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Fluorochrome choices for multi-color flow cytometry

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ABSTRACT

Fluorochrome selection is a key step in designing multi-color antibody panels. The list of available fluorochromes is continuously growing, fitting current needs in clinical flow cytometry to simultaneously use more markers to better define multiple leukocyte subpopulations in a single tube. Several criteria guide fluorochrome selection: i) the fluorescence profiles (excitation and emission), ii) relative brightness, iii) fluorescence overlap, iv) fluorochrome stability, and v) reproducible conjugation to antibodies. Here we used 75 samples (45 bone marrow and 30 blood) to illustrate EuroFlow strategies for evaluation of compatible fluorochromes, and how the results obtained guide fluorochrome selection as a critical step in the antibody-panel building process. Our results allowed identification of optimal fluorescence profiles (e.g. higher fluorescence intensity and/or resolution with limited fluorescence overlap into neighbor channels) for brilliant violet (BV)421 and BV510 in the violet laser and allophycocyanin (APC) hilite 7 (H7) or APC C750 in the red laser vs. other candidate fluorochromes generally applied for the same detectors and here evaluated. Moreover, evaluation of the same characteristics for another group of fluorochromes (e.g. BV605, BV650, PE CF594, AF700 or APC AF700) guided selection of the most appropriate fluorochrome conjugates to be combined in a multi-color antibody panel. Albeit this is a demanding approach, it could be successfully applied for selection of fluorochrome combinations for the EuroFlow antibody panels for diagnosis, classification and monitoring of hematological malignancies and primary immunodeficiencies. Consequently, sets of 8-, 10- and 12-color fluorochrome combinations are proposed as frame of reference for initial antibody panel design.

1. Introduction

1.1. Fluorochrome definition and main features

A fluorescent dye (i.e. fluorochrome) is a natural-occurring or artificial molecule that can be excited by light (energy) at a specific wavelength and consequently emits lower energy light with a longer wavelength (Baumgarth and Roederer, 2000; Macey, 2007). The wavelengths at which a fluorochrome is excited and the light spectrum that the fluorochrome emits (i.e. excitation and emission profiles, respectively), are distinctive features of each fluorochrome. In flow cytometry, the fluorescence profile of each fluorochrome needs to be compatible with the optical configuration of the flow cytometer to be properly detected (i.e. efficiently excited and its emission light

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optimally detected). Although a growing list of excitation sources (lasers with different emission wavelengths) are becoming available, current clinical (routine) flow cytometry instruments are generally restricted to Violet, Blue and Red lasers (VL, BL, RL) emitting at ≈405, ≈488, ≈633 nm, respectively. In turn, their collection optics are constructed to restrict ("filter") the spectrum of light that each detector can receive, limiting it to a rather narrow wavelength range.

According to their nature, fluorochromes used in multi-color flow cytometry (MFC) belong to one of the following groups: i) small molecules, e.g. fluorescein isothiocyanate (FITC), cyanines (Cy), the Alexa Fluor (AF) family of dyes, horizon V(HV) conjugates, eFluor dyes or Vio Dyes; ii) large protein-based molecules, e.g., phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP); iii) engineered polymeric antennae, e.g. the brilliant violet (BV), brilliant blue (BB) or Super Bright (SB) families of dyes; and iv) inorganic fluorescence nanocrystals, e.g. quantum dots (Qdots) (Abrams et al., 2013; Affymetrix eBioscience, 2017; Chattopadhyay and Roederer, 2012; Chattopadhyay et al., 2010; Oi et al., 1982; Resch-Genger et al., 2008; ThermoFisher Scientific, 2017b). In addition, a commonly applied strategy to increase the number of fluorochromes excited by the same laser but emitting at distinct parts of the spectrum is to generate tandem compounds. In brief, these tandem compounds increase the distance between the maximum excitation and emission wavelength (i.e. Stoke's shift) (McCoy Jr., 2002) vs the single dyes that form the tandem, by coupling two distinct fluorescent molecules. This approach profits from the fluorescence resonance energy transfer (FRET) principle (Macey, 2007), based on excitation of the first molecule (i.e. donor fluorochrome) which transfers its energy to the second one in such a way that the flow cytometer instrument collects the fluorescence emission of this second (i.e. acceptor) fluorochrome. Molecules most frequently used to construct tandems are: i) protein-based molecules or organic polymers as donor dyes, and ii) small organic molecules, particularly cyanines, used as acceptor fluorochromes (e.g., PE Cy7, PerCP Cy5.5, APC AF750, BV605 or BV785) (Baumgarth and Roederer, 2000; Berlier et al., 2003; Chattopadhyay et al., 2012, 2006; Oi et al., 1982; Roederer et al., 1996).

