Journal of Immunological Methods 475 (2019) 112680

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

Comments on EuroFlow standard operating procedures for instrument setup and compensation for BD FACS Canto II, Navios and BD FACS Lyric instruments

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ARTICLE INFO

Keywords:
Flow cytometry
Standardization
Instrument setup
EuroFlow
BD FACSLyric
Navios

ABSTRACT

This commentary discusses particularities of application of the EuroFlow standardization of flow cytometric analyses on three different flow cytometers. The EuroFlow consortium developed a fully standardized approach for flow cytometric immunophenotyping of hematological malignancies and primary immunodeficiencies. Standardized instrument setup is an essential part of EuroFlow standardization. Initially, the EuroFlow Consortium developed and optimized a step-by-step standard operating procedure (SOP) to setup 8-color BD FACSCanto II flow cytometer (Canto), with the later inclusion of Navios (Beckman Coulter) and BD FACSLyric (Lyric). Those SOPs were developed to enable standardized and fully comparable fluorescence measurements in the three flow cytometers. In Canto and Navios, mean fluorescence intensity (MFI) of a reference peak of Rainbow beads calibration particles is used to set up photomultiplier (PMT) voltages for each detector channel in individual instruments to reach the same MFI across distinct instruments. In turn, a new feature of Lyric instruments allows to share collection of attributes that are used to place the positive population at the same position among instruments in the form of assays, as one of its components integrated in the Cytometer Setup and Tracking (CS&T) module. The EuroFlow Lyric assays thus allow for standardized acquisition of 8-color EuroFlow panels on Lyric without the need to setup the PMT voltages on the individual instruments manually.

In summary, the standardized instrument setup developed by EuroFlow enables cross-platform inter- and intra-laboratory standardization of flow cytometric measurements. This commentary provides a perspective on the modifications of the standardized EuroFlow instrument setup of Canto, Navios and Lyric instruments that are described in detail in individual instrument-specfic SOPs available at the EuroFlow website.

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https://doi.org/10.1016/j.jim.2019.112680
Received 7 June 2019; Received in revised form 6 October 2019; Accepted 7 October 2019
Available online 23 October 2019
0022-1759/

1. Introduction

Standardization of flow cytometry measurements is crucial for obtaining robust and reproducible data, comparable at both intra- and inter-laboratory levels over time with automated data analysis tools.

The EuroFlow Consortium (www.euroflow.org) developed a fully standardized approach for flow cytometric immunophenotyping, from instrument setup through sample preparation, antibody panels for hematological malignancies (Flores-Montero et al., 2017; Kalina et al., 2012; Theunissen et al., 2017; van Dongen et al., 2012) and primary immunodeficiencies (Blanco et al., 2017, 2018, 2019; van der Burg et al., 2019; van der Velden et al., 2017) to innovative software tools and data analysis (Costa et al., 2010; Lhermitte et al., 2018; Pedreira et al., 2013).

Instrument setup is a key step in the EuroFlow standardization. Initially, the EuroFlow consortium developed and optimized a step-by-step standard operating procedure (SOP) for instrument setup for the 8-color BD FACSCanto II flow cytometer (Becton Dickinson (BD), San Jose, CA, USA), which at that time (2006) was the only 8-color flow cytometer available for clinical flow cytometry. This protocol was also applied to other ≥8 color instruments available at that time, such as the BD LSR II (Becton Dickinson) and Cyan ADP (DakoCytomation, Glostrup, Denmark/Beckman Coulter, Brea, CA, USA) which were mainly used for research purposes (Kalina et al., 2012). Since an increasing number of flow cytometers capable of ≥8 color measurements has become available in diagnostic laboratories, questions about the potential applicability of EuroFlow standardized protocols to other ≥8-color cytometers have emerged.

Overall, any instrument equipped with 405 nm, 488 nm and 633-640 nm excitation laser lines and at least two, four and two detectors with independent voltage settings for each excitation line, respectively, meets the technical requirements for acquisition of the 8 colors used in EuroFlow antibody panels (Nováková et al., 2017).

