Protocol 0.1 Instrument QC and Calibration with nanoRainbow Beads

Purpose

Assess laser alignment and fluorescence resolution and calibrate instrument flow rate and fluorescence intensity axes.

Materials

Kit Component	Size		Store
vCal™ nanoRainbow beads	2.5 mL		4°C
vCal™ nRB Report layout			

Materials to be provided by User

Gloves

Microwell plate (Sartstedt 82.1583.001)

Pipettes (5 uL – 300 uL)

Pipette tips

FCS Express Reader software (FCS Express Reader) –

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

Procedure

1. Create a New Experiment and Load the vCal Bead Settings_YYMMDD.ist file created during Instrument Set-up.
2. Define save file name as YYMMDD_*Cytometername*_ nanoRainbow.
3. Vortex nanoRainbow beads well.
4. Place two drops (~100 uL) undiluted nanoRainbow beads in a well and record for 30 seconds at the sample flow rate used for vFC™.

Analysis

1. Open the **vCal™ Bead Layout** in FCS Express and load the nanoRainbow Bead data file.
2. On the **nRB Report Tab**, inspect the time history and adjust the nanoRainbow gate on the population of single nanoRainbow Beads.

A. Laser Alignment and Fluorescence Resolution

1. Inspect the fluorescence histograms of a representative channel from each laser and adjust the markers to select each of the four bead populations.
2. Laser alignment is assessed by inspecting the CV of the bright bead population, which should be <8%. If CVs are higher, or there is an apparent leftward shoulder to the peak, alignment on that laser may be sub-optimal.
3. 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

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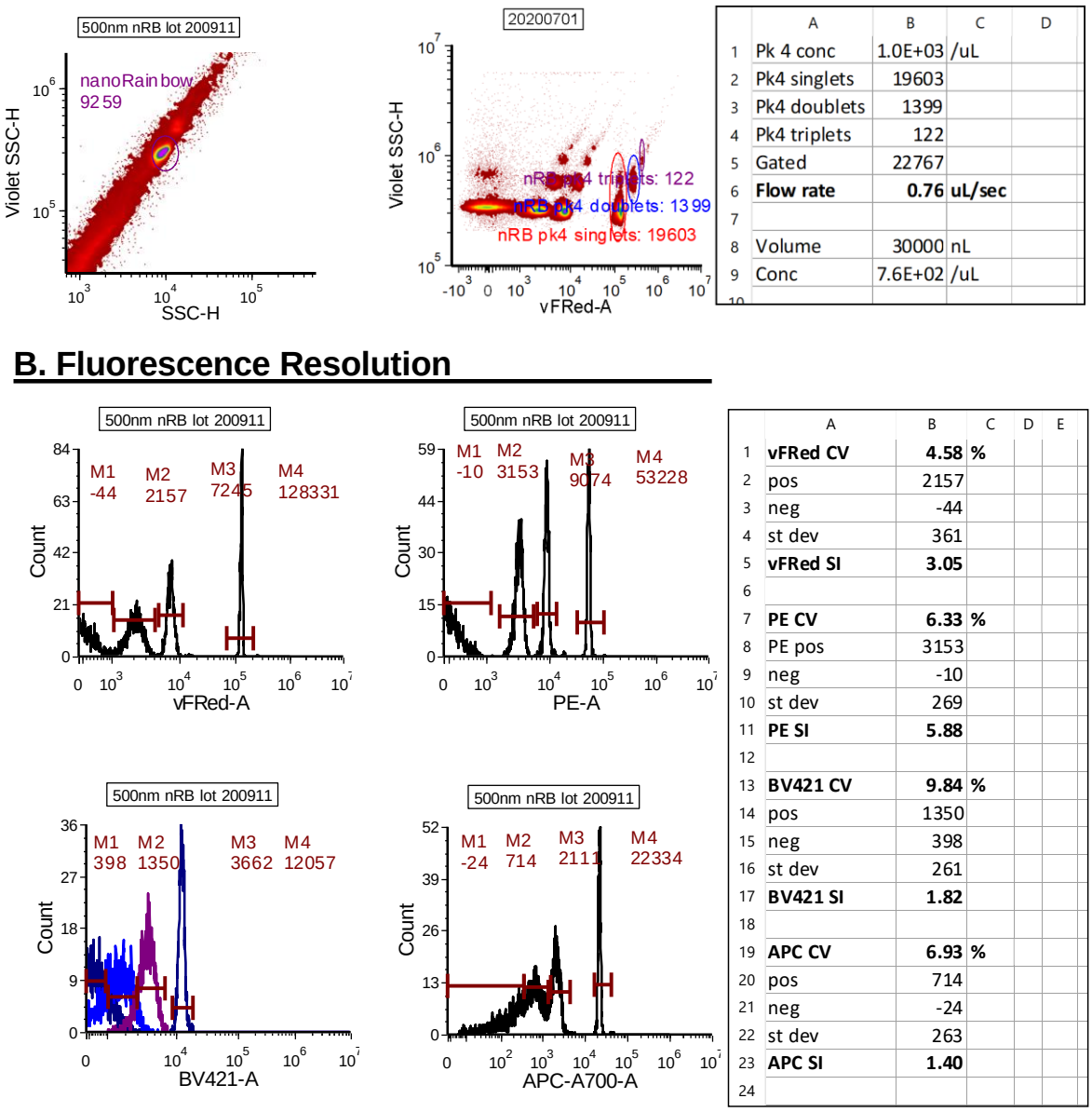
metric is useful for an initial analysis of instrument performance, and the vFRed™ SI should be >3.0 to ensure efficient EV detection.



