Instrument QC and Qualification

For the Cytek ImageStream

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 perform Instrument Setup and create a vCal™ Bead 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)
- FCS Express or 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 by opening the vCal™ Bead Template created during **Instrument Setup** (see **Appendix A**).
2. Define save file name as “YYMMDD CytoFlex nanoRainbow”.
3. Vortex nanoRainbow beads well.
4. Place five drops (~50 uL) undiluted nanoRainbow beads in a well (or tube).
5. Record the nanoRainbow bead sample for 60 seconds.
6. Load nanoRainbow.rif file into the IDEAs with the vCal Bead Ideas Template - ImageStream.ast

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Data Analysis

1. Open FCS Express.
   *Before the first use of FCS Express, select the instrument-specific options as instructed in **Notes**, below.*
2. Open the **Instrument QC and Qualification** data analysis Layout in FCS Express and load the nanoRainbow Bead data file.
3. On the **nRB Report Tab**, inspect the time history and adjust the nanoRainbow gate on the population of single nanoRainbow Beads (**Figure 1A**).

A. Laser Alignment and Fluorescence Resolution

1. Inspect the fluorescence histograms of the vFRed Plot to the left of the page under the **Bead Peak Gating** header. Adjust so that the appropriate markers include each of the 4 peaks (**Figure 1A**).
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 metric is useful for an initial analysis of instrument performance, and the vFRed™ SI should be >4.0 to ensure efficient EV detection.

B. Calibrate Sample Flow Rate

1. On the **Flow Rate Tab**, adjust the gates selecting the Peak 4 singlets, doublets and triplets (**Figure 1C**). The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (1e7/mL).

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vCal™ nanoRainbow Bead Report - ImageStream
Instrument Characterization and Calibration

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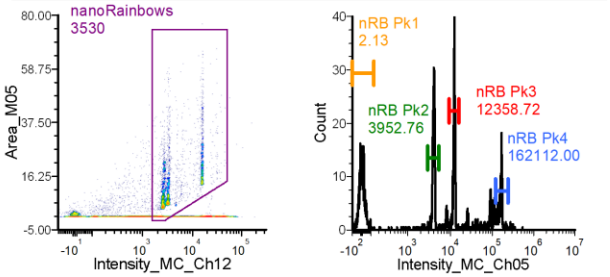
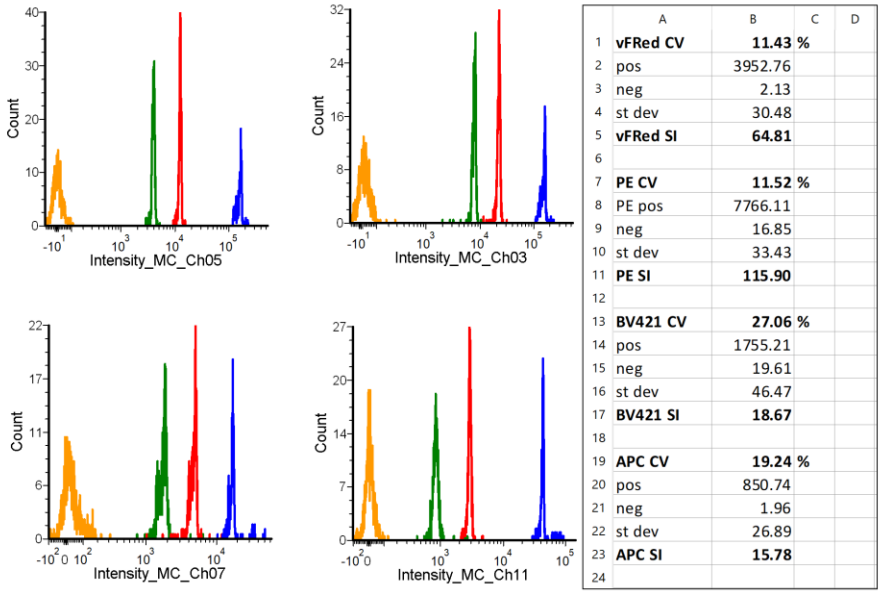
File: nRB_5minI-noBF_0.cif **Date:**
Sample:
Instrument: Image Stream
C:\Users\pjn97\Cellarcus Bioscience Dropbox\EV Analysis Products\FCSE Layouts\Data - MKII357 - 240530 min\Prot 0.1 nanoRainbow beads - 240530\Fluor Calibration - MKII357 - 230914 - A488 PEx3 BVx3 A647.cal

