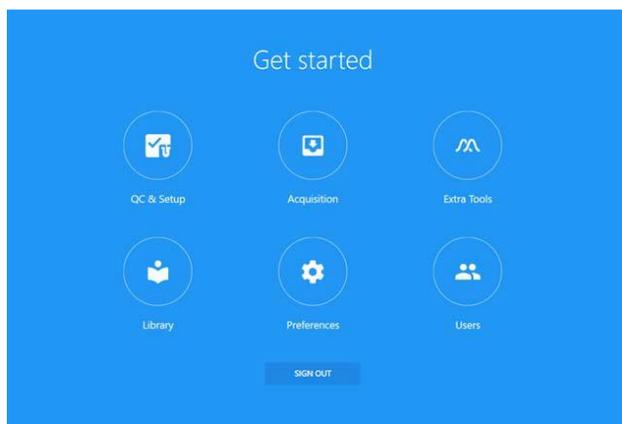


Preparing a Spectroflo Experiment

This document is intended to be a software guide only. It is not intended to teach flow cytometry theory or best practices — that will be discussed during your instrument training, but is not included here. For information on flow cytometry in general please contact the URM Flow Cytometry Resource staff.

Getting started and logging in:

1. Log in to the computer via the PPMS System:
 - a. This is the same login information you use to log in online for scheduling and training requests.
 - b. The lock screen will change and you'll be presented with a screen allowing you to update account info if needed.
 - c. Update the account info, or press “OK”, and you will be able to access the computer.
2. Log in to the Spectroflo Software:
 - a. The generic username for Spectroflo is “aurora user” and the password is “password”.



There are 6 major modules within the software and each contains a number of subsections which can be accessed from the left side of the window. A brief explanation of what these modules are is listed below, however this document will be focused on “Acquisition”.

QC & Setup: Daily QC ensures that the instrument is in optimal condition for use. Run SpectroFlo QC Beads daily to assess system performance and to adjust settings to account for day-to-day variation. Levey-Jennings reports keep track of trends in system performance.

Acquisition: The Acquisition module allows you to create experiments to acquire and analyze data. Experiments can be created through a guided wizard or created from previously saved templates.

Extra Tools: Here FCS files can either be unmixed or compensated using virtual filters. This is where you can Remove FCS parameters such as Area, Height, and Width as well.

Library: The library allows you to store experiment templates, worksheet templates, user settings, fluorescent tags, SpectroFlo QC bead information, label information, keywords, and Loader settings.

Preferences: Software preferences can be changed to customize the software. Default plot sizes, fonts, gate colors, print layouts, statistics box table option, and more can all be changed in the Preferences.

Users: The Users module contains user management options and administrative controls.

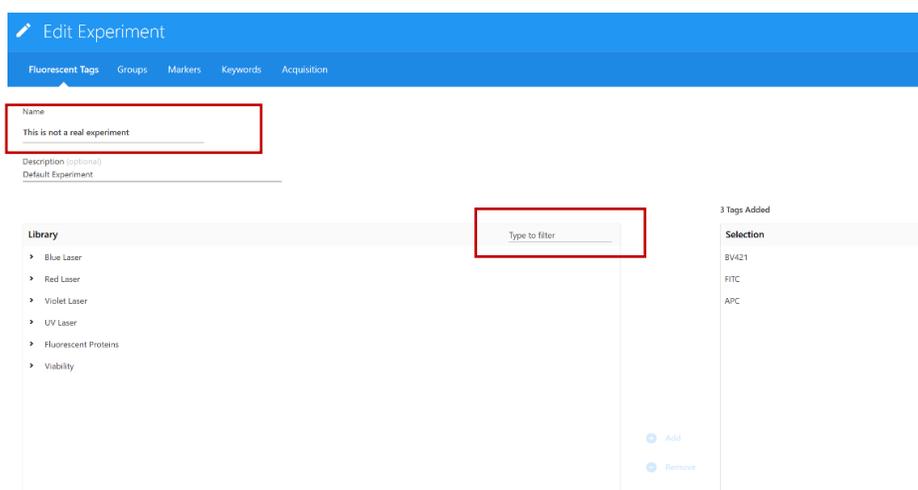
The process for creating and running an experiment in the software is broken into 4 major parts outlined below.

