6

Unmixing and Compensation

Spectral Unmixing

Spectral unmixing is an important concept to understand how data is generated and analyzed using the Aurora flow cytometer with SpectroFlo software. Spectral unmixing is used to identify the fluorescence signal for each fluorophore used in a given experiment.

Understanding Full Spectrum Flow Cytometry

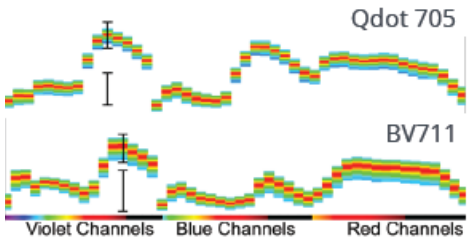
Because fluorophores emit light over a range of wavelengths, optical filters are typically used to limit the range of frequencies measured by a given detector. However, when two or more fluorophores are used, the overlap in wavelength ranges often makes it impossible for optical filters to isolate light from a given fluorophore. As a result, light emitted from one fluorophore appears in a non-primary detector (a detector intended for another fluorophore). This is referred to as spillover. In conventional flow cytometry spillover can be corrected by using a mathematical calculation called compensation. Single-stained controls must be acquired to calculate the amount of spillover into each of the non-primary detectors.

The Aurora's ability to measure a fluorophore's full emission spectra allows the system to use a different method for isolating the desired signal from the unwanted signal. The key to differentiate the various fluorophores is for those to have distinct patterns or signatures across the full spectrum. Because the system is looking at the full range of emission of a given fluorophore, and not only the peak emission, two dyes with similar emission but different spectral signatures can be distinguished from each other. The mathematical method to differentiate the signals from multiple fluorophores/dyes is called spectral unmixing and results in an unmixing matrix that is applied to the data. While not mathematically identical to conventional compensation, the overall principal is the same. Just as for compensation, single-stained controls, identified in SpectroFlo software as reference controls, are still necessary, as they provide the full fluorescence spectra information needed to perform spectral unmixing.

One advantage of unmixing is the ability to extract the autofluorescence of a sample and treat it as a separate parameter. This is especially useful when running assays with particles that have high autofluorescence and for which that high background has an impact in the resolution of the fluorescent signals. Per experiment, you can define one unstained control or multiple unstained controls (one per group), depending on whether the multicolor samples have the same or different autofluorescence signatures.

Spectrum plots from conventional spectrum viewer shows heavy overlap between Qdot 705 and BV711 peak emission spectra.

Spectrum plots from Aurora show distinct signatures for Qdot 705 and BV711.



Unmixing Workflows

Unmixing Overview

There are three unmixing workflows available in SpectroFlo software—two in the Acquisition module and one in the Extra Tools module:

- live unmixing during acquisition
- post-acquisition unmixing (in the Acquisition module)
- post-acquisition unmixing (in the Extra Tools module)

