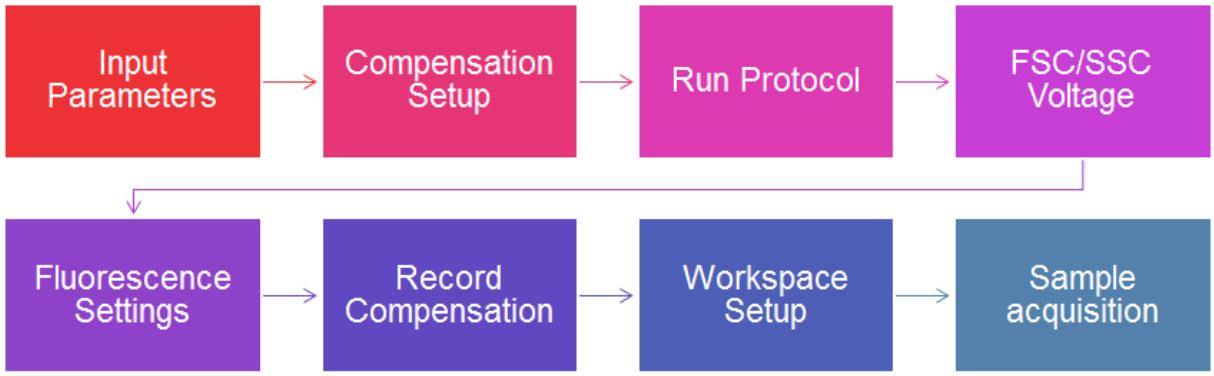
ATTUNE NXT BASIC TRAINING

PRACTICAL MODULE 3: MULTICOLOR ACQUISITION AND COMPENSATION

Multicolor experiments often require compensation to remove the spectral overlap of fluorochrome emissions into non-targeted detectors. This exercise is designed to demonstrate a 4 color experiment using fluorescently labeled beads for compensation. We will use the "Negative Gate" module for compensation for when negative and positive populations are present within the controls. If a negative control is available, or when there is no negative population in the single stained controls, the "Unstained Control" module can be used.

Learning Objectives:

1. Familiarize yourself with the workflow of setting up a multicolor acquisition experiment



2. Learn how to set up compensation controls as well as use on-plot compensation tools

For this Lab you will need:

- Human stabilized blood
- AbC™ Total Antibody Compensation Bead Kit
- Anti-human CD45 Alexa Fluor 488 antibody – specific to Leucocytes
- Anti-human CD3 R-PE antibody – specific to T Lymphocytes

- Anti-human CD19 PerCP-Cy5.5 antibody – specific to B Lymphocytes
- Anti-human CD14 APC antibody – specific to Monocytes
- Fix and Lyse solution
- 1x PBS
- Flow tubes (12x75mm tubes)

Lab Activity

Sample Preparation

1. Label 6 flow tubes and prepare samples according to the staining chart below; mix the beads and blood well before aliquoting.

Attune NxT Channel						
Tube #	Sample	CD45 AF488	CD3 PE	CD19 PerCP-Cy5.5	CD14 APC	← Target / ← Label
1	2 drops of AbC beads	5ul				
2			5ul			
3				5ul		
4					5ul	
5	100ul Blood					
6		5ul	5ul	5ul	5ul	

2. Incubate all samples for **>20 minutes** in the dark at room temperature.

3. While waiting, fill in the correct Attune NxT detector channel to be used in the table.

4. While waiting, set up Attune NxT flow cytometer in the next section.

5. OPTIONAL: For No-lyse-no-wash analysis of whole blood, take out 2 ul of Tubes 5 and 6 and add 4mL PBS.

6. To the rest of the blood in **tubes 5 and 6, add 2mL High Yield Lyse** to lyse RBCs. Mix and incubate in the dark for 10 minutes.