1. Creating the outline of your experiment
2. Adjusting setup and performing unmixing
3. Recording samples
4. Finishing the experiment

1. Creating the outline of your experiment: These steps are summarized from the process outlined beginning on page 53 of the Aurora Users Guide.

Note: New experiments, in addition to being created on the instrument computer before samples are run, can be created either on the Cytek Cloud or on the FCR's Spectroflo analysis computer and then imported to the copy of Spectroflo associated with the Aurora on which samples will be recorded. This can greatly streamline and reduce experiment setup time. It is important, however, that when making an experiment in this fashion that the right instrument configuration is selected. As mentioned at the end of this document, experiment files should remain zipped when importing or exporting.

1. From the “Get Started” page, click on the Acquisition module.
 - a. You can open an old experiment from the “My Experiments” list, or “Import” an experiment if the outline has been generated on a separate Spectroflo enabled computer or in the cloud.
2. To start from scratch click on the “New” button to open the Create New Experiment Wizard:
 - a. Always label your experiment with a name that is unique to you.
3. Add each of your fluorochromes by clicking on “Type to filter”.
 - a. If your fluorochrome is not listed it can be added via the “Library” module. This process is not covered in this document.



4. Click “Next” at the bottom right of the window to advance to the Groups tab. Within this tab you can create the basic outline of the tubes you’re going to run. Additional tubes/groups can be added during acquisition as well.

- a. To add a group with multiple tubes, first click to increase the tube number, followed by clicking “Group”.
 - i. The groups created consist of every tube included in the experiment other than those specifically part of your “Reference Group”.
- b. Click on “+ Reference Group” to create your reference controls.
 - i. **Please disregard any reference to “stored controls” when creating the reference group.**
 - ii. Determine if you want to include additional unstained controls and add if necessary by checking the “Define Universal Negative Controls”.
 - iii. Identify what Control Type (beads or cells) each reference control will be linked to. If only one unstained control is included this can be skipped.
 - iv. Label the antibody/target for each reference control if desired.
 - v. Label the Lot # information for each reference control if desired.
 - vi. Identify what Negative Control option applies for each reference control.
 1. Leaving this blank indicates that an internal negative will be used for this control.
 2. Switching to a specific negative/unstained control indicates that this single stained reference control will use that specific negative and does not need a negative gate to be included for unmixing.

✎ Create Reference Group

Define Unstained Control Matching Sample Autofluorescence

Define Universal Negative Control(s)

Name	Control Type	View Stored Unstained Controls
Unstained	Cells	1 Control

Name	Control Type
Negative_1	Cells

+ Add - Remove

Fluorescent Tags

Fluorescent Tag	Control Type	Label	Lot	Universal Negative	V
BV421	Cells				2
FITC	Cells				1
APC	Cells				2

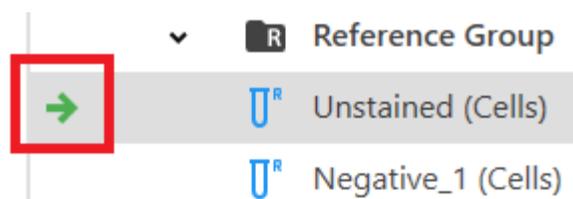
- c. Click “Save” at the bottom right of the window to create.
5. Click “Next” at the bottom of the Groups tab to advance to the Markers tab. Within this tab you will need to label each fluorochrome with the antibody or marker used.
 - a. **This labeling is critical because without it data analysis is extremely difficult.**
 - b. Labels are typically applied to the Experiment (click on the first empty square below the fluorochrome name) but can also be applied to a single tube or at the Group level.

