## 1. Preview and Acquire the Unstained Sample (beads or cells)

a. Go to the Status panel (upper left part of the screen) and click on the Load button.

b. Go to the Rack/Plate Layout panel (lower left). Ensure that the **current position (black arrow)** is on the unstained sample (A01). To change the current position, right click on the new location and click Set Current Position on the quick pick menu.
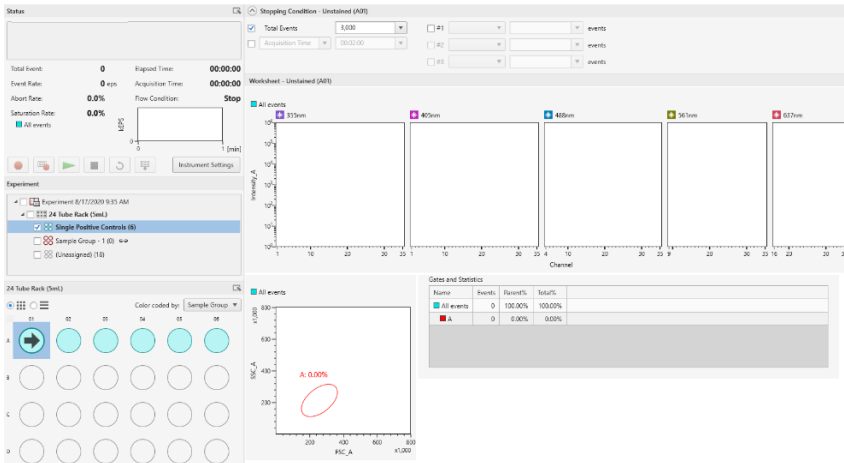
c. Optional: One can adjust the axis scale or zoom the plot, as required, if the gate is difficult to draw. Right-click on the FSC SSC plot, then click on Zoom to enlarge your population.

d. Go to the Status panel (upper left) and click on the Instrument Settings button.

e. Go to the Instrument Settings window and click on the Flow Control tab.
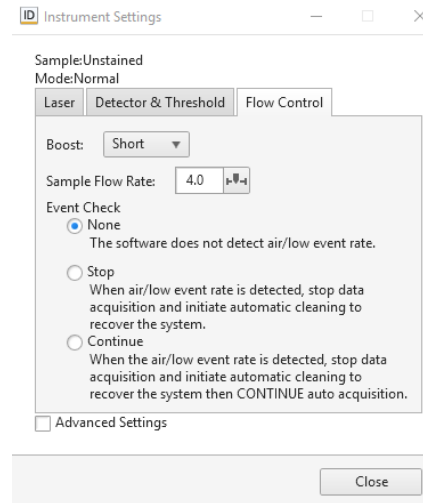
Boost: **Short** is recommended unless you have lots of sample you can waste.

Sample Flow Rate: 4.0 this can be adjusted while previewing a sample, but not during data acquisition or auto-acquisition.

Event Check: **ON** is recommended during unattended auto-acquisition.

	Sample Consumption Rate (μL/min) at each Sample Flow Rate Setting							
	1	2	3	4	5	6	7	8
	33	50	67	80	100	133	167	200
Sample Volume	Time to run the indicated Sample Volume at each Sample Flow Rate Setting							
	1	2	3	4	5	6	7	8
50 μL	01:30	01:00	00:45	00:37	00:30	00:22	00:18	00:15
100 μL	03:00	02:00	01:30	01:15	01:00	00:45	00:37	00:30
150 μL	04:30	03:00	02:15	01:52	01:30	01:07	00:55	00:45

h. Go to the Status panel and click on the Preview button ▶

i. Go to the Instruments Settings window and click on Detector & Threshold tab.

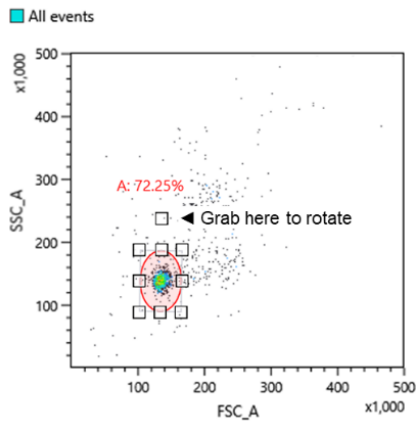k. Click on the Acquire button ● to record data from the unstained sample.
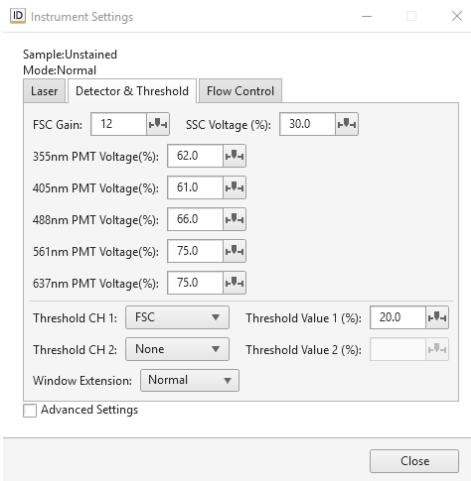
Adjust the FSC Gain and SSC Voltage settings to properly position the cell or bead population on the FSC SSC plot.

