How to make usage of the standardized EuroFlow 8-color protocols possible for instruments of different manufacturers

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Abstract

A critical component of the EuroFlow standardization of leukemia/lymphoma immunophenotyping is instrument setup. Initially, the EuroFlow consortium developed a step-by-step standard operating protocol for instrument setup of ≥ 8-color flow cytometers that were available in 2006, when the EuroFlow activities started. Currently, there are 14 instruments from 9 manufacturers capable of 3-laser excitation and ≥8 color measurements. The specific adaptations required in the instrument set-up to enable them to acquire the standardized 8-color EuroFlow protocols are described here. Overall, all 14 instruments can be fitted with similar violet, blue and red lasers for simultaneous measurements of ≥8 fluorescent dyes. Since individual instruments differ both on their dynamic range (scale) and emission filters, it is not accurate to simply recalculate the target values to different scale, but adjustment of PMT voltages to a given emission filter and fluorochrome, is essential. For this purpose, EuroFlow has developed an approach using Type IIB (spectrally matching) particles to set-up standardized and fully comparable fluorescence measurements, in instruments from different manufacturers, as demonstrated here for the FACSCanto II, and Navios and MACSQuant flow cytometers. Data acquired after such adjustment on any of the tested cytometry platforms could be fully superimposed and therefore analyzed together. The proposed approach can be used to derive target values for any combination of spectrally distinct fluorochromes and any distinct emission filter of any new flow cytometry platform, which enables the measurement of the 8-color EuroFlow panels in a standardized way, by creating superimposable datafiles.

Keywords: flow cytometry, standardization, fluorochromes, EuroFlow, fluorescent spectrum

Introduction

The EuroFlow consortium has been developing standardized ≥8-color immunophenotyping protocols since 2006, when only two manufacturers, Becton Dickinson (BD) Biosciences (San Jose, CA, USA) and DakoCytomation (Glostrup, Denmark), were producing instruments capable of ≥8-color measurements. Since complete standardization of instrument setup[1], sample preparation, antibody panels[1–4], and data analysis[5,6] are cornerstones of the EuroFlow approach for robust and reproducible immunophenotyping[2], the EuroFlow standard operating protocol (SOP) for instrument setup was developed in detail for the FACSCanto II flow cytometer (BD Biosciences), in a way, it could also be transferred and applied to BD LSRII, as well as to CyanADP (DakoCytomation)[1]. Later on, several other companies entered the multicolor flow cytometry market and at present there is a growing number of instruments capable of ≥8 color measurements. Thus, crucial questions have arised about the requirements for any cytometer to be used with the EuroFlow protocols and the parameters that need to be taken care of, to produce fully standardized (reproducible) data on different instrument platforms.

In general, EuroFlow compatible instrument must a) be equipped with excitation lasers and collection optics (emission filters) to detect at least the 8 colors used in the panel combinations; b) have stable fluidics, c) sensitivity to discriminate fluorescence signals, d) reasonably low background (noise) and e) signal linearity; and f) produce data conforming to Flow Cytometry Standard (FCS) data format without hidden features preventing their analysis by a third party software[7]. In addition, the features of instruments that require specific adjustments to generate standardized data include: a) emission filters'

specifications; b) data range (i.e., number of channels); and c) both data acquisition software and reliable fluorescence compensation calculation algorithms. Furthermore, should an instrument be used for diagnostic purposes it must conform to local legal requirements (e.g., CE marking, IVD regulatory status). Last, but not least, application and technical support from the vendor should be appropriate for the intended use at the user site.

For appropriate instrument set-up, different types of fluorescent beads are available. Reference internally dyed fluorescent particles (calibration standards) used by EuroFlow SOP are classified as Type IIIA beads[8], which are environmentally stable for over several years when properly stored. They contain bright signal beads in every detector but their fluorescence is not spectrally matched to individual fluorochromes used in ≥8-color panels. Spectrally matched beads (Type IIB) can be made ad-hoc by incubating immunoglobulin capture beads with fluorescence labeled monoclonal antibodies[9]. However, they must be made for each channel separately using specific fluorescent conjugates and their stability is limited.

