Fluorescence Intensity Calibration and Spectral Compensation

Protocol B. Fluorescence Calibration and Compensation

Objective

Calibrate the intensity and spectral response of the instrument for relevant fluorescence channels to report intensity in absolute units of antibody binding capacity (ABC), vesicle size (diameter via mean equivalent surface area, MESA) and account for fluorescence spillover between channels.

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) or
  - 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
- Gloves
- Microwell plate (v-bottom: Sartstedt 82.1583.001, other plates or tubes may be suitable)
- vCal™ Bead data acquisition template (created during **Protocol A Instrument QC and Qualification**)
- Protocol B Fluorescence Calibration Layout for FCS Express Reader or FCS Express
- Protocol B Vesicle Size Calibration Layout for FCS Express Reader or FCS Express
- Protocol B Fluorescence Compensation Layout for FCS Express Reader or 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. Generate and validate the intensity calibration for:

2a. Immunofluorescence using nanoCal™ beads and

2b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.

3. Determine the spillover matrix and create and validate compensation.

B1. Data Acquisition

B1a. nanoCal™ Beads

1. Reconstitute pre-stained nanoCal™ beads in vFC buffer according to the instructions (typically 100 uL)
2. Place each nanoCal™ bead in a different well of a 96-well plate for flow cytometer measurement.
3. Fill one well with 300uL of vFC buffer without beads
4. Measure using the vCal Bead Template at the same fluorescence channel gains as for vFC™ analysis.
5. Measure the Buffer-only well to estimate the system background.
   On conventional flow cytometers, including the CytoFlex and Aurora, lower the FCS trigger channel threshold until the system is triggered by the background noise and then collect data file. On the CellStream and ImageStream, measure the buffer as you would measure beads.
6. Save data files with informative names that include the data and antibody conjugate used to stain the bead (eg. 20210704 nanoCal CD9 PE.fcs, 20210704 Buffer-only.fcs, etc).

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. Data Analysis – Fluorescence Calibration

B2a. Data Analysis – Immunofluorescence Calibration

1. Open the vFC™ Fluorescence Calibration analysis layout using FCS Express or FCS Express Reader and load the appropriate data files into each plot.Use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate the population of single beads (**Figure 1**).
2. 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.

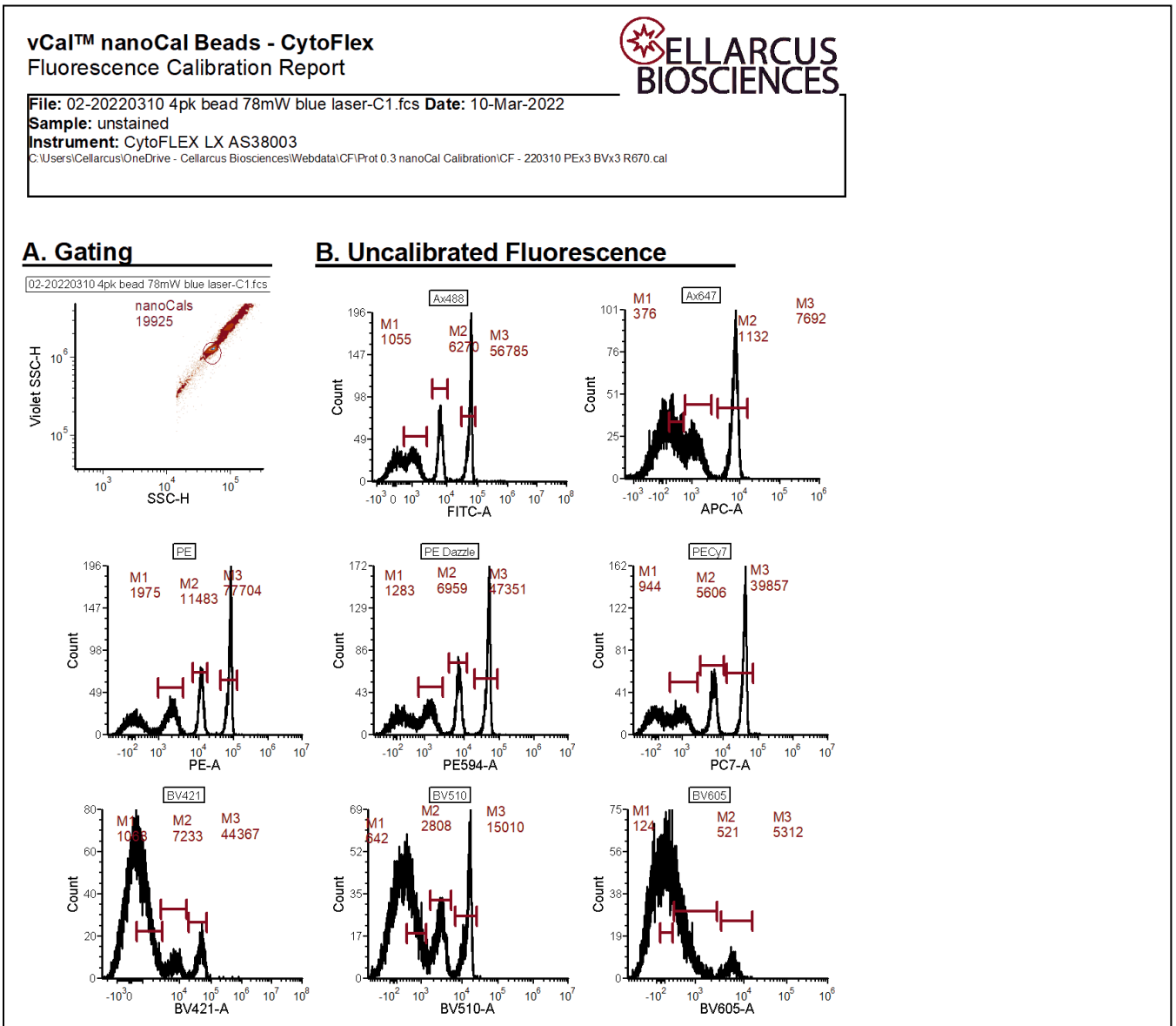


Figure 1. Fluorescence calibration using nanoCal™ beads. A. Single beads are gated by their light scatter. B. Individual peaks on each channel are gated and the median fluorescence intensity (MFI) values are used in the Channel Calibration dialogue in the Fluorescnce Calibratio Layout.

