Sponsored and reviewed by ICCS Quality and Standards Committee

Title: Compensation Tips for Beckman Coulter 10-Color Navios Platform

Written by:

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OUTLINE

Compensation is an important component of assay-specific optimization of a flow cytometer. Incorrect compensation has the potential to lead to false-positive or false-negative interpretation of antigen expression. This module will help the reader understand the technical background of compensation, provide guidance in optimizing instrument settings and some basic troubleshooting tips specific to the Navios.

A previous ICCS Module entitled "Instrument optimization - Adjusting PMT voltages and compensation"[1] should be read as a prerequisite to this module.

Applicable fluorochromes for the 10-color Navios flow cytometer:

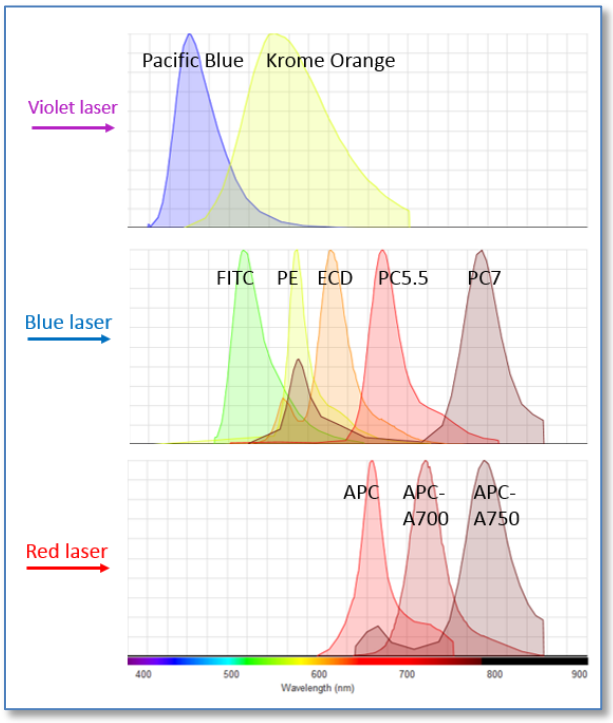
	Blue laser line				
Navios Detector	**FL1**	**FL2**	**FL3**	**FL4**	**FL5**
Fluorochrome	FITC	PE	ECD (PE-Texas Red)	PC5.5 (PE-Cy5.5)	PC7 (PE-Cy7)
	Red laser line			**Violet laser line**	
Navios Detector	**FL6**	**FL7**	**FL8**	**FL9**	**FL10**
Fluorochrome	APC	APC-A700	APC-A750	Pacific Blue	Krome Orange

The recommendations in this document are based on the spectral properties of these 10 fluorochromes. Other fluorochromes are commercially available that can be used in place of some of these fluorochromes, however their spectral properties may be different.

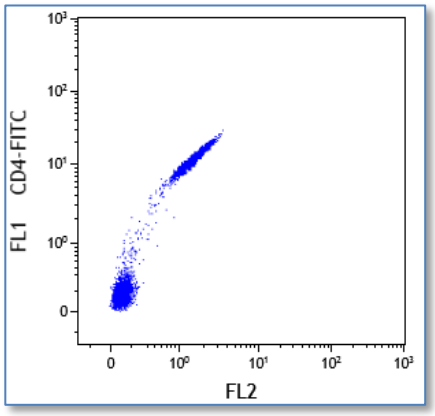
Spectral emission properties of applicable Navios fluorochromes:

Spectral emission graph obtained from:

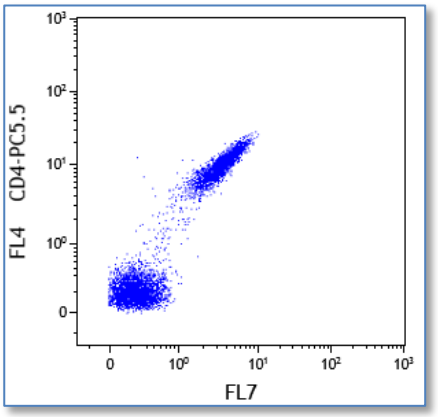
https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html



Spectral spillover occurs most significantly from a fluorochrome into an adjacent detector on the same laser line.
For example, light from the FITC fluorochrome will also be detected by FL2, which is used to detect PE. This is illustrated in this uncompensated plot of a FITC single-stained sample. The positive signal observed in FL2 is due to spillover from FITC.



