

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 download the vFC[™] SpectroFlo Experiment Template from the Cellarcus website (or perform Instrument Setup and create a vFC[™] Assay Experiment 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 TM Instrument QC and Qualification data analysis Layout		

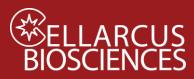
Materials to be Provided by user

- Gloves
- Microwell plate (Sartstedt 82.1583.001) or suitable tube.
- Cellarcus SpectroFlo Template Aurora.zip (Cellarcus web site)
- FCS Express or FCS Express Reader software (<u>FCS Express Reader</u>) Before first use, refer to Instrument-specific instructions in <u>Notes</u> (page 8).

Procedure

- Open a New Experiment by opening the Cellarcus SpectroFlo Template Aurora.zip (downloaded from the Cellarcus web site or created during Instrument Setup, see Appendix A).
- 2. In the *Acquisition* dialogue, set *Events to Display* to <u>500,000</u> to ensure that all data are displayed.

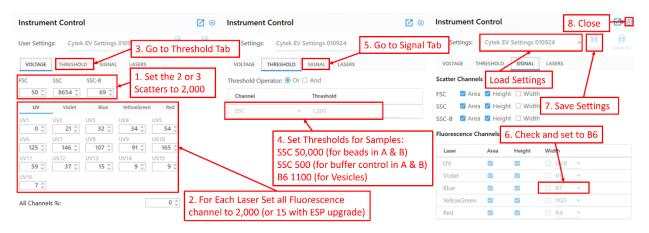




Under Instrument Control – Voltage set the gain for all of the scatters to 2000 and set the gain for all of the fluorescence channels to 2000.

Under Instrument Control – Threshold set the channel to the vFRed Parameter (B6-A) and set the threshold to 1100.

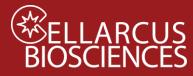
Under Instrument Control – Signal make sure B6-W (vFRed) is chosen for the width values.



You may choose to save/load the Instrument Control Settings file (see screenshot above). This shouldn't be necessary since the Instrument Control Settings are saved with the well/tube slots in the experiment.

Table A4				
Dialogue	Setting	vCal [™] beads	Buffer Bkgd	vFC [™] assay
Acquisition	Active Channels	All channels	All channels	All channels
Settings				
	Scatter gain	2000	2000	2000
	(FSC, SSC, SSC-B)	(SSC: 15 w/ESP)	(SSC: 15 w/ESP)	(SSC: 15 w/ESP*)
	Fluorescence gain	2000	2000	2000
	(all channels)			
	Primary Threshold:	SSC-H	SSC-H	В6-Н
	Manual Threshold:	50,000	500	1100
	Width Parameter:	B6-W	B6-W	B6-W
Stopping Rules	Time to Record	Checked: 30 sec	Checked: 30 sec	Checked: 120 sec
	Events to Record	Unchecked	Unchecked	Unchecked
Acquisition	Events to Display	500,000	500,000	500,000
	Sample Flow Rate	High	High	High
* Enhanced Sm	all Particle (ESP) detect	tor option installed.		

- 3. Click Save to save the new Settings.
- 4. Vortex nanoRainbow beads well.



- Place five drops (~250 uL) undiluted nanoRainbow beads in the Protocol A+B Group Well H3 (or tube).
- 6. Select Well H3 and click **Start**. Inspect the light scatter plot to make sure the beads are on scale and singlets can be gated.
- 7. Set the flow rate to **High** and **Record** the nanoRainbow bead sample for 30 seconds.
- 8. Save the Experiment with a descriptive file name that includes the date.



Data Analysis

Run SOP

Open the Aurora (including Northern Lights) **Instrument QC and Qualification** data analysis Layout in FCS Express and run the SOP by going to Tools>Run SOP.

Before the first use of FCS Express, select the instrument-specific options as instructed as specified in "READ ME FIRST" tab.

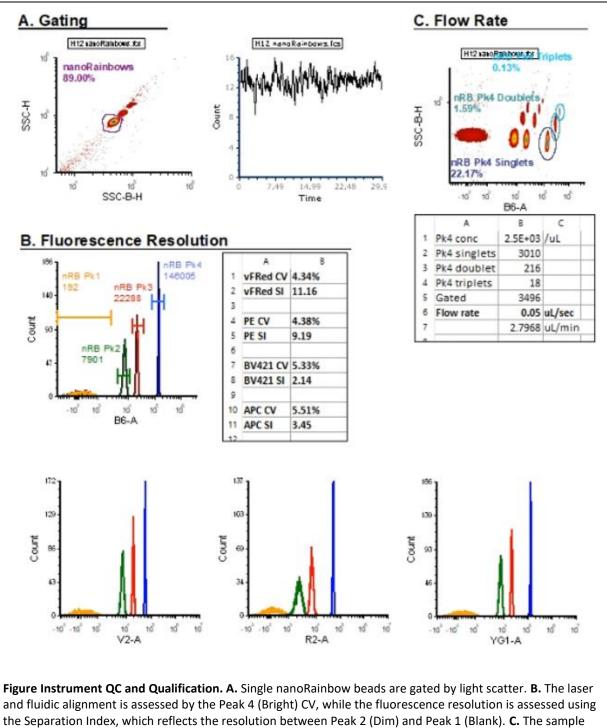
B			1. Tool	S			_		Format	N:\Lab	Operations\Products\\	/FC Protocols
File Ho	me Insert	Design	Gating Batch	& Export	Text Data	Tools	View	Help	Format			
	(Ö)		26				n SOF	,	1	5	靏	
Concentration Calculator	Channel Calibration	Parameter Labels *	Apply Iteration Snapshot	Snapshots	Compens and Unmixing	196 - 19 - 19 - 19 - 19 - 19 - 19 - 19 -	1001		rride horizations	Run SOP	Standard Operating Procedures	Permission Rules
Calco	late	Labels	Snapsh	nots	Trans	formatio	ns	0	Override		SOP	