	A	B	C	D
1	Pk 4 conc	1.0E+03	/uL	
2	Pk4 singlets	19603		
3	Pk4 doublets	1399		
4	Pk4 triplets	122		
5	Gated	22767		
6	**Flow rate**	**0.76**	**uL/sec**	
7				
8	Volume	30000	nL	
9	Conc	7.6E+02	/uL	

	A	B	C	D	E
1	**vFRed CV**	**4.58**	**%**		
2	pos	2157			
3	neg	-44			
4	st dev	361			
5	**vFRed SI**	**3.05**			
6					
7	**PE CV**	**6.33**	**%**		
8	PE pos	3153			
9	neg	-10			
10	st dev	269			
11	**PE SI**	**5.88**			
12					
13	**BV421 CV**	**9.84**	**%**		
14	pos	1350			
15	neg	398			
16	st dev	261			
17	**BV421 SI**	**1.82**			
18					
19	**APC CV**	**6.93**	**%**		
20	pos	714			
21	neg	-24			
22	st dev	263			
23	**APC SI**	**1.40**			
24					

Figure 0.1. Instrument Qualification for vFC™. 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 busing 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.

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B. Calibrate Sample Flow Rate

1. On the **Flow Rate Tab**, adjust the gates selecting the Peak 4 singlets, doublets and triplets. The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (1e7/mL). The Layout will also calculate the apparent Peak 4 bead concentration, assuming the instrument-reported volume estimate is correct.

C. Calibrate Fluorescence Channels

1. On the **nRB FL – all Tab** adjust the markers on each channel to calculate the median fluorescence intensity (MFI) for the three brightest beads in each channel.

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(If the brightest three beads cannot be resolved from background, calibrate using vCal™ AbCap beads stained with the desired fluorescence-conjugated antibody, as detailed in Protocol 0.3).