In order to control for the quality of data in individual laboratories, the EuroFlow Quality Assessment (EuroFlow QA) program has been developed[10]. This program identifies a parameter measured in the example Lymphoid Screening Tube-QA (LST-QA) that provides an abnormal readout and quantifies its distance from the expected value. The EuroFlow QA approach has also been applied to test the quality of the data acquired on multiple different flow cytometer platforms. By December 2016, there were 14 distinct flow cytometers that have ≥3 excitation lasers and allow for the detection of ≥8 different fluorochromes (Table 1). So far only four of them are CE IVD approved (however,

this may differ in different countries and will change over time). In principle, all instruments that have 405nm, 488nm and 633-640nm excitation lasers and at least two, four and two detectors for each excitation line, respectively, fulfill the technical requirements for acquisition of the complete set of 8 colors used in EuroFlow panels (Table 2). However, some instruments may be equipped with emission filters that are adjusted for optimal detection of manufacturer's proprietary fluorochromes (Table 2). If detection of the fluorescence emission of the EuroFlow reference and alternative fluorochromes is feasible by those filters, standardization can be achieved by adjusting the target MFI values for a given fluorochrome and its optical filter, as described below.

Here, we present an overview of currently available instruments capable of ≥8-color measurements together with the EuroFlow approach to standardization of data measured across multiple flow cytometer platforms. For this purpose, the Navios and MACSQuant flow cytometers were used as example, based on their availability in EuroFlow member laboratories and both type IIB and type IIIA beads were employed for fine-tuning the photomultiplier tubes (PMTs) voltage setup and for calibration of instrument- and fluorochrome-specific Target mean fluorescence intensity (MFI) measurements, respectively. Data generated on the Navios and MACSQuant® Analyzer flow cytometers were compared with the FACSCanto II flow cytometer data for which the EuroFlow SOP had been originally developed. The settings were adjusted for fluorochromes used in the LST-QA: fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin chlorophyll protein-cyanin5.5 (PerCP-Cy5.5), PE-cyanin 7 (Cy7), allophycocyanin (APC), APC-hillite7 (H7), Pacific Blue (PacB), and Orange Cytognos 515 (OC515).

Materials and methods

Instrument set-up for FACSCanto II flow cytometers. FACSCanto II flow cytometers were set up according to the EuroFlow SOP as published previously (Kalina et al.[1] and www.euroflow.org). Briefly, voltages of all PMTs were set to reach the target value for the 7th peak of the Euroflow-validated lot of Rainbow Calibration Particles, 8 peaks (Spherotech, Lake Forest, IL) Type IIIA calibration particles.

Evaluation of spectral differences among fluorochromes. Spectral differences of selected fluorochromes and emission filters were tested on the Sony SP6800 flow cytometer (Sony Biotechnology Inc., Tokyo, Japan) equipped with 405nm, 488nm and 638nm excitation lasers and detection of complete spectra on PMT array binned to 32 (for 488nm excitation) or 34 channels (for 405 and 638nm collinear excitation). Signal level of Pacific Orange (CD45 PacO, Exbio Praha, Prague, Czech Republic), OC515 (CD45 OC515, Cytognos SL, Salamanca, Spain) and Horizon V500 (BD Biosciences) was measured with either 550/40 (analogous to the filter set used in the Navios intruments) or 510/50 (analogous to the filter set of FACSCanto II cytometers) optical filters (AHF analysentechnik AG, Tuebingen, Germany) on a BD LSRII instrument (BD Biosciences) using the 405nm excitation laser line.

Instrument set-up of the MACSQuant analyzer and Navios flow cytometers. As a starting point, all PMTs of the two flow cytometers evaluated (MACSQuant® Analyzer, Miltenyi Biotec; and Navios, Beckman Coulter; Table 2) were set up so

that the signal of the 7th Rainbow particles peak matched the MFI of the reference flow cytometer, after rescaling to the common 18-bit scale. Thus, for Navios, that means that 7th peak target values were recalculated (multiplied by four) to its 20-bit scale (Table 2), and Navios software "True view set 1" was selected in configuration to display 20-bit scale.

As previously described, peripheral blood (PB) samples from adult volunteers were stained using the LST-QA[10]. Furthermore, single-antibody reagent stained tubes were prepared using antibodies conjugated with fluorochromes identical to those in the LST-QA combination (i.e., FITC, PE, PerCP Cy5.5, PE-Cy7, APC, APC-H7, PacB, and OC515). Either PB cells or capture beads were incubated with each single antibody-fluorochrome conjugated reagent used in the LST-QA for 30 minutes in the darkness, as described in Figure 1 and Figure 2 legends). The following capture beads were used during the study: BD™ CompBead (BD Biosciences) for adjustment of the spectra in the 488nm and 633nm excited channels, Simply Cellular® anti-Mouse beads for the Violet Laser (Bangs Laboratories, Inc., Fishers, IN) for adjustment of the spectra in 405nm excited channels where each single stained tube was measured on both instruments within 6 hours after staining was completed. UltraComp eBeads (eBiosciences Inc., San Diego, CA) were used for spectral cytometry measurements.