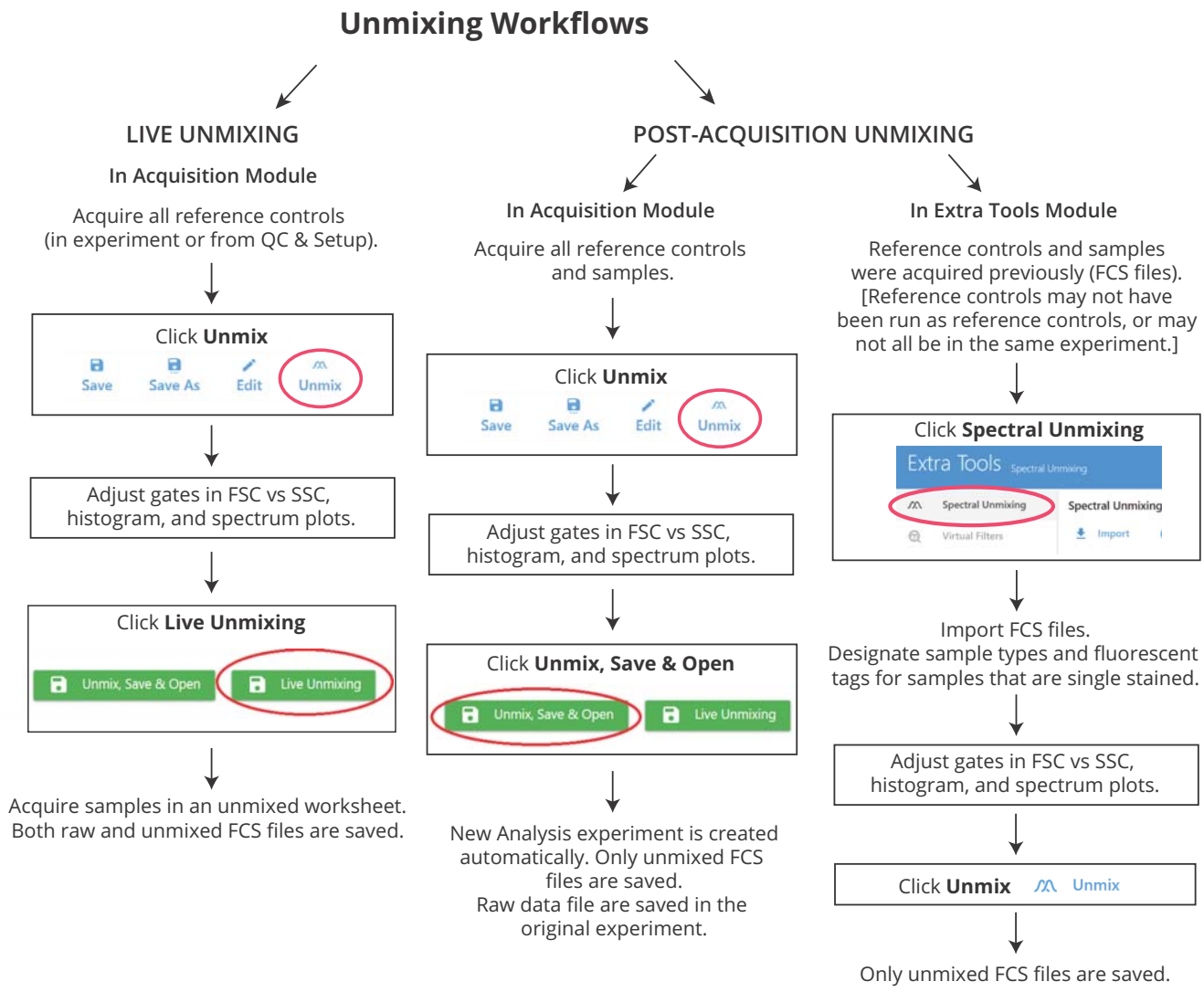
When data is acquired with live unmixing, references are acquired as raw data either in the experiment as part of the reference group or previously acquired in the QC & Setup module as reference controls. References for *all* fluorescent tags used in a given experiment must be present in the system in order for live unmixing of multicolor samples to occur. The live unmixing functionality allows you to visualize unmixed data during acquisition.

Reference Controls for Unmixing

Depending on when you unmix the data, you will use the following controls for unmixing.

Unmixing	Reference Controls
Live unmixing	Reference controls run in the experiment or reference controls run in QC & Setup
Post-acquisition unmixing in Acquisition module	Reference controls run in the experiment or reference controls run in QC & Setup
Post-acquisition unmixing in Extra Tools module	Any FCS files from samples run in any experiment

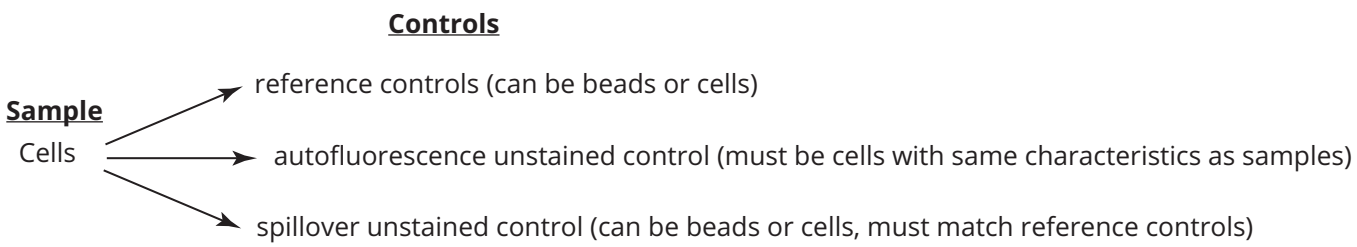
Multicolor samples can be acquired as raw data and unmixed post acquisition as well. This can be done in either the Acquisition module or the Extra Tools module.