3. Use the nanoCal™ Bead ABC 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 2**).

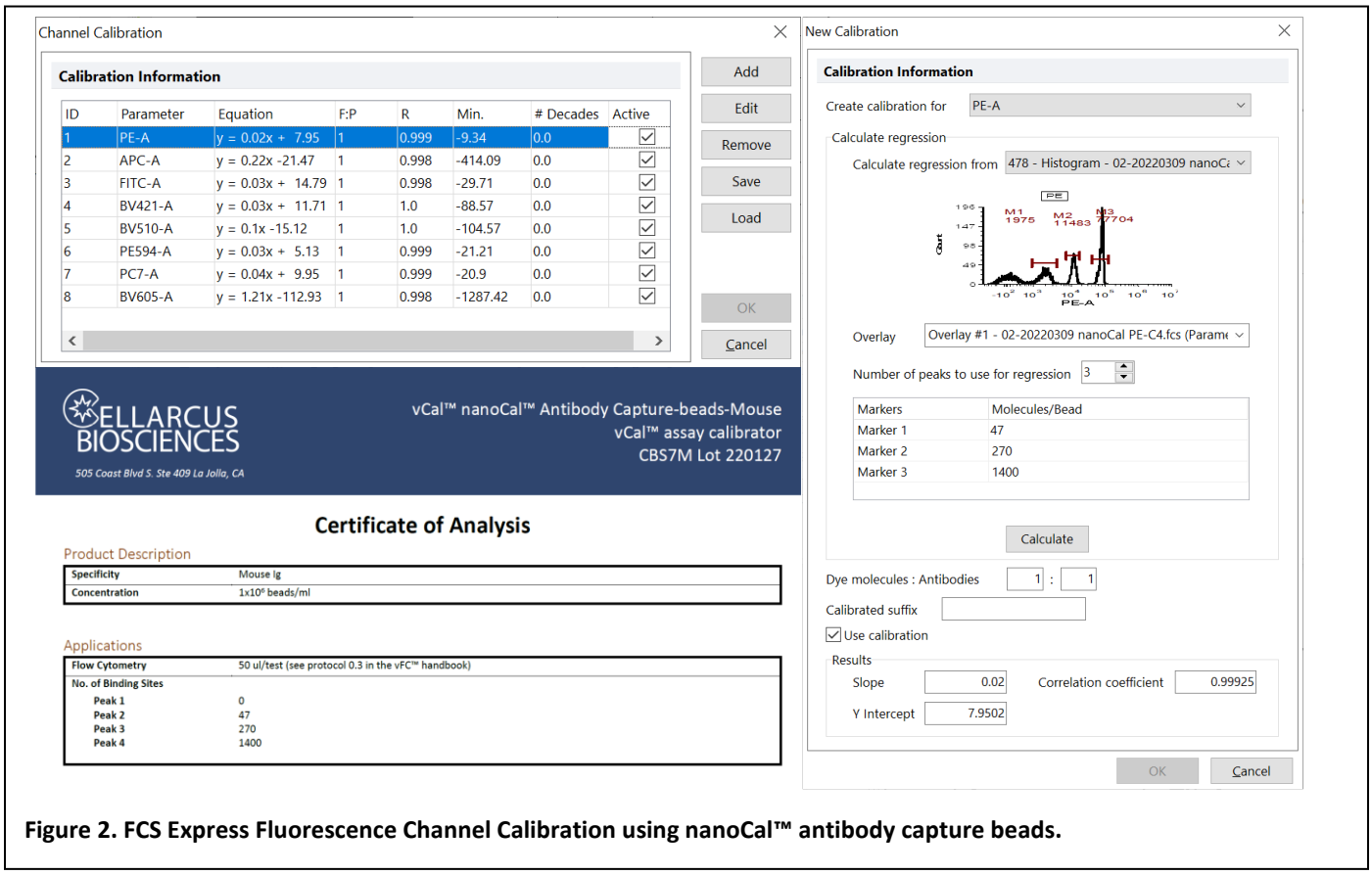


Figure 2. FCS Express Fluorescence Channel Calibration using nanoCal™ antibody capture beads.

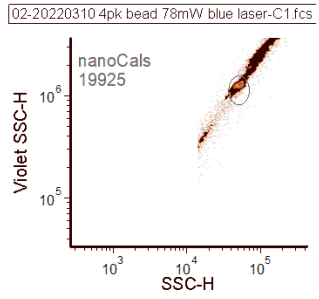
4. Save the Channel Calibration file: Fluor Calibration - Instrument - YYMMDD – Panel (eg. Fluor Calibration - CytoFlex - 220310 – PEx3 BVx3 APC FITC.cal)
5. Load the Channel Calibration file (so its name will appear in the File Information text box) and inspect the recovery of standards on the nanoCal - cal tab (**Figure 3**).

vCal™ nanoCal Beads - CytoFlex
Fluorescence Calibration Report

CELLARCUS BIOSCIENCES

File: 02-20220310 4pk bead 78mW blue laser-C1.fcs **Date:** 10-Mar-2022
Sample: unstained
Instrument: CytoFLEX LX AS38003
C:\Users\Cellarcus\OneDrive - Cellarcus Biosciences\Webdata\CF\Prot 0.3 nanoCal Calibration\CF - 220310 PEx3 BVx3 R670.cal

A. Gating



C. Calibrated Fluorescence

Parameter	Marker	Median	Arithmetic Mean	95%-ile
FITC-A	M1	18	17	32
FITC-A	M2	50	52	76
FITC-A	M3	215	218	277
FITC-A	M4	1826	1765	2083

