Operation Protocol Northern Lights 3L

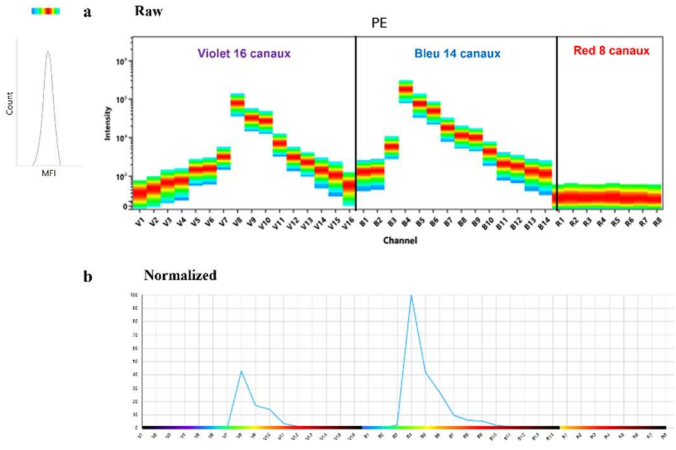
You will find the principles of spectral flow cytometry here:
https://cytobase.montp.inserm.fr/Cours/CytometrieSpectraleEN.html

1. Overview

- Cytometer : Northern Lights 3L
- 3 lasers: Violet, Blue, Red
- FSC : Photodiode, vSSC et bSSC : Avalanche PhotoDiode (APD)
- Fluorescences : 38 Avalanche PhotoDiode (APD)
- Fluidics system: sheath solution (milli-Q water), waste
- Software : SpectroFlo 3.3.0

Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Violet	V1	428	15	420	435
	V2	443	15	436	451
	V3	458	15	451	466
	V4	473	15	466	481
	V5	508		498	518
	V6	525	16 detectors	516	533
	V7	542	de 420 à 829	533	550
	V8	581		571	590
	V9	598	20	588	608
	V10	615	20	605	625
	V11	664	27	651	678
	V12	692	28	678	706
	V13	720	29	706	735
	V14	750	30	735	765
	V15	780	30	765	795
	V16	812	34	795	829
Blue	B1	508	20	498	518
	B2	525	17	516	533
	B3	542	17	533	550
	B4	581		571	590
	B5	598	14 detectors	588	608
	B6	615	de 498 à 829	605	625
	B7	661		653	670
	B8	679	18	670	688
	B9	697	19	688	707
	B10	717	20	707	727
	B11	738	21	728	749
	B12	760	23	749	772
	B13	783	23	772	795
	B14	812	34	795	829
Red	R1	661	17	653	670
	R2	679		670	688
	R3	697	8 detectors	688	707
	R4	717	de 652 à 829	707	727
	R5	738		728	749
	R6	760	23	749	772
	R7	783	23	772	795
	R8	812	34	795	829

An emission spectrum is generated for each fluorochrome. Each fluorochrome has a distinct spectral signature and the Unmixing allows to calculate the contribution of each fluorochrome in the full spectrum.



Example with PE signature

Exciting lasers: V, B, R

Main emission channel: B4

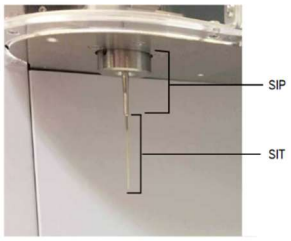
Secondary emission channels: V8

2. Starting the system

a) **Log in to MRI** with login and password (each user member of a team must have their own MRI account), select the account for billing > click ok

b) **Turn on the cytometer** by pressing the blue main button on the left-hand side of the unit.

Note: Ensure that a tube containing 1 mL of deionized (DI) water is loaded on the SIP before launching SpectroFlo software. The tube is required for the SIT depth calibration and to flush liquid through the flow cell to remove bubbles that may have formed.



Sample, contained in a standard 12 x 75-mm tube, enters the cytometer through the sample injection tube (SIT) that is contained within the sample injection port (SIP). The sample tube snaps into place under the SIP requiring no additional tube retention support. The SIT extends from the SIP during acquisition and retracts when the cytometer is not acquiring.

