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Title: Instrument optimization - Adjusting PMT voltages and compensation on a Beckman Coulter System

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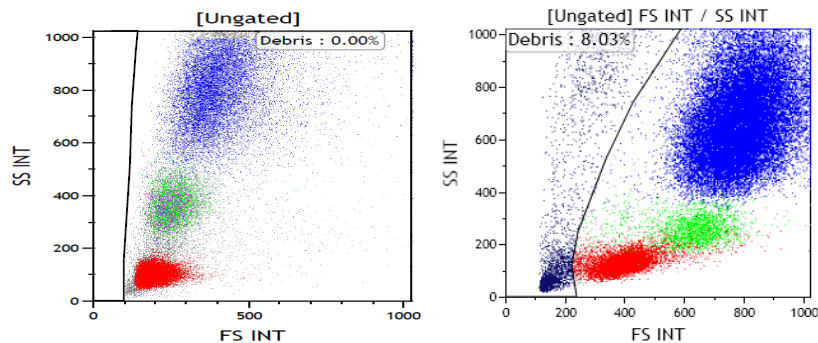
OUTLINE

Multicolor flow cytometry has evolved over the past years and has become more complex due to the number of PMT's and the associated potential for incorrect voltage and compensation settings. Instrument optimization is a much underestimated source of variability and it is important to optimize the voltages for each PMT in order to place the antigen-negative and antigen-positive population visibly "on-scale" and to maximize the potential resolution (signal/noise ratio). This is important to produce good resolution for dimly expressed antigens as well as visualization of antigen negative populations (e.g. PNH). This process should be followed at initial assay setup and this protocol can be used for QC purposes to document and track MFI and signal/noise ratio.

PROCEDURE/PROCESS

The first step in the instrument optimization is to ensure that the instrument is in good running condition, that QC values are within range and that a thorough cleaning cycle has been performed. Ideally, antibody selection and titrations were performed prior to optimizing the 10 color voltages and compensation matrix.

1. Use a protocol which includes the following dot plots:
 - a. SS vs FS to identify the major subsets and adjust that all populations are clearly visible and well separated from debris.

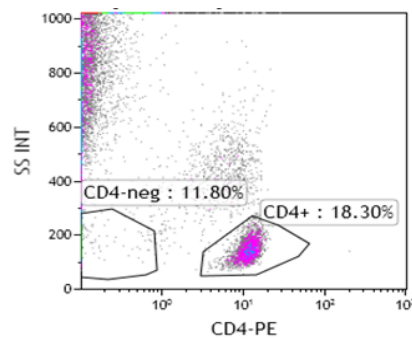


FS voltage/gain too low

Optimized FS Voltage/gain

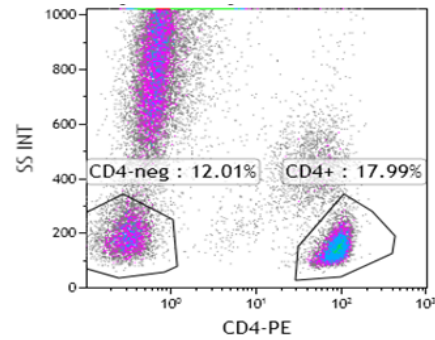
- b. Set up additional dot plots SS vs FL1, SS vs FL2 etc depending on the number of PMTs which need to be adjusted. Single color histograms gated on lymphocytes may also be used but these do not provide information of other subsets which may be helpful (e.g. dim to intermediate CD4 expression in monocytes)
- c. Draw a region around the antigen negative lymphocyte population and the antigen-positive population to determine the MFI and signal/noise ratio

- Set up control material (e.g. peripheral blood or cyto comp cells) in single tubes stained with single color antibodies for every PMT (recommend antibodies which identify "positive" and "negative" populations (e.g. CD2, CD3, CD4, CD5 etc)
- Use a protocol containing FS vs SS as well as dotplots with all the PMTs vs SS for this particular assay.
- Clear all compensation settings (important)
- Turn off "baseline offset" to avoid the negative population pushed on scale artificially. "Baseline offset" turned on may cause erroneous results. A well-optimized instrument should not result in a significant difference in the pattern between "baseline offset" turned on or off.
- For each tube adjust voltage so MFI of the negative population is between 0.3 and 0.4 (Figure 2 shows an example using CD4-PE. A shows the dotplot with the PMT at the start. B shows the dotplot after adjustment). For certain assays with a focus on antigen-negative populations (e.g. PNH), a slightly higher target MFI of 0.4-0.6 is preferred to make sure that the antigen negative population is clearly visible and not "crushed" against the axis.