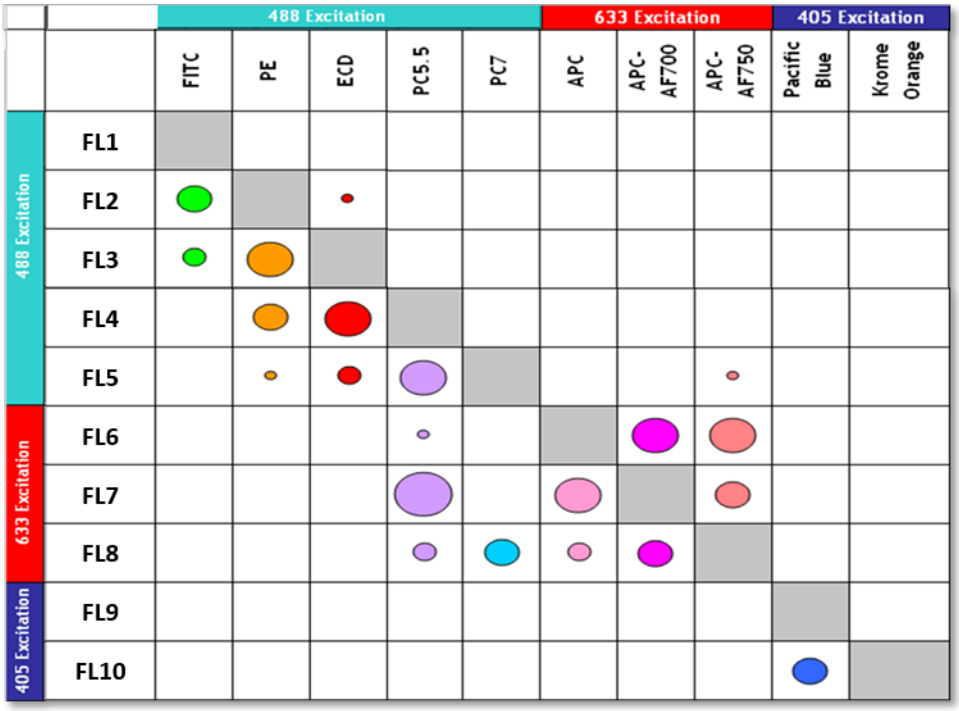
Spectral spillover also occurs between laser lines. For example, this is an uncompensated plot of a PC5.5 single-stained sample. Light emitted by PC5.5 spills over into FL7, which is the detector used for APC-A700.



Typical compensation matrix from a 10-color application on the Navios

To correct for spectral spillover, interfering light is subtracted from the total light entering each detector. The relative amount of light subtracted is represented as a percentage in a compensation matrix.

In the Navios software, the columns of the compensation matrix represent each fluorochrome, and the rows represent each detector (schematically shown below). Therefore, when reading down a column, these are the compensation values required to correct spillover *from* the fluorochrome listed (at the top of the column) *into* other detectors (listed as FL1 through FL10). The size of each bubble is directly proportional to the amount of spillover from the fluorochrome (column) into the detector (row).



(This schematic matrix was based on a compensation matrix obtained on a Navios flow cytometer following voltage setup as per the previous module.[1] Spillover values will vary depending on voltage setup.)

The percentage obtained for each position in the matrix is influenced by the voltages set for each detector. Therefore, voltages can be adjusted to minimize compensation values (refer to the previous module for guidance on setting voltages).[1] The larger the value in the matrix, the more distorted or spread-out the population may appear. Antibody conjugate selection and panel design will also influence how distortion/spreading affects sensitivity of detection of dim antigens.

Details of Navios-specific panel design can be found in the “Know Your Flow” Panel Design Resource from Beckman Coulter (Appendix A at the end of this document).

Single-stained controls for generating a compensation matrix

Historically, lymphocytes or CYTO-COMP/CYTO-TROL Cells stained with anti-CD45 conjugated to each of the 10 fluorochromes have been used to prepare single-stained controls, which are then used with the AutoSetup algorithm in the Navios software. The algorithm only requires a positively stained population to calculate a compensation matrix, and it assumes that the negative peak is located at 0.3 on the scale.

An antibody against an abundantly expressed antigen (e.g., CD3, CD4, CD8), or the same fluorochrome-antibody conjugate used in a lab's fully stained panel can also be used, as long as a bright positive population is generated. An advantage of using the actual conjugate present in the fully stained panel is that the spillover properties of tandem dyes will be accurately calculated.

