Protocol B2. vFC™ Vesicle Size Calibration using 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™ Report Layout (FCS Express)
g. vFC™ Vesicle Size Calibration Tool (Chrome)

Procedure

Prepare Working Solution

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

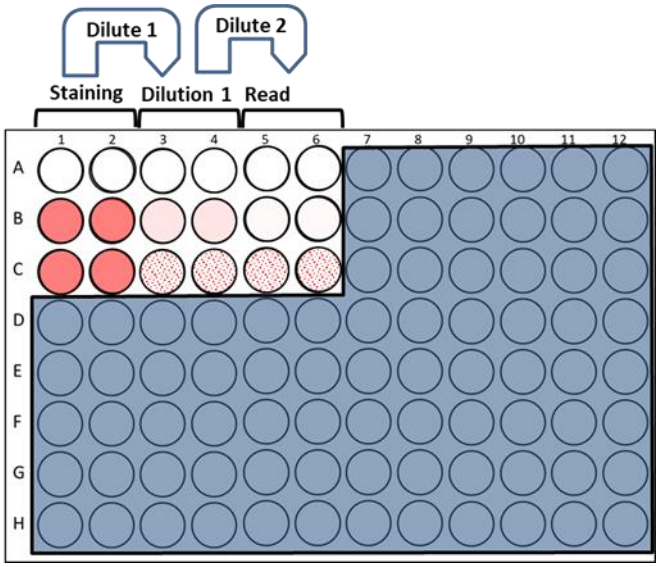
Prepare Samples

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 A).

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

Data Analysis

1. 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**).

