Data S1

MP protocol settings

This step aims at fine tuning the standardized PMP counting protocol in order to prepare the real PMP dosage experiments. It can be divided into 5 steps, including:

- Optimize scatter settings
- Optimize flow rate
- Optimize fluorescence settings
- Optimize compensation settings
- Optimize region boundaries

From this level on, the preferential choice for FCMr signals will be amplitude for all FCMrs (PEAK for BC FCMrs; HEIGHT/H for BC FCMrs; unknown for other brands).

a) Optimize scatter settings:

This requires checking the Megamix-Plus (**F**SC or **S**SC) protocol, up to switching to scatter-based (**F**SC or resp. **S**SC) threshold, same as for the instrument Q.C. operation, except for BC Gallios/Navios, where the use of PEAK signals will be required for plot display. This is a modification of the actual version of the box insert.
For **S**SC-optimized FCMrs, use HEIGHT signal for plot display as indicate in the box insert of Megamix-Plus **S**SC.
At the end of this step, MP-optimized scatter settings should be finalized/blocked.

b) Optimize flow rate:

- Make a working dilution of MP Count beads for testing, as follows:

30 µL MP Count beads + 45 µL Binding Buffer (BB) (or water) + 1 mL BB (or water).
This corresponds to the final concentration of MP Count beads in any future stained plasma sample.

- Select a priori the LOW flow-rate and run the bead suspension during 60 s (1 min) in the Mgx+ protocol, with compensation settings at zero and scatter threshold.

*MP Count beads appear in the upper part of **S**SC (and **F**SC) scale, well over the upper limit of the MP gate (Appendix 4, **F**SC FCMrs or Appendix 5, **S**SC FCMrs). They appear as FL2/PE+++ events with variable level of FL1 intensity, depending on compensation settings.*

- **Place a linear region around the count beads peak to get the number of events analyzed**

Conclusion : If total number of MP Count beads in 1 min ranges from 500 to 2,000, keep 1 min acquisition in the protocol.

If total number of MP Count beads is < 500, then increase acquisition time (automatic stop) to 2 min in the protocol.

c) Optimize fluorescence settings:

The aim of this step is to set-up FL1 and FL2 PMT voltages to reach pre-defined target values (median intensities) for single fluorescence positive beads.

This action makes use of a prototype reagent comprising a well balanced mixture of blank beads as well as relatively high intensity FITC-labeled and PE-labeled beads, called "Fluo Setting Beads" (FSB) which are mixed extemporaneously and receive the PMP staining reagents AnnV-FITC + CD41-PE at the same final doses as plasma samples, thus providing a comparable level of non-specific fluorescence background.

- Prepare the FSB tube:
    - **pipet 30 µL of FSB 1 (FITC-labeled beads)**
    - **add 30 µL of FSB 2 (Blank plus PE-labeled beads)**
    - **add 10 µL AnnV-FITC**
    - **add 5 µL CD41-PE**
    - **add 1 mL binding buffer (BB)**

This tube is immediately ready to read on the FCMr

To analyze the FSB tube:

- in the FL1 x SSC-H (resp. FL1 Peak x SS Peak) dual parameter plot, set a polygonal or rectangular gate e.g. FSB region (G) including all high scatter intensity beads, independent of their FL1 level (spanning the whole FL1 scale). (fig 1)

This gate may include several different tight clouds, including Blank (FL1 neg), PE-labeled (FL1 dim) and FITC-labeled beads (FL1 bright with varying levels of SSC, several peaks corresponding to singlets, doublets, triplets ..etc).

Note for SSC-optimized FCMrs :
In the FITC x **S**SC-H dual parameter plot, these beads appear off scale in SSC, due to their size (2.4 to 3µm) and the high SSC settings needed for SSC-based thresholding of MP analysis (Fig 1b). Prefer gating FSB beads using a FITC x FSC-H dual parameter plot, as shown below in Fig 1c.

