330 Abstract

Background: The size of cellular microparticles (MP), ranging from 0.1 to 1µm, places MP analysis at the very limits of flow cytometers' resolution in size. One option is to limit MP quantification to the fraction of the "biggest" MP using a standardized cut-off. Thus, reproducible counts of the "visible" fraction of platelet-derived MP (PMP) have been achieved on Beckman-Coulter (BC) FC500 instruments using a blend of fluorescent microbeads (Megamix, BioCytex, F) to define optimal settings, especially in the size-related Forward Scatter (FS) parameter. This tool including known amounts of two submicron beads (0.5µm and 0.9µm) permits individual instrument Q.C. and inter-instrument reproducibility (1, 2). Recently, new cytometers with increased FS sensitivity have appeared. In this study, we evaluated the potential of the BC Gallios for detection and standardized counting of MP in a wider size range. (1-Robert S, JTH 2009, 2-Lacroix R, JTH 2010). **Methods:** Megamix was enriched with fluorescent beads of 0.3µm and 0.1µm in well-known relative amounts (Megamix Plus prototype). 2 Gallios and 1 Navios were available for beads and MP analysis, adaptation of cut-off and inter-instrument reproducibility. For best sensitivity in the low size range, FS detection mode was "W". Platelet-free plasma samples (PFP) containing major MP subsets i.e. Platelet-, Erythrocyte- and Leucocyte-derived MP (PMP, Ery-MP, Leu-MP) were isolated from blood samples +/- purified MP. Various dual-color stainings were used to delineate PMP (e.g. AnnV-F/CD41-PE; CD41-F/CD31-PE or CD9-PE), Ery-MP (e.g. AnnV-F/CD235a-PE) and Leu-MP (e.g. AnnV-F/CD11b-PE or CD15-PE). 5µm counting beads (Cyto-Count®, Dako) were used for absolute counting. **Results:** Using FL1 discriminator (to eliminate bkgd) and FS W² option, Gallios provided clear FS resolution not only between 0.5µm and 0.9µm beads but also between 0.3µm and 0.5µm beads. The smallest 0.1µm beads were all counted, suggesting a potential for detecting highly fluorescent MP down-to 0.1µm, but partial FS overlap with 0.3µm beads limited resolution at about 0.3µm. Using FS discriminator (including dim MP and non fluorescent bkgd), the 0.3µm beads median FS appeared as a robust cut-off allowing routine analysis of MP on the 3 instruments. Under these "more open" conditions an additional subset of PMP appeared with lower staining intensities and FS (SS) values. Also, smaller (and less intensively stained) subsets of Ery-MP and Leu-MP were detected which remained hidden when gating at the previous 0.5µm cut-off level. Finally, PMP counts made on different PFP samples using our 3 instruments set-up at the same 0.3µm cut-off were similar (CV<15%) supporting inter-instrument reproducibility. **Conclusion:** The higher FS resolution and sensitivity offered by the W² option of Gallios/Navios permits the use of a standardized 0.3µm cut-off and detection of smaller MP than previously observed using FC500.

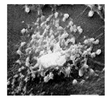
"SMALL SIZE" MICROPARTICLES: A NEW CHALLENGE FOR STANDARDIZATION OF FCM-BASED ANALYSIS AND COUNTING.

P. Poncelet[1], *S. Robert*[2], *J. Wischhusen*[1], *T. Bouriche*[1], *L. Arnaud*[3], *R. Lacroix*[2,3], *F. Dignat-George*[2,3] .

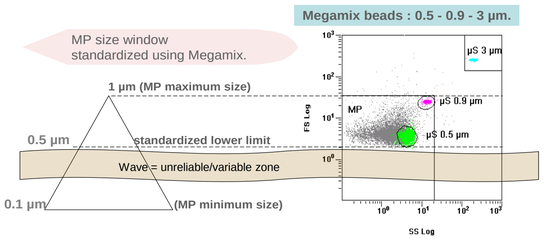
(1) BioCytex, Marseille (F) (2) INSERM UMR-S608, Aix-Marseille II University, UFR Pharmacie, Marseille (F)

(3) Hematology laboratory, CHU La Conception hospital, Marseille (F)

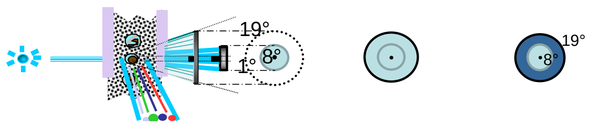
Background:

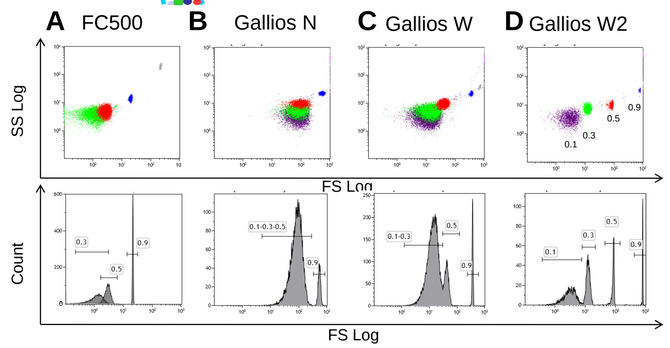


Microparticles (MP) are submicron vesicles released from cell membranes. Their reliable measurement in plasma is needed due to recognized patho-physiological roles in thrombosis, inflammation and cancer. Their low size range (0.1 – 1µm) creates a difficult challenge for detection, sizing and counting by flow cytometry (1), leaving only the emerging part of the "MP iceberg" to be routinely evaluated. One option is to limit MP quantification to the fraction of the "biggest" MP using a standardized cut-off. Thus, reproducible counts of the "visible" fraction of platelet-derived MP (PMP) have been achieved on Beckman-Coulter (BC) FC500 instruments using a blend of fluorescent microbeads (Megamix, BioCytex, F). These define optimal settings in the size-related Forward Scatter (FS) parameter used as trigger for MP analysis.



Recently, new cytometers with increased FS sensitivity have appeared (3).
Gallios (Beckman-Coulter) makes use of a high solid angle (1-19°) FS collection and a differential amplification of the external ring (8°-19°)
➔ improved sensitivity/resolution for submicron particles (see below).

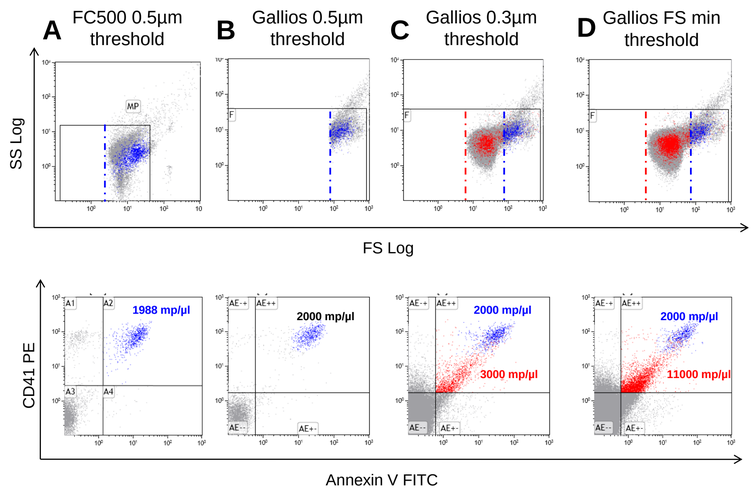




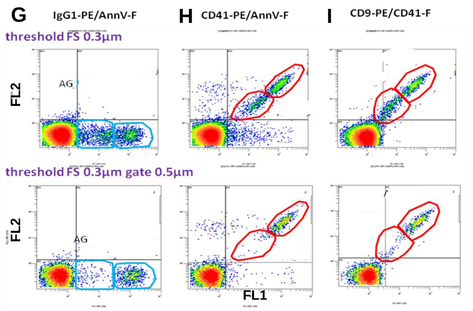
Resolution for submicron particles is illustrated here as a function of FS detector configurations (1-8° = B, 1-19° = A & C, and w² = D). The wider FS dynamic range of W2 requires an extended version of Megamix, enriched with 0.3 and 0.1 µm beads (Megamix-Plus prototype, BioCytex).
Note: Acquisition with FL1 threshold to remove background (BkGd) noise).

Methods:

Instruments: include several FC500 and 3 Gallios (Beckman-Coulter)
Beads: Prototype version of Megamix enriched with 2 smaller submicron fluorescent beads i.e. 0.3 µm and 0.1 µm (Megamix-Plus prototype, BioCytex). 1st threshold is done on FL1 to avoid noise (J). Then switched to FS at the desired cut-off level set-up with 0.5 and 0.3 µm beads (K, L, M).
Plasma samples: Platelet-Free Plasma (PFP) from cardiovascular disease patients included in official clinical studies (paper submitted). PFP obtained after serial centrifugations (1,500 g 15mn ➔ PPP & 13,000 g 2mn ➔ PFP) as described (1, 2).
Staining: Platelet-derived MP (PMP) defined as dual-positive AnnV-FITC+/CD41-PE+, alternatively CD41-FITC/CD9-PE (see below, I). Erythrocyte-derived MP (Ery-MP) defined as AnnV+/CD245a+ (E), leucocyte-derived MP (Leu-MP, F) as AnnV+/CD15+ (reagents from BioCytex and Beckman-Coulter).
MP counts: Absolute counts per µL PFP via 6µm counting beads (Cyto-Count, DAKO), read in FL3.



The new subset of "small" MP with less intense staining intensities for resp. AnnV-FITC, AnnV-FITC or CD41-PE, CD41-FITC & CD9-PE is lost when FS threshold is raised from 0.3 µm to 0.5 µm with a gate set-up using 0.5 µm Megamix (+) beads.

