Protocol B. Fluorescence Unmixing and Calibration

Objective

Calibrate the spectral and intensity response of the instrument to enable spectral unmixing to determine the intensity of each fluorochrome and calibrate the intensities to report fluorophore abundance in absolute units of antibody binding capacity (ABC) or mean equivalent surface area (MESA).

Materials

- nanoCal™ Antibody Capture Beads (800 nm diameter polystyrene, $1x10^6$/mL)
  - Prestained nanoCal beads, freeze-dried (CBS7*Fluor*-2T, where *Fluor* is the stain)
  - User-stained nanoCal beads (see **Appendix A**)
- vFRed™-stained Lipo100™ Vesicle Size Standard
  - prepared and measured using **Protocol B2** (**Appendix B**), or
  - measured as part of **Protocol 1** or **Protocol 2**
- vFC™ Staining and Dilution Buffer
- v-bottom plate for measurement (Sartstedt 82.1583.001)
- Cellarcus SpectroFlo Template - Aurora.zip
- Protocol B Fluorescence Calibration Layout for FCS Express Reader (Layout for FCS Express)
- Protocol B Vesicle Size Calibration Layout for FCS Express Reader (Layout for FCS Express)

Procedure

1. Acquire calibration data for:

1a. Immunofluorescence using nanoCal™ beads and

1b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.

2. Perform spectral unmixing of the data to calculate the intensity of each fluorochrome.

3. Calibrate the intensities of the unmixed fluorochromes and validate the intensity calibration for:

3a. Immunofluorescence using nanoCal™ beads and

3b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.

B1. Data Acquisition

B1a. nanoCal™ Beads

1. Open a **New Experiment** by opening the **vFC™ Assay Experiment Template** (downloaded from the Cellarcus web site or created during Instrument Setup, see Appendix A).
   The Experiment Template is set up to accommodate 10 different fluorophores, plus vFRed™ and buffer controls. If you plan to use fewer fluorophores, you will need to Edit Experiment and delete the unused fluorochromes.
2. Reconstitute pre-stained nanoCal™ beads in vFC buffer according to the instructions (typically 100 uL).
3. Place each nanoCal™ bead in the indicated wells of a 96-well plate for flow cytometer measurement (**Figure 1**).
4. Fill one well with 300uL of vFC buffer without beads.
5. If running Protocol B2 vFRed Vesicle Size Calibration, follow the instructions in Appendix B. If running Protocol 1 or Protocol 2, these samples will be generated and measured as part of those Protocols, and that data can be used during data analysis.
6. Select the first bead well on the sample list and click Start. Inspect the light scatter plot to make sure the beads are on scale and singlets can be gated.
7. Select Well A1, set the flow rate to High, and Record all samples.
   Note: The first well (A1 buffer SSC 500) will be collected with a low SSC scatter trigger to collect the instrument background, wells A2-F2 and A12 (nanoCal and nanoRainbow beads) will be collected with a high SSC trigger, to measure the bead signals, and wells G1 and G2 (Buffer and Lipo100+ vFRed) will be collected with a B6-A trigger, to measure signals from vesicles (if being run in this Protocol).
8. Save the Experiment with a descriptive file name that includes the date.

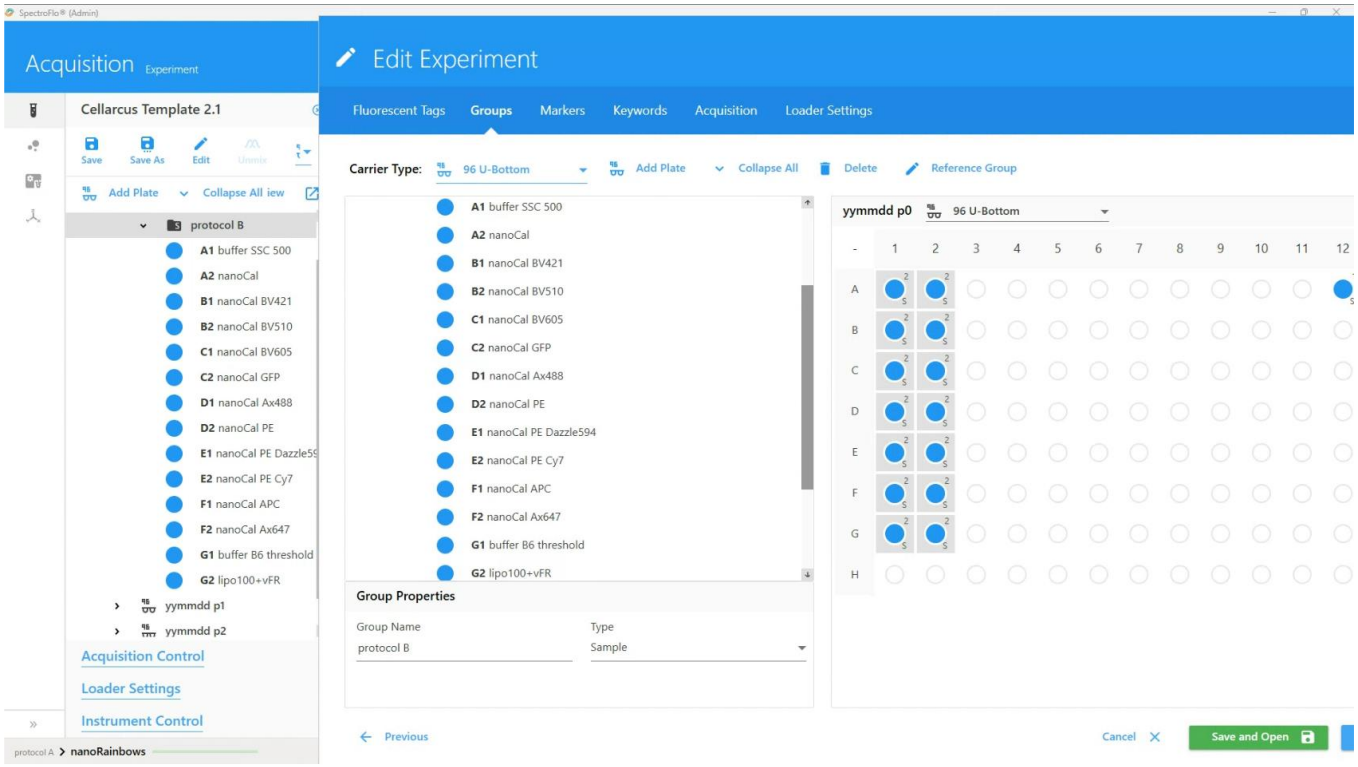


Figure 1. Sample list and plate layout for Aurora Protocol B.

B1b. Lipo100 Vesicle Size Standard

Measurement of the Lipo100 Vesicle Size Standard is part of every vFC™ Protocol and assay. In Protocol 1 Sample Dilution Series, Lipo100 serves as a vesicle positive control. In Protocol 2 and other Protocols that measure EV cargo, Lipo100 serves as a vesicle positive control and an immunofluorescence negative control.

For purposes of Fluorescence unmixing and Calibration, vFRed-stained Lipo100 fluorescence intensity data measured as part of Protocol 1 and Protocol 2 may be used. If you are performing Fluorescence Unmixing and Calibration prior to Protocols 1 and 2, use Protocol B2 (**Appendix B**) to prepare and measure vFRed-stained Lipo100.