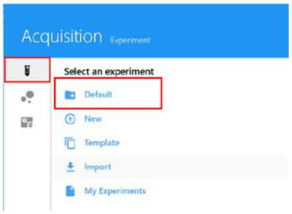
c) **Start the software SpectroFlo** and log in by entering your user name and password and clicking SIGN IN. The cytometer initialization procedure begins. Sheath fluid is flushed through the fluidics lines to prevent any saline buildup, and the system calibrates the SIT depth.

d) **Select Acquisition** from the Get started menu

e) **Check the status indicators** in the lower-right corner of the screen:

- Filling the sheath (milli-Q water): if insufficient, remove the sheath fluidics line cap from the sheath tank lid and replace the thank with a full one (a full sheat thank is always available)
- Emptying the waste: if full, disconnect the waste line orange from waste tank, disconnect the waste level sensor, replace the thank with an empty one and note the date on it. On the full thank: add 500mL of bleach and note on it "+ Javel" and the date.

f) Under the Experiment tab, open a default experiment, load a tube with approximately 3 ml water and run at high flow rate for 5 minutes.



3. Daily quality control (QC)

Run Daily QC using SpectroFlo QC beads prior to acquiring samples to ensure that the cytometer is performing optimally.

Note: SpectroFloQC beads are excited by all lasers and emit fluorescence in all detection channels. During the QC parameters are adjusted to account day-to-day instrument variability. rCV values and gain settings are measured and adjusted to place beads at the target locations for each detector. The aim is to have standardized settings during the time. Adjusted values are applied to all settings in the library as well as to Cytek Assay Settings (CAS).

Steps:

a) Prepare SpectroFlo QC beads: 1 drop of beads in 0.3 mL of sheath solution (milli-Q water)
b) Select QC & Setup from the Get started
c) Load a tube of the beads onto the SIP.
d) Select Start to begin acquisition.

A message is displayed when Daily QC passes. To view the QC report, click View Report.

Daily QC Report

QC Status:	PASSED	Date:	October 30, 2023 - 10:05 AM
Cytometer Name:	Aurora	User:	Admin
Serial Number:	S0144	Nozzle Size:	100 µm
Configuration:	4-Laser-V16-B14-R8-YG10	Sheath Pressure:	38 PSI
Software:	SpectroFloCS 1.0.7.1		

QC Beads
Lot ID: 2005 Expiration Date: December 31, 2026

Laser	Detector (nm)	Gain	Gain Change	Median (x1000)	% rCV	Status
Blue	FSC	421	72	1,911.3	0.98	✓
Violet	SSC	486	150	2,006.3	4.27	✓
Blue	SSC-B	527	141	1,986.7	4.39	✓
Violet	V1 (428)	302	67	466.1	2.92	✓
Violet	V2 (443)	370	79	1,080.7	2.98	✓
Violet	V3 (458)	347	70	1,226.7	2.98	✓
Violet	V4 (473)	251	52	1,203.5	2.73	✓
Violet	V5 (508)	297	62	1,134.4	2.71	✓
Violet	V6 (525)	295	61	837.4	2.81	✓
Violet	V7 (542)	382	81	962.1	2.60	✓
Violet	V8 (581)	629	114	1,029.8	1.97	✓
Violet	V9 (598)	486	79	1,234.4	1.36	✓
Violet	V10 (615)	508	77	2,152.9	2.10	✓
Violet	V11 (664)	430	81	1,014.9	2.15	✓
Violet	V12 (692)	364	75	517.8	2.43	✓
Violet	V13 (720)	329	74	498.9	2.42	✓
Violet	V14 (750)	475	115	1,248.6	4.71	✓
Violet	V15 (780)	623	156	1,791.4	6.45	✓
Violet	V16 (812)	457	120	1,330.8	8.73	✓
Blue	B1 (508)	1,269	311	142.9	3.26	✓
Blue	B2 (525)	672	158	134.9	2.92	✓
Red	R7 (783)	583	159	1,254.2	8.76	✓
Red	R8 (812)	397	131	897.9	15.15	✓