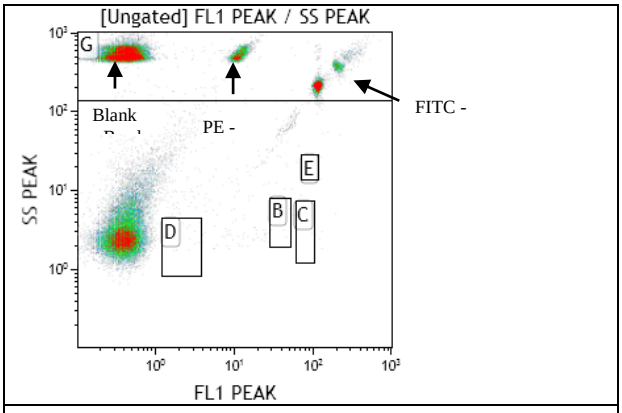


Fig 1a: FSB beads (G) with sub-optimal compensation (PE-beads >> to Blank beads in FL1 scale)

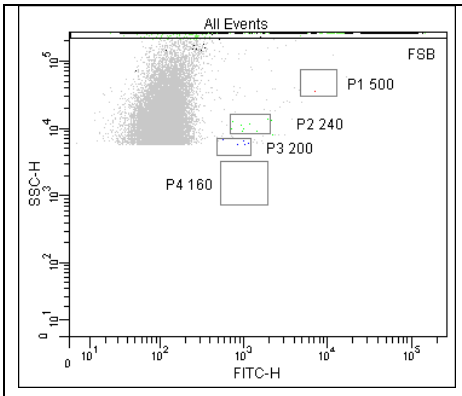


Fig 1b: FITC x SSC-H distribution of FSB beads. Grey cloud on the upper left side is background.

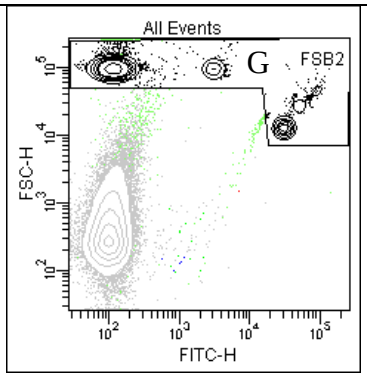
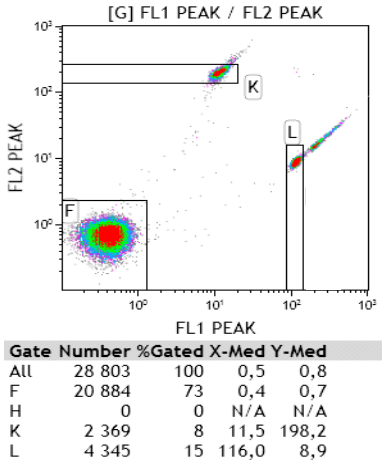


Fig 1c: FITC x FSC-H distribution of FSB beads with sub-optimal compensation (PE+++ beads >> to Blank beads in FITC scale). Grey cloud in the lower left side is MP-size background events.

- create a dual parameter density plot FL1 x FL2 gated on "FSB" region (G).

- finely tune FL1 PMTv so that the 1st (major) positive FL1 (FITC) peak is situated at
  ~100 a.u. for BC Gallios/Navios,
  ~3.10^4 a.u. for BD instruments using DIVA and 5.5 log scale

- finely tune FL2 PMTv so that the 1st (major) positive FL2 (PE) peak is situated at
  ~200 a.u. for BC Gallios/Navios,
  ~2.10^4 a.u. for BD instruments using DIVA and 5.5 log scale



Gate	Number	%Gated	X-Med	Y-Med
All	28 803	100	0,5	0,8
F	20 884	73	0,4	0,7
H	0	0	N/A	N/A
K	2 369	8	11,5	198,2
L	4 345	15	116,0	8,9

Fig 2a: Fluo Setting Beads to set target values (BC): Target FL2 Md ~200 a.u./FL1 Md ~100 a.u.

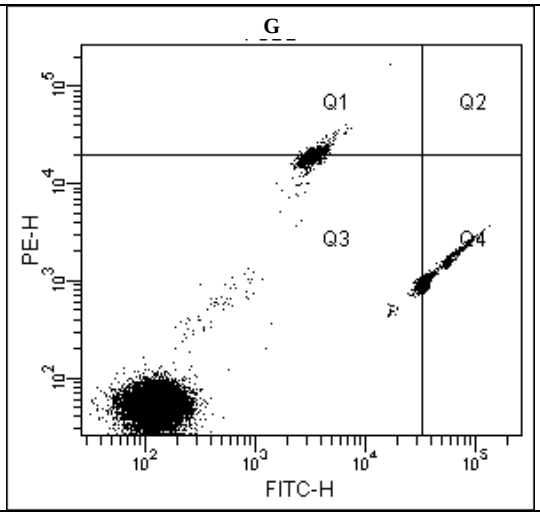


Fig 2b: Fluo Setting Beads to set target values (BD): FL2 Md ~2.10^4 a.u./FL1 Md ~3.10^4 a.u.