<u>Analysis</u>

- 1. Go to Data List (right side of page) and click the Add Data File icon to navigate to nanoRainbow data (.fcs) file.
- 2. In the "Data List" window click on the nanoRainbow (.fcs) file then open the "Change File" menu and select "Change Data On All Plots" from the dropdown menu.
- 3. Adjust nanoRainbows gate to include single beads (excluding background noise, doublets, etc).
- 4. Inspect the time history for any fluidics issues.
- 5. Inspect the fluorescence histograms of a representative channel from each laser and adjust the markers to select each of the four bead populations.
- 6. Laser alignment is assessed by inspecting the CV of the bright bead population, which should be <15%. If CVs are higher, or there is an apparent leftward shoulder to the peak, alignment on that laser may be sub-optimal.
- 7. 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 >3.0 to ensure efficient EV detection.
- 8. Set the "nRB Pk4 Singlets", "nRB Pk4 Doublets", and "nRB Pk4 Triplets" on the appropriate populations. The Layout will calculate the sample flow rate using the known concentration of the nanoRainbow beads (5e5/mL).
- 9. Go to File>Save As to save the layout with a descriptive name.



For the Cytek Aurora and Northern Lights



flow rate is estimated from the number of Peak 4 (Bright) beads, including doublets and triplets, measured for a fixed time.



<u>Notes</u>

Before loading data files, configure FCS Express Options to merge raw and unmixed parameters:

File>Options>Data Loading> Instrument Specific Settings> Cytek - SpectroFlo>

- 1. Cytek File Merge Rule: Merge both Raw and Unmixed Always
- 2. Delete fluorescence parameters after spectra merge: Uncheck

Click: OK

Note: You need to load the unmixed version of the file. If you just changed the settings then you will need to change files on all plots before changes take effect.

General	Instrument Specific Settings	
Plots	When loading data ECC Express will a	sutomatically detect the instrument used to acquire it
Statistics	and will apply the corresponding rule	from the list below. Select an instrument below to
Startup	review and modify the settings that w	rill be used for data from that instrument.
Gates	Accuri C6 CutoFLEX	
Quadrants	FACScan	
Files/Directories	MACSQuant	
Data List	BioRad ZE5	
Dialogs	BioRad S3 Fluidigm - Helios	1
Save	Fluidigm - XT	
Security	Cytek - SpectroFlo Sony - SA3800	
Spreadsheet Data	Lat	
Text Boxes	Restore Default Settings	Duplicate Add - Remove A V
User Information		
Advanced Publishing		
Performance	Instrument Name Cytek - Spectro	0F10
Customer Experience Program	Identification Rule ("<\$CYT>" = "S	SpectroFlo") or ("<\$CYT>" = "Aurora") or ("<\$CYT>" = "A
Data Saving		
Data Loading	General Options	
 FCS File Options 	Value to start for log parameters	Always start at 1 🛛 🗸
Instrument Specific Settings	Keyword To Use as Parameter Name	Stain
ImageXPress Velos Options		
CellProfiler Options	Keyword for Parameter Matching	Name ~
ICE Format Options	Merged File Import Options	
Perkin Elmer Acapella Options	Create File Identifier Column	
Life Technologies Tali Options		
Nexcelom Options	Create Classification Columns	
ImageJ Options	Classification Keywords (enter multip	ole keywords using commas):
Amnis DAF Reader Options		
Perkin Elmer Vectra Options		
Thermo Cytpix ACS Options	Cytek Specific Options	
	Import spectra as individual Para	
	Import spectra as Single Merged	
	Spectra Parameter Label Parameter	r Names 🗸 🗸
	Delete fluorescence parameters a	after spectra merge
	Merge Raw and Unmixed Cytek fi	iles during import



Appendix A. Instrument Setup – Cytek Aurora and Northern Lights

Step 1. Create an Experiment Acquisition Template.

The Cytek acquisition settings are stored in an .expt file, which contains data acquisition settings as well as the file and group naming rules applied to file names and Sample IDs. Frequently used plate layouts can be saved and used as templates for future experiments.

This Template will be used to measure both beads, including vCal[™] nanoRainbow Beads (Protocol A Instrument QC and Qualification) and vCal[™] Antibody Capture Beads (Protocol B Fluorescence Unmixing and Calibration) and vesicles, including vCal[™] Lipo100[™] Vesicle Standard (Protocol B2) as part of Fluorescence Unmixing and Calibration and for most vFC[™] assays, including measuring EV concentration and size (Protocol 1) and measuring EV surface markers (Protocol 2).

The beads are detected using light scatter triggering to measure the bead fluorescence intensity, while vesicles are detected using fluorescence triggering (vFRed Fluorescence, B6) and fluorescence measured under the same instrument conditions (flow rate, laser power, detector gain).

Before creating a template for vFC[™] you need to add the vFRed Fluorescence tag to the SpectroFlo Fluorophore Library (**Figure A1**). To do this go to Library then select the blue laser and finally click "Add fluorescence tag."

