Usage and maintenance of spectral flow cytometer NL3000

General rules:

Northern LIghts 3000 (Cytek®) is a spectral flow cytometer with automatic microsampling system (AMS). Spectral technology provides opportunity to design extensive panels of fluorochromes and detect wide variety of markers and probes within individual cells. We can also observe changes in fluorescent spectrum originated from changes in physiological state of the cell (autofluorescence) or in cumulative spectrum emitted by all fluorochromes.

Spectral technology and 3 lasers of NL3000 allows us to build panels of up to 24 fluorochromes. This is extremely helpful in phenotyping of leukocytes and other complex cell populations.

If you are interested in spectral flow cytometry, check out these links:

Spectral cytometry general:

https://www.thermofisher.com/cz/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-learning-center/flow-cytometry-resource-library/flow-cytometry-methods/spectral-flow-cytometry-fundamentals.html

Spectral flow cytometry Accela:

https://www.accela.eu/discover/webinar-advancing-spectral-flow-cytometry-data-analysis-with-new-spectroflo-2-2-fcs-express-7

Design of fluorochrome panel:

https://www.biorxiv.org/content/10.1101/784884v1.full

Spillover and spreading error:

https://voices.uchicago.edu/ucflow/2020/03/04/understanding-the-trumpet-effect-how-to-design-aurora-panels-around-spreading-error/

Before you start working with NL3000, please follow these steps:

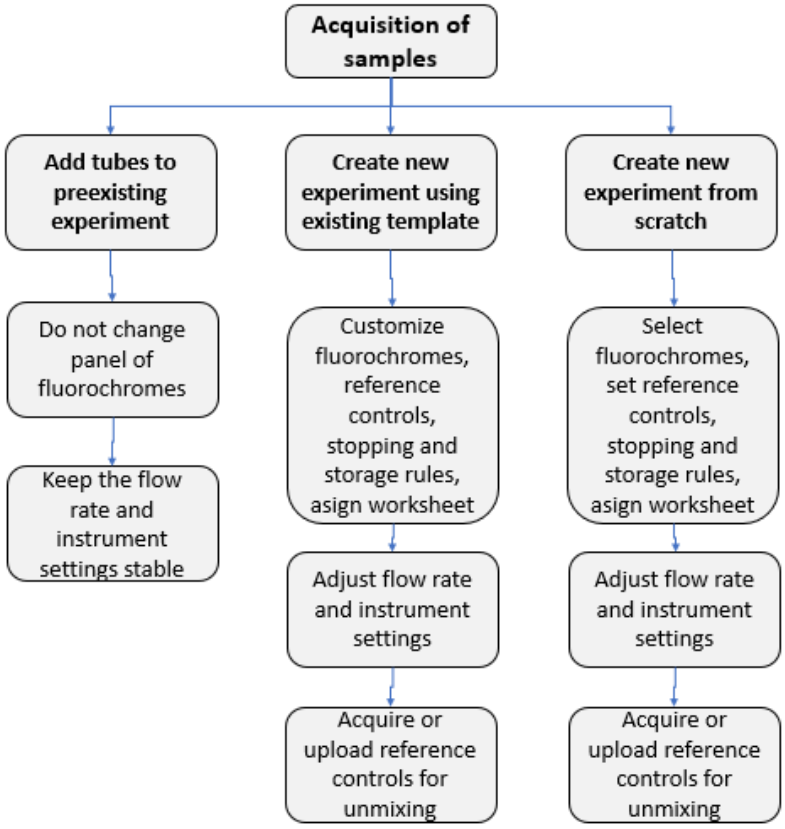
1. Inform the supeusers of NL3000 (Olga, Pája).
2. Arrange training in basic maintenance, operation of the machine and SpectroFlo software.
3. Your own user account in SpectroFlo will be created for you.
4. Use OFIZ booking system and adhere to your time frame.
5. Follow the rules in manufacturer manual Cytek Aurora Quick Reference Guide and rules on a poster.

6. Do not forget to perform required cleaning procedures (see section Plan of cleaning procedures below).
7. Do not contaminate computer area with infectious or toxic materials!
8. If there is any problem with the machine or its software, or if it is not performing as you expect, let the superusers know immediately!