For standardized instrument setup according to EuroFlow, mean fluorescence intensity (MFI) of a reference peak of Rainbow beads calibration particles (Spherotech, Lake Forest, IL, USA; hereafter referred to as Rainbow beads) is used to set up photomultiplier (PMT) voltages for each fluorescence detector in individual instruments to reach the same MFI in all instruments (Kalina et al., 2012)(www.euroflow.org). Rainbow beads are internally dyed fluorescent particles (hard dyed beads), containing bright signal beads in every detector, classified as type IIIA beads(Schwartz et al., 1998), with documented stability of fluorescence intensity. However, Rainbow beads fluorescence is not spectrally matched to the individual fluorochromes used in ≥8-color immunophenotyping panels (Nováková et al., 2017).

Since different instruments from the same or different manufacturers differ both in their dynamic range (scale) and emission filters, adjustment of PMT voltages (PMTV) for a given emission filter and fluorochrome pair is essential in order to obtain comparable data across different instrument platforms. To set up standardized and fully comparable fluorescence measurements in BD FACSCanto II, BD FACSLyric and Navios flow cytometers (hereafter referred to as Canto, Lyric and Navios, respectively), EuroFlow has defined specific MFI target values and tube target values (TTV) for each emission filter and fluorochrome pair for Navios and Lyric, respectively. Spectral adjustments were made using FC-beads, Type IIB (spectrally matching) particles (Schwartz et al., 1998) as illustrated elsewhere (Nováková et al., 2017) (for Navios). Adjusted TTV are available in the form of Lyrics' "assay files" (downloadable on www.euroflow.org) and CST beads (Type IIIA) are used for daily setup of BD FACSLyric. (Please see Table 1 for a summary of the most relevant technical features of the three instruments).

Thus, the standardized instrument setup protocols developed by EuroFlow enable cross-platform inter- and intra-laboratory standardization of flow cytometric measurements. The procedures for EuroFlow standardized instrument setup adapted to individual instruments (Canto, Lyric and Navios) are described step by step in SOPs available at the EuroFlow website (www.euroflow.org). We recommend that the EuroFlow website is regularly checked for newest versions of SOPs.

The specific adaptations required in instrument setup SOPs for specific instrument are discussed here, while a summary of the individual steps is provided in Table 2 and instrument specfic SOPs are also supplied for Canto (Supplementary document 1), Navios (Supplementary document 2) and for Lyric (Supplementary document 3).

Table 1
Overview of lasers and emission filters in Canto, Lyric and Navios.

Dynamic range (Number of channels)	BD FACS Canto II	BD FACS Lyric	Navios
	18-bit (262144)	18-bit (262144)	20-bit (1048576)
Excitation lines (emmission wavelengh, power)			
Violet laser	*405 nm*	*405 nm*	*405 nm*
	30 mW	*40 mW*	*40 mW*
Blue laser	*488 nm*	*488 nm*	*488 nm*
	20 mW	*20 mW*	*22 mW*
Red laser	*633 nm*	*640 nm*	*638 nm*
	17 mW	*40 mW*	*25 mW*
Emission optics for fluorochromes in 8-color EuroFlow panels			
Pacific Blue (V450)	450/50	448/45	450/50
Pacific Orange (OC515, V500)	510/50	**528/45**	**550/40**
FITC	530/30	527/32	525/40
PE	585/42	586/42	570/30
	–	–	620/30
PerCP-Cy5.5	670LP	**700/54**	695/30
PE-Cy7	780/60	783/56	**755 LP**
APC	660/20	660/10	660/20
	–	720/30	725/20
APC-C750 (APC-H7)	780/60	783/56	**755 LP**

FITC: fluorescein isothiocyanate, PE: phycoerythrin, PerCPCy5.5: Peridinin-chlorophyll-protein-cyanin5.5, Cy7: cyanin7, APC: allophycocyanin, H7: hilite 7, OC515: Orange Cytognos 515.
Bold: Filters where visible differences were noted when comparing spectrally unmatched and matched controls.

2. Commentary on EuroFlow SOPs for instrument setup

2.1. Instrument configuration

Canto, Lyric and Navios instruments equipped with 3 lasers (blue, red, violet) are used in a standard optical configuration. The cytometer optical configuration depends on the lasers, filters, mirrors and detectors installed on the instrument. Importantly, Navios is supplied with two alternative emission filters: 695BP30 and 675BP20 for the red laser channel FL4 in which e.g. PerCP-Cy5.5 is detected. For the Euroflow standardized setup, the 695BP30 filter has to be installed. Detailed information on lasers and emission filters for the three instruments is displayed in Table 1.

