BD FACSMatters Webinar – Panel Design Matters: Practical Aspects of Designing Flow Cytometry Panels
Questions and Answers

1. If you lower the voltage in the channel, where you see the spread, would this decrease the spread?

   > **Answer:** It will decrease the spread "value" but not the increase in spread relative to the double negative population. In the "spread" detector the ratio of the spread of the positive and negative populations stays relatively constant.

2. Double positive stain index table: the number meaning voltage or something else?

   > **Answer:** It is not voltage. The calculation of the Double-Positive is shown on Slide 14 (The Double Positive Stain Index) of the webinar. In short, for a given fluorochrome it is (MFI of the double positive population – the MFI of the single positive population) divided by two times the rSD (in MFI) of the single positive population. Completely analogous to the standard Stain Index. Since the units are MFI/MFI the stain index is unitless.

3. When we use brilliant violet colors, do we need to use the brilliant violet buffer? Why can't we use the normal buffer?

   > **Answer:** Definitely! Whenever you use two or more Brilliant Violet/UV/Blue reagents in a single cocktail you should add the Brilliant Stain Buffer to the cocktail. This will inhibit any non-specific interactions of the dyes. Normal buffer will make up most of the volume. Please follow the instructions in the TDS.

4. It's recommendable make a compensation again or try to fix when we have compensations problems?

   > **Answer:** It depends upon what the "compensation problem" is and why you have it. In the webinar we talked about ways of evaluating your compensation controls. If you look at your compensation and single-color controls and there seems to be a problem, then I would change my compensation controls. Again, making sure the compensation controls follow the four principles. If you have evaluated all of your compensation controls and they appear to be good but the compensation in your multi-color tube appears off, you can consider manually adjusting (correcting) the spillover values. But as noted in the webinar, great care should be used as you can make the problem worse not better.

5. I have been taught that to generate single colors/comp I should use my cell type, and other times that beads was the best. I am a bit confused what finally I should do or if there are specific situations where one is more convenient than the other (and vice versa).

> **Answer:** Which compensation controls you use is a balancing act between obtaining accurate spillover values and the ease/cost of preparing the controls. So, the concept of "best" is relative. In general, the best compensation control is the same cell type stained with the actual reagent as in the experimental sample. However, this is not always practical and rarely more convenient. The cell sample may be precious, the positive population too small or too dim. In such cases it makes sense to use an Ig-capture Compensation bead, which can be used with either the same reagent as in the assay (lot-specific control; especially for tandem dyes) or a generic fluorochrome antibody. The key here is to test any and all compensation controls you may use to insure they give you sufficient accuracy of the spillover values for your assay. Such Ig-capture beads may not give you the accuracy you need, in which case you will need to revert to the less convenient cellular compensation controls.

6. According to your lecture, do you think it would be easy to see false double positive population for CD45RA (BV650) and CD45RO (BV421)? I first ran the samples without the Brilliant buffers, and it didn't make sense at all, but after adding this buffer, the plot looked better, but still I see some double positive population in the plot.

> **Answer:** Unfortunately, without seeing the exact data I cannot give a specific answer. The fact that by adding the Brilliant Stain Buffer the data looked better indicates that it worked and was preventing interactions of the dyes and thus should prevent false double positives. Most multi-color panels have lots of double positive populations and they are likely real. In your specific case, although CD45RA and RO expression are inversely proportional, there is every reason to suspect that you could see double positive populations. Remember from the webinar, false double positive populations will typically be on a diagonal from the negatives (especially dead cells) or a double positive that is dimly positive for one maker and shows a diagonal expression as shown in the webinar.

7. Does the guide have any tips for co-localizing domains (such as in CD3) that might produce a FRET effect?

> **Answer:** I assume you are referring to subunits of CD3 such as CD3ε and CD3γ. A similar situation would by Ig heavy chains and light chains. I don't have any specific tips. For FRET to occur it requires very short distances between the epitopes/fluorophores. I have never

looked at CD3 subunits but can say I haven't seen evidence of FRET when staining Ig H and L chains. It would be hard to quantify with standard immune-fluorescent reagents since both fluorochromes would be simultaneously bound to the antigen. FRET is usually measured with specific probes. You could evaluate by looking at each epitope separately and comparing to the double stain. But that interpretation could be complicated by epitope cross-blocking.

8. With more channels/detectors available, I start wondering which channel can detect more autofluorescence by cells like macrophages?

