Instrument QC and Calibration with nanoRainbow Beads

Purpose

Assess fluorescence resolution and laser alignment and calibrate instrument flow rate using Cellarcus vCal™ nanoRainbow beads (nRBs). First-time users will need to download the vFC™ SpectroFlo Experiment Template from the Cellarcus website (or perform Instrument Setup and create a vFC™ Assay Experiment Template for data as described in **Appendix A).**

Materials

Kit Component	Size		Store
vCal™ nanoRainbow beads (CBS6 – 2.5 mL)	2.5 mL		4°C
vCal™ Instrument QC and Qualification data analysis Layout			

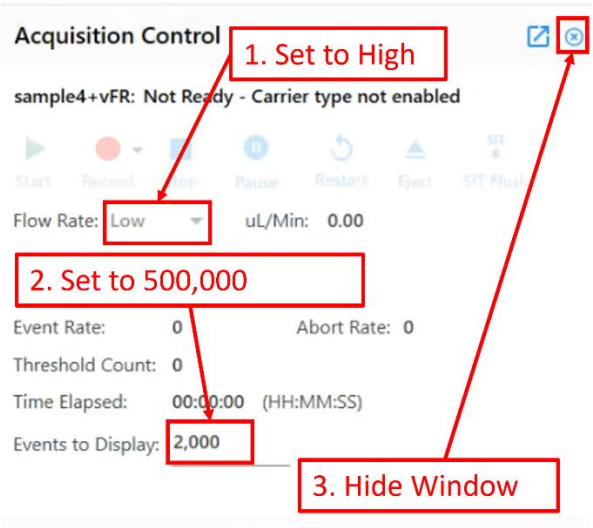
Materials to be Provided by user

- **Gloves**
- Microwell plate (Sartstedt 82.1583.001) or suitable tube.
- Cellarcus SpectroFlo Template – Aurora.zip (Cellarcus web site)
- FCS Express or FCS Express Reader software (FCS Express Reader) –

  *Before first use, refer to Instrument-specific instructions in **Notes** (page 8).*

Procedure

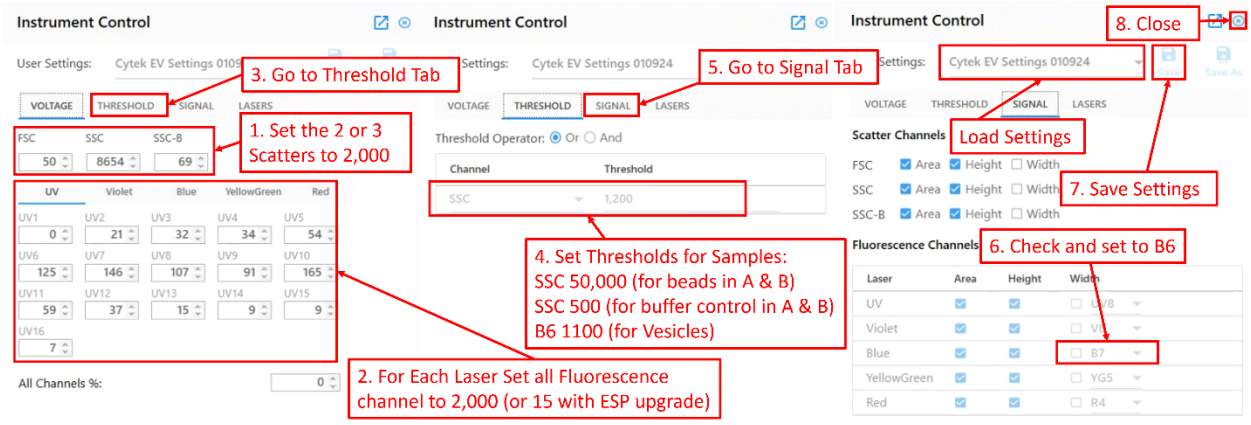
1. Open a New Experiment by opening the Cellarcus SpectroFlo Template - Aurora.zip (downloaded from the Cellarcus web site or created during **Instrument Setup**, see **Appendix A**).
2. In the *Acquisition* dialogue, set *Events to Display* to 500,000 to ensure that all data are displayed.



Under Instrument Control – Voltage set the gain for all of the scatters to 2000 and set the gain for all of the fluorescence channels to 2000.

Under Instrument Control – Threshold set the channel to the vFRed Parameter (B6-A) and set the threshold to 1100.

Under Instrument Control – Signal make sure B6-W (vFRed) is chosen for the width values.



You may choose to save/load the Instrument Control Settings file (see screenshot above). This shouldn't be necessary since the Instrument Control Settings are saved with the well/tube slots in the experiment.

Table A4				
Dialogue	**Setting**	**vCal™ beads**	**Buffer Bkgd**	**vFC™ assay**
Acquisition Settings	Active Channels	All channels	All channels	All channels
	Scatter gain (FSC, SSC, SSC-B)	2000 (SSC: 15 w/ESP)	2000 (SSC: 15 w/ESP)	2000 (SSC: 15 w/ESP*)
	Fluorescence gain (all channels)	2000	2000	2000
	Primary Threshold:	SSC-H	SSC-H	B6-H
	Manual Threshold:	50,000	500	1100
	Width Parameter:	B6-W	B6-W	B6-W
Stopping Rules	Time to Record	Checked: 30 sec	Checked: 30 sec	Checked: 120 sec
	Events to Record	Unchecked	Unchecked	Unchecked
Acquisition	Events to Display	500,000	500,000	500,000
	Sample Flow Rate	High	High	High
* Enhanced Small Particle (ESP) detector option installed.				

3. Click Save to save the new Settings.
4. Vortex nanoRainbow beads well.

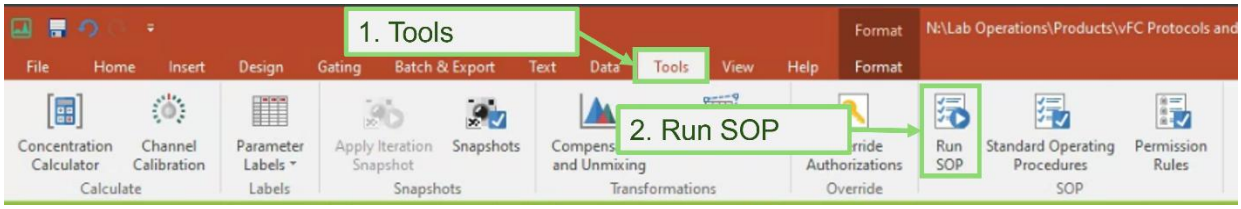
5. Place five drops (~250 uL) undiluted nanoRainbow beads in the **Protocol A+B Group** - Well H3 (or tube).
6. Select Well H3 and click **Start**. Inspect the light scatter plot to make sure the beads are on scale and singlets can be gated.
7. Set the flow rate to **High** and **Record** the nanoRainbow bead sample for 30 seconds.
8. Save the Experiment with a descriptive file name that includes the date.

