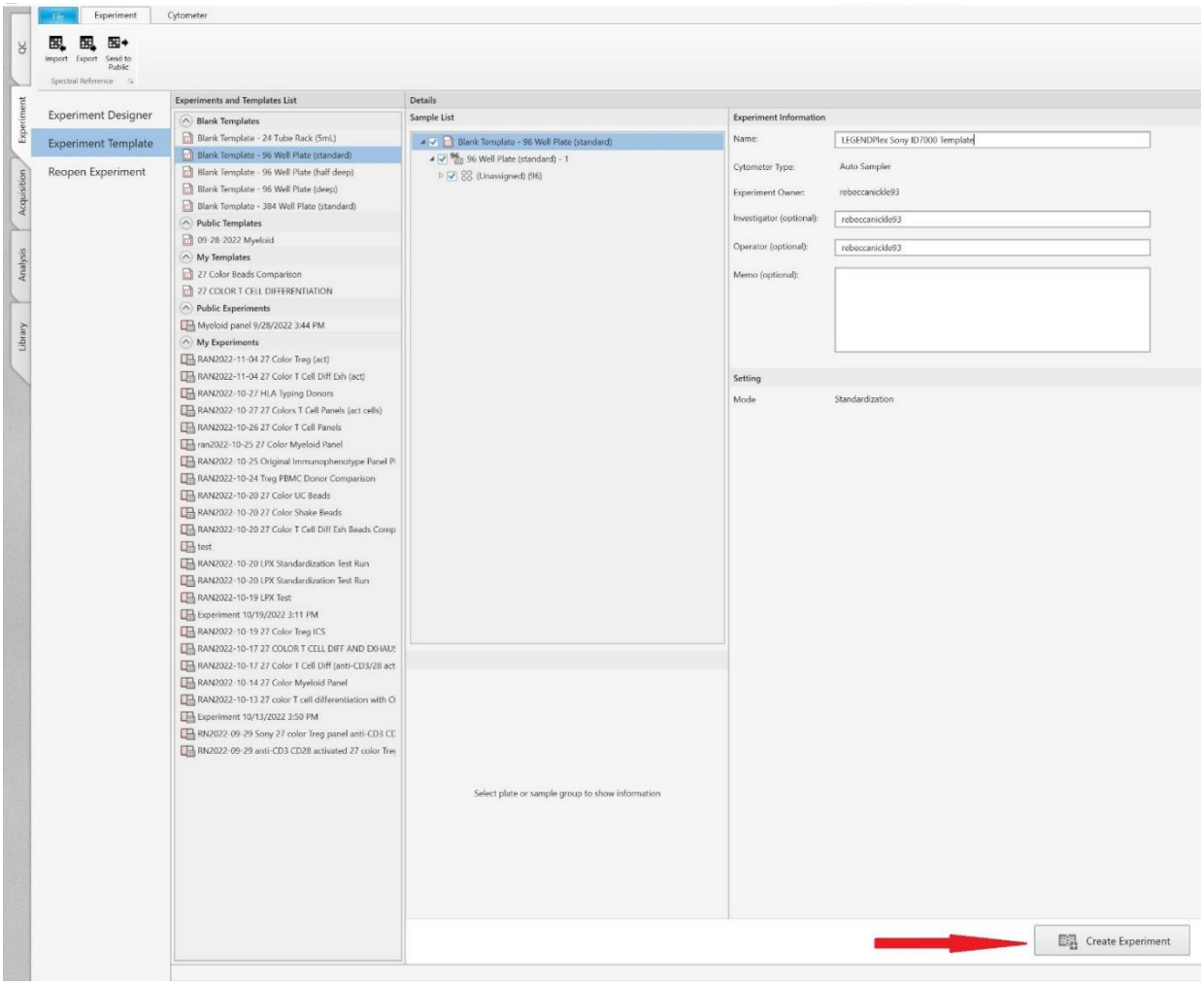
Sony ID7000™ Spectral Cell Analyzer setup guide for LEGENDplex™ Panels:

