

# Protocol B. Fluorescence Unmixing and Calibration

#### Objective

Calibrate the spectral and intensity response of the instrument to enable spectral unmixing to determine the intensity of each fluorochrome and calibrate the intensities to report fluorophore abundance in absolute units of antibody binding capacity (ABC) or mean equivalent surface area (MESA).

#### **Materials**

- nanoCal<sup>™</sup> Antibody Capture Beads (800 nm diameter polystyrene, 1x10<sup>6</sup>/mL) •
  - Prestained nanoCal beads, freeze-dried (CBS7Fluor-2T, where Fluor is the stain)
  - User-stained nanoCal beads (see Appendix A)
- vFRed<sup>™</sup>-stained Lipo100<sup>™</sup> Vesicle Size Standard
  - o prepared and measured using Protocol B2 (Appendix B), or
  - measured as part of Protocol 1 or Protocol 2
- vFC<sup>™</sup> Staining and Dilution Buffer
- v-bottom plate for measurement (Sartstedt 82.1583.001)
- Cellarcus SpectroFlo Template Aurora.zip
- Protocol B Fluorescence Calibration Layout for FCS Express Reader (Layout for FCS Express)
- Protocol B Vesicle Size Calibration Layout for FCS Express Reader (Layout for FCS Express)

### Procedure

1. Acquire calibration data for:

- 1a. Immunofluorescence using nanoCal<sup>™</sup> beads and
- 1b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.
- 2. Perform spectral unmixing of the data to calculate the intensity of each fluorochrome.
- 3. Calibrate the intensities of the unmixed fluorochromes and validate the intensity calibration for:
  - 3a. Immunofluorescence using nanoCal<sup>™</sup> beads and
  - 3b. Vesicle size using vFRed-stained Lipo100 Vesicle Size standard.



### B1. Data Acquisition

#### B1a. nanoCal<sup>™</sup> Beads

- 1. Open a New Experiment by opening the vFC<sup>™</sup> Assay Experiment Template (downloaded from the Cellarcus web site or created during Instrument Setup, see Appendix A). The Experiment Template is set up to accommodate 10 different fluorophores, plus vFRed<sup>™</sup> and buffer controls. If you plan to use fewer fluorophores, you will need to Edit Experiment and delete the unused fluorochromes.
- 2. Reconstitute pre-stained nanoCal<sup>™</sup> beads in vFC buffer according to the instructions (typically 100 uL).
- 3. Place each nanoCal<sup>™</sup> bead in the indicated wells of a 96-well plate for flow cytometer measurement (Figure 1).
- 4. Fill one well with 300uL of vFC buffer without beads.
- 5. If running Protocol B2 vFRed Vesicle Size Calibration, follow the instructions in Appendix B. If running Protocol 1 or Protocol 2, these samples will be generated and measured as part of those Protocols, and that data can be used during data analysis.
- 6. Select the first bead well on the sample list and click Start. Inspect the light scatter plot to make sure the beads are on scale and singlets can be gated.
- 7. Select Well A1, set the flow rate to High, and Record all samples. Note: The first well (A1 buffer SSC 500) will be collected with a low SSC scatter trigger to collect the instrument background, wells A2-F2 and A12 (nanoCal and nanoRainbow beads) will be collected with a high SSC trigger, to measure the bead signals, and wells G1 and G2 (Buffer and Lipo100+ vFRed) will be collected with a B6-A trigger, to measure signals from vesicles (if being run in this Protocol).



8. Save the Experiment with a descriptive file name that includes the date.

2



# **LLARCUS Fluorescence** Unmixing and Calibration

for the Cytek Aurora and Northern Lights

#### B1b. Lipo100 Vesicle Size Standard

Measurement of the Lipo100 Vesicle Size Standard is part of every vFC<sup>™</sup> 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.** Spectral Unmixing

Spectral unmixing determines the intensity of each fluorochrome used in a measurement and is performed before calibration data analysis.

Spectral unmixing can be performed using the Cytek SpectroFlo software using the Spectral Reference samples measured in above in B1, as described in Appendix C or using suitable thirdparty flow cytometry analysis software.

Control	Control Type	Fluorescent Tag
vFRed (Cells)	Single Stained	vFRed
BV421 (Beads)	Single Stained	BV421
BV510 (Beads)	Single Stained	BV510
BV605 (Beads)	Single Stained	BV605
GFP (Beads)	Single Stained	GFP
PE (Beads)	Single Stained	PE
PE-Dazzle594 (Beads)	Single Stained	PE-Dazzle594
PE-Cy7 (Beads)	Single Stained	PE-Cy7
Alexa Fluor 647 (Beads)	Single Stained	Alexa Fluor 647
Reference Group - Unstained (Beads)	Unstained	N/A
Reference Group - Buffer (Cells)	Unstained	N/A

Figure 2. Spectral References for Unmixing



# **ELLARCUS Fluorescence Unmixing and Calibration**

for the Cytek Aurora and Northern Lights

## **B3.** Data Analysis

#### B3a. Data Analysis – Immunofluorescence Calibration

- 1. Following spectral unmixing, open the vFC<sup>™</sup> Protocol B Fluorescence Calibration Layout using FCS Express or FCS Express Reader and load the appropriate data files into each plot.
- 2. Use the bivariate plot of scatter parameters (eg VSSC vs SSC) to identify and gate on the population of single beads (Figure 3).