Data Analysis

Run SOP

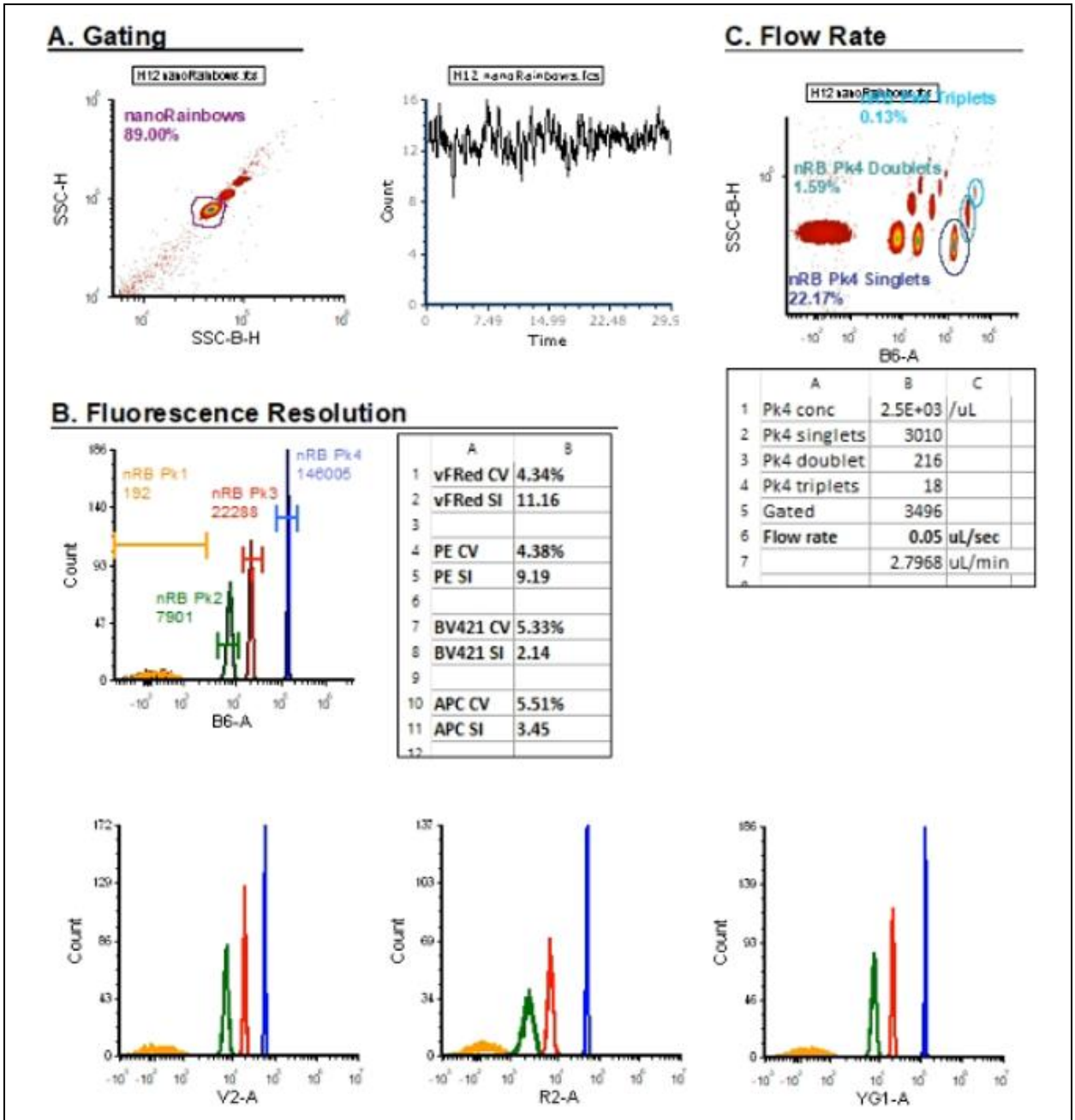
Open the Aurora (including Northern Lights) **Instrument QC and Qualification** data analysis Layout in FCS Express and run the SOP by going to Tools>Run SOP.

Before the first use of FCS Express, select the instrument-specific options as instructed as specified in "READ ME FIRST" tab.



Analysis

1. Go to Data List (right side of page) and click the Add Data File icon to navigate to nanoRainbow data (.fcs) file.
2. In the "Data List" window click on the nanoRainbow (.fcs) file then open the "Change File" menu and select "Change Data On All Plots" from the dropdown menu.
3. Adjust nanoRainbows gate to include single beads (excluding background noise, doublets, etc).
4. Inspect the time history for any fluidics issues.
5. Inspect the fluorescence histograms of a representative channel from each laser and adjust the markers to select each of the four bead populations.
6. Laser alignment is assessed by inspecting the CV of the bright bead population, which should be <15%. If CVs are higher, or there is an apparent leftward shoulder to the peak, alignment on that laser may be sub-optimal.
7. Fluorescence resolution is assessed via the Separation Index (SI), which reflects the resolution of dim signals from background by comparing the difference between the blank and dimmest bead to the standard deviation of the background. This semi-quantitative metric is useful for an initial analysis of instrument performance, and the vFRed™ SI should be >3.0 to ensure efficient EV detection.
8. Set the "nRB Pk4 Singlets", "nRB Pk4 Doublets", and "nRB Pk4 Triplets" on the appropriate populations. The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (5e5/mL).
9. Go to File>Save As to save the layout with a descriptive name.



	A	B	C
1	Pk4 conc	2.5E+03	/uL
2	Pk4 singlets	3010	
3	Pk4 doublet	216	
4	Pk4 triplets	18	
5	Gated	3496	
6	Flow rate	0.05	uL/sec
7		2.7968	uL/min

	A	B
1	vFRed CV	4.34%
2	vFRed SI	11.16
3		
4	PE CV	4.38%
5	PE SI	9.19
6		
7	BV421 CV	5.33%
8	BV421 SI	2.14
9		
10	APC CV	5.51%
11	APC SI	3.45
12		

Figure Instrument QC and Qualification. A. Single nanoRainbow beads are gated by light scatter. **B.** The laser and fluidic alignment is assessed by the Peak 4 (Bright) CV, while the fluorescence resolution is assessed using the Separation Index, which reflects the resolution between Peak 2 (Dim) and Peak 1 (Blank). **C.** The sample flow rate is estimated from the number of Peak 4 (Bright) beads, including doublets and triplets, measured for a fixed time.

Notes

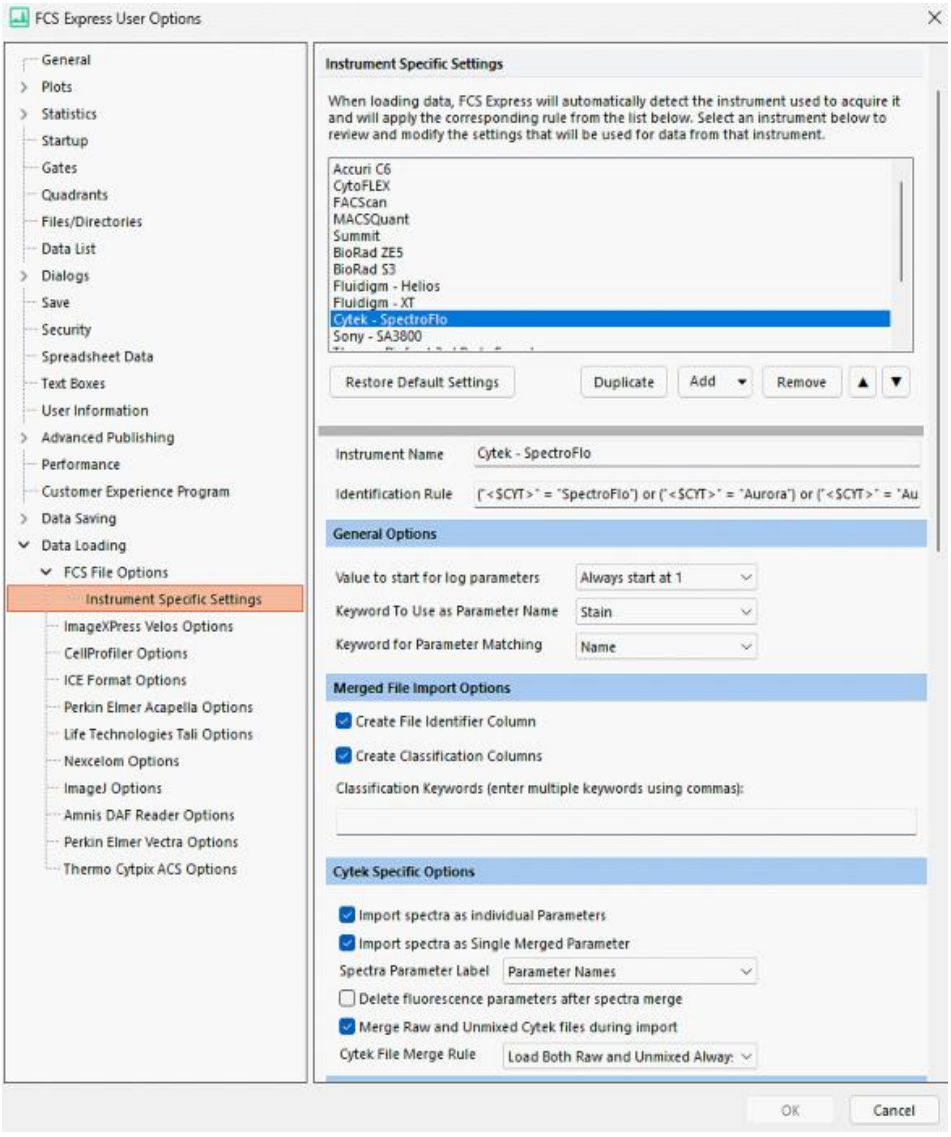
Before loading data files, configure FCS Express Options to merge raw and unmixed parameters:

File>Options>Data Loading> Instrument Specific Settings> Cytek - SpectroFlo>

1. Cytek File Merge Rule: **Merge both Raw and Unmixed Always**
2. Delete fluorescence parameters after spectra merge: **Uncheck**

Click: **OK**

Note: You need to load the unmixed version of the file. If you just changed the settings then you will need to change files on all plots before changes take effect.



Appendix A. Instrument Setup – Cytek Aurora and Northern Lights

Step 1. Create an Experiment Acquisition Template.

The Cytek acquisition settings are stored in an .expt file, which contains data acquisition settings as well as the file and group naming rules applied to file names and Sample IDs. Frequently used plate layouts can be saved and used as templates for future experiments.

This Template will be used to measure both beads, including vCal™ nanoRainbow Beads (Protocol A Instrument QC and Qualification) and vCal™ Antibody Capture Beads (Protocol B Fluorescence Unmixing and Calibration) and vesicles, including vCal™ Lipo100™ Vesicle Standard (Protocol B2) as part of Fluorescence Unmixing and Calibration and for most vFC™ assays, including measuring EV concentration and size (Protocol 1) and measuring EV surface markers (Protocol 2).