7. Add 2 mL PBS to **Tubes 1-4**.

8. Add 2 drops of negative beads to Tubes 1-4 before analysis.

9. Cells are ready for flow analysis

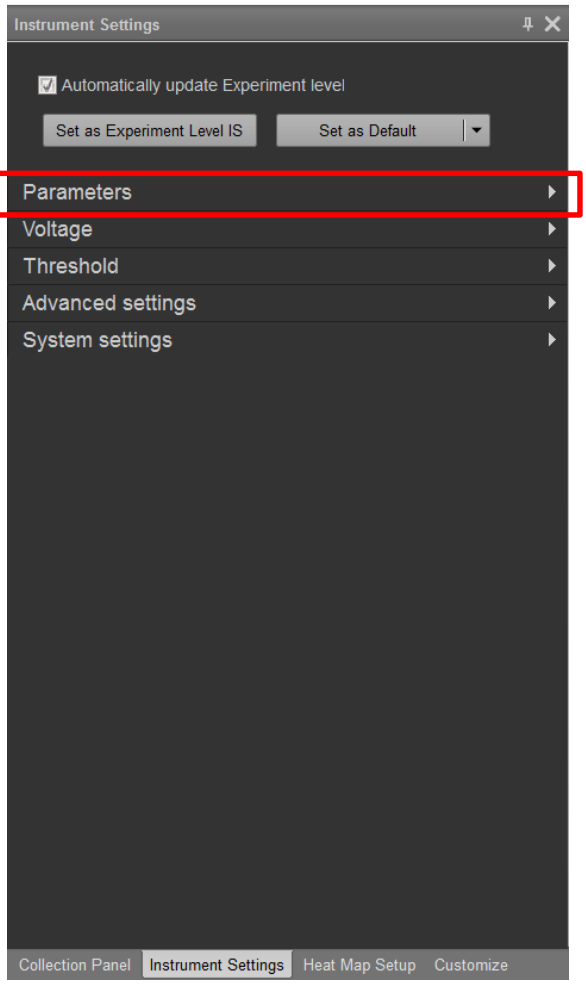
Flow Analysis

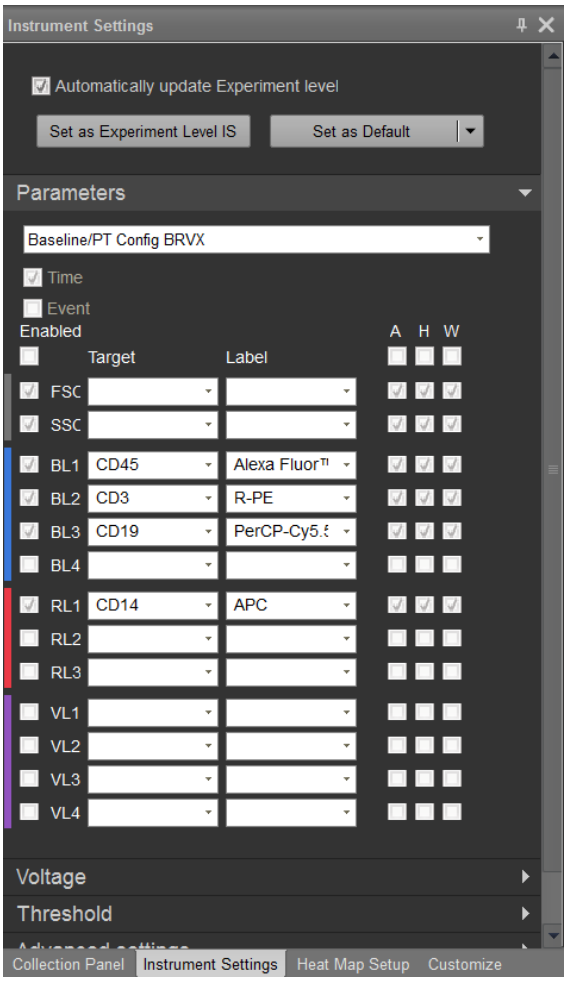
1. Turn on the Instrument and perform STARTUP and Performance test. Perform a SIP sanitize following Performance test. Instrument is ready for use.