Parameter	Marker	Median	Arithmetic Mean	95%-ile
APC-A	M1	-68	-79	-29
APC-A	M2	36	41	117
APC-A	M3	225	239	389
APC-A	M4	1649	1638	2049

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PE-A	M1	9	10	19
PE-A	M2	47	48	67
PE-A	M3	239	238	300
PE-A	M4	1575	1538	1798

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PE594-A	M1	8	8	20
PE594-A	M2	48	49	73
PE594-A	M3	238	239	298
PE594-A	M4	1586	1555	1834

Parameter	Marker	Median	Arithmetic Mean	95%-ile
PC7-A	M1	10	9	22
PC7-A	M2	46	47	75
PC7-A	M3	235	237	310
PC7-A	M4	1612	1585	1895

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV421-A	M1	10	7	26
BV421-A	M2	48	53	97
BV421-A	M3	280	291	449
BV421-A	M4	1528	1541	2133

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV510-A	M1	-13	-17	6
BV510-A	M2	40	46	93
BV510-A	M3	251	252	385
BV510-A	M4	1447	1387	1786

Parameter	Marker	Median	Arithmetic Mean	95%-ile
BV605-A	M1	-54	-64	424
BV605-A	M2	309	353	782
BV605-A	M3	693	728	1046
BV605-A	M4	6177	6280	9102

Figure 3. 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.

B2b. Data Analysis - Vesicle Size 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**).

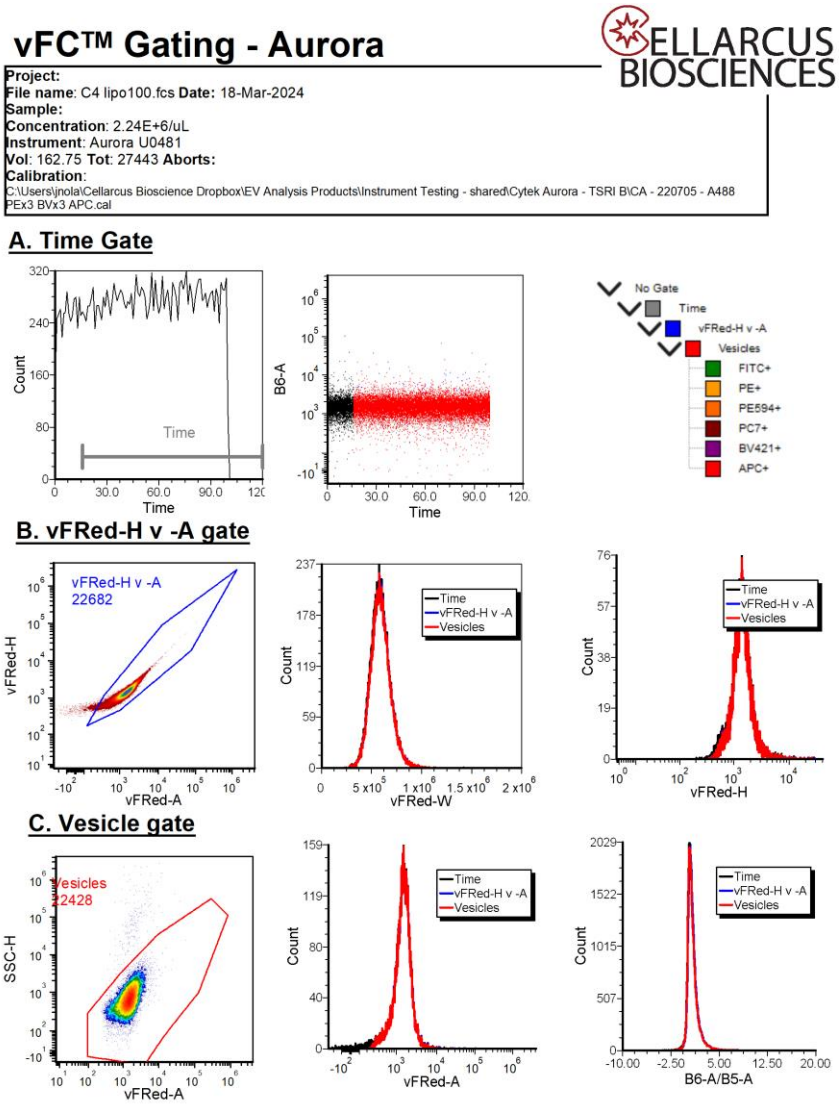


Figure 1. Vesicle Gating Tab.

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.
5. Open Transformations (Tools>Transformations) then select the Vesicle Size Calibration parameter math Transformation (**Figure 3**).