Warning : Before switching from beads to plasma samples (next chapter), take care to run a tube with water or PBS in between in order to avoid any carry-over effect and the impact of a few remaining stained beads in the tubings.

d) Optimize compensation settings:

This optimization should be operated once and should not need to be modified if the instrument remains stable over time and if no change is operated on PMT voltages/fluor. target channels. Compensation settings are best optimized using single fluorescence controls made on real samples[1].

- ✓ In order to create these controls, select PFP RED (4 aliquots available) and create the 2 following stained control tubes:

Control tube	PFP RED	AnnV-F	CD41-PE	IgG1-PE	BB	PBS wo Ca^{2+}
FITC-/PE+	30µL	10µL	5µL			1 mL
FITC+/PE-	30µL	10µL		5µL	1 mL	

- ✓ Analyze these tubes using the previously defined MP gate (with the help of Mgx+) to gate FL1 x FL2 plots e.g. "MP Peak" gate and optimized FL1/FL2 and FL2/FL1 compensations.

Optimized compensation on BC instruments:

- Create an additional region "E" including the 2 last decades of FL1 only to optimize compensation setting. *Indeed, despite inadequate compensation in Fig. 3a, the FL2 Md ("Y-Med") in quadrant D+- is not so different from that in D--, due to low FL1 intensity of most MP. Non rectangular quadrants are better than truly rectangular quadrants*

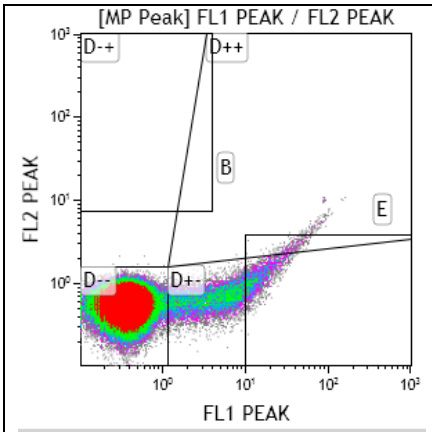
- Try to equalize Y-medians between E and D-- for adequate compensation setting (Fig 3b)

- Then, analyze the single PE positive control tube.

- Create a region "B" to measure PE medians on the most fluorescent MP events as illustrated in Fig3c.

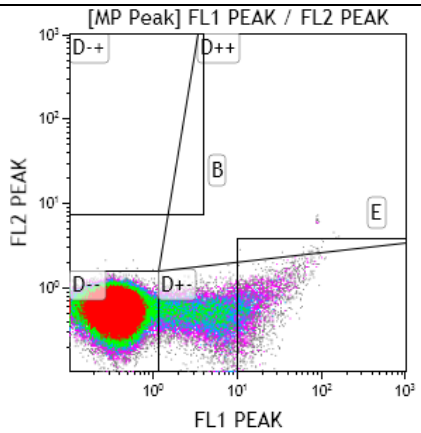
- Try to equalize X- medians between B and D-- for adequate compensation (Fig 3d)

[1] Using Fluo Setting Beads would induce apparent over-compensation of MP, due to much higher intensities on beads than the majority of MP.



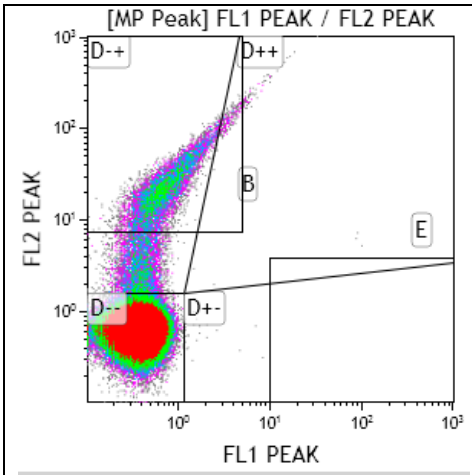
Gate	Number	X-Med	Y-Med
All	311 490	0,4	0,6
B	0	N/A	N/A
D--	297 771	0,4	0,6
D-+	6	0,6	1,7
D+-	13 250	4,8	0,7
D++	463	42,1	3,1
E	3 051	14,8	1,2

Fig. 3a: FITC+/PE-control tube without compensation.