The beads are detected using light scatter triggering to measure the bead fluorescence intensity, while vesicles are detected using fluorescence triggering (vFRed Fluorescence, B6) and fluorescence measured under the same instrument conditions (flow rate, laser power, detector gain).

Before creating a template for vFC™ you need to add the vFRed Fluorescence tag to the SpectroFlo Fluorophore Library (**Figure A1**). To do this go to Library then select the blue laser and finally click “Add fluorescence tag.”

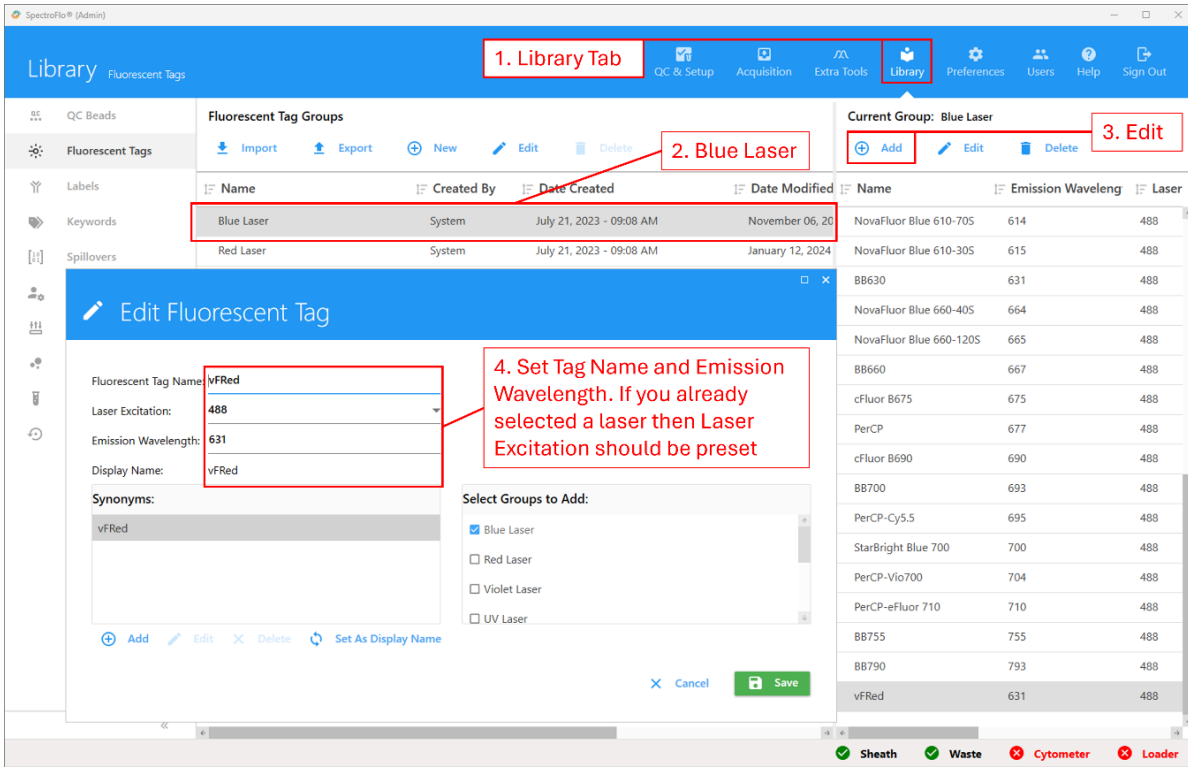
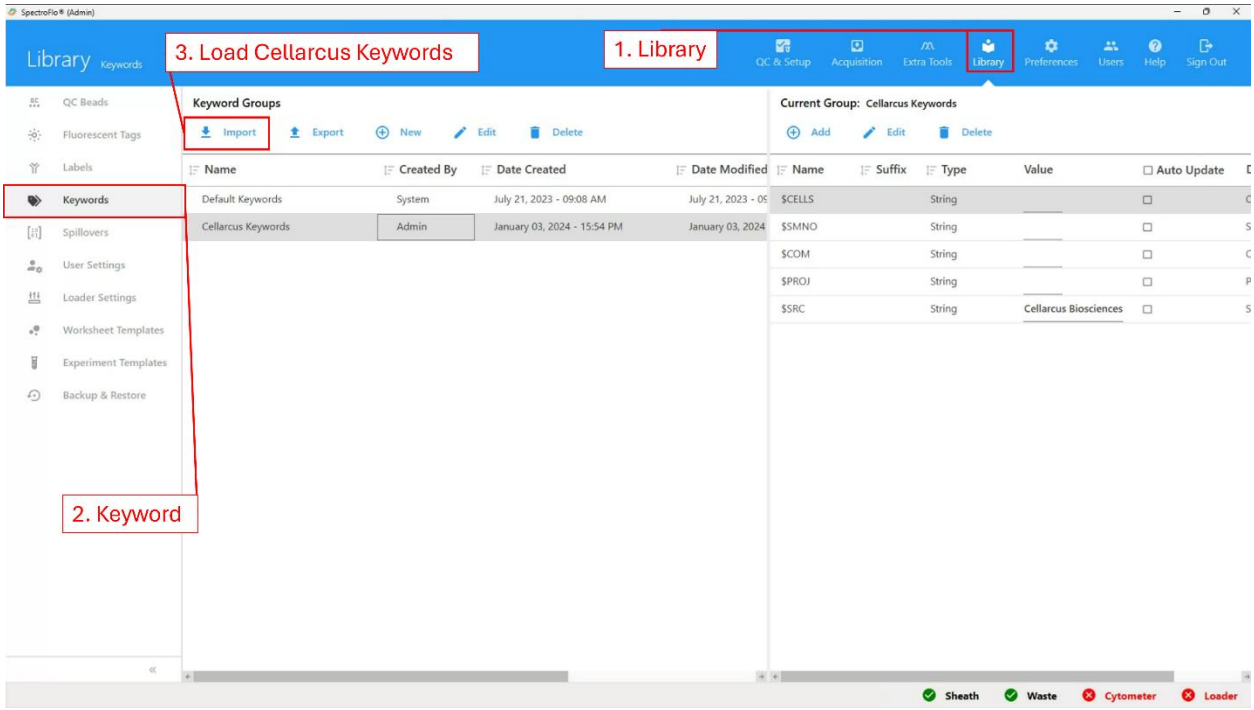


Figure A1. SpectroFlo Fluorophore Library.

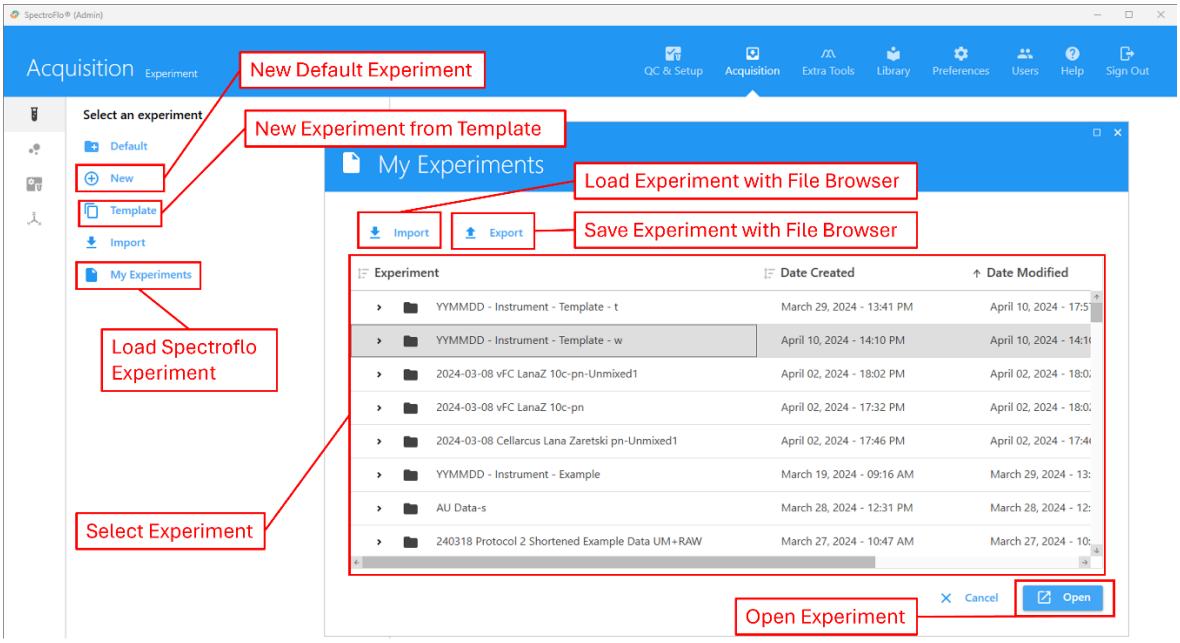
Table A1 shows the dyes that should be present in the Experiment Spectral Library. need

Table A1				
Detector	Parameter Name ($PnN)	Laser	EX-EM/BP	Stain Name ($PnS)
1	B06	Blue	B6	vFRed
2	B02	Blue	B2-525/17	AlexaFluor488
3	YG01	YellowGreen	YG1-577/20	PE
4	YG03	YellowGreen	YG3-615/20	PEDazzle
6	YG09	YellowGreen	YG9-780/30	PECy7
7	V01	Violet	V1-428/15	BV421
8	V07	Violet	V7-542/17	BV510
9	V10	Violet	V10-615/20	BV605
11	R01	Red	R1-660/17	APC
12	R02	Red	R2-678/18	AlexaFluor647

You will also need to import a set of keywords. To do this go to Library>Keywords then click Import under Keyword groups then load the .csv file containing the Cellarcus keywords in the proper format. You may also add additional keywords or create your own keyword groups but our layouts will work best with the Cellarcus keywords.