Names of all fluorochromes used in EuroFlow antibody panels have to be included in the cytometer configuration. The standardized EuroFlow nomenclature for fluorochromes and labels is available at the EuroFlow website (www.euroflow.org) and allows for unambiguous labeling of markers (fully) comparable to the EuroFlow databases. In Canto instruments, a custom "EuroFlow" configuration needs to be created (section 2 of Canto SOP). For Lyric instruments, it is necessary to add the EuroFlow names of detector channels in the format with underscore (e.g. "_PE") to the instrument configuration and assign them to the appropriate detectors. Importantly, Lyric software assigns "fluorochrome" as a name of detector channel, while "label" denominates the marker used (e.g. CD3) and "reagent" stands for a particular antibody conjugate (e.g. CD3 APC). For each reagent, its lot number and expiration date can be registered in the library. In Navios instruments, the EuroFlow parameter names can be assigned to detectors

Table 2
Summary of EuroFlow recommended approach to instrument setup and performance monitoring for Canto, Lyric and Navios flow cytometers.

Instrument setup	BD FACS Canto II	BD FACS Lyric	Navios
Fluorochromes	Use EuroFlow nomenclature (www.euroflow.org) type in manually	Use EuroFlow nomenclature (www.euroflow.org) type in manually into Library OR import reagent list from EuroFlow website into the Library	Use EuroFlow nomenclature (www.euroflow.org) type in manually
Cytometer Configuration	Create new Configuration with EuroFlow fluorochromes (SOP Section 2) Run new baseline (BD FACSDiva software manual)	Add fluorochromes to configuration and assign to detectors (SOP part B, Section 3)	Verify that the 695 BP30 filter is installed in FL4 channel (SOP Section 2)
Labels (e.g. CD3)	Use EuroFlow nomenclature (www.euroflow.org) Type Label names in the Labels tab of the Experiment Layout	Import the Label list from EuroFlow website (www.euroflow.org) into Library (SOP part B, section 3) OR add labels manually to the Library (Lyric manual)	Use EuroFlow nomenclature (www.euroflow.org) Type Label names in the Cytometer control window, change parameter Stain names $PnS in Edit FCS Header (SOP section 3, Fig. 3 and 6)
Reagents (e.g. CD3 APC)	–	Add reagents to the Library, assign expiration date and lot number (SOP part B, section 3)	–
Perform PMTV setup	Rainbow beads target MFI values	Import TTVs for EuroFlow panels	Rainbow beads target MFI values
Experiment/Assay	Create new experiment for cytometer setup Select parameters, labels	Import EuroFlow assays from the EuroFlow website Assign lots of the lot specific reagents in the library for each assay	Perform cytometer setup in a dedicated experiment (protocol)
Establish PMTVs for Experiment/Assay	Type in PMTVs from Rainbow setup Set FSC and SSC (EuroFlow target values for Lymphocytes)	Perform Tube settings and assay setup	Type in PMTVs from Rainbow setup set FSC and SSC (EuroFlow target values for lymphocytes)
Compensation	Create Compensation controls Run compensation control tubes (SOP Section 5)	Add compensation control tubes via “Add fluorochrome” to Lyse Wash reference settings Run compensation control tubes (SOP section 5)	Create Compensation experiment (protocol) Run compensation control tubes (SOP section 5)

Instrument performance monitoring	BD FACS canto II	BD FACS lyric	Navios
	Run CS&T daily performance check Run Rainbow beads daily monitoring (SOP section 6)	Run Performance QC and Assay/Tube settings Setup (SOP part B section 4)	Run FlowCheck Pro Fluorospheres Run Rainbow beads daily monitoring (SOP section 6)

EF: EuroFlow, SOP: standard operating procedure, MFI: mean fluorescence intensity, TTVs: tube target values, PMTV photomultiplier voltages, CS&T: Cytometer Setup and Tracking.

under “label” in the cytometer control window. To export the individual parameter names within the FCS file, the FCS header has to be edited as well (chapter 4.2 of EuroFlow Navios SOP).

