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Review

Quantitative flow cytometry: Concerns and recommendations in clinic and research

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Abstract

Background

Quantitative flow cytometry (QFCM) can be an important element within the developing toolbox for clinical diagnostics which relies on precise and rapid tests that provide a conclusive answer for physicians. The FC technology combines all of these features. Until recently, this imperative discipline was based on qualitative assessments of cell populations. However, due to the enormous advancement in FC technology, which allows the quantification of a number of antigens on cell surface and within the cells by units of median fluorescence intensity (MFI), this method becomes meaningful and fits the clinical needs. **Methods**: On the basis of our experience in the field of quantitative FC, we wish to highlight some of the key concerns related to this methodology and suggest possible solutions for achieving uniform and standardized QFCM tests based on MFI. **Results**: Several parameters are responsible for inter and intra laboratory variations. The standardization of quantitative FC relies on three major components; Samples and reagents handling, FC maintenance and data analysis. The use of specialized beads as a part of the routine calibration process lowers inter-test variability between different operators and different FC instruments. Similarly, the use of agreed biological controls contributes significantly to lowering test variability. **Conclusions**: The field of QFCM displays a significant part in the diagnostic clinical toolbox. We believe that the recommendations described herein can improve significantly the stability and accuracy of this method, thus assuring a more standardized cell analyses. © 2017 International Clinical Cytometry Society

INTRODUCTION

Flow cytometry (FC) allows fast and quantitative examination of the physical characteristics of thousands to millions of .]1individual cells in heterogeneous populations as they flow in a fluid stream through a beam of light [

The FC technology enables processing a large number of samples in a short period of time, producing significant amount of clinical and diagnostic data. Indeed, clinical diagnostics in recent years relays significantly on FC. The common use of FC tests in the clinic continues to grow annually, and develops together with the increased numbers of newly discovered markers]. Together with the availability of flow cytometer instruments in medical centers, multiple 2that can be tested simultaneously [.possibilities for immunophenotyping and/or analyses of additional cell parameters are accessible

Until recently, major differences between various medical centers and operators were evident due to the fact that the field of]. In the past decade, due to the growing needs, a 3FC relies on self-instrument settings and self-interpretation of the data [significant change in the FC community arose as numerous specialized groups and consortia were established and dedicated to]. These groups worked together and had identified the 4-6the analysis, standardization and education in the FC field [underlying obstacles in the FC field towards a standard and uniform evaluation, in order to set guiding principles that overcome these limitations. For instance, the ICSH/ICCS group of experts published a special addition of clinical Cytometry part B issue, focused on providing the flow cytometer community with guidelines and reference document for flow cytometry]. Similarly, the Euro-Flow consortium that provided clear guidelines for the immunophenotyping and 6laboratories [ ]. This approach relies almost exclusively on qualitative evaluation of 3diagnosis of most frequent leukemias and lymphomas [.]4 ,1cell types and specific subpopulations without taking into account the amount of antigen presence [

Another approach, although less common, is to use the FC in order to quantify the median fluorescence intensity (MFI). The obtained MFI correlates with the number of antibodies that recognize and attach accordingly to the cell antigens, thus allowing]. The field of clinical QFCM based on MFI evaluation, is relatively 2 ,1exact quantification of antigen expression per cell [new and less established. Moreover, it requires strict standardization procedures as compared to those needed for the qualitative FC analysis due to the requirements for precise quantitative and absolute results. The number of clinical tests]. As of today, there are several clinical MFI-based tests of biomarkers, which 2utilizing MFI is still low, but slowly growing [have been previously published. For instance, Gaucher disease is diagnosed on the basis of enzymatic activity of GC (*β*-glucocerebrosidase) on a specific florescent substrate. The ability of the enzyme to process the substrate is measured based on]. Information of other MFI-7cells' MFI; Patients with lower enzyme function tend to show significantly lower MFI values [It is important to stress, that although none of these tests were approved by the FDA, .1based tests is summarized in Table] and others are 14 ,7some have been already routinely used in clinical practice, as laboratory developed tests (LDT) [

currently being tested in clinical trials such as the CD247 biomarker. This protein, expressed in T and natural killer (NK) cells, is able to sense the immune system function. Lower CD247 MFI levels indicate lower biomarker expression which is]. Another example is STAT-3 and STAT-5 phosphorylation. Stevens et al. tested [19 ,9associated with immune dysfunction [the changes in markers' phosphorylation using ΔMFI of Acute myeloid leukemia patients samples at two different disease]. Many additional MFI-based tests are being performed routinely as part of [20stages in response to ligand stimulation [academic research studies. The regulation and standardization of these MFI-based tests are still in their early stage, although]. On the basis of our experience in the field of QFCM, we [21 ,6important steps towards their optimization have been taken [aim to shed some new light on this expanding field by highlighting some of the key concerns and possible solutions for .achieving uniform and standardized MFI-based tests