B2. Spectral Unmixing

Spectral unmixing determines the intensity of each fluorochrome used in a measurement and is performed before calibration data analysis.

Spectral unmixing can be performed using the Cytek SpectroFlo software using the Spectral Reference samples measured in above in B1, as described in **Appendix C** or using suitable third-party flow cytometry analysis software.

Control	Control Type	Fluorescent Tag
vFRed (Cells)	Single Stained	vFRed
BV421 (Beads)	Single Stained	BV421
BV510 (Beads)	Single Stained	BV510
BV605 (Beads)	Single Stained	BV605
GFP (Beads)	Single Stained	GFP
PE (Beads)	Single Stained	PE
PE-Dazzle594 (Beads)	Single Stained	PE-Dazzle594
PE-Cy7 (Beads)	Single Stained	PE-Cy7
Alexa Fluor 647 (Beads)	Single Stained	Alexa Fluor 647
Reference Group - Unstained (Beads)	Unstained	N/A
Reference Group - Buffer (Cells)	Unstained	N/A

Figure 2. Spectral References for Unmixing

B3. Data Analysis

B3a. Data Analysis – Immunofluorescence Calibration

1. Following spectral unmixing, open the **vFC™ Protocol B Fluorescence Calibration Layout** using FCS Express or FCS Express Reader and load the appropriate data files into each plot.
2. Use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate on the population of single beads (**Figure 3**).

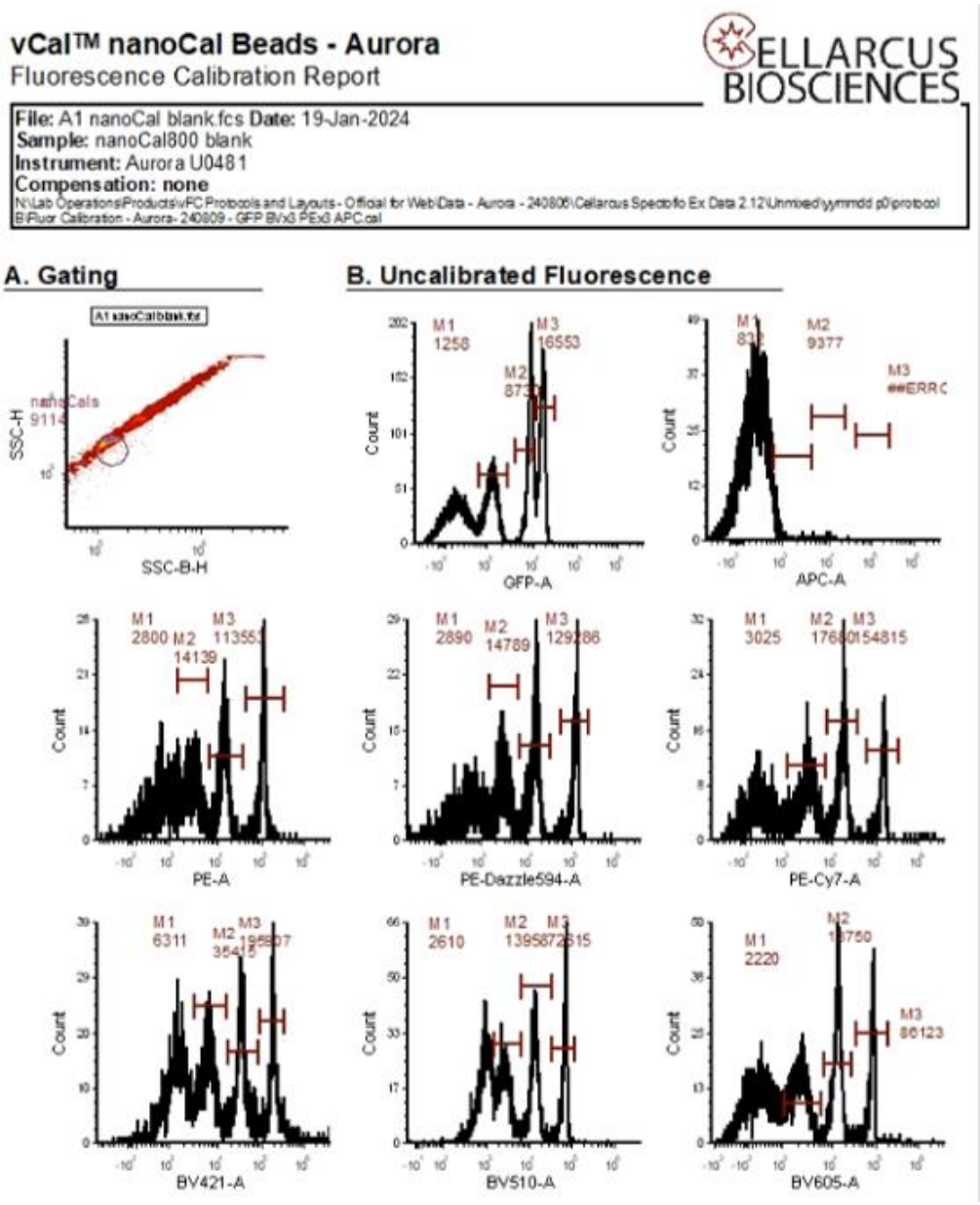


Figure 3. Fluorescence calibration using nanoCal™ beads. A. Single beads are gated by their light scatter. B. Individual peaks on each channel are gated and the median fluorescence intensity (MFI) values are used in the Channel Calibration dialogue in the vCal Bead Report layout.

3. Use the univariate plot of marker fluorescence intensity in the appropriate channel to visually inspect the separation of three bead populations and set the markers to report the medians of the individual peaks.
4. Use the nanoCal Bead ABV assignments (from the lot-specific Certificate of Analysis) and the FCS Express Channel Calibration Tool to generate a calibration file (Tools>Channel Calibration) to estimate the number of FL-mAbs bound per EV (**Figure 4**).