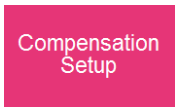
2. Select New Experiment on the Main menu or Right click on the user name in the experiment explorer and select “New experiment” from the drop down menu.

3. The new experiment dialogue box will open. Choose the experiment type (tube) and name the experiment “4 Colour Blood”. Choose to have 1 group and 2 samples (Right click on the sample names to rename them: Unstained and 4 colour blood).

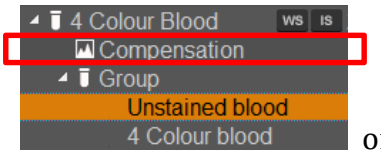
4. Input Parameters Under the “Instrument Settings” tab, open the “parameters” and turn on the appropriate channels. Input the Labels and Targets.

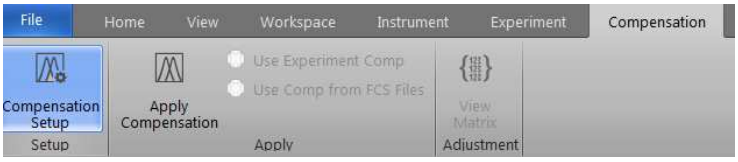




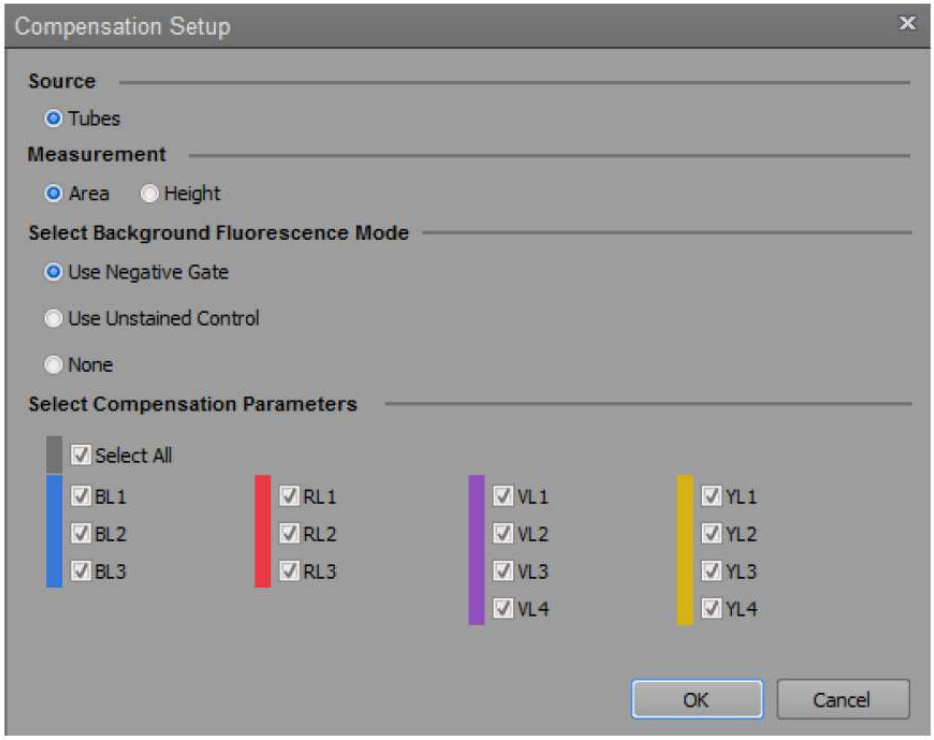
5. 

