SWOFF - the unrecognized sibling of FMO

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Background:

Fluorescence-Minus-One (FMO) controls are widely accepted and used to determine positive-negative thresholds for each antigen assessed within a multicolor panel. These limits of detection are specific for every population that is distinguishable by a given multicolor staining as specific fluorochrome spillover patterns are associated with each of these populations. Assuming homogeneous expression characteristics for population-defining antigens this approach accurately accounts for the data spreading observed for negative populations upon digital compensation, hence allows discrimination of specific positivity vs background data spreading. However, FMO analysis is not capable to reveal whether events with dim signal intensities are disguised by the data spreading. The "switch-off" approach (SWOFF) presented herein describes an additional way of analyzing data generated in FMO control measurements allowing to indicate hiding of positive events.

Methods:

2 pairs of 10 Color antibody panels - each pair varying the choice of dye conjugation for identical sets of markers - and respective FMO controls were stained in human peripheral blood (Figure1; all antibodies obtained from Beckman Coulter). Markers were selected based on their expression on lymphocyte subpopulations including CD4+ T cells. Thus, different spillover patterns associated with CD4+ T cells in the different panels were provoked in order to compare the impact of these different spillover patterns on the detection of dimly expressed CD117 on a small subpopulation of CD4+ T cells.

Staining was conducted according to the manufacturer's recommendations. In brief, recommended antibody volumes were mixed with 100 µl whole blood (EDTA) and incubated for 15min at RT in the dark. Erythrocytes were then lysed for 10min with a 40:1-mixture of Versalyse (Beckman Coulter) and IOTest Fixative solution (Beckman Coulter) and the samples were centrifuged at 300g for 5 minutes. The supernatant was discarded and the pellet resuspended in 3ml PBS. After further centrifugation at 300g and removal of the supernatant the pellet was resuspended in 300µl PBS and immediately acquired on a Gallios[$] flow cytometer (Beckman Coulter) equipped with 405/488/638 nm lasers and 10 fluorescence detectors (standard filter configuration). Setup of the Gallios[$] flow cytometer was conducted according to the manufacturer's recommendations.

Panel Pair 1	405 Excitation		488 Excitation					633 Excitation		
	Pacific Blue	Krome Orange	FITC	PE	ECD	PC5.5	PC7	APC	APC-AF700	APC-AF750
1	CD4	HLA-DR	CD45RA	CD25	CD45R0	CD117	CD127	CD69	CD8	CD3
2	CD4	CD3	CD8	CD45RA	HLA-DR	CD117	CD45R0	CD69	CD25	CD127

Panel Pair 2	405 Excitation		488 Excitation					633 Excitation		
	Pacific Blue	Krome Orange	FITC	PE	ECD	PC5.5	PC7	APC	APC-AF700	APC-AF750
1	CD19	CD8	CD6	CD25	CD45	CD117	CD127	CD4	CD7	CD49d
2	CD7	CD45	CD6	CD25	CD19	CD117	CD127	CD4	CD8	CD49d

Figure 1: Pairs of antibody panels with identical sets of clones but different sets of conjugations.

Data Analysis was done using KALUZA[$] analysis software (Beckman Coulter). In brief, files from complete staining and from FMO control staining were merged and events were color-coded according to their file parentage. Positive-Negative thresholds for selected markers gated on selected populations were determined by overlaying FMO parameters of complete and FMO control staining. In addition, in case of FMO controls assessing detection limits for brightly expressed and/or discrete antigens overlayed dot plots displaying parameters related to dimly expressed antigens (not containing FMO parameters) were reviewed. As this approach could be interpreted as to "switch off" spillover emissions potentially affecting the detection of dimly expressed antigens it is proposed to call this analysis method "switch-off" or SWOFF analysis.

*APC-Alexa Fluor750. Alexa Fluor is a registered trademark of Molecular Probes, Inc.

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Results:

For the different conjugate combinations used in pair 1 classical FMO analysis revealed - as expected - different positive-negative thresholds for dimly expressed antigens, hence different values for the portion of events gated as positive (Figure 2). Only this comparative approach can show that applying panel 1 out of pair 1 will results in loss of a considerable portion of the CD69+ positive CD8+ T cell fraction as compared to panel 2. Isolated FMO analysis conducted for panel 1 only will define respective positive-negative thresholds but cannot reveal the observed loss of positive events. Of note, in both panels bright APC (660/20) was used for conjugation of CD69 in order to avoid limitations related to conjugate brightness.

Panel Pair 1

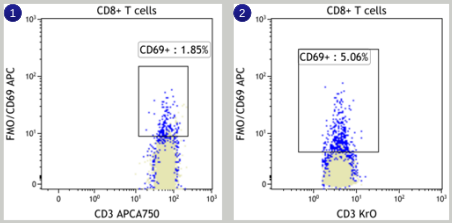

Figure 2: Depending on the multicolor panel configuration used (example from pair 1, configuration 1 vs 2; CD69-APC on CD8+ T cells) different percentages of events gated positive are obtained for each panel when comparing the complete staining (black dots) with the respective FMO control staining (light blue dots).