6. Click “Next” at the bottom to advance to the Keywords tab. Keywords must be created and saved in the Library module prior to being used. The process of creating and saving keywords in the Library is separate from this training document but can be discussed as needed.
 - a. For each keyword you’d like to include you can click and drag it to the sample(s) of choice.
7. Click “next” at the bottom to advance to the Acquisition tab. Within this tab it is possible to modify a number of factors controlling how your events are acquired in your experiment.
 - a. If save Assay settings are available they can be applied to the experiment here by selecting from the drop down menu at the top of the page.
 - b. Acquisition settings can be applied to a single tube, at the Group level, or at the Experiment level depending on what level you activate.
 - c. This instrument tracks the volume of sample run. You can use this as a stopping criteria if you choose, but it will track the volume regardless.
 - d. If you would like to apply a Stopping Gate or Storage Gate, you much assign a worksheet as well.
 - e. It is recommended that an Unmixed worksheet be applied to all but the reference group so visualization of those samples will default to the selected unmixed worksheet.
8. When the outline is complete it can be saved for use.
 - a. Click Save and Open if you are at the instrument and ready to begin running your experiment.
 - b. Click Save as Template if you are preparing the experiment at a separate computer and will import it into the instrument computer later.

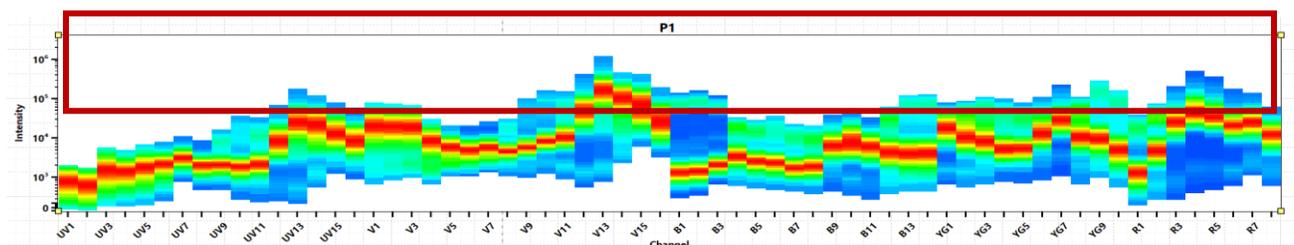
2. Adjusting Setup and performing unmixing: Only the scatter gains are adjusted in a typical experiment. The process for checking and adjusting the fluorescent detector gains will only briefly be addressed here.

- The gains pre-loaded in the software are derived from the “Cytek Assay Settings” which are based on the QC beads and are calculated on a daily basis during the quality control process.
- Cytek Assay Settings are a universal baseline for instrument performance to facilitate stability and normalization across instruments and time.
- In a typical experiment only the scatter gains need to be adjusted and the fluorescent gains are kept at the Cytek Assay Settings.
- This instrument has two side scatter measurements. The SSC measurement is generated from the Violet laser and can be slightly more sensitive for small particles. The SSC-B is generated from the Blue laser as is done on most older instruments.

1. Check and adjust the scatter gains.
 - a. Verify that the pointer is active for the unstained control and place the tube on the SIP. The “Start” button in the Acquisition Control window should become available when the tube is in place.