A. Gating:

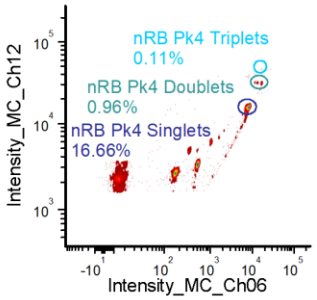


B. Fluorescence Resolution:



	A	B	C	D
1	**vFRed CV**	**11.43**	%	
2	pos	3952.76		
3	neg	2.13		
4	st dev	30.48		
5	**vFRed SI**	**64.81**		
6				
7	**PE CV**	**11.52**	%	
8	PE pos	7766.11		
9	neg	16.85		
10	st dev	33.43		
11	**PE SI**	**115.90**		
12				
13	**BV421 CV**	**27.06**	%	
14	pos	1755.21		
15	neg	19.61		
16	st dev	46.47		
17	**BV421 SI**	**18.67**		
18				
19	**APC CV**	**19.24**	%	
20	pos	850.74		
21	neg	1.96		
22	st dev	26.89		
23	**APC SI**	**15.78**		
24				

C. Flow Rate:



	A	B	C
1	Pk 4 conc	2.5E+03	/uL
2	Pk4 singlets	588	
3	Pk4 doublets	34	
4	Pk4 triplets	4	
5	Gated	668	
6	**Flow rate**	**0.00**	**uL/sec**
7			
8			
9			
10			

Figure 1. Instrument QC and Qualification. A. Single nanoRainbow beads are gated by light scatter and fluorescence intensity. **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.

Notes

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Appendix A. Instrument Setup - Cytek ImageStream

1. Startup the instrument and make sure ASSIST passed.

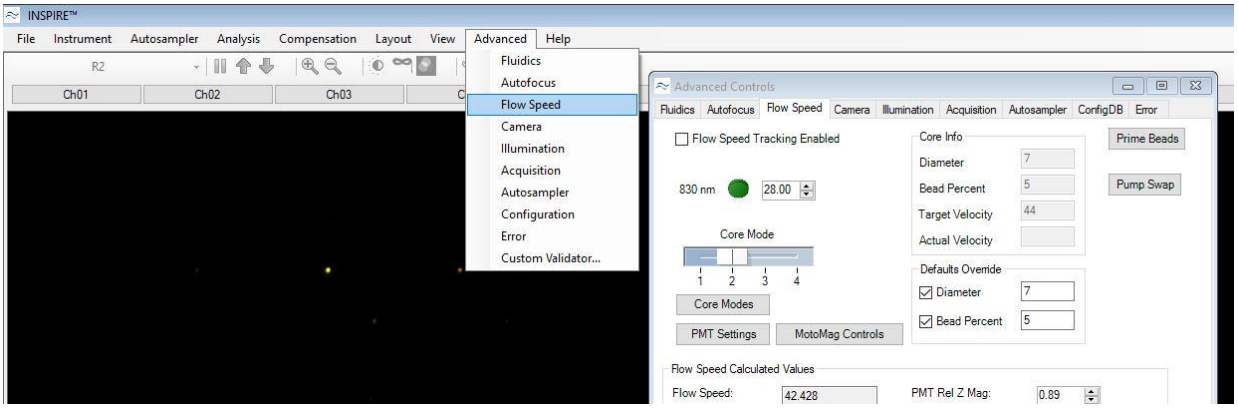
2. Illumination. Set all fluorescence excitation lasers at maximum power, the SSC laser on Ch12 at 0.75 mW power, and bright field (BF) channels turned Off. If your instrument is equipped with a 592 nm laser, turn off the laser. (Note: Some instruments are configured to collect SSC in Ch6)

3. Magnification. Set magnification to 60x, which provides the highest numerical aperture for light collection, resulting in the highest sensitivity.

4. High Gain Mode. Enable "High Gain" mode (if present), which operates the detector at high gain, increasing sensitivity for measurement of dim particles.

5. Fluidics. Set the instrument fluidics for "Lo" speed/"Hi" sensitivity.

6. Advanced Controls. In the **Advanced** menu, select **Flow Speed**. The Advanced Controls window will pop up. Under **Defaults Override**, select a core **Diameter** of "7" and a **Bead Percentage** of "5". This optimizes the vFC assay and detection of vCal beads.



