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**HARMONEMIA : a universal strategy for flow cytometry immunophenotyping.**

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To the editor,

Flow cytometry (FC), a keystone in the diagnosis of hematological malignancies, usefully complements morphology and enables early decisions for patient management.<sup>1,2</sup> Therefore, it is important that FC can be performed in many different centers, in the vicinity of the patients. Progress achieved in the past few decades in the stability and precision of flow cytometers and analysis software permit the acquisition of reliable data in any competent laboratory. Due to the sophistication and versatility of instruments now allowing for multi-color analyzes, and to the large number of reagents available, it remains necessary to define the most pertinent strategies to achieve comparable results between laboratories. A large amount of information is available in the literature concerning the choice of antigens to be tested in hematological diseases.<sup>1-4</sup> Recently, the Euroflow consortium published an extensive description of standardization in the FC diagnosis of hematological neoplasms.<sup>5</sup> However, application of these recommendations requires trained personnel for the maintenance of one type of instruments and a specific type of software for data analysis. The proposed panels, extensively tested and calibrated, besides being relatively expensive, are fixed and not likely to be changed if comparison with the pathology library proposed by the consortium is to be applied.

The Harmonemia project described in the current report was set up with the different aim of providing a simple, rapid and robust way to obtain comparable data on various types of FC instruments. It was set to verify that harmonization can be obtained between different instruments in different settings. Information is available in the literature indicating that instruments of the same make or even from different manufacturers can be cross-calibrated.<sup>6-8</sup> In the Harmonemia project, this concept was extended to an international series of platforms. This manuscript reports on the first two steps of this project, namely the harmonization of photomultiplier (PMT) settings and the staining of normal peripheral blood (PB) with a common antibody panel.

The Harmonemia project involved 23 instruments, i.e. 16 Navios® (Beckman Coulter (BC), Miami, FL) and 7 Cantoll® (BD Biosciences (BD), San Jose, CA) in 17 laboratories for PMT

settings. Compensation and labelling of normal PB involved tests in 8 colors on 4 Navios®, 10 colors on 13 Navios® and 8 colors on 5 Cantoll® instruments.

Ultimately, Harmonemia aims at working in lysis-no wash protocols. In such settings, normal cells in the sample act both as calibrators and controls. Autofluorescence provides internal calibration for PMT settings while intrinsic positive control is provided by proper labelling of cell populations targeted by the reagents used. It is thus important that the PMT be set in such a way that the majority of unstained cells appear above channel 1 for each applied fluorescence. In Harmonemia's primary laboratory, at Bordeaux University Hospital, ten 50µL samples of unstained PB lysed with Versalyse® (BC) were acquired while adjusting PMTs in order to have at least 80% of leukocytes fulfilling the channel 1 criterion described above. On the same instrument, FlowSet Pro® (BC) beads were the run with these PMT values, yielding target channel (TC) values for each fluorescence. The same batch of FlowSet Pro® were distributed, together with TC, to all participants to be applied directly on Navios® instruments and after multiplying the target values by 256<sup>6</sup> on Cantoll® instruments. The efficiency of this harmonization was checked by running 5 unstained-lysed normal PB samples in each facility. Listmode files were analyzed collectively during specific workshops. The 256 conversion factor was shown to be adequate for all channels except that used for KromeOrange or HorizonV500, emitting respectively in the 540-550 and 510-550 nm ranges, because of the difference in the 500-550 nm bandpass filters between the two types of instruments. A new conversion factor of 97 was calculated for this channel using stained lymphocytes as targets. Flow Set Pro® values obtained on each of the 23 instruments were analyzed and data expressed as a percentage of the initial target (normalization). As shown in Table 1, the values obtained were highly comparable with tight CVs(<10%).

Each laboratory used normal unstained lysed PB to check PMT settings. Each fluorescence results were plotted on a FL/SSC histogram, drawing a gate set to start FL assessment at channel 1 (Supplementary Figure 1A). Median percentages of cells above channel 1 were above 83% for all fluorescence results (Supplementary Table 1).

A panel devised within the French Groupe d'Etude Immunologique des Leucémies (GEIL<sup>9</sup> supplementary table 2) for the diagnosis of acute leukemias was then applied to perform compensations with Versacomp® (BC) beads and stain PB in each laboratory. Single staining of positive beads (coated with anti-mouse immunoglobulin antibodies) was performed in separate tubes, also containing negative (unstainable) beads. After incubation, all tubes were acquired without compensation with the PMT defined in the previous step. The wizard positive/negative option of Kaluza® (BC) was then applied to each facility's listmode files to obtain the respective individual matrices which turned out to be highly comparable (data not shown).

In the next step, 50µL of four normal PB were incubated with the antibody panel in each of the 22 platforms. After 15 minutes incubation and Versalyse® lysis, the samples were run using the PMT and compensation matrices previously established. All listmodes were analyzed collectively. The merge function of Kaluza and pre-set gates were used to confirm harmonization.