1.2. Optical configuration of flow cytometers and detection of multiple fluorochromes

Optical configurations of flow cytometer instruments are heterogeneous, and frequently even customizable -except for in vitro diagnostics (IVD) cleared cytometers-, in terms of excitation lines, set of combinations of mirrors and filters, and number and type of detectors. In addition, manufacturers are continuously introducing new alternatives for available channels and/or opening new positions by optimizing usage of fluorochromes excited on previously "underused" sections of the light spectrum (e.g. ultra violet laser-excited fluorochromes) (BD Biosciences, San Jose, CA, 2017a; Telford, 2015; ThermoFisher SCIENTIFIC, 2010). Nonetheless, a reasonable time gap exists before new fluorochromes become (widely) available and are incorporated to research or more importantly, to diagnostic flow cytometry. Table 1 summarizes selected (commonly available) fluorochromes grouped by their excitation profile in flow cytometer instruments with compatible (8-, 10- and ≥12-color) optical configurations.

Fluorochromes excited with a single laser line, need sufficiently similar excitation profiles. However, their fluorescence emission should be preferably well separated, so that they can be collected in different detectors (i.e. channels); despite this, fluorochromes with highly overlapping emissions would be hardly (or impossible) to resolve, forcing users to thereby select one single candidate per detector (Leach et al., 2013). Importantly, neither excitation nor emission curves are restricted to a narrow wavelength bandwidth; instead, they gradually decrease at both sides of the maximum emission/excitation peak, forming unique (fluorochrome specific) emission/excitation patterns. Consequently, emission curves of two or more distinct fluorochromes frequently overlap to some extent. Thus, a fluorescence detector intended to collect the emission from one fluorochrome will also receive light from secondary fluorochrome(s), contaminating primary measurements. This phenomenon is known as fluorescence spillover and it can be quantified and mathematically corrected (i.e. compensated). Finally, the compensated signal intensity collected in each channel reflects the amount of photons coming from the primary fluorochrome (Leach et al., 2013; Roederer, 2002), however, the secondary fluorochromes contribute to the compensation spread of the signal measured on negative events, which limits our ability to resolve weak signals from background fluorescence.

1.3. Basic principles of fluorochrome selection in panel design

Selection of appropriate antigen targets to be included in antibody panels mostly depends on their biological and clinical relevance. However, after a set of markers is properly selected, successful reagent panel design strongly depends on the antibody clones and fluorochrome conjugates chosen for specific antigens (Flores-Montero et al., 2017; Maecker et al., 2004; van Dongen et al., 2012). Thus, fluorochrome selection is a key step in the process of building an antibody panel (Chattopadhyay and Roederer, 2012). The main characteristics that describe fluorochrome performance and contribute to its utility in a panel, include: i) excitation and emission profile; ii) relative brightness as product of how efficiently light is absorbed (i.e. extinction coefficient) and how many photons are emitted per absorbed photon (i.e. quantum yield); iii) fluorescence emission overlaps; iv) antibody-fluorochrome conjugate stability; and v) reproducibility of antibody conjugation. An optimal fluorochrome should have a fluorescence profile matching the optical configuration of the instrument. Moreover, the selected fluorochrome should be: i) sufficiently bright to detect low density antigens or rather dim when used to detect highly express markers (i.e. low density antigens should be interrogated with bright fluorochromes and *vice versa*); ii) restricted overlap into other channels; iii) limited sensitivity to other reagents and procedures (i.e. quenching, different erythrocyte lysing solutions, pre-staining cell concentration steps, presence of fixative, or intracellular staining) or light exposure (i.e. photobleaching); and iv) be available for a large list of antibodies (Baumgarth and Roederer, 2000; Chattopadhyay and Roederer, 2012; Macey, 2007; Maciorowski et al., 2017; Mahnke and Roederer, 2007). Since multiple candidate fluorochromes exist for several fluorescence detectors in commonly used instruments (Table 1), these features must be evaluated for ≥2 fluorochromes to choose the right candidate for each channel-laser combination.

Despite the above principles to guide initial selection of fluorochromes, an optimal fluorochrome combination is rarely achieved *a priori*. Consequently, prospective evaluation with sequential cycles of optimization-revaluations are unavoidable (Flores-Montero et al., 2017; Mahnke and Roederer, 2007; van Dongen et al., 2012).

Here we describe the results of systematic evaluation of different fluorochrome options with a proposal for optimal baseline fluorochrome sets for distinct flow cytometer configurations.

2. Material and methods

A total of 75 anonymized samples −45 bone marrow (BM) and 30 peripheral blood (PB)- were included in the study. Samples were stained following either the EuroFlow standard or EuroFlow bulk lysis (BL) sample preparation standard operating procedures (SOP) available at www.EuroFlow.org (Flores-Montero et al., 2017; Kalina et al., 2012; Theunissen et al., 2016). Briefly, the EuroFlow conventional staining protocol consists of a stain-lyse-and-wash protocol that uses FACS Lysing solution -BD Biosciences, San Jose, CA, USA (BD)- while the BL protocol includes an initial lysing and cell concentration step based on the use of ammonium chloride lysing solution, prior to the stain-lyse-

Table 1
List of representative fluorochromes available for the most typical configuration (laser lines and fluorescence detector -channels-) of common flow cytometry instruments[a].