- 3. 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.
- 4. Use the nanoCal Bead ABV 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 FLmAbs bound per EV (Figure 4).

	lion mormat								
D	Parameter	Equation	F:P	R	Min.	# Decades	Active	Edit	Create calibration for PE-A ~
	PE-A	y = 0.02x + 7.95	1	0.999	-9.34	0.0	$\checkmark$	Remove	Calculate regression
	APC-A	y = 0.22x -21.47	1	0.998	-414.09	0.0	$\checkmark$		Calculate regression from $$ 478 - Histogram - 02-20220309 nanoCe $$ $$
	FITC-A	y = 0.03x + 14.79	1	0.998	-29.71	0.0	$\checkmark$	Save	
	BV421-A	y = 0.03x + 11.71	1	1.0	-88.57	0.0	$\checkmark$	Lood	<sup>196</sup> ] M1 M2 NB
	BV510-A	y = 0.1x -15.12	1	1.0	-104.57	0.0	$\checkmark$	LUau	147-1975 11483 17704
	PE594-A	y = 0.03x + 5.13	1	0.999	-21.21	0.0	$\checkmark$		ð •••
	PC7-A	y = 0.04x + 9.95	1	0.999	-20.9	0.0	$\checkmark$		
	BV605-A	y = 1.21x -112.93	1	0.998	-1287.42	0.0	$\checkmark$	ОК	-10 <sup>°</sup> 10 <sup>°</sup> 10 <sup>°</sup> 10 <sup>°</sup> 10 <sup>°</sup> PE-A
<							>	Cancel	Overlay Overlay #1 - 02-20220309 nanoCal PE-C4.fcs (Parame V
∰E BIC	:LLARC DSCIEN(	CUS CES		vCal	™ nanoCa	l™ Antibod	y Capture-b vCal™ assa CBS7M	eads-Mouse ay calibrator	Number of peaks to use for regression     3       Markers     Molecules/Bead       Marker 1     47       Marker 2     270
€ € BIC 505 Coat	LLARC DSCIEN( 1st Blvd 5. Ste 409 L	US CES 1 Jolia, CA		vCal	™ nanoCa	l™ Antibod	y Capture-b vCal™ assa CBS7M	eads-Mouse ay calibrator I Lot 220127	Mumber of peaks to use for regression     Image: Comparison of the second
BIC		CUS CES a Jolie, CA C	ertifi	vCal	™ nanoCa Analysi	I™ Antibod <sup>,</sup> S	y Capture-b vCal™ ass: CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression     3       Markers     Molecules/Bead       Marker 1     47       Marker 2     270       Marker 3     1400
505 Coal	LLARC DSCIENC 15t Blvd 5. Ste 409 L t Description	CUS CES a Jolia, CA Co Mouse in	ertifi	vCal	™ nanoCa Analysi	I™ Antibod <sup>,</sup> S	y Capture-b vCal™ assı CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression 3 Markers Molecules/Bead Marker 1 47 Marker 2 270 Marker 3 1400 Calculate
Specificit Concentr	LLARC DSCIENC set Blvd 5. Ste 409 L t Description ty ration	CUS CES a Jolia, CA C Mouse Ig 1.1.0° beads/ml	ertifi	vCal	<sup>™</sup> nanoCa Analysi	I™ Antibod <sup>,</sup> iS	y Capture-b vCal™ ass: CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 : 1
Sos coar	LLARC SCIENC and Blvd 5. Ste 409 L t Description ty ration	US p Jolio, CA Mouse Ig 1x10° beads/ml	ertifi	vCal	<sup>™</sup> nanoCa Analysi	I™ Antibod	y Capture-b vCal™ assa CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 : 1         Calibrated suffix       1
SOS Coal	LLARC DSCIENC and Blod 5. Site 409 L to Description ty ration	Dollo, CA Mouse Ig Ix10 <sup>e</sup> beads/ml	ertifi	vCal	™ nanoCa Analysi	I™ Antibod	y Capture-b vCal™ assa CBS7M	eads-Mouse ay calibrator   Lot 220127	Number of peaks to use for regression   Markers   Marker 1   47   Marker 2   270   Marker 3   1400     Calculate   Dye molecules : Antibodies   1   Calibrated suffix     Use calibration
Sos coas sos coas specificit concentr	LLARC SCIENC ast Blvd 5. Ste 409 L t Description ty ration tions toonetry	Mouse ig 1x10° beads/ml	ertifi	vCal cate of	™ nanoCa Analysi	I™ Antibod <sup>,</sup> is	y Capture-b vCal™ assi CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 :       1         Calibrated suffix
sos coas sos coas pecificit concentr pplicat	t Description ty rations tometry inding Sites	Mouse ig 1x10° beads/ml	ertific	vCal cate of	™ nanoCa Analysi	I™ Antibod S	y Capture-b vCal™ ass: CBS7M	eads-Mouse ay calibrator Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 : 1         Calibrated suffix
sos coal specificit Concentr Flow Cyto No. of Bin Peak	t Description ty tions tions tions tions timing Sites	Mouse (g 1x10° beads/ml	ertific	vCal cate of	™ nanoCa Analysi	I™ Antibod <sup>1</sup> S	y Capture-b vCal™ ass: CBS7M	eads-Mouse ay calibrator Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 : 1         Calibrated suffix
sos coas sos coas specificit Concentr Policat Flow Cyto No. of Bin Peak Peak Peak	t Description ty ration tions tions times tik times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times times ti ti ti ti ti ti ti ti ti ti ti	Mouse Ig 1x10 <sup>a</sup> beads/ml 50 ul/test (see prote 0 47 270	ertific	vCal cate of	™ nanoCa Analysi	I™ Antibod <sup>,</sup> is	y Capture-b vCal™ assa CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 :       1         Calibrated suffix
505 Coard 505 Co	t Description t Description ty tation tions tions tions k 1 k 2 k 3	Mouse ig 1x10° beads/ml 50 ul/test (see prote 0 47 270 1400	ertifi bcol 0.3 in t	vCal cate of	™ nanoCa Analysi	I™ Antibod <sup>,</sup> is	y Capture-b vCal™ assi CBS7M	eads-Mouse ay calibrator I Lot 220127	Number of peaks to use for regression       3         Markers       Molecules/Bead         Marker 1       47         Marker 2       270         Marker 3       1400         Calculate         Dye molecules : Antibodies       1 :       1         Calibrated suffix

