SWOFF - the unrecognized sibling of FMO

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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 result 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.

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 (Figure 1; 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 (ECD) and incubated for 10 min at RT in the dark. Erythrocytes were then lysed for 10min with a 4:1 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 1ml 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 405/488/633 nm lasers and 10 fluorescence detectors (standard filter configuration). Setup of the Gallios® flow cytometer was conducted according to the manufacturer’s recommendations.

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 dimmly expressed antigens (not containing FMO parameters) were reviewed. At this approach could be interpreted as “switch off” spillover emissions potentially affecting the detection of dimmly expressed antigens it is proposed to call this analysis method “SWOFF” or SWOFF analysis.

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 (660/20) and PC7 (755LP) (Figure 4). While superimposable CD4+ populations were observed plotting CD127-PC7 vs CD117-PC5.5 for pair 2 and panel 2 is a considerably higher PC5.5 background was detected when applying pair 2/panel 1 rather than the latter panel as not suited for detection of CD117 on CD4+ T cells.

SWOFF analysis of the APCCAPF700/FMO-controlled data regarding sensitivity for APC (660/20) and APCCAPF750® (755LP) shows that the complete staining is superimposable with the FMO dataset in case of pair 2/panel 1 but bears a much higher APC-background in case of pair 2/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 no even is affected when taking advantage of CD4/CD8 exclusion through gating accordingly (Figure 5, right plot).

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.