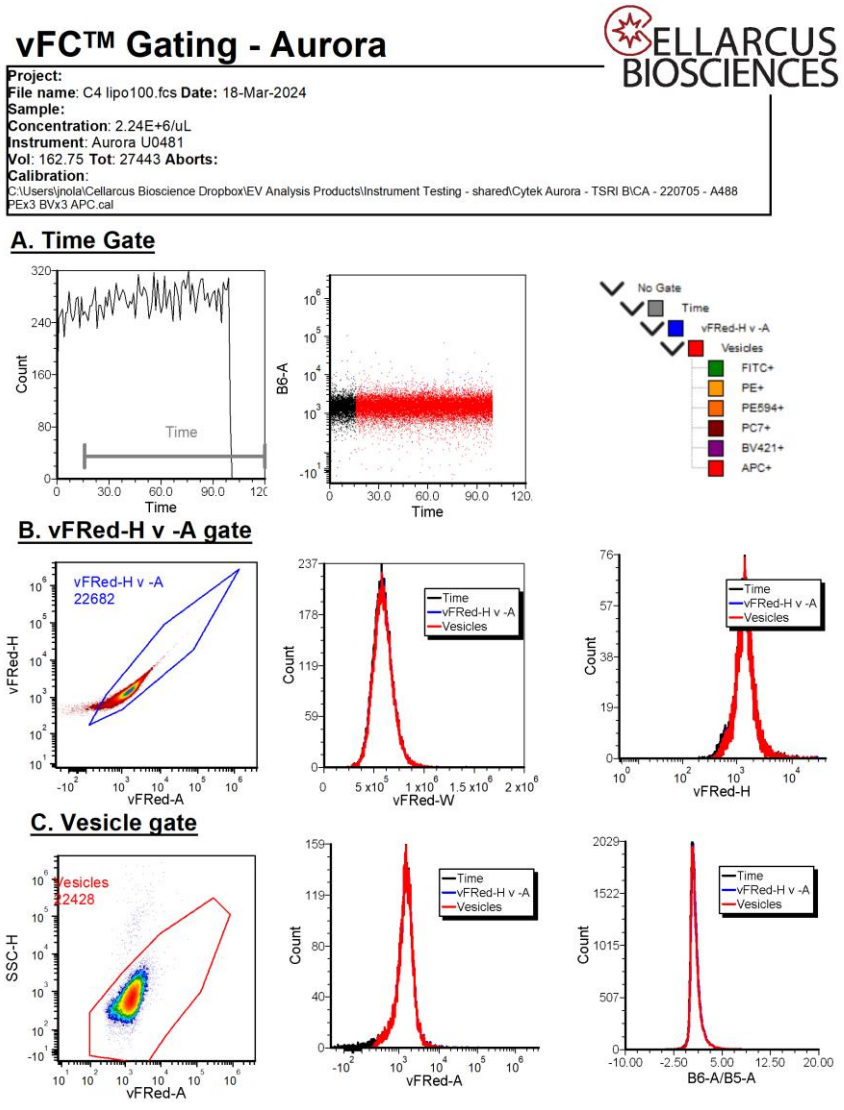
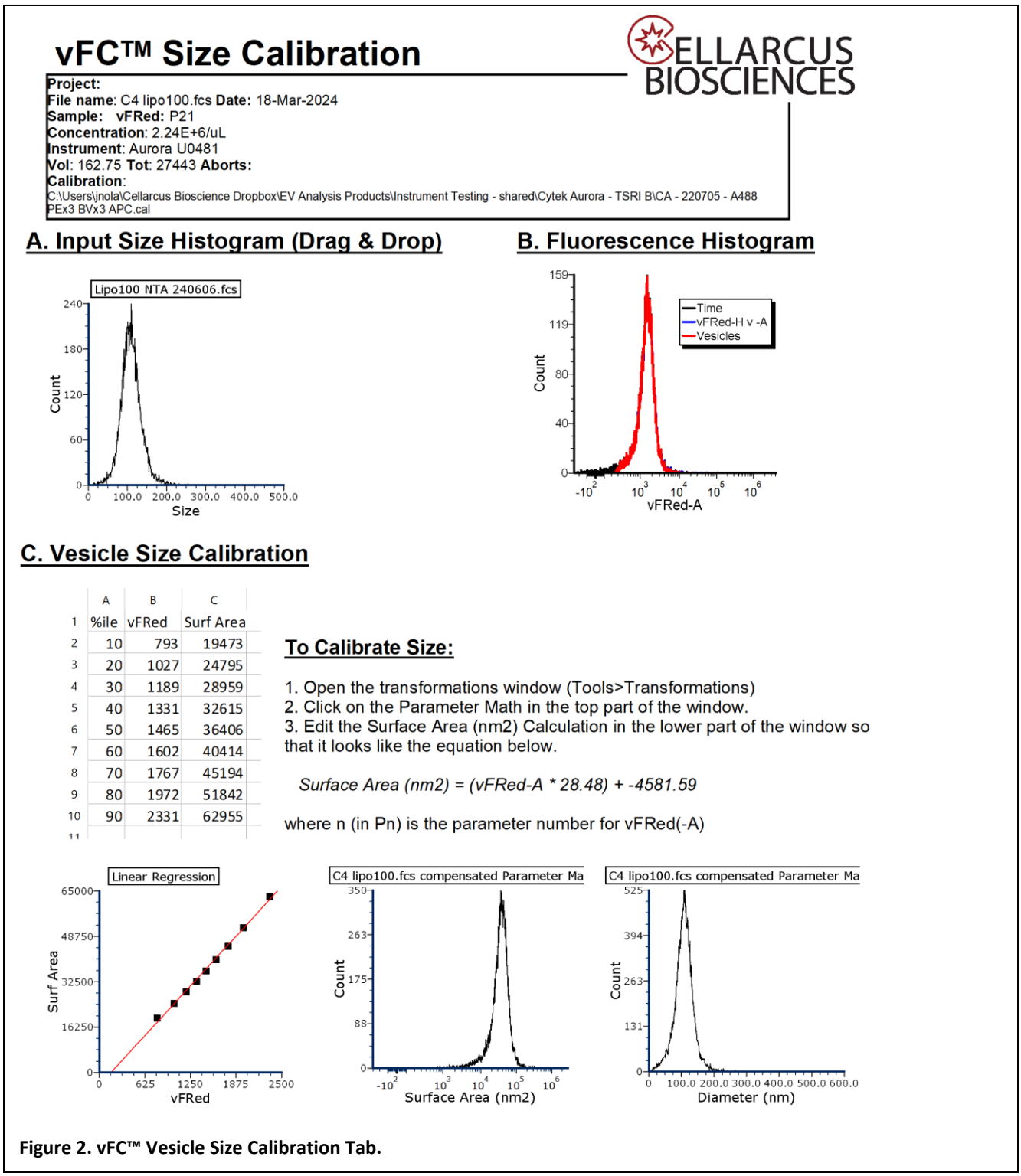


Figure 1. Vesicle Gating Tab.