Library Fluorescent Tags	1	Library Tab GC & Setup	Acquisition Extra		es Users Help	
QC Beads Image: Graph of the second transmission of transmission	Fluorescent Tag Groups temport temport Descent Tag Groups F Name F Name Blue Laser System	Edit Delets 2. Blu Deter Created July 21, 2023 - 09:08 AM	IE Laser	Current Group: Blue Laser Add	Delete 3.	Edit
Spillovers	Red Laser System Orescent Tag	July 21, 2023 - 09:08 AM	January 12, 2024	NovaFluor Blue 610-30S BB630 NovaFluor Blue 660-40S NovaFluor Blue 660-120S	615 631 664 665	488 488 488 488
 Fluorescent Tag Nan Laser Excitation: Emission Wavelengt Display Name: 	488 W	Set Tag Name and Em /avelength. If you alrea elected a laser then La xcitation should be pre	dy ser	BB660 cFluor B675 PerCP cFluor B690	667 675 677 690	488 488 488 488
Synonyms: vFRed	Select G	aser t Laser		BB700 PerCP-Cy5.5 StarBright Blue 700 PerCP-Vio700 PerCP-eFluor 710	693 695 700 704 710	488 488 488 488 488 488
Add 🧪	dit X Delete 🗘 Set As Display Name	X Cancel	Save	88755 88790 vFRed	755 793 631	488 488 488

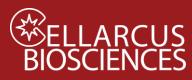


Table A1 shows the dyes that should be present in the Experiment Spectral Library. need

Table A1				
	Parameter Name	Laser		
Detector	(\$PnN)		EX-EM/BP	Stain Name (\$PnS)
1	B06	Blue	B6	vFRed
2	B02	Blue	B2-525/17	AlexaFluor488
3	YG01	YellowGreen	YG1-577/20	PE
4	YG03	YellowGreen	YG3-615/20	PEDazzle
6	YG09	YellowGreen	YG9-780/30	PECy7
7	V01	Violet	V1-428/15	BV421
8	V07	Violet	V7-542/17	BV510
9	V10	Violet	V10-615/20	BV605
11	R01	Red	R1-660/17	APC
12	R02	Red	R2-678/18	AlexaFluor647

You will also need to import a set of keywords. To do this go to Library>Keywords then click Import under Keyword groups then load the .csv file containing the Cellarcus keywords in the proper format. You may also add additional keywords or create your own keyword groups but our layouts will work best with the Cellarcus keywords.

	QC Beads	Keyword Groups				Current Group: Cellar	cus Keywords		
	Fluorescent Tags	🛓 Import 🏦 Export	🕀 New 🧨	Edit 🔋 Delete		🕀 Add 🧪 Ed	dit 🔋 Delete		
r	Labels	17 Name	I≓ Created By	17 Date Created	17 Date Modified	17 Name 17 Suf	fix ⊥≓ Type	Value	□ Auto Update
>	Keywords	Default Keywords	System	July 21, 2023 - 09:08 AM	July 21, 2023 - 09	\$CELLS	String		
i]	Spillovers	Cellarcus Keywords	Admin	January 03, 2024 - 15:54 PM	January 03, 2024	\$SMNO	String		
¢	User Settings					SCOM	String		
÷	Loader Settings					\$PROJ	String		-
	Worksheet Templates					\$SRC	String	Cellarcus Biosciences	
1	Experiment Templates								
I	Experiment Templates								
	Experiment Templates Backup & Restore								
D									
	Backup & Restore								
	Backup & Restore								
	Backup & Restore								



To configure the initial vCalTM and vFCTM Combined Acquisition Template (.expt file), create a New Experiment (*Acquisition > Select an experiment > New*), then follow steps below. To edit an existing experiment open the experiment then click "Edit."

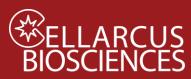
Acquisition Experiment New Default Experiment New Experiment from Template Mew Experiment Mew Experiment from Template Mew Experiment from Template Mew Experiment Mew Experiment Mew Experiment from Template Mew Experiment Mew Experiment Mew Experiment Mew Experiment Mew Experiment Mew Experiment from Template Mew Experiment Mew Expe	Ø SpectroFlo⊕ (Admin)				- 🗆 X
New Experiment from Template Image: I		/ Defau	It Experiment QC & Setup		
Open Experiment			My Experiments Load Experime Import Export Save Experime F Experiment Save Experime YYMMDD - Instrument - Template - t YYMMDD - Instrument - Template - t YYMMDD - Instrument - Template - w 2024-03-08 vFC LanaZ 10c-pn -Unmixed1 2024-03-08 vFC LanaZ 10c-pn 2024-03-08 cellarcus Lana Zaretski pn-Unmixed1 YYMMDD - Instrument - Example AU Data-s	IF Date Created March 29, 2024 - 13:41 PM April 10, 2024 - 14:10 PM April 02, 2024 - 18:02 PM April 02, 2024 - 17:32 PM April 02, 2024 - 17:32 PM March 19, 2024 - 09:16 AM March 28, 2024 - 12:31 PM March 27, 2024 - 10:47 AM	 ★ Date Modified April 10, 2024 - 17:5 April 10, 2024 - 14:1 April 02, 2024 - 18:0; April 02, 2024 - 18:0; April 02, 2024 - 17:4; March 29, 2024 - 12; March 28, 2024 - 12; March 28, 2024 - 10;

Note: Steps 1a and the first part of 1b to define the fluorescent tags and Reference Group are required for spectral unmixing in SpectroFlo, but not for conventional analysis with post-acquisition compensation (or unmixing).