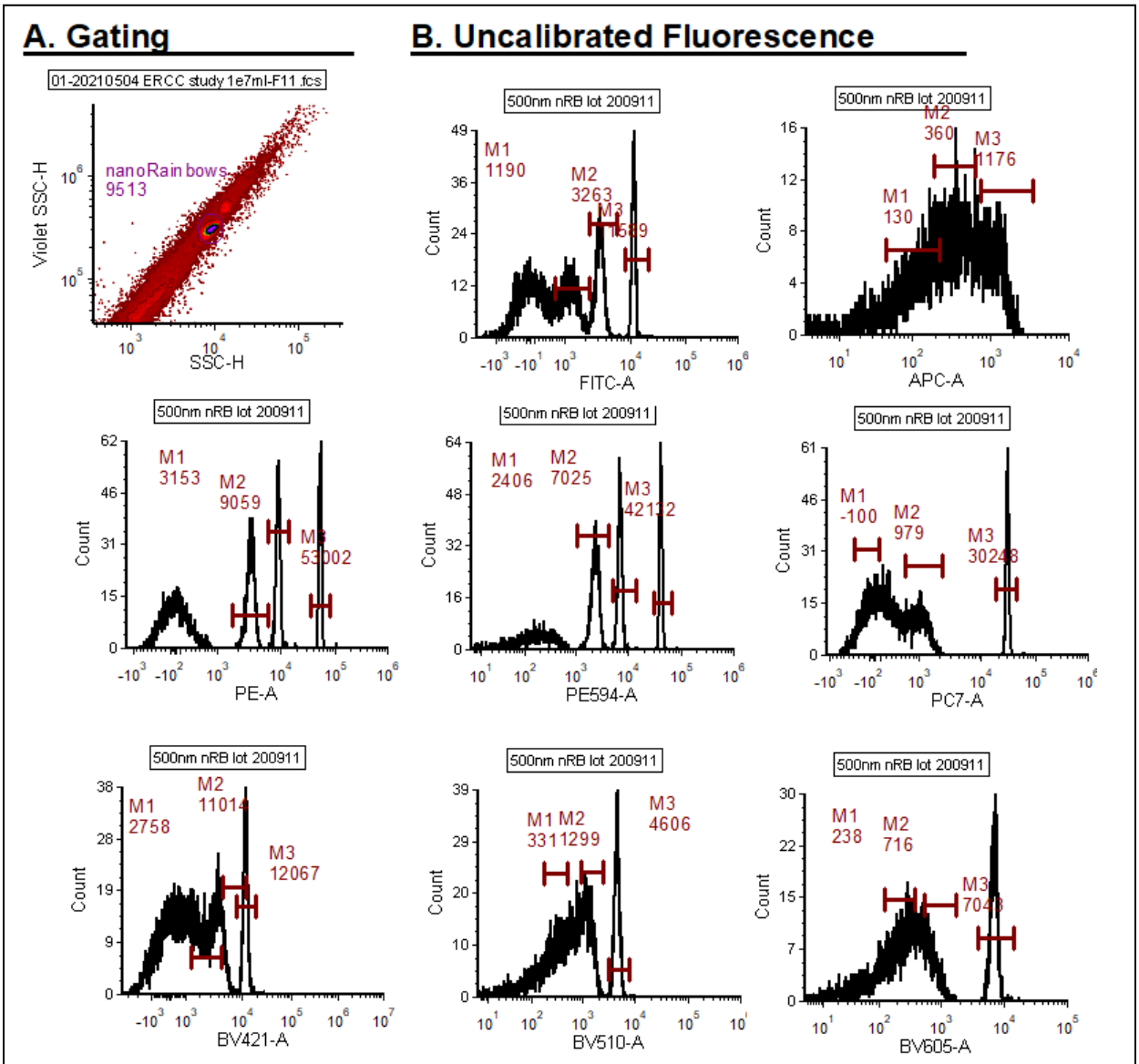

Figure 2. Fluorescence calibration using nanoRainbow beads. **A.** Single nanoRainbow beads are gated by their light scatter. **B.** Individual peaks on each channel are gated and the median fluorescence intensity (MFI) values are used in the Channel Calibration dialogue in the vCal Bead Report layout.

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2. Use the FCS Express Channel Calibration Tool (Tools>Channel Calibration to create a channel calibration file) using the nanoRainbow lot-specific MESF/ABV values (provided on the product data sheet).