Laser line (emission) Channel	Representative available fluorochromes	Instruments and collection channels						
		8-colors	10-colors			>10-colors		
		FACSCanto II (8 colors)[c]	FACSCanto II (10-colors)[c]	Navios[d]	FACSLyric (10-colors)[c]	FACSLyric/FACS Celesta (12-Colors)[c]	LSR II[c]	LSR Fortessa X-20[c]
Violet (≈405 nm)								
VL1	PacB, HV450, BV421, eFluor450, VioBlue, SB436	*	*	*	*	*	*	*
VL2	PacO, HV500C, OC515, BV510, eFluor506, KrO, VioGreen, BV480	*	*	*	*	*	*	*
VL3	BV570						*	
VL4	BV605, SB600		*		*	*	*	*
VL5	BV650, SB645						*	*
VL6	BV711, SB702					*	*	*
VL7	BV785, SB780					*	*	*
Blue (≈488 nm)								
BL1	FITC, AF488, BB515, eFluor520, Vio515	*	*	*	*	*	*	*
BL2	PE, eFluor570	*	*	*	*	*	*	
BL3	PE TR/ECD, PE CF594, PE Dazzle594, PE eFluor610, PEVio615, PE DyLight594			*			*	
BL4	PE Cy5/Tricolor, PE Cy5.5, PerCP, PerCP Cy5.5, PerCP eFluor710, PEVio700	*	*	*	*	*	*	*
BL5	PE Cy7, PE AF750, PEVio770	*	*	*	*	*	*	
Yellow-Green (≈561 nm)[b]								
YGL1	PE						*	*
YGL2	PE TR/ECD, PE CF594, PE Dazzle594, PE eFluor610, PEVio615, PE DyLight594						*	*
YGL3	PE Cy5/Tricolor							*
YGL4	PE Cy5.5, PEVio700							*
YGL5	PE Cy7						*	*
Red (≈633 nm)								
RL1	APC, AF647, eFluor660, Vio647	*	*	*	*	*	*	*
RL2	AF700, APC AF700, APC R700		*	*	*	*	*	*
RL3	APC Cy7, APC H7, APC C750, APC AF750, APC Fire750, APC eFluor780, APC Vio770	*	*	*	*	*	*	*

VL, violet laser; BL, blue laser; YGL, yellow-green laser; RL, red laser; PacB, pacific blue; HV, horizon violet; BV, brilliant violet; SB, super bright; PacO, pacific orange; OC, orange cytognos; KrO, krome orange; fluorescein isothiocyanate; AF, alexa fluor; BB, brilliant blue; PE, phycoerythrin; TR, texas red; Cy, cyanine; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; H7, hilite 7 (affymetrix eBioscience, 2017; BD Biosciences, San Jose, CA, 2017a, 2017b; Beckman Coulter Inc., 2017; BioLegend, 2017; Miltenyi Biotec, 2019; ThermoFisher SCIENTIFIC, 2017a, 2017b).

[a] Fluorescence channels are grouped according to the similar section of the spectrum of light they collect, and they are sequentially numbered for the purpose of this table. Actual optical detector configurations may vary.

[b] Most candidate fluorochromes available for the YGL are also (less efficiently) excited by the BL and can be collected on the BL line detectors with the appropriate optical configuration.

[c] From BD Biosciences.

[d] From Beckman Coulter.

and-wash (conventional) protocol. Samples were stained with the reagents listed in Tables 2 and 3, in addition to markers required for proper identification of positive reference cell populations (PRCP) and negative reference cell populations (NRCP). Selection of fluorochromes for further testing was based on broad availability, easy access to custom conjugates, strategic panel development requirements and compatibility with available clinical flow cytometer instruments. For each fluorochrome conjugated reagent we evaluated: i) PRCP brightness -mean fluorescence intensity (MFI); arbitrary units using fixed PMT settings calibrated following the EuroFlow instrument set-up and calibration protocol (Kalina et al., 2012)-; ii) stain index (SI; calculated as described elsewhere) (Maecker et al., 2004), and iii) spillover spreading matrix (Nguyen et al., 2013) and main overlaps into other channels. A complete list of the fluorochromes and fluorochrome conjugates evaluated is provided as supplemental material (Supplemental Table 1).

Data acquisition was performed on FACSCanto II, LSRFortessa X-20, BD FACSAria III and BD Celesta flow cytometers (hardware setup listed for the instruments used in this evaluation is summarized in Supplemental Table 2), all from BD. For all cellular measurements FACSCanto II instruments were calibrated and monitored following the EuroFlow instrument set-up and calibration SOP (Kalina et al., 2012), which was further adapted for the set-up and monitoring of LSRFortessa X-20 flow cytometers.

