BD FACSMatters Webinar – Compensation Matters: Considerations When Designing Your Flow Cytometry Panels
Questions and Answers

1. Does this mean that fluorochromes that have a high spillover value should not be used to separate populations? i.e. CD8 vs CD4

   Answer: Where you don't want to use fluorochromes with very high spillovers is on markers that are co-expressed on the same population.

   ANY two fluorochrome/CD reagents measured in different detectors (e.g. not FITC and AlexaFluor488) can be used to distinguish two populations IF the markers are exclusively expressed on different population e.g. CD4 on helper T cells and CD8 on cytotoxic T cells or CD19 on B cells and CD3 on T cells. The spread might be very large and the profile rather ugly, but the populations can still be cleanly gated.

2. How useful are Pseudocolor plots?

   Answer: Pseudocolor plots can be equally useful as straight non-colored contour plots. Both are giving you density information. I prefer straight contour plots with outliers, but it is a matter of taste between the two. Many people like pretty colors. Either are better than straight dot plots.

3. In the case of cross-laser excitation, why can't the software adjust for/correct it using the time delay between lasers? (APC is off the red laser, PerCP-Cy5.5 off the blue laser)

   Answer: This can't be corrected by laser-delay because although we measure PerCP-Cy5.5 from the excitation off the blue laser, in fact the fluorochrome tandem is being excited by both the blue (PerCP) and the red (Cy5.5) lasers.

   The time delay cannot distinguish between the signal generated by APC or by the Cy5.5 from the red laser. The Cy5.5 although having a higher Em max than APC still has significant spill into the APC detector.

   To see this in action use the BD Spectral Viewer. Set the laser to 640nm and select APC and PerCP. This is more easily seen with APC and PE-Cy5 as shown in the webinar.

4. We are always told that we are compensating the fluorochromes and not the beads/antibodies/cells. Why is it then that compensation using beads is not as good as using cells?

   Answer: You *are* compensating the fluorochrome and not the bead/cell.

   However, remember the #1 rule about compensation controls: "The spectra of the fluorochrome on the compensation control must be identical to that used in your experiment". So, having the same spectra profile is important. However, for reasons we don't fully understand, the spectra of a fluorochrome bound to a bead can be slightly different than the same fluorochrome bound to a cell. This leads to differences in spillover values.

   Depending upon the fluorochrome, these differences can be large or small. That is why it is important for you to test and understand these differences and how they may (or may not) affect your assay.

5. Where can I find out which fluorochromes do not work well with comp beads?

   Answer: For BD reagents, if we know that a fluorochrome will never work well with our BD CompBeads we put that information in the TDS.

   However, for many fluorochromes the SOV may have variable inaccuracies. For that reason, we cannot provide a blanket statement and therefore recommend that when working with a new reagent or developing a new assay you run single color compensation controls using both beads and cells to determine if the bead SOV values work in your system. This typically only needs to be done once. Refer back to the presentation for examples.

6. Does MFI mean Median FI or Mean FI?

   Answer: In this presentation and in Hi literature MFI refers to Median Fluorescence Intensity. In earlier days, it referred to Mean Fluorescence Intensity, however today Median is the more commonly used metric.

7. Would you recommend compensation before measurement or also suggest compensation in software like FlowJo on uncompensated data?

   Answer: The two approaches are essentially identical. If you want to look at your data while it's being collected then you want to run your Comp tubes first, then apply the compensation to your collection tubes. The advantage of this is also that you can make sure that your compensation controls are good before your have collected all your data and thrown out the tubes.

   However, running your compensation controls and sample tubes at the same time and then using a program like FlowJo to do compensation post data acquisition is equally valid.

8. Can you describe what information MFI measures? Area under curve in a histogram?

   Answer: MFI stands for Median Fluorescence Intensity.
   For each cell / event the fluorescence intensity is the number of photons emitted by the fluorochromes on the cell which fall within the filter window of the primary detector. For any population of cells the MFI is median of all the individual fluorescence intensities.

9. After doing the compensation, the machine did not come up with overspill messages, however, when the comp matrix is reviewed, some population still looked uncompensated. How do you deal with this?

