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# MIFlowCyt-EV: The Next Chapter in the Reporting and Reliability of Single Extracellular Vesicle Flow Cytometry Experiments

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## What Is MIFlowCyt-EV?

Following in the footsteps of the minimum information to report about a flow cytometry experiment (MIFlowCyt), an extracellular vesicle (EV) flow cytometry working group composed of members from the international societies of extracellular vesicles (ISEV), advancement of cytometry (ISAC), and thrombosis and haemostasis (ISTH) developed the recent MIFlowCyt-EV framework position statement, with the aim to improve reporting and reliability of single EV flow cytometry experiments (1,2).

## Why Is MIFlowCyt-EV Important?

MIFlowCyt-EV was developed in response to the growing body of single EV flow cytometry literature that lacks rigor and standardization, thereby leading to irreproducible results. MIFlowCyt-EV builds upon pre-existing frameworks for reporting and best practices in both flow cytometry and EV research; MIFlowCyt and MISEV, respectively (2–4). A need for MIFlowCyt-EV exists because (1) single EV flow cytometry experiments face specific technical challenges, which may lead to artifacts that are not covered by MIFlowCyt, and (2) in contrast to *Cytometry Part A*, which requires manuscripts to be supplemented with a MIFlowCyt template, journals in the field of EVs lack a formal requirement tailored to EV flow cytometry. To emphasize the need of MIFlowCyt-EV, Table 1 shows differences between the technical challenges and opportunities involved in EV and cell detection. This is mainly because EVs have signal intensities just below and above the detection limit, signal intensities overlap with buffer contaminants and unstained reagents, swarm detection may occur, and sensitivity differences strongly affect measured EV concentrations. On the other hand, the morphological properties of EVs offer new flow cytometric opportunities, such as particle sizing and refractive index estimation (5–8).

**Table 1.** Differences between the technical challenges and opportunities involved in EV and cell detection

| EXTRACELLULAR VESICLES (EVS) | CELLS |
|---|---|
| EVs <500 nm are spherical core-shell particles, allowing diameter and refractive index estimation | Cells have organelles and have varying shapes, complicating diameter, and refractive index estimation |
| Signals below and above detection limit | Signals exceed detection limit |
| Signal calibration is essential: sensitivity differences strongly affect measured EV concentrations | Signal calibration is optional: sensitivity differences weakly affect measured cell concentrations |
| Signal intensities overlap with buffer contaminants or unstained reagents | Signal intensities exceed buffer contaminants or unstained reagents |
| Swarm detection | Coincidence detection |

Coincidence detection: Simultaneous illumination and detection of two or a few particles. Swarm detection: Special case of coincidence detection, where instead of two or a few particles, multiple (tenths to hundreds) particles at or below the limit of detection are simultaneously and continuously illuminated and erroneously measured as single event.

As the field has progressed, essential controls have been identified to ensure (1) the detection of single EVs, and (2) providing reassurance that the detected events are EVs and not artifacts, such as free fluorescent labels. In line with the controls, calibration methods have been identified that allow for data to be reported in comparable, standardized units with a quantifiable limit of detection, which allows validation and comparison of data on flow cytometers that differ in sensitivity. Despite the identification and acknowledged importance of essential controls and calibrations, these currently lack widespread adoption by the scientific community, as evidenced by the absence of utilization in the published literature. Hence, now it is time to disseminate the solutions to these EV specific technical challenges in the form of a field standard: MIFlowCyt-EV.