Before Starting the Assay:

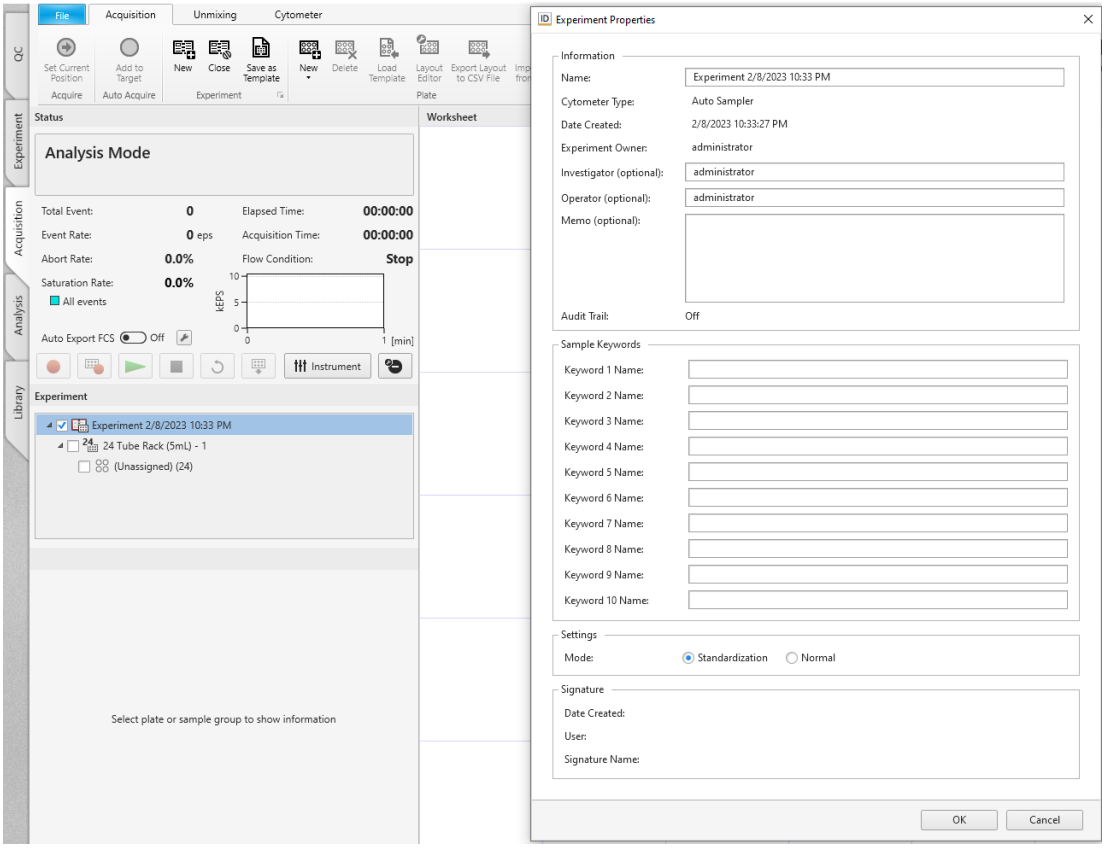
- For LEGENDplex™ analysis, the cytometer should be equipped with 488nm (blue) and/or 561nm (Yellow-Green) lasers, for PE detection, and 637nm (red) laser, for APC detection.
- Extra C7 standards might be needed for optimizing PMT voltages (see 8.2)

1. **Instrument Setup**
   1.1. Open Sony ID Software application and start up the ID7000™ instrument. Allow the instrument to go through set up processes and run daily and performance QC, if necessary, following the manufacturer's instructions.
2. **Create a New Experiment for Data Acquisition**
   2.1. In the **Experiment Tab** click **Experiment Template** → **Blank Template - 96 well plate (standard)**.
   2.2. Right click on **96 Well Plate (standard) - 1,** and select **New Sample Group**.
   2.3. In the **Experiment Information** field rename the experiment and click **Create Experiment** button below.