Flow cytometry data was analyzed using Infinicyt software (version 2.0, Cytognos SL, Salamanca, Spain). Comparisons between fluorochromes involved Wilcoxon's test or Friedman's test for comparisons of two or more groups of paired samples, and the Mann-Whitney's *U* test for comparisons of independent measurements. MFI values are expressed as mean, standard deviation (SD) and 95% confidence intervals and statistical significance was set as ≤ 0.05. For all statistical analyses the Statistical Package for Social Sciences (SPSS) software (version 23;

Table 2
Comparative performance of the different fluorochromes evaluated.

BD Canto II, 8-color optical configuration															**LSR Fortessa X-20, 16-color optical configuration**			
VL1				**VL2**						**BL1**		**RL3**					**VL2**		**RL3**	
Fluorochrome																				
Pac B	**HV 450**	**HV 450**	**BV 421**	**Pac O**	**OC 515**	**Pac O**	**HV 500C**	**OC 515**	**BV 510**	**FITC**	**BB 515**	**APC H7**	**APC C750**	**APC H7**	**APC C750**	**APC AF750**	**BV 510**	**BV 480**	**APC Cy7**	**APC Fire750**
Target marker																				
CD4		CD138		CD138		CD138		CD38		CD38		CD10		CD4			CD45RA		CD56	
Clone																				
RPA-T4	SK3	MI15		B-A38	LD38	B-A38	MI15	LD38	HIT2	LD38	HIT2	HI10a		SK3	GA20	13B8.2	HI100		HCD56	5.1H11
Type of sample tested (n)																				
PB (5)		BM (5)		BM (4)		BM (5)		BM (3)		BM (2)		BM (4)		PB (2)			PB (1)		PB (2)	
EuroFlow Staining protocol																				
Co		BL		Co		Co		BL		Co		Co		Co			BL		Co	
MFI (SD) of PRCP																				
7,290 (436)	7,494 (995)	4,986 (4,562)	31,703 (27,826)	6,574 (2,897)	4,649 (1,944)	2,934 (1,500)	7,034 (3,723)	3,798 (2,432)	66,806 (37,471)	32,952 (17,180)	96,199 (51,714)	5,270 (3,117)	12,408 (6,292)	8,982 (1,991)	4, 576 (2,014)	15,476 (3,241)	18,572 (17,977)	14,940 (14,504)	1,502 (921)	1,140 (681)
95% CI																				
6,748-7,832	6,499-8,489	0-10,651	3,877-59,529	1,385-11,763	1,210-8,682	1,048-4,820	3,600-10,468	1,611-5,985	11,656-121,956	29,315-36,589	9 427-182,971	2,623-7,917	7,902-16,914	4,892-13,072	837-8,315	7,767-23,185	NA	NA	0-4,549	0-2,948
SI																				
93.8	110	24.5	43.4	24.3	17.1	20	50	23.4	329.8	166.2	152.4	11	32.9	30.7	15.7	51.8	39.5	31	5.3	4.2
P value																				
NS		p = .04		NS		p = .04		p = .05		NS		NS		NS			NA		NS	
Reference population (PRCP/NRCP)																				
CD4 + T-cells / Non-T Lymphocytes		PC / T-cells		PC / Mature lymphocytes		PC / CD38- Lymphocytes		PC / NRC		PC / NRC		BCP / Mature B-cells		CD4 + T-cells / Non-T Lymphocytes			NK cells / Basophils		CD56 + NK cells / B-Cells	
Counterstaining markers																				
CD3		CD19, CD27, CD38, CD56, CD45, CD81, CD117		CD19, CD38, CD45, CD56, β2 micro, cyIgs		CD38		CD19, CD45, CD138		CD19, CD27, CD28, CD45, CD81, CD117, CD138		CD20, CD34, CD45		CD3, CD45			CD3, CD8, CD16, CD28, CD56, CD57, CD62L, CD137, TCRγδ, CyGranzB		CD3, CD16, CD19	
Main compensation requirements																				
PacB: VL2, 23% **HV450:** VL2, 21% **BV421:** VL2, 9%				**PacO:** VL1, 2.5% **OC515:** VL1, 7.2% **HV500C:** VL1, 10.6% **BV510:** VL1, 16.2%						**FITC:** BL2, 11.2%; BL3, < 5% **BB515:** BL2, 7.8%; BL3, < 5%		**APC H7:** RL1, 5.4%; BL5, < 5% **APC C750:** RL1 and BL5, < 5% **APC AF750:** RL1, 25%					**BV510:** VL1, 10.3%; VL4, 45.9%; VL5, 20.1%; VL6, 8.9%; BL1, 12%; VL7 and BL4, < 5% **BV480:** VL1, 28.9%; VL4, 17.5%; VL5, 5.4%; BL1, 15%; VL6, < 5% **APC Cy7:** VL7, 15.3%; BL5, 52.9%; RL1, 7.4%; RL2, 9.9%; VL4, VL5 and VL6, < 5% **APC Fire750:** VL7, 14.5%; BL5, 54.8%; RL2, 6.5%; VL1 and RL1, < 5%			