To configure the initial vCal™ and vFC™ Combined Acquisition Template (.expt file), create a New Experiment (*Acquisition > Select an experiment > New*), then follow steps below. To edit an existing experiment open the experiment then click "Edit."

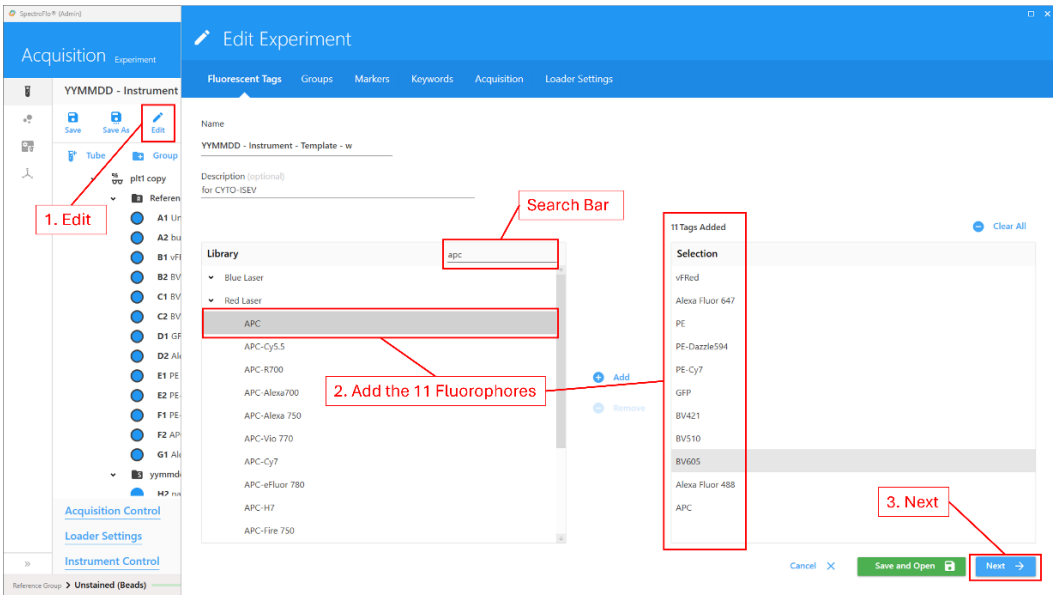


Note: Steps 1a and the first part of 1b to define the fluorescent tags and Reference Group are required for spectral unmixing in SpectroFlo, but not for conventional analysis with post-acquisition compensation (or unmixing).

a. Fluorescent Tags Tab – Add Fluorescence Tags (needed for unmixing in Spectroflo)

At the top of this first tab you can set a name and description for the experiment. Add vFC fluorescence tags from the Spectral Library. vFRed should have already been added to the spectral library (see screenshot above). To find tags quicker use the "Type to filter" search bar.

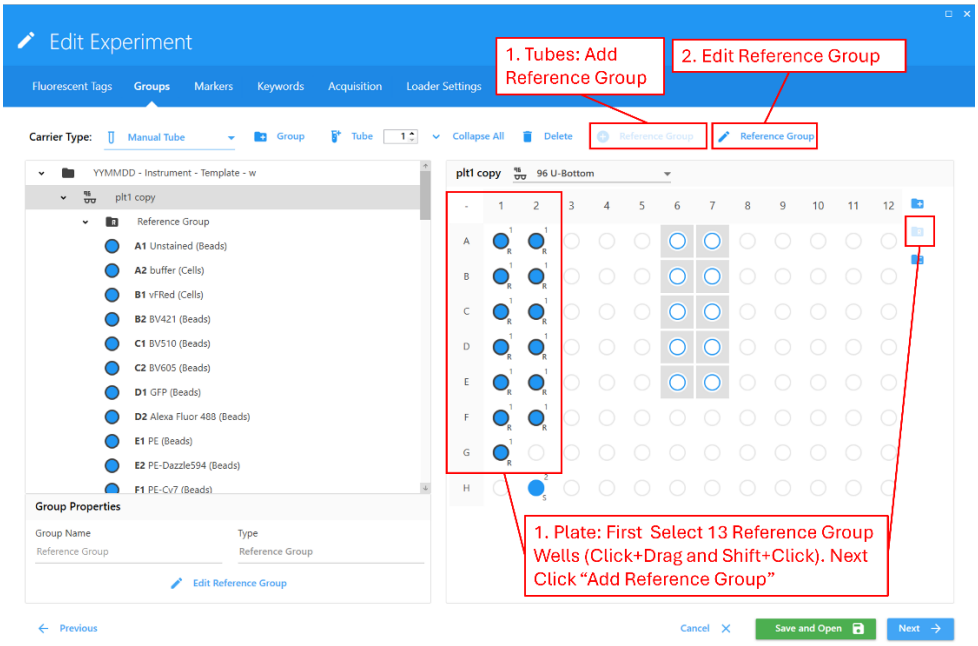
Click next.



b. Groups Tab – Create Reference Group and Sample Group(s)

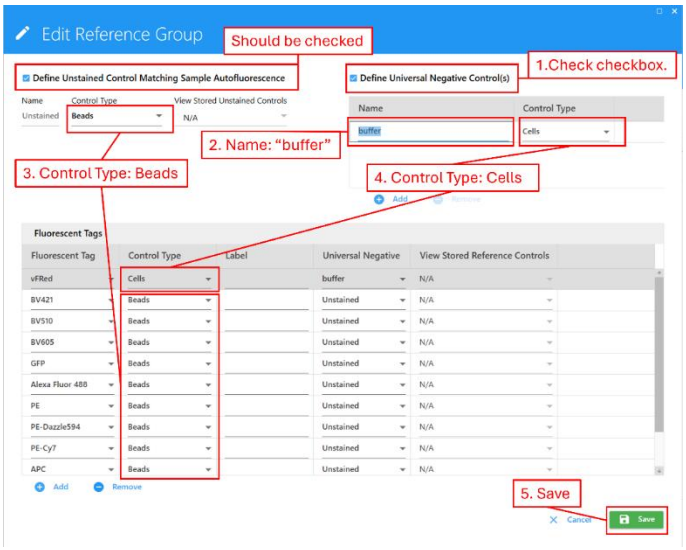
Tubes: If you are using tubes to collect data then Right click on the experiment group then select Add Reference Group and new window will open (see below).

Plates: If you using plates/wells change Carrier Type to “96 U-Bottom” then click “Add Plate.” Then select a group of wells one for each control (13 total). Hold click and drag to select multiple wells at once. Hold shift and click to add additional single wells. Once you have selected your wells then click the middle folder on the right side of the window (see below).



Check “Define Universal Negative Control(s) for Spillover Calculation” and a new section will appear. Click “Add” to create a new negative control. Name the new negative control “Buffer” and set the control type to “Cells.”

Under Fluorescent Tags section ensure that the positive controls have the appropriate control type and negative control set (vFRed should be Cells and Buffer and the rest should use the Unstained Bead control). Enter appropriate Labels for each positive control. Click Save.