Negative/Unstained Controls

In addition to positive reference controls needed for spectral unmixing, an unstained control is also necessary to assess **autofluorescence**. 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 extraction, if desired. Ideally, your reference controls, unstained control, and samples will all be the same sample type and prepared in the same way.

In addition to assessing autofluorescence, **fluorescence spillover** must also be determined. To correct for spillover, the unstained autofluorescence control can be used if it matches the sample and reference control type. However, if your reference controls do not match your sample type and do not contain a negative population in each tube (have only positive peaks), you must use a separate spillover unstained control that matches your reference control type.



Live Unmixing

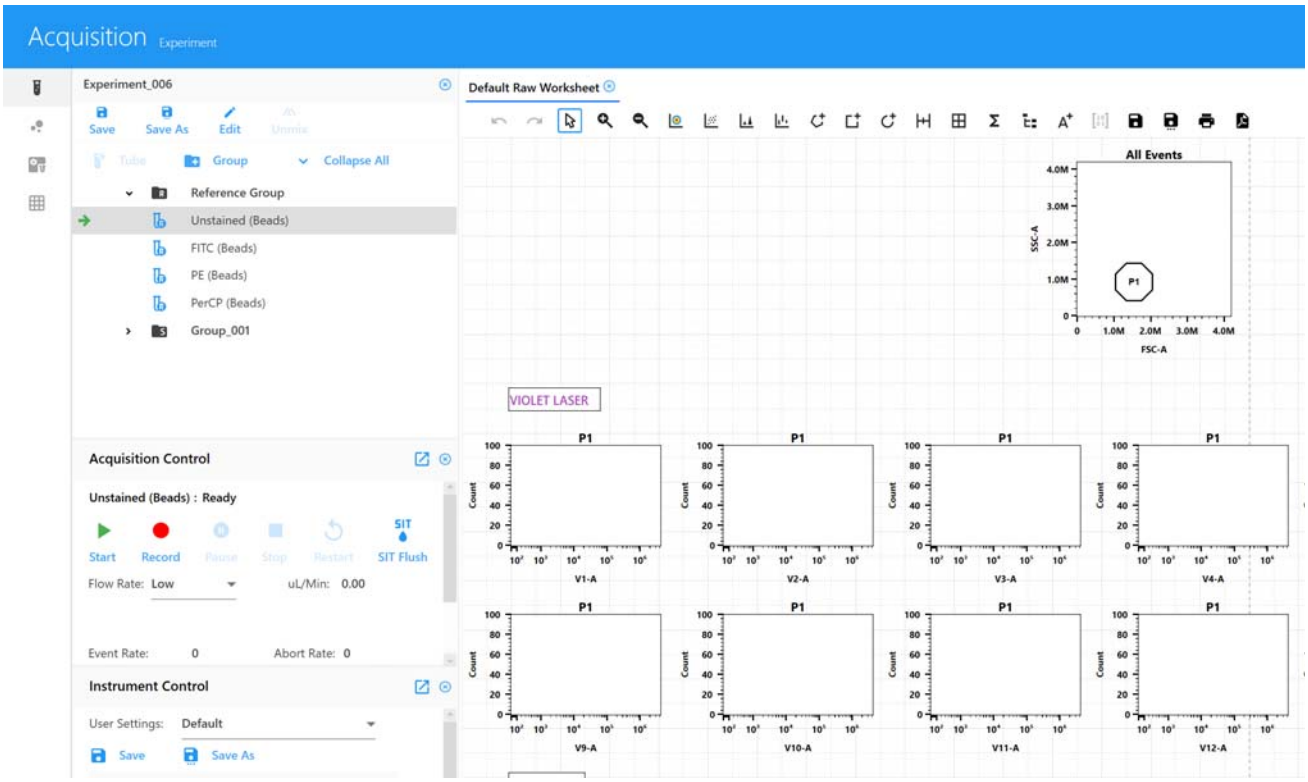
Samples can be unmixed during acquisition. Live unmixing can be performed with the reference group acquired during the experiment, the reference controls (run during QC & Setup and stored in the system), or a combination of both.