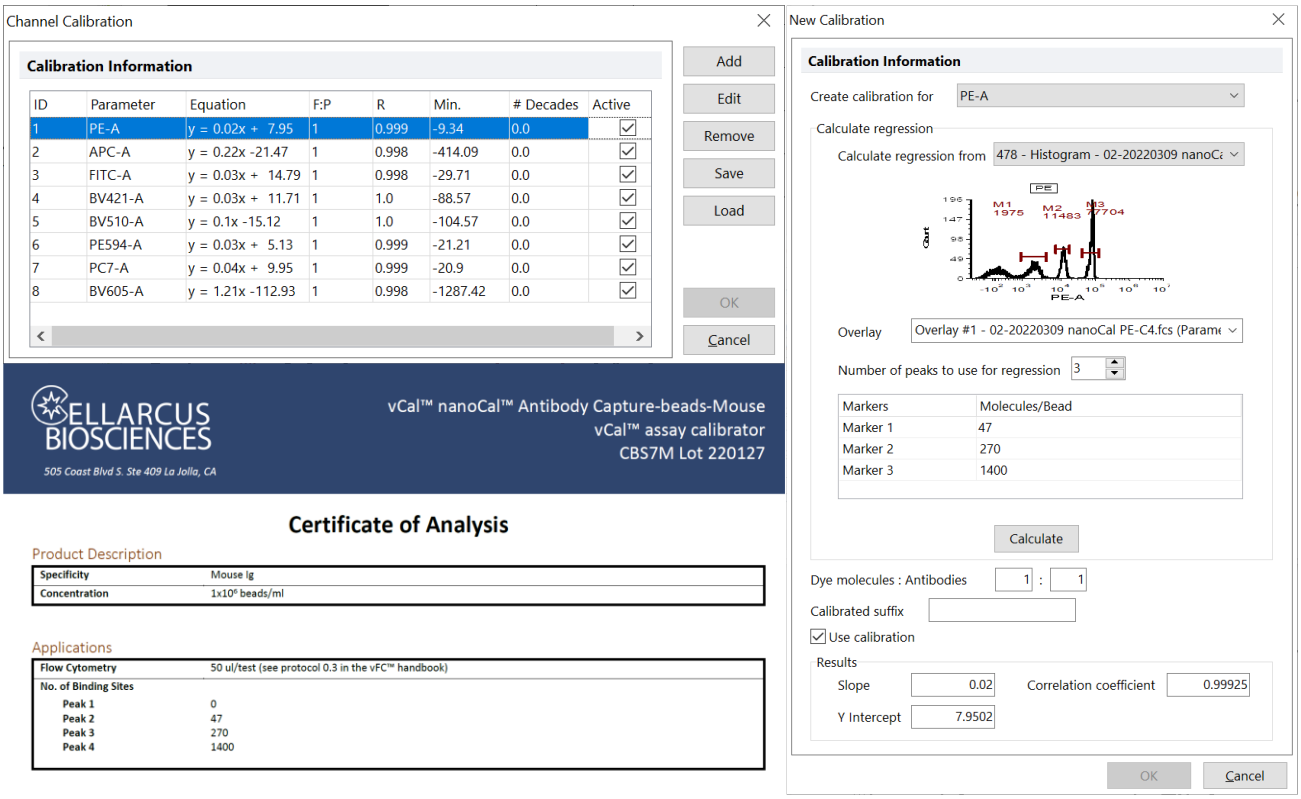
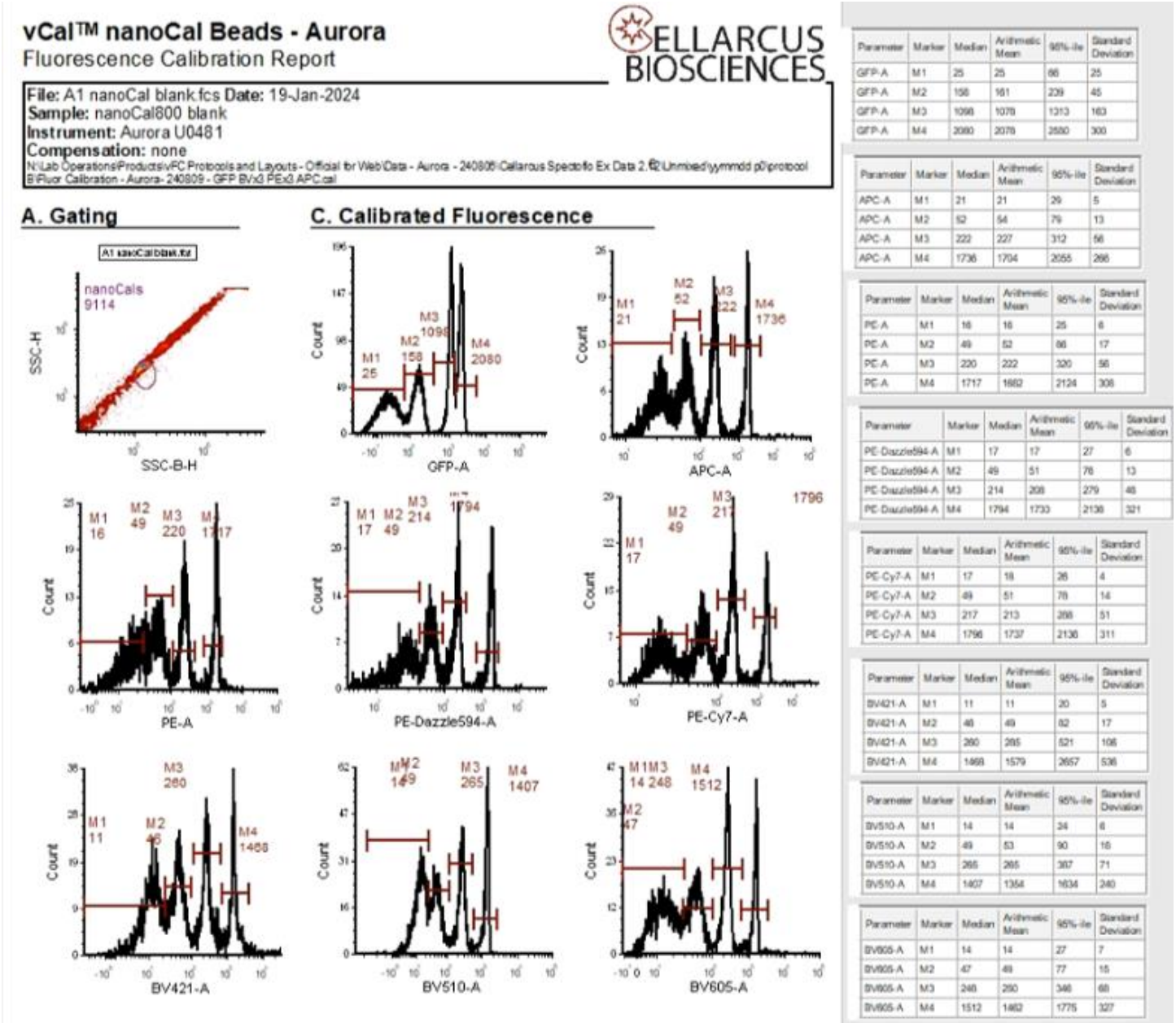


Figure 4. FCS Express Fluorescence Channel Calibration using nanoCal™ antibody capture beads.

5. Save the Channel Calibration file: Fluor Calibration - Aurora - YYMMDD – Panel (eg. Fluor Calibration - Aurora - 220310 – PEx3 BVx3 APC FITC.cal)
6. Load the Channel Calibration file *Channel Calibration >Load), so that its name will appear in the File Information header) and inspect the recovery of standards on the nanoCal - cal tab (**Figure 5**).



Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
GFP-A	M1	25	25	66	25
GFP-A	M2	158	161	229	45
GFP-A	M3	1098	1078	1313	163
GFP-A	M4	2080	2078	2580	300

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
APC-A	M1	21	21	29	5
APC-A	M2	52	54	79	13
APC-A	M3	222	227	312	56
APC-A	M4	1736	1704	2055	266

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
PE-A	M1	16	16	25	6
PE-A	M2	49	52	86	17
PE-A	M3	220	222	320	56
PE-A	M4	1717	1682	2124	306

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
PE-Dazzle594-A	M1	17	17	27	6
PE-Dazzle594-A	M2	49	51	78	13
PE-Dazzle594-A	M3	214	208	279	48
PE-Dazzle594-A	M4	1794	1733	2136	321

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
PE-Cy7-A	M1	17	18	26	4
PE-Cy7-A	M2	49	51	78	14
PE-Cy7-A	M3	217	213	288	51
PE-Cy7-A	M4	1796	1737	2136	311

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
BV421-A	M1	11	11	20	5
BV421-A	M2	46	49	82	17
BV421-A	M3	260	265	521	106
BV421-A	M4	1468	1579	2657	536

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
BV510-A	M1	14	14	24	6
BV510-A	M2	49	53	90	18
BV510-A	M3	265	265	387	71
BV510-A	M4	1407	1354	1634	240

Parameter	Marker	Median	Arithmetic Mean	95%-ile	Standard Deviation
BV605-A	M1	14	14	27	7
BV605-A	M2	47	49	77	15
BV605-A	M3	248	250	346	68
BV605-A	M4	1512	1462	1775	327

Figure 5. Fluorescence calibration using nanoCal™ beads. A. Single beads are gated by their light scatter. C. The fluorescence axes calibrations are assessed via recovery of standards.

B3b. Data Analysis - Vesicle Size Calibration

7. On the Aurora, unmix the data using the Cytek SpectroFlo software as described in **Fluorescence Unmixing and Calibration**.
1. Open the vFC™ Analysis Protocol B Vesicle Size Calibration Layout with FCS Express (or FCS Express Reader).
   Note: The vFC Analysis Layout has additional notes and tips to guide the data analysis.
2. From the Data List, click the Add File (+) and navigate to the data directory and select the Protocol 0 data files.
   a. Select a **Buffer + vFRed™** data file and click Change Data On All Plots. Inspect the Gating Plots. Adjust Time Gate, Area/Pulse Gate, and Vesicle Gate as needed to minimize background events.
   b. Select a **Lipo100™** data file and click Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed™-positive events (**Figure 1A-C**).

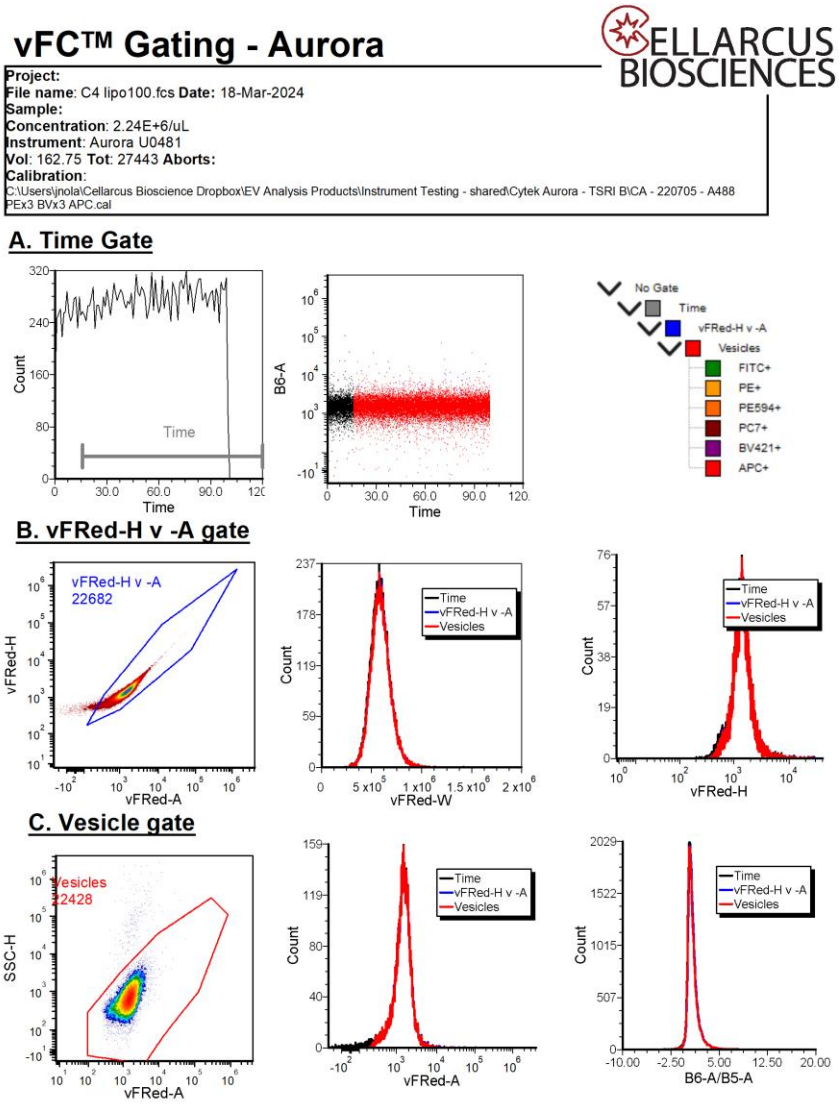


Figure 1. Vesicle Gating Tab.

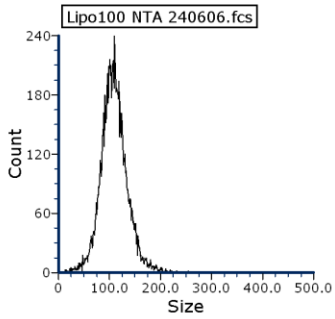
3. Open the **vFC™ Vesicle Size Calibration Tab** and load (drag and drop) the Lipo100 Reference size data onto the Input Size Histogram Plot.
4. View the resulting Vesicle Size Calibration plot of vFRed Intensity vs Surface Area (**Figure 2A**) and the resulting regression equation and coefficients.

vFC™ Size Calibration

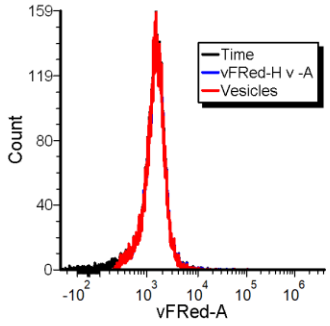
ELLARCUS BIOSCIENCES

Project:
File name: C4 lipo100.fcs **Date:** 18-Mar-2024
Sample: **vFRed:** P21
Concentration: 2.24E+6/uL
Instrument: Aurora U0481
Vol: 162.75 **Tot**: 27443 **Aborts:**
Calibration:
C:\Users\jnola\Cellarcus Bioscience Dropbox\EV Analysis Products\Instrument Testing - shared\Cytek Aurora - TSRI B\CA - 220705 - A488 PEx3 BVx3 APC.cal

A. Input Size Histogram (Drag & Drop)



B. Fluorescence Histogram



C. Vesicle Size Calibration

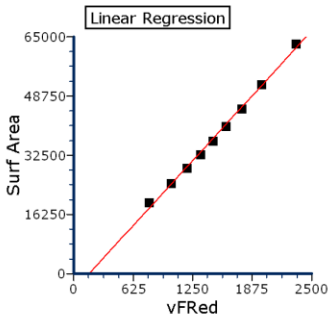
	A	B	C
1	%ile	vFRed	Surf Area
2	10	793	19473
3	20	1027	24795
4	30	1189	28959
5	40	1331	32615
6	50	1465	36406
7	60	1602	40414
8	70	1767	45194
9	80	1972	51842
10	90	2331	62955
11			

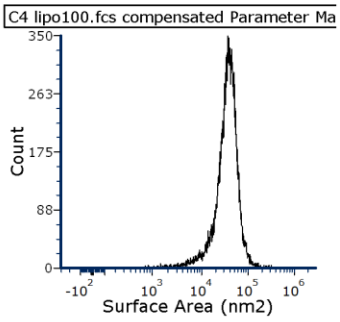
To Calibrate Size:

1. Open the transformations window (Tools>Transformations)
2. Click on the Parameter Math in the top part of the window.
3. Edit the Surface Area (nm2) Calculation in the lower part of the window so that it looks like the equation below.