2.2. Setup of FSC and SSC parameters

For setup of scatter parameters, fresh peripheral blood of a healthy donor should be used and processed as described in the Euroflow SOP for instrument setup and compensation, Section 3. Since the optimal staining protocol includes BD FACS Lysing solution (BD Biosciences), scatter settings should be defined using that lysing buffer; other lysing solutions might give different results and therefore should not be used for setup of scatter parameters.

Signal area and height (integral and peak in Navios, respectively) should be activated and recorded for at least FSC and optionally for SSC, to allow for doublet discrimination.

The forward scatter detection in the Navios flow cytometer is possible in three software controlled settings: wide (1 to 19° angle collection), narrow (1 to 8° angle collection) and wide enhanced. For IVD applications, the wide setting should be used. As the FSC collection by Navios slightly differs from Canto and Lyric, the full inter-instrument standardization of FSC and SSC signals remains a challenge. Thus Navios users should verify that all populations of interest are visible within the scale in the FSC/SSC dot plot.

It should be noted, that FSC/SSC setup adjusts the position of lymphocytes, but position of other cell types with higher FSC or SSC cannot be user adjusted relative to lymphocytes, since the scatter parameters are dependent on particular construction features of each instrument. Consequently, even if lymphocytes are well positioned with respect to FSC and SSC, other populations (e.g. neutrophils and monocytes) may have somewhat different positions in the scatter plot.

2.3. Setup of PMT voltages for target fluorescence channels

The MFI of the 7th peak of Rainbow beads is used to set up the PMTV for each fluorescence detector to reach the same MFI in Canto and Navios instruments. Target MFI values may vary for different lots of Rainbow beads and may differ from those listed in the EuroFlow SOPs for Canto and Navios cytometer setup, respectively (Table 1, Section 4). Updated information on lot-specific target MFI values is available at the EuroFlow website (www.euroflow.org). When using other fluorochromes than those listed in Table 1 of the SOPs, for which the target MFI values were defined, standardization will be compromised.

Lyric users have the possibility to import 8-color EuroFlow assays that are available for download at the EuroFlow website (www.euroflow.org). The assay contains all information necessary for standardized acquisition of the EuroFlow antibody panels except for the compensation that needs to be created on each individual instrument. The detailed procedure for importing and running EuroFlow assays is described in the EuroFlow SOP for Lyric instrument setup and compensation, Part B.

While fluorescence intensity scale used in software by Canto and Lyric uses 18-bit scale, the Navios instrument uses 20-bit scale. However, to display data on 20-bit scale on the Navios, a TrueView Settings Set 1 should be selected. Upon loading the Navios LMD files in Infinicyt software, the scale can be adjusted by selecting “convert data to the scale of Canto files” so that the MFI values from Canto and Navios files can be compared on the same scale.

Next, for correct setting of fluorescence PMTV, it is crucial to perform the setting in a dedicated experiment (Canto, Lyric) or protocol (Navios) without compensation. For Canto and Lyric instruments, “Enable Compensation” option has to be deselected in cytometer settings window and tube properties, respectively. Lyric Tube settings include a collection of attributes that are used to place the positive population at the same position (brightness) whenever the tube settings are applied to tubes. These values are called Tube target values (TTVs). TTVs determine the median target channel of the positive population for each fluorescence and scatter parameter. In fact, TTVs are the ratio of median fluorescence intensity to the assigned BD unit (ABD) and determine the voltage of a detector. TTVs are created and saved within each tube settings. Thus, tube settings allow the system to produce comparable results from day to day and from cytometer to cytometer. Importantly, tube settings can be exported and shared among various Lyric cytometers.

When importing the tube settings or assays, any new fluorochrome has to be assigned to the appropriate detector in cytometer configuration (refer to BD FACSLyric Reference Manual for details). Subsequently, new fluorochromes and label-specific reagents have to be added to Lyse/Wash reference settings through Add fluorochrome (refer to Section 2.4 below) so that the appropriate Spillover values (SOVs) can be calculated.

2.4. Fluorescence compensation settings

Fluorescence compensation setup on Canto and Navios may only be performed after the Target MFI settings have been established.

2.4.1. Creation of compensation setup control tubes

Spillover values on all cytometers should be determined using single-color compensation controls. An unstained control is required as well, in a separate tube or in the same tube as the single-stained controls. Strikingly, most errors in calculating SOVs are due to the use of inappropriate compensation controls (Kalina et al., 2018; Glier et al., 2017; Cossarizza et al., 2017).