Laser Settings

Laser	Laser Delay	Area Scaling Factor
Red	-19.68	1.09
Blue	0.00	1.21
Violet	20.93	1.16
YellowGreen	40.08	1.10

FSC Area Scaling Factor: 1.21 Window Extension: 3

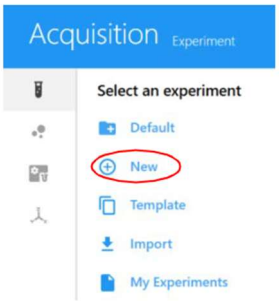
Temperature: 24.4 °C

Specifications

FSC	% rCV:	< 6	(Recommended)
SSC-B	% rCV:	< 8	(Recommended)
R3	% rCV:	< 6	(Recommended)
B3	% rCV:	< 6	(Recommended)
V3	% rCV:	< 6	(Recommended)
YG3	% rCV:	< 6	(Recommended)
All Channels	% Gain Change:	< 100	(Recommended)

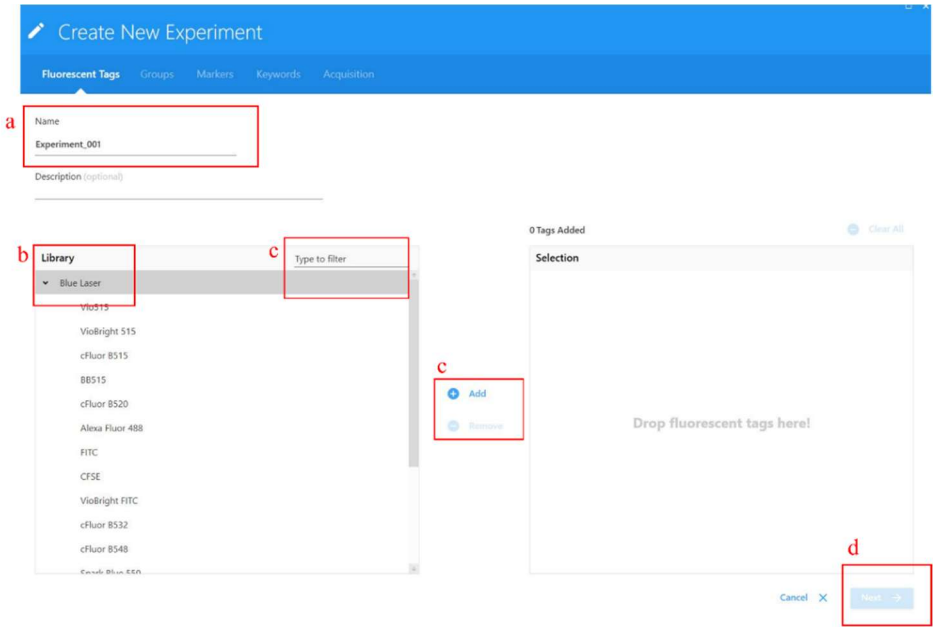
4. Creating a new experiment

1. Create New experiment
   Click New in the Acquisition Experiment menu. This opens the New Experiment wizard.

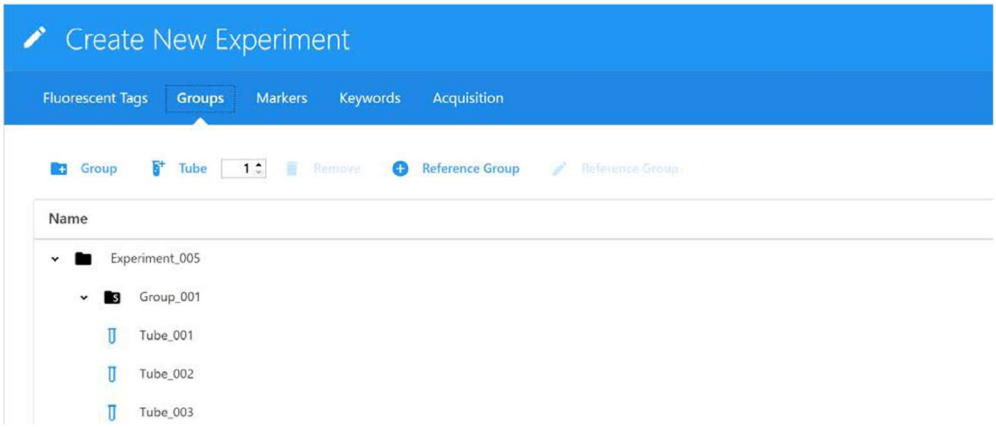


2. Specify a name for the experiment and select the fluoroschromes of the panel

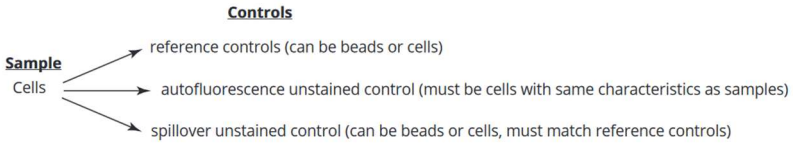
   a) Name the experiment: LastnameUser, Date(year-month-day), fluorochromes. If your panel contains more than 3 colors, after user name and date, note the number of fluorochromes followed by « C » (colors). Examples : « Leccia 2023-11-3 FITC PE APC » ou « Leccia 2023-11-13 4C »
   b) Click the arrow to the left of the group name (laser) in the Library pane on the left to display the list of its fluorescent tags.
   c) Select the fluorescent tags used in the experiment and click Add to add them to the Selection list on the right. You can also double-click the tag to add it to the Selection list. To quickly find a fluorescent tag, type the tag name in the Type to filter text box. A default list of fluorescent tags for each group is available in the library.