Gate	X-A-Mean
All	2.74
CD4+	11.73
CD4-neg	0.06

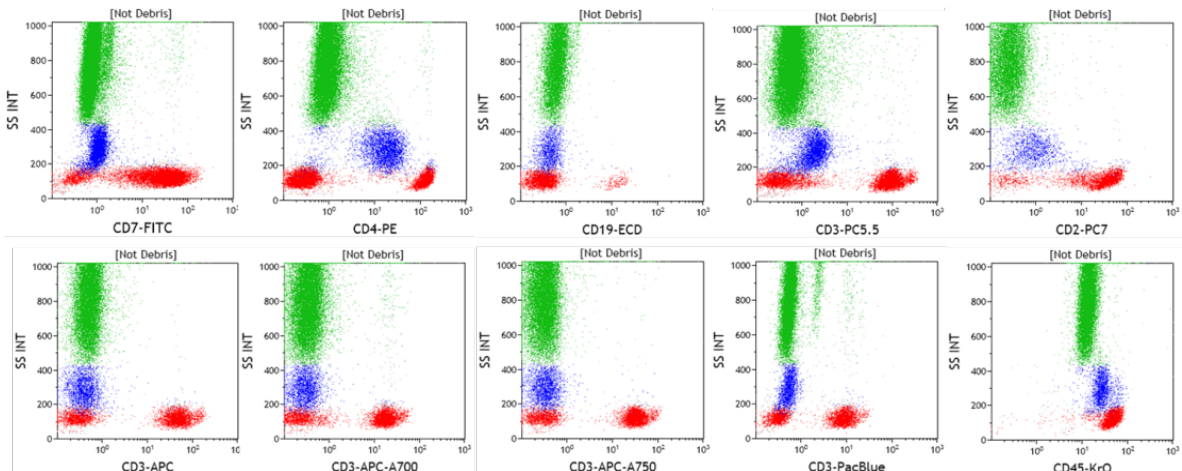
PMT-2 voltage too low



Gate	X-A-Mean
All	19.78
CD4+	88.81
CD4-neg	0.34

Optimized PMT-2 voltage

- The figure below shows an example of a correct adjustment of PMTs for a ten color protocol.



In this laboratory the CD7-FITC was used to set PMT for FL1 and compensation autoseup. However, CD7 does not show very bright expression and may be substituted by other antibodies conjugated with FITC which show brighter expression including but not limited to CD2-FITC, CD3-FITC, or CD4-FITC.

8. For protocols that contain CD45, adjust the voltage to place the lymphocyte, monocyte and granulocyte population approximately into the third decade. As CD45 is positive on all WBC populations, there typically is no CD45-negative population present and the main purpose is for gating only.
9. The above process should determine the voltage to be used for each PMT. These voltages can then be used for the next step which is the compensation process.
10. Save this "Voltage setup" protocol and print out the cytosettings (status page) containing the optimized voltages for each PMT
11. Run FlowSet with the voltages determines for each of the optimized PMT voltages determined in the previous steps to determine the target channel for the specific lot of FlowSet used.
12. Run the assay-specific Autoscheduler with:
 - a. FLOWSET
 - b. All single color tubes for each PMT other than the CD45 (preferably CD2, CD3, CD4, CD5) and the CD45 tube
 - c. Assay-specific verify tube
13. Immediately check "autoadjust disabled" when the wizard starts with the Flow Set tube and unselect "automatically approve step". This puts Flow Set in the setup mode and allows the user to rerun a tube prior to the next one in case of errors.
14. Enter PMT voltage(s) based on the previously determined voltage on the cytosettings printout
15. Adjust target regions tightly (+/- 10%) around the peaks of each histogram.
16. Save the Flow Set protocol ("standardization" protocol)
17. Click on "acquire".
18. Continue through the Autoscheduler tubes and check the dotplots on each tube/PMT. Verify that there is an antigen-negative and an antigen positive population. If necessary, move the region to fully encompass only the positive population. Also see the spillover into the other PMTs which allows for better understanding of the concept of spillover and necessary compensation.
19. When running the verify tube, the compensation settings may be adjusted at this time if necessary. However, the more PMT's are utilized the more complex compensation becomes and it is highly recommended to perform adjustment post-autosetup. This can be performed on the instrument acquisition CXP or Navios software; however, this may also be done through other software (e.g. Kaluza, FlowJo, FCSExpress and others).
20. Click on "Approve" and then click on "finish" to approve the Autoscheduler process. This now has updated the "settings.PRO" file with preferred voltages and compensation settings.

As the voltages and compensation settings are connected, the position of the antigen-negative and positive populations may move once combined into a single tube. Therefore, fine-tuning of the PMTs may be needed to optimize the protocol. The objective of the compensation adjustment is to achieve the normal staining patterns of all cell populations as well as ensure that both antigen-positive populations and antigen negative populations are clearly visible with multiple combinations of PMTs.

If there are some remaining compensation issues and some adjustment is necessary, this may be performed through other software. The example below is performed through Kaluza™ software.