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.

To Perform Live Unmixing

1 Create a new experiment with fluorescent tags defined. Create a reference group in the experiment with the fluorescent tags, if there are any that have not already been stored as reference controls. See "Creating a New Experiment" on page 50 for details.



2 To view the data for the reference control tubes, make sure CytekAssaySetting is selected, then click **Start**. If necessary, use the Instrument Controls to adjust the settings so that all events are on scale. View all the controls, as well as the multi-color tube, and make any instrument adjustments to ensure populations are on scale before you begin recording.

To edit the acquisition criteria, click **Edit** at the experiment level and select the Acquisition tab. Or, to edit the properties of a single tube, right-click a tube and select Tube Properties.

■ **NOTE:** Keep in mind the more events you acquire, the longer it takes to unmix the data.

3 Click **Record** when you are ready to begin acquisition. Acquisition stops when the first stopping criterion is met.

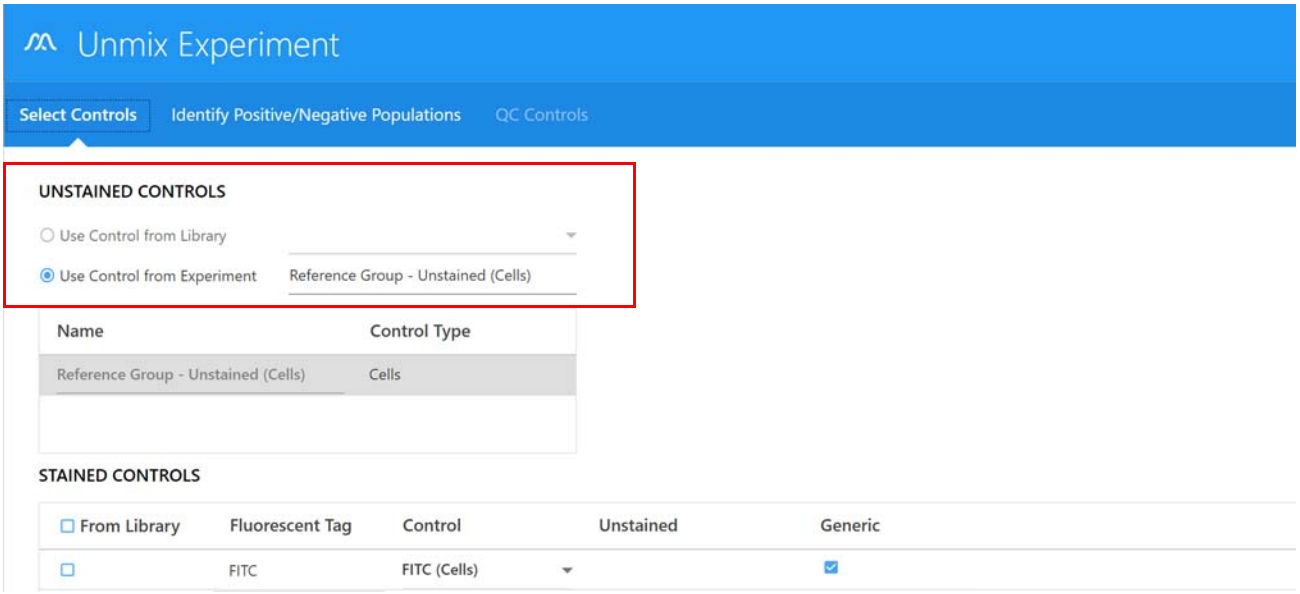
■ **NOTE:** If necessary, you can pause to change the flow rate.

4 When all reference controls are acquired, click **Unmix** in the upper-left toolbar.

5 For the unstained controls, we recommend selecting **Use Control from Experiment** if unmixing with controls you acquired in the experiment.

6 If necessary, for the stained controls, select **Use Control from Library** if unmixing with reference controls run in QC & Setup.

Checkmarks appear for those controls coming from QC & Setup. The checkbox is only active if reference controls for those fluorescent tags are already saved with the reference controls from the QC & Setup module.