2.4.In the Experiment box of the Acquisition tab, right click on the experiment title and select **Properties**.

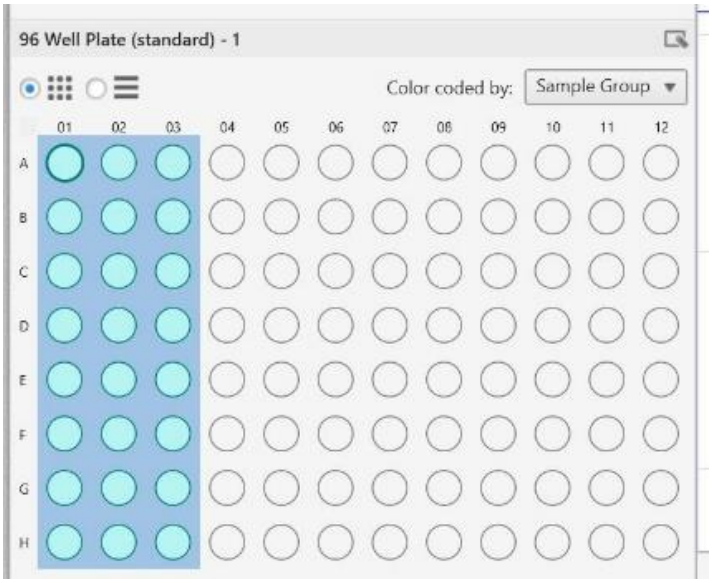
2.5.In the pop-up window, select **Standardization** in the **Settings – Mode** portion.



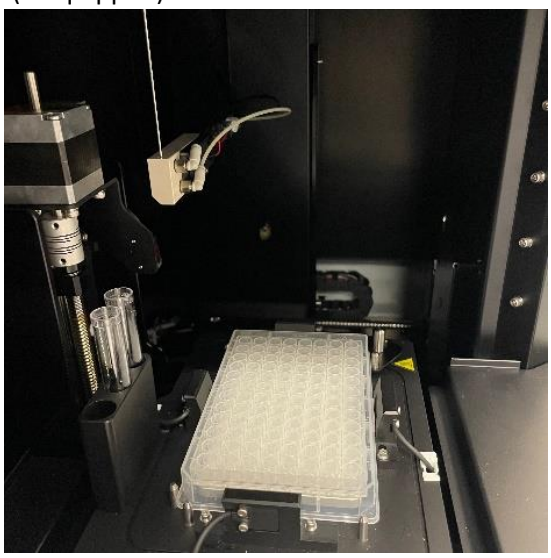
3. **Plate Layout**

3.1.In the **Acquisition Tab** select wells with samples in them, right click, move to sample group, and select **Sample Group – 1**.

3.2.Right click on selected wells and add to auto acquisition target.