For correct performance of compensation setup, it is critical to adhere to the rules for good compensation, as described in detail by Cossarizza et al. (2017). These rules can be summarized as follows: 1) the fluorescence spectrum of the compensation control fluorochrome-conjugated reagent should be identical to the reagent used in the experiment. This is important especially for tandem reagents (e.g. PE Cy7, APC Cy7) where there can be significant spectral differences among various reagent lots and/or different vendors, leading to requirement of reagent specific SOV; (Tung et al., 2004) 2) autofluorescence levels of positive and negative populations must be equivalent, in other words, the same material type (cells-cells or beads-beads) and/or the same cell type and cell subset (lymphocytes-lymphocytes) should be used as positive and negative reference populations in single-stained tubes; 3) the positive population should be as bright as possible, but never dimmer than the actual measured fluorescence on the investigated cells; 4) enough events need to be collected to obtain meaningful accurate SOVs. In Diva software, at least 1000 events for both negative and positive populations are required. The compensation calculation is optimal under the assumption that detectors perform linear measurements (linearity measurements are either performed by the dedicated software module or can be characterised by Perfetto's Quality Assurance protocol (Perfetto et al., 2012)).

A list of recommended fluorochrome-conjugated antibody reagents for compensation controls for each individual fluorochrome and tandem dye used with the EuroFlow panels on the Canto and Navios is provided in Table 2 of the EuroFlow SOP for instrument setup. However, each user must verify that compensation reagents match with the actual reagents used in the panel since e.g. alternative reagents are not listed in the SOP, individual users might not need some compensation controls if they do not use particular panels and divergent spectral properties of different manufacturing lots should be taken into account).

The names of compensation control tubes should follow the EuroFlow nomenclature for labels and antigens for Canto, Lyric and Navios instruments (www.euroflow.org).

In Canto instruments (FACSDiva software), one compensation setup experiment may contain several compensation control tubes for each fluorescence channel. Thus, all fluorochrome-conjugated reagents used

in various antibody panels may be included in one compensation setup experiment as long as the same PMTVs are used. In contrast, in Navios instruments, only one fluorochrome can be assigned to each fluorescence channel within the experiment. As a result, several "compensation setup experiments" have to be created for Navios, each for a given combination of fluorochromes.

In Lyric, reference settings contain SOVs based on measured single-stained tubes. For tube settings without associated reference settings, the compensation matrix is calculated using the SOVs from the Lyse/ Wash reference settings. SOVs for individual fluorochromes can be added to Lyse/Wash reference settings by measuring single-stained compensation control tubes. Thus, SOVs for all fluorochromes used within various antibody panels may be included in Lyse/Wash reference settings.

Next, when creating compensation control tubes in Lyric, it is necessary to indicate the control type used for each compensation control tube: "Fc Beads" (BD One Flow assays, BD Biosciences), "BD Comp Beads" (BD Biosciences) or "FC" (fluorescent control, user defined, EuroFlow SOP recommends UltraComp eBeads Compensation beads (Thermo Fisher Scientific (Waltham, MA, USA)). It is recommended that only one type of compensation control is used at a time, e.g. either BD Comp Beads or FC. Different compensation control types can be added subsequently in a separate step. Importantly, in Lyric the lot-specific SOVs are applied on a measured tube only when label and lot ID have been entered in the Reagents tab of the Tube Properties dialog.

2.4.2. Calculation of compensation matrix

In Canto and Lyric instruments, the compensation matrix is calculated within the acquisition software. For Navios instruments, the compensation matrix should be calculated in Kaluza software v1.2 or later or in third-party software programs such as FlowJo, Infinicyt v2.0 or Summit v5.2 and then applied post-acquisition. Alternatively, the calculated compensation matrix may be transferred to and saved within a corresponding acquisition protocol in Navios Acquisition software. Importantly, to be able to calculate the compensation matrix in Kaluza v1.2, each compensation control tube must contain both positive and negative populations. For automatic calculation of compensation in Kaluza v1.2 or later, use "negative-positive "method. When other methods of calculating compensation matrix with Kaluza are used, a fluorescence value of 100 is arbitrarily assigned to the negative population, which is not a correct assumption with EuroFlow set target MFI. This results in incorrect compensation matrix calculation due to violation of the rule 2 for a good compensation (Cossarizza et al., 2017). The calculated compensation matrix has to be transferred to and saved within a corresponding acquisition protocol in Navios Acquisition software.