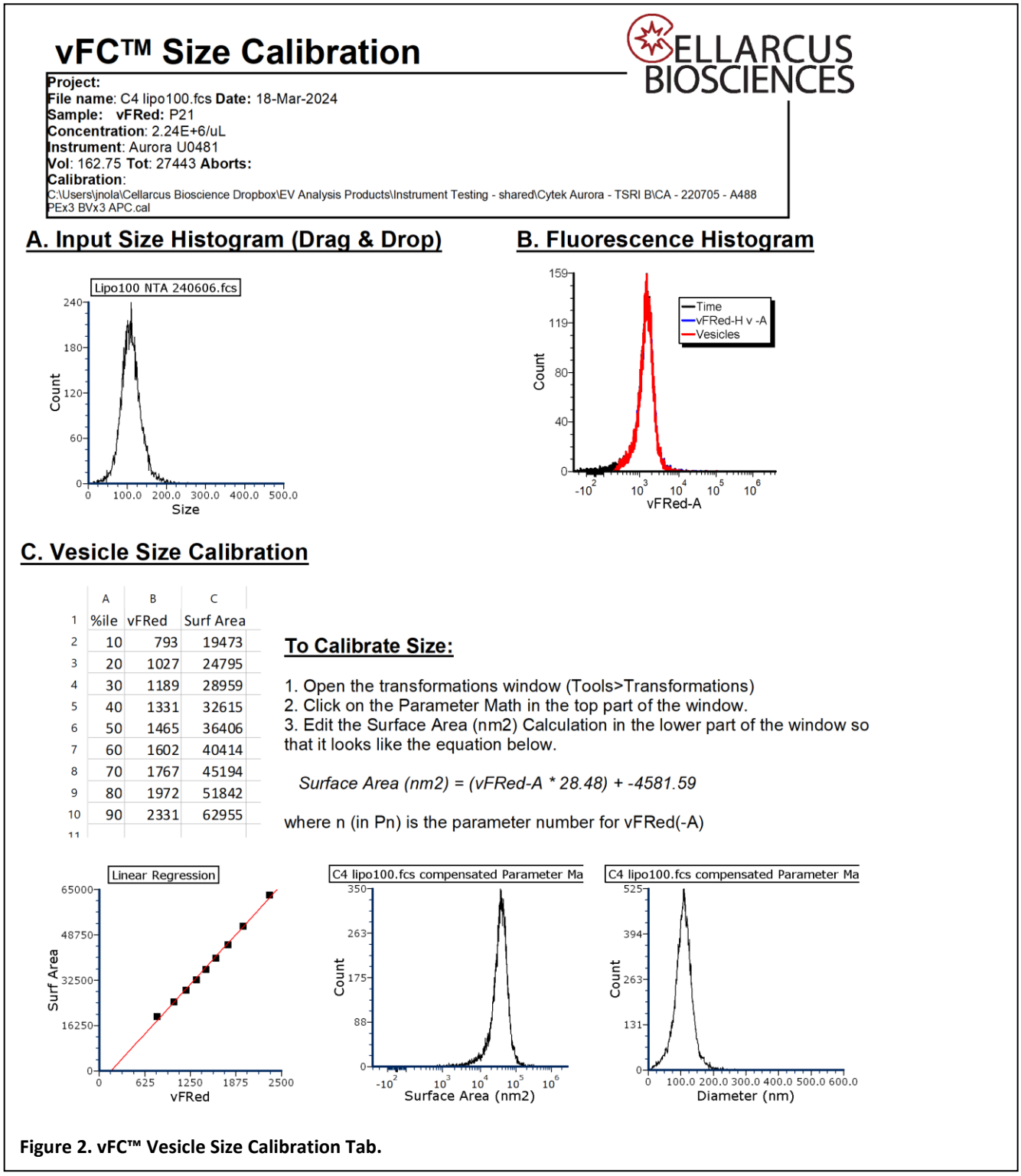


Figure 2. vFC™ Vesicle Size Calibration Tab.

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.”

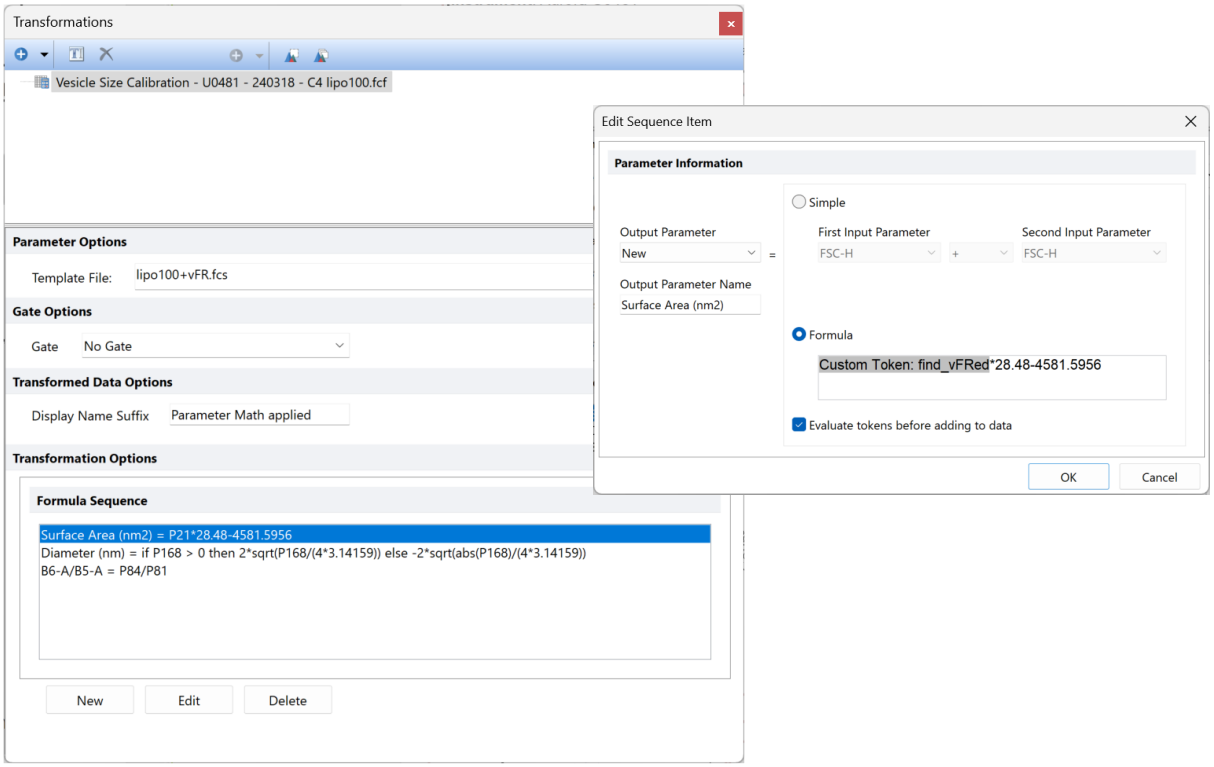


Figure 3. Transformations and Parameter Math Dialogue.

B3. Data Analysis – Fluorescence Spectral Compensation

This step creates the spectral spillover and compensation matrices using the stained nanoCal™ Beads and scatter-triggered buffer data, together with the vFRed-stained Lipo100 data collected in step B2, or as part of Protocols 1 or 2).