VersaComp antibody-capture beads (For Laboratory Use Only, and not for use in diagnostic procedures) may also be used. Further details on this topic, as well as how to use Kaluza (For Research Use Only. Not for use in Diagnostic Procedures) as an alternative method for generating a compensation matrix (instead of AutoSetup), can be found in a previous ICCS module.[1]

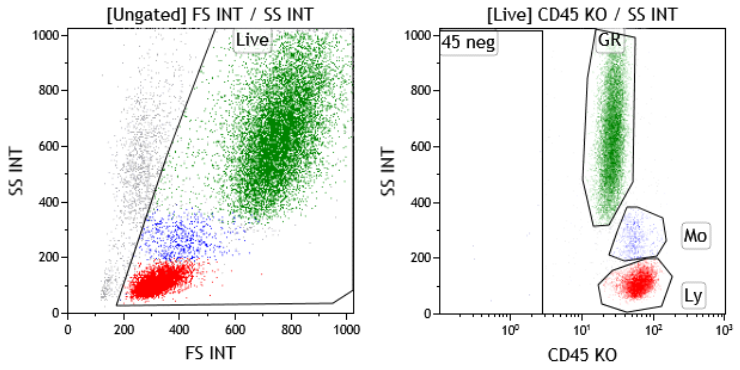
Adjusting/troubleshooting compensation after AutoSetup

For a 10-color application, the compensation matrix automatically generated by AutoSetup must be manually fine-tuned and validated. After the single-stained samples have been acquired during AutoSetup, there is an opportunity for a verify tube to be acquired. It is possible to adjust compensation while the verify tube is acquiring. However, due to the more complicated nature of 10-color compensation, alternative analysis programs such as Kaluza can be used to adjust compensation on the acquired LMD file. A major advantage to this is that logicle scales can be used to properly visualize the data and examine potential compensation issues. Also, the lab staff can take their time to adjust compensation, instead of being rushed to complete it as the tube is actively acquiring.

The following examples demonstrate common fluorochrome combinations that typically require manual adjustment. The advantages of using logicle scaling in Kaluza are also shown.

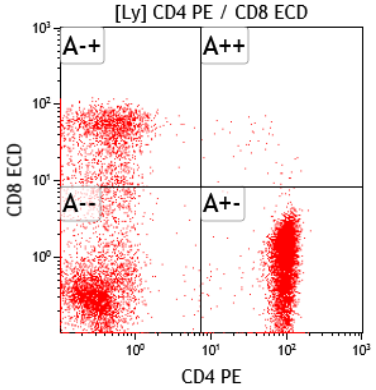
Example #1: PE & ECD

Initial gating plots:



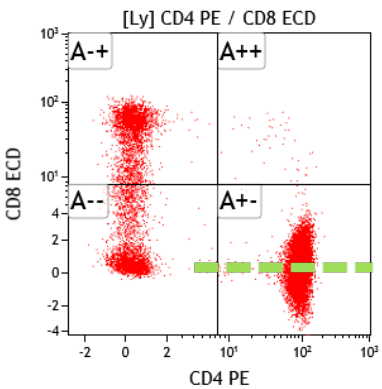
Correct compensation:

Log scale



Gate	X-Med	Y-Med
All	71.1	0.6
A--	0.3	0.4
A-+	0.3	46.9
A+-	93.9	0.4
A++	48.5	29.3

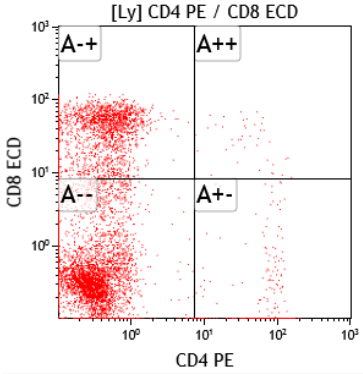
Logicle



Gate	X-Med	Y-Med
All	71.1	0.6
A--	0.3	0.4
A-+	0.3	47.0
A+-	93.9	0.4
A++	48.4	29.4

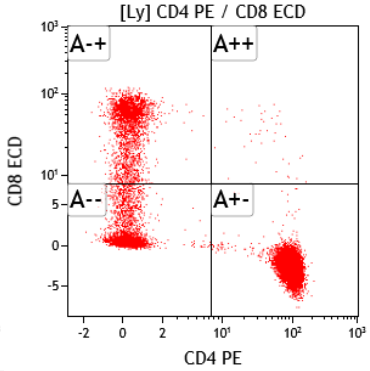
Over-compensation:

Log scale



Gate	X-Med	Y-Med
All	71.3	0.1
A--	0.3	0.4
A-+	0.3	47.0
A+-	94.2	0.1
A++	38.1	29.1

Logicle



Gate	X-Med	Y-Med
All	71.3	-1.0
A--	0.3	0.4
A-+	0.3	47.0
A+-	94.2	-2.5
A++	38.1	29.2

Correct compensation can be visually confirmed by the appearance of symmetrical populations. Logicle scaling is required for such visual judgment. A more accurate way of determining correct compensation can be accomplished by observing the means or medians of the populations.