After the 7th peak Rainbow Calibration particles (type IIIA beads) setup had been completed, single stained BD Comp Beads (type IIIB capture particles) were measured on a reference flow cytometer (FACSCanto II) and the two flow cytometers under study. Files from reference versus each of the evaluated flow cytometers were merged together, recalculated to the same scale (divided by 4,

to reach 18-bit from 20-bit) in case of the Navios instrument (MACS Quant uses 18-bit scale), and compared using Infinicyt™ software. In case of a difference in the MFI of the positive particle population, the PMT voltage for the particular fluorochrome was increased or decreased on the evaluated flow cytometer to reach the same MFI as that defined as target value for the reference flow cytometer. This process was performed on 3 different Navios and one MACSQuant instruments iteratively in different laboratories (Prague, Brno, Aarau-Zurich) until a match was reached. After all PMTs were adjusted as necessary, Rainbow calibration particles were measured again on the evaluated flow cytometer to establish the final target values for the 7th peak (specific for each flow cytometer and fluorochrome). These settings were then used to measure the LST-QA tube on 4 different FACSCanto II instruments (14 PB samples), 6 Navios instruments (17 PB samples) and 1 MACSQuant cytometer (3 PB samples).

Evaluation of LST-QA data on different flow cytometers. All data were analyzed with the Infinicyt™ software. Data acquired derived from the Navios instrument were first rescaled to 18-bit. Then, data were merged, a common assignment of parameters was used and the analysis performed. LST-QA data was analyzed using Principal Component Analysis (PCA) and visualized through the Automatic Population Separator (APS) graphical representation, as previously described[1].

Results

Scale of fluorescence intensity measurements. Each of the instruments analyzed here uses a different data range (number of channels, referred to as "dynamic range"; Table 2). Thus, data generated on each instrument were rescaled by software to a common scale for further analyses. After rescaling of the FCS data, the analysis of the merged files from the reference Rainbow calibration particles measurements showed similar patterns for the 7th peak fluorescence emissions on the three instruments tested (Figure 1 A). Indeed, for those channels that used similar emission filters (centered on the maximum emission wavelength of the fluorochrome used), the standard instrument setup according to the EuroFlow SOP and rescaling to a common scale (if needed) were sufficient to achieve the same level of signal without any additional adjustment required. This is documented by the results obtained for peripheral blood samples single-stained with CD8 FITC, as illustrated in Figure 1B.

Adjustment for different spectra and distinct emission filters. Whereas different instruments use similar excitation lines (violet, blue and red) there can still be differences in some of the emission filters (Table 2). When filters transmitting different (non-identical) bands of the emission spectra are used (e.g., the FACSCanto II 510/50 filters vs the Navios 550/40 filters for OC515 fluorescence here collected), the instrument setup using the EuroFlow Target MFI value for the Rainbow calibration particles is no longer sufficient because they are not spectrally identical to the actual fluorochromes (Figure 2A). In that case, a shift in the signal intensity corresponding to the proportion of the fluorophore's emission spectra transmitted by a distinct filter, is observed

(Figure 2A). Thus, for each different optical filter and each spectrally different fluorochrome, a specific target MFI correction factor was required, calculated and applied. For this purpose, we empirically adjusted the PMT voltages on the Navios and MACSQuant instruments using OC515-stained capture beads, to exactly match the signal obtained on the FACSCanto II instrument for the same fluorochrome. Next, we measured the Rainbow calibration particles and established OC515/Navios and OC515/MACSQuant specific Target MFIs. When PB samples stained with an anti-CD45 reagent conjugated with OC515 were measured on the three different type of instruments with the specific correction factors applied, we could see in the merged data files that the staining pattern was fully comparable and thus, standardized (Figure 2B).

Standardized data acquisition on different instruments. Finally, three EuroFlow laboratories have used the Navios flow cytometer in parallel to the FACSCanto II instrument during the EuroFlow QA rounds, with fully comparable results (see Kalina et al. in this issue[11]). Results showed that by applying the channel/fluorochrome specific Rainbow calibration particles target values, the fluorescence staining patterns were fully matched and standardized. The resulting FCS files could also be merged an analyzed jointly and they were also fully comparable to a reference image of the expected staining/fluorescence emission patterns (Figure 3A). In addition, the complete immunophenotypes analyzed using APS projections of fluorescent parameters of the merged files acquired in different laboratories and in different instruments, were also fully comparable (Figure 3B). Merged files from FACSCanto II, Navios and MACSQuant instruments showed the same staining pattern in all 8 channels for both the

positive and negative cell populations coexisting in the sample (Supplementary Figure 1).