- 5. Save the Channel Calibration file: Fluor Calibration Aurora YYMMDD Panel (eg. Fluor Calibration Aurora -220310 - PEx3 BVx3 APC FITC.cal)
- 6. Load the Channel Calibration file \*Channel Calibration >Load), so that its name will appear in the File Information header) and inspect the recovery of standards on the nanoCal - cal tab (Figure 5).



# **Fluorescence Unmixing and Calibration**

for the Cytek Aurora and Northern Lights



Figure 5. Fluorescence calibration using nanoCal<sup>™</sup> beads. A. Single beads are gated by their light scatter. C. The fluorescence axes calibrations are assessed via recovery of standards.



#### B3b. Data Analysis - Vesicle Size Calibration

- 7. On the Aurora, unmix the data using the Cytek SpectroFlo software as described in Fluorescence Unmixing and Calibration.
- 1. Open the vFC<sup>™</sup> 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<sup>™</sup>** 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<sup>™</sup> data file and click Change Data On All Plots. Inspect the Gating Plots. Adjust Gates as needed to select vFRed<sup>™</sup>-positive events (Figure 1A-C).





- 3. Open the vFC<sup>™</sup> 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.



8



- 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 (yint).
- 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<sup>™</sup> Layout as: vFC Protocol B Vesicle Size Calibration Instrument Date Samples (eg. "vFC Protocol B Vesicle Size Calibration – Aurora – 220401 – lipo100 B9.fey."

Transformations	×					
	2					
📲 Vesicle Size Calibration - U0481 - 240318 - C4 lipo100.fcf						
	Edit Sequence Item					×
	Parameter Information					
		Simple				
Parameter Options	Output Parameter	First Inpu	Parameter	+ ~	Second Input P	Parameter
Template File: lipo100+vFR.fcs	Output Parameter Name					
5ate Options	Surface Area (nm2)					
Gate No Gate ~		Formula				
Fransformed Data Options		Custom	Token: find_	vFRed*28.4	18-4581.5956	
Display Name Suffix Parameter Math applied		Evaluate to	ens before ac	lding to data		
Fransformation Options						
Formula Sequence					OK	Cancel
Surface Area (nm2) = P21*28.48-4581 5956						
Diameter (nm) = if $P168 > 0$ then $2^{*}$ sqrt( $P168/(4^{*}3.14159)$ ) else $-2^{*}$ sqrt(abs( $P168)/(4^{*}3.14159)$ )						
B6-A/B5-A = P84/P81						
New Edit Delete						
in a Transformation and Domainstan Math. Dist.						
igure 3. Transformations and Parameter Math Dialo	gue.					



# **ELLARCUS** BIOSCIENCES Fluorescence Unmixing and Calibration for the Cytek Aurora and Northern Lights

### Notes



## Appendix A. Staining nanoCal antibody capture beads

Specific antibody conjugates can be evaluated using nanoCal antibody capture beads and used for intensity and spectral calibration. This is recommended when using custom conjugates or antibodies from venders other than Cellarcus.