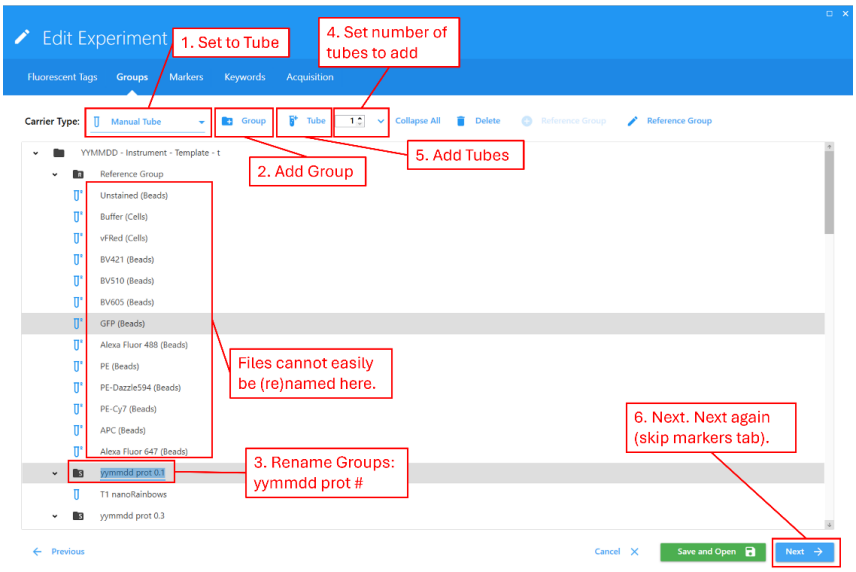
Tubes: Set Carrier type to “Manual Tube”

Protocol A: Add a group. Name it “yymmdd Prot A” then add 1 tube.

Protocol B: Add a group. Name it “yymmdd Prot B” then add 13 tubes.

Protocol 1: Add a group. Name it “yymmdd Prot 1” then add 16 tubes.

Protocol 2: Add a group. Name it “yymmdd Prot 2” then add 32 tubes.



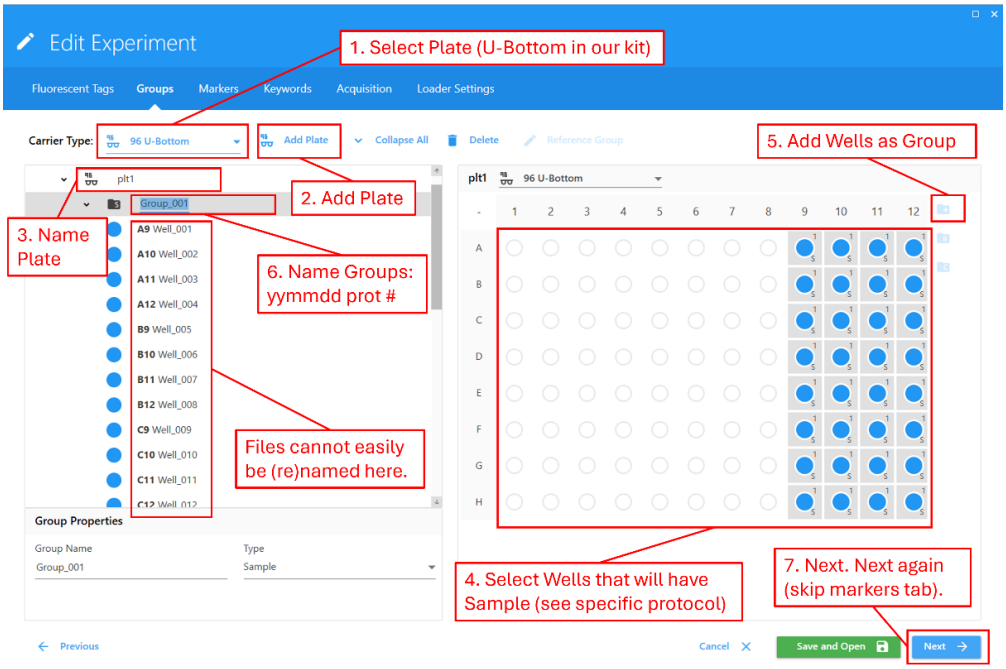
Plates: Set Carrier type to “96 U-Bottom” (or whatever plate type you are using). Add 3 plates. Name them something like “plt1,” “plt2,” and “plt3” (shorter is better).

Protocol A: Add a group to plt1. Name it “yymmdd Prot A” then add 1 tube.

Protocol B: Add a group to plt1. Name it “yymmdd Prot B” then add 13 tubes.

Protocol 1: Add a group. Name it “yymmdd Prot 1” then add 16 tubes.

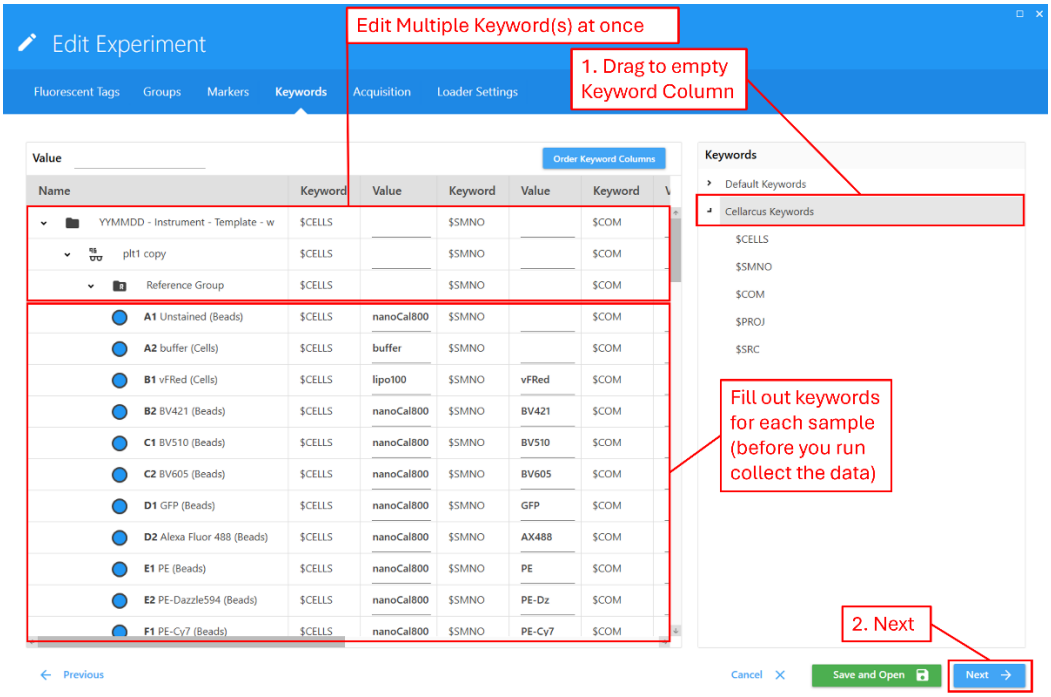
Protocol 2: Add a group. Name it “yymmdd Prot 2” then add 32 tubes.



Click Next. Click Next (Skip Markers tab).

c. Keywords Tab – Keywords

Drag the "Cellarcus Keywords" group over to the top row of the empty keyword column.



Click Next.

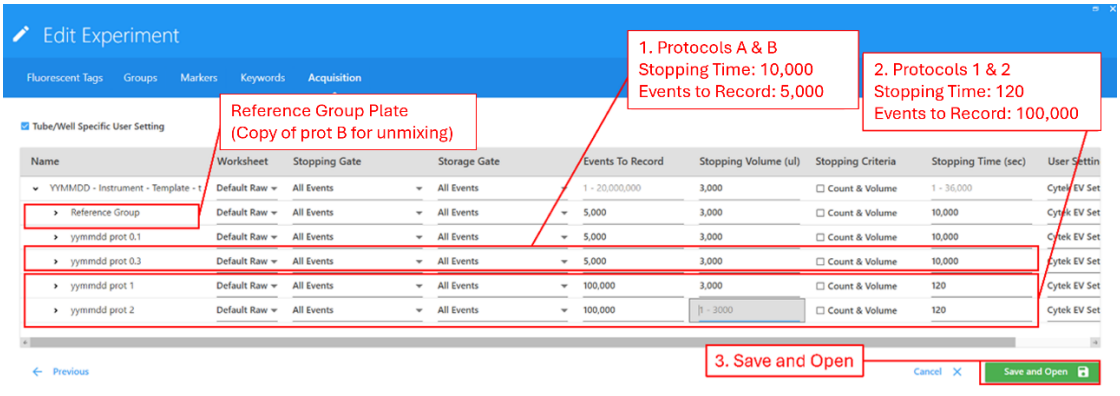
d. Acquisition Tab – Set Acquisition Settings

Protocol A - Set Stopping Time to 30 seconds. Set Events to Record to 100,000.