## What Are the Contents and Objectives of MIFlowCyt-EV?

The MIFlowCyt-EV reporting framework is composed of seven main components which include: (1) preanalytical variables and experimental design, (2) sample preparation, (3) assay controls, (4) instrument calibration and data acquisition, (5) EV characterization, (6) flow cytometry (FC) data reporting, and (7) FC data sharing. Together, these components ensure measurement of single EVs, data reproducibility, and the facilitation of data interpretation. Each component of the reporting framework aids in different areas of increasing reported data integrity. Components 1 and 2 provide the necessary details to repeat a reported assay. Component 3 provides evidence that single EVs of interest are being detected, and not labeling/purification artifacts or coincidence events. Components 4 and 5 allow for validation of assays irrespective of differences in instrument settings and/or limits of detection. Finally, Components 6 and 7 provide evidence of how the data was analyzed proof of raw data quality. Whereas the framework has a focus on EVs, it is also applicable to and useful for flow cytometry experiments on submicrometer particles other than EVs, such as viruses.

## What Is the Impact of MIFlowCyt-EV Being Used in the Literature?

First, the completion of the MIFlowCyt-EV components will aid the peer-review process by allowing reviewers and readers to have access to essential information about the conducted experiment to assess the quality of data and validity of findings. Second, the field will get insight into the actual concentration of EVs in biofluids within reported detection ranges. Because hitherto detection ranges are unreported, the concentration of EVs in biofluids is virtually unknown. Third, the framework raises awareness about the essential criteria to consider when designing and performing a single EV flow cytometry experiment to researchers who may be new to single EV flow cytometry.

## Steps toward Adoption of MIFlowCyt-EV Framework

The MIFlowCyt-EV framework was established to address key issues identified in the field of EV research, specifically pertaining to the quality of flow cytometry data. For this framework to have the intended impact on improving transparency and data quality in literature, ubiquitous adoption and integration into the research workflow is required. Figure 1 shows a diagram containing eight steps toward adoption of the MIFlowCyt-EV framework. *Step 1 and 2*, which involve the identification of key issues and the establishment and publication of the framework, have been carried out. Although this framework has been endorsed and acknowledged by international societies and key opinion leaders alike in flow cytometry and EV research fields (Fig. 1, Step 3), a clear understanding of the rationale and methods behind each component of the framework is required (Fig. 1, Step 4) to achieve widespread adoption. To educate the field, several recent initiatives led by the ISEV-ISAC-ISTH EV FC Working Group have contributed to a growing body of online educational resources (www.evflowcytometry.org). Software tools to facilitate data calibration are available both commercially and free-of-charge online with resources and tutorials on how to use these tools (5,9,10). A concerted effort has also been made by the ISEV, ISAC, ISTH communities to dedicate time for disseminating and discussing developments within the EV flow cytometry community at international meetings and workshops for several years, which will continue to aid understanding and uptake of best practices (11).

We envision that the next step toward adoption of the MIFlowCyt-EV framework is the involvement of shared resource labs (SRLs). SRLs can substantially contribute to the