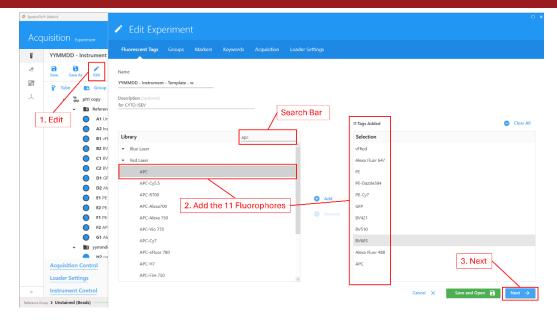
a. Fluorescent Tags Tab – Add Fluorescence Tags (needed for unmixing in Spectroflo)

At the top of this first tab you can set a name and description for the experiment. Add vFC fluorescence tags from the Spectral Library. vFRed should have already been added to the spectral library (see screenshot above). To find tags quicker use the "Type to filter" search bar.

Click next.



For the Cytek Aurora and Northern Lights



b. Groups Tab – Create Reference Group and Sample Group(s)

Tubes: If you are using tubes to collect data then Right click on the experiment group then select Add Reference Group and new window will open (see below).

Plates: If you using plates/wells change Carrier Type to "96 U-Bottom" then click "Add Plate." Then select a group of wells one for each control (13 total). Hold click and drag to select multiple wells at once. Hold shift and click to add additional single wells. Once you have selected your wells then click the middle folder on the right side of the window (see below).

Edit Expe						D	. Tub efere			un	2.	Edi	t Ref	eren	ce G	Fou	р	
luorescent Tags	Groups Markers	Keywords		Loader	Settings		cicic	nee		up								
arrier Type: 🗍 M	lanual Tube 👻	+ Group	5* Tube	1.	Collap	se All	👕 Del	ete	0	leferenc	e Group		Refer	ence Gro	up			
 YYMMDD 	- Instrument - Template - w			*	plt1 c	ору	96 U	Bottor	n		*							
י ינה plt1 פ	сору				-	1	2	3	4	5	6	7	8	9	10	11	12	
_	Reference Group			11							\circ	\bigcirc						R
•	A1 Unstained (Beads)				A			\bigcirc			0	0					\bigcirc	
-	A2 buffer (Cells)				в		O	\bigcirc			0	0						
-	B1 vFRed (Cells) B2 BV421 (Beads)				с			\odot			0	0						
-	C1 BV510 (Beads)				D			\sim			0	0						
-	C2 BV605 (Beads)				U			\cup			0	0					\cup	
0	D1 GFP (Beads)				E	O		\odot			0	0					\odot	
\bigcirc	D2 Alexa Fluor 488 (Beads)				F			\bigcirc									\odot	
•	E1 PE (Beads)				G		Ő											
•	E2 PE-Dazzle594 (Beads)					R	2										1	
Group Properties	F1 PE-Cv7 (Beads)			4	н	Q	S										9	
Group Name	Туре					1	1.	Plate	e: Fi	ist S	elec	t 13	Refe	erend	te G	rour		
Reference Group		erence Group					V				raga							
	Edit Reference	Group									erend				1			
																	_	



Check "Define Universal Negative Control(s) for Spillover Calculation" and a new section will appear. Click "Add" to create a new negative control. Name the new negative control "Buffer" and set the control type to "Cells."

Under Fluorescent Tags section ensure that the positive controls have the appropriate control type and negative control set (vFRed should be Cells and Buffer and the rest should use the Unstained Bead control). Enter appropriate Labels for each positive control. Click Save.

Define Unstained Cor	trol Matching Sample Autofluo	rescence 🛛 🖾 Define Univ	ersal Negative Control(s)	checkbox
ame Control Type	View Stored Unstaine	ed Controls Name	Control Type	
nstained Beads	• N/A	buffer	Cells	-
/	2. Nar	me: "buffer"		
. Control Type	e: Beads	4.00	ontrol Type: Cells	
1		4. CO		
		G Ada	Contractions	
Fluorescent Tags				
Fluorescent Tag	Control Type Label	Universal Negative	View Stored Reference Controls	
vFRed	Cells -	buffer 👻	N/A	
8V421	Beads 👻	Unstained 👻	N/A ~	
BV510 -	Beads +	Unstained 👻	N/A	
	Beads -	Unstained 👻	N/A	
BV605 -				
	Beads -	Unstained 👻	N/A. T	
GFP 👻		Unstained + Unstained +	N/A ~	_
GFP + Alexa Fluor 488 +	Beads 👻			
GFP Alexa Fluor 488 PE	Beads -	Unstained v	N/A ~	
Alexa Fluor 488 ¥	Beads	Unstained * Unstained *	N/A * N/A *	

Tubes: Set Carrier type to "Manual Tube"

Protocol A: Add a group. Name it "yymmdd Prot A" then add 1 tube. Protocol B: Add a group. Name it "yymmdd Prot B" then add 13 tubes. Protocol 1: Add a group. Name it "yymmdd Prot 1" then add 16 tubes. Protocol 2: Add a group. Name it "yymmdd Prot 2" then add 32 tubes.