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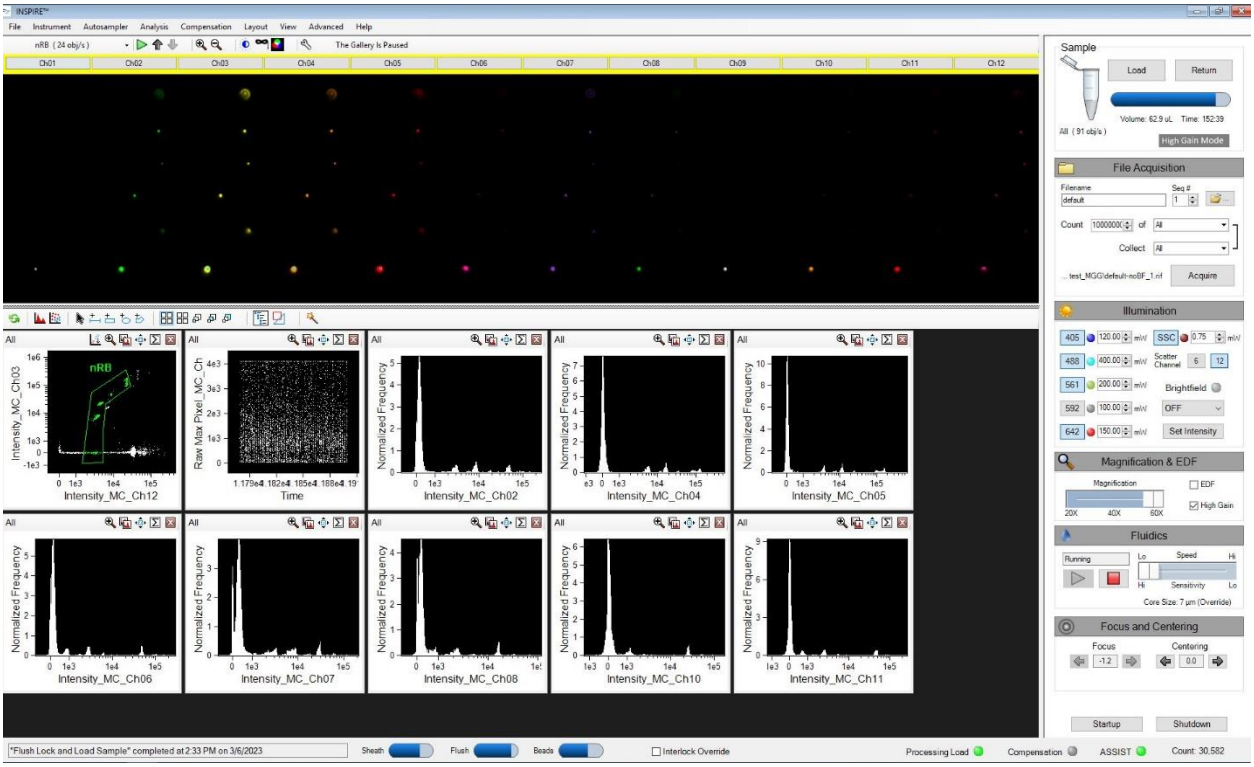
7. Acquisition Plots. Once Acquisition settings are set correctly as described above, place 3 drops of vCal™ nanoRainbow beads in a 0.65 ul tube, load, and run, and add the following 1- and 2-parameter plots and gates to view data:

Plot/Gate: Description	Purpose
Plot 1: Intensity_MC_Ch5 vs _Ch12	Separate Speed Beads from vCal beads
(Optional) Gate 1: Bead gate (nRB, nanoCal; Not Speed Beads)	Gate vCal beads (nRB, antibody capture beads, nanoComp, etc.)
Plot 2: Intensity_MC_Ch12 vs Time	Monitor fluidic stability
Histograms: Intensity of any/all channels	Display objects/peaks detected in all channels



Note that these plots are for visual inspection of the data during data acquisition, ALL objects are collected. Gating and data analysis do not take place during acquisition.

8**. Save Bead acquisition template.** Under the File menu, click on Save template. Name the template: "vCal Bead Ideas Template - ImageStream.ast".