4. Load plate on the plate loader (if equipped) or tubes on the tube rack.



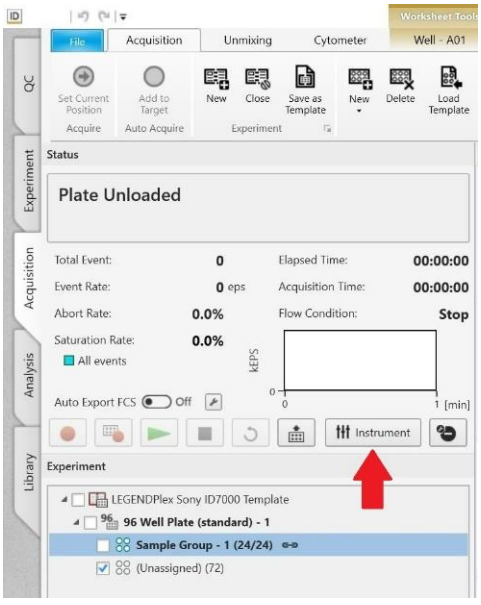
5. **Instrument Settings**

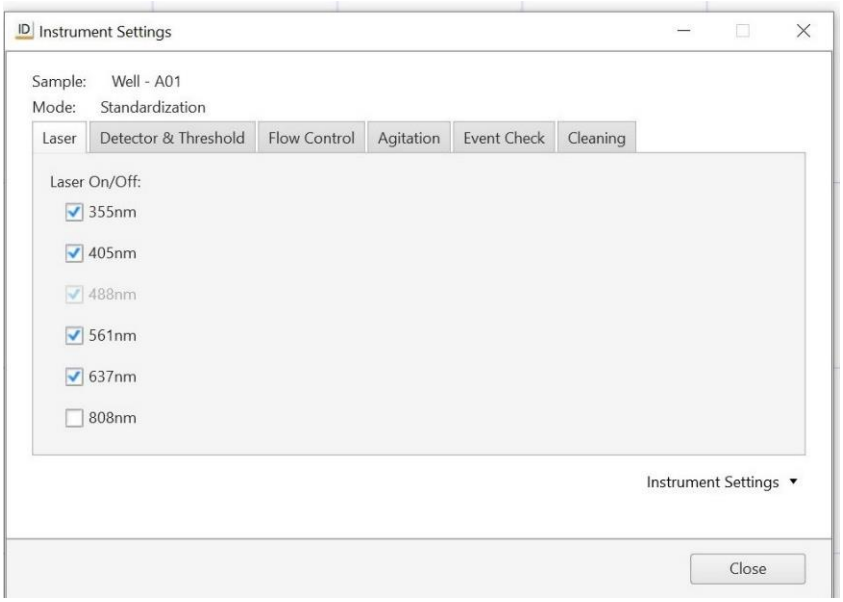
5.1. Click on **Instrument** and check the following lasers – 355nm, 405nm, 561nm and 637nm (6 laser configuration pictured below).

5.1.1. If using 3 laser configuration, ensure 405nm, 488nm, and 637nm are checked.

5.1.2. May also check 320nm laser if applicable.

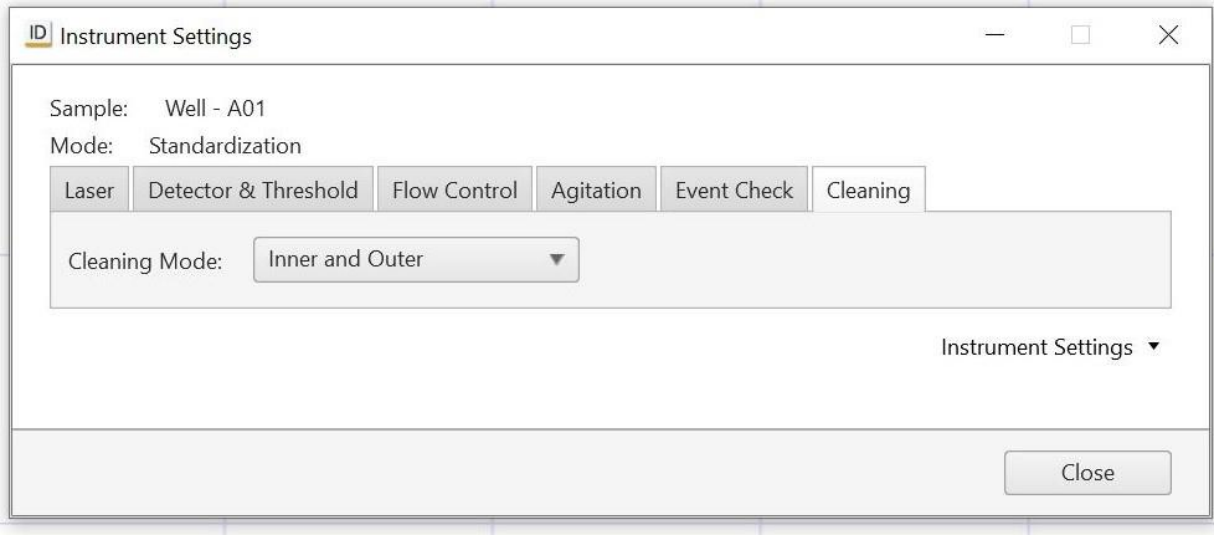
5.1.3. **Uncheck 808 nm (if applicable):**