Discussion

The EuroFlow concept is built on the analysis and direct comparison of flow cytometry data files against well-defined databases of standardized data files acquired in different laboratories with different instruments[1–4]. At present, at least fourteen different instruments from nine distinct manufacturers, potentially enable acquisition of e.g. the EuroFlow 8-color panels for the diagnostic screening and classification of hematologic malignancies[2]. However, to take full advantage of the EuroFlow approach, acquisition of data must be performed under standardized signal settings, and data must be saved in a common data format. Unfortunately, most manufacturers do not consider cross-platform standardization in their instrument development plans and even a compliance with a common FCS data format[7] doesn't guarantee that the data can be analyzed in a third party software (most FCS files contain additional features that need to be specifically handled by a third party software).

Flow cytometry instrument manufacturers could support standardization by using the same (or identical) optical filters (the benefit of a filter optimized to a particular fluorochrome in 8-color setup is minor, while the negative impact on the standardization is major) or by supporting custom optical filters that could be exchanged by the user whenever needed. Also, an embedded feature of any instrument's software that would allow the user to easily export FCS data in a particular scale range, without (additional) features that prevent analysis in

third party software and that facilitate evaluation of academically developed R-project based algorithms,[12] would greatly enhance collaboration and joint analysis of large data sets[13].

Since different filters are used on distinct instruments, to achieve standardization across different flow cytometers, the optimal standardization tools would involve internally dyed beads which are spectrally matched to the actual reagents. However, such beads are not widely available (fluorochromes are often proprietary to the manufacturer) and they are rather difficult to produce (fluorescence emission spectra can shift when a given fluorochrome is conjugated to a particle, instead of an antibody molecule)[8]. To circumvent these limitations, we used capture beads with spectrally matching fluorescence to the antibody-conjugated dyes (Type IIB beads); to adjust the level of the signal to the desired value provided by the reference instrument. We then proceeded to establish specific target MFI values for each individual combination of fluorochromes and optical setup, using the Rainbow calibration particles (Type IIIA beads). Thus, new target MFI values had to be established and used for each optical filter that deviated from the standard one, as well as for each spectrally distinct fluorochrome. Thus, target MFI values adjusted for the Navios instrument and the following FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-H7, PacB, and OC515 fluorochromes, were developed. Similarly, other alternative fluorochromes used in recently developed EuroFlow protocols (e.g. Brilliant Violet 421, Brilliant Violet 510)[3] would also require fluorochrome specific adjustment for any instrument with a different optical filter setup.

At this point, Navios is the only non-reference cytometer used by EuroFlow members. We have created a SOP for its setup (available at

www.euroflow.org). By using this protocol, Navios users have shown to perform very similar to FACSCanto II users in the EuroFlow QA scheme (Kalina et al, this issue[14]). By adhering to this SOP, data from FACSCanto II, Navios and MACSQuant, once merged together, showed the same staining patterns for all 8 fluorescence channels, as illustrated in Supplementary Figure 1 for the fluorochromes used for the development of the SOP. In the GEIL study, Solly et al. reported superimposable data measured on FACSCanto and Navios[15]. They used Type IIIA beads only and observed some histogram shifts in the PE-Cy7 and APC-Cy7 channels (e.g., OC515/Pacific Orange/VH500 histogram was not shown) consistent with the emission filter differences specified in Table 2. Other published inter-laboratory studies did not attempt to standardize the measured signal[16–18].

Of note, the presented list of instruments does not intend any endorsement of particular cytometers, nor does intends to evaluate their technical performance (e.g., linearity, sensitivity, background noise, speed of acquisition) or other important variables (e.g., ease of use, technical and application support) guiding a cytometer selection. In contrast, we tried to address the question whether the same standardized approach (PMT voltage setup to reach specific target MFIs) could be used for ≥8-colors experiments, which proved to be feasible and successful. An identical strategy is currently being evaluated with similar good results at the moment, in other flow cytometry instruments, such as the Attune Nxt (Thermo Fisher Scientific, Waltham, Massachusetts) and the FACSLyric (BD Biosciences). In fact, we have successfully adopted the same standardization scheme in a 16-color inter-laboratory study on 4-laser instruments (561nm excitation line added) (see Blanco et al. in this