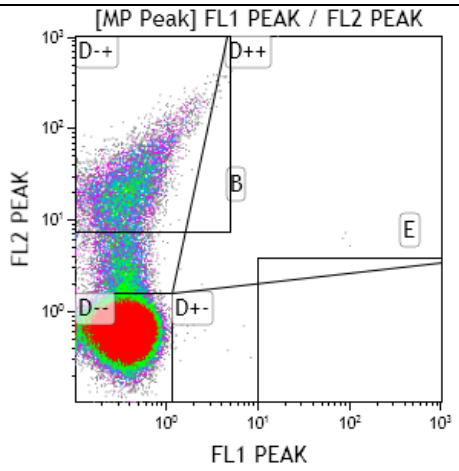
Gate	Number	X-Med	Y-Med
All	311 490	0,4	0,6
B	0	N/A	N/A
D--	297 786	0,4	0,6
D-+	6	0,6	1,7
D+-	13 662	5,0	0,5
D++	36	87,9	3,1
E	3 157	15,1	0,6

Fig. 3b: FITC+/PE-control tube after optimized (FL2-x%FL1) compensation. Here, x=4.2



Gate	Number	X-Med	Y-Med
All	291 751	0,4	0,7
B	8 540	0,7	21,3
D--	278 057	0,4	0,6
D-+	13 350	0,5	12,3
D+-	24	1,5	0,6
D++	320	3,9	135,9
E	0	N/A	N/A

Fig. 3c: FITC-/PE+ control tube without compensation.



Gate	Number	X-Med	Y-Med
All	291 751	0,4	0,7
B	8 607	0,4	21,4
D--	278 052	0,4	0,6
D-+	13 652	0,4	12,8
D+-	24	1,5	0,6
D++	23	4,3	319,5
E	0	N/A	N/A

Fig. 3d: FITC-/PE+ control tube after optimized (FL1-y% FL2) compensation. Here, y=1.6

Optimized compensation on BD instruments:

- Select the bi-exponential options for both FL1 and FL2.
For this step, bi-exponential scales (Fig 4) are more appropriate than fixed 4 log decade scales.

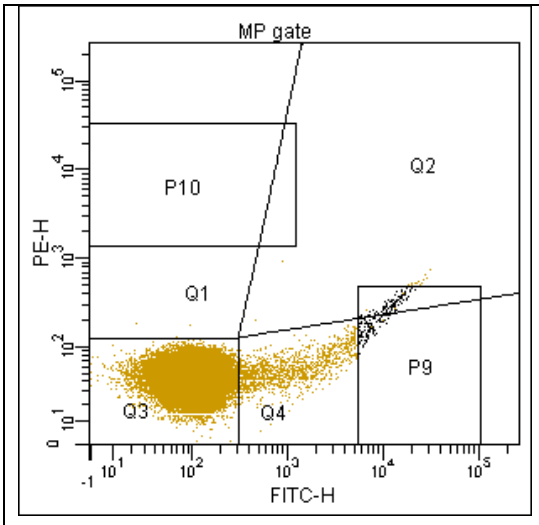
- Create an additional region "P9" including the 2 last decades of FL1 only to optimize compensation setting, as illustrated in Fig 4a. *Indeed, despite inadequate compensation in Fig 3a, the FL2 Md ("PE-H Median") in quadrant Q4 is not so different from that in Q3, due to low FL1 intensity of most MP. Non rectangular quadrants are more appropriate than truly rectangular quadrants.*

- Try to equalize PE-H medians between Q3 and P9 for adequate compensation setting (Fig 4b).

- Then, analyze the single PE positive control tube.