Double click the "Compensation" word under your experiment to open the compensation setup module. OR Under the compensation ribbon, select compensation setup.



or


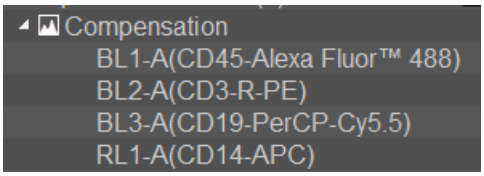
6. In the compensation setup, select **Tubes, Area, Negative Gate** and leave all the channels checked (only the channels turned on in the Parameter list will appear).



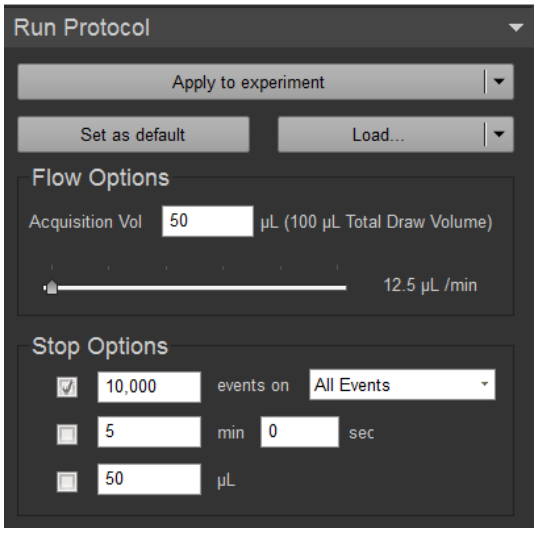
About Background Fluorescence mode

- Selecting **Use Unstained Control** adds Unstained Control as an additional Compensation control to the Compensation node in the Experiment.
- Selecting **Use Negative Gate** provides an additional Histogram gate or Rectangle gate on the Compensation Workspace for defining the negative population.
- When **None** is selected, compensation is calculated without correcting for background auto-fluorescence.

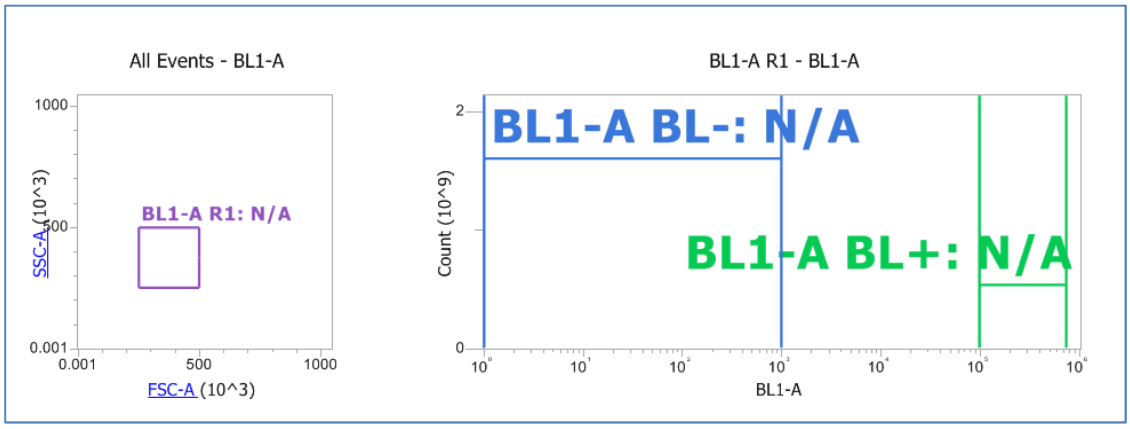
7. After confirming, single colour controls for each channel will appear.