Table 1. List of Clinical Biomarkers Tests Based on Quantitative Flow Cytometry and MFI

Reference	Test reference	Kit available	Used in clinic	Biomarker/enzyme activity	Disease	Test name
[32]	Internal Control	licensed) + from trillium (diagnostic	+	CD64 expression in neutrophils	Sepsis	LeukoDx (formally known as leuko 64)
[10]	Internal control (negative cell population) & Biological Control (commercially available preserved blood)	In development	Clinical trials	CD247 in T cells	Cancer/chronic inflammation	CD247
[33]	Internal control (negative cell population) & Biological Control (commercially available preserved blood)	In development	–	SNX9 In T cells	Cancer/chronic inflammation	SNX9
[29 ,25]	Internal Control based on negative and positive cell population within the sample	–	–	ZAP-70 expression in CLL B-cells	chronic lymphocyte leukemia (CLL)	ZAP-70
[34]	Healthy donor	–	–	CD25 expression in CD4+ cells	Acute lymphocyte leukemia (ALL)	CD25
[7]	Healthy donor	–	+	GC (β-glucocerebrosidase) enzyme activity	Goucher	Goucher diagnosis
[8]	Healthy donors	In clinical labs The OxyBurst assay kit (DCFDA)	+	NADPH activity	CGD	Dihydrorhodamine (DHR) flow cytometric test
[14]	Healthy donors	–	+	Perforin content/cell	Chronic fatigue syndrome	Perforin content
[36 ,35]	Healthy donors/family members	–	+	SAP/XIAP/XLP in NK and T cells	X-linked lymphoproliferative disease	SAP/XIAP/XLP
[37]	Healthy donors/family members	–	+	T cell proliferation test	Immunodeficiency diseases	CFSE proliferation test

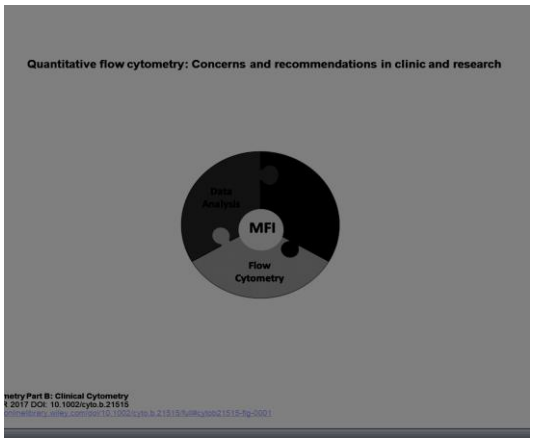
REPRODUCIBILITY OF MFI VALUES ACROSS EXPERIMENTS

There are few key components that form the basis of any quantitative test and each of them is crucial for proper test validation;]. If MFI evaluation is successful in meeting 22accuracy, precision, sensitivity, specificity, stability, and control materials [these criteria, it can serve as a powerful tool for assessment of cell state, cell activity, and various disease conditions affecting]. However, several studies have previously shown that there is a considerable variation in the [15 ,7such cell parameters [obtained MFI values using the same test between different centers (inter-laboratory differences). These variations can be monitored using the coefficient of variation percentage (CV%) values, which measure the inter-test imperfections and the tests' measuring range. CV% is calculated as the standard deviation of the tests divided by mean value of the tests ×100.]. In fact, even when using the same equipment in the same [23 ,5Lower values indicate that the variability in the assay is low [ ]. We [25 ,24laboratory, a variation occurs and the CV% values can vary between 7 and 33% depending on the tested marker [

found that MFI values of various markers (CD3, CD4, CD56 etc.) in blood samples derived from the same donor can be .significantly altered when tested in different days or even hours with CV% reaching up to 30%

