident that under the given experimental conditions, flow cytometry and TEM detect EVs of different size.

This finding is particularly important, because the guideline for minimal information for studies of extracellular vesicles 2018 (MISEV2018) states that “EV characterization by multiple, complementary techniques is important to assess the results of separation methods and to establish the likelihood that biomarkers or functions are associated with EVs.”<sup>1</sup> To bring good practice into use, many publications provide TEM images of EVs with the aim to confirm that indeed EVs were detected by flow cytometry. By calibrating an A50-Micro flow cytometer, which belongs to the most sensitive flow cytometers with a throughput >5000 particles per second, and by quantifying TEM data, however, it can be shown that both methods detect EVs within different size ranges. Hence, TEM does not provide the experimental confirmation that a flow cytometer detects EVs. A crucial insight like this can save the field wasting resources and would have been impossible without calibration.

## 1.6 | Why a reporting framework?

A working group with EV flow cytometry experts would ideally publish guidelines for the use of flow cytometry to study EVs. However, it is currently unclear what the optimal procedures are to confirm the detection of EVs, to isolate and stain EVs, and to quantify the LoD. This knowledge gap is partly because publications contain insufficient data to interpret and reproduce EV flow cytometry experiments. As a first step toward the development of reliable procedures and perhaps even guidelines, a reporting framework was published as a consensus document between the flow cytometry experts of the three societies on the minimal information to report about a flow cytometry experiment on EVs.<sup>2</sup>

## 2 | MIFlowCyt-EV REPORTING FRAMEWORK

The main objectives of the MIFlowCyt-EV reporting framework are to (1) improve reproducibility, (2) provide experimental evidence that signals originate from single EVs, and (3) improve standardization. To achieve these goals, the MIFlowCyt-EV reporting framework contains seven components: (1) pre-analytical variables and experimental design according to MISEV guidelines (ISEV), MIFlowCyt guidelines (ISAC), and other relevant guidelines;<sup>1,25-27</sup> (2) sample preparation; (3) assay controls; (4) instrument calibration and data acquisition; (5) EV characterization; (6) data reporting; and (7) data sharing. The MIFlowCyt-EV is expected to improve the quality of publications, because reviewers and readers will be able to independently assess the quality of the experiments, data, and conclusions as well as reproduce experiments (components 1, 2, 6, and 7). Furthermore, the implementation of assay controls should ensure that signals really originate from single EVs and not from artefacts, such as non-EV particles, platelets, reagents, or swarm detection (component 3). Moreover, calibration is recommended to (1) convert the arbitrary units of flow cytometry signals into comparable units, thereby improving data interpretation, and (2) express the measured EV concentrations within known fluorescence and size ranges, thereby improving data comparison (components 4 and 5). The MIFlowCyt-EV reporting framework was published in 2020 in the *Journal of Extracellular Vesicles*, which is the official journal of ISEV.<sup>2</sup> To promote the framework among members of ISAC, in parallel an article was published in 2021 in *Cytometry Part A*, one of the official journals of ISAC.<sup>15</sup> In turn, the goal of this SSC Communication is to promote the MIFlowCyt-EV reporting framework among members of the ISTH.

To circumvent miscommunication, the MIFlowCyt-EV is not a guideline, but a reporting framework. The supplemental material of the MIFlowCyt-EV contains a template with logically ordered questions that should be answered systematically. Although filling out the template takes effort, two additional advantages will be that (1) reviewers have an instrument to judge the quality of the performed experiments in a structured and therefore more efficient way, and (2)

all experimental details are documented, which improves the overall reproducibility of experiments, externally as well as internally. In addition, the questions in the template offer a structure to design robust and reproducible experiments. Targeting flow cytometry experts rather than beginners, however, the MIFlowCyt-EV does not provide the background that beginners need to design a reliable EV flow cytometry experiment. To address the needs of beginners, the EV flow cytometry working group is writing a compendium on single extracellular vesicle flow cytometry, which will be published in 2022.

Step-by-step and probably within the near future, standardization initiatives like EV-TRACK, MIFlowCyt, MIFlowCyt-EV, MISEV will help the field establish the first reference ranges of cell-type-specific EV concentrations in plasma, performing multicenter studies on EVs, and introducing EVs into the clinic routine. Hopefully, in the future we can look back and conclude that EV science is reproducible and EV diagnostics are reliable and clinically useful. With this in mind, we recommend the use of MIFlowCyt-EV and the supplemented template.

### CONFLICTS OF INTEREST

JAW is an inventor on patents and patent applications (National Institutes of Health, Bethesda, Maryland, USA) related to extracellular vesicle assays. EvdP is co-founder and shareholder of the company Exometry BV (Amsterdam, the Netherlands). RN does not have conflicts of interest to declare.

### AUTHOR CONTRIBUTIONS

E. van der Pol designed the experiments, performed the data analysis and interpretation, and wrote the manuscript. J.A. Welsh and R. Nieuwland critically wrote and revised the content. All authors gave their final approval for publication.

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