 Edit Experiment 1. s 		o x
Fluorescent Tags Groups Markers		
Carrier Type: Manual Tube	😰 Group 👔 Tube 📑 🗸 Collapse All 👔 Delete 💿 Reference Group 🖍 Reference Group	1
Reference Group	2. Add Group 5. Add Tubes	
Unstained (Beads)		
Buffer (Cells)		
vFRed (Cells)		
BV421 (Beads)		
BV510 (Beads)		
BV605 (Beads)		
GFP (Beads)		
U* Alexa Fluor 488 (Beads)		
U* PE (Beads)	Files cannot easily	
PE-Dazzle594 (Beads)	be (re)named here.	
U* PE-Cy7 (Beads)	6. Next. Next again	
OPC (Beads)	(skip markers tab).	
Alexa Fluor 647 (Beads)	3. Rename Groups:	
v yymmdd prot 0.1	yymmdd prot #	
T1 nanoRainbows	- Mininga brock	
 yymmdd prot 0.3 		4
← Previous	Cancel X Save and Open	

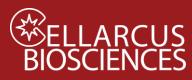


Plates: Set Carrier type to "96 U-Bottom" (or whatever plate type you are using). Add 3 plates. Name them something like "plt1," "plt2," and "plt3" (shorter is better).

Protocol A: Add a group to plt1. Name it "yymmdd Prot A" then add 1 tube. Protocol B: Add a group to plt1. Name it "yymmdd Prot B" then add 13 tubes. Protocol 1: Add a group. Name it "yymmdd Prot 1" then add 16 tubes. Protocol 2: Add a group. Name it "yymmdd Prot 2" then add 32 tubes.

Edit Experiment	arkers Keywords Acquisition Load	late (U		tom	in o	ur ki	t)								
arrier Type: 📲 96 U-Bottom	▼ Add Plate ✓ Collapse All	📋 Delet	e	🕅 Refe	rence Gi	roup				5.	Add	Wel	ls as	Grou	р
	2. Add Plate	plt1	୩ <u>୫</u> ୨	6 U-Bott	om		*							\searrow	
Group_001 A9 Well 001	2. Add Flate	- ·	1	2	3	4	5	6	7	8	9	10	11	12	0
Name		А	\bigcirc	0	0	0	0	0	0	0					
A10 Well_002 A11 Well_003	6. Name Groups:	в	\circ												
A12 Well_004	yymmdd prot #		\cup								S I	S	S I	s	
B9 Well_005		С	\odot								● s	●_s	●_s	●_s	
B10 Well_006		D	\bigcirc												
B11 Well_007		E	\circ												
B12 Well_008			č								S I	S I	s s	s 1	
C9 Well_009	Files cannot easily	F	0								S	⊂ _s	⊂ _s	⊂_s	
C10 Well_010	be (re)named here.	G	\bigcirc								O			⊂,	
C12 Well 012		н	\bigcirc												
Group Properties]							-	_		-s	S	-s	S	
Sroup Name Sroup_001	Type Sample 👻			t We (see					ol)					again tab).	

Click Next. Click Next (Skip Markers tab).



c. Keywords Tab – Keywords

Drag the "Cellarcus Keywords" group over to the top row of the empty keyword column.

Edit Evporiment		Edit Mult	iple Key	word(s)) at once	
• Edit Experiment Fluorescent Tags Groups Markers Ke	eywords		Loader Settin		1. Drag to Keyword C	
/alue						Keywords
Name	Keyword	Value	Keyword	Value	er Keyword Columns	Default Keywords
YYMMDD - Instrument - Template - w	\$CELLS	Value	\$SMNO	value	scom	 Cellarcus Keywords
						\$CELLS
✓ ^{NB} ob plt1 copy	\$CELLS		\$SMNO		\$COM	\$SMNO
✓ ■ Reference Group	\$CELLS		\$SMNO		\$COM	\$COM
A1 Unstained (Beads)	\$CELLS	nanoCal800	\$SMNO		\$COM	\$PROJ
A2 buffer (Cells)	\$CELLS	buffer	\$SMNO		\$COM	\$SRC
B1 vFRed (Cells)	\$CELLS	lipo100	\$SMNO	vFRed	\$COM	Ell autobre and a
B2 BV421 (Beads)	\$CELLS	nanoCal800	\$SMNO	BV421	\$COM	Fill out keywords for each sample
C1 BV510 (Beads)	\$CELLS	nanoCal800	\$SMNO	BV510	\$COM	(before you run
C2 BV605 (Beads)	\$CELLS	nanoCal800	\$SMNO	BV605	\$COM	collect the data)
D1 GFP (Beads)	\$CELLS	nanoCal800	\$SMNO	GFP	\$COM	
D2 Alexa Fluor 488 (Beads)	\$CELLS	nanoCal800	\$SMNO	AX488	\$COM	
E1 PE (Beads)	\$CELLS	nanoCal800	\$SMNO	PE	\$COM	
E2 PE-Dazzle594 (Beads)	\$CELLS	nanoCal800	\$SMNO	PE-Dz	\$COM	
F1 PE-Cy7 (Beads)	\$CELLS	nanoCal800	\$SMNO	PE-Cy7	\$COM	2. Next

Click Next.

d. Acquisition Tab – Set Acquisition Settings

Protocol A - Set Stopping Time to 30 seconds. Set Events to Record to 100,000.

Protocol B – For bead wells (A1-F2), set Stopping Time to 10,000 seconds. Set Events to Record to 5000. For vFC Buffer and Lipo100+vFRed wells (F1,F2) set Stopping Time to 120 seconds. Set Events to Record to 100,000.

Protocols 1 & 2 – Set Stopping Time to 120 seconds. Set Events to Record to 100,000.