MFI, mean fluorescence intensity; SD, standard deviation; CI, confidence interval; SI, stain index; PRCP, positive reference cell population; NRCP, negative reference cell population; VL, violet laser; BL, blue laser; RL, red laser; HV, horizon V; BV, brilliant violet; PacB, pacific blue; PacO, pacific orange; OC, orange Cytognos; FITC, fluorescein isothiocyanate; BB, brilliant blue; APC, allophycocyanin; H7, hillite 7; C750, C750 dye; AF, alexa fluor; Cy, cyanin; BM, bone marrow; PB, peripheral blood; BL, bulk lysis; Co, conventional staining protocol; NS, not significant; NA, not applicable; PC, plasma cells; NRC, nucleated red cells; BCP, $CD10^{+}/CD34^{+}$ B-cell precursors; b2-micro, β2 microglobulin; cyIgs, cytoplasmic immunoglobulins; TCR, T-cell receptor; cyGranzB, cytoplasmic Granzime B.

Table 3
Performance of individually evaluated reagents in LSRFortessa X-20 (16-color configuration) instrument.

Detector channel									
Fluorochrome	Target market	Clone	Type of sample tested (n)	MFI (SD) of PRP	95% CI	SI	Reference population (PRCP/NRCP)	Counterstaining markers	Main compensation requirements
VL4									
BV605	CD117	104D2	BM (1)	1,382 (980)	NA	9.6	Myeloid precursors/NRC	CD19, CD27, CD38, CD45, CD56, CD138, sIgs	VL5, 59%; VL6, 23%, VL7, 7.9%; BL3, 6.5%; YL1; 21.3%; YL2, 16.2%; VL1 and YL5, <5%
	CD45	HI30	BM (3)	24,761 (10,335)	4,478–45,044	107.1	Mature lymphocytes/ NRC	CD19, CD27, CD38, CD45, CD56, CD81, CD138, sIgs	
VL5									
BV650	CD45	HI30	BM (3)	26,303 (10,507)	14,858-37,748	57.7	Mature lymphocytes/ NRC	CD19, CD38, CD138, CD56, cyIgs	VL4, 14.8%; VL6, 42%; VL7, 11.3%; RL1, 11.1% BL3, 7.1%; VL1, YL5, RL2 and RL3, <5%
YGL2									
PE CF594	CD8	RPA-T8	PB (5)	11,036 (4,012)	8,566–13,506	183.8	$CD8^{hi}$ T-cells/B-cells	CD3, CD16, CD56, CD57, CD161, TCRγδ	VL4, 19.7%; VL5, 9.1%; BL3, 49.9%; YL1, 45.4%; YL5, 20.4%; VL6 and VL7, <5%
RL2									
AF700	Igκ	G20-193	BM (2)	34,525 (24,991)	0–106,372	72.3	CyIgκ+ PC/T-NK-cells	CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, sIgs	VL2, 5.8%; VL6, 12.5%; VL7, 6.2%; BL4, 5.9%; YL1, 5.7%; YL5, 15.8%; RL1, 5.7%; RL3, 29.8%; VL1, VL4, VL5, BL1, YL2, <5%
APC AF700	CD81	M38	BM (3)	8,342 (2,544)	0–17,327	39.7	BCP/NRC	CD27, CD38, CD45, CD56, CD117, CD138, sIgs	VL6, 21.3%; VL7, 11.3%; BL3, 5.3%; YL5, 15.8%; RL1, 10.3%; RL3, 29%; BL4 and VL5, <5%

All samples were processed following the bulk lysis staining protocol.
MFI, mean fluorescence intensity; SD, standard deviation; CI, confidence interval; SI, stain index; PRCP, positive reference cell population; NRCP, negative reference cell population; VL, violet laser; YGL, yellow green laser; BL, blue laser; RL, red laser; BV, brilliant violet; PE, phycoerythrin; APC, allophycocyanin; AF, alexa fluor; BM, bone marrow; PB, peripheral blood; NRC, nucleated red cells; PC, plasma cells; BCP, B-cell precursors; sIgs, surface membrane immunoglobulins; cyIgs, cytoplasmic Immunoglobulins; TCR, T-cell receptor.

IBM, Armonk, NY, USA) was used. Spillover spreading matrix (SSM) (Nguyen et al., 2013) was calculated using FlowJo software (version 9.9.6; Ashland, OR, USA) from single stained capture beads (UltraComp Compensation Beads, Invitrogen|ThermoFisher, Carlsbad, CA, USA) incubated with fluorescent conjugates for 15 min and acquired on either FACSAria III or Celesta flow cytometers.