   d) Once all fluorescent tags have been chosen from the Library list, confirm the list in the selection pane, then click Next.



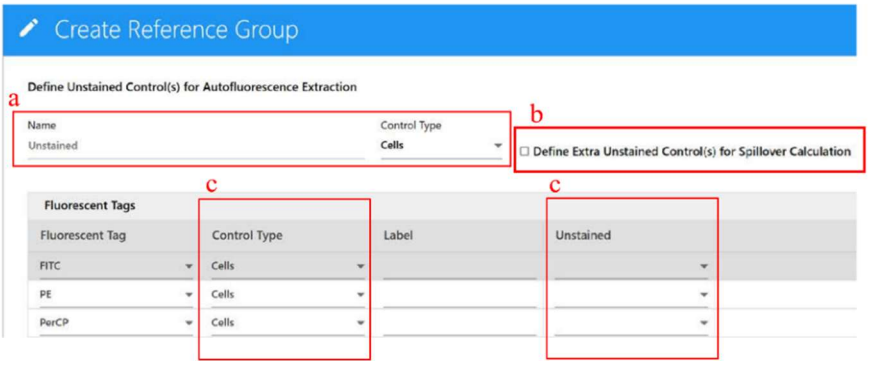
3. Create groups for your samples by selecting + Group. Add tubes to the groups.



4. Create your reference controls by selecting + Reference Group, this creates a list of reference control tubes for each fluorescent tag specified as part of the experiment
   a) Define an unstained control for autofluorescence by selecting its control type (beads or cells).
   Note: The unstained control needs to be of the same type and prepared in the same way as the samples, as this will ensure accurate unmixing and autofluorescence quantitation. Ideally, your reference controls, negative control, and samples will all be the same sample type and prepared in the same way.
   b) If your reference controls do not match your sample type and do not contain a negative population in each tube (for example if test samples are cells and the reference controls are beads, all with only positive peaks) you must use a separate spillover unstained control that matches your reference control type for the spillover calculation. Select Define Additional Negative Control(s) for Spillover Calculation to use a different unstained control to calculate spillover for your reference controls. Then enter a name and control type for this extra negative control.



   c) Select the control type (beads or cells) for the single-stained reference controls. (it is possible to have in the same experiments some controls on cells and others on beads). Click Save.