*Surface Area (nm2) = (vFRed-A * 28.48) + -4581.59*

where n (in Pn) is the parameter number for vFRed(-A)





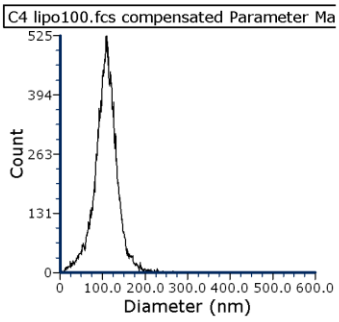


Figure 2. vFC™ Vesicle Size Calibration Tab.

5. Open Transformations (Tools>Transformations) then select the Vesicle Size Calibration parameter math Transformation (**Figure 3**).
6. Edit the Surface Area Transformation by entering the values in the equation to the slope (mA) and intercept (y-int).
7. Save Transformation by right-clicking on the box with the equations and saving as: Vesicle Size Calibration – CytoFLEX – YYMMDD – Calibrator Filename (eg. "Size Calibration – Aurora – 220401 – Lipo100 B9.fcf"). This Transformation (.fcf file) will be loaded into the vFC Report Layout for analysis of vFC Assay results.
8. Save vFC™ Layout as: vFC Protocol B Vesicle Size Calibration - Instrument - Date – Samples (eg. "vFC Protocol B Vesicle Size Calibration – Aurora – 220401 – lipo100 B9.fey."

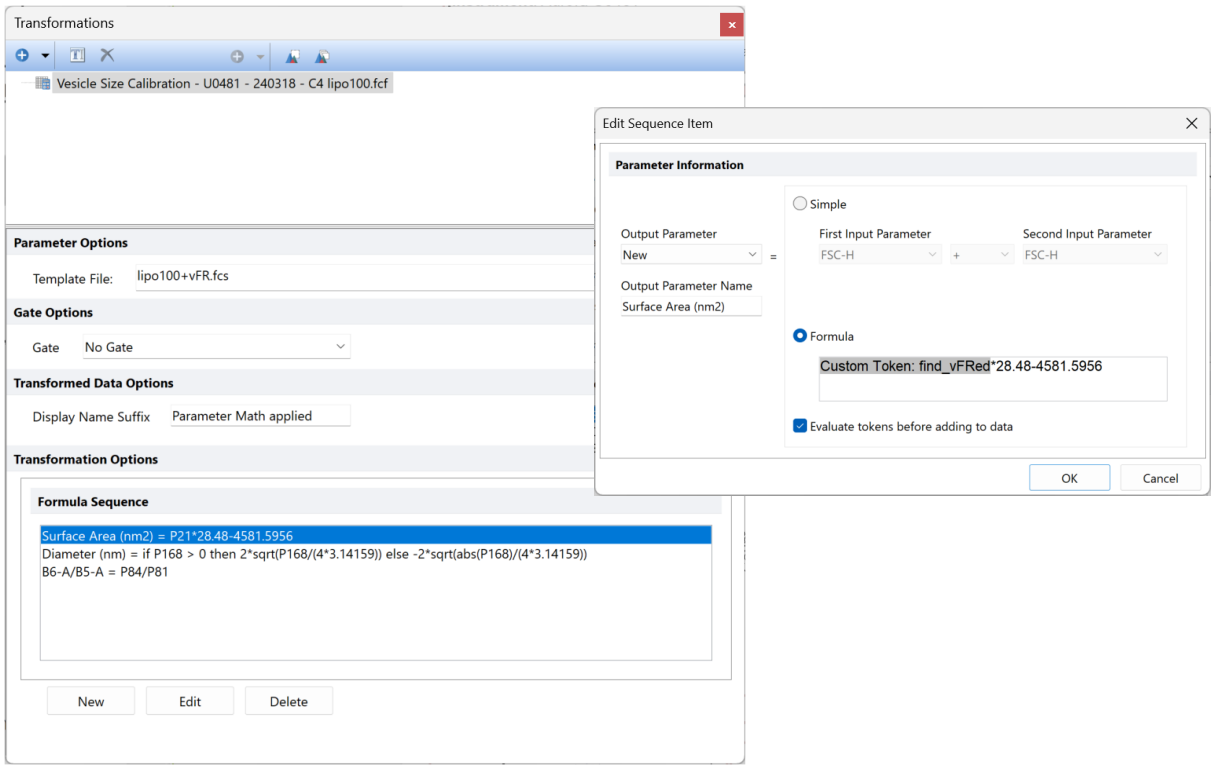


Figure 3. Transformations and Parameter Math Dialogue.

Notes

Appendix A. Staining nanoCal antibody capture beads

Specific antibody conjugates can be evaluated using nanoCal antibody capture beads and used for intensity and spectral calibration. This is recommended when using custom conjugates or antibodies from venders other than Cellarcus.

Materials

- nanoCal™ Antibody Capture Beads (800 nm diameter polystyrene, $1x10^6$/mL)
  Note: Calibrated beads are available for each of mouse, rat, rabbit, and hamster antibodies.
- Fluorescent antibody conjugate(s)
- vFC™ Staining and Dilution Buffer
- 1.5 mL microfuge tubes for staining and washing

Procedure

1. Vortex Capture Beads for 10 seconds.
2. Add one drop (~50 uL) of nanoCal™ antibody capture beads to a microfuge tube. You will need one tube for each fluorophore to be calibrated.
3. Add 5 uL of 10x fluorescent antibody conjugate to each tube. Mix well by vortex.
4. Incubate for 1 hour at RT in the dark.
5. Wash beads by two cycles of centrifugation and resuspension. To the stained beads add 1000 uL vFC™ staining buffer followed by centrifugation at 10,000 xg for 10 min (*note: there will not be a visible pellet*). Aspirate buffer carefully by removing buffer from the top of the tube and stopping when about 50uL remains. Repeat wash 1 more time. After second aspiration, add 200uL of vFC staining buffer to tube and resuspend beads by vortexing for 10 secs.

Appendix B. Protocol B3. vFRed™-stained Lipo100™

Objective

Protocol B2 calibrates the relationship between vesicle membrane surface area and vFRed™ fluorescence using Lipo100™, a synthetic vesicle size standard whose size distribution has been determined using orthogonal methods, including nanoparticle tracking analysis (NTA) and resistive pulse sensing (RPS). Lipo100™ will be stained with vFRed™, measured on the flow cytometer, and linear regression performed to determine the fluorescence brightness per unit surface area (F/nm2). These regression coefficients are used to estimate the size of individual EVs.