#### Materials

- nanoCal<sup>™</sup> Antibody Capture Beads (800 nm diameter polystyrene, 1x10<sup>6</sup>/mL) Note: Calibrated beads are available for each of mouse, rat, rabbit, and hamster antibodies.
- Fluorescent antibody conjugate(s) .
- vFC<sup>™</sup> Staining and Dilution Buffer
- 1.5 mL microfuge tubes for staining and washing

#### Procedure

- 1. Vortex Capture Beads for 10 seconds.
- 2. Add one drop (~50 uL) of nanoCal<sup>™</sup> 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<sup>™</sup> 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 B3. vFRed<sup>™</sup>-stained Lipo100<sup>™</sup>

#### Objective

Protocol B2 calibrates the relationship between vesicle membrane surface area and vFRed<sup>™</sup> fluorescence using Lipo100<sup>™</sup>, 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<sup>™</sup> Staining Buffer
- b. Lipo100<sup>™</sup> Standard
- c. vFRed<sup>™</sup> membrane stain
- d. Microwell plate (Sartstedt 82.1583.001)
- e. Gloves
- f. vFC<sup>™</sup> Vesicle Size Calibration Layout (FCS Express)

#### Procedure

#### **Prepare Working Solution**

- Prepare vFRed<sup>™</sup> membrane stain working solution (5 uL/well, +1 well) 1.
  - a. 25 ul/5 wells: 2.5 µL vFRed<sup>™</sup> (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<sup>™</sup> vesicle size standard

Table 1.1 Staining reactions									
Well	Buffer	Lipo100 <sup>TM</sup>	vFRed <sup>TM</sup>	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  $\mu$ L 10x vFRed<sup>TM</sup> to Rows B and C.
- 4. Mix well, incubate 1 hour at RT in the dark.

Dilute 2 Dilute 1 Staining Dilution 1 Read B D Ε F G

12



- 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 D).

#### **Dilute and Read**

- Dilute the staining reaction according to Table 1.2. 4.
  - 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.
- 5. Run the wells in Columns 5-6 at the indicated sample flow rate for 120 seconds each.

Table 1.2 Post	t-stain dilution and run
Wells	Aurora
Staining	50 uL
Dilution 1	8 μL → 245 uL
Dilution 2	8 μL → 245 uL
Post-stain Dilution	1000
Run	High 60 uL/min



# Appendix C. Unmixing in SpectroFlo spectral mode (v3.2.1)

SpectroFlo spectral mode uses the proprietary unmixing matrices and algorithms of the SpectroFlo software and the appropriate single component controls, to generate new fcs files with parameters containing the estimated fluorochrome abundances, as described in the following sections. These new fcs files are then used for intensity calibration (Protocol B) and EV analysis (Protocols 1 and 2). You should have set up the fluorescent tags and added the reference group (see step 2a and step 2c on pages 2 to 3 above). These are needed for the unmixing.

#### a. Load Experiment then Start Unmixing

Make sure the experiment to be unmixed is loaded. If you have just acquired data on current worksheet you probably don't need to load anything. Otherwise load experiment to be unmixed.

SpectroFlo®	(Admin)			- 0
Acqı	uisition Experiment	QC & Setup	Acquisition Extra Tools Library	
8 ••	Select an experiment	My Experiments		. x
۰,	New     Template	± Import ± Export	1. Acquisition Tab	
	• Import	I≓ Experiment	‡∃ Date Created	↑ Date Modified
	My Experiments	> 2024-03-08 Cellarcus Lana Zaretski pn-Unmixed1	April 02, 2024 - 17:46 PM	April 02, 2024 - 17:46 PM
		YYMMDD - Instrument - Example	March 19, 2024 - 09:16 AM	March 29, 2024 - 13:40 PM
	2. Experiment Browser	> AU Data-s	March 28, 2024 - 12:31 PM	March 28, 2024 - 12:31 PM
		> 🖿 240318 Protocol 2 Shortened Example Data UM+RAW	/ March 27, 2024 - 10:47 AM	March 27, 2024 - 10:47 AM
		> 240318 Protocol 2 Shortened Example Data-Unmixed	1 March 26, 2024 - 14:22 PM	March 26, 2024 - 14:22 PM
		> 240119 Prot 0.3 Example APC GFP-Unmixed1	March 26, 2024 - 12:23 PM	March 26, 2024 - 12:23 PM
	3. Select Experiment	> 240119 Prot 0.3 Example APC GFP	January 23, 2024 - 13:28 PM	March 26, 2024 - 12:20 PM
		> 240119 Prot 0.3 Example All Colors	March 26, 2024 - 12:15 PM	March 26, 2024 - 12:15 PM
		> 240208 vFC Assay FSEV workshop+KWs	February 13, 2024 - 16:22 PM	March 25, 2024 - 17:27 PM
		> yymmdd Houdini Protocol 0.3 nanoCal MGG3	March 21, 2024 - 22:16 PM	March 21, 2024 - 22:16 PM
		> yymmdd Protocol 2 EV Cargo	March 21, 2024 - 22:15 PM	March 21, 2024 - 22:15 PM 4
		¢		4. Open
>				X Cancel 🖸 Open

#### b. Load Reference Controls

In addition to the data you have collected there is a separate group called "Reference Group" which wells/tubes for each file needed for unmixing. To load a file into the "Reference Group" click on the well/tube to select it then right click on it again and choose "Import FCS File" to open file selection dialog.