Importantly, FACSDiVa software will assign proper reagent (lot) specific compensation whenever the string used to label the fluorochromes in a given acquisition exactly matches the label used in the compensation control tubes. If a lot specific compensation cannot be linked to the individual tube because of name mismatch or non-existing SOVs, a generic compensation is applied. In Lyric instruments, tube settings without associated reference settings use Lyse/ Wash SOVs. When applying reference settings to a tube, the generic values are applied to any parameter that does not have the correct reagent and lot ID selected in the reagents tab of the tube properties dialog. Noticeably, in Lyric, the compensation matrix is automatically recalculated any time the PMTVs for a tube are adjusted (which is currently not possible in Canto and Navios).

Importantly, the same tandem-conjugated reagent (e.g. CD19 PE Cy7) produced by various manufacturers may require different SOVs. Moreover, different lots of a given tandem-dye conjugated reagent from the same manufacturer may require different SOVs. Exposure to light and aging of a tandem dye will result in different SOV requirements. It should always be verified that the appropriate SOV is used for a given lot of tandem-conjugated reagents.

Occasionally, errors during calculation of the compensation matrix by the software may occur, resulting in improper SOVs. If this is the case, simple recalculation of the compensation matrix by the software would resolve the issue. It is recommended to perform appropriate visual compensation control to identify possible errors in compensation matrix.

2.5. Monitoring of instrument performance

2.5.1. Daily instrument performance check

An adequate consistency of data with time is ensured by monitoring the instrument's performance. According to the EuroFlow SOP for the Canto cytometer setup and compensation, a simple one tube test with Rainbow beads is run daily (at each cold start of the instrument). Subsequently, a gated reference peak of Rainbow beads is used to track CV and fluorescence intensity (MFI), low intensity peaks are watched to monitor resolution. As long as the MFI and CV values are within the predefined acceptance limits (see Table 1, Chapter 4 of the EuroFlow SOP for instrument setup), no action is needed. In contrast, whenever the MFI values repeatedly fail to fulfil the criteria, PMTVs need to be re-adjusted to reach the Target MFI. Importantly, a dedicated experiment/ protocol without compensation should be used for monitoring instrument performance. It is recommended to prepare a fresh Rainbow beads suspension before each use and keep it protected from light at 4–8 °C. During acquisition of Rainbow beads, the flow rate should be kept at „low "(i.e. event rate below 200 events/s). Only EuroFlow validated lots of Rainbow beads should be used. The appropriate MFI target values for a particular lot of Rainbow beads can be found at the EuroFlow website (euroflow.org).

Monitoring instrument performance with Rainbow beads is possible also on Navios. However, due to differences in emission filters on Navios instruments, specific Rainbow bead MFI target values for each fluorochrome and channel were defined. That is, e.g. specific MFI for Pacific Orange, OC515, V500, BV510 and BD Horizon V500 that are all measured in the same fluorescence channel. As a result, it would be necessary to monitor multiple MFI values for each channel on each instrument. It is acceptable to select just one, most frequently used, primary fluorochrome for each channel for monitoring.

In a Lyric SOP, performance monitoring relies on the CS&T module rather than on Rainbow bead MFI target values. During Performance QC, Lyse Wash/Lyse-no-Wash tube settings setup is performed in order to determine the PMTVs needed to reach the median fluorescence intensity dictated by the TTVs associated with Lyse Wash/Lyse-no-Wash tube settings. It is optional to define instrument specific values by measuring Rainbow beads in EuroFlow panels Tube settings, and track those daily.

The EuroFlow SOP for Canto was developed prior to the industrial solution (i.e. CS&T) system was integrated in FACS Diva software on Canto, which explains why the monitoring and setup on Canto is defined as a manual procedure in the SOP, in contrast to Lyric. CS&T defines and characterizes baseline performance, optimizes and standardizes cytometer setup and tracks cytometer performance. Daily performance check with CS&T should be performed at least once a day. It standardizes the cytometer measurements by adjusting the PMTV values to keep the median fluorescence target values of a bright bead population in each detector. Further, the CS&T module checks and adjusts laser delays, area scaling factors, measures performance parameters (Qr, Br, rCVs, PMTV) and records (and tracks) performance parameters with Levey-Jennings graphs. Application settings in FACSDiva software allow to update PMTV settings in individual applications (experiments) based on CS&T daily performance check, thus allowing for standardized measurements over time.