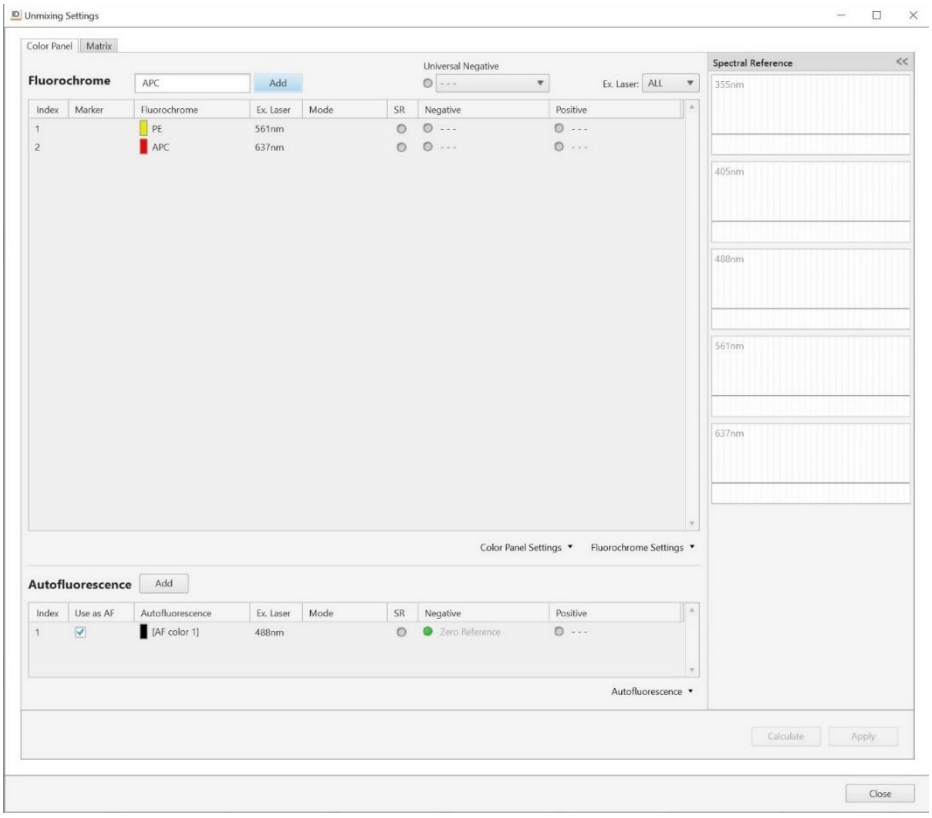
5.2. Under **Flow Control** tab, set **sample flow rate** to **1** while setting up voltages to prevent all of sample from being consumed.

5.3.Select Cleaning mode **Inner and Outer**.