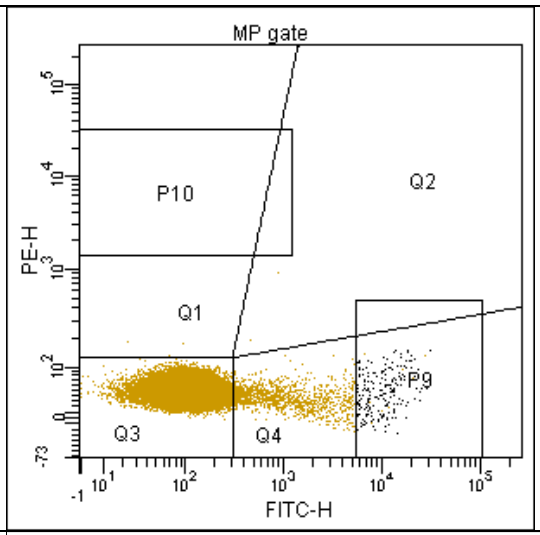
- Create a region "P10" to measure PE medians on the most fluorescent MP events as illustrated in Fig 4c.

- Try to equalize PE-H medians between Q3 and P10 for adequate compensation (Fig 4d).



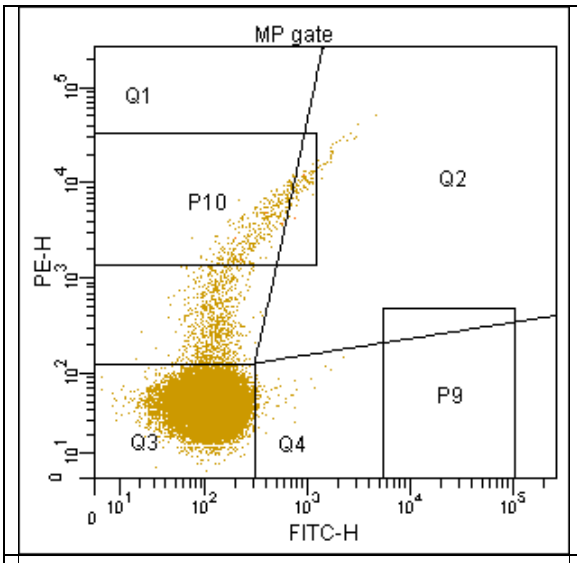
Population	#Events	FITC-H Median	PE-H Median
All Events	337 903	105	45
P9	406	9 009	214
P10	0	####	####
Q1	8	95	194
Q2	263	12 650	309
Q3	330 478	104	45
Q4	7 154	1 233	57

Fig 4a: Single FITC positive control without compensation.



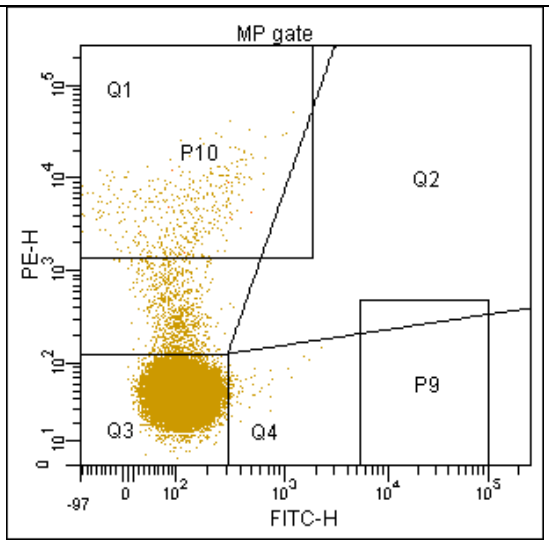
Population	#Events	FITC-H Median	PE-H Median
All Events	337 903	105	42
P9	423	9 277	43
P10	0	####	####
Q1	8	95	192
Q2	10	3 400	239
Q3	330 469	104	43
Q4	7 416	1 300	32

Fig 4b: Single FITC positive control with optimal compensation. (FL2-1.9% FL1)



Population	#Events	FITC-H Median	PE-H Median
All Events	288 053	109	49
P9	0	####	####
P10	793	371	4 174
Q1	5 934	152	902
Q2	270	948	10 335
Q3	281 576	108	48
Q4	273	340	57

Fig 4c: Single PE positive control without compensation.



