Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop

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Running Head : Standardization of MP counts by flow cytometry

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Essentials

- The clinical enumeration of microparticles (MPs) is hampered by a lack of standardization.
- A new strategy to standardize MP counts by flow cytometry was evaluated in a multicenter study.
- No difference was found between instruments using forward or side scatter as the trigger parameter.
- This study demonstrated that beads can be used as a standardization tool for MPs.

Summary

Background: Microparticles are extracellular vesicles resulting from the budding of cellular membranes that have a high potential as emergent biomarkers; however, their clinical relevance is hampered by methodological enumeration concerns and a lack of standardization. Flow cytometry (FCM) remains the most commonly used technique with the best capability to determine the cellular origin of single MPs. However, instruments behave variably depending on which scatter parameter, (Forward (FSC) or Side scatter (SSC)), provides the best resolution to discriminate submicron particles. To overcome this problem, a new approach, based on two sets of selected beads adapted to FSC or SSC optimized instruments, was recently proposed to reproducibly enumerate platelet-derived MP counts among instruments with different optical systems. **Objective**: The objective was to evaluate this strategy in an international workshop that included 44 laboratories accounting for 52 cytometers of 14 types. **Methods/Results:** Using resolution capability and background noise level as criteria to qualify the instruments, the standardization strategy proved to be compatible with 85% (44/52) instruments. All instruments correctly ranked the PMP levels of two platelet-free plasma samples. The inter-laboratory variability of PMP counts was 37% and 28% for each sample. No difference was found between instruments using forward or side scattered light as the relative sizing parameter. **Conclusions:** Despite remaining limitations, this study is the first to demonstrate a real potential of bead-based strategies for standardization of MP enumeration across different FCM platforms. Additional standardization efforts are still mandatory to evaluate MP clinical relevance at a multicenter level.

Keywords

Cell-derived microparticles, extracellular vesicles, flow cytometry, multicenter study, standardization.

Introduction

Among extracellular vesicles, microparticles (MPs) are sub-micron sized vesicles released by blebbing from cell membranes in response to activation or apoptosis. MPs originate from blood and vascular cells, and plasma levels are elevated in a variety of prothrombotic and inflammatory disorders, cardiovascular diseases, autoimmune disorders, infectious diseases, and malignancies [1].

Although MP counts may provide useful diagnostic/prognostic information, assessment of their pathophysiological relevance in multicenter studies is hampered by methodological concerns and a lack of standardization. Among the various methodologies available to measure MPs in biological samples, flow cytometry (FCM) remains the most commonly used technique with the highest potential to determine the cellular origin of single MPs [2]. Over the past few years, significant improvements have been made in the sensitivity of flow cytometers to detect vesicles of smaller size, and thus have confirmed this methodology as the most promising for routine enumeration of MP subsets [3-5].

Six years ago, a first collaborative workshop defined the inter-laboratory reproducibility of platelet MP (PMP) counts using FCM [6]. The standardization strategy was based on the forward light scatter (FSC) signal of size-calibrated latex beads to set a common MP window of analysis [7]. However, the variety of optical designs among flow cytometer (FCMr) sub-types impeded a universal standardization strategy for PMP enumeration. Since a better resolution and a more homogeneous response of instruments was observed in a subgroup of FCMrs using the light scatter signal measured at 90° (Side scatter, SSC) rather than FSC, a new set of beads was selected to better suit the design of these SSC-oriented instruments [8]. Correspondence between the two sets of beads was accurately determined so that similar PMP counts were obtained on both types of FCMrs. Thus, a new standardization strategy is proposed based on the use of two types of beads, each adapted to instruments of

different optical design. Based on this strategy, the International Society on Thrombosis and Haemostasis (ISTH) Vascular Biology Standardization Subcommittee organized an additional workshop to evaluate the inter-instrument reproducibility of PMP counts among different platforms.