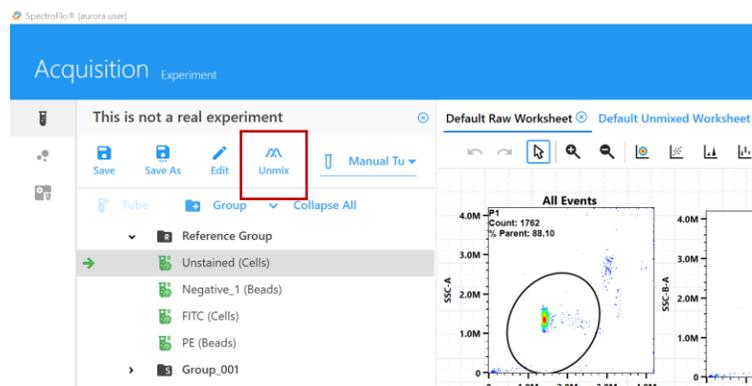


- b. Click on “Start” to begin running the sample. The data should begin populating the plots in the “Default Raw Worksheet” but these events are not being recorded and saved at this time.
 - c. Adjust the FSC, SSC and SSC-B(lue) gains found in the Instrument Control window such that the populations are on scale as desired. ***Do not record the data until you are confident that no further adjustments are needed. While scatter gains can be adjusted during the experiment, the fluorescent gains must remain fixed once recording of data begins*.**
 - i. The first adjustment will almost always be to decrease the FSC voltage to move your events from right to left on the graph.
 - ii. Once an acceptable FSC gain is established, the two SSC gains can be adjusted. These may have to go up or down depending on a number of factors so FSC should always be set first. ***Note that the scatter gains for beads and cells will not necessarily match and that is okay. It’s most important to have all populations be on scale, not to have matching scatter gains*.**
 - iii. If faster refreshing of the data in the plots is needed the “Restart” button can be clicked or the “Events to Display” can be adjusted in the Acquisition Control window.
 - iv. The P1 gate on the FSC/SSC plot does not need to be perfectly set, but gating on the major populations of interest is helpful in visualizing the fluorescent signal.
 - d. When the scatter gains are determined, click on “Stop” and remove the Unstained control tube from the SIP.
2. Check and adjust the fluorescent gains. This step is only necessary the first couple times something new is run. Once established, the fluorescent gains do not need to be verified every time.
 - a. Place a fully stained sample on the SIP. This should be a sample that is expected to have positive signal for all/most of the fluorescent targets of interest.
 - b. Click on “Start” to begin running the sample. The data should begin populating the plots in the “Default Raw Worksheet” but these events are not being recorded and saved at this time.
 - c. Review the fluorescent signal in the spectral signature plot.
 - i. If any of the fluorescence goes off scale to the top of the spectral signature plot (Y axis is intensity of fluorescence) then the gains have to be reduced.



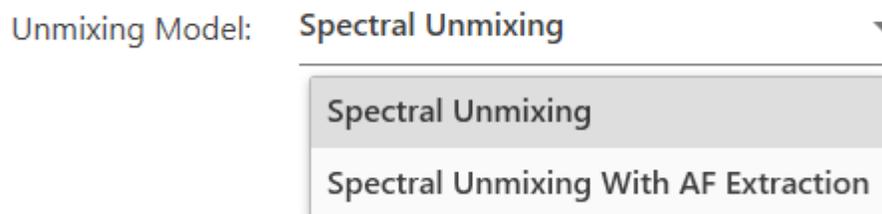
- ii. If all of the fluorescence is on scale no further adjustments are needed or recommended. Proceed to **step d** below.
- iii. To reduce fluorescent gains it is recommended to reduce all of the gains for a single detector set, by a percentage of the total.
- iv. In the “All Channels %” option at the bottom of the Instrument Control window a negative value can be entered. ***Only reduce the gains enough such that all positive signal is on scale*.**

- v. Once established, this negative reduction in gains will be consistent for all future runs of this panel/experiment.
 - d. When the fluorescent gains are set, click “Stop” and remove the tube from the SIP. ***Note that no data has been recorded yet***.
3. Record all reference controls.
- a. Verify the pointer is still on the unstained control and place the unstained control back on the SIP.
 - b. Click on “Start” to begin acquisition of the data.
 - c. Once the acquisition is started and stable, click on “Record” to begin saving data.
 - d. When recording is completed remove the tube from the SIP.
 - i. Recording can be stopped for reference controls or samples as needed by clicking on “Stop” at any point. Data will be saved up until the button is clicked.
 - e. Repeat steps a-d for the remainder of the reference controls.
 - i. When a sample runs to the stopping point established in the acquisition tab (step 7 in Creating the Outline above) the pointer will advance automatically.
4. Perform unmixing
- a. Click on the “Unmix” button once all reference controls are recorded.