Load the scatter buffer only into the buffer control well (or tube). Load a lipo100+vFRed only into the vFRed well (or tube). Load the stained bead controls into the appropriate well (or tube).

Click on the Unmix button (under acquisition tab).



# ELLARCUS Fluorescence Unmixing and Calibration

for the Cytek Aurora and Northern Lights



#### c. Select Controls – Set Control Type and Assign Negative Controls

If you created the worksheet properly then you should see two negative controls, one for "Cells" (EVs) and one for Beads. Make sure the vFRed positive control has the "Cells" negative control EVs assigned to it. The rest of the positive controls should use the unstained bead as a negative.

Ensure Unmixing Model is "Spectral Unmixing" (not "Spectral Unmixing with AF Extraction").



# LLARCUS Fluorescence Unmixing and Calibration OSCIENCES for the Cytek Aurora and Northern Lights

for the Cytek Aurora and Northern Lights

Let Control       Mantify Positive/Negative Populations       QC Controls         UNSTAINED CONTROL MATCHING SAMPLE AUTOFLUORESCENCE       •         • Use Control form Library       •         • Use Control form Experiment       Reference Group - Unstained (Beads)         • Use Control form Experiment       Reference Group - Unstained (Beads)         • Use Control form Experiment       Beads         • Use Control form Experiment       Beads         • Use Control form Experiment       Beads         • StatNED CONTROLS       • Universal Negative         • From Library       Fluorescent Tag       Control         • VfRed       vfRed (Cells)       • Reference Group - Dustained (Beads)       ©         • BV421       BV421 (Beads)       • Reference Group - Unstained (Beads)       ©         • BV510       BV510 (Beads)       • Reference Group - Unstained (Beads)       ©         • BV505       BV605 (Beads)       • Reference Group - Unstained (Beads)       ©         • BV505       BV605 (Beads)       • Reference Group - Unstained (Beads)       ©       Unmixing Model should be "Spectral Unmixing"         • GFP       GFP       GFP (Beads)       • Reference Group - Unstained (Beads)       ©	Unmix E     Unmix     Unmix E     Unmix E	kperiment			
UNSTAINED CONTROL MATCHING SAMPLE AUTOFLUORESCENCE          Use Control from Library       •         Ise Control from Experiment       Reference Group - Unstained (Beads)         Name       Control Type         Reference Group - Unstained (Beads)       Beads         StainED CONTROLS       Buffer Only control for EV controls (usually only vFRed). Unstained control for bead controls.         STAINED CONTROLS       •         • From Library       Fluorescent Tag       Control         • VFRed       vFRed (Cells)       •         • BV421       BV421 (Beads)       •         BV510       BV510 (Beads)       •       Reference Group - Unstained (Beads)         • BV605       BV605 (Beads)       •       Reference Group - Unstained (Beads)       •         • GFP       GFP (Beads)       •       Reference Group - Unstained (Beads)       •       •         • GFP       GFP (Beads)       •       Reference Group - Unstained (Beads)       •       •       •         • os       BV605       BV605       •       Reference Group - Unstained (Beads)       •       •       •       •         • os       BV605       BV605       •       Reference Group - Unstained (Beads)       •       •       •       •       •         • os	lect Controls Iden	ntify Positive/Negative	Populations QC Cont		
UNSTAINED CONTROL MATCHING SAMPLE AUTOFLUORESCENCE     Use Control from Libray     Reference Group - Unstained (Beads)     Name   Control Type   Reference Group - Unstained (Beads)     Buffer Only control for EV controls   (usually only vFRed). Unstained   (usually only vFRed). Unstained   STAINED CONTROLS     STAINED CONTROL     Buffer Only control for EV controls   (usually only vFRed). Unstained					
Name Control Type   Reference Group - Unstained (Beads) Beads     Buffer Only control for EV controls   Usually only vFRed). Unstained   STAINED CONTROLS     From Library Fluorescent Tag   Control Universal Negative   Generic   VfRed vFRed (Cells)   Reference Group - Unstained (Beads)   BV510 BV510   BV510 BV510   BV510 BV510   BV510 BV605   BV605 BV605   BV605 BV605   BV605 BV605   BV605 BV605   BV605 Reference Group - Unstained (Beads)   BV510 BV510   BV510 BV510   BV510 BV605   BV605 BV605   BV50 Reference Group - Unstained (Beads)   BV50 BV605   BV50 Reference Group - Unstained (Beads)   BV50 BV50   BV50 Reference G	UNSTAINED CONTRO	DL MATCHING SAMPL	E AUTOFLUORESCENCE		
Buse Control from Experiment       Reference Group - Unstained (Beads)         Name       Control Type         Reference Group - Unstained (Beads)       Beads         Buffer Only control for EV controls (usually only vFRed). Unstained control for bead controls.         STAINED CONTROLS         Prom Library       Fluorescent Tag       Control         VFRed       vFRed (Cells) <ul> <li>Reference Group - Buffer (Cells)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV421</li> <li>BV421 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV510</li> <li>BV510 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605</li> <li>Reference Group - Unstained (Beads)</li> <li>Beforence Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV</li></ul>	O Use Control from Lib	rary		Ψ.	