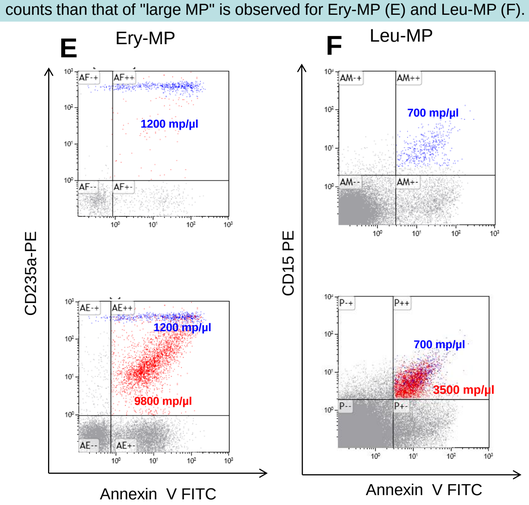


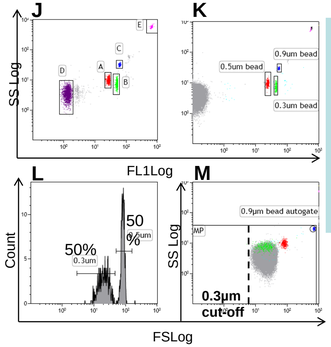
Results:

Due to higher FS sensitivity and lower BkGd on Gallios, threshold can be lowered from 0.5 µm (B, same as on FC500-based "old" protocol, A), down-to 0.3 µm in routine use (C), and even ~ 0.2 µm in extreme conditions (D), reaching the limits of instrument's electronics (significant electronic aborts from 8,000 evts/s up-to 15,000 evts/s and thus loss of information).

Below the 0.5 µm cut-off (blue dotted line), a new subset of so-called "small PMP" appears (red points), in addition to the already known "large PMP" (blue dots). PMP counts for large and small PMP appear in blue and red, respectively. These PMP subsets can be observed with various markers (see G,H, I).

Same, a new "small MP" subset characterized by significantly higher MP counts than that of "large MP" is observed for Ery-MP (E) and Leu-MP (F).





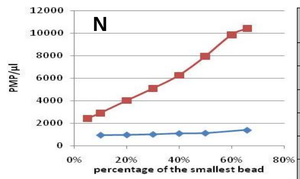
Construction of a 0.3 µm cut-off standardized MP protocol:

1) Classify bead subsets in FL1 x SS using FL1 threshold (J)
2) Show FS histograms of both 0.3 and 0.5 µm beads (L). Measure % of 0.3 µm among the sum of both (66% - 2:1 ratio - originally).
3) Switch to FS threshold (K). Optimize FS settings to reduce 0.3µm % at 50%. Then half the total amount of 0.3 µm beads pass the threshold ⇔ cut-off at the median, nominal value of 0.3 µm-eq.
4) Define new MP gate with upper limit near 1 µm-eq. i.e. at the end of 0.9 µm beads distribution. This is automatically done on Gallios by tangenting the autogate (0.01% sensitivity).

References: *1-Robert S, JTH 2009, 2-Lacroix R, JTH 2010, 3 - Kirouac B, CYTO 2011 (abst. # 525). Poster on-line at www.beckman.com*

Reproducibility with the new standardized MP protocol:
In contrast with the "old" 0.5 µm-eq. cut-off protocol which benefits from a rather empty space between the large and small PMP subsets and thus defines quite stable PMP counts on Gallios (blue curve in N), the new 0.3 µm-eq cut-off is located on a steep slope (red curve in N) and thus requires very precise set-up.

Inter-instrument reproducibility of PMP counts was challenged on multiple similar platforms. 3 independent Gallios/Navios instruments were similarly setup using Megamix-Plus beads and used to count PMP on the same PFP samples, split just after staining and analyzed the same way, as shown in C.
On n = 10 samples, PMP counts were comparable with an overall CV of 10%, as illustrated in O.



Sample	PMP /µl (mean +/- SD)	PMP count CV
1	989 (+/-46)	5%
2	771 (+/-83)	10%
3	878 (+/-110)	12%
4	1097 (+/-185)	17%
5	904 (+/-114)	13%
6	760 (+/-58)	8%
7	387 (+/-53)	14%
8	2841 (+/-179)	6%
9	1572 (+/-39)	2%
10	1356 (+/-197)	15%
O	Mean CV :	10%

This inter-instrument reproducibility observed on Gallios is a good 1st step forward towards future multi-centric studies. Extension to other instruments will likely require adaptations since the relative scatter position of MP as compared to beads depends on many factors, including solid angle of FS collection (see poster 329, CYTO 2011).

Conclusion:

Gallios/Navios flow cytometers offer higher FS dynamic range and lower background. This is illustrated with:
1) a new extended bead-based tool (Megamix-Plus)
2) newly appearing MP subsets of smaller size than previously seen..
This permits a deeper insight into the "MP iceberg", even on a routine basis.
A new standardized MP protocol is proposed to take the best from technology improvements, with a cut-off close to 0.3 µm-eq..