Fluorescent Tags Groups Marl	ers Keyword	s Acquisition					Stoppi	ocols A & B ng Time: 10,000 to Record: 5,00			otocols 1 & 2 ping Time: 120	
Tube/Well Specific User Setting		nce Group Plate of prot B for unn									ts to Record: 10	0,000
Name	Worksheet	Stopping Gate	Storag	e Gate		Events To	Record	Stopping Volume (ul)	Stopping	Criteria	Stopping Time (sec)	User Set
YYMMDD - Instrument - Template - t	Default Raw 👻	All Events	→ All Eve	ts	1	1 - 20,000,0	00	3,000	Count 8	Volume	1 - 36,000	Cytel EV
 Reference Group 	Default Raw 👻	All Events	+ All Eve	its	/-	5,000		3,000	Count 8	Volume	10,000	Cytek EV
 yymmdd prot 0.1 	Default Raw 👻	All Events	→ All Eve	ts	-	5,000		3,000	Count 8	k Volume	10,000	Cytek EV
 yymmdd prot 0.3 	Default Raw 👻	All Events	→ All Eve	ts	+	5,000		3,000	Count 8	k Volume	10,000	Cytek EV
 yymmdd prot 1 	Default Raw 👻	All Events	✓ All Eve	ts	Ť	100,000		3,000	Count 8	k Volume	120	Cytek EV
 yymmdd prot 2 	Default Raw 👻	All Events	→ All Eve	ts	*	100,000		1 - 3000	Count 8	Volume	120	Cytek EV



For the Cytek Aurora and Northern Lights

Plates:

uorescent Tags Groups	Markers Keywo	rds Acquisition	Loader Settings		1. pl Stop Ever	ping	g Tin	ne: 1	10,0	00						
late List	(C	opy of plt0 fo	or unmixing 🗸 Collapse A	pl	t1 96 U-Ba	ttom										
YYMMDD - Instrument	Template - w				1	2	3	4	5	б	7	8	9	10	11	12
> to plt1 copy				-/ .												
> 👸 plt2						•										
> 🐮 plt3																
			2. plt1 & plt2 (Protocols 1 Stopping Time: 120	& 2)		•2 •5										
			Events to Record: 100,00			● s										
quisition Settings		L														
me Works	heet It Raw Worksheet (Ra	aw)	*		3	0										
	e Gate	Stopping Time (sec) 10,000		,	0											
300	ing Volume (ul)	Stopping Criteria														
er Setting rtek EV Settings 010924 👻													3	. Nex	ĸt	

Click Next.

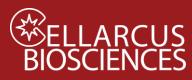
e. Loader Settings Tab – Set Run Order (Plate Only)

Click on Loader Settings on the right side of the screen (Plates only).

Specify the Run Order to be by Columns $\downarrow \downarrow \downarrow \downarrow$ (Plates only).

🖍 Edit Exper	iment	•
Fluorescent Tags G	roups Markers Keywords Acquisition Loader Settings	
Plate List		Collapse All
YYMMDD - In	istrument - Template - w	
ິ່ງອີ່ vor plt1.cop	у	
Refe	erence Group	1. Select Experiment Group to
s yym	nmdd Prot 0.1 nanoRainbows Copy	change all protocols at once
✓ 10 plt1		
s yym	nmdd prot 0.1	
s yym	nmdd prot 0.3	
ע שם plt2		
Loader Settings		
YYMMDD - Instru	2. Set to Run by Columns	
Default (System)	, 8	
	Save	
∃ Ш ≓ 1		
Mix Time	Mix Speed	
1	▼ 1500 ▼	
SIT Flush Times	Sample Recovery	
Single Flush	▼ Off ▼	
Stage Temperature Disabled	Record Data Delay Time	3. Save and Open
← Previous		Cancel X Save and Open

Click Save and Open (Plates only)



f. Setup vCal[™] Beads Worksheet – Define Acquisition Plots and Gates

Start with the default raw worksheet. Create the histograms and gates to assess the sample during data acquisition (**Table A2**). During data acquisition, the Singlet beads (Gate 1) will be adjusted to facilitate visual inspection during acquisition. Analysis of the data will take place in the appropriate vCal[™] Bead Report layout.

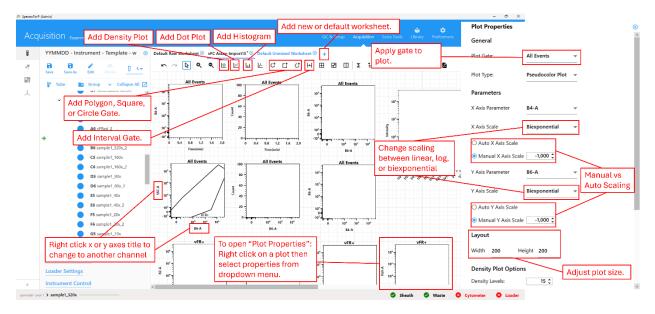


Table A2	
Plot/Gate: Description	Purpose
Plot 1: Time	Monitor fluidic stability
Plot 2: SSC-A vs FSC-A	Gate singlet beads
Gate 1: Singlet gate	Select single beads and exclude doublets and background
Plots 3-n: Bright Channels*	Individual fluorescence channel intensity resolution

*Bright Channels (5 Laser): R1, R2, B1, B2, B5, YG1, YG3, YG9, V1, V7, V10

*Bright Channels (3 Laser): R1, R2, B1, B2, B4, B6, B13, V1, V7, V10

*Bright Channels (1 Laser): B2, B4, B6, B13

g. Setup vFC Assay Worksheet – Define Acquisition Plots and Gates.

Start with the default worksheet since it includes all the parameters. Ensure the following 1and 2-parameter plots are present and draw gates to view data during acquisition (**Table A3**). These plots are only for visual inspection during acquisition. Analysis of the data will take place in the appropriate vFC[™] Report layout.