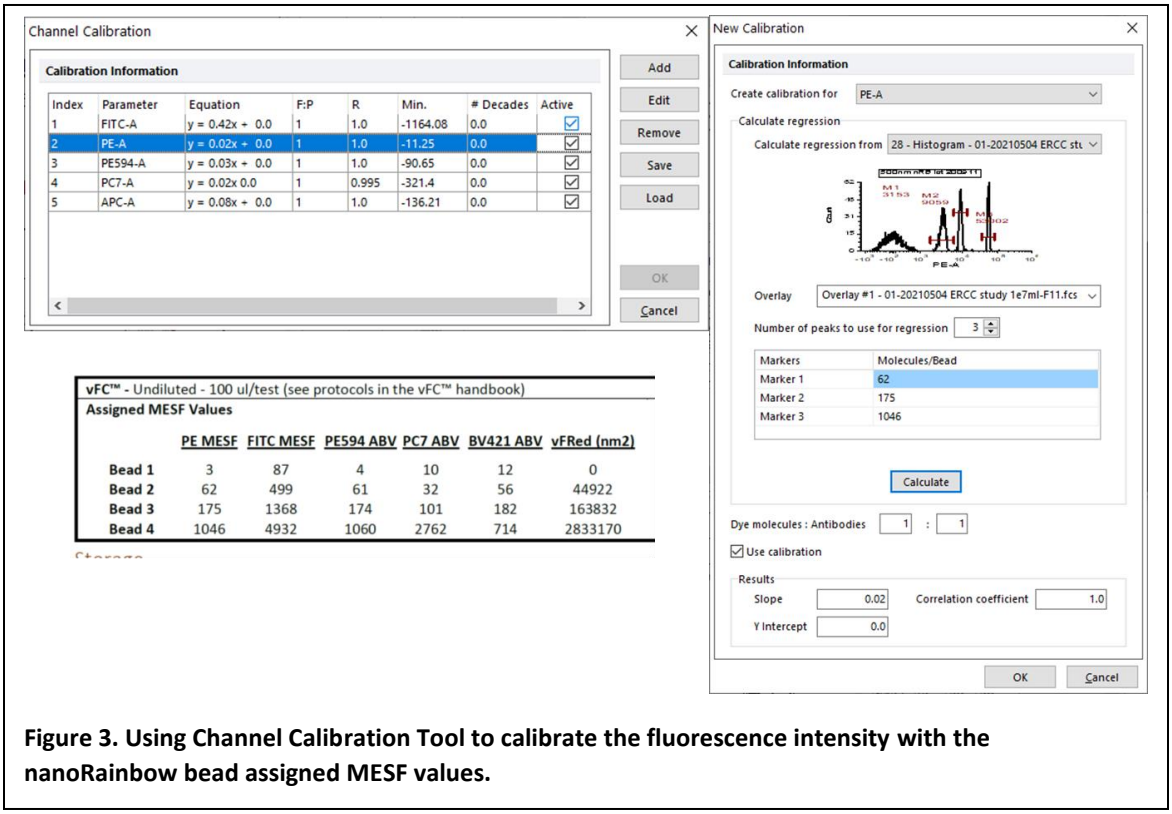

Figure 3. Using Channel Calibration Tool to calibrate the fluorescence intensity with the nanoRainbow bead assigned MESF values.

3. Apply the calibration to the selected plots to validate calibration.
4. On the **nRB FL – cal Tab** examine the recovery of the standard values on plots displaying calibrated axes.
5. Save the Channel Calibration file (Tools>Channel Calibration> Save) as

Instrument_nRB FL Calibration_YYMMDD.cal

eg. ***FC01_nRB FL Calibration_210704.cal***

This Channel Calibration file will be loaded into the vFC Report layouts

Note that these MESF assignments are nominal and are likely to be similar across similarly configured instruments (lasers, filters, detectors, etc.), but may vary when configurations are dissimilar. When the highest accuracy is required, the instrument response and nRB assignments should be determined using appropriate MESF and/or antibody capture standards as described in Protocol 0.3.