Materials and methods

Study design

The study was conducted in two stages over a two-year period. The first stage was aimed at qualifying the instruments for the standardization strategy according to required performance levels of scatter resolution and background noise. This step led to acceptance or rejection of the tested instrument(s). In the second stage, the inter-instrument reproducibility of 3 different platelet free plasma (PFP) samples, prepared by the core laboratory and featuring defined levels of PMP subsets, was evaluated using common reagents and the standardized protocol.

Cytometers

The study included 44 laboratories from 17 different countries, accounting for 52 registered cytometers. The tested instruments included 11 FACSCanto (I/II), 6 FACSCalibur, 2 FACSVerse, 5 FACSAria (I/II), 4 LSRII, 3 LSR Fortessa, 1 Influx and 2 Accuri C6 from Becton-Dickinson (BD, Franklin lakes, NJ, US), 1 EPICS XL, 2 FC500 and 12 Gallios/Navios from Beckman-Coulter (BC, Miami, FL, US), 1 Apogee A50 micro (Apogee System, Hertfordshire, UK), 1 Guava EasyCyte (Millipore, Hayward, CA, US) and 1 Stratedigm S1000 EXi (Stratedigm, San Jose, CA, US).

Standardization beads

Megamix-Plus FSC or SSC beads were provided by BioCytex (Marseille, France) to the core-lab that distributed them to participants according to their instrument's characteristics. Megamix-Plus SSC is a ready-to-use mix of fluorescent polystyrene beads of various diameters (0.16 µm, 0.20 µm, 0.24 µm and 0.5 µm) dedicated to flow cytometers using SSC as the best resolving size-related parameter. Megamix-Plus FSC is a mix of fluorescent polystyrene beads of various diameters (0.1 µm, 0.3 µm, 0.5 µm and 0.9 µm) dedicated to FCMrs using FSC as the best resolving size-related parameter. The intrinsic numerical ratio of 2:1 from the 0.3 µm to 0.5 µm beads facilitates fine-tuning of the FSC threshold [3]. According to the instrument characteristics, standardization beads were tested as follows: 1) Megamix-Plus FSC: Gallios, Navios, FC500, Epics XL and Guava. 2) Megamix-Plus SSC: FACSAria (I/II), LSR II (+/- Fortessa), FACSCanto (I/II), FACSVerse, FACSCalibur, Accuri C6 and Megamix-Plus FSC and Megamix- Plus SSC: Influx, Apogee A50 and Stratedigm.

Flow cytometry reagents

The common flow cytometry reagents for PMP staining were annexinV-FITC (fluorescein) (Tau Technologies, Kattendijke, Netherlands) and its associated binding buffer, CD41-PE (phycoerythrin; clone PL2-49) and its concentration-matched isotype control IgG1-PE (clone 2DNP-2H11/2H12), both from BioCytex. Counting beads (3 µm, MP-Count beads, prototype version) were from BioCytex.

Platelet-free plasma preparation

All Platelet-free plasma (PFP) were prepared at the core laboratory. Briefly, blood from healthy donors, who signed an informed consent, was collected with a 21-gauge needle in 0.129 M citrated tubes after discarding the first 2 ml. PFP was prepared according to a

published protocol using two successive centrifugations, each of 15 minutes at 2,500g [9, 10] with the following modifications: Sample A was prepared after agitation of the blood tubes at room temperature on a rotating wheel for 2 hours. Sample C was prepared after a 2 hour delay without agitation. Samples A and C were prepared from a pool of 10 donors, whereas sample B was from a unique donor. Aliquots (200 µl) of PFP were stored at -80ºC until use (less than 6 months). Inter-aliquot variability of PFP samples was measured on a single instrument (Gallios) by the core-lab over a 2 month period, yielding values with CVs of 14%, 8% and 24% (n=18) for samples A, B and C, respectively. The variability of a triplicate measurement of one aliquot was also found to be acceptable, resulting in CVs of 10%, 8% and 16% (n=6). Given the high variability of PMP counts on sample C, results with this sample were retrospectively excluded from the study. The preparation of this sample as a mixture of plasma from different blood groups may have generated MP aggregates with an impact on MP count reproducibility.