For instance, correct compensation of PE spillover into FL3 (ECD) can be visually estimated by whether the population in quadrant A +- appears symmetrical (e.g., around the dashed green line provided for reference), when logicle scales are used. Note that this is more difficult to confirm when log scales are used.

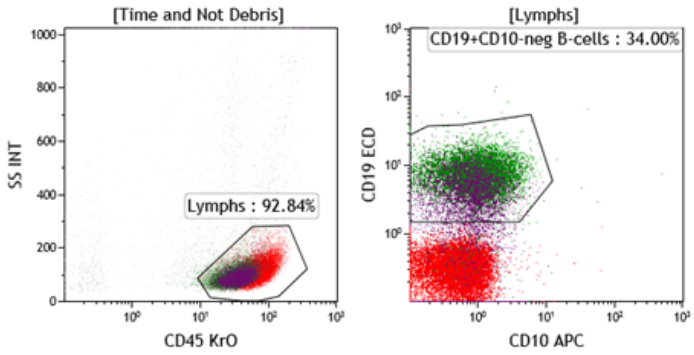
In this example, correct compensation can be further confirmed by looking for similar Y-medians between quadrants A - - and A +- (green arrows).

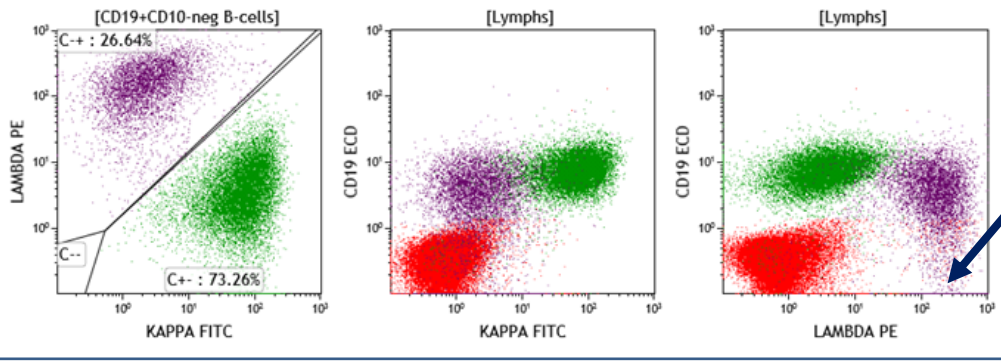
Laboratories should develop their own criteria for acceptability for compensation settings, e.g.,

1. Visual: Populations should appear symmetrical; populations not be "crushed" against axis or "bleed" into the A++
2. Numerical: develop acceptable range for medians

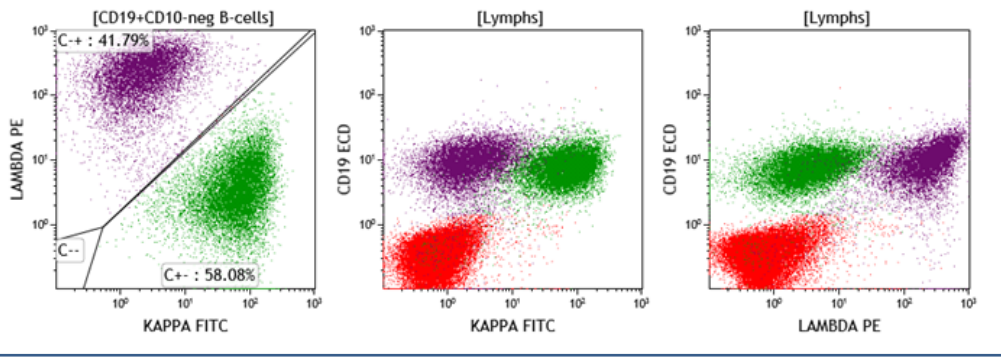
Example #2: PE & ECD

Initial gating plots:





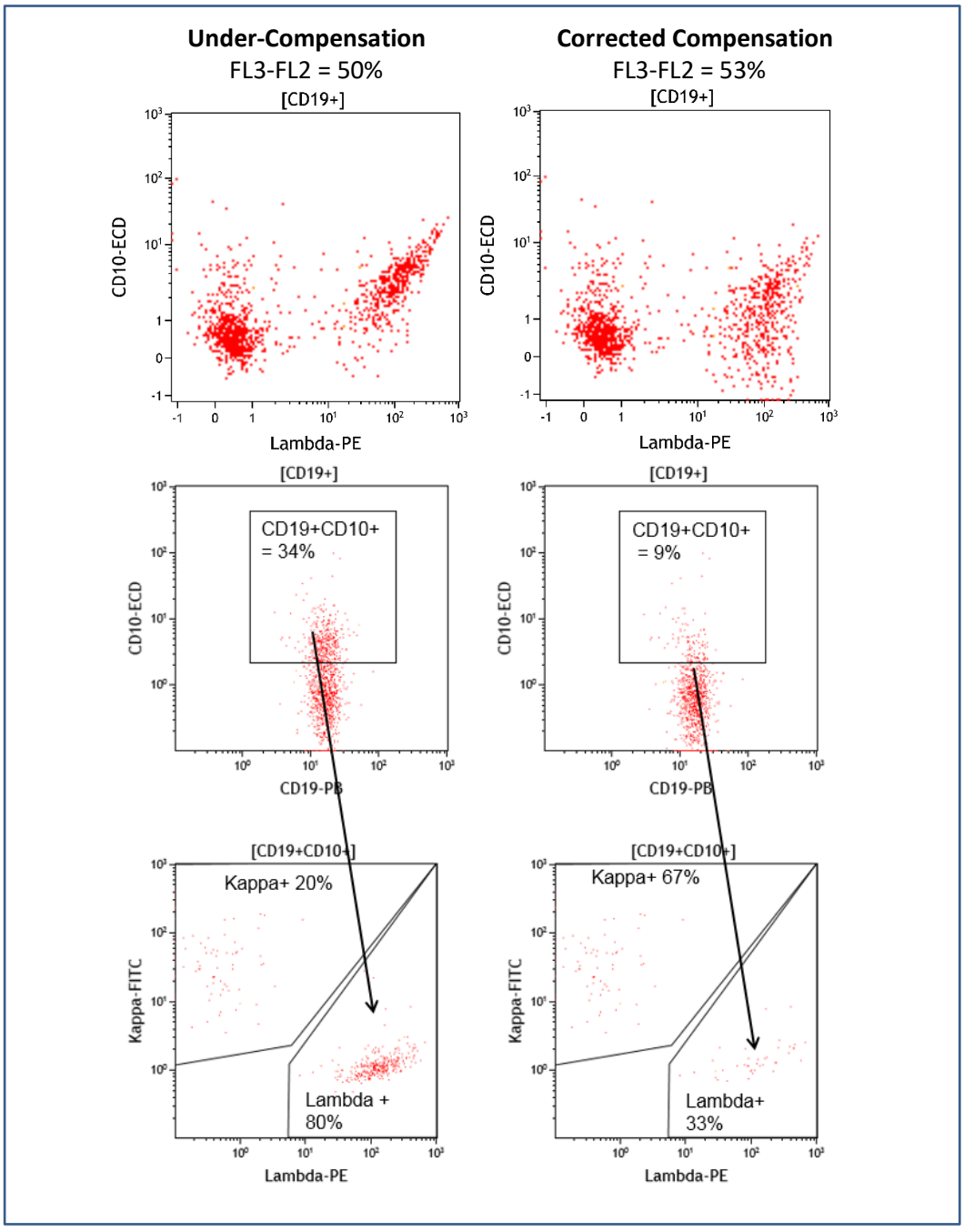
Over-compensation of PE spillover into FL3 (ECD) leads to loss of CD19+ Lambda+ cells and leads to a Kappa/Lambda ratio of 3/1