> **Answer:** This is easy to test for yourself. Run unstained Lysed Whole Blood and put FSC vs SSC gates around the lymphocytes, monocytes and granulocytes. Then look at the autofluorescence of each population in each detector. In general monocytes and granulocytes will have higher autofluorescence compared to lymphocytes. Typically, the auto-fluorescence emission (irrespective of the population analyzed) is higher at the lower wavelengths (400 to 550/600nm) and decreases at higher wavelengths; >600nm. This is why you can increase the PMT gains for far red detectors (e.g. PE-Cy7, APC-Cy7, APC-R700). The autofluorescence is so low that the increased gain does not significantly increase the background MFI while increasing the positive population MFI. However, for a "bluer" dye like FITC as you increase the gain the autofluorescence MFI increase proportionally. Of course, the MFI of what you "measure" as autofluorescence will depend upon the gain of the individual PMTs.

9. Is it possible to add in viability stains using GPS?

> **Answer:** You can add them manually in the Antigen Tab, the antigen named is the viability dye. But you also have to add the fluorescence intensity and the detector that the dye is seen in. However, when thinking about Panel Design viability dyes need to be considered differently. There really aren't populations that are functionally double positive for the viability stain. If a cell is positive for the viability stain, then it will be gated out as a dead cell. Therefore, you don't have to consider the spillover of the viability dye into other detectors and the "antigen density" should be specified as dim or zero. GPS should be able to take this into account. Of course, spillover of other antigens into the viability dye detector is important as it would cause spread and would impact what is called positive.

10. If there are two steps of staining for one ANTIGEN (PRIMARY+SEC Ab), what is the FMO?

> **Answer:** It depends on why you are using the FMO. Let's refer to your Antigen as AgX. Remember FMO stands for Fluorescence *MINUS* One. The one being a given fluorochrome. If you are using the FMO to identify the spread of that reagent (Primary + Secondary) into other detectors, then remove both steps of staining for AgX. If you are using the FMO to determine what is positive (i.e. above background) in terms of AgX you can do one of two things. First you could remove the Primary and Secondary stains. This would show the background due to autofluorescence and spillover/ spread. Second you could just remove the Primary Stain. This would show the background due to autofluorescence, spillover/ spread and non-specific staining of the secondary. In higher multi-color panels spillover/spread is a significantly greater contributor to background than autofluorescence and non-specific staining so a straight FMO is probably sufficient. However, it is always good practice to test the assumption in your assay.

11. Is BD planning to incorporate Antigen density information into the GPS?

> **Answer:** This is something we are looking into. However, BD (or any company) cannot provide information for Ag density for every antigen on every sub-population. (Remember antigen density information is only meaningful at the level of individual sub-populations.) So, in many / most studies outside of classical T cell / B cell sub-populations you are going to have to get the antigen density info yourself from experiments or the literature.

12. Thanks for the webinar. I am facing a situation where my CD62L PECF594 is spreading into my CD4 PerCP. Both are expressed on the same cells. However, the compensation value for PECF594 spreading into PerCP is very little. I will appreciate any help or advice about this.

> **Answer:** You bring up a very important point which is just touched upon in the webinar. Look at the slide which shows that changing the PMT voltage can dramatically change the spillover (i.e. compensation) values but does not change the actual amount of the spread. What this means is that spillover values are not necessarily an accurate indication of the level of spread. PE-CF594 definitely spills into PerCP. So, in your case the lower spillover value may be due to the relative PMT voltages of the two detectors. The best way to evaluate the amount of spread for a given reagent is to simply look at the single stained control and visually look at the spread into other detectors (or measure the spread as the rSD of the positive cells in the other

detectors). You might consider moving either CD62L or CD4 to a dim fluorochrome off of the Red laser such as APC-Cy7.

13. How will your panel design change if you are using Phospho-proteins? Is there anything special I should consider?

> **Answer:** It is important to remember that in Panel Design you are focusing on two things. 1) Making sure you are using bright enough reagents to resolve dim populations. 2) Minimizing spread due to spillover. The spread is related to the amount of spillover and staining brightness. Staining brightness is a function of fluorochrome brightness and antigen density/abundance. In these regards all markers are equivalent. However, there are special considerations when dealing with intra-cellular reagents like an anti-Phospho-protein. First it is hard to estimate antigen density / abundance. Second, fluorochrome brightness rankings based on surface staining do not translate well when those fluorochromes are used intra-cellularly. So you can estimate the overall fluorescence intensity of your phosphor-protein staining by comparing it to CD4. About the same brightness would be a +++; 1/10th the intensity ~= ++; 1/100th ~= +. These values can be put into the GPS.