Instrument qualification

Instrument qualification to enable the proposed strategy was based on 2 criteria [8]. First, a sufficient resolution was required to resolve small beads whose size depends on the selected scatter parameter (0.3µm and 0.5 µm for FSC and 0.16 µm and 0.2 µm for SSC). This was attested by a scatter sensitivity index > 3 (SSI = (Median bead A- Median bead B)/ (SD bead A+ SD bead B) [11]. The second criterion was based on a background noise ratio (BNR) which was defined as the ratio between the number of events per second measured in the protocol settings and the maximal number of events per second acceptable by the instrument without significant abort rate (FACSCanto I/II = 4000, FACSCalibur = 2000, FACSAria I/II = 4000, LSRII (+/- Fortessa) = 4000, FACSVerse = 4000, Apogee A50 = 2000, Stratedigm = 4000, Gallios/Navios = 5000, Influx = 15,000, defined according to both instrument

specifications and core lab validation). BNR was evaluated on filtered distilled water and should be lower than one in order to avoid impeding the instrument's electronic system.

Protocol setting

The standardization protocols were set according to the manufacturer's instructions for SSC and FSC Megamix beads. For FSC-optimized instruments, the MP analysis region was defined as follows: 1. the upper boundary was determined by the edge of the 0.9 µm bead cloud, and 2. the lower boundary was defined by the threshold on FSC that allowed inclusion of 50% of the 0.3 µm beads in the analysis. A range of 48% to 52% was considered acceptable [3]. For SSC-optimized instrument, the upper boundary of the MP analysis region was determined by the end of the 0.5 µm bead peak (e.g. 99th percentile). The lower boundary was set according to the product insert following the formula: Low SSC-H level = Median 0.16 µm beads + (0.3 x (Median 0.20 µm beads – Median 0.16 µm beads)) [8]. The MP protocol settings were optimized as follows: a) Scatter settings were optimized recording PEAK (= HEIGHT) signals. b) Low flow rate was selected and acquisition time was optimized according to the MP count beads (60 s when the total number of MP Count beads in 1 minute ranged from 500 to 2,000 or 120 s if MP Count beads were < 500). c) Fluorescence settings were optimized by setting FL1 and FL2 PMT voltages to reach pre-defined target values (median intensities) for single fluorescence positive beads ("Fluo-Setting-Beads" (FSB), designed by BioCytex for this exercise). Briefly, blank beads as well as high intensity FITC-labelled and PE-labelled beads were mixed extemporaneously, and staining reagents (AnnV-FITC + CD41-PE) were added at the same final concentrations as in plasma samples, thus providing a comparable level of non-specific fluorescence background. d) Compensation settings were set up using single fluorescence labelling of PFP samples. e) Positive and negative region boundaries were defined using concentration-matched isotype

control and AnnV-FITC in filtered PBS without calcium so that <0.1% of events were included in the positive gates. Detailed instructions for optimization of the MP protocol setting can be found in supplemental document 1.

PMP counting experiments

Three PMP counting experiments were performed for each PFP sample operated in independent series. Before running each series of samples, standardized scatter settings were checked with Megamix-Plus and fluorescence target channels assessed with Fluo-SettingBeads. 30µl of PFP were incubated for 20 minutes with 10 µl of AnnV-FITC and 5 µl of CD41-PE, and then diluted in 1 mL of Binding Buffer. A negative control was performed for each PFP, by incubating 30µl of PFP with 10µl of AnnV-FITC and 5 µl of IgG1-PE, and diluting the sample in PBS without calcium. In order to derive absolute PMP counts per µL of plasma, 30µl of counting beads (MP-Count beads) were added before running the samples. PMP concentration in plasma was calculated according to the formula: events/µL = Double positive events x Counting bead concentration/ Number of Counting Beads. Non-specific events/µL in the control tube were subtracted from the PMP counts.