In summary, MFI measurement can be affected by multiple parameters such as: (1) Sample and reagent handling including]. These [26 ,23 ,21 ,1[1standard operating procedure (SOP), (2) FC instrument and (3) Data analysis as depicted in Figure .parameters are reviewed below

.Figure 1



.Graphic presentation of the different aspects that impact flow cytometer-based quantitative fluorescence

SAMPLE HANDLING AND REAGENTS' INTEGRITY

Differences in MFI values may arise, in many cases, from biological variables that can be avoided. As a first line of flow cytometry standardization, the Euro-Flow consortium has specified guidelines for flow cytometry samples' handling. These guidelines relate to many aspects of sample handling including flow cytometer settings, recommended antibody panel to be]. It is highly recommended to follow these guides for all FC experiments 3used, compensation settings and antibody titration [.in order to get unified baseline settings and conditions

In addition, the importance of consistent and meticulous sample handling, was previously shown at length; The composition of cell populations, markers expression levels and cell function, all can be effected depending on the time elapsed from blood drawing or cell isolation, type of the used anti-coagulant, sample storage conditions (temperature, light, etc.) and interval until .][27 ,2samples are processed [

Additional obstacle in obtaining steady MFI acquisition is the use of improper reagents and antibodies. There is a considerable significance to a proper antibody titration prior to its routine use. For example, using a non-optimal concentration of an]. In addition, different fluorochromes' [23 ,3antibody can result in inaccurate staining of the positive cell populations [sensitivity and brightness should be taken into account when designing FC assays. Similarly, the MFI is highly affected by .]. For example a lot-to-lot variation may be the cause for the MFI variations[21reagent integrity and proper quantities [

Implementing strict guidelines regarding sample processing from the time of blood withdrawal through sample storage and .reagents handling until sample staining, will significantly contribute to the results' stability

STAINING PROCEDURE AND CROSS-EXPERIMENTAL STANDARDIZATION

Inter-laboratory differences are one of the important issues in diagnostics. The ability to compare and standardize results between various laboratories and analyses done in different days is a crucial issue. Similarly, when comparing MFI values across different tests, it is critical to obtain consistent values as diagnosis is based on such parameters with specific threshold. During clinical trials, most of samples analyses are performed during different days and usually multiple medical centers are involved. At the end of the process, all the data obtained from the different test points and centers is gathered and compared. Therefore, the obtained MFI values must be standardized. From our experience, MFI values vary considerably when testing, .even the same subject, during different days without the proper standardization of the staining procedure (Unpublished Data)

Defining clear SOPs for each procedure and supplying suitable training, unifies the work process between different laboratories and operators and consecutively minimizes deviations from the used protocol. When adding to the SOP regular quality control (QC) tests (e.g., comparison between the values obtained from different operators when processing the same sample), one can monitor inter-laboratory performance. When done properly, QC tests can even detect and identify]. There are organizations that provide external quality [21mishandling in reagents, staining procedure or data acquisition [assessment and training by issuing stabilized patients' blood samples for specific flow cytometry procedures [e.g., UK

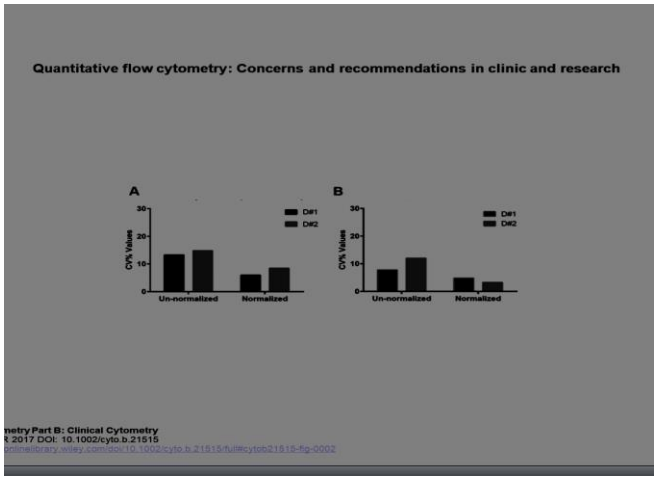
National External Quality Assurance Scheme (UK NEQUAS)]. This valuable service evaluates laboratory's ability to correctly diagnose a particular condition. In addition, there are several organizations that currently operate to guarantee adequate laboratory performances [e.g., as Clinical Laboratory Improvement Amendments (CLIA) or The College of American]. 28-30Pathologists (CAP)], via special accreditation programs, that aid laboratories to operate at high working standards [.Getting the assistance of these organizations will significantly improve the capability to maintain standard MFI results