Click on the Restart button ↻ as needed to refresh the data.

Ensure that the right Instrument Settings is loaded

Adjust Threshold Value to eliminate excess debris downscale from the cell or bead population in FSC SSC plot.

j. Go to the FSC SSC plot. Adjust the size and position of Gate A to be tightly around the cell or bead population.
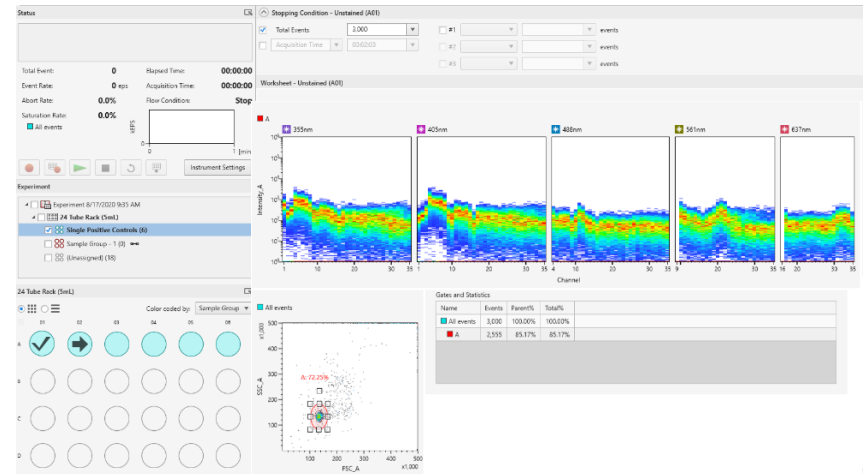

Click inside Gate A to select.

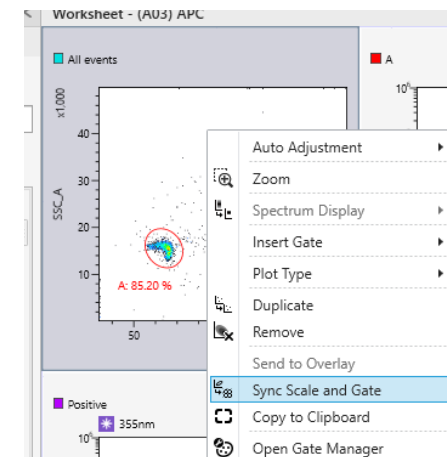Grab and drag any corner or side handle to re-size the shape.

Grab and drag the top handle to rotate the shape.

Grab and drag the label to move it.

l. To **apply the position and scaling of Gate A** to all the single-color controls, right click on the FSC SSC Plot, and click on Sync Scale and Gate (Synchronizes the scale and gates of all plots within a sample group).

3. Auto Acquire the Single-color Controls

Select the wells that you want to acquire automatically in the [Plate] panel, and right click [Add to Auto Acquisition Target]
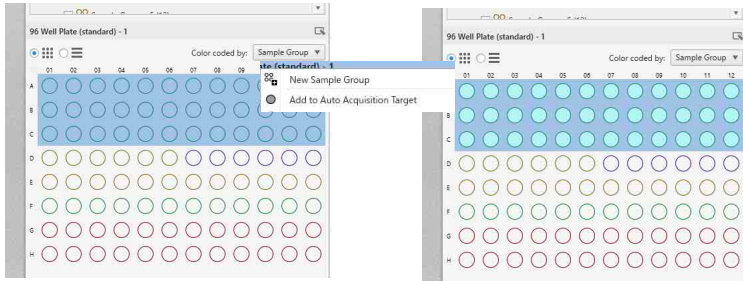

a. Stopping conditions: Select for Compensation Beads: 5000 events

b. click on the Auto Acquire button

c. check is positive gate sits tightly around the positive peak in the Spectral Blot or histogram.

d. **Repeat step b for each single-color control.**
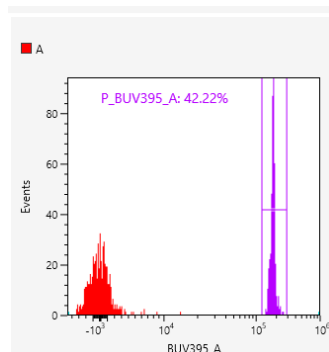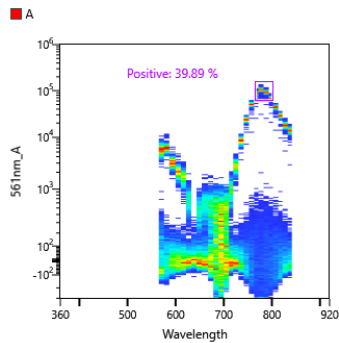
### 4. Calculate the Unmixing Settings

a. Go to the Unmixing tool ribbon (top of screen) and click on Unmixing Settings.