Population	#Events	FITC-H Median	PE-H Median
All Events	288 053	105	49
P9	0	####	####
P10	841	110	4 628
Q1	6 204	92	974
Q2	36	621	272
Q3	281 556	105	48
Q4	257	342	56

Fig 4d: Single FITC positive control with optimal compensation. (FL1- 6.4% FL2)

e) Optimize region boundaries:

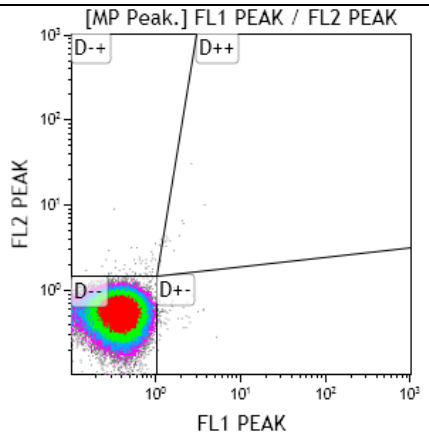
- ✓ In order to optimize region boundaries, select PFP RED (use the same aliquot as for compensation setting) and create the 2 following stained control tubes:

Control tube	PFP RED	AnnV-F	CD41-PE	IgG1-PE	BB	PBS wo Ca^{2+}
FITC-/PE-	30µL	10µL		5µL		1 mL
FITC+/PE+	30µL	10µL	5µL		1 mL	

- ✓ Using dual negative control (FITC-/PE-), optimize quadrant positioning, mainly to include all dual negative MP-gated events in D--. Typically, D-+, D+- and D++ should contain < 0.1% of D-- counts (fig5a)

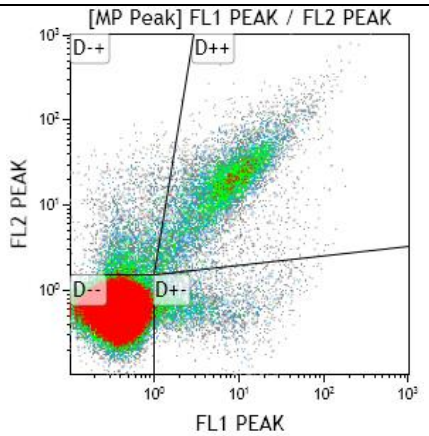
- ✓ Using dual stained positive control (FITC+/PE+), optimize quadrant D++ and check the position of PMP (fig 5b)

Illustrations on Gallios (Fig 5a/b)



Gate	Number	X-Med	Y-Med
All	291 113	0,4	0,5
D--	290 938	0,4	0,5
D-+	62	0,6	1,8
D+-	99	1,1	0,6
D++	14	1,5	4,1

Fig. 5a : FITC-/PE- dual negative control tube.



Gate	Number	X-Med	Y-Med
All	285 057	0,4	0,6
D--	267 397	0,4	0,6
D-+	3 507	0,5	2,8
D+-	3 962	2,1	0,7
			17,3

Fig. 5b : FITC+/PE+ positive (control) test tube

Illustrations on FACS CantoII (BD) (Fig6a/b)

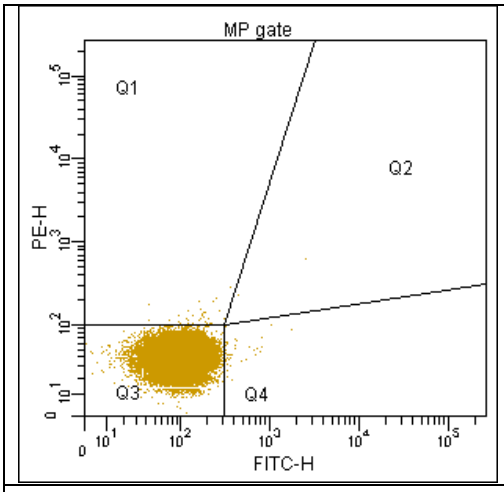


Fig 6a : Dual negative control with appropriate pseudo-quadrant boundaries.