Materials

a. vFC™ Staining Buffer
b. Lipo100™ Standard
c. vFRed™ membrane stain
d. Microwell plate (Sartstedt 82.1583.001)
e. Gloves
f. vFC™ Vesicle Size Calibration Layout (FCS Express)

Procedure

Prepare Working Solution

1. Prepare vFRed™ membrane stain working solution (5 uL/well, +1 well)
    a. 25 ul/5 wells: 2.5 µL vFRed™ (100x) into 22.5 µL Staining Buffer.

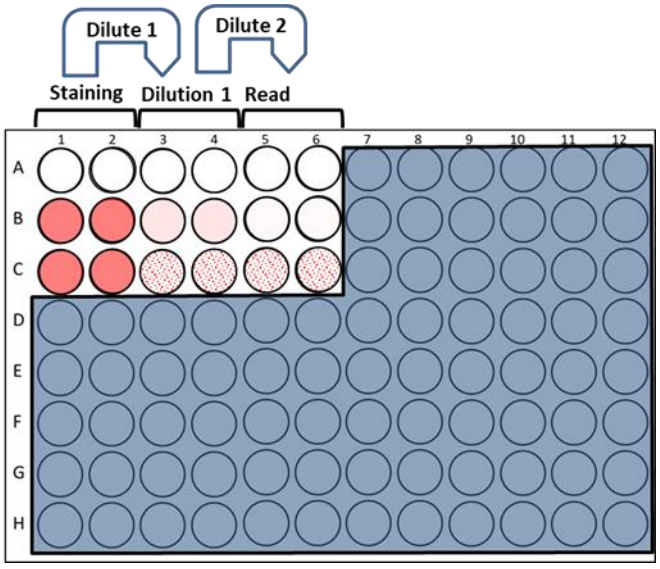
Prepare Samples

2. Prepare EV samples (Table 1 and Plate Map below):
    A. Buffer-only control
    B. Buffer +reagent control
    C. Lipo100™ vesicle size standard

Table 1.1 Staining reactions

Well	Buffer	Lipo100™	vFRed™	Total
A1,2	50	0	0	50 uL
B1,2	45	0	5	50 uL
C1,2	40	5	5	50 uL

3. Add 5 µL 10x vFRed™ to Rows B and C.
4. Mix well, incubate 1 hour at RT in the dark.



4. During the staining incubation, ready the flow cytometer to run load the vFC Assay data acquisition Template (prepared in Protocol A Instrument QC and Qualification).
5. If not previously done, set that optimal trigger threshold (**Appendix D**).

Dilute and Read

4. Dilute the staining reaction according to Table 1.2.
    a. Add the indicated amount of Staining Buffer to Columns 3-6
    b. Transfer the indicated amount of stained sample from Columns 1-2 to Columns 3-4, and mix well.
    c. Transfer the indicated amount of the Dilution 1 samples in Columns 3-4 to Columns 5-6, and mix well.
5. Run the wells in Columns 5-6 at the indicated sample flow rate for 120 seconds each.

Table 1.2 Post-stain dilution and run

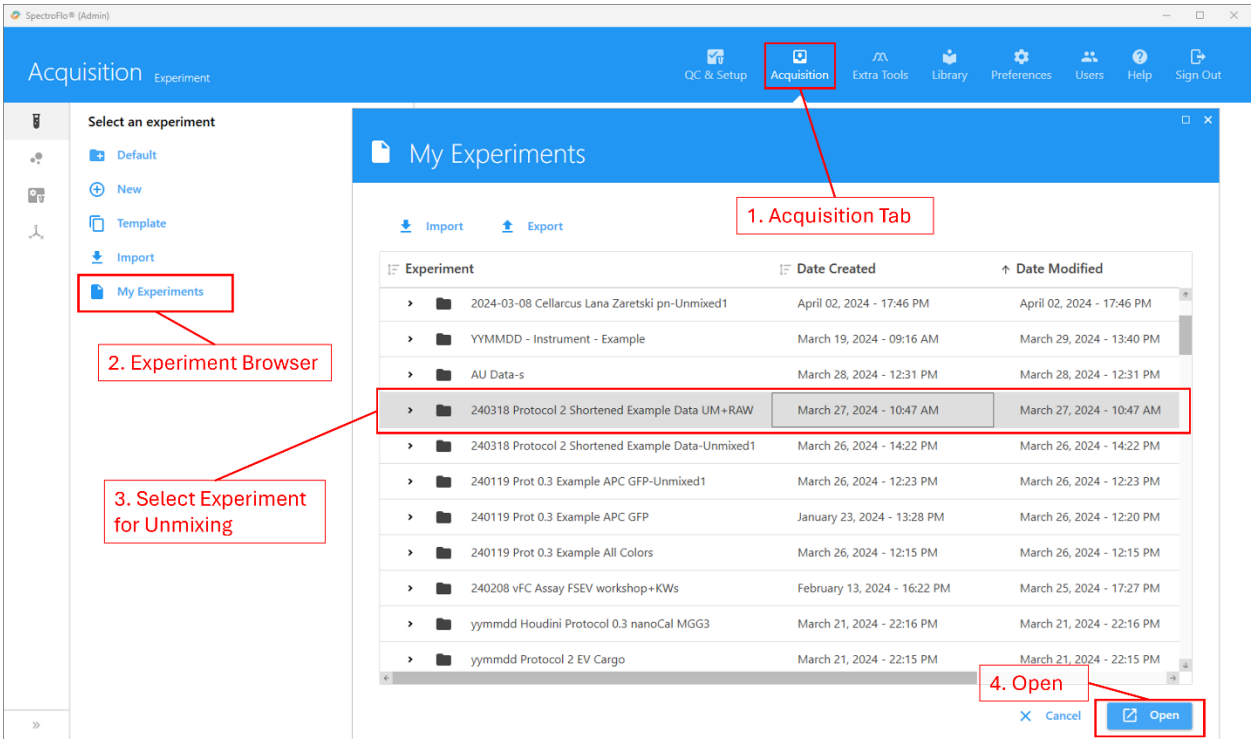
Wells	**Aurora**
Staining	50 uL
Dilution 1	8 µL → 245 uL
Dilution 2	8 µL → 245 uL
Post-stain Dilution	1000
Run	High 60 uL/min

Appendix C. Unmixing in SpectroFlo spectral mode (v3.2.1)

SpectroFlo spectral mode uses the proprietary unmixing matrices and algorithms of the SpectroFlo software and the appropriate single component controls, to generate new fcs files with parameters containing the estimated fluorochrome abundances, as described in the following sections. These new fcs files are then used for intensity calibration (**Protocol B**) and EV analysis (**Protocols 1 and 2**). You should have set up the fluorescent tags and added the reference group (see step 2a and step 2c on pages 2 to 3 above). These are needed for the unmixing.

a. Load Experiment then Start Unmixing

Make sure the experiment to be unmixed is loaded. If you have just acquired data on current worksheet you probably don't need to load anything. Otherwise load experiment to be unmixed.