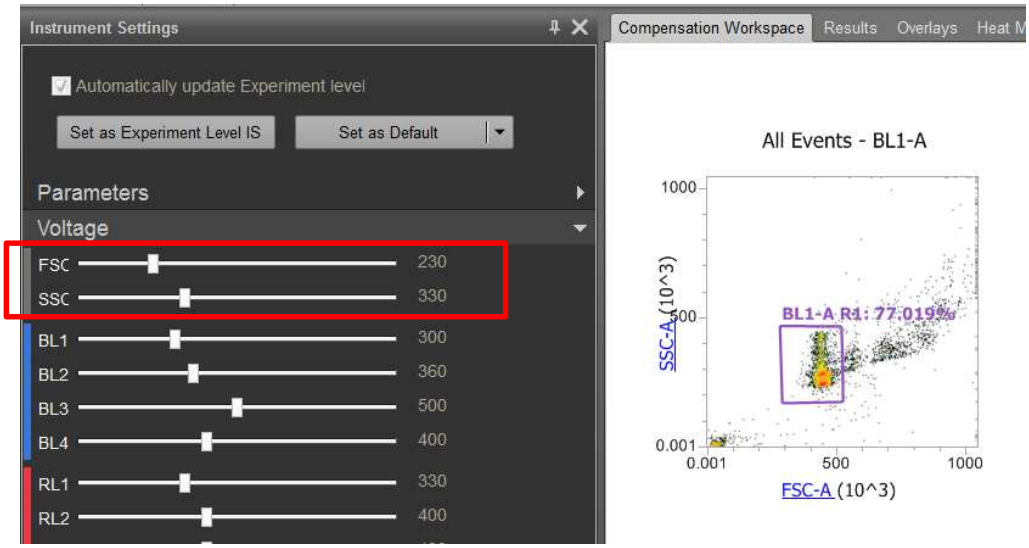
8. Run Protocol In the collection panel, set up the acquisition settings as follows. We are using low speed to allow more time for setting adjustment. Remember to click "Apply to experiment"



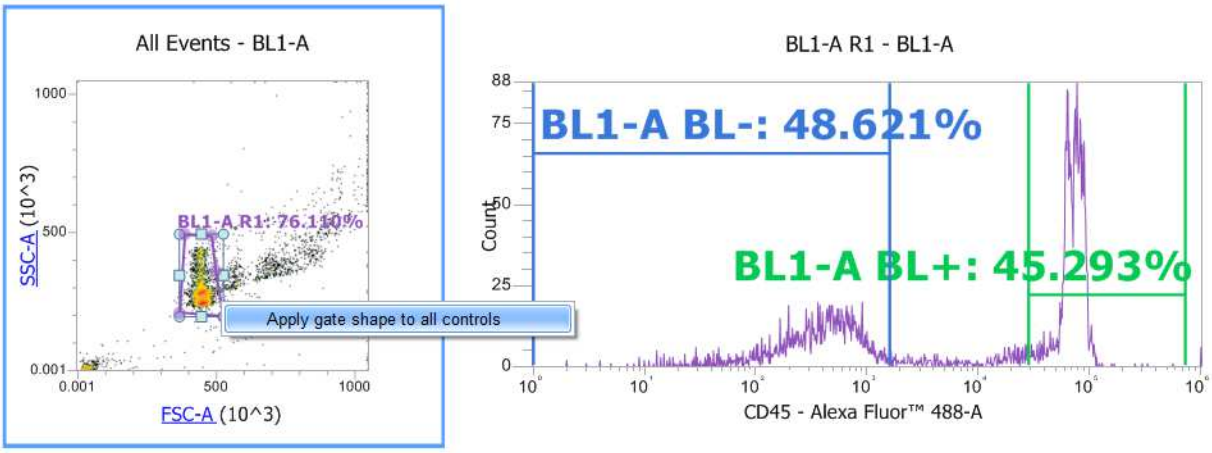
9. FSC/SSC Voltage Double click on the first compensation control (BL1-A) to open the workspace.



10. Load Tube 1 and click RUN. Open Instrument settings -> Voltage tab. Adjust the FSC and SSC voltages until the beads are on scale (the values in the picture are not accurate). Click STOP.