Procedure

1. Open the vFC™ Fluorescence Compensation data analysis Layout using FCS Express or FCS Express Reader
2. Load the data files from the stained nanoCal beads (plus the unstained bead control) into the Data List. Load the data files from vesicle spectral reference standards (vFRed-Lipo100 and plus buffer-only).
3. On the **nanoCal Tab,** use the bivariate plot of scatter parameters to identify and gate on the population of single beads. Load the single-color bead data into the appropriate individual histograms. Inspect the positive and negative populations in each histogram.
4. On the Gating Tab, load vFRed-Lipo 100 data into the appropriate histograms and inspect the data
5. Click on the **Tools** tab→**Compensation and Unmixing** command to open the **Compensation and Unmixing** navigator (Figure 1).
6. The **Compensation and Unmixing** navigator can be moved anywhere on the screen; it is dockable and pinnable, and you can move it around the screen or close and reopen as needed.
7. Click on the blue plus button,  , to **Add a new compensation** (Figure 1, red outline).
8. A text box highlighted in blue will appear with the text **New Compensation** (Figure 1).

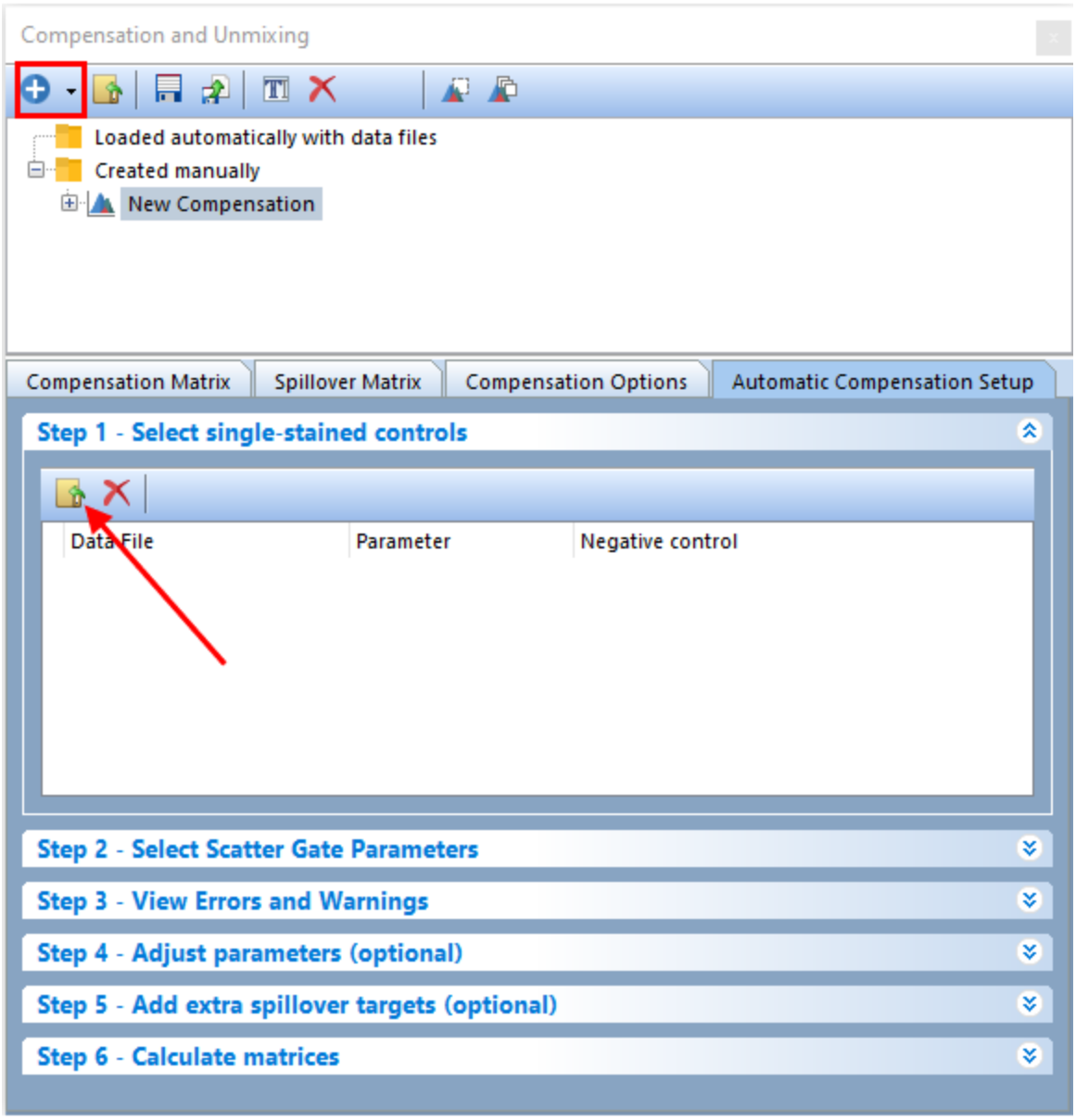
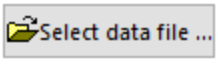


Figure 1. Compensation and Unmixing navigator: use to add new compensations, edit definitions, create Automatic Compensations, and unmix spectral signatures

9. Click on the Add data for compensation button,  , in the **Automatic Compensation Setup→Step 1 - Select single-stained controls** window (Figure 1, red arrow).
10. The **Standard Open Data Dialog** will appear (Figure 2).
11. Note: based on your User Options, the Advanced Open Data Dialog may appear. If the **Advanced Open Data dialog** appears, please select the 

button to access the **Standard Open Data Dialog**.
12. Select and load the single stain and negative control data files appropriate for your instrument (**Table 1**).
13. Click **Open file**.

14. Check that all of the single-stained control files and the unstained control file have been brought into the **Select single-stained control** window. In some cases, the **Parameters** will need to be set manually. Assign each single-stained control the appropriate **Negative Control (Table 1)**.