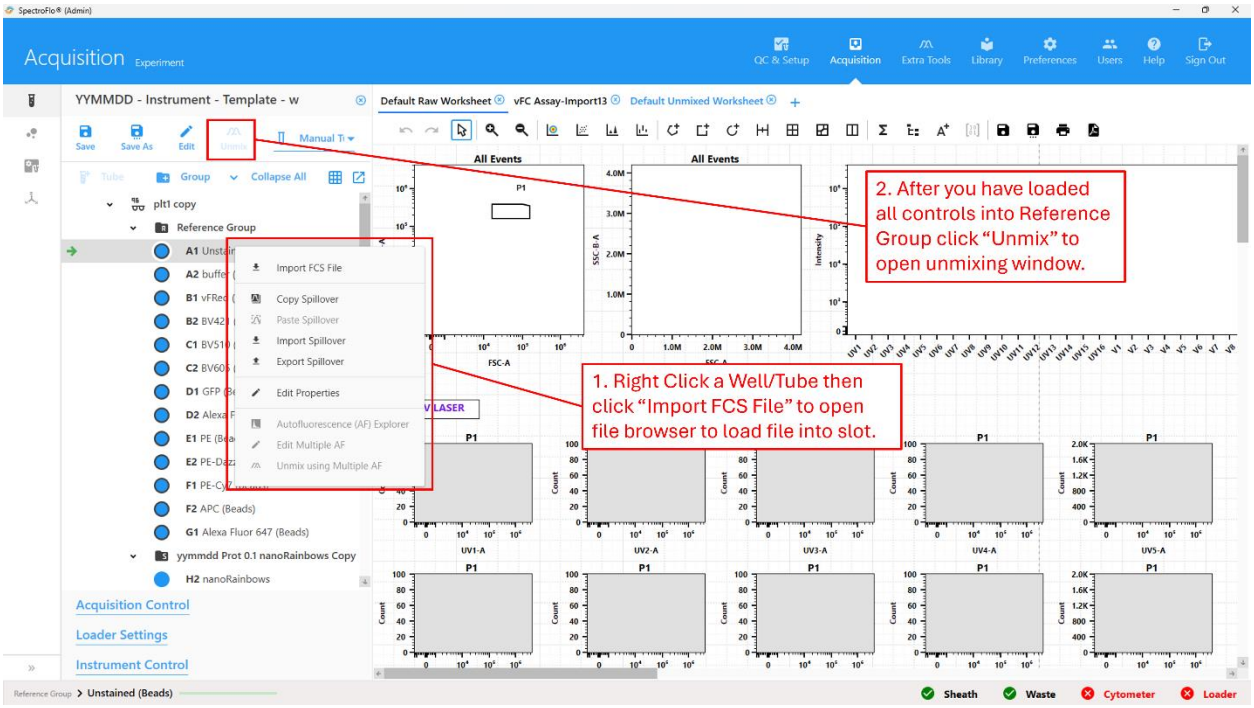


b. Load Reference Controls

In addition to the data you have collected there is a separate group called "Reference Group" which wells/tubes for each file needed for unmixing. To load a file into the "Reference Group" click on the well/tube to select it then right click on it again and choose "Import FCS File" to open file selection dialog.

Load the scatter buffer only into the buffer control well (or tube). Load a lipo100+vFRed only into the vFRed well (or tube). Load the stained bead controls into the appropriate well (or tube).

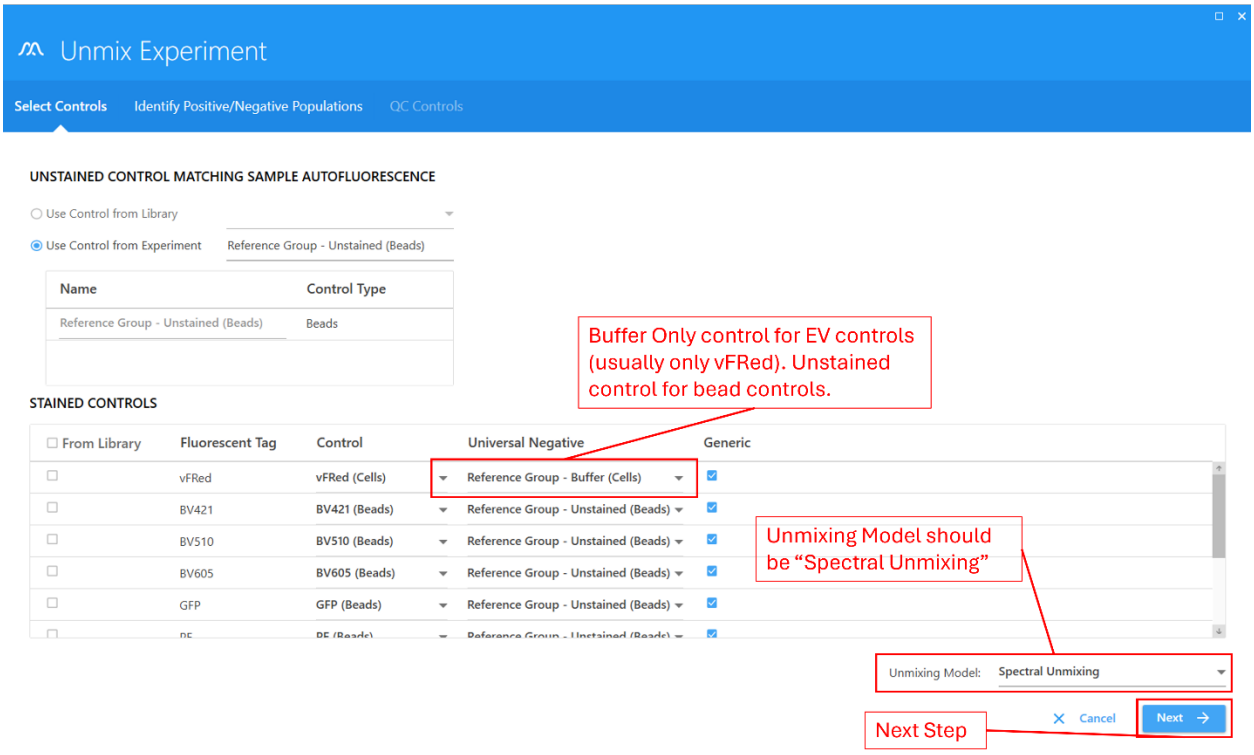
Click on the Unmix button (under acquisition tab).



c. Select Controls – Set Control Type and Assign Negative Controls

If you created the worksheet properly then you should see two negative controls, one for "Cells" (EVs) and one for Beads. Make sure the vFRed positive control has the "Cells" negative control EVs assigned to it. The rest of the positive controls should use the unstained bead as a negative.

Ensure Unmixing Model is "Spectral Unmixing" (not "Spectral Unmixing with AF Extraction").