   Answer: If the spillover matrix has been correctly calculated with appropriate compensation controls, then there should not be any populations that look ***un***compensated.

   It is possible that some biological populations may be partially ***under*** compensated especially if the compensation control did not perfectly match the biological sample.

10. I am often told that there is no need to compensate for the dead cell exclusion dyes. I have always compensated with these, but am I wasting my time doing this?

    Answer: This is partially true (theoretically). Since you are going to gate out (remove) the viability dye positive cells from the analysis, leaving only the dye negative cells, which should have no spillover into the other detectors.

    The caveat to this is that you must gate out all viability dye positive cells. Dimly stained cells could give spillover into other detectors. In general, it is good practice to also include a compensation control for your viability dye. However, any errors in the spillover values are easily tolerated.

    It is important to correctly compensate all other fluorochromes which spill ***into*** the viability dye detector. Otherwise you could get false viability dye positive cells which would be incorrectly gated out in your analysis.

11. Something that I always struggle with is deciding which samples to use when setting voltages. Should I set my voltages based on my uncompensated samples first (to get them on scale) and then acquire compensation bead controls?

    Answer: First of all, you probably don't want to be setting your voltage on an experiment by experiment basis. You probably only need 2 or 3 different sets gain (PMTV) settings for all your experiments. Each set of gains can be saved as an Application setting (FACSDiva) or Assay Setting (FACSuite). These application settings can then be reused for multiple experiments.

    Gain settings should always be done with uncompensated samples. Please contact your local Application Scientist to get advice on how to determine good PMTV and create application settings.

12. Will the data still be valid if you adjust the PMT voltage when you acquire your samples after you have applied compensation?

    Answer: On the FACSuite™-based systems such as the BD FACSVerse™ and BD FACSLyric™, you can adjust the PMT voltage and the system will automatically adjust the spillover values to be correct and the new compensation will be applied to the sample data being collected.

    On other BD FACSDIVA™-based systems such as the BD FACSCanto™ or BD LSRFortessa™ X-20 you cannot adjust the PMT voltage after you have applied the compensation to the experiment.

13. Will the EuroFlow 8 peak rainbow bead target values be available for Lyric soon? If so, how should we tackle the additional detector standardization?

    Answer: This is currently under development at BD. Please check with your local Applications Scientists for any updates.

14. In some cases, when compensating using beads (UltraComp), the positive population looks fine and they are located at let's say 10^4 MFI. However, when collecting the actually stained cells, the positive population is very dim (although i know that they should be bright population). How could I solve this? Should I stain compensation controls using cells in those cases?

    Answer: This is probably due to the fact that the compensation bead is capturing a specific number of Ab molecules. Therefore, the brightness is similar for most antibodies. The brightness of the same Ab on the cells depends upon the Ag density on the cell. So, if the specificity of the Ab is to a low-density Ag then you would expect lower fluorescence compared to the Compensation bead.

    You state that when you used the Ab as a single stained control the population is very dim even though you expect it to be bright. This should have nothing to do with compensation but could be due to a number of factors. A high probability is that the amount of reagent used for the cell staining was not at saturation.

15. Sometimes when matching bright fluorochrome to highly expressed antigen by mistake or due to absence of antibody with dimmer dye, the spread of data (I guess resolution as well?) after compensation is sometimes so high that it is difficult to analyze. The spread of negative data is high, so it becomes difficult to distinguish from positive population. Is there anything you can do to analyze accurately without redesigning a new panel?

    Answer: Unfortunately, at that point there is really nothing you can do that will actually change the spread you see. As discussed in the webinar, the amount of spread is inherent in the biology of the assay (i.e. what antigens are co-expressed and their expression level) and the reagents used (i.e their spillover/spread).

    That is why it is critical to spend the time designing the assay ahead of time. There is one option you have in designing the assay. If you have to use a bright fluorochrome with high spillover on a highly expressed antigen. You can titrate the antibody (lower than saturation) and use a lower concentration which would result in less intense staining while maintaining resolution. In this case it is important to be careful with subsequent dilution of your antibody since you are no longer working at saturation.