1. Manually adjust compensation in Kaluza and record the specific changes
2. Go into Resource Explorer on Navios and click on the folder which contains the cytosettings protocol
3. Select the appropriate settings file and drag this cytosettings protocol into the "grey space"
4. Go into cytometer controls and update the compensation matrix

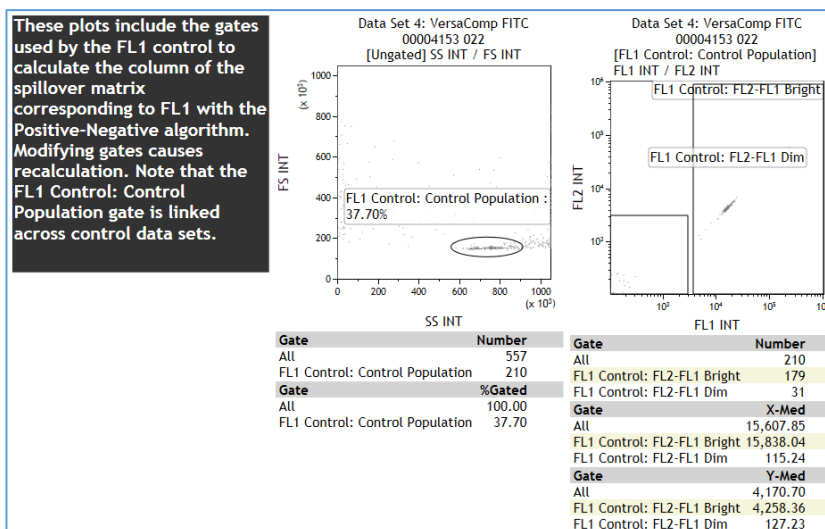
- Go into the file folder and "Save protocol as"
- Replace the previous protocol with the current protocol containing the adjusted settings.

Using VersaComp Beads (RUO only)

The compensation matrix can also be calculated using antibody capturing beads (e.g. VersaComp) stained with single antibody conjugates. This approach is highly recommended as it would provide a higher level of standardization. Using the optimised PMT voltages as described above, a listmode file will be acquired for each single color stain of the capture beads. Each tube will also include a negative non-capturing beads population. Lastly, the Kaluza™ software (RUO use only) is used to calculate the compensation matrix.

Procedure using VersaComp Antibody Capture bead Kit:

- Prepare single color tubes containing the positive capture and negative capture beads. Follow the manufacturer's instructions to label the VersaComp beads with single antibodies (using optimized/titrated amount of antibodies)
- Prepare an assay-specific verify tube.
- Run all the tubes (compensation single color tubes and the assay specific verify tube) on a flow cytometer using the optimised voltage for each PMT as above. Ensure the compensation matrix is clear. Note if the discriminator is on FS then this may need to be lowered to view all the beads.
- Label the tubes in the acquisition software with the fluorochrome used in each tube.
- Firstly open the listmode files containing the capture beads labeled with the single color antibodies using Kaluza software.
- Select all the files then click on "Add selected to new Compensation"
- Select "Method" as Positive-Negative", and click on "Detect Controls".
- Confirm that all files are selected with the right fluorescence. (cross check with the listmode file name)
- Click on "Generate Compensation"
- Select sheet "FL1" and position the "FL1 Control" gate on FSC/SSC plot to include the singlet capturing beads.



- Confirm that all rectangular regions on the fluorescence dot plots are optimised for all sheets.
- Save the compensation matrix

13. Click the “Save compensation as” icon from the Compensation pane.
14. Using Kaluza software, open the LMD file of the assay-specific verify tube collected using the optimised voltage for each PMT.
15. Import the saved Kaluza compensation matrix into the LMD file. Fine-tune the compensation to achieve normal staining patterns of all cell populations.
16. Manually transfer the final compensation matrix from Kaluza to the flow cytometer cytosettings protocol.

SUMMARY

Optimizing the instrument is an important part of achieving optimal results showing accurate display of antigen expressions. In addition, targeted antibody and fluorochrome selection and optimizing settings for each flow cytometry assay are essential next steps. Please refer to the general rules of antibodies and fluorochrome selection available through the cytometry literature. A more detailed ICCS Quality and Standards Module dedicated to Navios -specific fluorochrome spectral properties and compensation tips will also be posted in the near future.

References:

1. Cytometry B, Vol 84B, No5, Sept 2013: Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS”
2. Internal laboratory SOP at Dahl-Chase Diagnostic Services, Aug 2016
3. Communications with Amr Rajab at University of Toronto
4. Beckman Coulter training manuals

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Reviewed and Approved by:

1. Mike Keeney and Ben Hedley, London Health Sciences Centre and St. Josephs Health Care
2. Rob Sutherland, Toronto General Hospital