It is possible to combine both approaches, CS&T daily performance check and Rainbow beads monitoring of instrument performance. However, as the CS&T automatically adjusts several parameters of flow cytometer instruments, it is recommended to perform the Daily

performance check with CS&T, prior to monitoring daily instrument performance with Rainbow beads.

In the Lyric instrument, the integrated CS&T system evaluates the cytometer performance, similarly to what is described above for Canto. Performance QC and Assay/Tube settings setup should be performed each day before data acquisition. It ensures that target values for flow cytometer, assays and experiments are maintained.

The QC process in Navios encompasses multiple steps using different fluorescence particles. Flow-Check pro Fluorospheres are used to verify fluidics and laser alignment, whereas Flow-Set pro Fluorospheres are used to ascertain target mean position (fluorescence intensity) based upon particular application to (daily) adjust high voltage and gain to that target.

Instrument specific EuroFlow recommended approaches to instrument setup and monitoring are summarized in Table 2.

2.5.2. Troubleshooting

Whenever MFI values obtained during monitoring of the performance of the flow cytometers are outside those described in Table 1 of the SOP for Canto and Navios instrument setup and compensation, for the same lot of Rainbow beads, actions to improve the instrument's performance are required as described in the EuroFlow SOP for instrument setup.

After each service visit, it is important to run a performance check with Rainbow beads to check out with the service engineer whether the MFI values for any detector have changed and fall out of the desired target values as described in Table 1 of the SOP. If the MFIs are not acceptable, PMT target MFI and fluorescence compensation should be set again from the beginning.

3. General remarks

Currently, several tools for standardized instrument setup exist for BD flow cytometers. The CS&T system is a fully automated software and reagent system for BD digital flow cytometers, to optimize and standardize cytometer setup and track cytometer performance. CS&T in conjunction with application settings in FACSDiva software allow for standardized measurements by maintaining MFI target values by means of updating PMTV settings in individual applications (experiments) based on the CS&T daily performance check.

Instrument setup and (performance) monitoring using Rainbow beads was developed by EuroFlow to standardize flow cytometry measurements. The Rainbow bead standardization was proposed already before the CS&T system was available explaining why CS&T is not part of the EuroFlow SOPs for Canto. Untill now, EuroFlow developed SOPs for standardized setup of Canto, Lyric and Navios instruments (www.euroflow.org) allowing for cross-platform standardization of flow cytometry measurements. By using these protocols, Navios users have shown to perform very similar to Canto users in the EuroFlow QA scheme (Kalina et al., 2015). Moreover, data from the last two rounds of the EuroFlow QA scheme in 2018, document that Lyric users also perform very close to Canto and Navios users (data not shown). Rainbow beads can be used independently of CS&T for standardized set up of the flow cytometer and to monitor the instrument performance, and also to ensure reproducible data at different times and among instruments. Importantly, this approach is not linked to a specific software or equipment and it can be applied to various flow cytometry platforms after slight modification/adaptation(Nováková et al., 2017). In spite of successfull standardization of fluorescence signals, cross-platform standardization of FSC and SSC still remains a challenge.

Although it is possible to combine both CS&T and Rainbow beads for standardization, it has to be taken into account that CS&T performance check should be performed prior to monitoring instrument performance with Rainbow beads.

Recently, the BD OneFlow solution has been developed by BD Biosciences to allow for standardized measurement of flow cytometry data through standardized setup of the instrument. BD OneFlow uses one-peak fluorescence beads MFI target values to setup assay-specific PMTV for fluorescence channels. The conjunction with CS&T and application settings allows for standardized and reproducible measurements by adjusting the flow cytometer settings to reach the target MFI. Similarly to EuroFlow protocols, lysed washed blood is used to set cytometer FSC and SSC voltages to a target value range. The detector settings are then saved as Application Settings. BD OneFlow setup beads are intended to set voltages appropriate for the BD multicolor tube assay when used with a BD FACSCanto II flow cytometer set with the 4-2H-2 V optical configuration and BD FACSDiva software v8.0.1. or later. BD One Flow is a closed CE IVD solution that should be performed strictly according to the manufacturers recommendations, and should only be applied to BD OneFlow assays.