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C. ImageStream Data Analysis

1. Convert ImageStream rif files to fcs files. For analysis of the vFC assay, data will need to be in .fcs format. Prior to data export, analysis templates (.ast files) are applied to .rif files in IDEAS

The nRB template displays the relevant parameters needed to identify and gate nanoRainbow beads, and to display and inspect their resolution on each fluorescence channel.

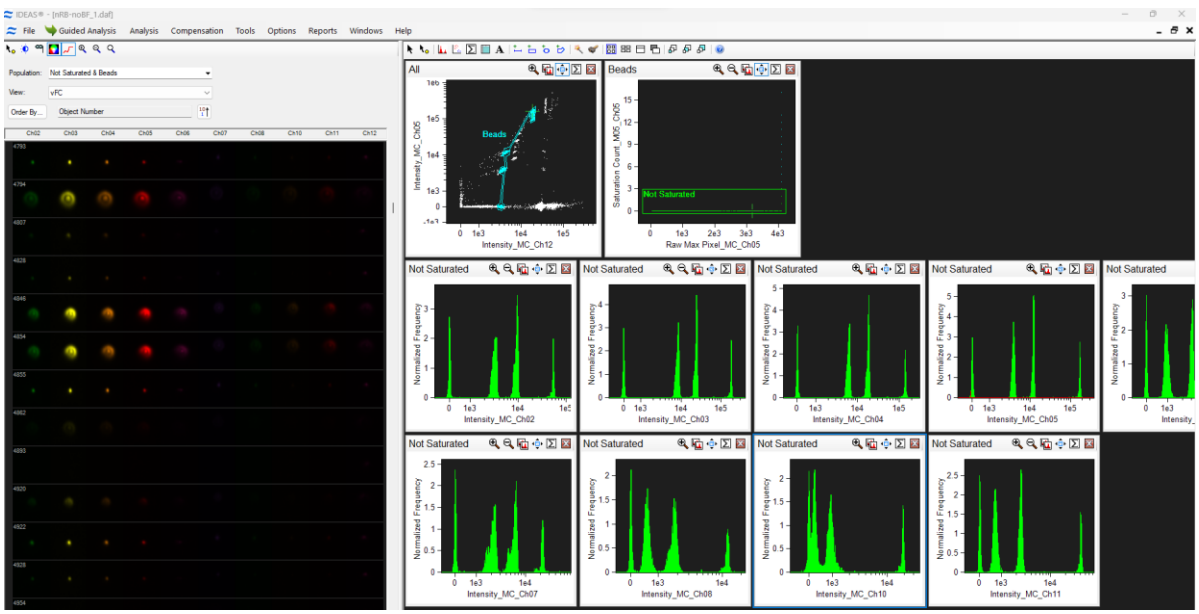

Figure 3-1. nRB.ast template includes parameters and gates to identify vCal calibration beads, exclude SpeedBeads and saturated images, and assess fluorescence resolution.

b. nanoCal Antibody Capture and nanoComp beads.

The nanoCal template displays the relevant parameters needed to identify and gate Antibody Capture beads, and to display and inspect their resolution on a specific fluorescence channel.

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The initial plot and "Bead" gate must be adjusted depending on the fluorophore used. The example below is for the PE fluorophore.

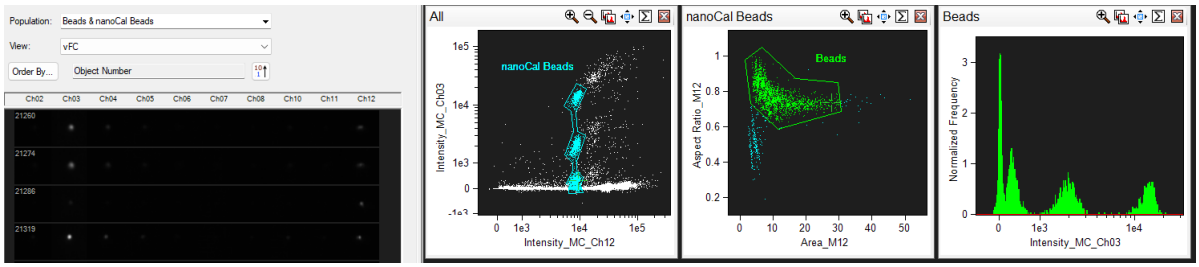

Figure 3-2. ACB-PE.ast template includes parameters and gates to identify nanoCal Antibody Capture beads stained with the Tetraspanin mix-PE marker, exclude SpeedBeads and clipped objects, and assess fluorescence resolution.