3. Results

3.1. Comparison of fluorochromes measured in the same detector

Different candidate fluorochromes for single detectors were evaluated and compared in parallel measurements (Table 2). In brief, for the VL1 detector, comparison of Pacific Blue (PacB) and HV450 using CD4 conjugates, showed similar fluorescence patterns and overlap profiles. In turn, BV421 showed a six-fold brighter signal than HV450 for the PRCP (Table 2), with almost twice stain index values and lower SSM values on the VL2 detector when CD138 conjugates were compared (Table 2 and Supplemental Fig. 1). Similarly, for the VL2 detector, comparison of Pacific Orange (PacO) and Orange Cytognos 515 (OC515), revealed a similar performance (Table 2). In turn, HV500C, showed a 2.4-fold increase in brightness and resolution in this channel, compared to PacO. Interestingly, BV510 was 17.6-fold brighter with 14-fold higher resolution than OC515. The main spillover channels for this group of fluorochromes were VL1, VL3 and BL1, with less impact on VL1 and BL1 than in VL3 (Supplemental Fig. 1). The recently available BV480 fluorochrome, which might also be used in this channel, showed an increased spillover to VL1 and BL1 than in other detectors collecting longer wavelength light (i.e. VL3), particularly when compared to BV510 (Supplemental Fig. 1). However, in these same comparison experiments, the BV480 conjugate evaluated resulted in slightly dimmer fluorescence vs BV510 (Table 2).

BB515 was three-fold brighter than FITC, but with a similar (slightly lower) resolution (Table 2), due to increased background and heterogeneity (higher SD) within the reference populations for the reagent evaluated. In turn, for the 3rd detector of the RL (i.e. RL3), APC AF750 displayed a somewhat brighter fluorescence intensity and greater resolution when compared to APC H7 and APC C750 (1.7-fold and 3.4-fold for both brightness and resolution, respectively, on CD4 conjugates comparison), but at the expense of increased spillover into to the RL1 detector (Fig. 1 and Supplemental Fig. 1). Performance of APC H7 and APC C750 was similar in terms of overlap, while they differed in brightness and resolution depending on the specific reagent evaluated. Finally, APC Fire750 displayed a similar performance to APC Cy7 for the conjugates evaluated. Of note, APC H7 and APC Fire750 were designed to be resistant to fixation, which might be beneficial in assays with post-staining fixation.

3.2. Evaluation of other individual fluorochromes

Evaluation of single reagents in channels for which there are limited fluorochrome options, provided baseline information to assess their potential utility and decide about inclusion into specific antibody combinations. Thus, when choosing between BV605 and BV650 for CD45 conjugates, similar brightness and resolution profiles were found for both fluorochromes, while they had different compensation requirements into neighbor channels (Table 3), particularly when low density markers are placed in these neighbor channels and when these are expressed in $CD45^{hi}$ cell populations (e.g. lymphocytes). Similarly, performance of PE CF594 in the yellow-green laser (YGL) 2 and BL3 detectors and of AF700 and APC AF700 in the RL2 detector, demonstrated comparable fluorescence profiles, with relatively bright fluorescence and high SI. While PE CF594 compensation requirements are sometimes high (i.e. SSM value into the BL4 detector is the highest in the dataset), AF700 and APC AF700 spillovers into the RL3 detector are acceptable with appropriate panel design (Table 3 and Supplemental Fig. 1).

3.3. Fluorochrome combinations to be included in 8- 10- and 12-color panels

From all alternatives evaluated, the most efficient options for the VL1 and VL2 detectors are BV421 and BV510, while for the other VL channels appropriate fluorochromes might be selected from several candidates, for example BV605, BV650, BV711 and BV785 for VL4, VL5, VL6 and VL7, respectively. In turn, for the blue laser, BB515 was not clearly superior to FITC as regards to resolution power; therefore FITC, PE, PerCP Cy5.5 and PE Cy7 remain one of the most useful combinations of fluorochromes, although alternative fluorochromes to potentially replace PerCP Cy5.5 and PE Cy7 were not investigated. If an YGL is available, both PE and its tandems can be detected on this laser line, and PE CF594 might be additionally incorporated, which yields more favorable background fluorescence profiles and thereby improved resolution (data not shown). Finally, APC and APC H7 continue to be the most frequently used dyes for RL1 and RL3, while among the evaluated options for RL2, APC AF700 is better for dim markers (at the

