329 Abstract

Background: Microparticles (MP) are submicron vesicles released from cell membranes. Their reliable measurement in plasma is needed due to recognized patho-physiological roles in thrombosis, inflammation and cancer. Their low size range (0.1 – 1µm) creates a difficult challenge for detection, sizing and counting by flow cytometry.1.A standardized protocol for PMP counts in the limited size range of 0.5 to 1µm2 had been developed on Beckman-Coulter (BC) FC500 cytometers (FCMrs).Recent studies3 showed that this strategy, optimized on BC FCMrs measuring Forward Scatter (FS/FSC) at high solid angle (1-19°), did not totally fit with FCMrs using low angle (1-8°) collection, including Becton-Dickinson (BD) FACS and LSR.Our study looked for alternative MP settings more adapted to BD FCMrs. **Methods:** Platelet-free-plasma samples frozen in multiple aliquots were used as common sources of platelet MP (PMP), defined via dual staining with CD41-PE and Annexin V-FITC. BD FCMrs: 3 FACS-Canto II, 1 LSRII and 1 LSRII-Fortessa equipped with a PMT for FSC. BC FCMrs: 4 FC500 and 6 Gallios. All passed our stringent QLC, based on the resolution between Megamix 0.5 and 0.9µm beads2. The position of PMP in dual-scatter plots was compared to that of calibrated beads on each machine. To extend the scope of analysis, Megamix was enriched with smaller, 0.3 and 0.1µm, fluorescent beads. **Results:** Using FS/FSC, Gallios and Fortessa clearly differentiated 0.3µm beads from the 0.5µm subset present in our Megamix-Plus prototype but not fully 0.1µm from 0.3µm beads. Other BD FCMrs only discriminated 0.5µm from 0.9µm beads, as did FC500.Using side scatter (SSC) however, a clear discrimination was obtained between all bead subsets (even 0.1µm to 0.3µm beads) in all BD FCMrs. Added to a better rejection of background, this suggested SSC might be preferable to FSC in BD FCMrs. However, since in FCM the SSC intensity of beads is always higher than that of similarly-sized cells, the relative positions of PMP in both FSC and SSC were scrutinized. Gallios and Fortessa discriminated 2 subsets among PMP both in terms of fluorescence intensities and dual scatter. "Large PMP" were located, as in FC500, between the median FS of 0.5µm beads and the end of 0.9µm peak (so-called 0.5-1µm "MP gate") and the "small PMP" spanned below the 0.5µm beads, down-to the selected threshold value. In SSC, the "large PMP" were located on all FCMrs below the SSC level of 0.5µm beads down-to a lower level between 0.1 and 0.3µm beads. **Conclusion:** Using SSC as the preferred triggering signal for MP analysis on BD FCMrs required a threshold level far below the SSC of 0.3µm beads to avoid accidental loss of PMP. Based on these observations, optimally sized fluorescent beads will be selected to provide an appropriate standardized cut-off reference material tailored to the technical specifications of routinely used BD FCMrs. Ref.1) Lacroix R, Semin Thromb Hemost 2010, 2) Robert S., JTH 2009, 3) Lacroix R, JTH 2010.

Optimization of FCM-based microparticle analysis protocols for cytometers using small-angle forward scatter for size measurements.

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Background:

Microparticles (MP) are submicron vesicles released from cell membranes. Their reliable measurement in plasma is needed due to recognized patho-physiological roles in thrombosis, inflammation and cancer. Their low size range (0.1 – 1µm) creates a difficult challenge for detection, sizing and counting by flow cytometry (1).A standardized protocol for PMP counts in the limited size range of 0.5 to 1µm (2) had been developed on Beckman-Coulter (BC) FC500 cytometers (FCMrs), using submicron beads (Megamix, BioCytex, Marseille, F).