Maintenance and operation rules:

1. Before start, check waste and sheet fluid containers and put tube with MQ water on SIT.
2. Follow the manufacturer's manual Cytek Aurora Quick Reference Guide. Switch on the machine and computer. Start SpectroFlo and log in to your account. There is approximately **45 min needed for warm-up** of the machine. It is running only when SpectroFlo is on.
3. Perform QC control according the manual and software guidance (QC&Setup). Vortex the vial with QC beads and add 2 drops of beads to 1 ml PBS. Store in fridge (room 1S16) for up to one week or till there is enough events to successfully run QC control. If the QC control fails – vortex the tube with beads, rerun it or prepare a new QC control – let the supervisors know that QC did not pass.
4. Decide if you will use plate or tubes for your samples. Switch on the loader, change the selection tubes to plate in Preferences-Acquisition and immediately drag silver lever from the front to the back (**Fig.3**). Proceed with plate calibration (automatically during SIT calibration).
5. At this stage **check SIT and run automatic setting of it in Acquisition-Cytometer-Calibrate SIT** (how far it extends itself down into the tube or plate). Recommended value for tubes is 1, for plates it depends on type (usually 1,2). Check alignment of a pipeline and distance of SIT from the bottom of chosen vessel. If it is bend on the bottom, it is not correct (**Fig.2).** Calibrate the SIT again. If it is not corrected, contact supervisors and do not proceed with acquisition!
6. For acquisition, use experiment from template, modify older one or build experiment from scratch (**Fig.1**).
7. New experiment from the scratch – follow the steps in Acquisition-Experiment-New and choose fluorochromes of interest, adjust number of groups and tubes, keywords, names of markers and be particularly careful to entitle right worksheet (customize the general ones and save them under your name) and adjust collecting and stopping gates, stopping time and volume of acquisition.
8. Once the experiment setup is finished, run control sample for a short time and set the flow rate, FCS and SSC to get anticipated values. Adjust gain at detectors (all detectors the same way preferentially) and flow rate if needed - **Fig.3**. You may choose to adjust the worksheet at this stage, name it and save it. **Be careful not to overwrite the default raw worksheet**! If it accidentally happens, go to Library and reset default worksheet settings.
9. After running all the samples and reference controls, do not forgot to save experiment with a fitting name.
10. If you do not use more than one fluorochrome or if you are interested only in counts or FCS/SSC, you can perform batch analysis in SpectrFlo software straight away. Alternatively, open raw fcs file in any other flow cytometric software (NovoExpress, FlowJo).

11. If you have more than one fluorochrome and want to unmix the signals and extract fluorescence intensities that correspond to single fluorochromes/expression of the markers in individual cells, you should perform spectral unmixing (see Spectral unmixing part below and Unmixing Workflows in Cytek Guide).
12. After you measured all samples and reference controls, check if you have saved final version of experiment, exported raw and unmix FCS files in CytekExport directory (disc C) or export them manually elsewhere.
13. If you used plate mode, set the machine back to tube mode. Change it in Preferences-Acquisition (**Fig.3**) and drag the silver lever back to the front position. Turn off the loader.
14. Let run tube with MQ water for couple of minutes to wash the pipes or perform **Clean the flow cell in Acquisition-Cytometer menu** (**Fig.2**).
15. If you are the last one using the machine, follow the Shutdown manual in software (run 10% contrad/SAVO, MQ water, 30% contrad, MQ water). Turn the machine (and autosampler), software and computer off.
    Check waste and sheet fluid tanks again and empty/refill it with MQ water if it is needed.



Material:

1. Sheet fluid – use always MQ deionized water from western blot room. It is stored in a tank on the bench next to the cytometer.
2. Contrad – detergent daily clean, VWR, # DECN1003.5L (at the bench)
3. QC beads – SpectroFlo QC Beads 2000 Series, # N7-97355-0A, Cytek (fridge 1S16)
4. Compensation beads – AbC™ Total Antibody Compensation Bead Kit, # A10513, Invitrogen 122/4)