Name       Control Type         Reference Group - Unstained (Beads)       Beads         Buffer Only control for EV controls (usually only vFRed). Unstained control for bead controls.         STAINED CONTROLS         • From Library       Fluorescent Tag       Control         • VFRed       vFRed (Cells)       • Reference Group - Buffer (Cells)       •         • WFRed       WFRed (Cells)       • Reference Group - Buffer (Cells)       •         • WFRed       BV421 (Beads)       • Reference Group - Unstained (Beads)       •         • BV510       BV510 (Beads)       • Reference Group - Unstained (Beads)       •       Unmixing Model should be "Spectral Unmixing"         • GFP       GFP (Beads)       • Reference Group - Unstained (Beads)       •       •         • Def (Beads)       • Reference Group - Unstained (Beads)       •       •	Use Control from Exp	periment Reference G	iroup - Unstained (Beads)		
Reference Group - Unstained (Beads)       Beads         Reference Group - Unstained (Beads)       Buffer Only control for EV controls (usually only vFRed). Unstained control for bead controls.         STAINED CONTROLS       Iniversal Negative       Generic         vFRed       vFRed       vFRed (Cells)       Reference Group - Buffer (Cells)       Image: Centrol Buffer Cells)         BV421       BV421 (Beads)       Reference Group - Unstained (Beads)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)         BV510       BV510 (Beads)       Reference Group - Unstained (Beads)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)         BV510       BV510 (Beads)       Reference Group - Unstained (Beads)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)         BV510       BV510 (Beads)       Reference Group - Unstained (Beads)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)       Image: Centrol Buffer Cells)         BV605       BV605 (Beads)       Reference Group - Unstained (Beads)       Image: Centrol Buffer Cells)	Name		Control Type		
STAINED CONTROLS         Image: From Library       Fluorescent Tag       Control       Universal Negative       Generic         Image:	Reference Group -	Unstained (Beads)	Beads	Buffer Only control for EV controls	
control for bead controls.         STAINED CONTROLS       Fluorescent Tag       Control       Universal Negative       Generic         Image: Im				(usually only vFRed). Unstained	
From Library       Fluorescent Tag       Control       Universal Negative       Generic         Image: Strain St	STAINED CONTROLS			control for bead controls.	
vFRed       vFRed (Cells)       r       Reference Group - Buffer (Cells)       s         BV421       BV421 (Beads)       r       Reference Group - Unstained (Beads)       s         BV510       BV510 (Beads)       r       Reference Group - Unstained (Beads)       s       Unmixing Model should be "Spectral Unmixing"         BV605       BV605 (Beads)       r       Reference Group - Unstained (Beads)       s       be "Spectral Unmixing"         GFP       GFP (Beads)       r       Reference Group - Unstained (Beads)       s       s         ns       DF (Beads)       r       Reference Group - Unstained (Beads)       s       s	From Library	Fluorescent Tag	Control	Universal Negative Generic	
BV421       BV421 (Beads) <ul> <li>Reference Group - Unstained (Beads)</li> <li>BV510</li> <li>BV510 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605</li> <li>Reference Group - Unstained (Beads)</li> <li>BC605</li> <li>BV605</li> <li>Reference Group - Unstained (Beads)</li> <li>BC605</li> <li>Reference Group - Unstained (Beads)</li> <li>Refe</li></ul>		vFRed	vFRed (Cells)	Reference Group - Buffer (Cells) 👻 🛛	<b>†</b>
BV510       BV510 (Beads) <ul> <li>Reference Group - Unstained (Beads)</li> <li>BV605</li> <li>BV605 (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>GFP</li> <li>GFP (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>Beference Group - Unstained (Beads)</li></ul>		BV421	BV421 (Beads)	🗸 Reference Group - Unstained (Beads) 👻 🗹	
BV605       BV605 (Beads) <ul> <li>Reference Group - Unstained (Beads)</li> <li>GFP</li> <li>GFP (Beads)</li> <li>Reference Group - Unstained (Beads)</li> <li>BE (Beads)</li> <li>BE</li></ul>		BV510	BV510 (Beads)	Reference Group - Unstained (Beads) 👻 🛛 Unmixing Model should	
GFP     GFP (Beads) <ul> <li>Reference Group - Unstained (Beads)</li> <li>DE</li> <li>DE (Peads)</li> <li>Peference Group - Unstained (Reads)</li> <li>Instained (Reads)</li> <li>Instained (Reads)</li> <li>Instained (Reads)</li> </ul>		BV605	BV605 (Beads)	Reference Group - Unstained (Beads) 🗸 🛛 be "Spectral Unmixing"	
DE DE (Basde) - Dafaranca Group - Unetsinael (Basde) - 🔯		GFP	GFP (Beads)	🗸 Reference Group - Unstained (Beads) 👻 🔽	
		DE	DE (Roade)	- Deference Grown - Unetsined (Reade) - 🛛	4
				Next Step × Cancel Next	÷

#### Click Next.

#### d. Identify Positive/Negative Populations – Adjust gates for each control

Adjust the bead scatter gates to include only singlets. If you hold down shift while adjusting the scatter gates for one bead it will apply that scatter gate to all beads.

