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Scientific Comment

Scientific comment on: “Quantitative flow cytometric evaluation of CD200, CD123, CD43 and CD52 as a tool for the differential diagnosis of mature B-cell neoplasms”<sup>☆</sup>



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Since the 1980s, multiparametric flow cytometry (FCM) was brought to the clinical laboratory for diagnostic immunophenotyping. The expression of antibodies bound to the membrane or intracellular receptors has been generally defined as positive or negative with a cutoff set relative to a nonstaining control population. It is known that the intensity of the fluorescent signal is proportional to the amount of antibody bound per cell, i.e., it is proportional to the number of antigen sites expressed. This means that the quantity of molecules per cell can be measured by the intensity of antigen expression.

One of the most important clinical utilities of fluorescence intensity measurements is the diagnosis of hematological malignancies, by which aberrant phenotypes are identified according to the over- or under-expression of various cellular proteins compared to those expressed in normal cells.

In principle, antigen detection by FCM is described qualitatively as having absent, bright or dim expressions. Advances over the past two decades have resulted in the development of FCM methods and materials that permit measurements of quantitative fluorescence with improved levels of control and interlaboratory precision. With these advances, interest in quantitative flow cytometry (QFCM) has grown as a

method to quantify the expression and activities of a variety of proteins and enzymes for diagnostic, prognostic, and therapeutic purposes.<sup>1</sup> Over the years, it has been recognized that quantifying the number of antigens per cell provides useful information that should be considered an intrinsic part of the cellular phenotype process.<sup>2</sup> A current example of the utility of QFCM is the therapeutic use of high doses of the monoclonal antibody (MoAb) anti-20 (Rituximab) for chronic lymphocytic leukemia (CLL), which has a dim expression of CD20 on the cell membrane.

QFCM based on mean fluorescent intensity (MFI) is relatively new and pending standardization which requires strict procedures compared to those needed for qualitative FCM analysis due to the requirements of precise quantitative and absolute results. MFI is proportional to the number of antibodies that recognize and bind to the cell antigens, allowing exact quantification of antigen expression per cell.<sup>3</sup> Quantitative measurement is based on the calibration of the fluorescence axis of the number of fluorochrome molecules attached to the cell or directly of the antibody binding capacity using standard beads.<sup>2</sup>

The regulation and standardization of MFI-based tests are still in their early stage although many MFI-based tests

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are being performed routinely as part of academic research studies.<sup>3</sup>

Some studies show that assays to evaluate MFI were not reproducible, intra- and inter-laboratory. MFI values have been reported in a variable range of the coefficient of variation percentage (CV%) between 7 and 33% depending on the MoAb tested, even on using the same equipment in the same laboratory, and also using the same donor blood on different days or at different times of the day.<sup>4,5</sup> This means that the whole process of FCM assays must be properly standardized and validated to allow reliable and reproducible MFI measurements that include pre-analytical and analytical procedures.

The strict application of the sample preparation protocols, the adequate MoAb titration, controls of reagent stability, the use of better MoAb-fluorochrome combinations and accurate data analysis are some of the most important tools to guarantee accurate results in FCM. In this sense, previous reports showed the effectiveness of standardization guidelines to achieve reproducible results.<sup>6,7</sup>

Despite all the variables involved, calibration of instruments for quantification of fluorescence measurements by FCM should be considered a routine and absolutely necessary procedure. Calibration beads are used to assure control of flow cytometer performance and laser stability. Furthermore, some kinds of beads can be used to set a fixed output value (target MFI) for each fluorescent channel. The setting of these target values in the instrument prior to each experiment ensure a more accurate and reproducible assay.<sup>6</sup>

Other methods for standardization of MFI are:

- (i) Converting MFI to known fluorescent units such as molecules of equivalent soluble fluorochrome (MESF). MESF beads can be used to create a standard curve of MFI versus MESF values and can translate MFI to more universal MESF units;
- (ii) Transforming MFI into antibody binding capacity units (beads with a known number of antibody binding sites are stained together with the cell samples so the MFI of the beads should reflect the number of molecules bound to a single cell);
- (iii) By using quantitative fluorescent beads labeled with a known number of phycoerythrin (PE) molecules, the curve of fluorescence intensity can be extrapolated to evaluate the number of PE molecules bound per cell.<sup>2,3</sup>

Regarding the article “Quantitative flow cytometric evaluation of CD200, CD123, CD43 and CD52 as a tool for the differential diagnosis of mature B-cell neoplasms” by Arlindo et al. in this issue of the *Revista Brasileira de Hematologia e Hemoterapia*, the authors should be encouraged to continue with this research. QFCM represents a developing field within FCM, which can provide an important understanding about cellular mechanisms in hematological and immunological diseases and consequently the use of such knowledge in their control.<sup>8</sup>

The authors showed their discernment by using protocols to maintain quality control, including instrument calibration, the process of sample preparation, data acquisition and analysis. However, the process of validation and titration

of the MoAb used, which led to the choice of these clones and fluorochromes for the research in question, was not shown.

Furthermore, it is important to improve the methods of MFI evaluation to make the analysis less subjective. FCM is a semi-quantitative method for diagnostic immunophenotyping, and sometimes it is criticized as being an ‘intuitive’ method of analysis. Thus, the rigorous maintenance of the standard operational proceedings in all the phases of the immunophenotyping process is essential to ensure reliable and reproducible results, minimizing the subjectivity of the method.

Regarding the sample, it is recommendable to increase all the study groups, except the CLL group, to allow a better evaluation of the variability of expression of each marker within each group and compare it with that of other groups. Despite of this, the results obtained with the expressions of the four AbMo chosen in the study were similar to those previously described in the literature.

Concerning the final diagnosis of the mature B-cell neoplasms (MBCN), it is important to emphasize the current limitations of FCM alone to establish the diagnosis of some of these neoplasms, such as lymphoplasmacytic lymphoma (LPL) and marginal zone lymphoma (MZL); the differential diagnosis between them by FCM is difficult and cannot be assumed without other auxiliary diagnostic methods, until new FCM strategies are designed. One attempt at comparing cases with the profile of similar diseases is the MBCN case database that is being developed by the Euroflow group.

Concluding, the initiative to implement QFCM in the diagnosis of MBCN is commendable and avant-garde in Brazil and should be stimulated. Congratulations to the authors!

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## Conflicts of interest

The author declares no conflicts of interest

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