Job Aid

BD FACSDiva™ software
Determining Initial PMT Voltages

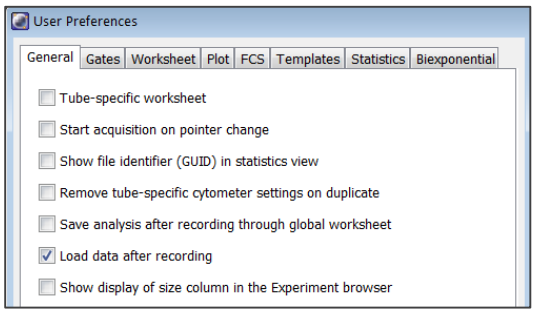
This job aid contains instructions for determining voltage settings that will optimize the stain index (SI) of cells stained with a reference reagent for each detector. After PMT voltages have been determined, they can be saved as Application Settings and used as a starting point for subsequent experiments.

Before you Begin

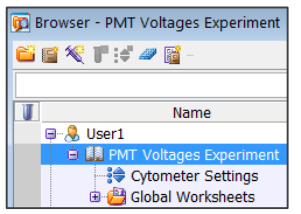
- Prepare single-color cells, one for each fluorochrome on your instrument, plus one tube of unstained cells. The BD® CD4 Evaluation kit (BD Catalog No. 566352) can be used to stain lymphocytes with CD4 in each color.
- Ensure that the instrument has been started up and a CS&T performance check has been performed.

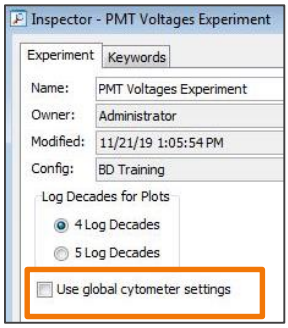
Setting Up an Experiment

1. From the **Edit** menu, select **User Preferences**.
   In the **General** tab:
   - Clear the checkbox for **Remove tube-specific cytometer settings on duplicate.**
   - Clear the checkbox for **Save analysis after recording through global worksheet**.

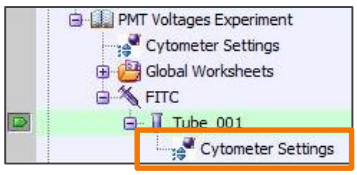


2. In the Browser:
   - Create and rename a new experiment.
   - Verify that the experiment is selected and then in the Inspector, clear the **Use global cytometer settings** checkbox.

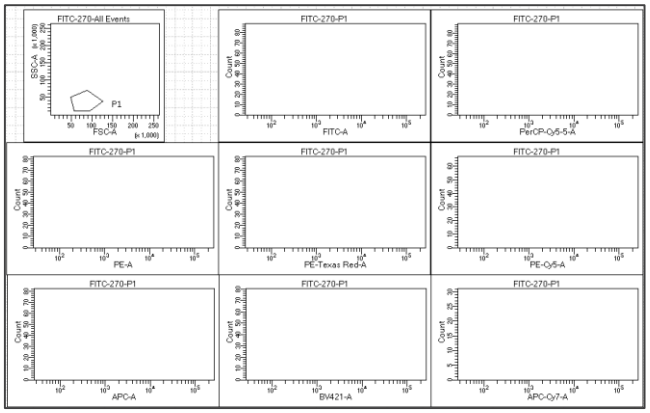




3. In the Experiment:
   - Add a specimen and rename it with the first fluorochrome you will be running.
   - Select Tube_001 and click the **New Cytometer Settings** button to add tube-specific cytometer settings to Tube_001.
   - Set PMT voltages for each fluorescence detector to 210.



4. On the global worksheet:
   - Create an FSC vs SSC dot plot.
   - Create histograms for each detector on your instrument.
   - Create a P1 gate on the FSC vs SSC plot and set all histograms to show P1.



5. While acquiring a tube of unstained cells:
   - Adjust FSC and SSC voltages to place the cells on scale.
   - Set FSC threshold, if needed.
   - Adjust P1 to identify the population of interest.

6. From the **Experiment** menu, select **Experiment Layout**.
   In the **Acquisition** tab:
   - Select the new worksheet from the Global Worksheet menu.
   - Select P1 as the Stopping Gate.
   - Enter 2,000 in the Events to Record field.

Name	Events to Record	Global Worksheet	Stopping Gate
488nm PMT Setup			
FITC			
Tube_001	2,000	Global Sheet1	P1 (GW)

7. Duplicate Tube_001 and increase the PMT voltages for each fluorescence detector by 30 volts.

8. Repeat step 7 to create tubes for voltages up to 690, for a total of 17 tubes.

Status | Parameters | Threshold

Parameter	Voltage
FSC	310
SSC	421
FITC	210
PerCP-Cy5-5	210
PE	210
PE-Texas Red	210
PE-Cy5	210
PE-Cy7	210
APC	210
APC-Cy7	210

Status | Parameters | Threshold

Parameter	Voltage
FSC	310
SSC	421
FITC	240
PerCP-Cy5-5	240
PE	240
PE-Texas Red	240
PE-Cy5	240
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Status | Parameters | Threshold

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APC	270
APC-Cy7	270

9. Duplicate the specimen and rename it for each fluorochrome.

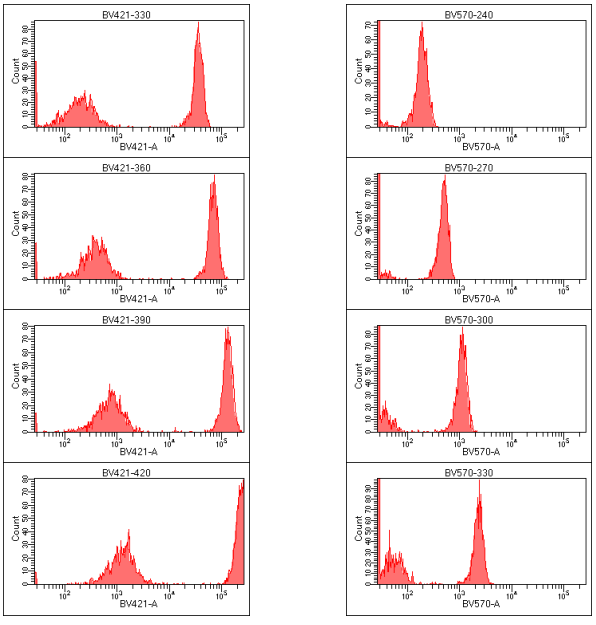
Tip: Save the first specimen as a panel template for future use.