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D. vFRed™ Vesicle Size Calibration

1. On the nRB – Size Tab, adjust the markers to calculate the vFRed MFI for the three brightest peaks and inspect the resulting regression fit and results.

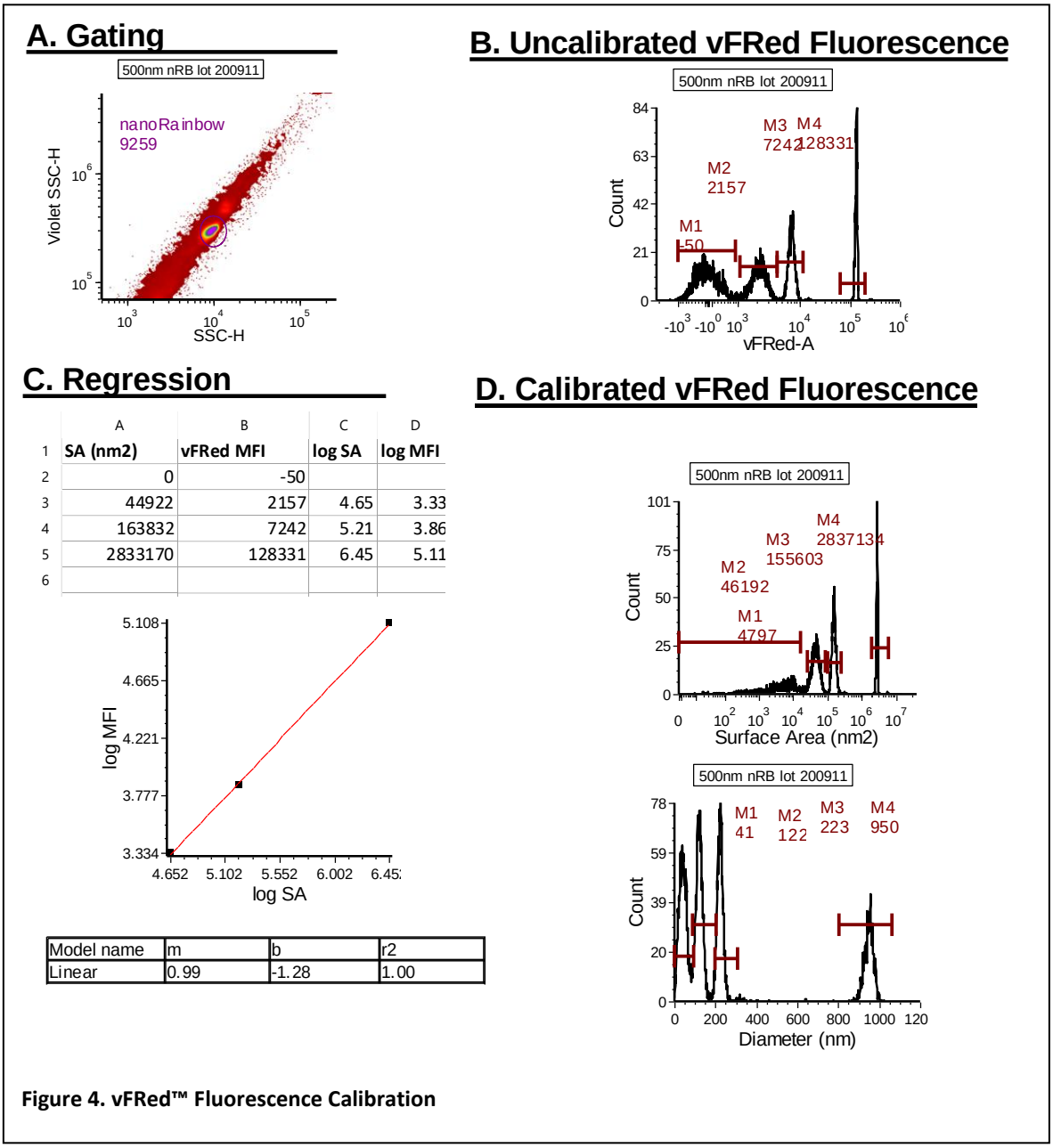

Figure 4. vFRed™ Fluorescence Calibration

2. Use the FCS Express Transforms Tool (Tools<Transforms<nRB Size Calibration) to edit the nRB Size Calibration Parameter Math estimation of Surface Area (nm2) by inputting the slope and intercept values from the regression fit.

