PROCEDURE A3:   Optimising Cytometer Settings (PMT Voltages & Threshold)

1.   Create a new Experiment. Add a Specimen and activate the Tube.

In the Cytometer window, delete the fluorescence parameters that are not required (so you are not storing unnecessary data).

For FSC and SSC parameters, tick the boxes for Height (H) and Width (W) allowing for doublet discrimination.

2.   Right-click *Cytometer Settings --> Application Settings --> Create Worksheet.*

A new global worksheet with dot-plots specifically configured for each parameter is created.

Each plot contains an **Application Target Box** to help you **objectively** optimise voltages for resolution of dim populations. You may need to load the tube for Unstained Cells and click Acquire to display the application target boxes.
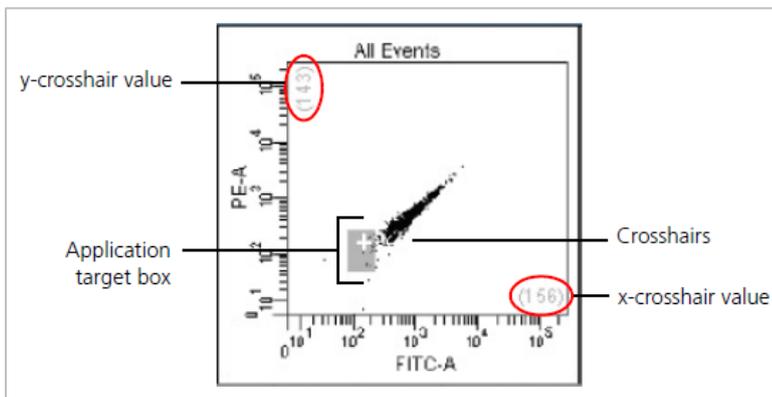
The white crosshairs in each application target box represent MFI values that are 10 times the standard deviation of electronic noise (rSDEN) for each detector. These MFI values are displayed on each axis.

The size of the application target box represents 2 x rSD of the CS&T dim beads in each detector.

3.   Load the tube of Unstained Cells and click Acquire. Optimise your PMT voltages by following steps **a - d**:

   a.   Ensure you are using LOW flow rate.

   b.   Adjust FSC and SSC voltages to place the cells on scale. Adjust the FSC Threshold if necessary.

   c.   Draw a polygon gate (P1) around the lymphocytes. In the dot-plots with target boxes, show only P1.

   d.   Adjust your fluorescence PMT voltages until population P1 resides within each target box. You do not need to record data.

   e.   Remove the tube of Unstained Cells.

4.   Load the tube of Full Stained Cells and click Acquire. Confirm that population P1 is *on scale (can be visualised on the plot)* for all fluorescence parameters. Reduce PMT voltages if necessary. You do not need to record data.

5.   Add a new Specimen and acquire data for all each tube of Single Stained Cells. *Record 5,000 events in the Lymphocyte gate using a LOW flow rate. Note down the MFI of the brightest staining cells for each fluorochrome* – this will be compared to the MFI of the equivalent positive CompBead.

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QUICK REFERENCE: Optimising Cytometer Settings with Application Target Boxes

Unstained Cells

Adjust FSC and SSC settings to place populations on scale and adjust the threshold.

Adjust voltages for fluorescence channels to overlay cells onto target boxes.
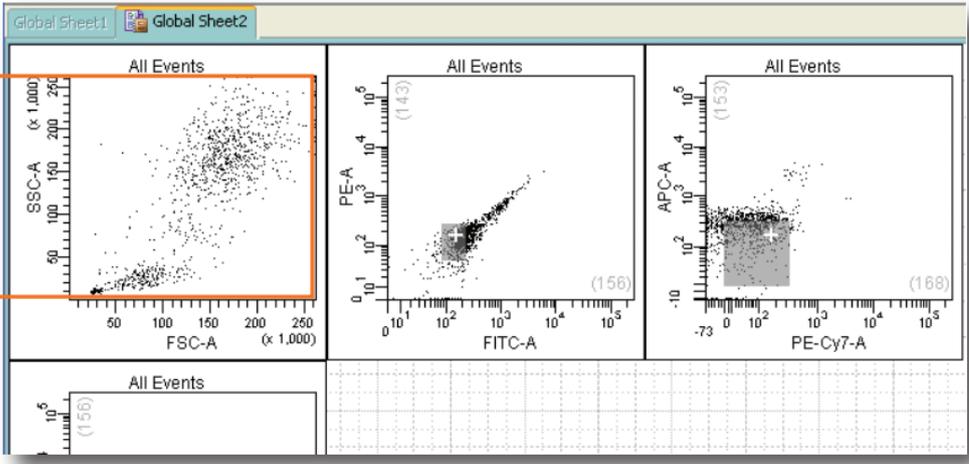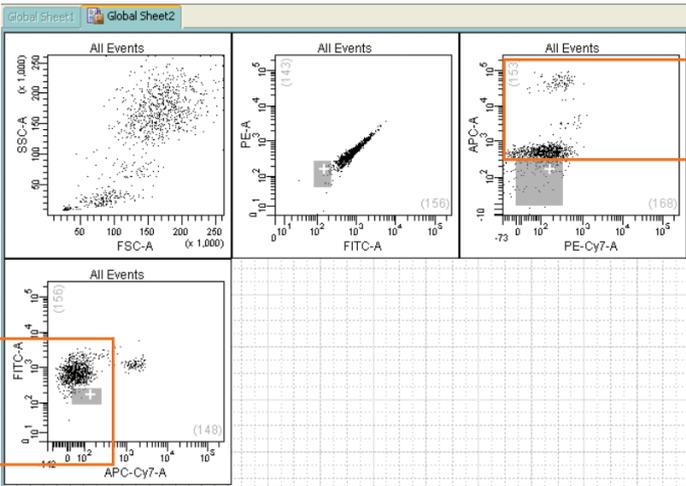

Stained Cells

Confirm that stained populations are in the linear range of each detector.

If needed, decrease the PMT voltages to place the positive populations on scale.

Use the gray boxes as a guide when decreasing the PMT voltages.

Additional Notes about Application Target Boxes

The application target boxes act as guides for adjusting PMTVs for your samples. The software sets the PMTVs to provide appropriate resolution for dimly fluorescing populations. However these settings may not be optimal for all sample types and may require adjustment. Use the application target boxes to decide whether to adjust the PMTVs. Placing the negative population below these application target boxes might compromise low-end resolution. However, this may be required to bring bright staining populations on scale.

The starting PMTVs generated by the software may place some of the negative populations below the application target boxes. This is normal for some parameters. Note that this behaviour, in general, applies more to lymphocytes than other cell types.

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