Population	#Events	%Parent	FITC-H Mean	PE-H Mean
Q1	106	0,0	190	162
Q2	605	0,2	3 219	19 082
Q3	401 290	99,8	100	40
Q4	258	0,1	489	52

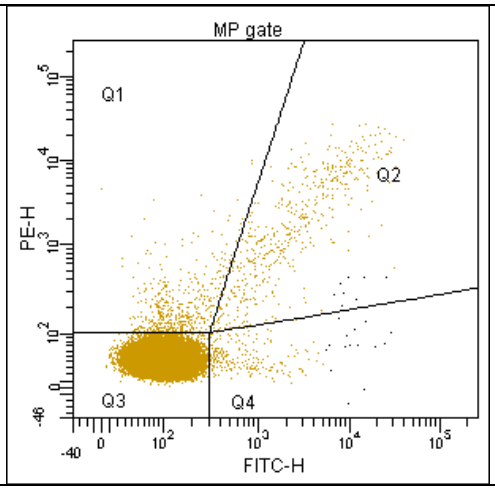


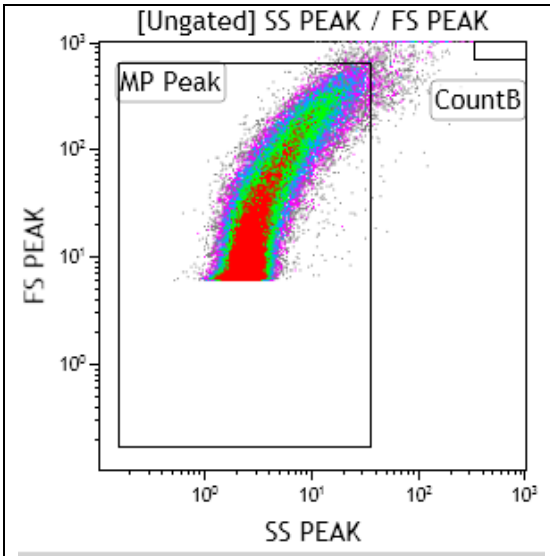
Fig 6b : Dual staining for PMP in RED control with ad hoc pseudo-quadrant boundaries.

Optimize Counting beads region boundaries :

Warning : MP Counting Beads are highly fluorescent in FL2 not in FL3.

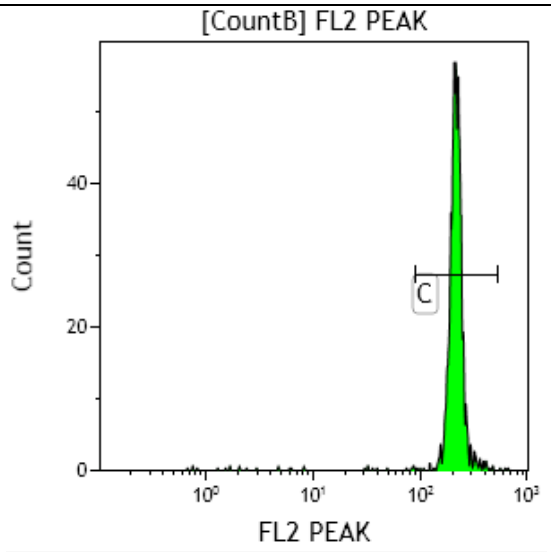
- ✓ Select MP Count beads using the same FL1 x SSC gate as for fluorescent setting beads (FSB) and a mono-parametric histogram gated on FSB. The counting region (C in fig 7b) corresponds to the high level FL2 peak.

Illustrations on Gallios (Fig 7a/b)



Gate	Number
All	75 862
CountB	1 797
MP Peak	73 010

Fig 7a: Dual scatter distribution of total events and position of the gate for counting beads (here "CountB").



Gate	Number
All	1 797
C	1 765

Fig 7b: FL2 PEAK distribution of counting beads (here gated on "CountB").

Illustrations on FACS CantoII (BD) (Fig 8a/b/c/d)