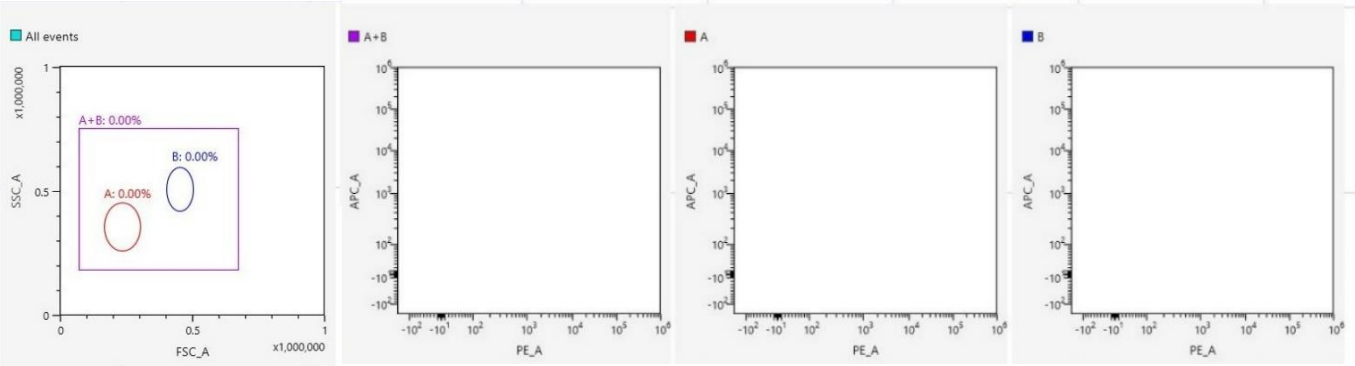
6. **Add Fluorophores to Experiment**

6.1.Under **Unmixing Tab** open **Unmixing Settings** → add PE and APC at the top search bar → close the window:



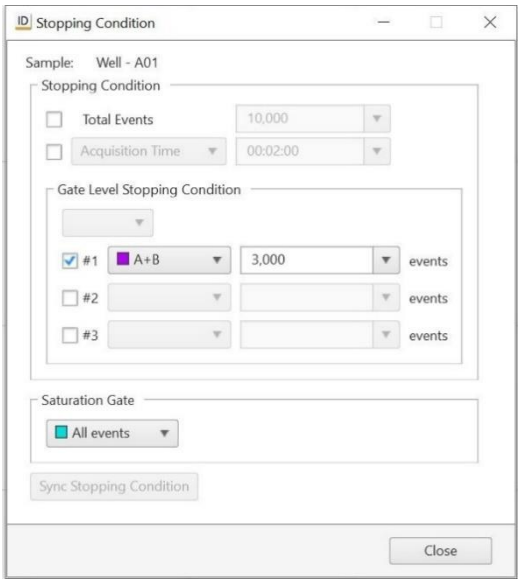
7. **Worksheet Set Up**

7.1. On FSC vs SSC density plot draw A, B, and A+B gates. Open density plots for each gate and display PE on X-axis and APC on Y-axis. Right click on FSC vs SSC plots and select **sync scale and gate**.



7.2. Open stopping conditions → Uncheck total events → Under **Gate Level Stopping Condition** select the A+B gate and enter **300 events per analyte** with a 3,000-event maximum.

7.2.1. (e.g., if your LEGENDplex™ Panel is 13-plex, enter 3,000).



8. **Gate and Gain Set Up**

8.1. Select **Load** to load plate.

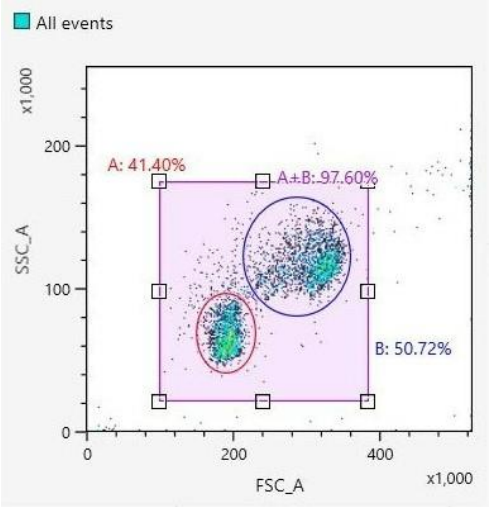
8.2. Right click well with Standard C7 and select **set current position**. Ensure sample flow rate is set to 1 prior to previewing.