Cave: **Make sure the correct gate is selected for each marker**

b. Click on the **Calculate** button.
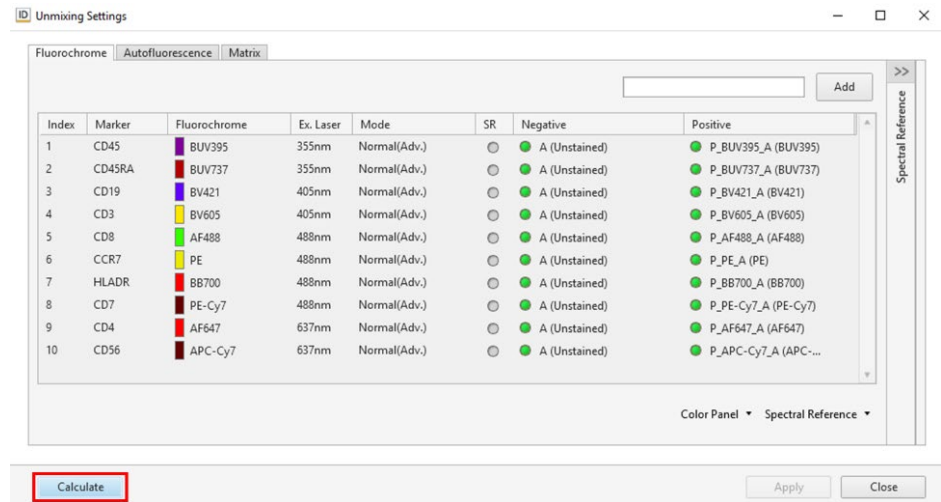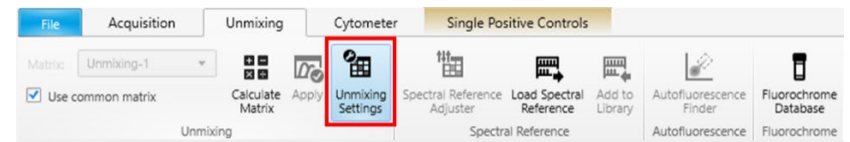
c. Click on yes to confirm applying the unmixing settings to **Sample Group - 1.**

5. Acquire Data from the Unstained Cell Sample

a. Go to the Rack/Plate Layout panel (lower left) and select positions C01 and C02.

b. Right-click on the selected positions, hover over **Move to Sample Group** and click on Sample Group – 1.
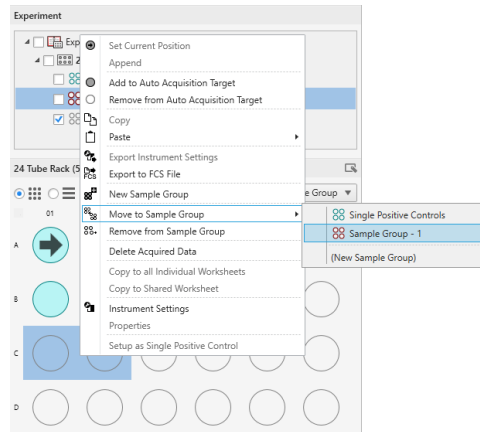
c. Right-click on position C01 and click on **Set Current Position.**

d. Copy and paste the Worksheet settings from A01 to C01: click on A01, then right-click, and click on Copy. Click on C01, then right-click, hover over Paste, and click on Worksheet.

e. Click to change from Map view to List view and overwrite the sample names:

f. Change the Stopping Condition to ▮A and 5,000:

g. Go to the Status panel and click on the Preview button ▶

h. Go to the Instruments Settings window and click on Detector & Threshold tab.

i. Adjust the FSC Gain and SSC Voltage settings to properly position the cell population on the FSC SSC plot.

j. Click on the Restart button ↻ as needed to refresh the data.

k. Adjust FSC Threshold Value 1 to eliminate excess debris downscale from the cell population on the FSC SSC plot.

l. Move, size, and rotate gate A around the cell population.

m. Click on the Acquire button ● to record data from the unstained cell sample.