Table 1. Compensation Setup

Data File	Parameter	Negative control
Buffer	Negative control	
Lipo100	vFRed-A	Buffer-only
Bead-FL1	FL1	Use self as negative or blank
Bead-FL2	FL2	Use self as negative or blank
Bead-FL3	FL3	Use self as negative or blank
Bead-FLn	FLn-A	Use self as negative or blank

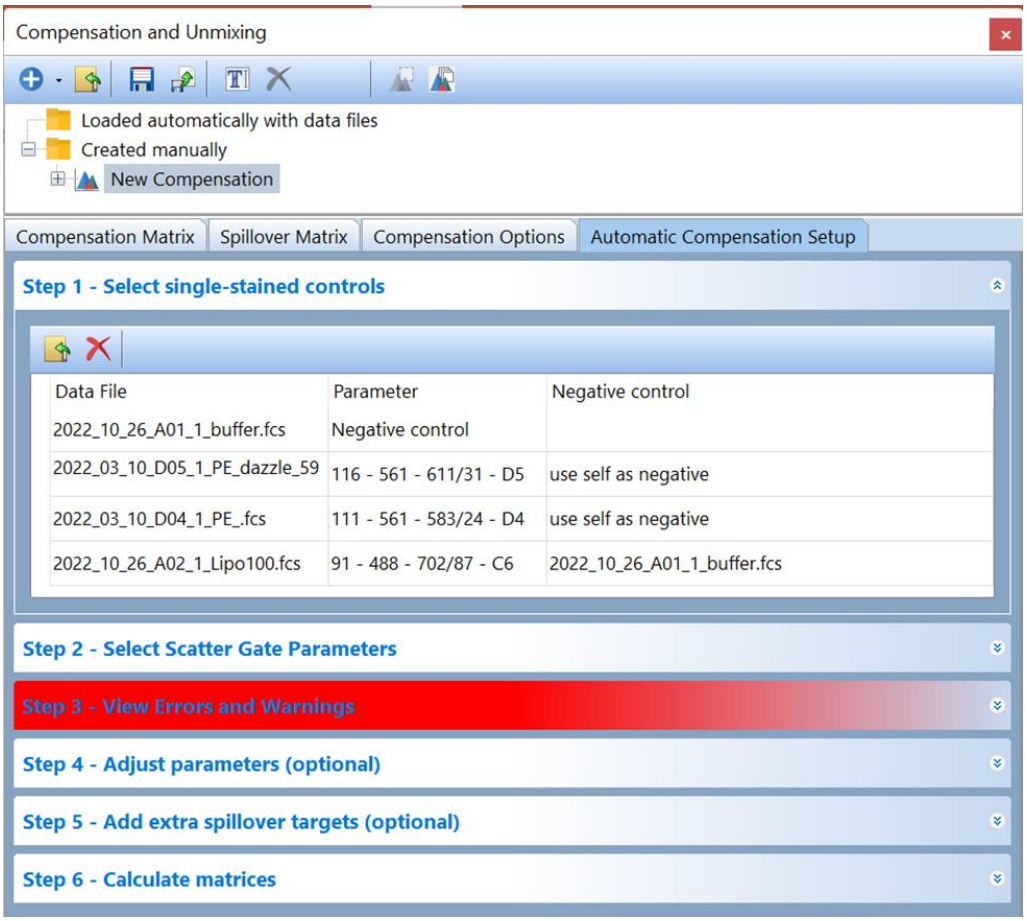


Figure 2. Files for controls have been added to Step 1 in the Automatic Compensation Setup and are typically matched automatically to their respective parameters. Note: For some instruments this may need to be done manually (See instruments specific protocol 0).

15. Set the Scatter Gate Parameters (Step 2) to SSC-H and VSSC-H (Figure 3). FCS Express may regenerate charts each time something changes but this shouldn't affect the compensation results.
    a. CytoFlex: SSC-H vs VSSC-H
    b. CellStream: Raw Max Pixel_SSC – 773/56 – A1 vs SSC – 773/56 – A1
    c. ImageStream: Raw_Max_Pixel_MC_CH12 vs Intensity_MC_CH12

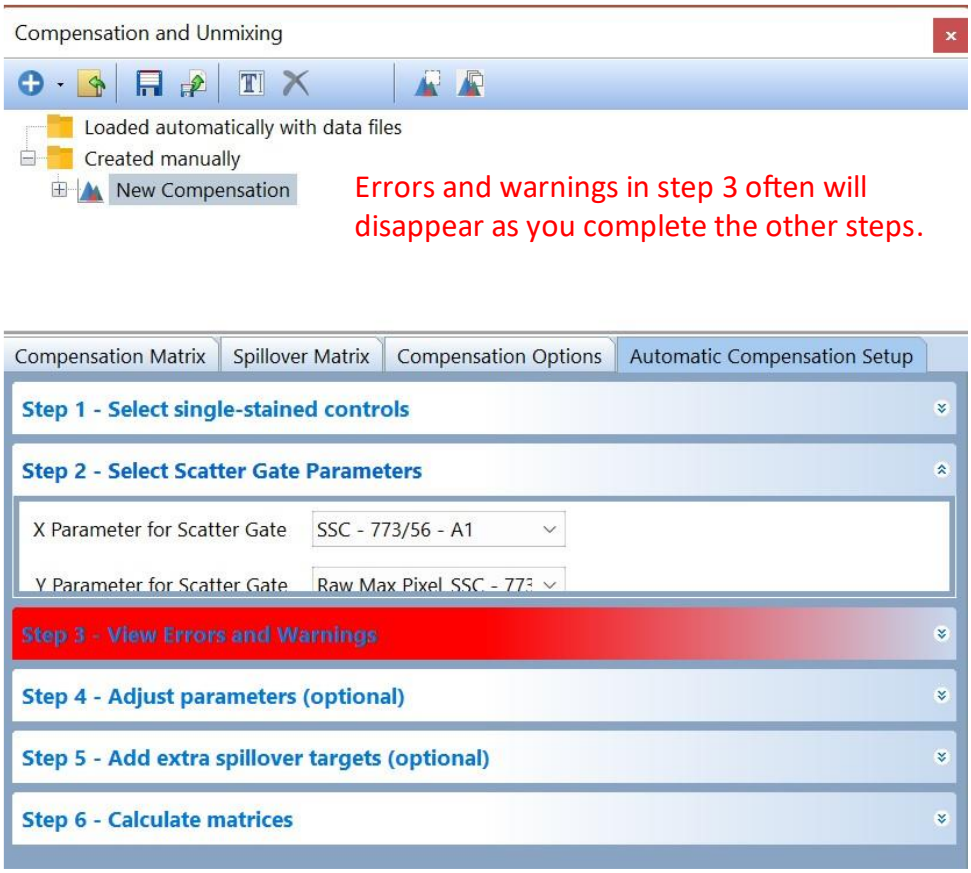
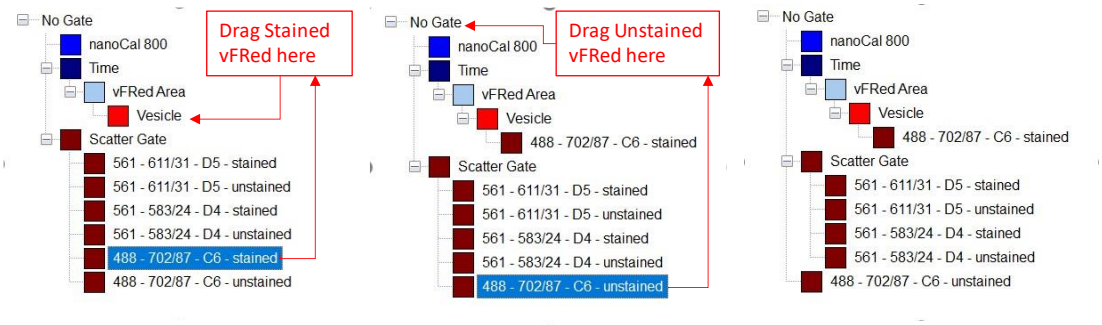