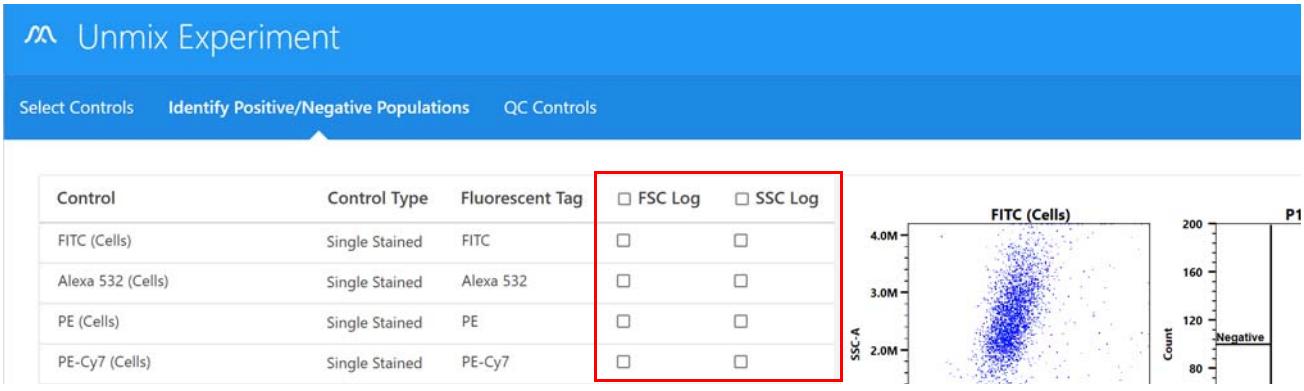


7 Click **Next**.

8 Use the Identify Positive/Negative Populations tab to include the positive and negative populations for each fluorescent tag in the appropriate gate.

Only the data plots for the samples you acquired are displayed, not for reference controls that you chose to use from the library.

■ **NOTE:** If you need to set the FSC and/or SSC axis to a log scale, select the Log checkbox.



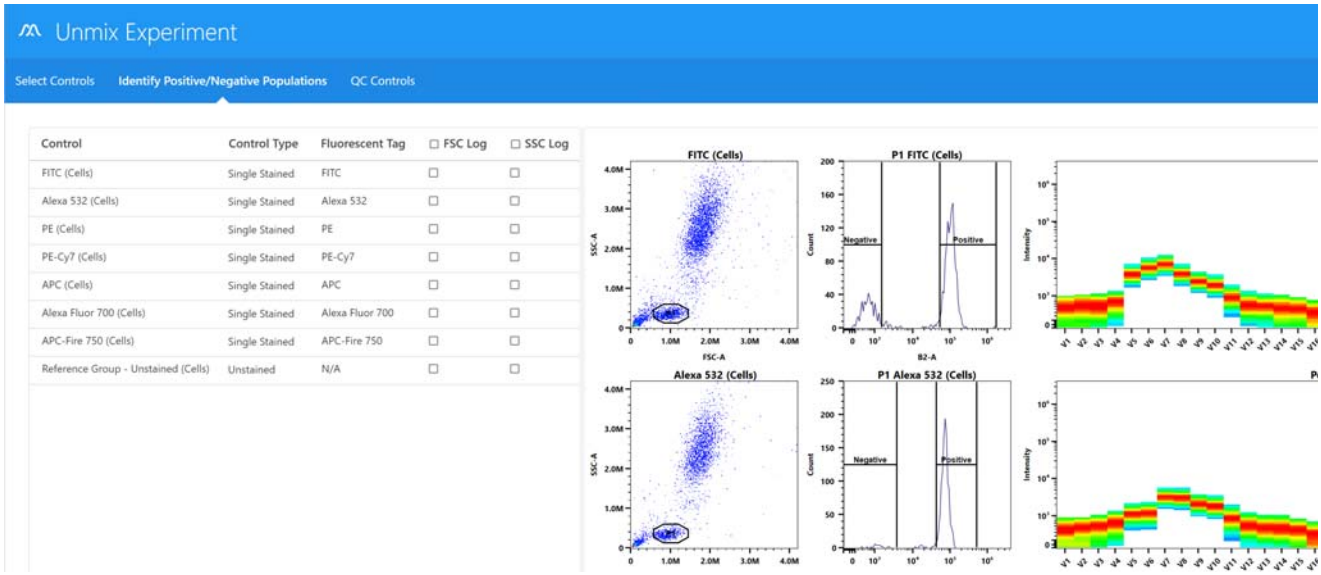
You may find it more efficient to view the data in columns—adjust the gates in the FSC vs SSC plots first, then adjust the histogram gates, finally adjust the gates on the spectrum plots.