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Appendix B. Prepare an IDEAS analysis template (.ast)

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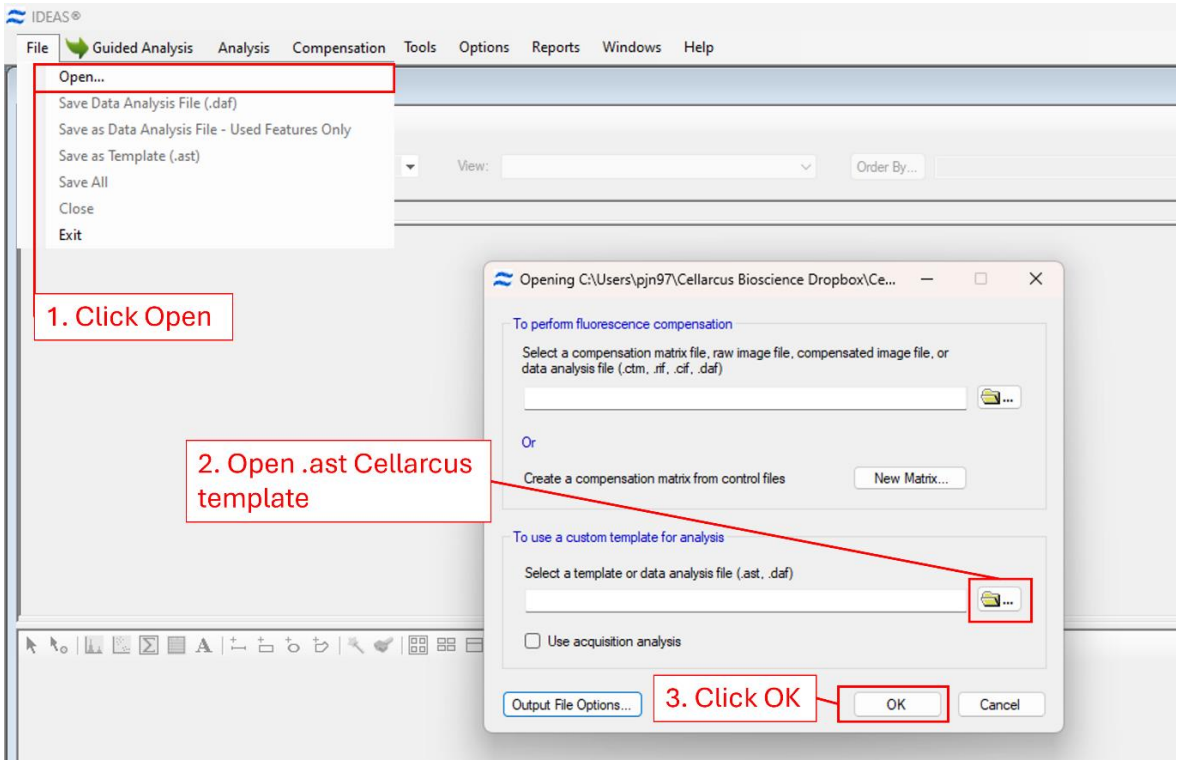
Appendix C. Batch conversion of rif files to fcs format

4. Opening a File

1. Go to File>Open and select a RIF file
2. In the window that opens load the appropriate template (vCal Bead Ideas Template - ImageStream.ast or vFC Assay Ideas Template - ImageStream.ast) for analysis (see screenshot below)
3. Click OK to view the data