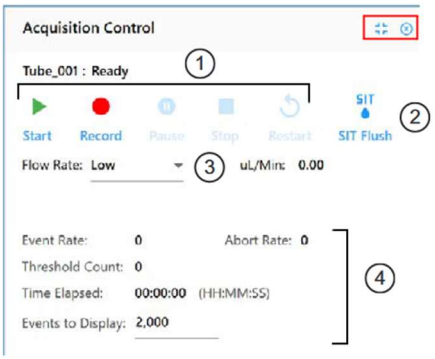


5. Select the acquisition settings and worksheet(s):
   a) Select the Default Raw Worksheet (Raw) for the Reference Group.
   b) Select the Default Unmixed Worksheet (Unmixed) or any user-created unmixed worksheet for your sample groups.
   c) Select the stopping gate, storage gate, number of events to record, stopping time (in seconds), or stopping volume (in µL). Acquisition stops when the first of the stopping criteria is met. If acquiring beads, we recommend collecting 5,000 singlet events. If acquiring cells, we recommend collecting 10,000 to 20,000 events of the desired population.
   Note: The number of events to acquire depends on the target population. For example, you may need to acquire 10,000 to 20,000 events to get 2,000 of the desired population. Approximately 1,000 to 2,000 events is needed in both the negative and positive populations of each control for accurate unmixing.

   d) Once the worksheet and stopping criteria have been defined, click Save and Open to open the new experiment. To make any changes to the experiment, click Edit above the group/tube hierarchy.

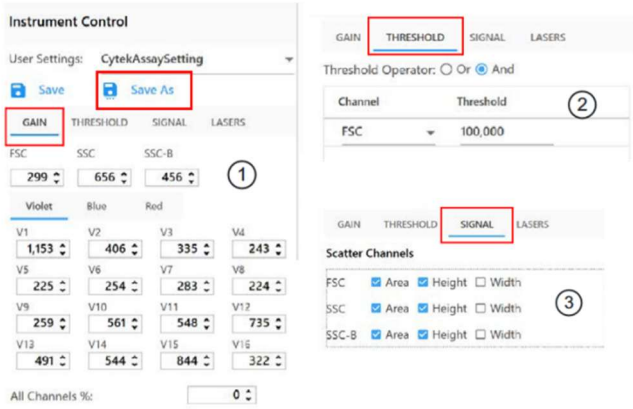
5. Record reference controls, unmix and record samples

1. Record the unstained control by clicking Start.



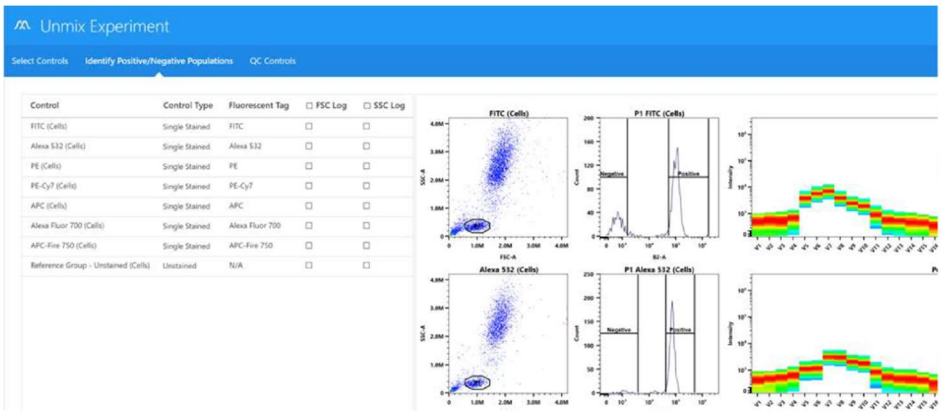
Acquisition Control pane allows you to :
(1) Start, stop, pause acquisition, record data and restart acquisition counters.
(2) Perform a SIT Flush
(3) Choose flow rate: Low (Faible, 15 µl/min), Medium (Moyen, 30 µl/min) ou High (Élevé, 60 µl/min).
(4) Display the real-time counts during acquisition: Event Rate, Abort Rate, Threshold Count, Time Elapsed

2. Make sure that CytekAssaySetting is selected. Use Instriment Controls to adjust FSC and SSC parameters. Click on Record



The Instrument Control pane allows you to adjust your User Settings: This setting provides the optimal resolution for each channel, accommodates bright signals, and minimizes spread. While using CytekAssaySetting, you will need to only adjust FSC, SSC gains (1), and Threshold (2). Use the Signal tab to select area, height, or width for each signal (3).