Fig.1

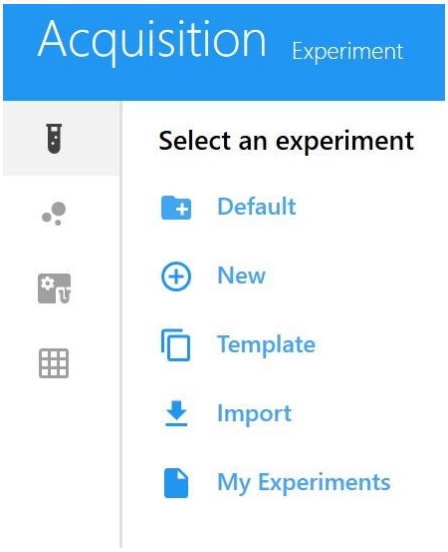


Fig.2

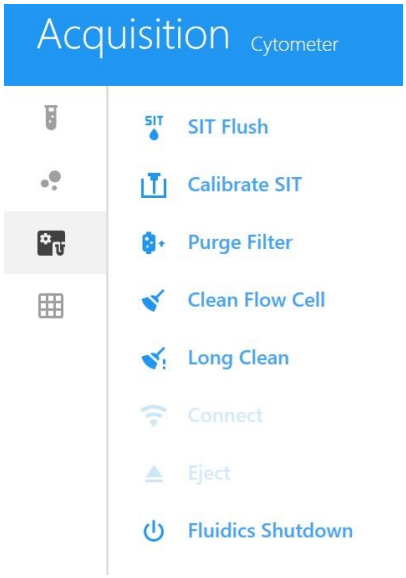
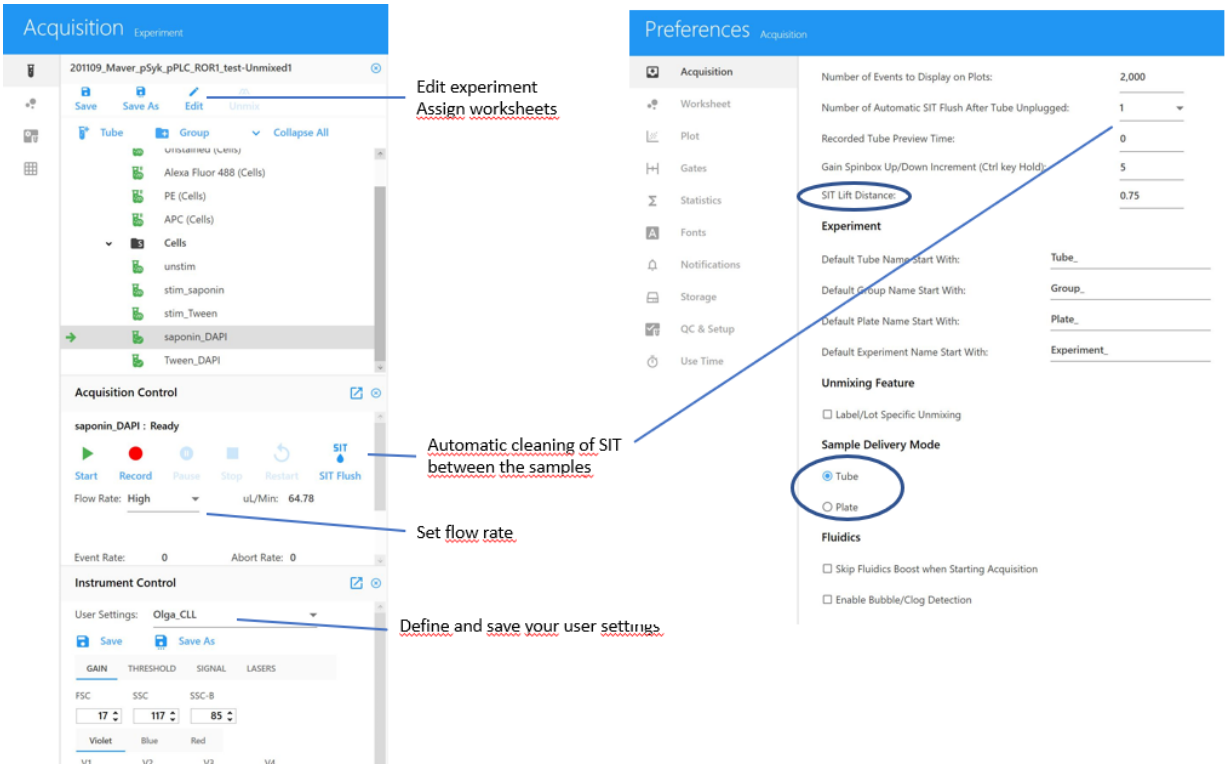


Fig.3



Plan of cleaning procedures:

WHEN	WHAT	WHO	MATERIAL
Warming up after start of NL	45 min run DI water (high flow rate)	everybody	DI water
Between individual sessions	Clean flow cell/run DI water on high	everybody	10% bleach
At the end of the day	Fluidics shutdown	everybody	bleach, Contrad
Once a week	Contrad soak	Pája M.	Contrad
Once a month (cleaning day)	Long clean	PJ/Olga/Marek	10% bleach
Once a month (cleaning day)	Upload QC protocol on OneDrive	Olga	

These procedures are obligatory.