issue)[19]. Further increase in the number of colors will likely require an addition of UV excitation (350-355nm), with (currently) up to 6 PMT detectors for UV excited fluorochromes[20]. Of note, the ability to reach a specified target MFI-based instrument setup requires that PMT voltages are adjusted independently of each other (this is not the case for instruments that share PMT detectors for two differently excited fluorochromes). As a concept, the Target MFI standardization can be expanded, but it requires stable Type IIIA beads excited by all lasers and emitting in all fluorescence channels. Here, the Rainbow calibration particles come short at violet laser excited, far-red emitting fluorescence. While we show here that standardization of signal on instruments equipped with different emission filters is possible, it is advisable to attempt emission filter unification as it becomes very complex and challenging to maintain and correctly apply Target MFI when several filters differ and multiple spectrally different dyes are used.

The key question when executing the EuroFlow protocol on any flow cytometer instrument is whether the data can be superimposed against the data in the EuroFlow databases[21]. Ideally, the data generated on any instrument should pass the EuroFlow QA without a systematic deviation from the expected values for a given fluorochrome and cell subset. We have shown here that this is indeed possible when SOPs are developed to adjust for differences in the emission filters and data scaling. Thus, we show that a concept of standardized data generation can be applied across multiple instruments from different manufacturers.

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Table Legends

Table 1. List of instruments with the hardware capable of measuring 8-color EuroFlow panels.

Information was collected from technical specifications given by the manufacturers. In the case where more similar instruments are produced by a manufacturer, only a representative is listed.

Table 2. Instruments that were used to evaluate the feasibility of standardized measurements across different flow cytometry platforms.

Differences in the data dynamic range (number of channels) and differences in the emission filters are highlighted in bold.

Figure Legends

Figure 1

Rescaling of channels allows for comparable pattern of fluorescence.

Merged data files of the 7th peak of Rainbow calibration particles measured on 3 instruments listed in Table 2 (FACSCanto II, orange; Navios, blue; MACSQuant, green) are shown. After rescaling, the target value of the 7th peak beads is comparable across the three flow cytometers. The FITC channel is shown as an illustrating example **(A)**. Peripheral blood from a healthy volunteer was stained with a CD8 FITC antibody. Using Rainbow calibration particles for setting target PMT values as described above, comparable signals on all three flow cytometers was detected **(B)**.

Figure 2

Differences in filters lead to detection of different parts of the spectra and influence the signal intensity.

Rainbow calibration particles and UltraComp eBeads (stained with Pacific Orange, Horizon V500 and Orange Cytognos 515 tagged antibodies) were measured on the Sony SP6800 Spectral Cytometer. Distinct filters for Pacific Orange/OC515/H-V500 are highlighted: blue rectangle shows the wavelength detected using the emission filter of the Navios flow cytometer, while the orange rectangle represents the FACSCanto II filter. For each fluorochrome, a different band of the emission spectra is detected leading to differences in MFI values as shown in the bottom row, when measured on the same detector of the BD LSRII flow cytometer using either 550/40 or 510/50 filters **(A)**. A comparable signal is yielded when peripheral blood stained with CD45 OC515 is measured using a correction factor for PMT voltages. The correction factor applied was devised empirically to fit the signal on all instruments to the same position. FACSCanto II, orange; Navios, blue; MACSQuant, green **(B).**

Figure 3

Results of the EuroFlow QA.

Comparison of the same PB sample measured on two cytometry platforms. Reference images depicting the expected position of analyzed cell subsets are shown for FACSCanto II instrument **(A)** and Navios flow cytometer **(B).** APS plot of LST-QA stained with adult PB samples measured on FACSCanto II (4 different

instruments, n=14), Navios (6 different instruments, n=17) and MACSQuant (single instrument, n=3) flow cytometers **(C).**

Table 1

Instrument name	Manufacturer	Lasers	Fluorochrome parameters	Acquisition speed (events/s)	CE IVD clearance
BD FACSCanto II	BD Biosciences	3	10	10000	CE IVD
Navios	Beckman Coulter	3	10	25000	CE IVD
BD FACSLyric™	BD Biosciences	3	10	35000	CE IVD
Novocyte	ACEA Biosciences	3	13*	35000	CE IVD
Cyan ADP	Beckman Coulter	3	9	70000	
MACSQuant	Miltenyi Biotec	3	8	10000	
BD LSR Fortessa	BD Biosciences	4	18	70000	
BD FACSCelesta	BD Biosciences	3	12	25000	
Cytoflex	Beckman Coulter	3	13	30000	
Attune Nxt	Invitrogen™/ ThermoFisher	4	14	35000	
Yeti / ZE5	Propel Labs / Bio-Rad	5	30	100000	
SE520EXi	Stratedigm	4	18	10000	
SP6800	Sony Biotechnology Inc	3	2x PMT array*	20000	
CyFlow® Space	Sysmex / Partec	5	15	n.a.	