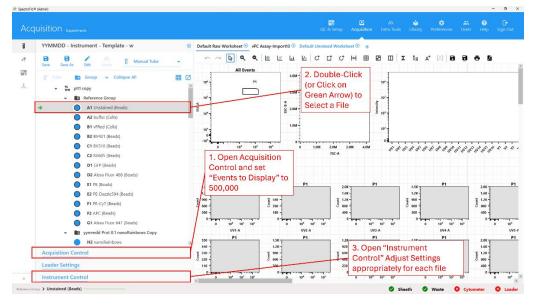


For the Cytek Aurora and Northern Lights

Table A3	
Plot/Gate: Description	Purpose
Plot 1: VSSC-A vs Time	Monitor fluidic stability
Plot 2: Time	Monitor fluidic stability
Gate 1: Time gate	Exclude the first 20 sec and collect the final 100 sec.
Plot 3: vFRed-A vs vFRed-H	Monitor event pulse characteristics
Gate 2: Pulse width gate	Exclude short pulse width background events
Plot 4: vFRed-A vs VSSC-H	Monitor event vFRed and VSSC intensities
Gate 3: Vesicle gate	Select events characteristic of single vesicles, exclude high- and low-VSSC events.
Plot 5: PE-A vs vFRed-A	Monitor PE immunofluorescence
Gate 4: PE positive	Estimate number of "PE positive" events
Additional Plots and Gate	For additional immunofluorescence channels as needed/desired

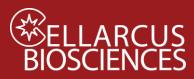
h. Instrument Control – Set Instrument Settings

Click on Acquisition Control below the Plate Window under Acquisition.



In the Acquisition dialogue, set Events to Display to 500,000 to ensure that all data are displayed.

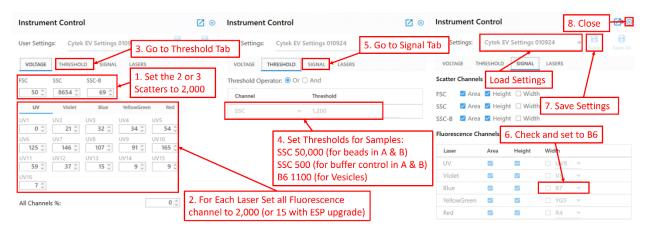




Under Instrument Control – Voltage set the gain for all of the scatters to 2000 and set the gain for all of the fluorescence channels to 2000.

Under Instrument Control – Threshold set the channel to the vFRed Parameter (B6-A) and set the threshold to 1100.

Under Instrument Control – Signal make sure B6-W (vFRed) is chosen for the width values.



You may choose to save/load the Instrument Control Settings file (see screenshot above). This shouldn't be necessary since the Instrument Control Settings are saved with the well/tube slots in the experiment.

Table A4				
Dialogue	Setting	vCal [™] beads	Buffer Bkgd	vFC [™] assay
Acquisition	Active Channels	All channels	All channels	All channels
Settings				
	Scatter gain	2000	2000	2000
	(FSC, SSC and SSCB)	(SSC: 15 w/ESP)	(SSC: 15 w/ESP)	(SSC: 15 w/ESP*)
	Fluorescence gain	2000	2000	2000
	(all channels)			
	Primary Threshold:	SSC-H	SSC-H	В6-Н
	Manual Threshold:	50,000	500	1100
	Width Parameter:	B6-W	B6-W	B6-W
Stopping Rules	Time to Record	Checked: 30 sec	Checked: 30 sec	Checked: 120 sec
	Events to Record	Unchecked	Unchecked	Unchecked
Acquisition	Events to Display	500,000	500,000	500,000
	Sample Flow Rate	High	High	High
* Enhanced Sm	all Particle (ESP) detect	tor option installed.		

i. Save As – Save the data acquisition template

Click Save As to save a Template. Give the template a descriptive name so it won't be confused with any other templates.



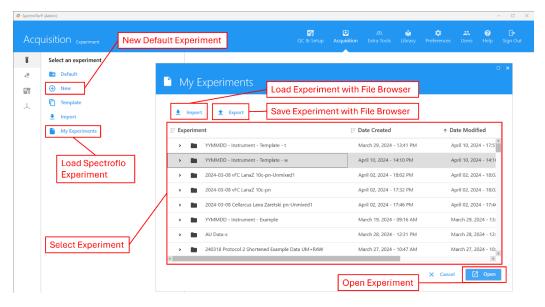
Various settings that should be associated with the template are summarized below (if you did the previous steps correctly).

Appendix B. Data Acquisition

1. vCal nanoRainbow and nanoCal antibody capture bead measurement

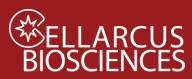
a. Load Assay Template and Settings

If you downloaded a Cellarcus SpectroFlo Experiment Template and haven't yet saved it as a template, load it then click Save As to save a template. Give the template a descriptive name so it won't get confused with any other templates.



Load an experiment template configured for either plates or for tubes (downloaded from Cellarcus or produced as described in Appendix A). The template should have your instrument set up with the appropriate Instrument Control Settings applied to the relevant sample tubes/wells.

• At the Import Options dialog box, check "Import as new item" to avoid overwriting previous versions.