SWOFF analysis for detection of CD117 PC5.5 on CD4+ T cells is conducted for both panels through merging of ECD-FMO control and complete staining data sets and subsequent review of CD4+ vs CD117 clusters. SWOFF analysis demonstrates that a considerable portion of the CD117+ subpopulation of CD4+ T cells cannot be detected when using panel 1. This conclusion is based on the different shapes observed for the CD4+ vs CD117 clusters in case of panel 1. The complete staining disguises positive events due to a much larger (symmetrical) PC5.5 background data spread than observed for panel 2 where superimposable population shapes are obtained. The different PC5.5 (695/30) sensitivities are related to the marked co-expression of CD45R0 (ECD-label, panel 1) versus the dim co-expression or absence of HLA-DR (ECD-label, panel 2) on CD4+ T cells, respectively. Of note, SWOFF analysis reveals the suboptimal sensitivity for CD117 on CD4+ T cells inherent to panel 1 without the need to compare different panel configurations as described above for FMO analysis.

Panel Pair 1

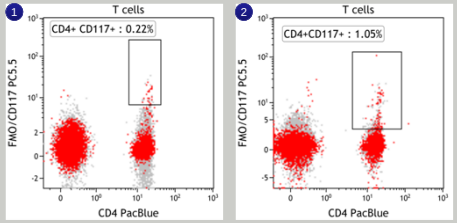

Figure 3: SWOFF analysis of pair 1/ panel 1 (left plot) demonstrates a larger PC5.5 data spread for the complete staining (grey dots) as compared to the FMO control devoid of CD45R0-ECD-staining (red dots). In contrast, for pair 1/panel 2 (right plot) isuperimposable shapes for CD4+ vs CD117 clusters are obtained indicating panel 2 as appropriate configuration for assessment of CD117 expression on CD4+ T cells.

Analysis of complete staining vs FMO-staining for the second pair of panels further elucidates the SWOFF approach. The ECD-FMO controls were compared to complete staining with regard to their data spread for PC5.5 (695/30) and PC7 (755LP) (Figure 4). While superimposable CD4+ populations were obtained plotting CD127-PC7 vs CD117-PC5.5 for pair 2/panel 2 a considerably higher PC5.5 background was detected when applying pair 2/panel 1 rating the latter panel as not suited for detection of CD117 on CD4+ T cells.

Panel Pair 2

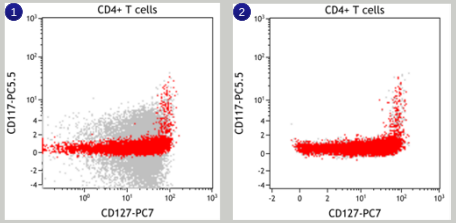

Figure 4: SWOFF analysis indicates poor sensitivity inherent to pair 2/panel 1 regarding detection of CD117-PC5.5 on CD4+ T cells (complete staining with grey dots, FMO-ECD staining with red dots).

SWOFF analysis of the APCAF700[§]-FMO-control data regarding sensitivity for APC (660/20) and APCAF750* (755LP) shows that the complete staining is superimposable with the FMO dataset in case of pair2/panel 1 but bears a much higher APC-background in case of pair2/panel 2 (Figure 5). However, it cannot be concluded that panel 2 is not suited for detection of CD4-APC as the discrete population separation is not at risk and furthermore not even is affected when taking advantage of CD4/CD8 exclusion through gating accordingly (Figure 5, right plot).

Panel Pair 2

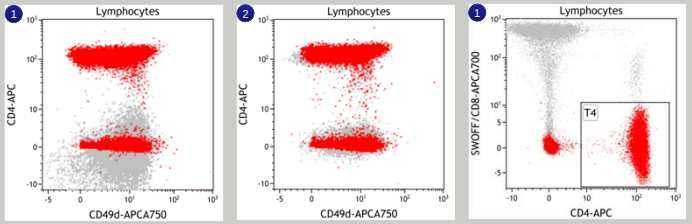

Figure 5: SWOFF analysis indicates a much higher APC background for pair 2/panel 2 than for pair 2/panel 1. However, the identification of CD4+ T cells is not affected due to the exclusion gating strategy applied. Minor data spread effects can be observed for CD49d-APCAF750* background staining either specifically for CD8+ T cells (panel 1, CD8-APCAF700[§] spillover) or for the majority of all T cells (panel 2, CD7-APCAF700[§] spillover).

Conclusion:

SWOFF analysis of FMO control data sets is a new useful concept in order to assure and test for sensitive detection of dim antigen expressions within given multicolor panel configurations. While FMO analysis determines the detection limit for each antigen within the spillover pattern of a given multicolor panel SWOFF analysis - by using the same FMO control data sets – indicates unfavorable conjugate combinations with compromised sensitivity for modulated and dim markers. The autonomous benchmark set by SWOFF analysis for each individual multicolor panel configuration can be visualized appropriately through analysis techniques such as data set merging and dot plot overlay.