Corrected compensation: Kappa/Lambda ratio is now 1.4/1

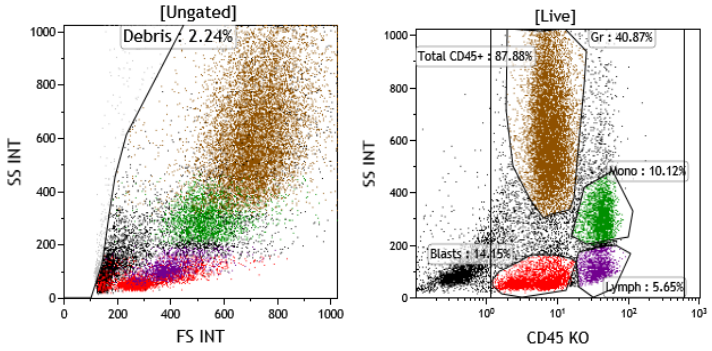
Example #3: PE & ECD

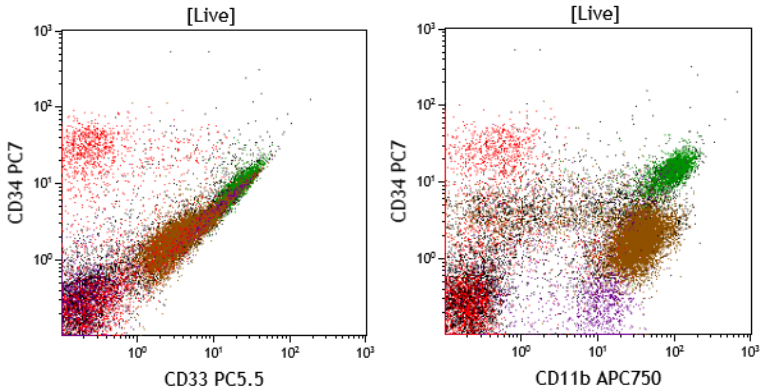
In this example, a bright lambda-PE positive population causes compensation issues with CD10-ECD. If this spillover is under-compensated, a CD10+Lambda+ population could be erroneously reported:



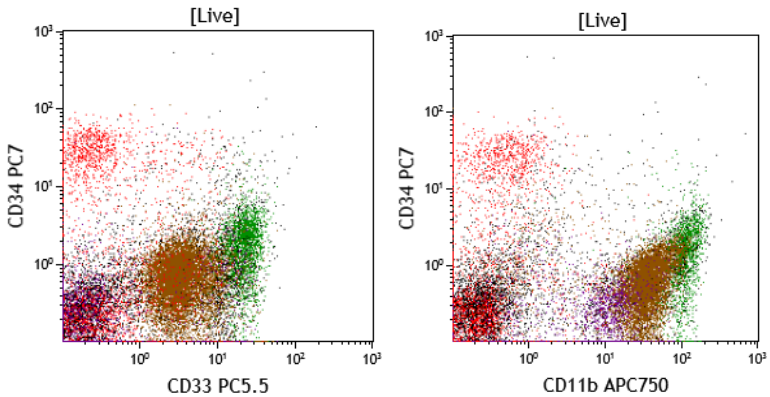
Example #4: PC5.5 & PC7

Gating plots:





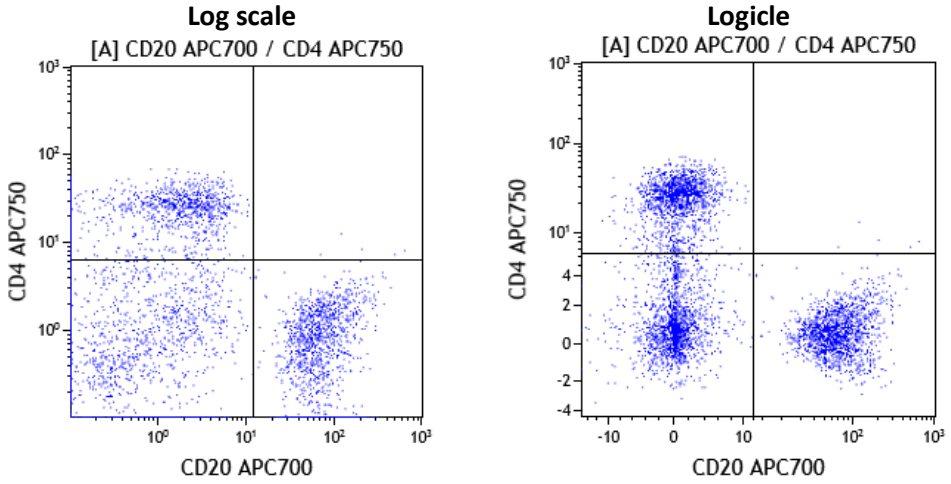
Excess (under-compensated) spillover signal from PC5.5 into FL5 (the detector used for PC7) (left plot) leads to CD11b monos (green) and grans (brown) to appear artificially CD34-positive (right plot).



Optimal compensation between PC5.5 & PC7

Example #5: APC-A700 & APC-A750

The following example illustrates the "spread-out" nature of populations stained with APC-A700 and APC-A750. These two fluorochromes exhibit spillover into each other's detectors, leading to a post-compensation spreading effect. In addition, these fluorochromes emit light at relatively long wavelengths, which is less efficiently detected by photomultiplier tube (PMT) detectors. This leads to a greater error in the measurement of fluorescent light, and therefore a more spread-out appearance of the population. Logicle scaling enables the full population to be viewed onscale, which helps determine if compensation appears correct.