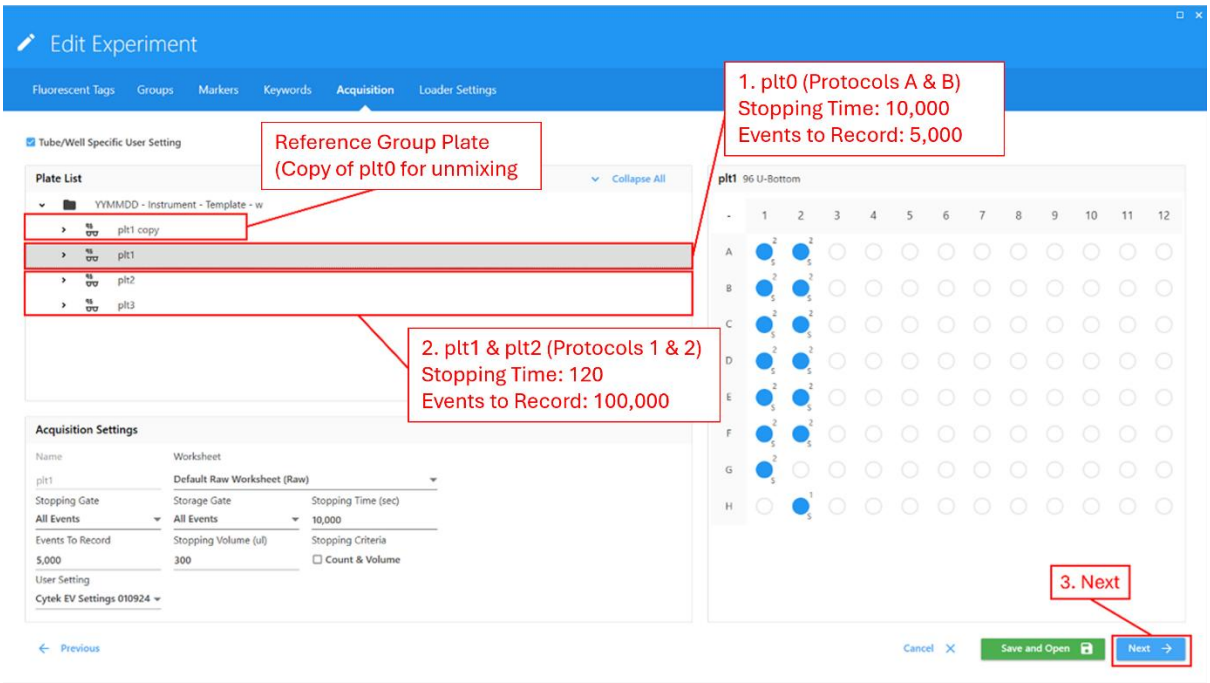
Protocol B – For bead wells (A1-F2), set Stopping Time to 10,000 seconds. Set Events to Record to 5000. For vFC Buffer and Lipo100+vFRed wells (F1,F2) set Stopping Time to 120 seconds. Set Events to Record to 100,000.

Protocols 1 & 2 – Set Stopping Time to 120 seconds. Set Events to Record to 100,000.

Tubes:



Plates:

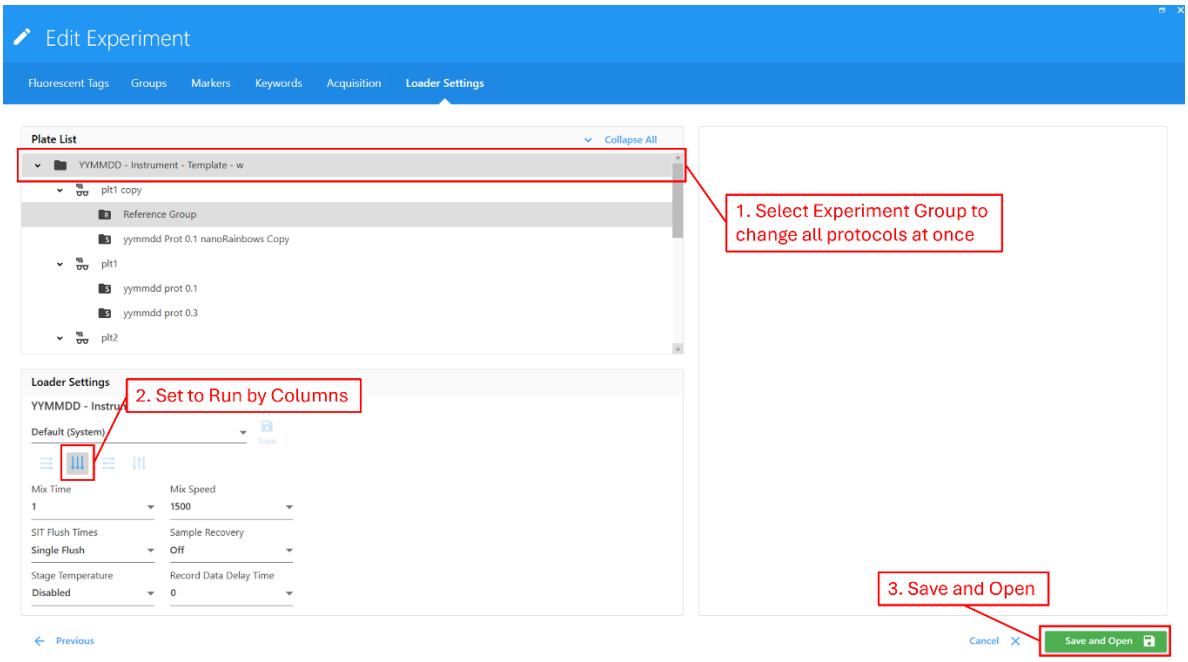


Click **Next**.

e. Loader Settings Tab – Set Run Order (Plate Only)

Click on Loader Settings on the right side of the screen (Plates only).

Specify the Run Order to be by Columns ↓↓↓ (Plates only).



Click Save and Open (Plates only)

f. Setup vCal™ Beads Worksheet – Define Acquisition Plots and Gates

Start with the default raw worksheet. Create the histograms and gates to assess the sample during data acquisition (**Table A2**). During data acquisition, the Singlet beads (Gate 1) will be adjusted to facilitate visual inspection during acquisition. Analysis of the data will take place in the appropriate vCal™ Bead Report layout.

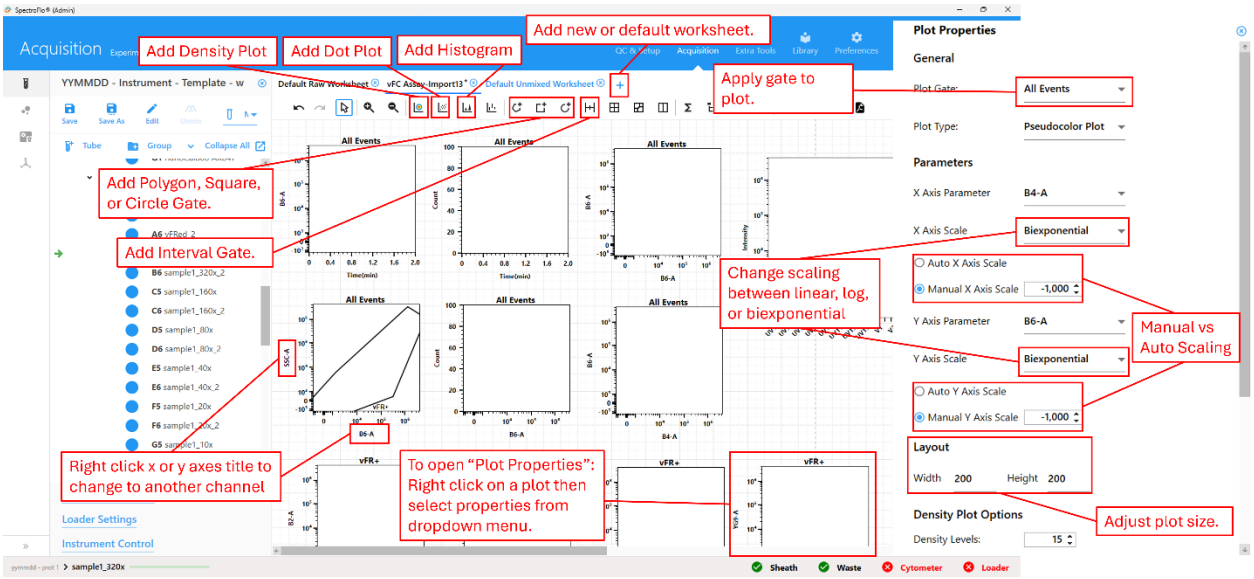


Table A2	
Plot/Gate: Description	**Purpose**
Plot 1: Time	Monitor fluidic stability
Plot 2: SSC-A vs FSC-A	Gate singlet beads
Gate 1: Singlet gate	Select single beads and exclude doublets and background
Plots 3-n: Bright Channels*	Individual fluorescence channel intensity resolution

*Bright Channels (5 Laser): R1, R2, B1, B2, B5, YG1, YG3, YG9, V1, V7, V10
*Bright Channels (3 Laser): R1, R2, B1, B2, B4, B6, B13, V1, V7, V10
*Bright Channels (1 Laser): B2, B4, B6, B13