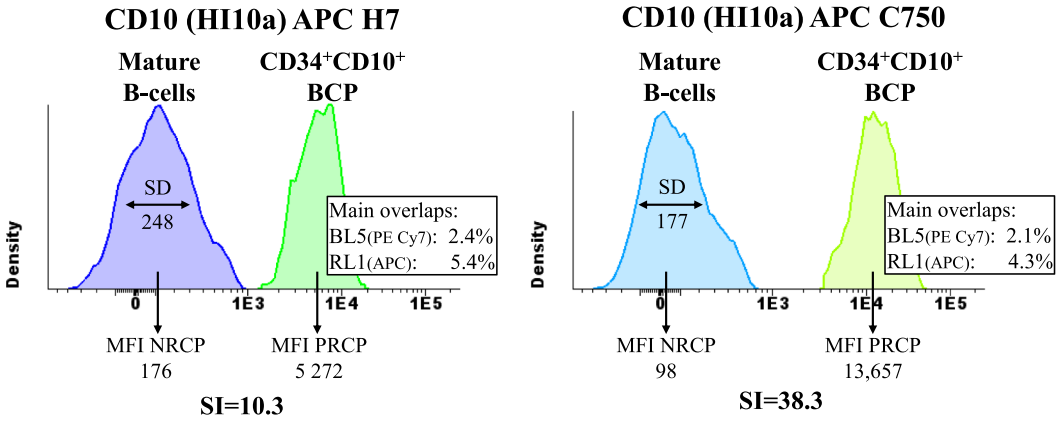

Fig. 1. Illustrating example of direct comparison between two fluorochromes conjugated with the same antibody clone to be measured in the same detector. Two CD10 conjugates of the same clone (i.e. HI10a) but distinct fluorochromes with similar fluorescent profile -i.e. allophycocyanin (APC) hilite7 (H7) and APC C750- are compared in two aliquots of the same BM sample. Under this condition, differences observed are mainly related to the fluorochrome. In this example, the APC C750 conjugate displays a brighter relative fluorescence -mean fluorescence intensity (MFI) of positive reference cell population (PRCP)-, which provides a better resolution -i.e. stain index (SI)-, with a very similar compensation requirement (shown in boxes). BCP, B-cell precursor; SD, standard deviation; NRCP, negative reference cell population; BL5, fifth blue laser channel; RL1, first red laser channel; PE, phycoerythrin.

Table 4
Combination of candidate fluorochromes selected for baseline antibody panel design according to the number of parameters and configuration of distinct flow cytometry instruments.

Number of parameters													
Fluorescence channels													
8-color configuration													
VL1[a]	VL2[b]	BL1[c]	BL2	BL4	BL5	RL1	RL3[c]						
BV421	BV510	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC H7						
10-color configuration													
VL1[a]	VL2[b]	VL4	BL1	BL2	BL3	BL4	BL5	RL1	RL2	RL3[c]			
FACSCanto/FACS Lyric 10-color optical configuration													
BV421	BV510	BV605	FITC	PE		PerCP Cy5.5	PE Cy7	APC	APC AF700	APC H7			
NAVIOS optical configuration													
BV421	BV510		FITC	PE	PE CF595	PerCP Cy5.5	PE Cy7	APC	APC AF700	APC H7			
12-color - 3 laser (VL5, BL5, RL3) configuration													
VL1[a]	VL2[b]	VL4	VL5	VL6	VL7	BL1	BL2	BL4	BL5	RL1	RL2	RL3[c]	
FACS Lyric 12-color optic configuration													
BV421	BV510	BV605		BV711	BV785	FITC	PE	PerCP Cy5.5	PECy7	APC	APC AF700	APC H7	
12-color - 4 laser (VL6, BL2, YGL5, RL3) configuration													
VL1[a]	VL2[b]	VL4	VL5	VL6	VL7	BL1	BL4	YGL1	YGL2	YGL5	RL1	RL2	RL3[c]
Option 1													
BV421	BV510	BV605		BV711		FITC	PerCP Cy5.5	PE	PE CF594	PE Cy7	APC	APC AF700	APC H7
Option 2													
BV421	BV510		BV650		BV785	FITC	PerCP Cy5.5	PE	PE CF594	PE Cy7	APC	APC AF700	APC H7

Fluorescence channels are labeled following Table 1's structure.
VL, violet laser, BL, blue laser; RL, red laser; YGL, yellow green laser; BV, brilliant violet; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; Cy, cyanin; APC, allophycocyanin; H7, hilite 7; AF, alexa fluor; BB, brilliant blue.
[a] HV450 or PacB can be consider as alternative options for this channel.
[b] HV500 and PacO, specially for Navios instruments, are suitable alternatives.
[c] APC AF750 and APC Fire750 may be used to replace APC H7.

cost of higher spillover into RL1) and AF700 lends itself for high expression markers with low spillover into RL1. Table 4 summarizes the proposed fluorochrome combinations according to the instrument configuration.

4. Discussion

The hematopoietic compartment consists of highly heterogeneous cell populations (Blanco et al., 2019; Orfao et al., 2019; Perfetto et al., 2004). Nowadays, clinical flow cytometry progressively uses more markers simultaneously to better define multiple (normal and/or abnormal) leukocyte subpopulations in a single tube. Subsets within the same differentiation pathway and cell compartment might differ only slightly in a limited number of markers, and they might be present at relatively low frequencies in the sample (Braylan et al., 2001; Szczepanski et al., 2006; Wood et al., 2007). Current flow cytometry approaches to study leukocytes rely on optimal antibody combinations to clear-cut discriminate all target populations and their subsets in a single measurement, and accurately assess their phenotypic and functional features independently of their relative distribution in the sample (Flores-Montero et al., 2017; Orfao et al., 1999; Sewell and Smith, 2011; van Dongen et al., 2012). The efficiency of antibody panels does not only depend on the selected CD markers, but also on the fluorochromes they are conjugated to and their compatibility with the optical configuration of the flow cytometer instrument. As a general rule, there is no “bad” or “good” fluorochrome *per se*, but rather fluorochromes that fit better in a panel than others.