FC CALIBRATORS AND BIOLOGICAL CONTROLS

The standardization and normalization of flow cytometry experiments relies on two key components; the staining procedure (control) and the FC instrument (calibrator). In many cases, confusion occurs between the two concepts. To clarify this]. Briefly, the definition of control is a 23ambiguity, we followed the definition as described previously by Hoffman et al. [stable material, which gives reproducible results at each analysis. A control sample is usually prepared in same manner as the test sample which produces an expected result (not necessarily with specific values). A calibrator is a prepared sample of particles that have known value of chosen characteristics, which are used for FC adjustment and determining accurately the .amount of florescence of a specific sample

The ideal reference and normalizing agent for each experiment is a biological control. This type of control can serve as QC and normalization value for the staining process, the antibodies and the flow cytometer, all at once. In this methodology, the biological control is being analyzed with the test samples, using the same process. Later, it can serve as a normalizing reference when comparing analyses from different days/laboratories. To this end, many laboratories currently use fresh blood]. However, due to the lack of availability of healthy donors' blood samples 31 ,22 ,2samples from random healthy donors [together with the physiological differences between various healthy donors, this method gives only a partial solution. Taking this approach one step further, involves the establishment of a "bank" of healthy donors. The values of an antigen expression in the healthy (controls) samples together with the characterization of a tested sample, allows representation of the obtained MFI as a percentage out of 100%. In this method, the 100% is calculated according to the average MFI values of the constant healthy subjects group, which serve as a relative reference between different analyses. A more controlled methodology, which allows a better test reproducibility, is based on available biological control products including: Preserved control blood samples as CD Chex Select, (Streck) and lyophilized peripheral blood mononuclear cells (PBMCs). The use of preserved control blood samples, in order to normalize the MFI values of blood tests analyzed by different operators, significantly lowers Similar outcomes could be achieved when normalizing .2the inter-test differences and CV% values as demonstrated in Figure results from the same donor in different days. However, inter-laboratory variance is a more complex mission when comparing simultaneously data from different medical centers and should be carefully addressed. This can be achieved by using the same]. Another 32lot of commercially available biological controls as early mentioned or a custom-manufactured reference [method for validating and normalizing FC analyses is the use of an internal control; in some samples there is a cell population in which the tested biomarker is not present or expressed at a low level. The obtained MFI values of this population can be used as a control reference or alternatively, can be integrated in a specialized algorithm to normalize the procedure as demonstrated by Shankey et al., ZAP-70 expression index, which can indicate patient's disease severity in B cell chronic lymphocytic leukemia (CLL), is calculated based on an integration of the marker's MFI values in a number of cell populations present in patient's blood; CLL cells, B cells (negative population) and T cells (positive population). Consequently, ZAP-70]. Indeed, the use of 11expression is represented by a numeric value between 0 and 100 that indicates disease severity [biological controls can significantly improve tests accuracy and reproducibility and allow proper comparison between different .experiments

.Figure 2



Quantitative FC normalization using biological control. CV% of fresh whole blood staining MFI values of 2 healthy subjects (D#1 and D#2) performed by three different operators before and after normalization using preserved blood (Streck). (**A**) .Intracellular staining for the CD247 marker in T cells. (**B**) Surface staining for the CD3ε marker in T cells

THE FLOW CYTOMETER INSTRUMENT

Flow cytometer is a highly complex instrument, which has to be routinely calibrated and maintained in order to provide]. To obtain the finest results, a system calibration based on various parameters such as optical [1accurate and precise outputs filter alignment, laser power check, photomultiplier tubes (PMT) voltage linearity and so forth, must be performed with instrument setup. Moreover, following initial set-up, the FC must be routinely monitored, as it has a great impact on the validity of the results. Even when the system is calibrated, FC output tend to vary due to differences in the cytometers' .]33configuration and adjustment [