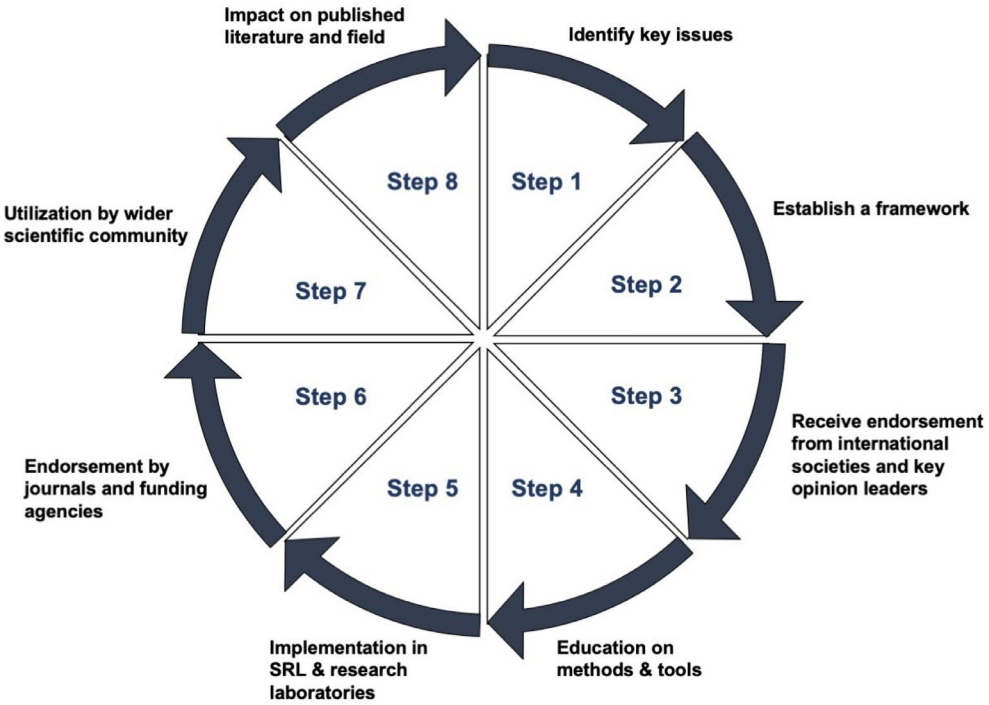


**Fig 1.** Strategy consisting of eight steps to realize adoption of the MIFlowCyt-EV framework. SRL, shared resource laboratory.

effort to standardize EV measurements, as SRLs are a source of both education and instrument expertise in the research setting (Fig. 1, Step 5). SRLs play a crucial role in instrument maintenance, calibration, and educating users on instrument best practices, such as instrument settings, calibration methods, experiment controls, FC data sharing, and reporting. For example, calibrating light-scatter, which is not required to be performed on an individual experiment basis, can potentially be a service performed by the SRL whenever instrument alignments have been adjusted. Components of the MIFlowCyt-EV framework, such as sample preparation and assay controls, instrument calibration, and data reporting can also be incorporated into user training for EV flow cytometry. As more EV researchers turn to flow cytometry for the analysis of EVs, the onus will be on flow cytometry core facilities to adequately support this application. The MIFlowCyt-EV framework provides an ideal reference for core facilities to provide these services and on the other hand, SRLs have the role to support and promote best practices; making them ideal candidates to disseminate the MIFlowCyt-EV framework for adoption in the wider scientific community.

The implementation of MIFlowCyt-EV will enable clearer interpretation and increased confidence in the reported single EV flow cytometry literature. However, to reach the goal of increasing transparency and confidence in the published literature (Fig. 1, Step 7–8), widespread adoption and implementation is required by academia, industry, and commercial vendors. Similar to the requisite by *Cytometry Part A* with compliance to MIFlowCyt guidelines for publication, endorsement for MIFlowCyt-EV by major journals and funding agencies for manuscript and grant submissions containing single-EV flow cytometry data would be crucial for the success of this initiative (Fig. 1, Step 6). We further believe that industry should take responsibility and participate in this effort, for example, by incorporating calibration procedures that lead to data in comparable units into instrument data acquisition and analysis software. Moreover, additional investment in research and development of reference materials resembling EV properties as well as calibration materials would significantly enhance standardization efforts.

In summary, to improve reporting and reliability of flow cytometry experiments on EVs, we have developed the MIFlowCyt-EV framework and a strategy to disseminate and adopt the framework. While no gold standard exists for single EV flow cytometry assays, the field is at a stage where methods have been identified that enable confirmation of single EV detection and calibration of results across instruments. Improvements to existing procedures and expansion of current knowledge about EVs in biofluids can only be achieved with proper reporting. We believe the endorsement of MIFlowCyt-EV by *Cytometry Part A*, like MIFlowCyt for cellular analysis, is a big step forward for the small particle field and will aid in the development of optimal instrumentation, settings, staining assays, and future standards. This progress can only be made if it is built upon transparent, reproducible data, for which the MIFlowCyt-EV framework will aid researchers to identify.

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## Conflict of Interest

EvdP is shareholder of the company Exometry B.V., Amsterdam, The Netherlands.

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