6. Add a Reference Spectrum for Cellular AF to the Unmixing Settings

Do this section if you used capture beads for your single-color controls. You may skip this section if you used the cells for your single-color controls.

a. Make sure you are viewing the <u>worksheet for unstained cells.</u>

b. Click [Autofluorescence Finder] in the [Autofluorescence] group on the [Unmixing] tab of the ribbon.Go to the Autofluorecence Finder ribbon (top of screen)
NOTE: The Unmixing Settings needs to be OFF.
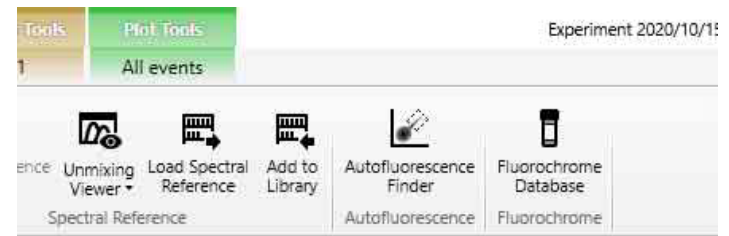
1: In [Step 1], add the appropriate gates to the FSC/SSC plot. The added gate automatically is reflected as the parent gate in [Step 2].

**Note:** Gate the corresponding populations to make later adjustment easier
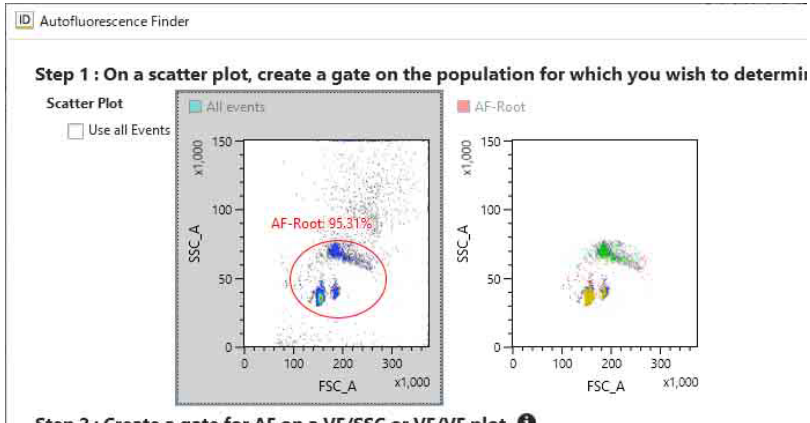
2: In [Step 2], search for the autofluorescence population while changing the virtual filter ranges.

Note: You can change the wavelength band of the virtual filters to any range using the mouse.

If you want to change the Y-axis of the plot to the wavelength range selected by the virtual filter, select the virtual filter of the desired laser axis from [Y Axis].
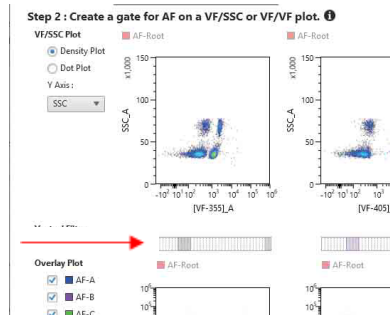
3. Add a gate for the population to register as the autofluorescence population.
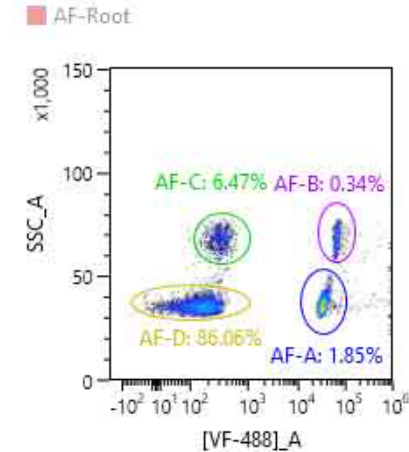
Note: Up to eight autofluorescence components can be registered as fluorochromes named "AF color 1" up to "AF color 8."

4. Click [OK].

7. Apply the Unmixing

a. Go to the Unmixing tool ribbon (top of screen) and click on **Apply.**

b. **WLSM** will appear in the upper-right corner of each data plot to indicate that the unmixing is applied to the data.

8. Acquire Data from the Stained Cell Sample

a. Go to the Rack/Plate Layout panel (lower left) and make sure the current position (black arrow) is on C02.

b. Click on C02 to view the worksheet for C02.

c. Change the stopping conditions to ■ A and 10,000.

d. Click on the Acquire button or Autoacquire button to record data from the stained cell sample.