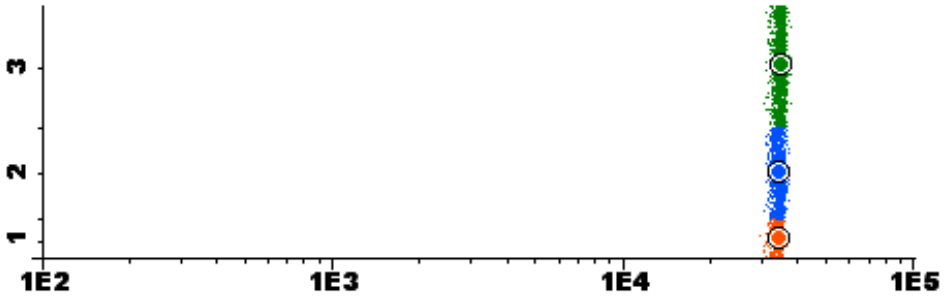
*does not allow for independent PMT voltage settings

Table 2

Instrument features	**BD FACSCanto II**	**Navios**	**MACSQuant**
Dynamic range (Number of channels)	18-bit (262 144)	20-bit (1 048 576)	18-bit (262 144)
Excitation lines			
Violet laser	405nm, 30mW	405nm, 40mW	405nm, 40mW
Blue laser	488nm, 20mW	488nm, 22mW	488nm, 30mW DPSS
Red laser	633nm, 17mW	638nm, 25mW	638nm, 20mW
Emission optics for EuroFlow fluorochromes			
Pacific Blue (HV450)	450/50	450/50	450/50
OC515 (PacOr, HV500)	510/50	550/40	525/50
FITC	530/30	525/40	525/50
PE	585/42	570/30	585/40
no EF fluorochrome*		620/30	
PerCP-Cy5.5	670LP	695/30	655-730
PE-Cy7	780/60	755 LP	750 LP
APC	660/20	660/20	655-730
no EF fluorochrome*		725/20	
APC-C750 (APC-H7)	780/60	755 LP	750 LP

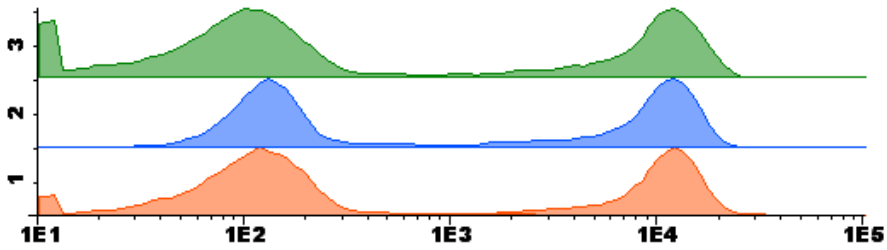
*No EuroFlow fluorochrome is used in this position for the 8-color EuroFlow panels.