For high quality and reproducible data acquisition, it is crucial to avoid any errors when performing standardized instrument setup. Several errors in cytometer setup resulting in erroneous data acquisition have previously been reported (Glier et al., 2017; Kalina et al., 2018). Among other errors, target MFI values for the 7th peak of the Rainbow beads were applied to the 8th peak of the beads instead during instrument setup, resulting in incorrect PMTV settings. In turn, failure to collect data on the height values for the light scatter signal (FSC-H) hinders gating of singlets and discrimination of doublets, respectively. Furthermore, compensation errors resulting from violation of any of the rules for good compensation were the most frequently found user dependent mistake.

The compensation matrix is generally valid as long as the instrument setup and the fluorochromes used are stable. The stability of the instrument over time can be ensured by performing quality control (QC) as described in the EuroFlow SOP for instrument setup and/or daily performance check using CS&T (BD instruments) or other similar system (e.g Flow Check Pro/Flow Set Pro Fluorospheres for Navios instrument). CS&T daily performance check allows for reproducible data measurements by adjusting instrument PMTVs in order to reach the target MFI values. Similarly, monitoring instrument setup using Rainbow beads as described in SOP for Canto instruments allows for instrument QC.

The stability of fluorochromes depends on their characteristics. Some fluorochromes, such as FITC and PE are usually stable over several years when properly stored, whereas fluorochromes emitting in the red part of the spectra are generally sensitive to light, which is even more noticeable for the tandem dyes. Aging of tandem dyes leads to their desintegration and changes in the emission spectra resulting in a need for adjustment of spillover values. Moreover, differences in spectral properties of tandem dyes among various lots from the same manufacturer, as well as from different manufacturers, resulting in particular SOV requirements, has to be considered. As the SOVs for individual tandem dye lots are not declared by the manufacturer, it is advised to assess the stability of tandem dyes and their SOVs and recalculate the compensation matrix whenever needed. In the SOP for Canto instrument setup, EuroFlow recommends to rerun the compensation controls and recalculate the compensation matrix every month. Based on the experience gathered so far, it is not necessary to rerun the compensation matrix as long as the fluorochrome and instrument setup is stable. For the Lyric instrument it is recommended by the manufacturer to rerun the compensation matrix every 60 days. In the future, a routine protocol for assessing dye SOV stability needs to be developed and tested for most optimal handling of compensation matrix recalculation times.

A new feature of the Lyric instrument is the possibility to export and share tube settings and/or assays among Lyric users. This may be of great advantage when inter-laboratory standardization of flow cytometry measurements is needed. EuroFlow provides assays for the individual EuroFlow antibody panels for download at the EuroFlow website.

In summary, EuroFlow has developed SOPs for standardized

instrument setup of Canto, Lyric and Navios instruments, enabling cross-platform inter- and intra-laboratory standardization of flow cytometry measurements. The feasibility of the procedures and acquisition of highly comparable data among various instruments in various laboratories have already been documented (Glier et al., 2017; Kalina et al., 2015; Lhermitte et al., 2018).

Grants

TK and MN were supported by Ministry of Health of the Czech Republic, grant nr. NV18-03-00343, Ministry of Education, Youth and Sports of the Czech Republic, NPU I project nr.LO1604 and EU-Prague project CZ.2.16/3.1.00/24505. JFM and AO were supported by CB16/12/00400 grant (CIBER-ONC, Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Madrid, Spain and FONDOS FEDER).

Declaration of Competing Interest

JvD and AO report an Educational Services Agreement from BD Biosciences (San José, CA) and a Scientific Advisor Agreement with Cytognos; all related fees and honoraria are for the involved university departments at Leiden University Medical Center and University of Salamanca. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

Authors wish to thank to Michael Wahl, Dennis Tielemans and Ondrej Pelak (BD Biosciences) for their advice on Lyric instrument setup. All members of the EuroFlow consortium are acknowledged for their contribution to the network and fruitful discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.112680.

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