Adjust the Cells (EV) scatter gate to include the main population. It helps to check the checkbox for log for these controls to switch to log scaling. This makes it easier to adjust the gates for dimmer particles.

Adjust the positive and negative gates. If possible, it is better to adjust positive gates to include only the brighter half of the positive peak. Similarly, it is better to adjust negative gates to include only the dimmer half of the negative peak. This improves Spectroflo's unmixing algorithm.



# ELLARCUS Fluorescence Unmixing and Calibration

for the Cytek Aurora and Northern Lights



Click "Live Unmix." Live unmixing can be done before collecting data to unmix as data is collected. Live Unmix can also be done after data is collected and in either case it includes both a raw and an unmixed copy of each data file. "Create New Unmixed Experiment" will create a new experiment with only unmixed data it will not however delete the old experiment which will still include the raw data. It is recommended that you use "Live Unmix."

#### Other SpectroFlo Help

#### a. Export an experiment along with data files (any experiment)

Saved experiments are stored inside spectroflo and if you want to send the whole experiment to someone or save it in a folder somewhere then you will need to export it first.

Start with your experiment open in the acquisition tab. Click Save in upper left corner of the screen to save any changes to your experiment before exporting it.

Click the x on the left side of the screen (next to the experiment name) to close your current experiment and go back to the Select an experiment screen.

Go to "My Experiments" then select the saved experiment that you wish to export then click Export to choose a save location and name.