To better appreciate the level of harmonization, mean fluorescence intensity (MFI) of neutrophils stained with CD11b and CD16 as well as CD65 for Navios 10C, monocytes stained with CD11b and CD33 and T-lymphocytes stained with CD7 for Navios 10C were plotted (Supplementary Table 3). Data analysis of the 90 samples tested revealed a non-normal distribution. This was due to the presence of one or more outlier in 16 samples identified as originating from clinically normal patients containing aberrant PB subpopulations. The distribution was normal when these samples were omitted. MFI of these 74 samples were compared for CD16, CD11b and CD33 staining of granulocytes and monocytes. As shown in Figure 1, means did not significantly differ between laboratories and all data were included in the interval defined by the global mean $\pm$ 2SD.

In summary, this study of the Harmonemia working group reports on a robust strategy allowing for a successful cross-instrument harmonization on a large scale. It extends former

studies<sup>5-9</sup> and provides an operating procedure applicable anywhere, independently of the instrument available.

Indeed we have shown that any instrument can be set-up by acquiring unstained lysed blood and getting over 80% of the cells above channel 1 within the first log decade for each fluorescence. An alternative would be to send an aliquot of any type of beads to one of the participating laboratories and determine TC values for this specific set of beads. Self-generation of TC can then be obtained locally at each change of beads batch.

Beads provide fluorescence signals over the whole range of wavelengths and minimize bandpass filter-related variations between instruments by yielding the best signal. The use of beads to establish PMTs avoids the inter-individual variability of PB. It also circumvents the limitations of fluorochrome-conjugated antibody-based assays which are more sensitive to handling and thus operator-dependent. The merges of stained blood samples yielded extremely reproducible patterns, where predefined gates for the major subsets could be established and applied to any sample. Fluorescent beads are thus excellent and cost-effective to harmonize instruments with comparable optical filters. The only specific adjustment that proved necessary between instruments was for the violet laser, with a different conversion factor.

Checking the validity of this strategy for PMT settings demonstrated several previously not well-described features. One is that there is some degree of inter-individual variation in the autofluorescence of unstained cells, making mandatory the use of several samples to check/establish valid PMT values.

Labelling of local samples in all participating laboratories yielded highly comparable results with very little dispersion for monocytes and slightly more for CD11b on granulocytes. The broader distribution of CD16 labelling of granulocytes is likely to reflect the known polymorphism of this molecule. The use of logarithmic scales to collect fluorescence signals, and the proper compensations applied, erasing spillover, made feasible the comparison and superimposition of signals obtained with different instruments independent of their make. This first part of the Harmonemia project demonstrated the feasibility of a simple, robust and

economically-justifiable way of harmonizing instruments in a large array of different platforms. It allows the comparison of data from distinct laboratories using different instruments as well as a simple harmonization of flow cytometers in a given facility. The sophistication of 8 and 10 colors flow cytometers now common in FC laboratories, allow to obtain detailed information from such complex samples as diseased bone marrow. This can be applied to a refined study of hematopoiesis in the diagnosis and follow-up of myelodysplastic syndromes or in the analysis of minimal residual disease. In both these examples, the detection of abnormalities will depend on a good knowledge of, or direct comparison with, normal bone marrow. Thus, the Harmonemia project now aims at providing reference material available for such assessments overcoming inter-individual variability.

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**Table 1.** Mean and SEM of bead values obtained after PMT adjustment and normalization to expected target on 23 instruments.

**Figure 1.** Comparison of MFI (geometric mean) of granulocytes (CD11b (A) and CD16 (C)) and monocytes (CD11b (B) and CD33 (D)) on both types of instruments. Each tick in the abscissa represents a different instrument. The dotted lines indicate 2SD of the mean. For each instrument, the triangle indicates the mean of the local series of peripheral blood. The monoparametric histograms in inserts are from merged samples

**Supplementary Table 1.** Combined percentages of unstained leukocytes (autofluorescence) above channel 1 on 23 instruments.

**Supplementary Table 2.** AML-1 tube of the GEIL panel. Configuration and volume for 10 and 8 colors instruments. All reagents in this study were from Beckman Coulter except V500-conjugates obtained from BD Biosciences.

Supplementary Table 3. Comparison of mean fluorescence intensity (MFI) of Granulocytes (CD11b, CD16, CD65), Monocytes (CD11b and CD33) and Lymphocytes (CD7).

**Supplementary Figure 1. 1A.** Gating strategy for evaluating the percentage of unstained lymphocytes (autofluorescence) above channel 1 on bi-parametric histograms. **1B.** Overlay display of the same unstained lysed blood sample acquired on the same day on a Navios (green) and on a Cantoll (red). This display shows the high comparability achieved for both instruments, in spite of the broader peaks obtained with Canto II.

Table 1.

<b>Laser/PMT</b>	<b>N</b>	<b>Mean</b>	<b>SEM</b>	<b>CV%</b>
Blue 1	23	100.87	0.29	1.4
Blue 2	23	100.26	0.50	2.4
Blue 3	16	100.76	0.45	1.8
Blue 4	23	100.70	0.50	2.4
Blue 5	23	101.12	0.66	3.2
Red1	23	101.58	0.83	3.9
Red 2	16	100.57	0.213	0.9
Red 3	23	100.10	0.31	1.5
Violet 1	23	100.77	0.50	2.4
Violet 2	23	100.07	0.27	1.2

Figure 1