8.3. Click green preview button and adjust FSC to max 17, and SSC around 3.78.

8.3.1. Optimal FSC and SSC values may vary for each cytometer.

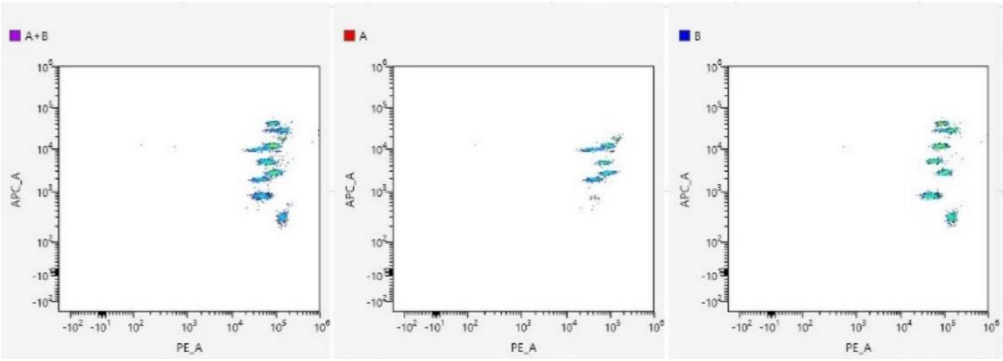
8.3.2. Can adjust X and Y axis by hovering over axis until double arrows appear, then drag away from 0 to zoom in on bead populations.

8.4. Adjust A, B, and A+B gates around main populations. Right click on the plot and select **sync scale and gate** to synchronize gates across all wells.

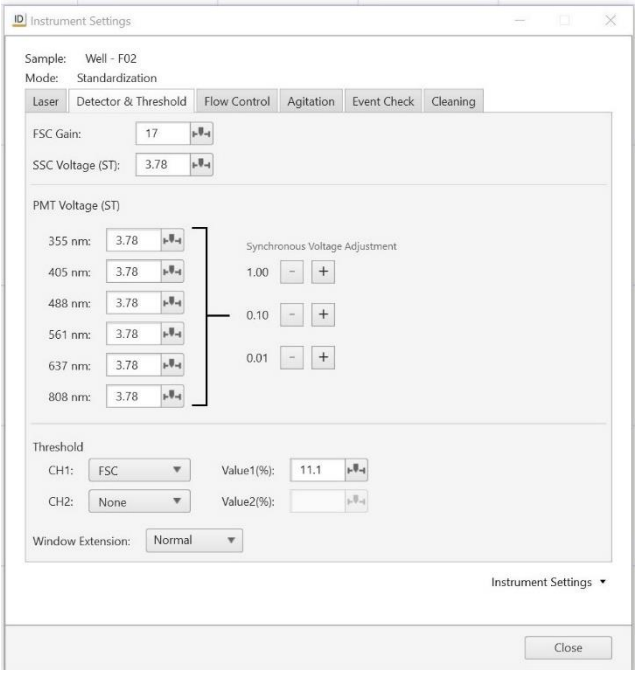


8.5. Ensure beads are on scale in regards to PE and APC signals using the **Instrument Settings** window.

8.5.1. The highest stained C7 beads PE fluorescence should be around 10^5.



8.5.2. For all PMT Voltages, 3.78 should be sufficient, if not, use **Synchronous Voltage Adjustment** to adjust all PMTs equally by +/- 0.01, 0.1, or 1.0 until the PE signal falls within the desired range.



8.6. Once PMT voltages are optimized, stop previewing well.

9. **Plate Acquisition**

9.1. Right click on the first well and **set current position**.

9.2. Adjust Sample Flow Rate as preferred in the **Instrument Settings**, **Flow Control** tab.

9.3. Click **Auto Acquire** and let samples be acquired.

10. **Exporting Data**
    10.1. After acquisition has completed, unload plate and remove from cytometer.
    10.2. Right click on experiment name, select **Export to FCS File**.
    10.3. Deselect pulse type height and width.
    10.4. Select **Browse** to select place to save on computer and click export.