For the Cytek Aurora and Northern Lights

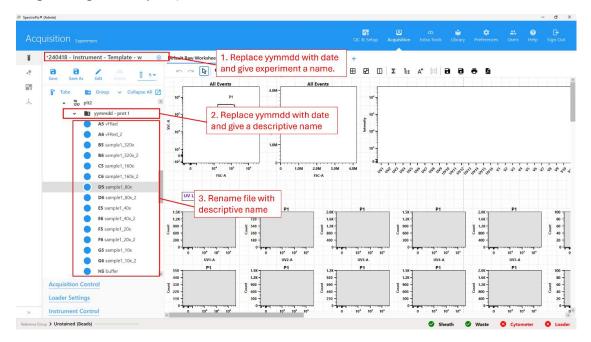
Select an experiment Default		🛛 Expe	eriment Templates			
New Template		Experime	ent	I≓ Created By	I≓ Date Created	Description
Import Im		> m	3 Laser Template	Admin	March 15, 2024 - 10:42 AM	
My Experiments		> m	Cellarcus Spectroflo Template	Admin	March 21, 2024 - 08:18 AM	From SBP Experiment
			Default	Admin	November 05, 2023 - 15:27 PM	Default Experiment
		> m	Experiment Template_001	Admin	April 19, 2024 - 09:23 AM	
2. Select either Well or		> m	Protocol 0.1 nanoRainbows Template	Admin	December 23, 2023 - 11:40 AM	
Tube Template to use to		> m	Template Test	Admin	April 19, 2024 - 09:51 AM	
Create New Experiment	\searrow	> 🖿	YYMMDD - Instrument - Template - t	Admin	April 19, 2024 - 09:25 AM	Cellarcus Spectroflo Template for Tube:
		> m	YYMMDD - Instrument - Template - w	Admin	April 19, 2024 - 09:24 AM	Cellarcus SpectroFlo Template for Wells
		> •	yymmdd Houdini Protocol 2 EV Cargo MGG	Admin	January 31, 2024 - 19:33 PM	
						3. Open Template as New Experiment

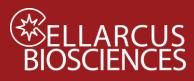
b. Set Experiment Name, Group Name(s), and File Names

Give your experiment a name. Include the date of the experiment at the beginning in the form of YYMMDD (so sorting by name sorts by date of experiment). Include some information about the experiment as well.

Edit group names so that the YYMMDD part is replaced with the date the group (possibly same as experiment date above) will be run.

Double-click each well/tube and rename to include the sample, any dyes added, and the date (note that there is an option to auto-add the date but it will also include time and group, lengthening the file path).





c. Fill in Keyword Values

Open the Edit Experiment window then go to the Keywords tab.

YYMMDD - Instrument	Fluorescent Tags Groups Markers Keyv	vords Acquis			values with sa	тріе туре
Save As	Value yymmdd prot 2 EV Cargo				Order Keyword Columns	Keywords
Tube Group	Name	Keyword	Value	Keyword	Value	 Default Keywords
E9 san	C9 lipo100+vFR	\$CELLS	lipo100	\$SMNO	viRed *	 Cellarcus Keywords
1. Edit • E11 58	C10 lipo100+vFR_2	\$CELLS	lipo100	\$SMNO	vFRed	
E12 sa	C11 lipo100+marker	\$CELLS	lipo100	\$SMNO	vFRec; [list other mark	
 F9 san F10 sa 	C12 lipo100+marker_2	\$CELLS	lipo100	SSMNO	vFRec; [list other mark	3. Replace [list other
F11 sa	D9 PLT+vFR	\$CELLS	PLT	SEMNO	vFRec	markers] with the markers
F12 sa	D10 PLT+vFR_2	\$CELLS	PLT	\$SMNO	vFRec	included in the sample.
G9 sar	D11 PLT+marker	\$CELLS	PLT	\$SMNO	vFRec; [list other mark	
G11 s	D12 PLT + marker_2	\$CELLS	PLT	\$SMNO	vFRec; [list other mark	
G12 s	E9 sample1+vFR	\$CELLS	[type1 of ev]	\$SMNO	vFRec	
H9 sar	E10 sample1+vFR_2	\$CELLS	[type1 of ev]	\$SMNO	vFRec	
H10 sa	E11 sample1+marker	\$CELLS	[type1 of ev]	\$SMNO	vFRec; [list other mark	
H12 si	E12 sample1+marker_2	\$CELLS	[type1 of ev]	\$SMNO	vFRec; [list other mark	
Acquisition Control	F9 sample2+vFR	\$CELLS	[type2 of ev]	\$SMNO	vFRec	4. Save and Open

The first keyword to edit is \$CELLS which should include particle type (eg. type of ev, type of bead, or type of cell). If you are using the Cellarcus SpectroFlo Template, edit any values in brackets which correspond to tube/well of each sample.

The second keyword is \$SMNO which should contain the fluorescent markers included in the sample. If you are using the Cellarcus SpectroFlo Template, replace the brackets with the list of dyes.

The third keyword is \$COM is for comments and you may choose to include any extra information that you want included in the data file for a given sample.

The fourth keyword is \$PROJ which is for the experiment name and will likely be the same for all samples of a given experiment.

The fifth and final keyword is \$SRC which is for the source of the sample. For standards provided with our kit it could be something like "Cellarcus Biosciences" (which is the default).

d. [Protocols 1&2 Only] Adjust Trigger Channel Threshold.

Load a well of vFC[™] Staining Buffer and allow system to run for 15 seconds on high flow rate (60ul/min). 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. Apply this threshold to all wells/tubes in Protocols 1 and 2 and to the vFRed control well/tube in Protocol B. Save the Template file (.expt) for future use. In general, once optimized the threshold does not need to be changed every time you run an experiment.

e. Auto Record data.

You are now ready to run samples. Click Auto Record to run the selected Sample wells.