Figure 3. Select scatter gate parameters

16. Once Step 1 and Step 2 of the FCS Express Compensation wizard are completed, charts and gates will be automatically generated for the compensation.
17. Step 3 View Errors and Warnings – Don't worry about this step until the end. The errors should be resolved by the end of the protocol.
18. In the gate hierarchy (on gating tab) adjust so that the positive and negative control for vFRed are not gated (See diagram below).



19. Inspect the plots on the newly created pages (Figure 4). Inspect and adjust the Scatter Gate to select the population of single stained beads. For the antibody-stained beads, confirm that the positive and negative populations are correctly identified. Adjust positive and negative gates/markers as needed. The software should identify the brightest of the four peaks in the nanoCal bead set. The vFRed control charts will need to have their

gate adjusted as shown below (Figure 4). The chart for the stained vFRed control should be set to use the vesicle gate while the chart for unstained vFRed should be set to no gate.

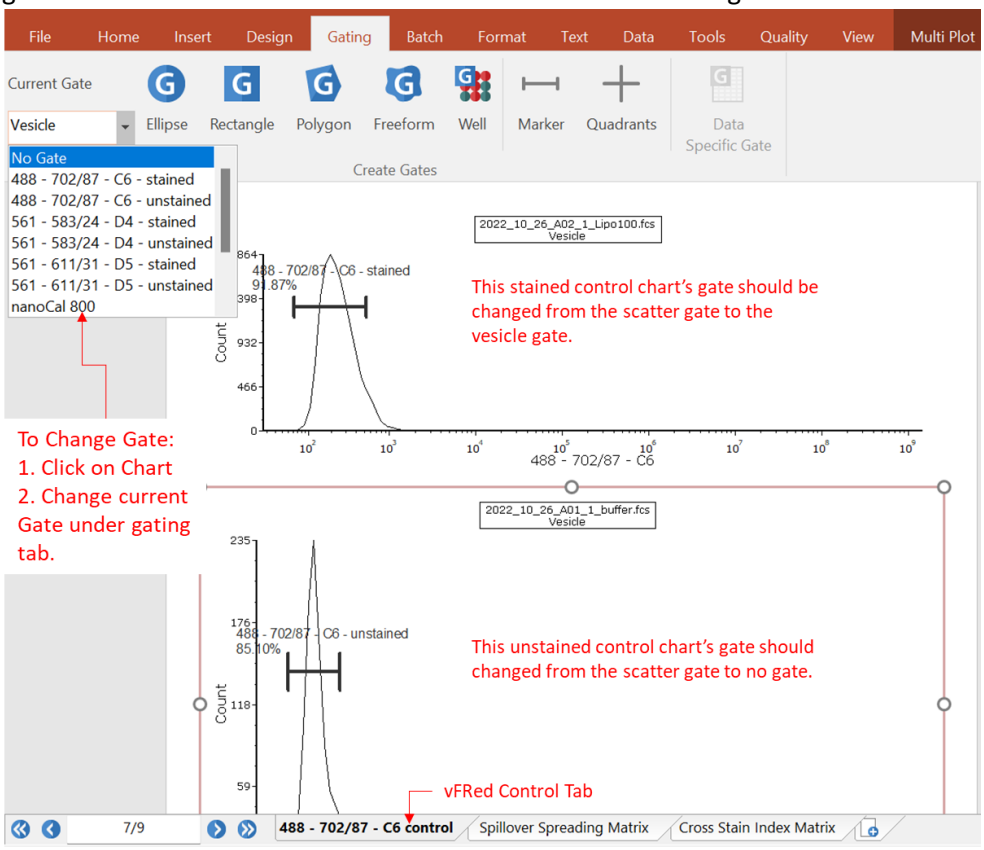
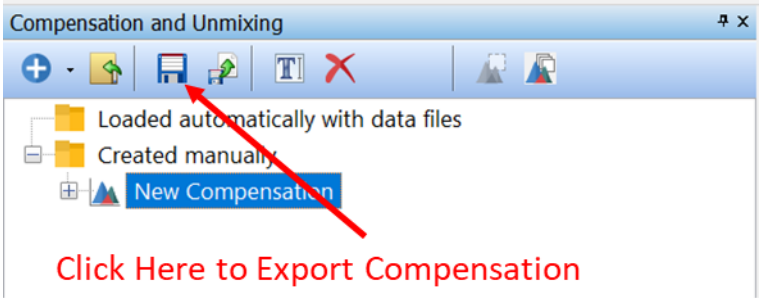


Figure 4. Adjust markers and gates on generated compensation plots (vFRed charts shown).