You can find them in the menu (Acquisition-Cytometer, Fig.2). Follow instructions on the screen.

See document Instrument Cleaning Recommendation (2nd document #63).

Long procedures (Long Clean and Contrad soak) should be booked in reservation system.

Spectral unmixing

Spectral unmixing is a process that unmixes complex emission fluorescent spectrum consisting of all used fluorochromes and denotes intensity of fluorescence for individual fluorochromes for each event (cell). It is based on spectra of single fluorochromes. For proper unmixing, we need unstained cells and a set of single stained samples, aka **compensation/reference controls**.

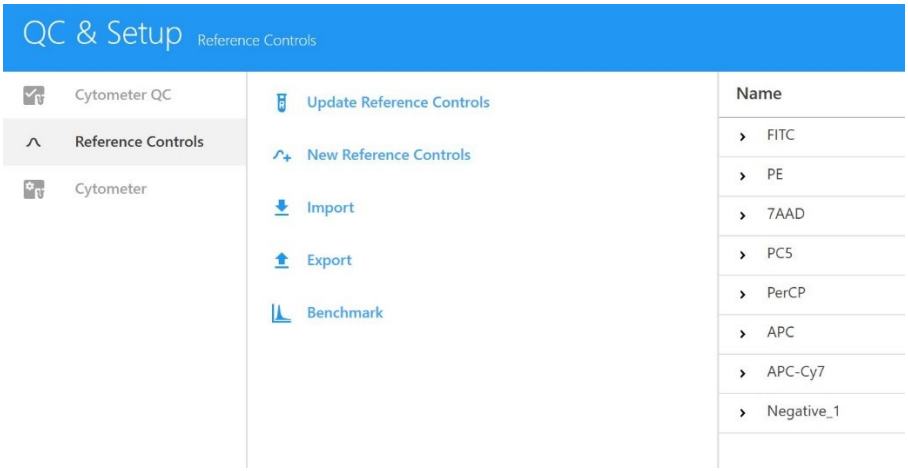
> CAUTION! Use proper reference controls. Check if you set the fluorochromes in experiment correctly.
> You can use live unmixing, unmix spectra after finishing the acquisition of all samples in experiment file or in analysis module. (Check NL3000 manual - Unmixing workflows.)
> You can customize unmix template, save it and associate it with the experiment (press edit button and change it).
> Press unmix button and follow the instructions. You can use reference control from experiment our you can use reference controls saved in library of the software. Adjust graphs, check unmixing matrix and complexity of your panel (for panels up to 9 fluorochromes, complexity in single digits is OK).
> You can also unmix raw FCS files any time afterwards in any other software with unmixing tool.

Compensation rules – same for reference controls:

1. Compensation control must be as least as bright as the stained sample.
2. Negative and positive populations must have matched autofluorescence: cells to cells, beads to beads, etc.
3. The fluorochrome used for the control must exactly match the fluorochrome used in the experiment: FITC cannot be used instead of GFP, tandem dyes must be from the same vial as the experimental stain.

If it is not possible to generate bright positive control in system with similar autofluorescence (e.g. marker is not expressed or weakly expressed in the cells and positive control is not within reach), you can bind the antibody to compensation beads (Ultra Comp beads BD) or antibody conjugated with exactly the same fluorochrome for strongly expressed marker (new one or previously established **Fig.4**).