5. Batching Data Files (Converting multiple RIF's to DAF's)

1. Go to Tools>Batch Data Files…
2. In the new window that opens click "Add Batch"

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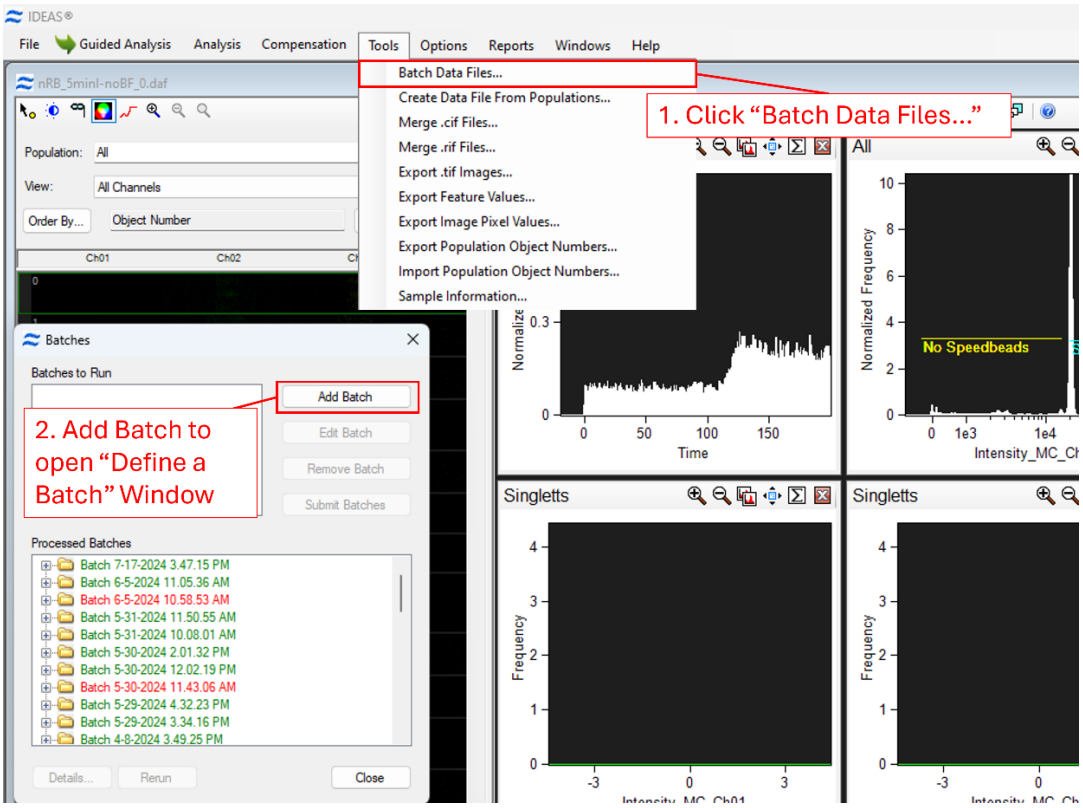
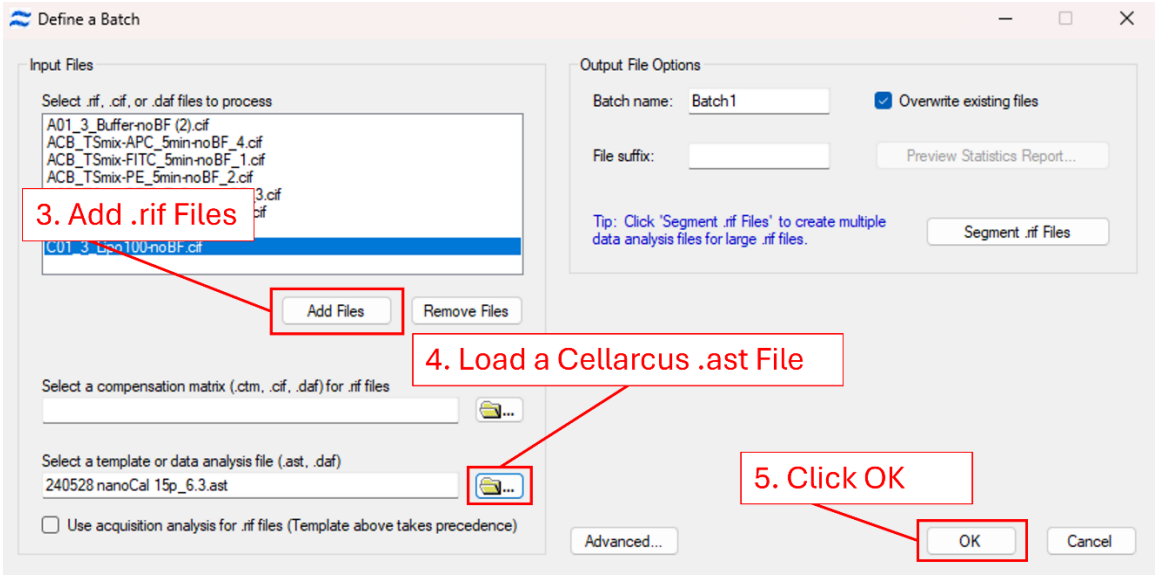