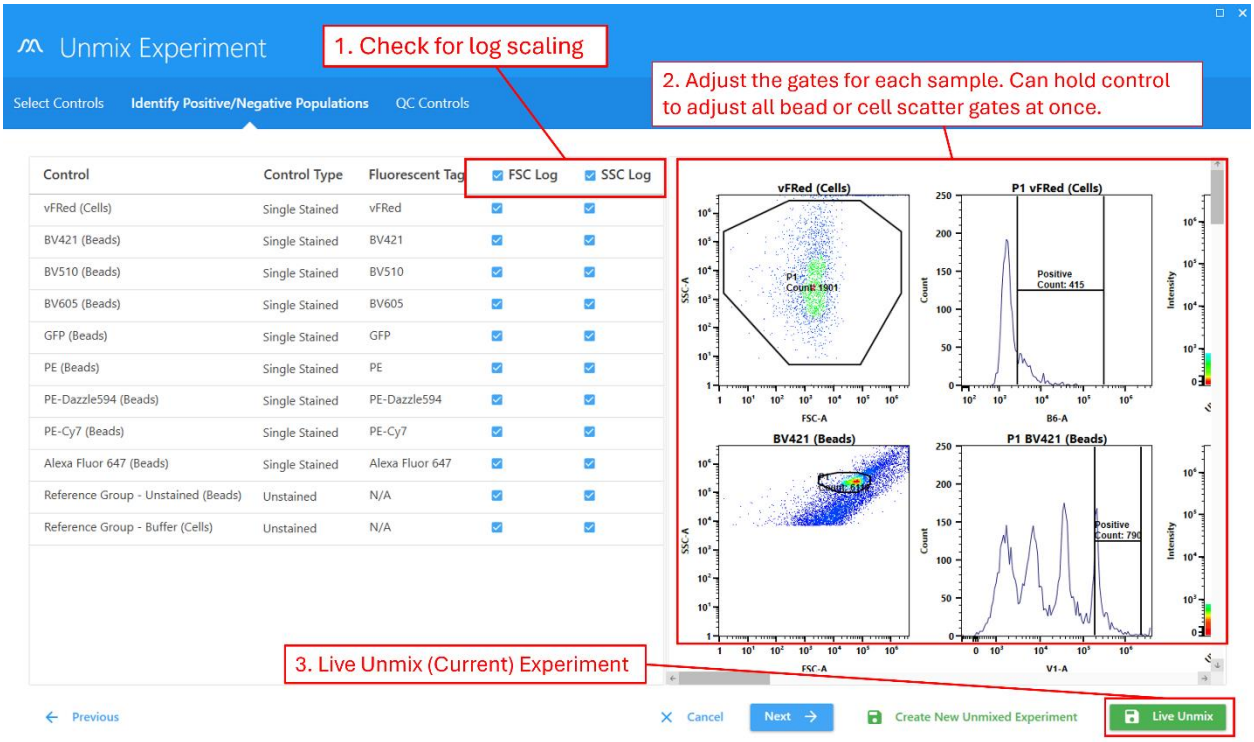
Click Next.

d. Identify Positive/Negative Populations – Adjust gates for each control

Adjust the bead scatter gates to include only singlets. If you hold down shift while adjusting the scatter gates for one bead it will apply that scatter gate to all beads.

Adjust the Cells (EV) scatter gate to include the main population. It helps to check the checkbox for log for these controls to switch to log scaling. This makes it easier to adjust the gates for dimmer particles.

Adjust the positive and negative gates. If possible, it is better to adjust positive gates to include only the brighter half of the positive peak. Similarly, it is better to adjust negative gates to include only the dimmer half of the negative peak. This improves Spectroflo's unmixing algorithm.



Click “Live Unmix.” Live unmixing can be done before collecting data to unmix as data is collected. Live Unmix can also be done after data is collected and in either case it includes both a raw and an unmixed copy of each data file. “Create New Unmixed Experiment” will create a new experiment with only unmixed data it will not however delete the old experiment which will still include the raw data. It is recommended that you use “Live Unmix.”

Other SpectroFlo Help

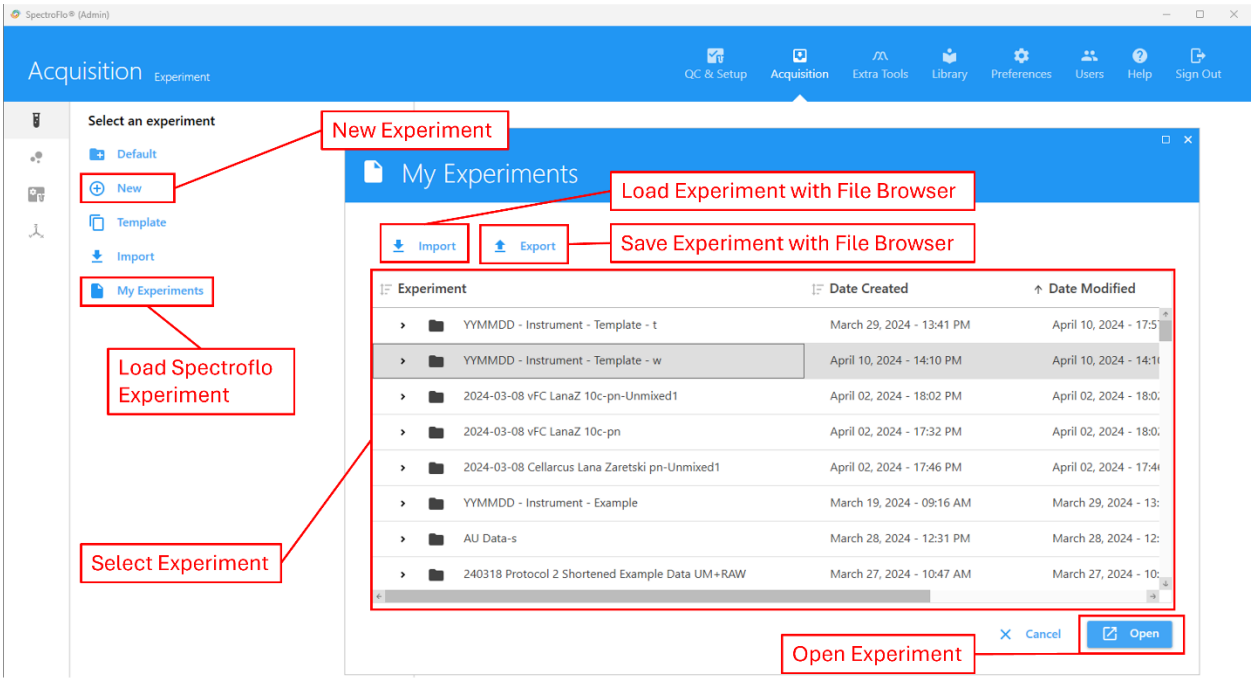
a. Export an experiment along with data files (any experiment)

Saved experiments are stored inside spectroflo and if you want to send the whole experiment to someone or save it in a folder somewhere then you will need to export it first.

Start with your experiment open in the acquisition tab. Click Save in upper left corner of the screen to save any changes to your experiment before exporting it.

Click the x on the left side of the screen (next to the experiment name) to close your current experiment and go back to the Select an experiment screen.

Go to “My Experiments” then select the saved experiment that you wish to export then click Export to choose a save location and name.



This will save an experiment as a compressed folder that contains the data files of the experiment as well as the various Spectroflo files (such as .xitm).

Appendix D. Setting the Trigger Channel Threshold

Purpose

This Protocol instructs on the setting of an optimal detection trigger threshold for vFRed-based EV detection.

Procedure

1. Load a well of vFC™ Staining Buffer and allow system to run for 15 seconds on high flow rate (60ul/min).
2. 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.
3. Save the new Settings file (.expt) for future use. In general, once optimized the threshold does not need to be changed every time you run an experiment.

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