After editing the compensation matrix in Kaluza:

Once the compensation has been adjusted in Kaluza, this edited matrix can be entered into the appropriate cytosettings protocol in the Navios software, so that future LMD files will be recorded with this edited matrix (see detailed instructions in a previous ICCS module).[1] If the fluorescence emitted by all 10 fluorochromes is stable day to day, this edited compensation matrix should not need to be adjusted. This can be confirmed by running a verify tube routinely.

Verify tube

Compensation issues can be identified and corrected by observing populations in the single-positive quadrants of a bivariate plot. Therefore, it is ideal to use a combination of antibodies that yield as many single-positive populations as possible on plots that will have significant compensation applied. Ideally, a lab would use antibodies that are already in their inventory that yield clearly defined and bright populations.

A Kaluza template can be used to facilitate compensation verification on a routine basis. This template should consist of all possible bivariate plots representing spillover between all channels. The plots most likely to require adjustment can be placed at the top of the protocol (refer to the Compensation Bubble Matrix above). Another advantage of using Kaluza software for routine verification of compensation is that the data can be exported in an electronic PDF format as documentation of the compensation verification and to meet accreditation and regulatory requirements.

Also, it is recommended to incorporate all possible dot plots in specimen analysis templates to evaluate compensation per specimen, as the applied compensation may not be appropriate due to specimen-to-specimen variation. For example, in cases of bright over-expression of a marker, care should be taken to observe dot plots displaying the over-expressed marker versus parameters that receive spillover from that marker. These plots may require compensation adjustments per specimen.

A reference verify tube data file (e.g., one that was acquired immediately after the assay and compensation were optimized) can be used to compare to future verify tube samples for routine monitoring purposes. Verify tubes should be acquired and evaluated on a routine basis.

Re-compensation should be considered according to manufacturer's instructions, potentially including any changes in voltages, preventative maintenance visits, or major instrument repairs. If the verify tube sample appears significantly different from the reference verify tube data file, and no major hardware issues can be identified as the cause, the lab should not jump into re-compensation until the underlying cause is rectified. For example, reagents (e.g., lot changes) and sample preparation procedures (e.g., automated sample preparation instruments) should be investigated first.

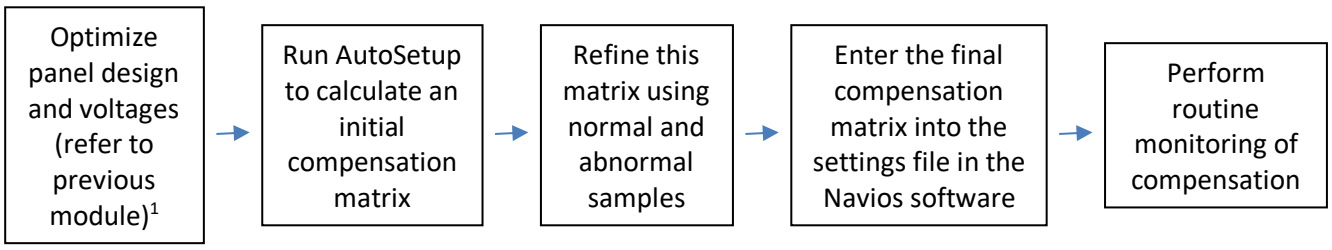
There are many options for types of samples that can be used for a verify tube. Examples are listed below with advantages and disadvantages:

Sample Type	Advantages	Disadvantages
Normal Fresh Peripheral Blood	Cellular autofluorescence closely matches fresh samples; acts as a process control for RBC lysis reagents as well as antibody reagents	Normal samples may be difficult to obtain day to day; specimen-to-specimen variability is expected
CYTO-COMP/CYTO-TROL Cells (lyophilized lymphocytes)	Day-to-day appearance of cells is consistent within a lot	Cells may not express all markers present in a peripheral blood sample
IMMUNO-TROL Cells (stabilized whole blood product)	Day-to-day appearance of cells is consistent within a lot; acts as a process control for RBC lysis reagents as well as antibody reagents	Background autofluorescence is higher than fresh blood samples due to the solution used to stabilize the product

This is not a complete list of possible sample types. Other commercial control samples are also available, such as CD-CHEX and CRISP control cells. In addition to the advice above, when considering which control sample to choose for a verify tube, labs should investigate whether the sample possesses an adequate percentage of positive cells for the antigen of interest (e.g., rare antigens).