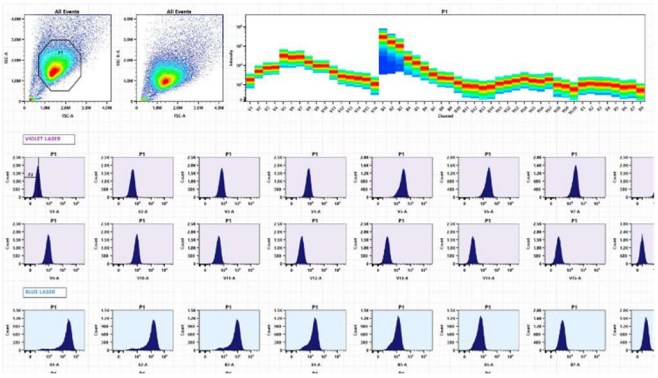
3. Acquire all your reference controls. If necessary, use the Instrument Controls to adjust the settings so that all events are on scale. If necessary, use All Channels % to increase/decrease all gains for a selected laser by the percentage you select.
   Important: To save your Instrument settings, click on Save As in the Instrument Control pane and name it: LastnameUser, Date(year-month-day), fluorochromes. If your panel contains more than 3 colors, after user name and date, note the number of fluorochromes followed by « C » (colors). Examples : « Leccia 2023-11-3 FITC PE APC » ou « Leccia 2023-11-13 4C ».
4. Clik Unmix in the upper left toolbar.
5. Select Use control from experiment for the Unstained control
6. Click on Next
7. Use the Identify Positive/Negative Populations tab to include the positive and negative populations for each fluorescent tag in the appropriate gate.
   a) Move the polygon gate in the FSC vs SSC plot on the left to include the singlet population. Hold down Ctrl to move all the polygon gates at once.
   b) Move the positive interval gate in the histogram to include the positively stained population.
   c) Move the negative interval gate to include the negative population if not using a separate negative control.
   d) Move the interval gate on the spectrum plot on the right to select the channel that exhibit the brightest fluorescence intensity. This channel is the peak emission channel for the fluorescent tag.



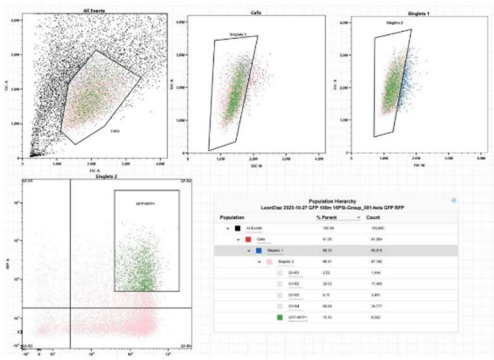
8. Use the QC Controls tab to check the quality of your reference controls.
9. Click on Live Unmixing.
10. The wizard closes and the experiment reappears. The reference group now has the unmixed icon to the left of the tube(s). Select an unmixed worksheet to view the unmixed data.
11. Select the sample tube you wish to acquire. The green arrow indicates the tube is selected.
12. Click on Start, then Record.

Note: For each sample tube that is live unmixed, two FCS files are generated, one that is composed of raw data and one that is composed of unmixed data. Live unmixed data can be analyzed in unmixed worksheets in the Acquisition module. Unmixed worksheets are different from raw worksheets, as they only display fluorescence information categorized into the defined fluorescent tags for each of the experiments

Raw data :
48 paramètres
+ FSC et SSC
visualisés dans une worksheet raw

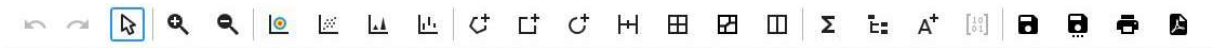


Ummixed data :
nombre de fluorochromes
+ FSC et SSC
visualisés dans une worksheet unmixed