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. Move the negative interval gate to include the negative population.

c. Move the interval gate on the spectrum plot on the right to select the channel that exhibits the brightest fluorescence intensity. This channel is the peak emission channel for the fluorescent tag.

■ **NOTE:** If one of the controls is questionable or does not contain sufficient data, you can reacquire it or append to it, then unmix again.



9 (Optional) To see how the reference controls run in the experiment compare to the benchmark reference controls, click **Next**.

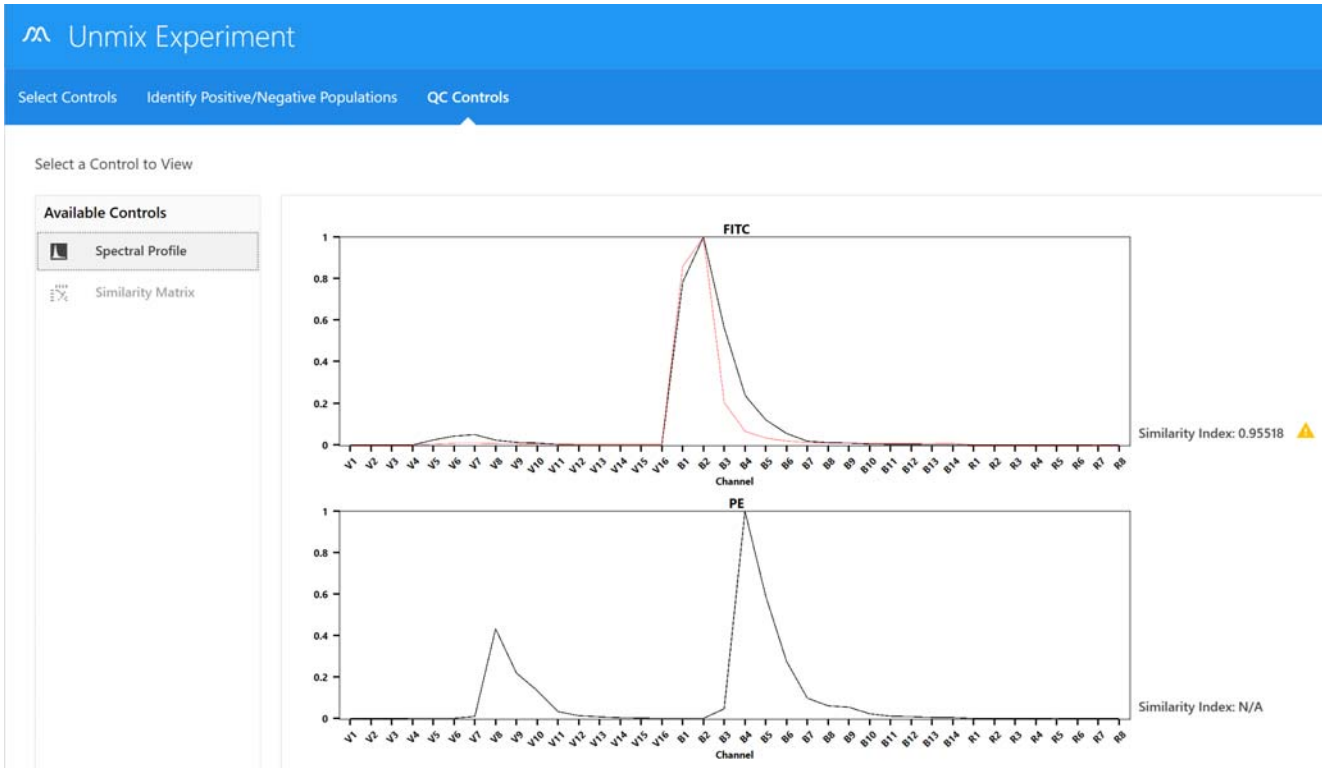
■ **NOTE:** For information on creating benchmarks, see "Setting Reference Controls as Benchmarks for Reference Control QC" on page 37.