14. What is the benefit of using and unstained with an FMO, I'm not sure I get what information the unstained would add to the process of panel design?
15. In our study, we always have this problem. We have non-transduced cells and transduced cells. We examine that one marker expression. We always find that the negative population of this marker is not the same between these two populations. What is the problem? It's hard to compare if this the case.

> **Answer to Q14 and Q15:** Unstained controls are most useful when designing a panel. In designing a panel, it is useful to understand the sources of your background because they a vary independently. Once you have done the unstained control once you may or may not need to do it again. The situation described in Q16 is probably a perfect example of where Negative controls are very informative. In this case they were looking at transduced and non-transduced and noted that the "negative" population is not the same brightness in the two samples. Why? The process of transducing cells often results in a significant increase in background autofluorescence. That is why the best controls for transduced cells are mock-transduced cells. Running unstained controls for the transduced and non-transduced / mock-transduced cells would easily identify if the increased MFI of the "negative" transduced cells was due to such auto-fluorescence. A similar situation is true for intra-cellular staining. The fix-perm step often results in both increased auto-fluorescence and increased

background staining. In developing assays, I find it useful knowing where my background is coming from. Especially early in development.

16. How can I know what is the best voltage?

Answer: There is no best voltage. It depends upon the instrument and reagents you are using. This is not a trivial subject and can be viewed at many levels. In general, a good voltage/gain for any detector meets two criteria. 1) Keep the gain high enough such that the negative population is out of the electronic noise. 2) Make sure the gain is not TOO high where your bright positive populations go off-scale. A presentation our website gives a fuller explanation for setting gain /PMTV for instruments. You can find it here: *https://go.bd.com/horizon-webcasts.html?_ga=2.257731979.1260257832.1551375325-592203342.1543953157*

17. Will changing the Ab titer help with the spread?

Answer: Yes, for the reasons described in the webinar. For any Ab-fluorochrome conjugate, the brighter the stain/fluorescence the greater the spread. One way to reduce the brightness for a given marker is to lower the Ab concentrations. This then will reduce the spread of that markers into other detectors. However, this does mean the you are no longer at saturation and the data can be more variable. In general, this is only done for high density antigens like CD3, CD4, CD8, and CD45RA etc.

18. Can I use the same compensation with 6 colors and 8 colors?

Answer: A spillover/compensation matrix created for 8 colors can be used for 6 colors. But a spillover/compensation matrix created for 6 colors cannot be used for 8 colors.

19. In which cases it is necessary to use isotype controls?
20. FMO and isotype control, which one is better for the negative control?

Answer to Q19 and Q20: The question of the use of isotype controls has been a source of controversy for many years. This is my take on the issue. Others may differ. The purpose of all these "negative" controls is to help you identify for any marker what is a true positive, that is, above background. The question is, "What is the background?" Background comes from three major sources. 1) Autofluorescence, 2) Non-specific staining, and 3) Spread due to spillover. Unstained controls will tell you about #1, isotype controls will tell you about #1 and 2, FMOs tell you about #1 and 3. In most multi-color experiments the major contributor to the background is #3 = spillover and these days most (but not all) reagents have much less non-specific binding.

For these reasons, I find isotype controls generally of minimal value and strongly prefer FMOs, which give more relevant information. It is theoretically possible to have a combination FMO/Isotype Control. Just add an isotype control for the reagent that is missing in the FMO. However, again since the major source of background is the spillover this is rarely if ever necessary. It should be noted, however, that reviewers for many publications still request relevant isotype controls be run. In such cases an FMO/Isotype control would be preferable.

A full set of FMO controls for a >12 color assay can be cumbersome to prepare and expensive. For that reason, they should only be used when necessary in an ongoing assay. In particular, you would want to use them for any detector where you are identifying dim populations. Assay FMOs are not really necessary for detectors measuring CD8 or CD4.

21. You showed a slide with CD4 PE-Cy5 vs CD4 APC and talked about being mutually exclusive. Was this a typo? Was one of those supposed to be CD8?

Answer: Since we know that CD4 and CD8 are effectively mutually what I was showing, but did not make clear, was a mock-up- a combination of two individually stained samples; one stained with PE-Cy5 CD4 and the other with APC CD4. This mimics the case of two mutually exclusive populations. What I wanted to demonstrate was that the spread of the PE-Cy5 into the APC detector would not prevent the accurate gating of either the APC+ or PE-Cy5+ populations. I could have labeled one axis CD8 but that would not have been an accurate representation of the data.