Optimal use of all different channels/detectors implies a balance between high sensitivity for detection of dim markers and acceptable loss of resolution for highly expressed antigens. Thereby, in depth evaluation of the performance of alternative fluorochromes is critical to generate initial antibody panel combinations (Mahnke and Roederer, 2007; Sewell and Smith, 2011) and to subsequently optimize them (Flores-Montero et al., 2017; Kalina et al., 2012; van Dongen et al., 2012).

Efficient selection of candidate fluorochromes depends on many different factors (e.g. fluorescence profile, match with instrument's optical configuration, relative brightness and resolution power, fluorescence spillover), including reagents used in sample processing protocols (e.g. red cell lysing or fixation solutions) (Johansson and Macey, 2011; Macey, 2007). In fact, sample processing (i.e. reagents and procedures) might affect performance of specific fluorochromes because of direct effects on the dye molecule or as a consequence of changes on the targeted epitope configuration and/or expression levels. Therefore, differences in the brightness of a given fluorochrome reagent can be observed after different protocols are followed (Kalina et al., 2012; Le Roy et al., 2009; Macey et al., 1995; McCarthy et al., 1994; Szöllosi et al., 1998; van Dongen et al., 2012; Workgroup, 2013). Because of this, once a favorable performance profile is identified for a given fluorochrome, particularly when there are several suitable fluorochrome candidates reagents for a given detector, availability and costs might have a major impact on final fluorochrome selection. Nonetheless, complete characterization of the performance of a given fluorochrome against another similar molecule might require extensive testing involving enough numbers of samples. Thus, while direct comparison between distinct fluorochromes in a limited number of samples might point to the most appropriate candidate for initial selection, some particularities of individual fluorochromes might only emerge after more extensive testing due to e.g. greater levels of data spread.

Albeit the evaluation of the whole list of alternative options per channel seems unfeasible, here an important number of the most common fluorochromes for 8-, 10- and 12-color stainings we had access to at the moment of evaluation, were compared.

Overall, for 8-color instruments, the BV conjugates (Chattopadhyay et al., 2012) BV421 and BV510 are the first choice options for the two first detectors of the VL line (i.e. VL1 and VL2). Similarly, despite their lower brightness vs other available fluorochromes, APC H7 or APC C750, are more appropriate for the RL3 detector because of their more limited overlap with RL1. Regarding ≥10-color instruments, the choice of alternative fluorochromes is restricted due to less reagent availability. However, assessment of the general characteristics of these more limited fluorochromes can guide selection of the most appropriate

antibody-fluorochrome conjugates. For example, when considering CD45 as the target antigen for BV605 or BV650, comparative evaluation of both reagents will help selecting the one that performs better in combination with the other fluorochrome conjugates in the panel, depending on brightness and co-expression of markers placed in channels with high spillover (Table 3). Thus, in such case the relative performance of the two fluorochromes depends more on the combination of markers and labeled cell populations, than on the precise fluorochromes used. In turn, AF700 is regarded to be the first option for the RL2 detector, because of its more limited spillover into other channels.

For some "classical" laser line/detector pairs -i.e. BL (≈488 nm) and BL1 or RL (≈633 nm) and RL1-, few fluorochromes are available, but enough data and experience has been generated over time about them (e.g., FITC, PerCP or APC), some even being among the best performing fluorochromes (e.g., PE or APC) with a wide portfolio of good quality antibody conjugates. The few alternative fluorochrome options that are currently available, have not yet succeeded to replace them. Still, these new alternative fluorochromes -e.g. newer members of BB (BD) family of dyes- might be valid alternatives but their advantages must be demonstrated.

In summary, this study provides a solid proposal for baseline fluorochrome combinations, based on evaluated alternatives, to be used in the design of antibody panels for 8-, 10- or 12-colors format. However, fine tuning might be needed for specific applications, depending on the availability of the required antibody clones, their fluorochrome conjugates and the performance of these conjugated antibodies. Practice has shown that no claims can be made about the efficiency of a given theoretical combination until fully tested and validated (Flores-Montero et al., 2017; Theunissen et al., 2016; van Dongen et al., 2012). Optimally, a fair comparison between fluorochromes would require usage of the same CD markers (and same clones) conjugated with fluorochromes of similar quality, stained in the same sample(s) and under the exact same conditions and measured in the same/identical instruments. Unfortunately, this is not always feasible. New developments in fluorochromes and the introduction of spectral flow cytometry (Nolan and Condello, 2013) will provide new opportunities for antibody panel design.

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