File transfer and re-analysis

All electronic raw data (listmode) files corresponding to instrument qualification, protocol setting and PFP analysis were sent to the core laboratory in fcs (flow cytometry standard format) 2.0 or fcs 3.0. Files were re-analyzed by the core-lab using the same software (Kaluza v1.2 software, Beckman Coulter). In the event of irreversible discrepancies with the protocol instructions, data were not accepted for final analysis.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software v.5.0 (GraphPad Software, San Diego, CA). Each PFP was analyzed in triplicate and the mean of this triplicate (xi) was considered for further analysis. The robust mean (X*) and robust standard deviation (SD*) of these data were calculated, taking into account only the results from cytometers with values between median +/- SD [12]. The Mann-Whitney test was used to compare instrument families. A result was considered significant if $p<0.05$.

Results

Instrument qualification

Instruments were qualified for the standardization strategy according to their resolution capability and low background noise. As illustrated in table 1, with the exception of two Accuri C6 and one FACSAria, most instruments using exclusively the SSC strategy showed an SSI > 3, indicating that the resolution was sufficient to enable the proposed strategy. Among these instruments, LSRII (+/- LSRII Fortessa) showed the best resolution (SSI = 7.9 +/- 1.3, n=7). All SSC-FCMrs (except a FACSCalibur) showed a background noise that was acceptable in the standardization protocol settings. Overall, combining both criteria, 87% of the instruments that used SSC as the preferred parameter were found to be qualified. Regarding instruments that used FSC, only the last generation of FCMr (Gallios/Navios, Stratedigm and BD-Influx) showed a SSI >3 (table 1). Among these instruments, BD influx and Apogee A50 showed the best resolution (SSI = 11.3, n=2). Regarding background noise, five (of twelve) Gallios/Navios showed a BNR > 1. This noise proved to be of optical origin, and was reversible by externally cleaning the flow cell from dust deposits. Therefore, these instruments were incorporated in the second stage of the study. The qualification step resulted

in a 75% qualification rate for FSC-optimized instruments. Finally, the standardization strategy proved to be compatible with 44/52 instruments (85%).

Inter-instrument variability

In the second stage of the protocol, participating labs with qualified instruments enumerated PMPs on 3 PFP samples prepared by the core laboratory. Because of the one year delay between the two stages of the workshop, it was required to check SSI and BNR of the instruments again before analyzing the samples. The qualification criteria were same as in first stage. As a result, with the exception of two Navios with significant background noise, all instruments re-qualified. The standardized protocol was set up optimizing the scatter settings, flow rate, fluorescence and compensation settings, and region boundaries as detailed in Methods. After analysis of the FCM raw data files by the core- lab, data from 3 instruments were rejected due to irreversible discrepancies with the protocol instructions. Also, plasma sample C was excluded from analysis due to its inherent heterogeneity leading to high PMP count CVs at the core-lab. Each PFP was analyzed in triplicate. The mean CV for each triplicate of the validated PMP counts were 15% and 12% for samples A and B, respectively. Individual results showing a triplicate CV > 50% -- suggestive of a manipulator-dependent bias -- were not considered valid (3 instruments for sample A and no instrument for sample B). Finally, among the 32 results received by the core lab for samples A and B, 81% and 91% were considered valid, respectively.