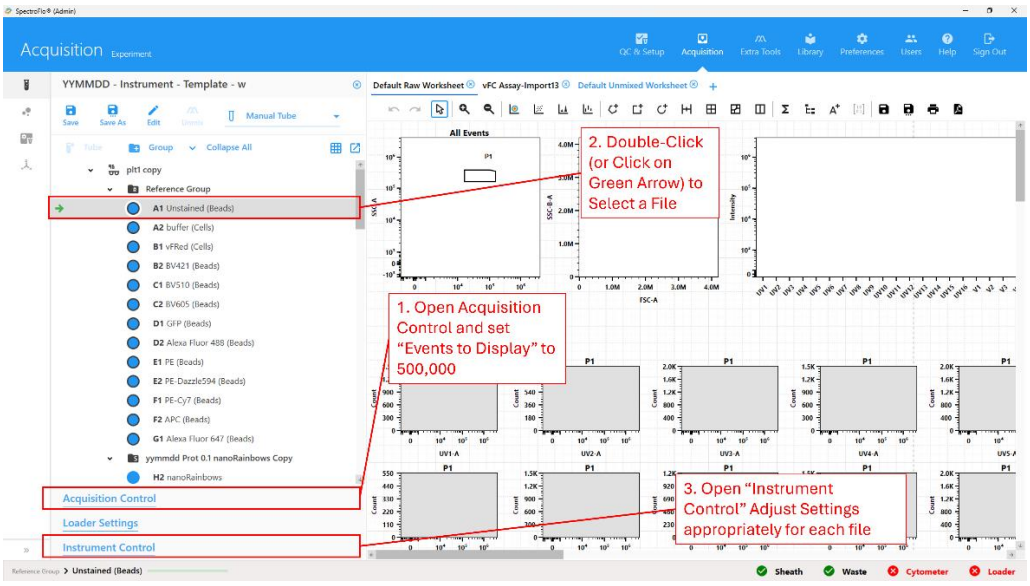
g. Setup vFC Assay Worksheet – Define Acquisition Plots and Gates.

Start with the default worksheet since it includes all the parameters. Ensure the following 1- and 2-parameter plots are present and draw gates to view data during acquisition (**Table A3**). These plots are only for visual inspection during acquisition. Analysis of the data will take place in the appropriate vFC™ Report layout.

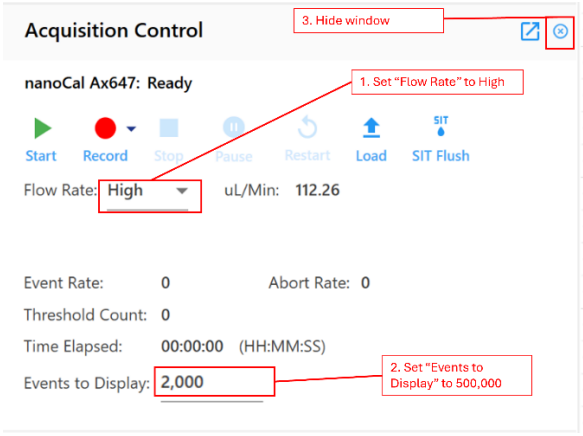
Table A3	
Plot/Gate: Description	**Purpose**
Plot 1: VSSC-A vs Time	Monitor fluidic stability
Plot 2: Time	Monitor fluidic stability
Gate 1: Time gate	Exclude the first 20 sec and collect the final 100 sec.
Plot 3: vFRed-A vs vFRed-H	Monitor event pulse characteristics
Gate 2: Pulse width gate	Exclude short pulse width background events
Plot 4: vFRed-A vs VSSC-H	Monitor event vFRed and VSSC intensities
Gate 3: Vesicle gate	Select events characteristic of single vesicles, exclude high- and low-VSSC events.
Plot 5: PE-A vs vFRed-A	Monitor PE immunofluorescence
Gate 4: PE positive	Estimate number of "PE positive" events
Additional Plots and Gate	For additional immunofluorescence channels as needed/desired

h. Instrument Control – Set Instrument Settings

Click on Acquisition Control below the Plate Window under Acquisition.



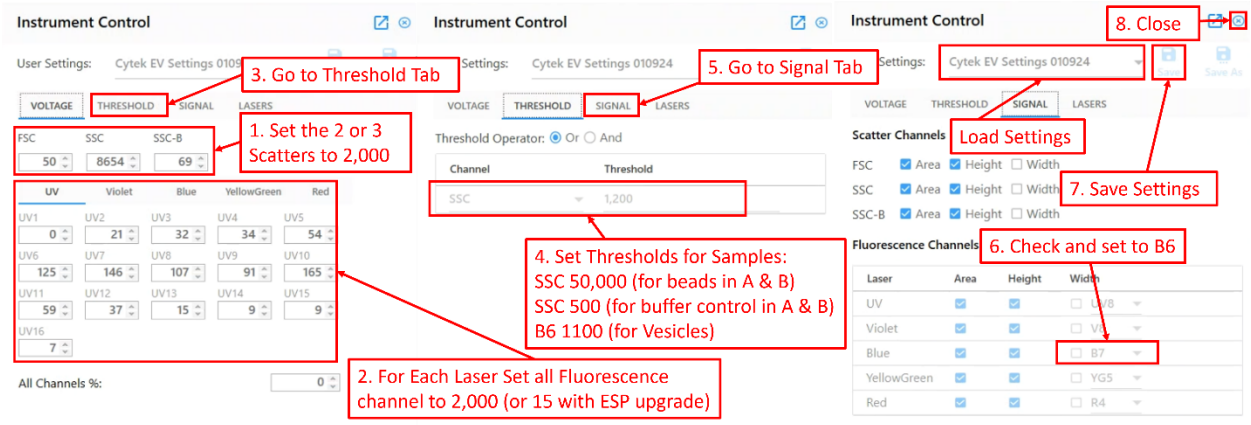
In the *Acquisition* dialogue, set *Events to Display* to <u>500,000</u> to ensure that all data are displayed.



Under Instrument Control – Voltage set the gain for all of the scatters to 2000 and set the gain for all of the fluorescence channels to 2000.

Under Instrument Control – Threshold set the channel to the vFRed Parameter (B6-A) and set the threshold to 1100.

Under Instrument Control – Signal make sure B6-W (vFRed) is chosen for the width values.



You may choose to save/load the Instrument Control Settings file (see screenshot above). This shouldn't be necessary since the Instrument Control Settings are saved with the well/tube slots in the experiment.

Table A4				
Dialogue	**Setting**	**vCal™ beads**	**Buffer Bkgd**	**vFC™ assay**
Acquisition Settings	Active Channels	All channels	All channels	All channels
	Scatter gain (FSC, SSC and SSCB)	2000 (SSC: 15 w/ESP)	2000 (SSC: 15 w/ESP)	2000 (SSC: 15 w/ESP*)
	Fluorescence gain (all channels)	2000	2000	2000
	Primary Threshold:	SSC-H	SSC-H	B6-H
	Manual Threshold:	50,000	500	1100
	Width Parameter:	B6-W	B6-W	B6-W
Stopping Rules	Time to Record	Checked: 30 sec	Checked: 30 sec	Checked: 120 sec
	Events to Record	Unchecked	Unchecked	Unchecked
Acquisition	Events to Display	500,000	500,000	500,000
	Sample Flow Rate	High	High	High
* Enhanced Small Particle (ESP) detector option installed.				

i. Save As – Save the data acquisition template

Click Save As to save a Template. Give the template a descriptive name so it won't be confused with any other templates.

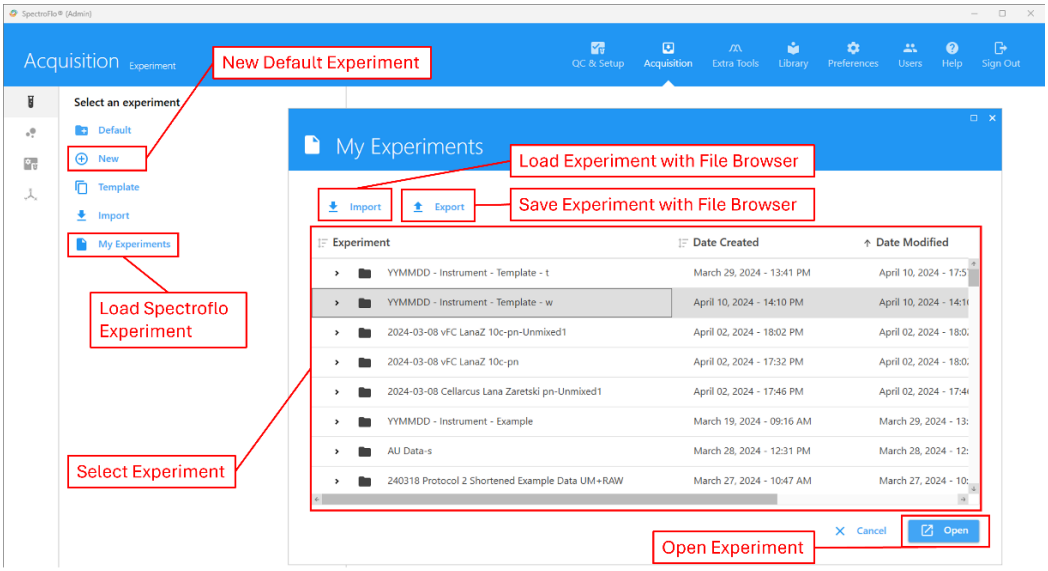
Various settings that should be associated with the template are summarized below (if you did the previous steps correctly).