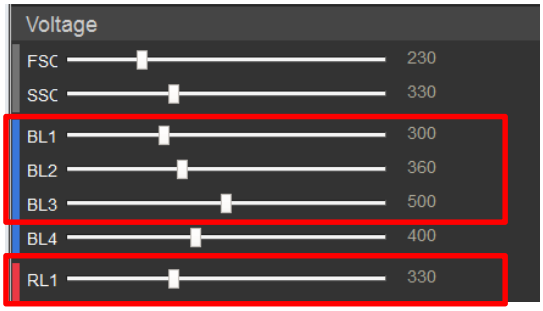
11. Shift the gate to include the bead population, excluding the multimers. See the plot below for an example. Right click on the gate to Apply gate shape to all controls.



12. Fluorescence Settings Click Run again and adjust the voltage of BL1 channel so that the negative beads are around 10^2-10^3 region, making sure that the positively stained beads are within scale (see image above).

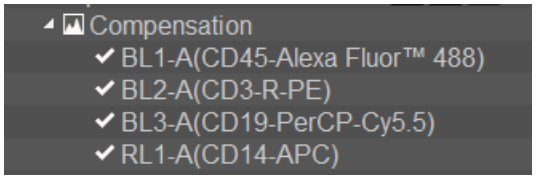
IMPORTANT: The first two decades is essentially the 'noise' region of the PMT. Positioning the negative population above 10^2 in the fluorescence log scale ensures that you set the correct voltage to allow resolution of the negative and positive populations above the noise.

13. Double click to open the sample workspace of the next compensation control (BL2). Adjust the voltage of the next fluorescence channel like in step 12. Do this for all 4 controls. <u>All voltages must be adjusted</u> before compensation is recorded.

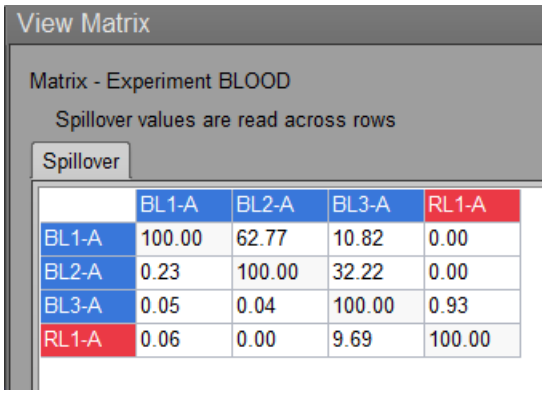


14. 

In the collection panel, increase the flow rate to 200ul/min and click "Apply to experiment". Record 10,000 events for each control. A white tick will appear next to each of the samples as shown below.