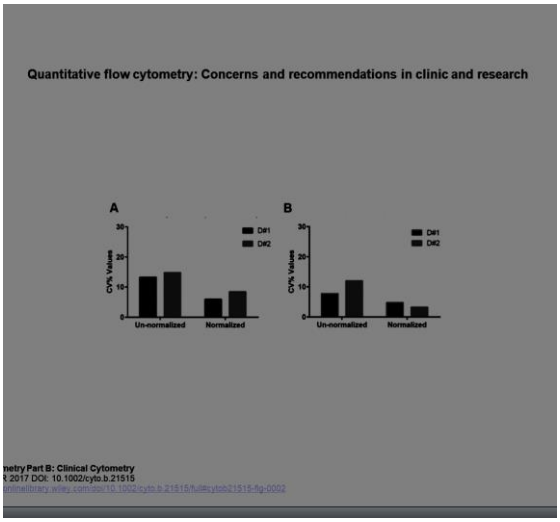
The immense progress in different combinations of fluorochromes availability allowed the use of diverse panels of markers. However, the use of multiple fluorochromes can increases the risk for spectral overlap, where a particular fluorochrome is detected in more than one fluorescent channel and thus, leading to false negative readings. On the other hand, suboptimal settings can result in incorrect data interpretation or skewed values. Hence, proper compensation subtracting the spectral .overlap and proper settings must be applied to achieve accurate results

In addition, setting the appropriate parameters prior to flow analysis in different instruments by different operators will surely]. Moreover, reading the same [3result in different MFI values, even for the same sample, due to flow cytometers variability sample analyzed in different days at the same cytometer with the same settings (PMT voltage), can also result in a variation in .]1the sample's MFI, which can be attributed to loss of sensitivity and instrument aging due to long term usage [

FLOW CYTOMETERS STANDARDIZATION: CALIBRATION BEADS

The use of calibration beads such as Rainbow beads (Sperotech) can significantly improve results' stability. These beads are]. The beads can be used for a [23particles labeled with a mixture of fluorochromes at different fluorescent intensity levels variety of FC calibrations; starting with quality control for FC performance and laser stability, as previously recommended by]. In addition, rainbow beads allow setting a fixed output value (target MFI) for each [30 ,29 ,3 ,1EuroFlow and ICSH/ICCS]. The target MFI is set prior to each experiment, ensuring that the reading is done in the correct [34 ,33 ,3fluorescent channel and standardized settings that are more accurate than using the same PMT settings through different experiments. In some flow cytometers from the "new generation," an automated calibration process is integrated in the instrument, which enables accurate calibration across different days or even various cytometers from the same model. Moreover, laser intensity tends to change throughout the day, sometimes even during data acquisition, especially when a large number of samples is being analyzed. Therefore, rainbow beads can be used to monitor FC performance during data acquisition. The beads alongside with the samples and confirming target MFI stability will ensure that no deviation in the results will occur due to FC demonstrates the beneficial effect of using the target MFI approach (set with Rainbow beads), [3malperformance. Figure which enables obtaining similar MFI values, when the same samples had been acquired across different flow cytometers. It is important to stress, that variances between different flow cytometers (such as BD and Beckman coulter flow cytometers), .]35should be factored in, when setting target MFI values [

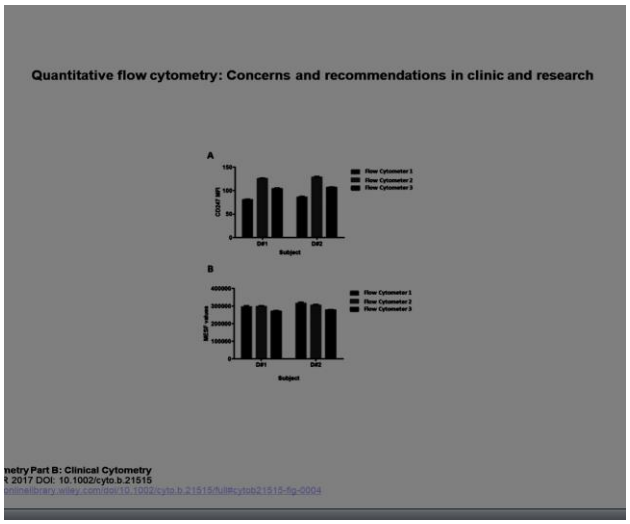
.Figure 3



MFI values with and without target MFI normalization. Normalization of fresh whole blood staining acquisitions using two different models of BD FACSCalibur (BD biosciences) flow cytometer according to target MFI set with rainbow beads (Biolegend) or PMT values. Staining was performed for surface (CD4, CD8, CD15, and HLA-DR) and intracellular (CD247) markers. Significant differences were observed between MFI values of the same sample in different instruments before .normalization (CV% 25–50%) and after normalization with target MFI (CV%- 3–15%)