Appendix B. Data Acquisition

1. vCal nanoRainbow and nanoCal antibody capture bead measurement

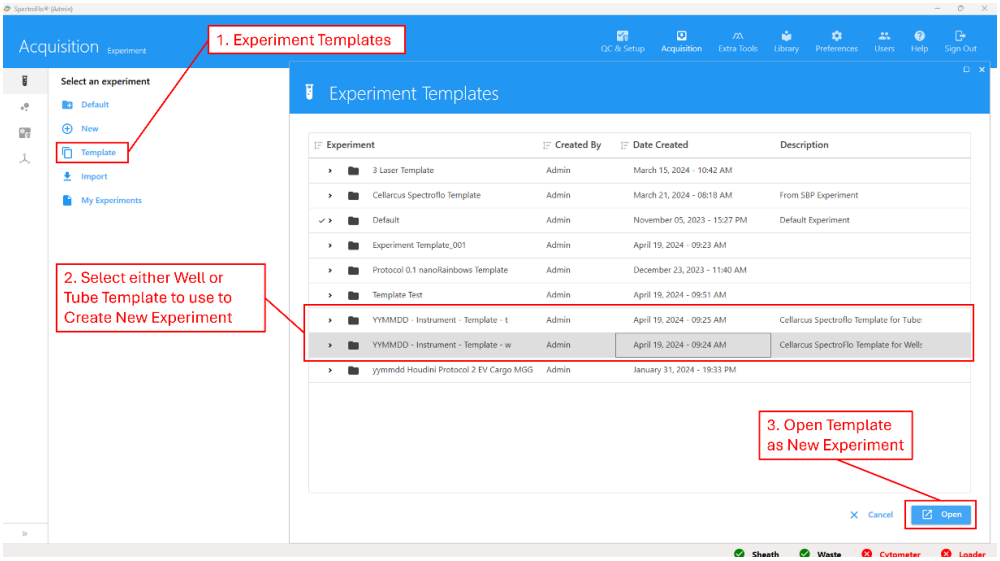
a. Load Assay Template and Settings

If you downloaded a Cellarcus SpectroFlo Experiment Template and haven't yet saved it as a template, load it then click Save As to save a template. Give the template a descriptive name so it won't get confused with any other templates.



Load an experiment template configured for either plates or for tubes (downloaded from Cellarcus or produced as described in Appendix A). The template should have your instrument set up with the appropriate Instrument Control Settings applied to the relevant sample tubes/wells.

- At the Import Options dialog box, check "Import as new item" to avoid overwriting previous versions.

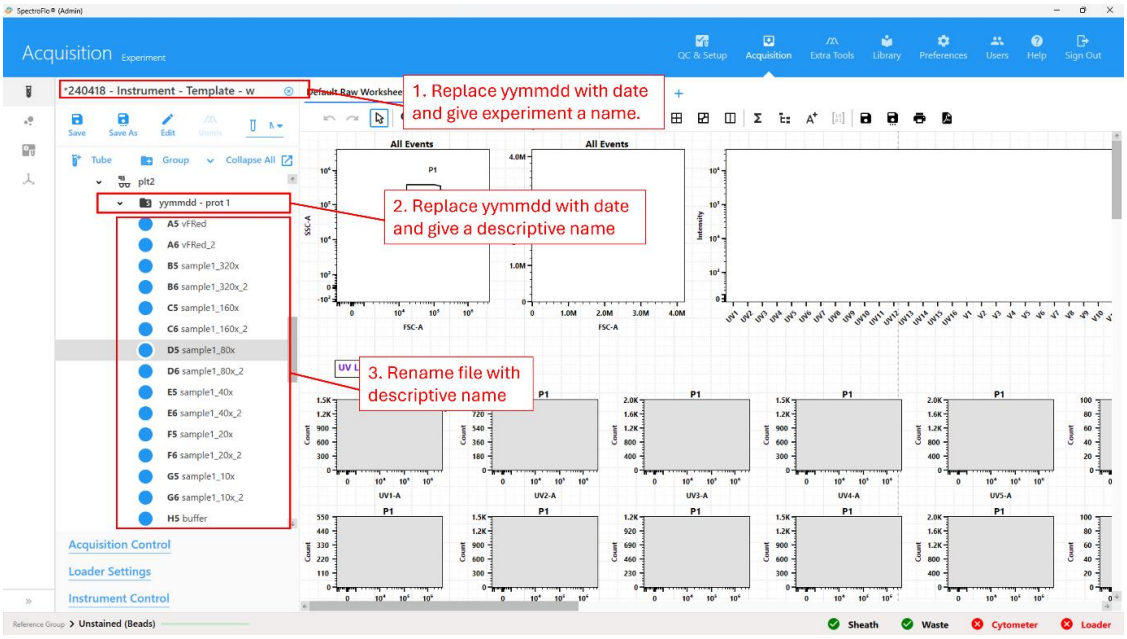


b. Set Experiment Name, Group Name(s), and File Names

Give your experiment a name. Include the date of the experiment at the beginning in the form of YYMMDD (so sorting by name sorts by date of experiment). Include some information about the experiment as well.

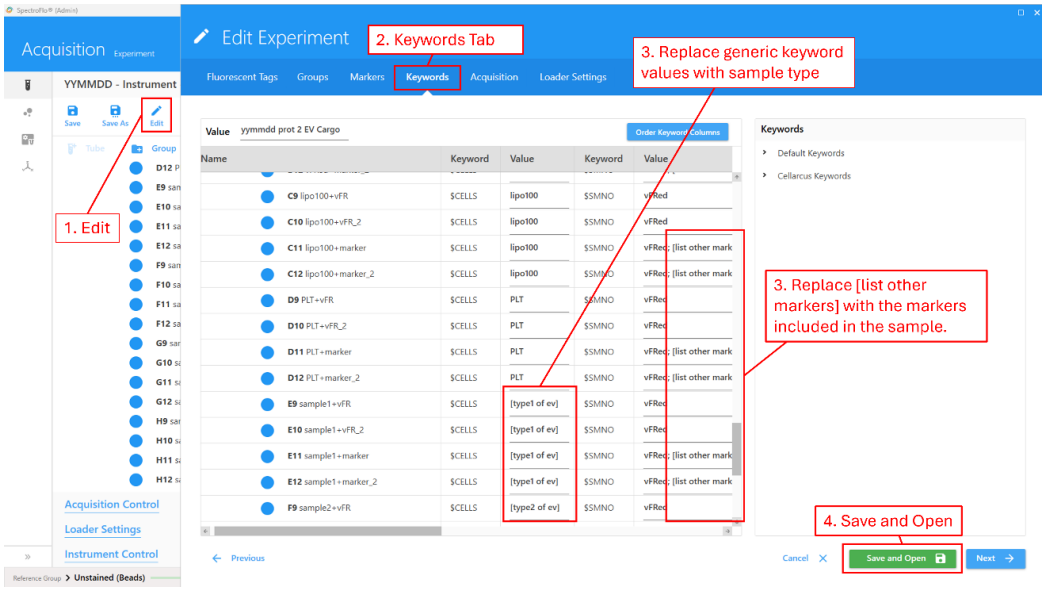
Edit group names so that the YYMMDD part is replaced with the date the group (possibly same as experiment date above) will be run.

Double-click each well/tube and rename to include the sample, any dyes added, and the date (note that there is an option to auto-add the date but it will also include time and group, lengthening the file path).



c. Fill in Keyword Values

Open the Edit Experiment window then go to the Keywords tab.



The first keyword to edit is $CELLS which should include particle type (eg. type of ev, type of bead, or type of cell). If you are using the Cellarcus SpectroFlo Template, edit any values in brackets which correspond to tube/well of each sample.

The second keyword is $SMNO which should contain the fluorescent markers included in the sample. If you are using the Cellarcus SpectroFlo Template, replace the brackets with the list of dyes.

The third keyword is $COM is for comments and you may choose to include any extra information that you want included in the data file for a given sample.

The fourth keyword is $PROJ which is for the experiment name and will likely be the same for all samples of a given experiment.

The fifth and final keyword is $SRC which is for the source of the sample. For standards provided with our kit it could be something like “Cellarcus Biosciences” (which is the default).

d. [Protocols 1&2 Only] Adjust Trigger Channel Threshold.

Load a well of vFC™ Staining Buffer and allow system to run for 15 seconds on high flow rate (60ul/min). Ensure that the blue laser-excited, B6-H (vFRed) fluorescence channel is set at a threshold of 1100, then adjust the manual threshold until you get 10-15 events/second in a Buffer Only sample. Apply this threshold to all wells/tubes in Protocols 1 and 2 and to the vFRed control well/tube in Protocol B. Save the Template file (.expt) for future use. In general, once optimized the threshold does not need to be changed every time you run an experiment.

e. Auto Record data.

You are now ready to run samples. Click Auto Record to run the selected Sample wells.