As shown in figure 1, all instruments with validated results for the two samples (n=26) correctly discriminated the two PMP levels. The inter-instrument variability of the ratio between sample A and B was 30.2% and was not significantly different between the instruments using SSC or FSC as the preferred scatter parameter (35.1 +/- 4.9 *vs.* 39.3 +/- 14.3, respectively, p = 0.7). As illustrated in figure 2A for sample A, 58% of instruments

provided comparable PMP counts within a restricted range of values (robust mean +/- robust SD). This result was better with sample B (69%, figure 2B). 15/26 instruments (58%) gave results within the robust mean+/- robust SD range. However, some individual instruments (LSR II Fortessa, Apogee A50) were systematically outside the robust mean +/- robust SD range for both samples. In the specific case of Apogee A50, the discordance with expected values was clearly due to an inappropriate choice of the set of beads (FSC instead of SSC). Finally, the inter-laboratory variability of PMP counts was 37% and 28% for samples A and B, respectively (figure 2C) with mean and 95% confidence interval at 8490 [7190-9790] PMP/µl and 3075 [2745-3400] PMP/µl for samples A and B, respectively. Interestingly, we found no significant difference in PMP counts between instruments using SSC or FSC as the preferred trigger (sample A: 8900 +/- 4000 PMPs/µl *vs.* 8000 +/- 2000 PMPs/µl, p = 0.8; sample B: 3100 +/- 980 PMPs/µl *vs.* 2800 +/- 550 PMP/µl, p = 0.5). However, the inter-instrument variability was higher for SSC instruments compared to FSC-oriented instruments (sample A: 46% *vs.* 25%; sample B: 31% *vs.* 19%) probably due to a greater diversity of tested models.

Discussion

This study is the first to demonstrate that standardization is possible for MP enumeration by flow cytometry. We also demonstrated that size-calibrated polystyrene beads can be used as a standardization tool for MP enumeration, provided that instrument intrinsic behaviors for size-related measurements have been taken into account. Bead-based strategies have been criticized because the relationship between bead and MP sizes is not obvious and highly depends on the size-related scatter parameter used and on the refractive index [13-16]. Therefore, the beads should not be used as calibrators to derive absolute size values for MPs. Other standards with refractive indices closer to those of MPs may be a better alternative. However, such a standardization strategy awaits similar multi-center validation. Moreover,

whereas we focused in this study on the use of scatter for triggering MP analysis, several other groups focus on fluorescence as a preferred threshold [4, 17-20]; however, thresholding on fluorescence currently encounters several practical limitations. Although generic labels have been proposed, e.g. lipophilic fluorescent labels such as PKH dyes, the labeling procedure of MPs in complex body fluids such as plasma is hardly applicable, necessitating protocols that use specialized lab equipment to get rid of free dye and prevent measurement of artifacts. Indeed, non-specific fluorescent background due to the staining of lipoprotein particles present in plasma added to the variability in fluorescence sensitivity among instruments, remain two major limitations to define any clear-cut, reproducible, fluorescent threshold level that could be generally applied. Most probably, both fluorescence and scatter triggering strategies will have to be combined.

In contrast to the previous ISTH standardization study [6], the proposed bead-based strategy is now applicable on most commercially available instruments. No significant variability was observed between instrument families measuring PMPs with different optical systems. These results open the way for multicenter studies comparing MP counts in clinical samples. Although only PMP were measured in this workshop, it can be anticipated that the same strategy could be extended to other clinically-relevant MP subsets. However, this standardization strategy displays several limitations: 1) It still addresses only a small fraction of MPs, a large part being below the detection limit of instruments; 2) Homogeneous re-treatment of raw data by the core laboratory was still required. Thus specific training is still needed for data treatment; 3) It was mainly focused on harmonizing the scatter-based MP gates. Although the conditions of fluorescence detection were tentatively harmonized in this study using specifically designed Fluo-Setting-Beads to be set in similar target channels, the complete standardization of fluorescence measurements would require more sophisticated

approaches [21]; 4) The strategy has to be challenged on future instruments with different optical design.

Despite having still significant limitations, this study is the first to demonstrate a real potential for standardization of MP enumeration across different FCM platforms. Additional standardization efforts are mandatory to allow the evaluation of the clinical relevance of MP counts at a multicenter level, and should accompany the continuous improvement in the sensitivity of instruments to detect progressively smaller MPs.