3. When a third window opens click "Add Files" and load RIF's from your experiment
4. Click on the folder to load an AST or a DAF (see screenshot)
5. Click OK



6. Click "Submit Batches" to batch the files you loaded
7. Once batch is finished click "Close"

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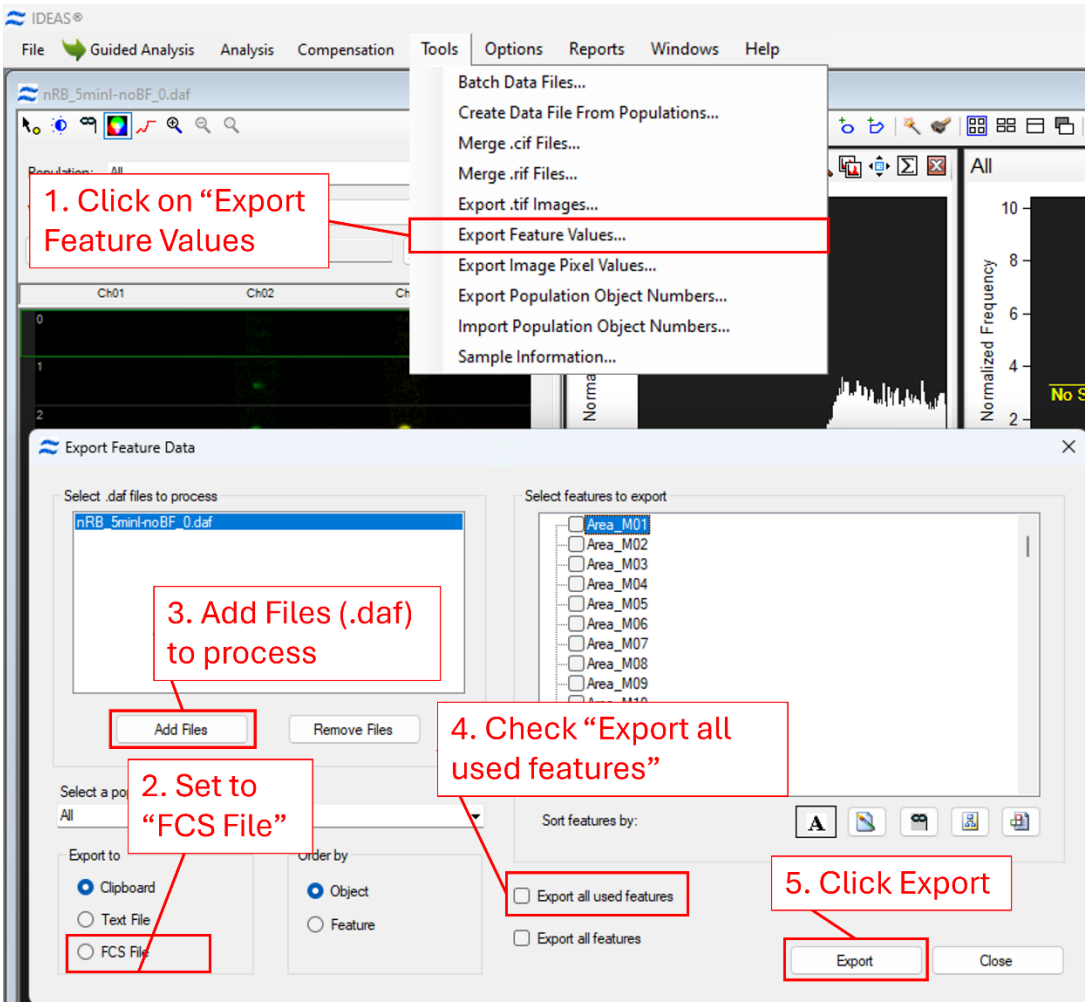
6. Export ImageStream feature data to fcs-format files:

1. Make sure you have a .daf file opened for export
2. Under the **Tools** menu, click on "**Export Feature Values.**"
3. Under **Export to**, select "**FCS file**".
4. Add .daf files from the experiment (created after batching)
5. Check "Export all used features'
    a. Note: If "Export all used features" doesn't work you may check each of the 15 features individually: Time, Area_M05, Raw Max Pixel_MC_Ch12, and the 12 Intensities (Intensity_MC_Ch01 - Intensity_MC_Ch12).



The newly created fcs files will be analyzed using the protocol-specific FCS Express layouts, as described in individual Protocols.

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