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.



	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

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 – CytoFLEX – 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 – CytoFLEX – 220401 – lipo100 B9.fey.”

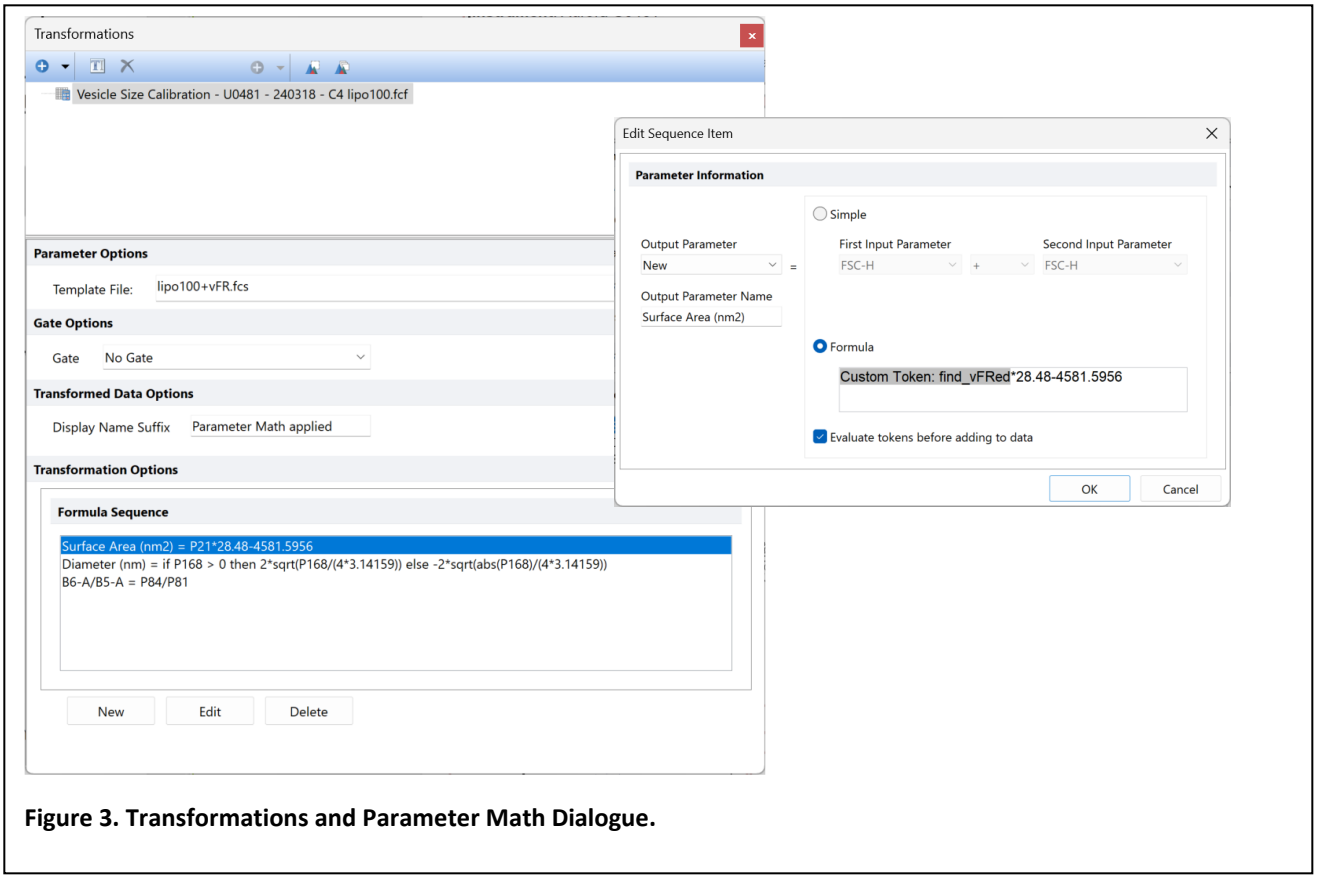


Figure 3. Transformations and Parameter Math Dialogue.

Notes

Appendix A. Setting the Trigger Channel Threshold

Purpose

This Protocol instructs on the setting of an optimal detection trigger threshold for vFRed-base 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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