Addendum: Authors' contribution

S. Cointe, C. Judicone, and S. Robert performed the research, collected the data and analyzed and interpreted the data. M. Mooberry, and P. Poncelet designed the research and reviewed the manuscript. M. Wauben and R. Nieuwland reviewed the manuscript, N. S. Key and F. Dignat-George supervised the work and reviewed the manuscript and R. Lacroix designed the research, supervised the work, analyzed and interpreted the data and wrote the manuscript.

Appendix: [x]ISTH SSC Workshop

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Disclosure of conflict of interests

C. Judicone and P. Poncelet are full-time employees of BioCytex.

For the workshop, BioCytex provided Megamix beads, and non commercial reagents including counting and fluo setting beads. Separate from this study; F. Dignat-George and R Lacroix declare a collaboration contract with Stago (on fibrinolytic microparticles: licensed patent).

Figure legends

Figure 1: Inter-instrument variability of the PMP ratio between samples.

PMP counts of sample A was fixed at 100% and counts in sample B was displayed as a percentage of sample A for both groups of instruments, using side scatter (SSC) or forward scatter (FSC) as preferred parameter to define the MP gate of analysis.

Figure 2: Inter-instrument variability of PMP counts.

A and B: Platelet-derived microparticle (PMP) counts determined as events/µl in sample A (A) or sample B (B) by each qualified flow cytometer using either side scatter (SSC) or forward scatter (FSC) as the preferred parameter. The grey area is defined by the robust mean (X*) +/- the robust standard deviation (SD*). X* and SD* were calculated taking into account only results from cytometers with values between the median +/- SD. **C:** Inter-instrument variability (CV) of PMP counts. $p < 0.05$ was considered significant.

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Table

	Instrument type	SSI mean [min-max]	BNR mean [min-max]	Qualified instruments
SSC instruments	Accuri C6	0	ND	0/2
	Apogee A50[a]	7.7	0.03	1/1
	FACSAria	5.2 [2.8-7.1]	0.07 [0-0.22]	4/5
	FACSCalibur	4.6 [3-6.5]	0.58 [0-2.59]	5/6
	FACSCanto	4.5 [3.4-7]	0.07 [0.01-0.2]	11/11
	FACSVerse	7.1 [7-7.3]	0.12 [0.06-0.19]	2/2
	Influx[a]	2.4	0.43	0/1
	LSR Fortessa	8 [7.2-9.1]	0.02 [0.02-0.05]	3/3

	LSR II	7.9 [5.7-10]	0.02 [0-0.03]	4/4
	Stratedigm[a]	4.3	0.02	1/1
FSC instruments	Apogee A50[a]	11.3	0	1/1
	Epics XL	0	ND	0/1
	FC500	0	ND	0/2
	Guava	0	ND	0/2
	Influx[a]	11.3	0.01	1/1
	Navios/Gallios	5.6 [2.8-7.6]	1.53 [0-6.2] (0.03)[b]	7/12 (12/12)[b]
	Stratedigm[a]	4	1	1/1
Total				39/52 (44/52)[b]

[a] Instruments tested both in SSC and FSC; [b] Results after flow cell wash; Sensitivity index (SSI) = (Median bead A- Median bead B)/ (SD bead A+ SD bead B) where bead A = 0.2µm and bead B = 0.16µm for SSC FCMrs, and bead A = 0.5 µm and bead B = 0.3 µm for FSC FCMrs. SSI > 3 was required to be compatible with the standardization strategy. Background noise ratio (BNR) is the ratio between the number of events per second measured in the protocol settings and the instrument specific maximal number of events per second keeping abort rate at a low level (FACSCanto I/II = 4000, FACSCalibur = 2000, FACSAria I/II = 4000, LSRII (+/-Fortessa) = 4000, FACSVerse = 4000, Apogee A50 = 2000, Stratedigm = 4000, Gallios/Navios = 5000, Influx = 15 000, defined according to both instrument specifications and core lab validation).

Table 1: Instrument qualification according to resolution and background noise.