The MP gate was thus reproducibly set-up between:

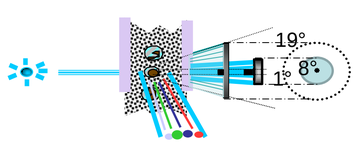
• 0.5µm beads-equivalent (eq.) in FS, as the lower limit defined by FS threshold,

• 1µm beads-eq. in FS, as the upper limit defined by the end of 0.9µm beads

Recent studies (3) showed that this strategy, optimized on BC FCMrs measuring Forward Scatter (FS/FSC) at high solid angle (1-19°), did not totally fit with FCMrs using low angle (1-8°) collection, including Becton-Dickinson (BD) FACS and LSR. Indeed, the following points were observed.

Forward scatter collection:

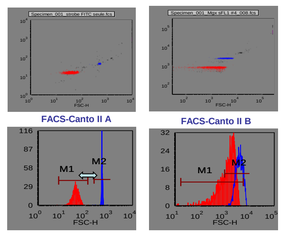
High solid angle (1-19°) may be more adapted to study submicron particles with FS/FSC.



Scatter resolution:

FSC resolution is very heterogeneous from one BD instrument to the other, with frequent cases of inadequate separation of Megamix 0.5 µm from 0.9µm beads.

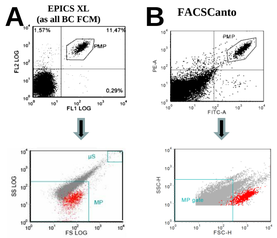
In contrast, side scatter (**SSC**) is generally consistent among BD instruments and Megamix submicron beads always fully discriminated.



Scatter threshold:

In BD instruments, threshold on SSC (alternatively combined on SSC & FSC) is mandatory to get rid of background noise as opposed to FS alone on BC XL, FC500 or Gallios.

➔ how to best set-up SSC threshold on BD instruments without loosing MP ?



Scatter position of MP:

In BD instruments with ad hoc FSC resolution, dual positive PMP (AnnV+/CD41+) may be variably positionned in FSC, anywhere from inside, fully or partly outside the MP gate (illustrated in B), in contrast to consistent position in BC FCMrs (Cf. A, adapted from ISTH international workshop (3)).

Aim: Our study looked for alternative MP settings more adapted to BD FCMrs.

Question: Can we use SSC parameter (rather than FSC) as the prime size-related parameter for standardization of MP analysis on platforms using low angle FSC detection ?

Methods:

Multi-instrument comparison includes BC Gallios (left), FACS-CANTO II (middle left), LSRII (middle right and far right) and LSR-FORTESSA (right) with FSC detection by photodiode or PMT.
Beads: Prototype version of Megamix enriched with 2 smaller submicron fluorescent beads i.e. 0.3 µm and 0.1 µm (Megamix-Plus prototype), and alternatively a 3rd 0.24 µm beads (see far-right plots).
1st threshold is done on FL1 to avoid noise. Then switched to FSC or SSC or both depending on FCMr ➔ how to best set-up SSC threshold on BD instruments without loosing MP ?
Plasma samples: One common, well-known, PFP sample available in multiple frozen aliquots ("Blue" PFP as in ref. 3). Additional sample ("Red" PFP, see 3) for comparison of Gallios & LSRII (far left & right).
Staining: Platelet-derived MP defined as dual-positive AnnV-FITC+/CD41-PE+. **MP counts:** Absolute counts per µL obtained by adding counting beads in stained/diluted samples (FL3).

Results:

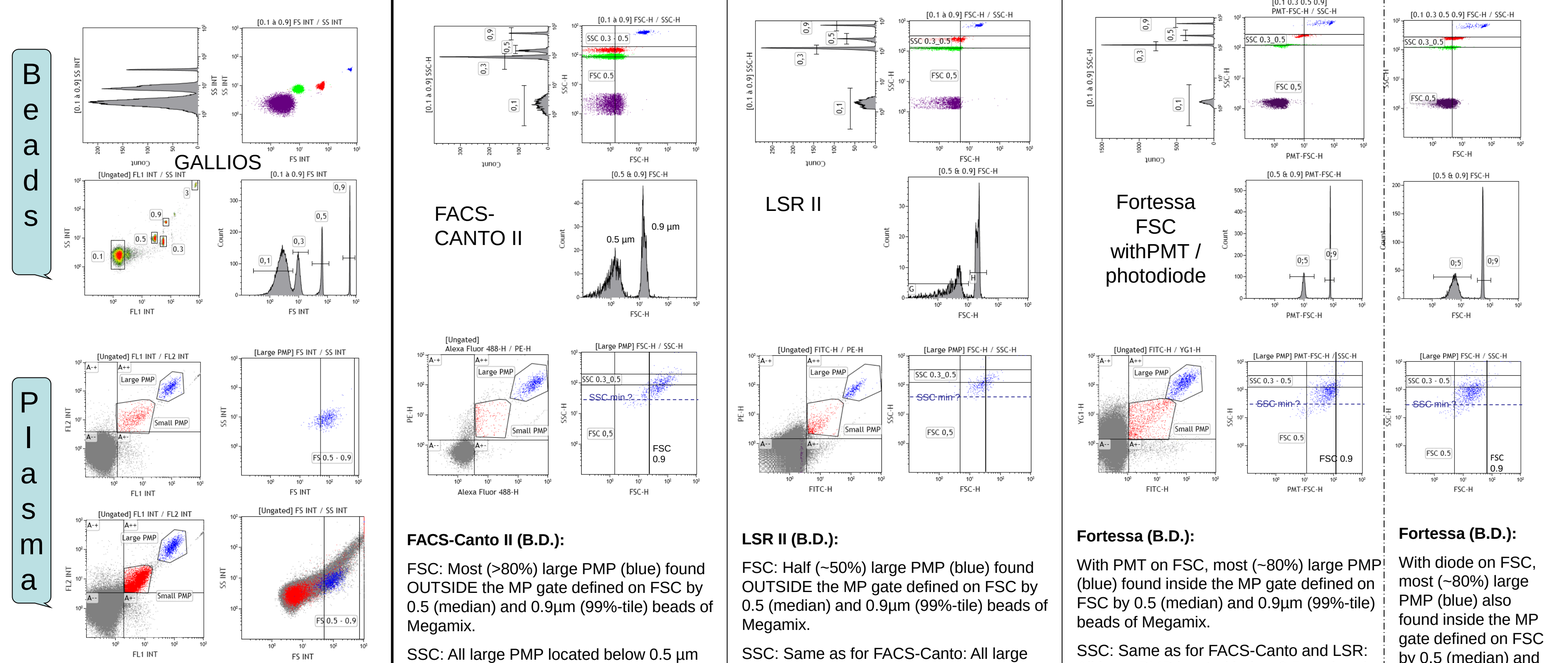
Gallios (B.C.): Increased FS resolution via W² option (see poster 330, CYTO 2011). All beads of Megamix-Plus resolved from 0.1 µm to 0.9 µm. Poor SS resolution.

FACS-Canto II (B.D.): Best possible FSC resolution shown here (see poster 325, CYTO 2011), but limited to 0.5 - 0.9µm. ➔ Clear SS resolution. All beads of Megamix-Plus resolved from 0.1 µm to 0.9 µm, with major gap from 0.3 to 0.1 µm beads.

LSR II (B.D.): Typical FSC resolution shown here but limited to 0.5 - 0.9µm. ➔ Clear SS resolution. All beads of Megamix-Plus resolved from 0.1 µm to 0.9 µm, with major gap from 0.3 to 0.1 µm beads. SSC distribution rather consistent.

Fortessa (B.D.): Fine FSC resolution with PMT. Can even discriminate 0.3 from 0.5 µm beads. 0.5 - 0.9µm range shown here. ➔ Clear SS resolution. All Megamix-Plus beads resolved, with major gap from 0.3 to 0.1 µm beads. SSC distribution rather consistent with previous generations.