Fig.4

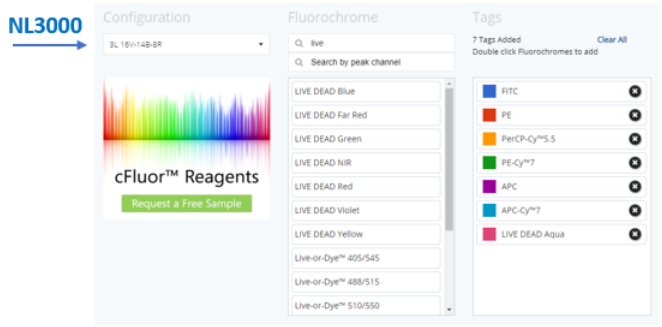


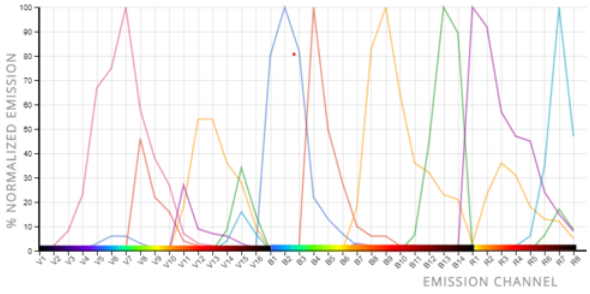
Antibody/Fluorochrome panel design (Fig.5)

1. Create list of markers to detect. Are they surface or intracellular?
2. For panel of surface markers, it is advisable to include viability probe and analyze only viable cells.
3. Do you expect high or low expression?
4. Check availability of conjugated Ab.
5. Test spectral similarity of available fluorochromes. Use Cytek Spectral similarity tool (https://spectrum.cytekbio.com/). It works properly only in Google Chrome. Set NL configuration 3L 16V-14B-8R. You will get complete matrix of similarity indices and overall complexity index of the panel. For simple panels (up to 10 fl.), it is OK to be less than 5, for more complex panels it can be way above 10. Unmixing of critically similar fluorochromes (e.g. ALEXA Fluor 647 and APC) will lead to increased spread of the data (check Spillover and spreading error in General rules section).
6. Highly expressed markers – dim fluorochrome
   Weakly expressed markers – strong fluorochrome
   https://www.bu.edu/flow-cytometry/files/2013/06/Fluorochromes-Brightness-Chart.pdf
7. If it is unavoidable to use highly similar fluorochromes, assign them to markers expressed on distinct cell populations.

Fig.5

https://spectrum.cytekbio.com/

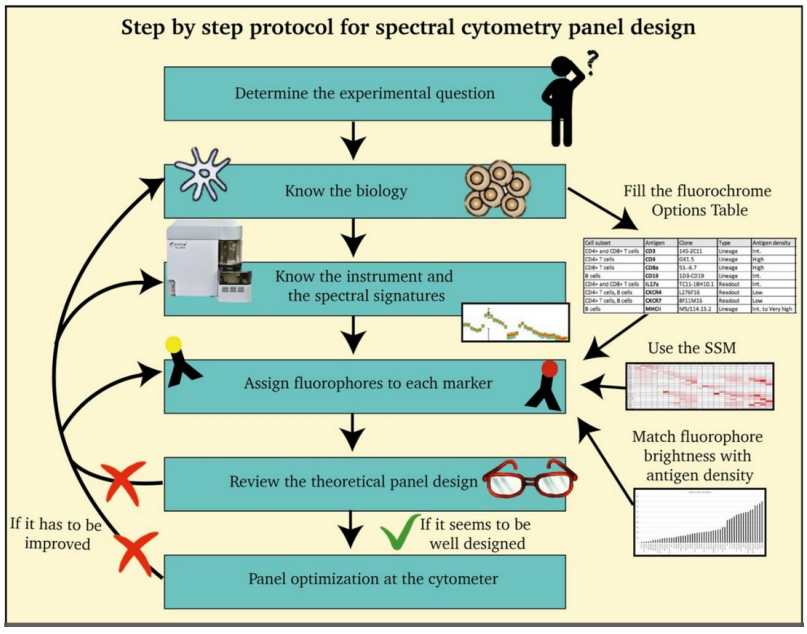




Similarity™ Indices

	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7	LIVE DEAD Aqua
FITC	1	0.17	0	0	0	0	0.06
PE	0.17	1	0.06	0.01	0.01	0	0.19
PerCP-Cy5.5	0	0.06	1	0.27	0.29	0.13	0
PE-Cy7	0	0.01	0.27	1	0.03	0.19	0
APC	0	0.01	0.29	0.03	1	0.19	0.01
APC-Cy7	0	0	0.13	0.19	0.19	1	0
LIVE DEAD Aqua	0.06	0.19	0	0	0.01	0	1

Complexity™ Index: 1.95



Ferrer-Font et al. 2019