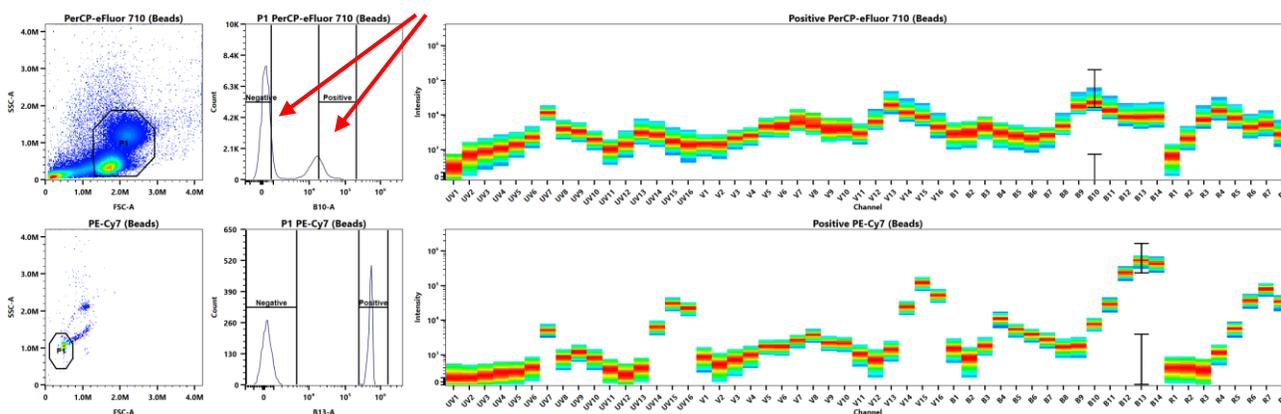
- b. In the “Select Controls” tab you will ignore almost everything shown other than the Unmixing Model dropdown menu in the bottom right corner of the window, which will allow you to choose between “Spectral Unmixing” and “Spectral Unmixing with AF Extraction.”

****AF = Autofluorescence****



- i. Activating AF Extraction is often helpful, but the full details regarding this decision, and how to optimize its implementation are beyond the scope of this document.

- c. Click on the “Identify Pos/Neg Populations” tab to adjust gating for your reference control data.
- All of the plots and gates needed have been created automatically based on the decisions made in setting up your reference group. (step 4B in Creating the Outline above).
 - First adjust the P1 gate in the FSC and SSC plots to include your cells of interest. By holding the control key while adjusting the gate, it will be moved in all related plots.
 - Note that the position of the P1 gate has a significant impact on the unmixing. This is something that can adjusted or fine tuned when unmixing is suboptimal.
 - Next adjust each positive and negative gate to include only the brightest (pos) or dimmest (neg) events as highlighted below.



- When all gates are set the unmixing can be calculated.
 - Create New Unmixed Experiment: the current experiment will be closed and a new experiment that includes only unmixed data will be created and opened. This is usually only used when unmixing is understood to be correct and no further modifications are intended.
 - Live Unmix: continuing in the same experiment the unmixed data will be displayed and saved, but the raw data will remain available in case further adjustments to the unmixing are intended. This is often the preferred option when experiments are being developed and optimized.
- d. Once unmixing is applied it is time to advance to recording sample data.

3. Recording Samples:

- If you chose, Live Unmixing above, you will be brought back to the main Acquisition module within the same ongoing experiment.
- If you chose Create New Unmixed above, a new experiment will be opened with “Unmixed” added to the title. Both the original “raw data” experiment and the new “unmixed” experiment will be saved.
- The unmixing is automatically applied to both reference controls as well as real samples after calculation.
- After unmixing is calculated a “Default Unmixing Worksheet” will become available automatically for use in visualizing data while recording. It is not necessary to analyze data in real time, but the worksheet will be available to do so.