FSC with photodiode resolution limited to 0.5 µm (comparable to FC500)



FACS-Canto II (B.D.):

FSC: Most (>80%) large PMP (blue) found OUTSIDE the MP gate defined on FSC by 0.5 (median) and 0.9µm (99%-tile) beads of Megamix.

SSC: All large PMP located below 0.5 µm bead-eq. SSC level (~50% below 0.3 µm bead-eq.). A minimum (?) SSC level below 0.3 µm bead-eq may be found to create appropriate standardized cut-off on SSC.

LSR II (B.D.):

FSC: Half (~50%) large PMP (blue) found OUTSIDE the MP gate defined on FSC by 0.5 (median) and 0.9µm (99%-tile) beads of Megamix.

SSC: Same as for FACS-Canto: All large PMP located below 0.5 µm bead-eq. SSC level (~50% below 0.3 µm bead-eq.). A minimum (?) SSC level below 0.3 µm bead-eq may be found to create appropriate standardized cut-off on SSC.

Fortessa (B.D.):

With PMT on FSC, most (~80%) large PMP (blue) found inside the MP gate defined on FSC by 0.5 (median) and 0.9µm (99%-tile) beads of Megamix.

SSC: Same as for FACS-Canto and LSR: All large PMP located below 0.5 µm bead-eq. SSC level (~50% below 0.3 µm bead-eq.). A minimum (?) SSC level below 0.3 µm bead-eq may be found to create appropriate standardized cut-off on SSC.

Fortessa (B.D.):

With diode on FSC, most (~80%) large PMP (blue) also found inside the MP gate defined on FSC by 0.5 (median) and 0.9µm (99%-tile) beads of Megamix. Suggests a different FSC collection optics in Fortessas (?).

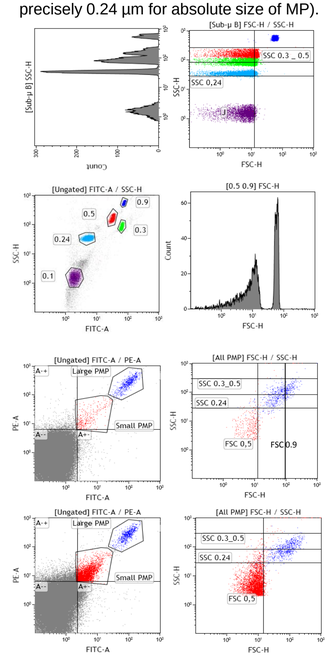
Gallios (B.C.): 2 PMP subsets clearly resolved: Large (blue) and Small (red). All (>90%) large PMP found in the MP gate defined by 0.5µm (median) and 0.9µm (99%-tile) beads of Megamix.

Upper plot: PFP sample "blue" .
Lower plot: PMP sample "Red"

Conclusion:

SSC may be used as size-related parameter on BD FCMrs provided that: smaller reference beads are used (0.24 µm seems ad hoc). 0.9µm beads set at upper SSC

LSR II (B.D.): 2nd test on the same LSRII (AMF). A new 0.24 µm bead added to Megamix-Plus provides ad hoc resolution in SSC to add a new reference point (0.24 µm bead-eq. which means that this may not be precisely 0.24 µm for absolute size of MP).



LSR II (B.D.):

SSC: All (>90%) large PMP (blue) found inside a new MP gate defined on SSC by 0.5 (75%-tile ? upper level) and 0.24µm (25%-tile? lower level) beads of BD-extended Megamix-Plus prototype.

FS-standardized Gallios versus SSC-standardized LSR II (same day / samples) ➔ comparison of "large PMP" counts:

➔ **Gallios:** Large PMP in standard MP gate (FS 0.5µm-1µm): "blue": **1650 MP/µL**. "Red": **2350 MP/µL**

➔ **LSRII** in new MP gate (SSC 0.24µm-0.5µm): "blue": **1620 MP/µL**; "Red": **2150 MP/µL** (940 & 1240 MP/µL in standard MP gate, resp.)