Figure 1A



FITC channel

Figure 1B



FITC fluorescence

Figure 2A

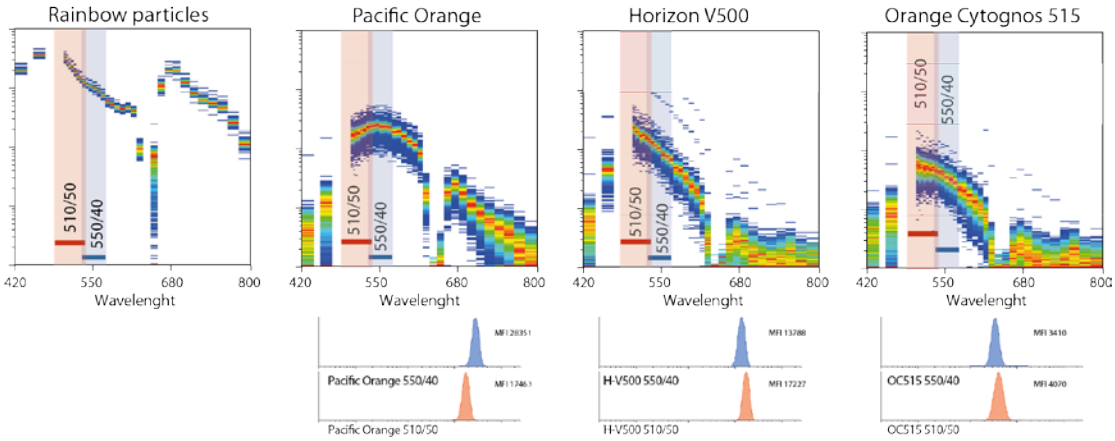


Figure 2B

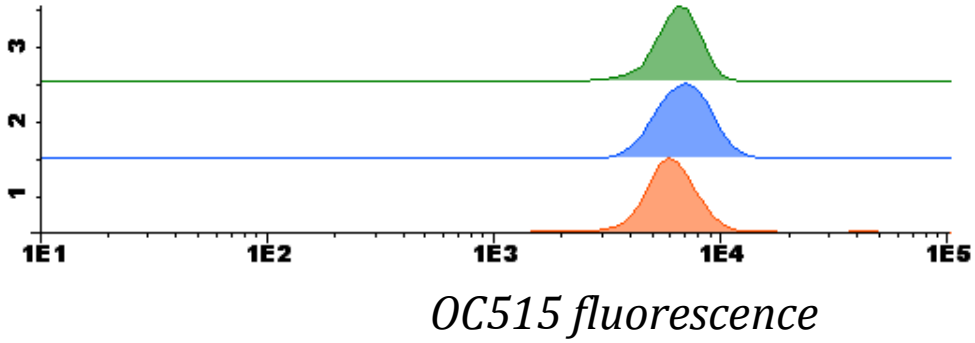


Figure 3

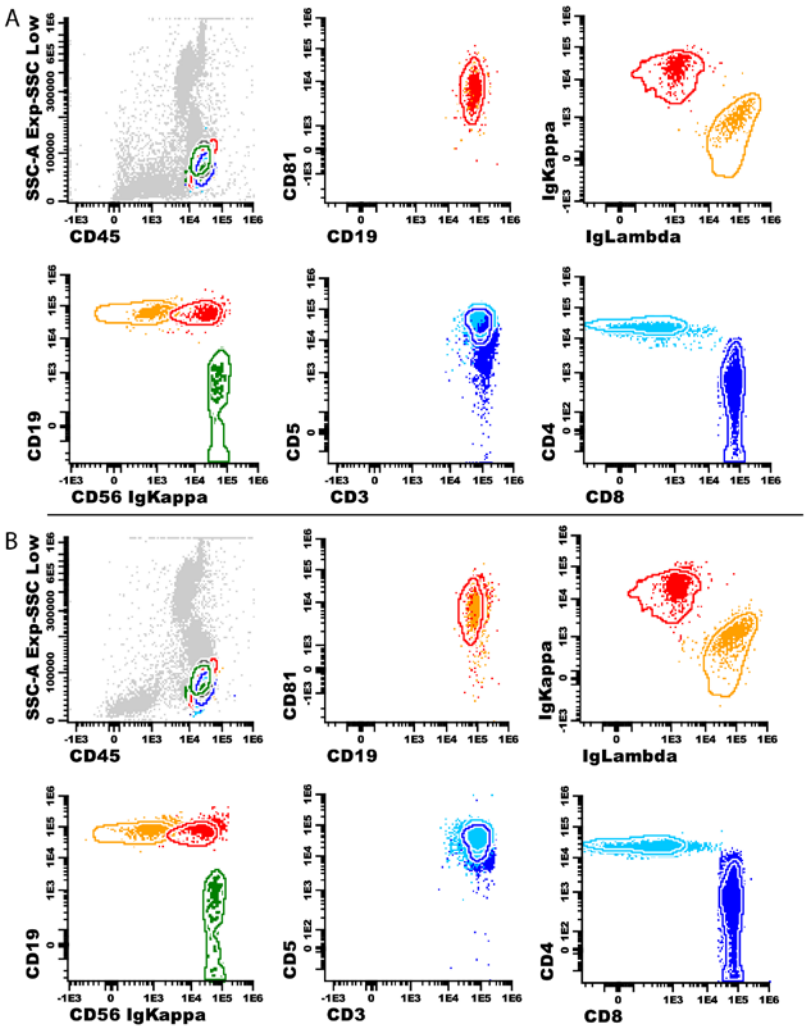
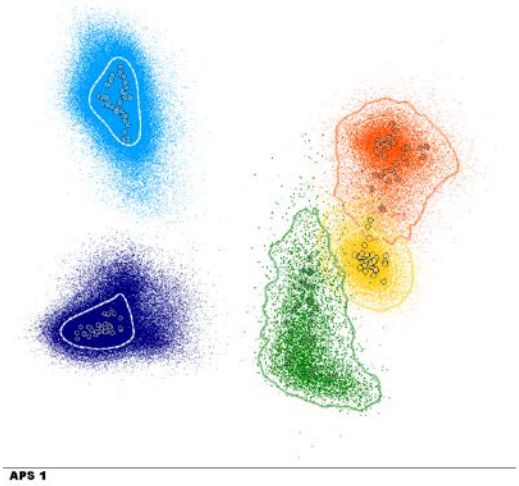
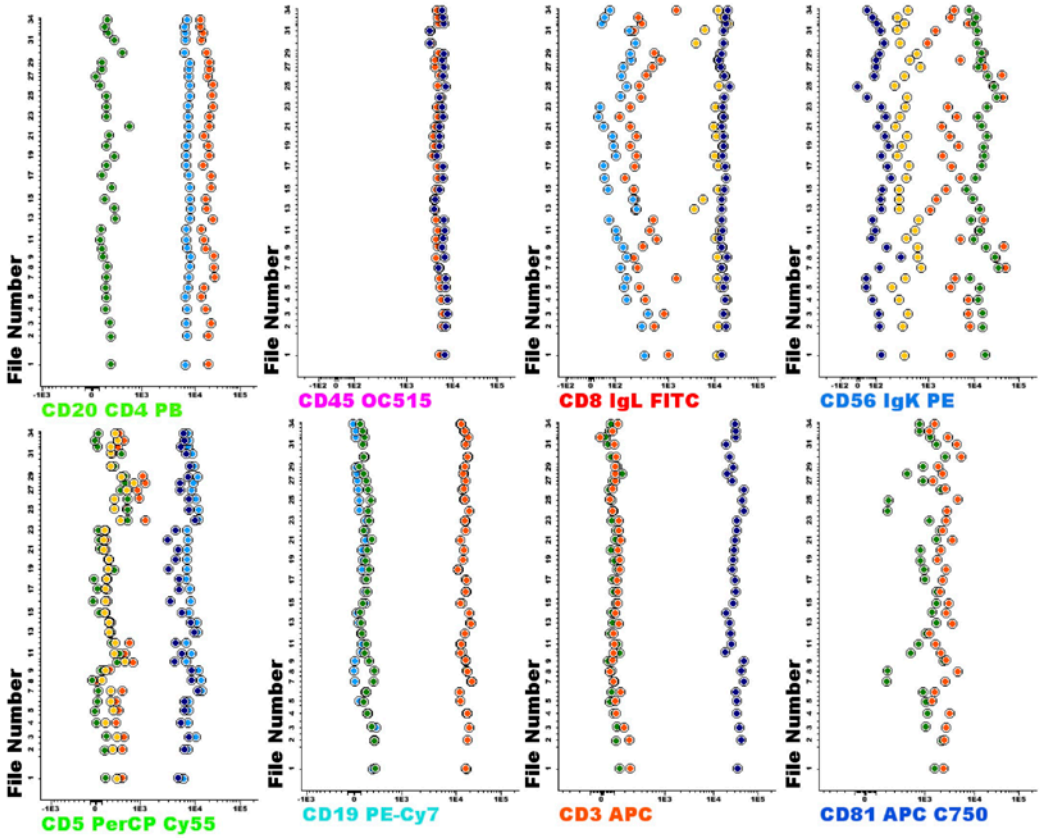

A
SSC-A Exp-SSC Low
CD45
CD81
CD19
IgKappa
IgLambda
CD19
CD56 IgKappa
CD5
CD3
CD4
CD8
B
SSC-A Exp-SSC Low
CD45
CD81
CD19
IgKappa
IgLambda
CD19
CD56 IgKappa
CD5
CD3
CD4
CD8

Figure 3C



Supplementary Figure 1



Supplementary Figure 1

Analysis of 34 LST-QA files measured in different laboratories on different instruments. Circles represent MedFI values for each corresponding (gated) peripheral blood lymphocyte subset. Color coding: Igλ⁺ B-cells, orange; Igκ⁺ pos B-cells, red; CD56 bright NK-cells, green; CD4⁺ T-cells, light blue; CD8⁺ T-cells, dark blue. Files 1-14 were acquired using FACSCanto II instruments files 15-31 using Navios instruments and files 32-34 using a MACSQuant instrument.