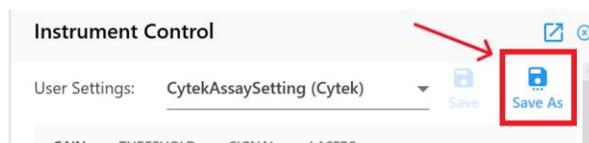
1. Activate the pointer for the first sample you'd like to record and place the sample on the SIP.
2. Click on "Start" to begin acquisition of the data.
3. Once the acquisition is started and stable, click on "Record" to begin saving data.
4. When recording is completed remove the tube from the SIP.
 - a. Recording can be stopped for reference controls or samples as needed by clicking on "Stop" at any point. Data will be saved up until the button is clicked.
5. Repeat Steps 1-4 for all tubes remaining to be recorded.

4. Finishing the Experiment:

- All FCS files (Raw and Unmixed) are automatically saved to the D: drive so there is no need to "export" them out of the software.
1. There are 3 components that can/should be saved before closing an experiment. These are the assay setting, the worksheets, and the experiment outline.
 - a. **Always click the "Save As" button for assay settings and worksheets such that you do not save over the existing defaults.**
 - i. Click on "Save As" in the worksheet (Raw or Unmixed) to save it as a template if you created graphs and a gating strategy that you'd like to access in the future.



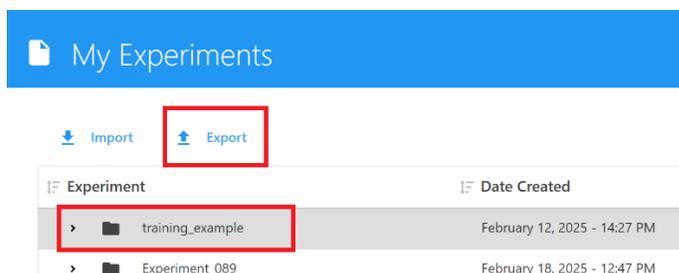
- ii. Click on "Save As" in the assay settings to save if you made adjustments to scatter or fluorescence that you'd like to maintain for the future.



- iii. Click on the "Save" icon below your experiment title to lock all changes to the outline and link saved worksheets/settings.
 1. The experiment can also be saved as a template by clicking "Save As" if you want an empty template to access in the future though this is less common.
2. Once you've saved the components you needed, click on the small x to the right of the experiment title to close the experiment.



3. It is also recommended that the Experiment be exported as a Zip file such that it can be archived for future use.
 - a. From the Acquisition module start page click on "My Experiments"
 - b. In the list of experiments that opens, single click on the title of the experiment you're interested in.



- c. Once highlighted, click on the “Export” button at the top of the window.
 - i. When prompted, double click on the “Exp Zip File - Temp” folder before clicking save to export your experiment.
 - ii. Zipped experiment files should be exported to the Exp Zip Files - Temp folder for temporary storage only before transferring.
4. Transfer the zipped experiment file along with your FCS (raw and unmixed) files.
5. Once closed you can transfer your FCS files to perform data analysis offline.
 - a. On the computer desktop open FCS File – Shortcut/Experiments/”current date”/”current user”/”your experiment title.
 - b. The FCS files can be transferred to the Transfer Pipeline, Box or other cloud based storage program as desired. Keep in mind that if future adjustments or unmixing will be performed you may end up with multiple copies of the same files which can cause confusion.
6. Additionally, at all Spectroflo enabled computers there is a folder called Aurora Pipeline that allows files to be shared between each of those computers. This is to facilitate easy access to those users that want to work between multiple computers and/or instruments.
7. Begin the cleaning procedure as described on the front of the machine and in the Aurora Operation document.
8. When cleaning is complete you can log out of both the Spectroflo software and the PPMS tracker to complete your session.

Thank You.