Another method for standardization of MFI is converting MFI to known fluorescent units such as molecules of equivalent soluble fluorochrome (MESF). The MESF concept is based on the fact that a sample labeled with a specific fluorochrome has]. By this 12the same MFI value as a fluorochrome solution with an equivalent number of free fluorochrome molecules [principle, designated MESF beads can be used to create a standard curve of MFI versus MESF values can translate MFI to more universal MESF units. This in turn, allows comparison between analysis at different days and/or different flow cytometers. It should be noted that it is recommended to further calibrate the assay with cells or particles labeled with a known shows that the results from different flow cytometers with MESF values 4]. Figure 36 ,5 ,2 ,1number of fluorochromes [acquired with MESF beads (Bang laboratories), had similar outcome as opposed to settings set based on negative population expression. Furthermore, the two methodologies can be combined to receive the best inter-assay precision; first setting target .]34MFI with the aid of the rainbow beads followed by conversion of the MFI values of the sample to universal MESF values [

.Figure 4



Quantitative florescence normalization between different flow cytometers. Normalization of fresh whole blood samples using MESF beads (Bang laboratories). (**A**) MFI values of blood samples from two healthy subjects (D#1 and #2) were acquired

using three different flow cytometer instruments (BD FACSCalibur (BD biosciences, Gallios flow cytometer (Beckman coulter) and Mylteniy flow cytometer (Mylteniy Biotec) without any normalization process. (**B**) MFI levels were normalized by converting them to MESF values as per the manufacturer instructions. Significant differences were observed between MFI .(values of the same sample in different instruments (CV% 19–21%) and after conversion to MESF units (CV%- 3–15%

Similarly, MFI can be transformed into antibody binding capacity (ABC) units. In this approach, beads with a known number of antibody binding sites are stained together with the cells/blood samples. Under optimal conditions, the MFI of the beads]. In this method, it is recommended as well to verify the 36 ,2should reflect the number of molecules bound to a single cell [results using cells with a known number of surface molecules. Another approach to quantitate fluorescence is by utilizing QuantiBRITE beads. These beads are labeled with a known number of PE molecules. Standard curve of fluorescence intensity can be generated if the antibody-fluorophore ratio is known and can be extrapolated to evaluate number of PE molecules bound per cell. In addition, specialized micro particles with capture antibodies that bind antibodies in a homogenous mode combined with low auto-fluorescence values are commercially available. These in turn, can be used as calibrators for each .]33fluorescent channel enabling proper adjustment of compensation in more controlled and homogenous system [

DATA ANALYSIS

The final, yet crucial step, in flow cytometry is the data analysis. In this process, gates are defined based on the positively and negatively stained cell populations. MFI values are calculated for the desired cell populations. In many cases, a dramatic MFI variation results from incorrect or nonunified gating strategy thus, data analysis should be done by a qualified and trained]. In addition, there is growing evidence that data analysis should be done in a central location to ensure minimum 12operator [ ]. As of today, with the advancement in the field of FC, great amount of data is generated during clinical 26 ,5deviations [trials. This data is then often processed using automated analyzing software. This method, although eases and expedite the analysis process, can introduce many downstream errors due the fact that the populations are not properly aligned in all the analyses. For this reason, analysis strategies should be carefully carried out and included in the QC process. It is important to .]37note that several computational tools have been developed to normalize data via suited algorithms [

CONCLUSIONS

QFCM has a major impact in the field of diagnostics. This review highlights the need for standardization of quantitative clinical cytometry and focuses both on the major obstacles that prevent a wider use of this discipline and on possible solutions based on the following aspects: (A) Samples and reagents handling; including working with strict SOPs as per biological sample handling and reagents integrity, propounding QC process, well-tittered antibodies and biological control as reference in each test. (B) Flow cytometer; including the instrument maintenance and daily QC tests, its calibration using relevant beads and setting of target MFI values prior to each test. (C) Data analysis; we recommend that the data analysis will be performed in .a central location by a qualified operator

.Adoption of these recommendations will increase the impact of this method both for research and its clinical implementation

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