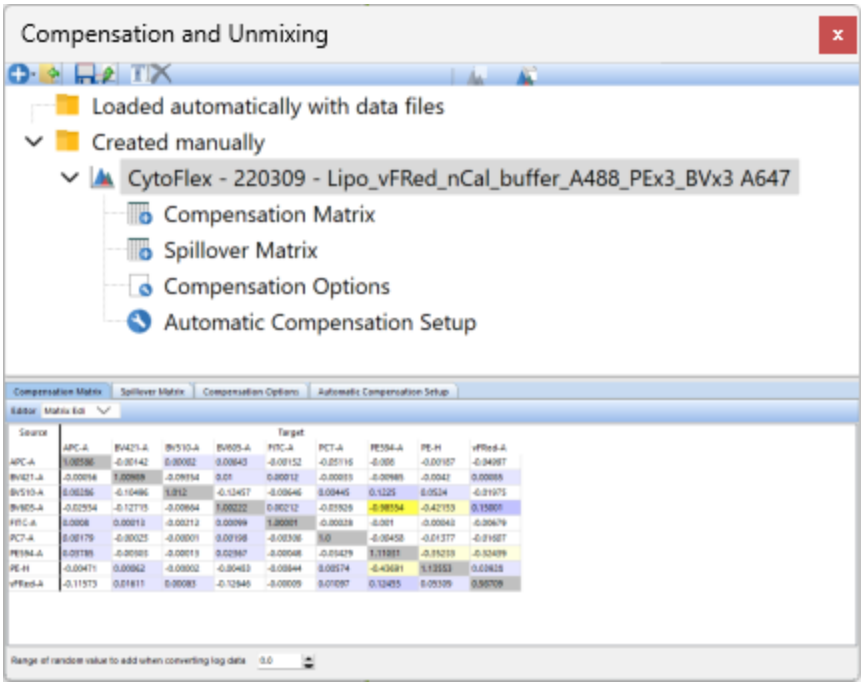
20. Click Calculate Matrices (Step 6) using the median fluorescence intensity values. The Spillover and Compensation matrices will be calculated (Figure 5).

Figure 5. The calculated Compensation matrix.

21. Export the compensation matrices as a **.compensation* file with an informative name (eg. CytoFlex - 220309 – buffer lipo100+vfred PEx3 BVx3 B525 R670.compensation", describing the particles and fluorophores used).



22. Copy the name of this file, and rename the New Compensation (right click, F2, Rename) by pasting.



23. Save the Layout with a similarly informative name (eg "vFC Protocol 0.4d Compensation - CytoFlex - 220309 – buffer lipo100+vFRed PEx3 BVx3 B525 R670.fey") as documentation of the calculations that produced the Spillover and Compensation matrices.

Notes

For more information about using FCS Express for Compensation and Spectral Unmixing, see the FCS Express manual, Tutorials, and videos at denovosoftware.com.

Developed, Manufactured, and Distributed By
Cellarcus Biosciences, Inc.
Telephone: +1 (858) 239-2100
Customer Care: cellarcus@cellarcus.com
Technical Support: technical@cellarcus.com

Appendix A. Staining nanoCal™ Antibody Capture Beads

Cellarcus nanoCal™ Antibody Capture Beads have calibrated antibody binding capacities that can be stained with fluorescent antibody conjugates of interest and used to calibrate the intensity and spectral responses of an instrument. Cellarcus offers pre-stained, freeze-dried nanoCal™ beads, as well as unstained for use with 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 nanoCal™ Antibody 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 B2. 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.

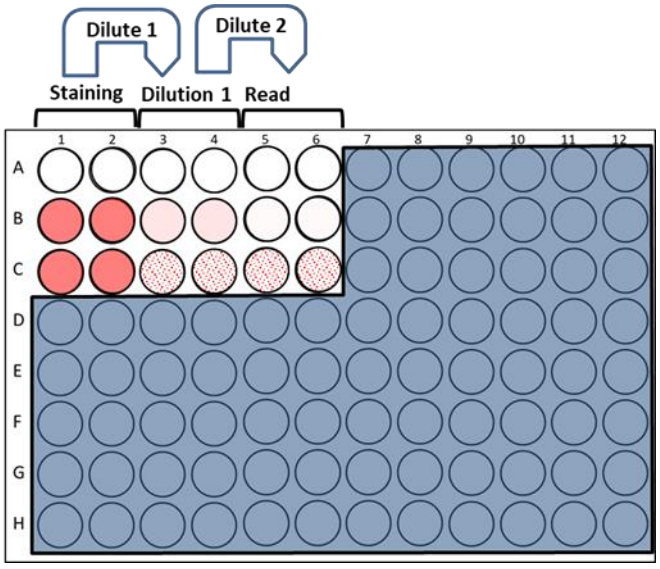
Prepare Samples

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 C**).

Dilute and Read

6. 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.
7. 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	**CytoFlex**	**CellStream**	**ImageStream**
Staining	50 uL	50 uL	50 uL
Dilution 1	8 µL → 245 uL	8 µL → 105 uL	5 ul→ 145 uL
Dilution 2	8 µL → 245 uL	8 µL → 105 uL	None
Post-stain Dilution	1000	200	30
Run	High 60 uL/min	Slow 3.7 uL/min	Slow x.x uL/min

Appendix C. 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.

Developed, Manufactured, and Distributed By
Cellarcus Biosciences, Inc.
Telephone: +1 (858) 239-2100
Customer Care: cellarcus@cellarcus.com
Technical Support: technical@cellarcus.com