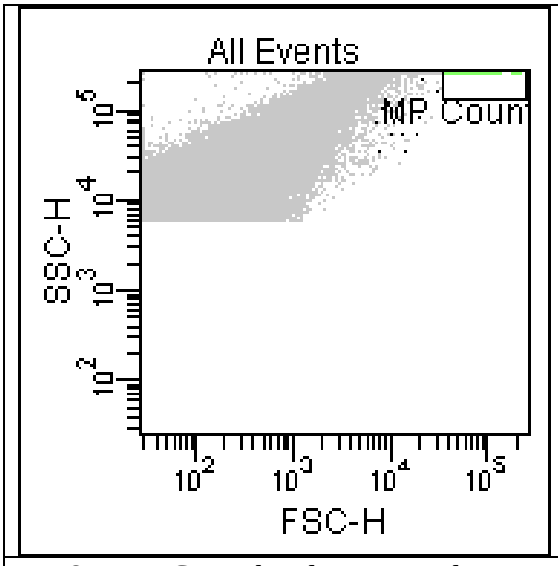


Fig 8a: MP Count beads in stained PFP
These 3 µm beads are located off-scale of SSC in MP settings, included in the "MP Count B" gate, colored here in green but hardly visible in this plot.

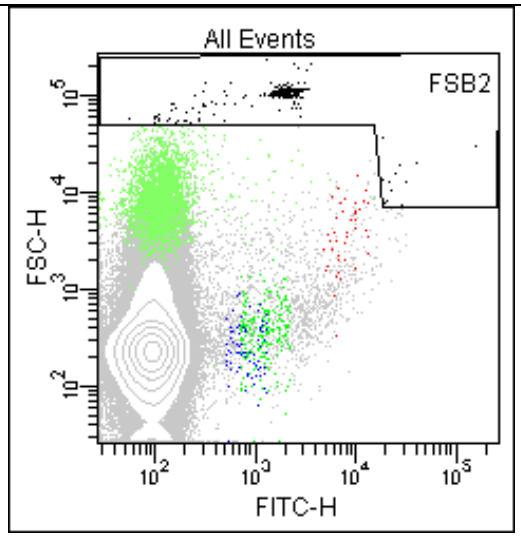


Fig 8b: MP Count beads in a stained PFP
These 3 µm beads can be found in any place in "FSB2" region, depending on compensation (null here).

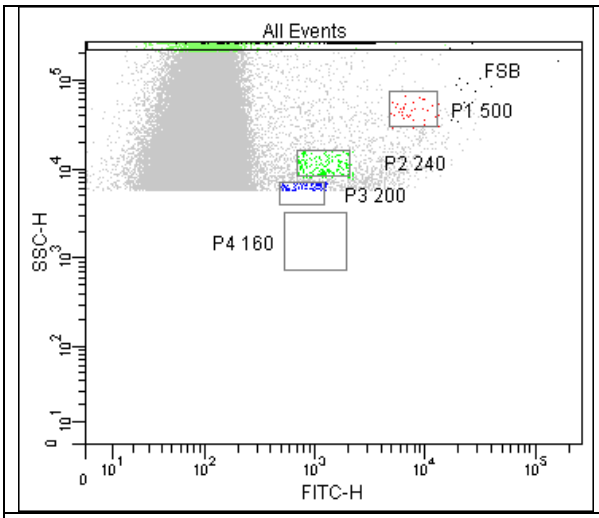


Fig 8c: MP Count beads in a stained PFP.
Can be found anywhere in the "FSB" region, depending on compensation (null here).

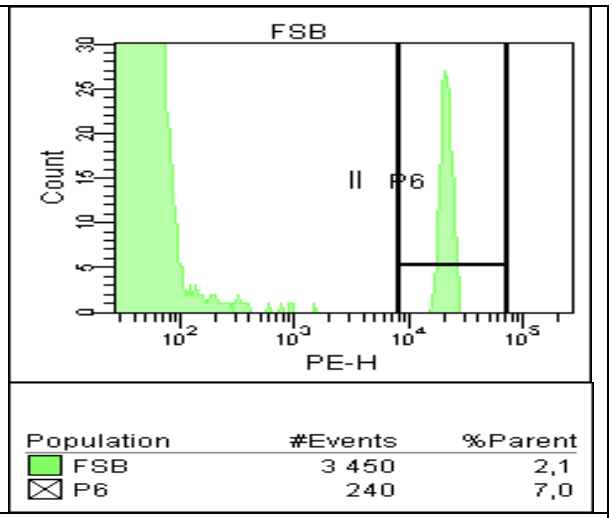


Fig 8d: MP Count beads gated by "FSB" gate, as seen on the FL2 scale. Note low number of beads (P6<400) in a 1 mn run.

At the end of these 5 actions of MP protocol setting, the standardized protocol is optimized.