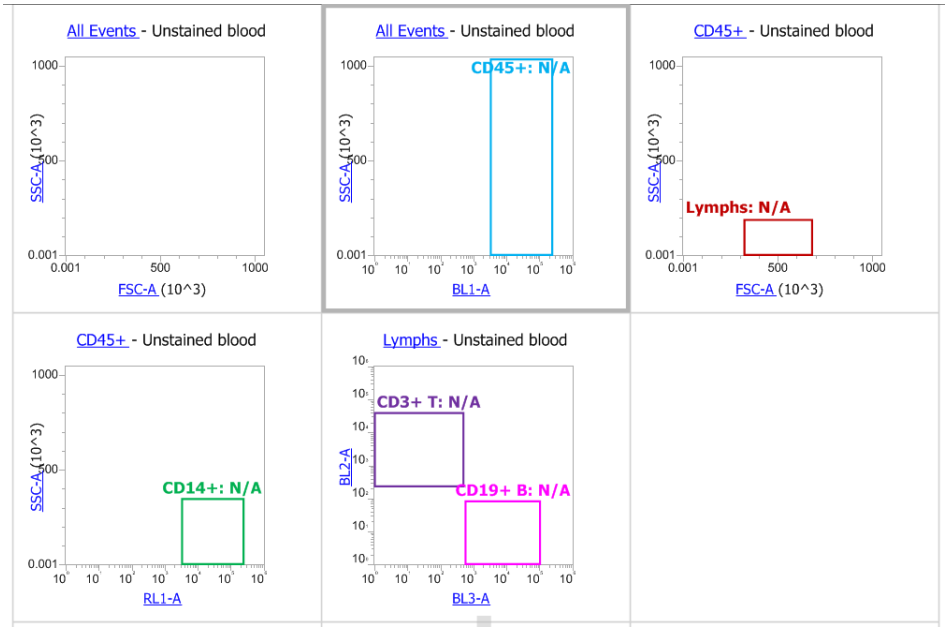


15. Review the compensation matrix by clicking on the Compensation ribbon -> "View matrix". Once all compensation is recorded, the fluorescence voltages will be locked and you will only be able to change the FSC and SSC voltages.



	BL1-A	BL2-A	BL3-A	RL1-A
BL1-A	100.00	62.77	10.82	0.00
BL2-A	0.23	100.00	32.22	0.00
BL3-A	0.05	0.04	100.00	0.93
RL1-A	0.06	0.00	9.69	100.00

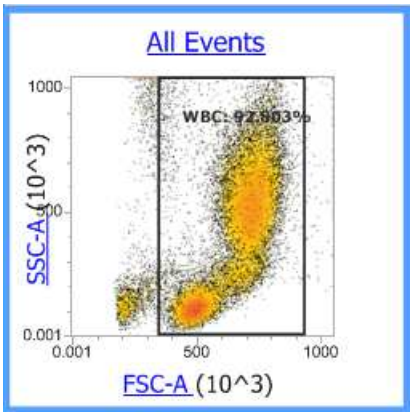
16. Workspace Setup Set up the workspace using tools in the Workspace ribbons to generate a similar workspace as below.



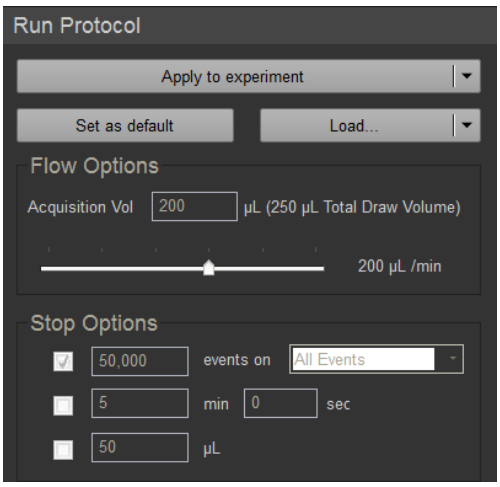
17. Once this is ready, we now need to optimize the FSC/SSC for your actual sample. This is because your compensation control uses beads while your actual cells have different forward and side scatter. If your compensation controls and samples are the same cells, then you would not need to change these values.

18. First, change back the flow rate to 12.5 to allow time to optimize the settings (50ul will take 4minutes to go through the system).

19. Change the FSC and SSC settings to put all 3 WBC types on scale such as this shown below.



20. Sample acquisition It is now time to acquire your samples. Change the run protocol to that shown below and acquire both the unstained and stained 4 colour blood samples.



21. Move the gates to include the correct populations. Change gate type in the customize menu if needed.

22. Following acquisition, perform a SIP sanitize to clean the system.

DATA ANALYSIS. FIND OUT THE PERCENTAGES AND ACTUAL CELL CONCENTRATIONS OF THE FOLLOWING POPULATIONS:

a. T lymphocytes _____% _____cells/ul (in blood diluted 21x in buffer)
b. B lymphocytes _____% _____cells/ul
c. Monocytes _____% _____cells/ul

Print the results into PDF by right-clicking on the experiment name and selecting Print.