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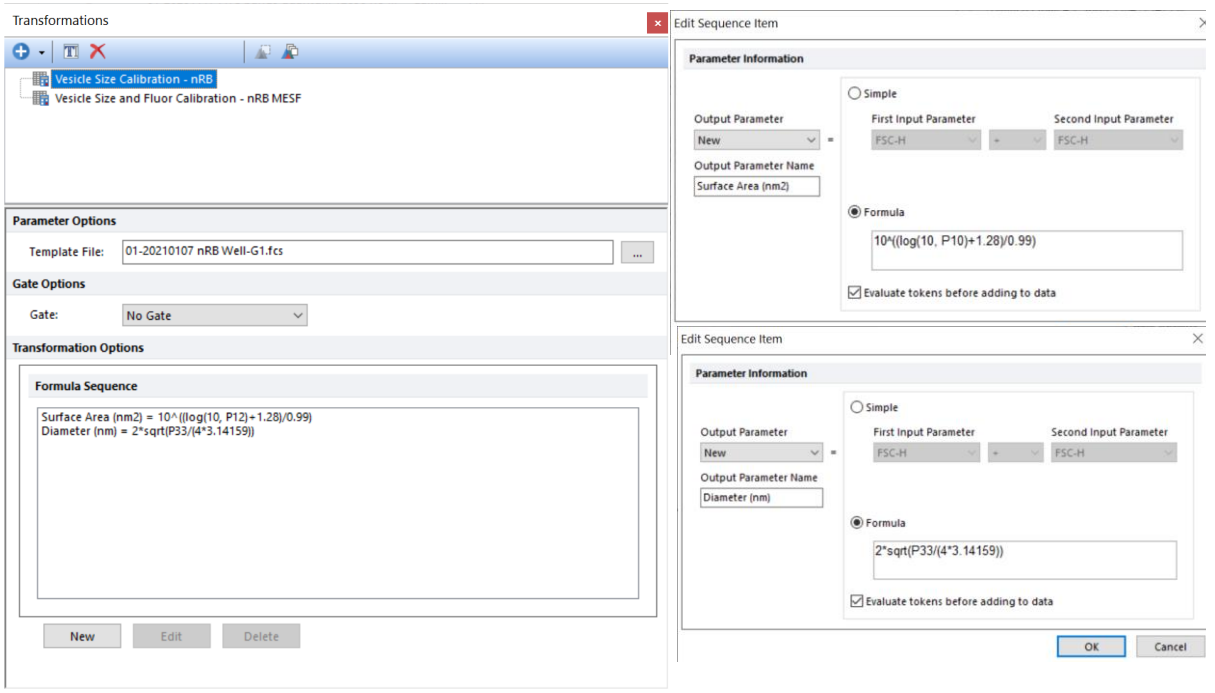

Figure 5. Vesicle Size Calibration Parameter Math Dialogue

3. Save the updated Parameter Math formula sequence file (right click<Save) as Instrument_nRB Size Calibration_YYMMDD (eg. CytoFlex_nRB Size Calibration_200704.fcf). This formula sequence file will be loaded into the vFC Report layouts used in the vFC Assays.
4. Close the Transforms dialogue box. Inspect the recovery of the standards in the resulting Surface Area histograms.
5. Save the vCal™ Bead Report layout (vCal Bead Report_CytoFlex_nRB FL and Size Calibration_200704.fey).

Note that these Surface Area assignments are nominal and are likely to be similar across similarly configured instruments (lasers, filters, detectors, etc.), but may vary when configurations are dissimilar. When the highest accuracy is required, the instrument response and nanoRainbow Bead assignments should be determined using appropriate vesicle size standards as described in Protocol 0.2.