Summary

Achieving optimal compensation is crucial for the correct interpretation of flow cytometry results, summarized in the following workflow:



Further resources for laboratories running flow cytometry lab-developed tests can be found at http://www.cytometry.org/web/quality.php

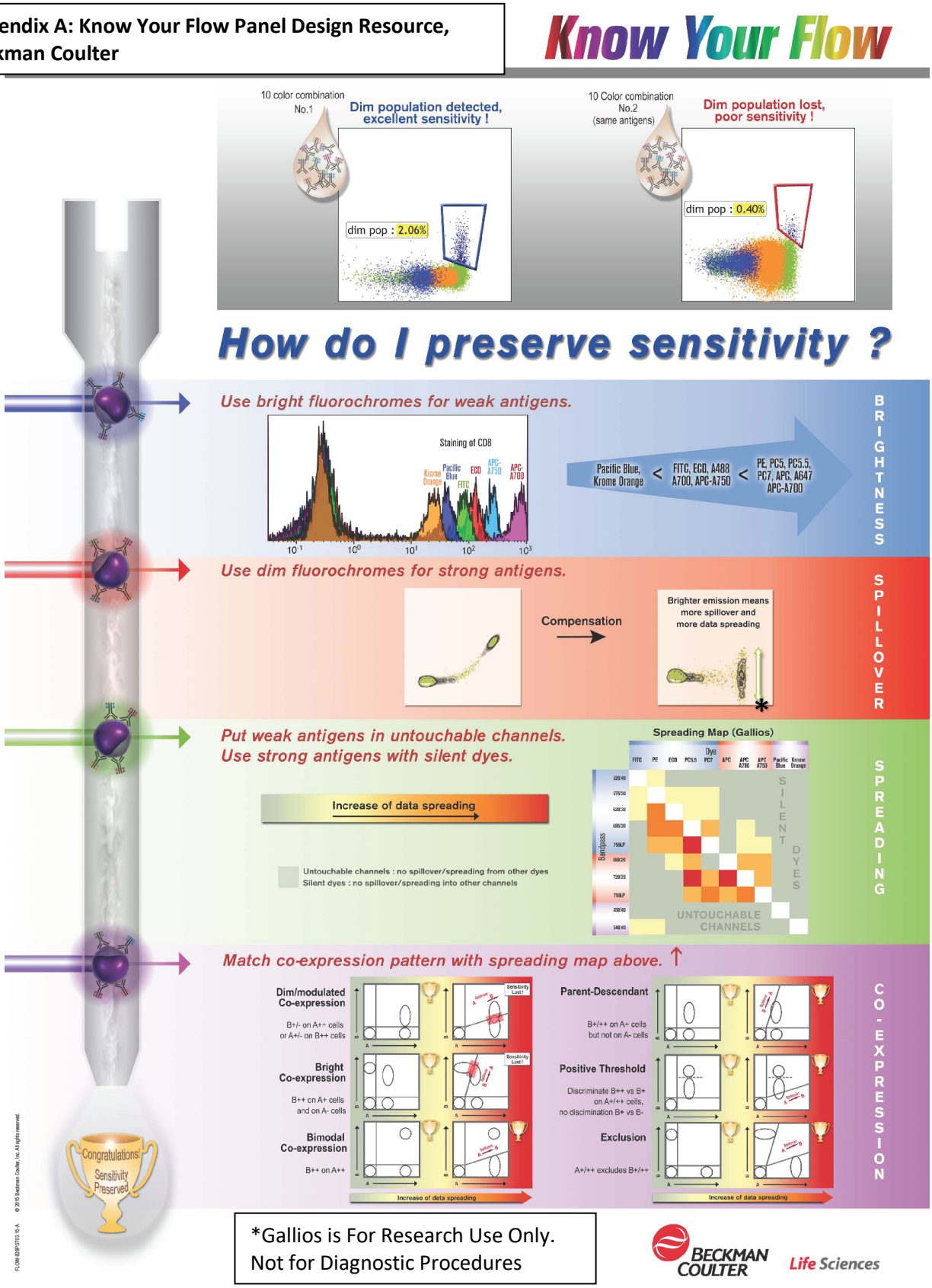
For any questions on this module or any other suggestions, please email info@cytometry.org

References:

1. "Instrument optimization - Adjusting PMT voltages and compensation" https://www.cytometry.org/web/modules/module2.pdf

Reviewed and approved by: Ben Hedley and Ahmad Al Samman

Appendix A: Know Your Flow Panel Design Resource, Beckman Coulter



*Gallios is For Research Use Only. Not for Diagnostic Procedures

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