Acquiring Voltage Titration Data Files

1. Load the first single color control and record a data file for Tube_001.

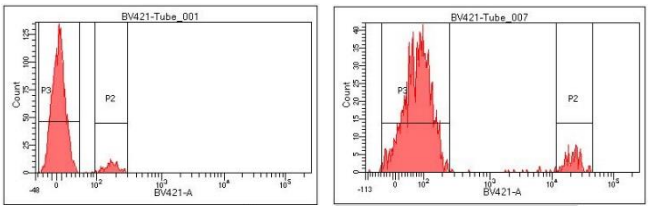
2. Record data files for all tubes in the specimen using the same single-color control sample.
   - Keep the same flow rate for all tubes.
   - When the positive peak starts to go off scale, you do not need to continue recording data files.

3. Repeat steps 1 and 2 with the next fluorochrome and detector being evaluated.

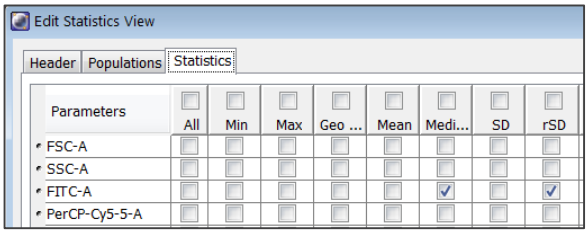


Analyzing Data

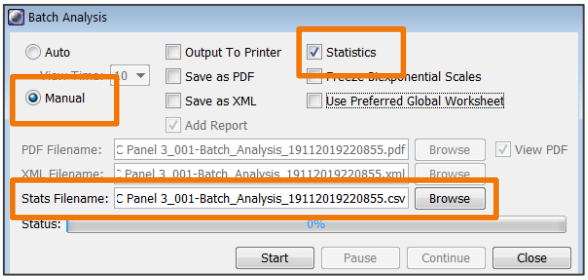
1. For Tube_001 of the first fluorochrome, create gates around the positive and negative populations.
   - If two distinct populations are not visible, create two gates as placeholders, ensuring that you have a few data points in each gate.



2. Create a statistics view to display median and rSD for the appropriate fluorochrome.



3. Batch analyze the specimen to export statistics to a CSV file.
   - Right-click the specimen and select Batch Analysis.
   - Select Manual and Statistics.
   - For Stats Filename, select a file location for the CSV file and rename it with the name of the fluorochrome you are analyzing.
   - Start the batch analysis, adjusting the gates to capture the positive and negative populations for each data file.

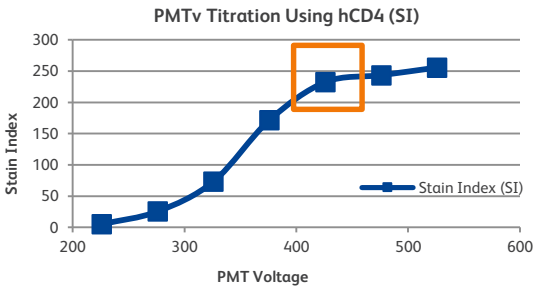


4. Calculate the stain index for each PMT voltage data point using the following equation, where MFI is Median Fluorescence Intensity.

$$\text{Stain Index (SI)} = \frac{\text{MFI}_{\text{positive}} - \text{MFI}_{\text{negative}}}{2 \times \text{rSDnegative}}$$

Tip: Open the exported CSV file in a spreadsheet program and create a formula to calculate stain index.

5. Create a chart showing Stain Index vs PMT Voltage.



6. Identify PMT voltages where the stain index for a biological sample is at or near the maximum value.

7. Repeat steps 1 through 6 for each fluorochrome or detector to be analyzed.

If you need assistance identifying the optimal PMT voltages, contact ResearchApplications@bd.com.

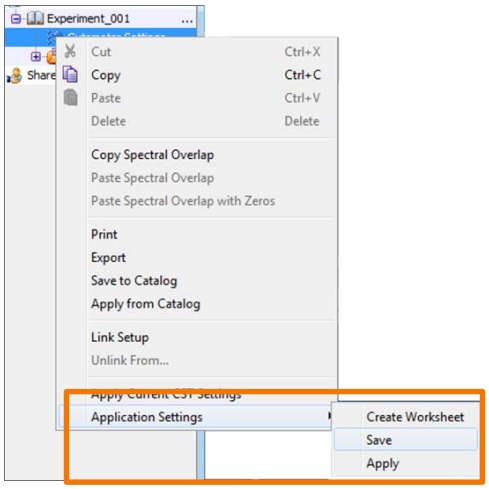
Saving as Application Settings

1. Create a new experiment in the Browser and enter the voltages determined in the previous section.

   Right-click the cytometer settings and select **Application Settings** > **Save**.

2. The application settings are saved in the Cytometer Catalog and can be applied to future experiments with the same configuration.

   Application settings will be updated automatically when a performance check has been run.



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