# SellarcusFluorescence Unmixing and CalibrationBIOSCIENCESfor the Cytek Aurora and Northern Lights

Select an experiment     Default     Import	equisition Experiment			ूरि QC & Setup	Acquisition		📫 Library	🔅 Preferences	<b>XX</b> Users	? Help Sig
<ul> <li>Code Experiment with the browser</li> <li>Import</li> <li>My Experiments</li> <li>Load Spectroflo Experiment</li> <li>YVMMDD - Instrument - Template - t</li> <li>YVMMDD - Instrument - Template - w</li> <li>April 10, 2024 - 13:41 PM</li> <li>April 10, 2024 - 14:10 PM</li> <li>April 10, 2024 - 14:10</li> <li>April 10, 2024 - 17:5</li> <li>YVMMDD - Instrument - Template - w</li> <li>April 10, 2024 - 14:10 PM</li> <li>April 10, 2024 - 17:5</li> <li>YVMMDD - Instrument - Template - w</li> <li>April 10, 2024 - 14:10 PM</li> <li>April 10, 2024 - 14:10</li> <li>April 02, 2024 - 17:32 PM</li> <li>April 02, 2024 - 17:32 PM</li> <li>April 02, 2024 - 17:46 PM</li> <li>April 02, 2024 - 17:47 M</li> <li>April 02, 2024 - 12:31 PM</li> <li>April 02, 2024 - 10:47 AM</li> </ul>	Select an experiment Default (+) New	Nev	• Experiment My Experiments	ad Experime	ent with	File Brow	sor			• ×
My Experiments       IF       Date Created               P Date Modified          Load Spectroflo Experiment              YYMMDD - Instrument - Template - t       March 29, 2024 - 13:41 PM       April 10, 2024 - 17:5                 YYMMDD - Instrument - Template - w       April 10, 2024 - 14:10 PM       April 10, 2024 - 14:11                 YYMMDD - Instrument - Template - w       April 10, 2024 - 14:10 PM       April 10, 2024 - 14:11                 YYMMDD - Instrument - Template - w       April 10, 2024 - 14:10 PM       April 10, 2024 - 14:11                 YOMMDD - Instrument - Template - w       April 10, 2024 - 14:10 PM       April 10, 2024 - 14:11                 YOMMDD - Instrument - Template - w       April 10, 2024 - 14:10 PM       April 02, 2024 - 17:40                 YOMMDD - Instrument - Template - w       April 02, 2024 - 17:52 PM       April 02, 2024 - 17:40                 YOMMDD - Instrument - Example       March 19, 2024 - 09:16 AM       March 29, 2024 - 13:                 YOMMDD - Instrument - Example       March 29, 2024 - 12:        March 28, 2024 - 12:                 YOMADD - Instrument - Example       March 27, 2024 - 10:47 AM       March 27, 2024 - 10:47	<ul> <li>Template</li> <li>Import</li> </ul>		₹ Import € Export Sa	ve Experime	ent with I	File Brow	ser			
Load Spectroflo         Experiment            • • • YYMMDD - Instrument - Template - v          April 10, 2024 - 1341 PM         April 10, 2024 - 14:10 PM         April 02, 2024 - 18:02 PM         April 02, 2024 - 18:02 PM         April 02, 2024 - 17:32 PM         April 02, 2024 - 17:40 PM         March 19, 2024 - 09:16 AM         March 29, 2024 - 12:         Au Data-s         March 28, 2024 - 12:         Au Data-s         March 27, 2024 - 10:47 AM         March 27, 20	My Experiments		Experiment		te d	ate Created	2.41 DM	↑ Da	te Modifi	ed
Experiment <ul> <li>             2024-03-08 vFC LanaZ 10c-pn-Unmixed1             </li></ul> 2024-03-08 vFC LanaZ 10c-pn                  2024-03-08 vFC LanaZ 10c-pn                April 02, 2024 - 18:02 PM             April 02, 2024 - 18:02 <ul> <li>             2024-03-08 vFC LanaZ 10c-pn         </li> <li>             2024-03-08 vFC LanaZ 10c-pn         </li> <li>             2024-03-08 Cellarcus Lana Zaretski pn-Unmixed1         </li>                Pi 2024-03-08 Cellarcus Lana Zaretski pn-Unmixed1               April 02, 2024 - 17:46 PM             April 02, 2024 - 17:44                 Pi YYMMDD - Instrument - Example          March 19, 2024 - 09:16 AM             March 29, 2024 - 13:                 Pi AU Data-s          March 28, 2024 - 12:31 PM          March 28, 2024 - 12:                   240318 Protocol 2 Shortened Example Data UM + RAW          March 27, 2024 - 10:47 AM</ul>	Load Spectrof		YYMMDD - Instrument - Template     YYMMDD - Instrument - Template	- w	A	pril 10, 2024 - 14	:10 PM	A	oril 10, 2024	4 - 14:1(
Select Experiment       > 2024-03-08 vFC LanaZ 10c-pn       April 02, 2024 - 17:32 PM       April 02, 2024 - 18:0.         > 2024-03-08 Cellarcus Lana Zaretski pn-Unmixed1       April 02, 2024 - 17:46 PM       April 02, 2024 - 17:47         > YYMMDD - Instrument - Example       March 19, 2024 - 09:16 AM       March 29, 2024 - 13:         > AU Data-s       March 28, 2024 - 12:31 PM       March 28, 2024 - 12:         > 240318 Protocol 2 Shortened Example Data UM+RAW       March 27, 2024 - 10:47 AM       March 27, 2024 - 10:47 AM	Experiment	Ĩ	> 🖿 2024-03-08 vFC LanaZ 10c-pn-Unr	nixed1	A	pril 02, 2024 - 18	:02 PM	Aş	oril 02, 2024	¥ - 18:0ï
>       2024-03-08 Cellarcus Lana Zaretski pn-Unmixed 1       April 02, 2024 - 17:46 PM       April 02, 2024 - 17:41         >       YYMMDD - Instrument - Example       March 19, 2024 - 09:16 AM       March 29, 2024 - 13:         >       AU Data-s       March 28, 2024 - 12:31 PM       March 28, 2024 - 12:         >       240318 Protocol 2 Shortened Example Data UM+RAW       March 27, 2024 - 10:47 AM       March 27, 2024 - 10:			> 2024-03-08 vFC LanaZ 10c-pn		A	pril 02, 2024 - 17	:32 PM	Aş	oril 02, 2024	4 - 18:0;
Select Experiment         March 19, 2024 - 09:16 AM         March 29, 2024 - 13:           >         AU Data-s         March 28, 2024 - 12:31 PM         March 28, 2024 - 12:           >         240318 Protocol 2 Shortened Example Data UM+RAW         March 27, 2024 - 10:47 AM         March 27, 2024 - 10:47 AM			> 💼 2024-03-08 Cellarcus Lana Zaretsk	pn-Unmixed1	Ą	pril 02, 2024 - 17	:46 PM	Ap	oril 02, 2024	+ - 17:4(
Select Experiment         >       AU Data-s         March 28, 2024 - 12:31 PM       March 28, 2024 - 12:         >       240318 Protocol 2 Shortened Example Data UM+RAW       March 27, 2024 - 10:47 AM         March 27, 2024 - 10:47 AM       March 27, 2024 - 10:47 AM			> YYMMDD - Instrument - Example		Μ	arch 19, 2024 - 0	9:16 AM	М	arch 29, 20	24 - 13:
Select Experiment			> 🖿 AU Data-s		Μ	arch 28, 2024 - 1	2:31 PM	М	arch 28, 20	24 - 12:
	Select Experimen	t j	240318 Protocol 2 Shortened Exam	ple Data UM+RAW	Μ	arch 27, 2024 - 1	0:47 AM	М	arch 27, 20	24 - 10:
								× ~ 1	<b>C</b> 7	0

This will save an experiment as a compressed folder that contains the data files of the experiment as well as the various Spectroflo files (such as .xitm).



# **ELLARCUS** BIOSCIENCES Fluorescence Unmixing and Calibration for the Cytek Aurora and Northern Lights



# Appendix D. 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<sup>™</sup> 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