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Notes

CytoFlex-specific Instructions:

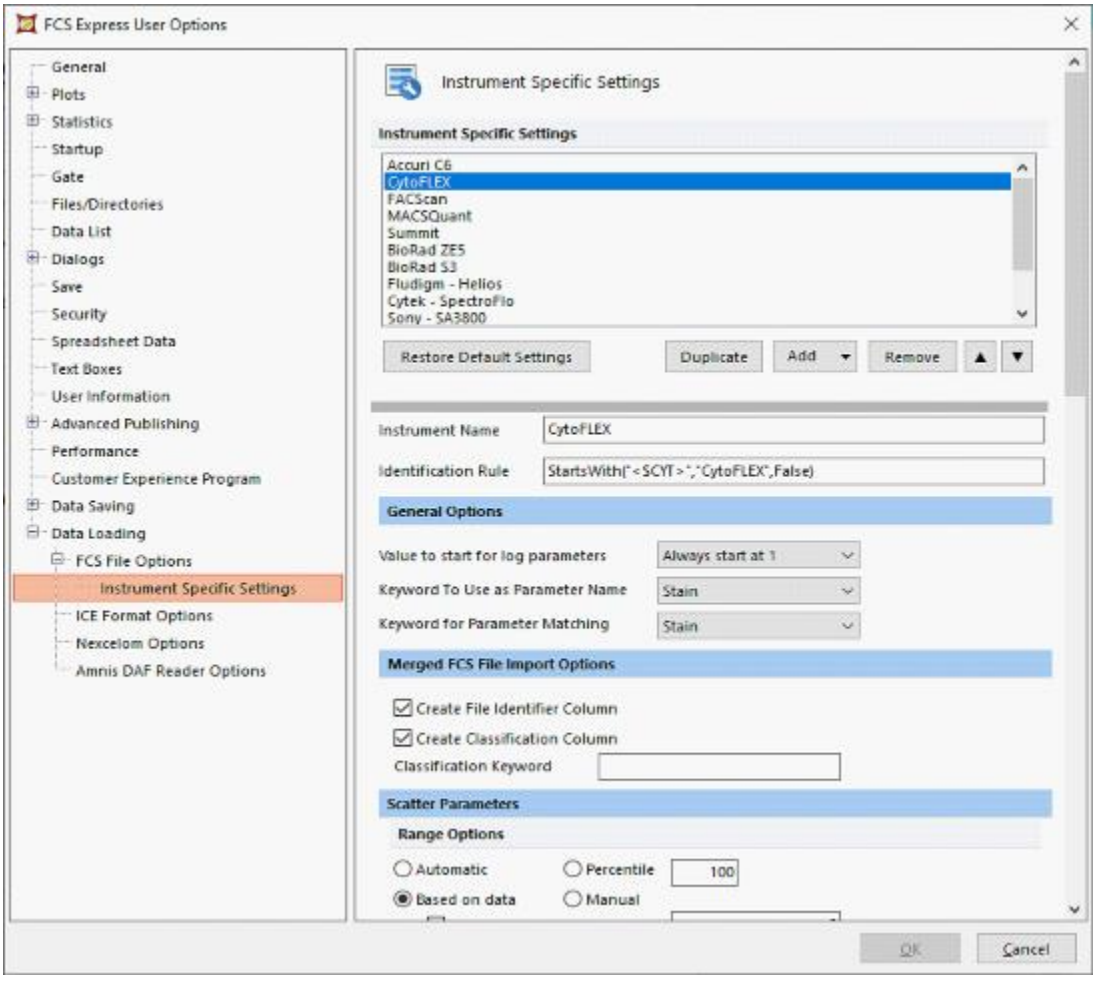
Before loading data files

Configure FCS Express Options to recognize CytoFlex data file parameters by name:

File>Options>Data Loading> Instrument Specific Settings> CytoFLEX>General Options>

Keyword to Use as Parameter Name: **Stain**
Keyword for Parameter Matching: **Stain**

Click: **OK**



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