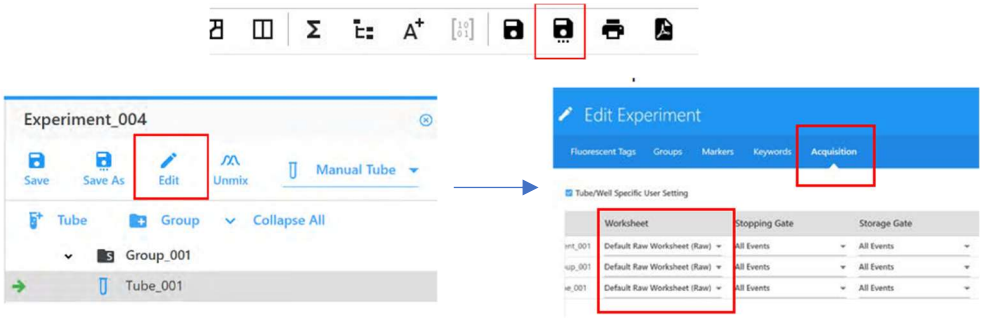
6. Unmixed data analysis with SpectroFlo

A toolbar at the top of the worksheet area allows you to create plots, gates, statistics, population hierarchy, annotations; and save, print, and save a PDF of the worksheet.



- Plots: Four plot types can be created in the worksheet: dot plots, pseudocolor plots (density plots), histograms, spectral plots. To change the properties of a plot, right-click the plot and select Properties. You can select the plot type, parameters, scale, background color, and labels.
- Gates: Gates types include: rectangle, ellipse, polygon, interval, quadrants and hinged quadrants (select and drag an offset handle to move the quadrant segment up or down), binary. Gate properties can be changed by right-clicking on the gate and selecting gate properties. You can change the name of the gate, the color, and gate boundary line weight. You can also select whether to display the count and/or the % parent events within the gate, as well as the gate parameters
- Statistics: To create a statistics box, click the Statistics icon in the worksheet toolbar, then click in the worksheet area. Select the population checkbox next to the populations that have stats to display. To add a statistic, select the statistic

from the Statistics Variable list. Select the parameter you would like to add for the statistics. Multiple parameters can be selected at once. The software offers a counts/µL statistic that can be calculated for any gate.
Important: Once you have created your analysis worksheet from the Default Unmixed Worksheet, remember to save it and rename it by clicking on the corresponding icon in the toolbar and to link it to your experiment in Edit > Acquisition.



7. At the end of your experiment:

1) **Before closing your experiment, remember to save:** a) the experiment b) your unmixed worksheet c) your instrument settings.

2) **Export and save your data**
In My Experiment right-click on the experiment > Export > Disk D > NL Analyseur > NL Analyseur 2025 > NL Analyseur Export.
Important: Remember to recover your data using a USB key or external hard disk or transfer them in your folder in FileZilla. Delete them from the analysis software and from disk D. Data will be kept for a maximum of **3 months** before deletion without prior warning.

3) **Systematically check the MRI reservation website to see if another slot is reserved after yours. Leave the cytometer on standby or switch it off, depending on reservations.**

a) If there is no slot reserved after you, so you are the last user for the schedule of the day, shutdown the system:
- In the Cytometer menu from Acquisition module, select Fluidics Shutdown
- Load a tube containing 3 mL of 10% bleach on the SIP and click Continue.
- Load a tube containing 3 mL of DI water and click Continue.
- Load a tube containing 3 mL of 30% Contrad 70 and click Continue.
- Load a tube containing 3 mL of DI water and click Continue.
- Allow the shutdown procedure to complete, then click Done and Turn off the cytometer
- Exit SpectroFlo software
- Log out of your MRI session (logout icon on desktop)
- Make sure the SIT is submerged in the DI water at the end of the procedure.
- Leave the computer on.

b) <u>If there is a slot reserved after you:</u>

- Under the Cytometer tab, click on Clean Flow Cell and follow the instructions. After completing Clean Flow Cell, leave the tube of DI water on the sample injection port (SIP) for the next user.
- Log out of your MRI session (logout icon on desktop)