Two options allow you to view how the two reference controls compare—spectral profile and similarity index.

- **Spectral Profile** displays the emission spectrum of the unmixing controls against benchmark spectra designated by the user. The benchmark reference control spectra appear in red and the reference controls appear in black.

  A similarity index appears to the right of the plots. If the value is below 0.97, it will be flagged with a yellow warning symbol. This indicates a mismatch of the unmixing control spectra with the benchmark spectra. If the similarity index falls below this value, it is imperative to check the unmixing control against the reference spectra provided in the fluorochrome guideline found in the Help menu. See "Similarity Matrix" below.

If no benchmark control is established for a particular dye, that plot will only display a black line that represents the spectra of the unmixing control. The similarity index will display N/A.

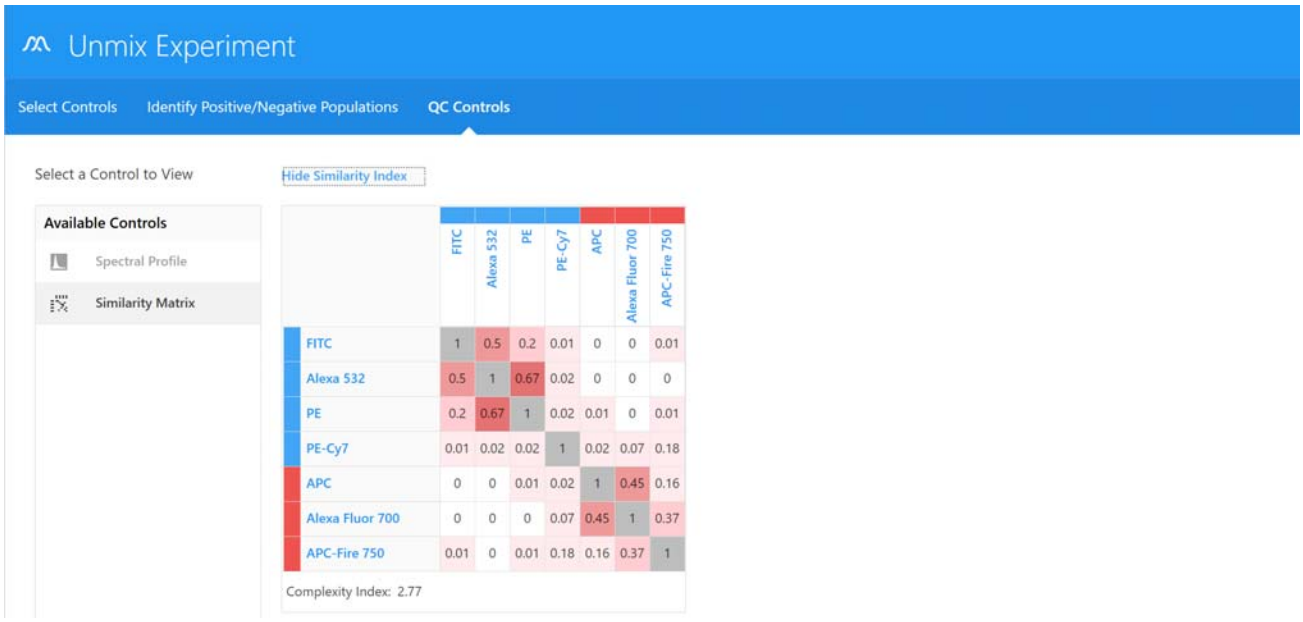


- Similarity Matrix displays a similarity index matrix and a complexity index value.

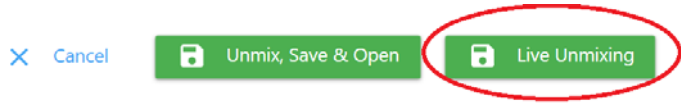
  The similarity index is a number between 0-1 that measures how closely the unmixing control's spectral signature matches the benchmark control's spectral signature. Click View Similarity Index above the matrix to display the indexes for each dye. The Similarity Matrix will display the similarity index for each dye against itself and all the other dyes to be unmixed in the experiment.

  The complexity index is a measure of how distinguishable a collection of spectral signatures are from each other when unmixed together. It calculates this by looking at the ratio of the similarity index of the worst overlapping combination of signatures to the best overlapping

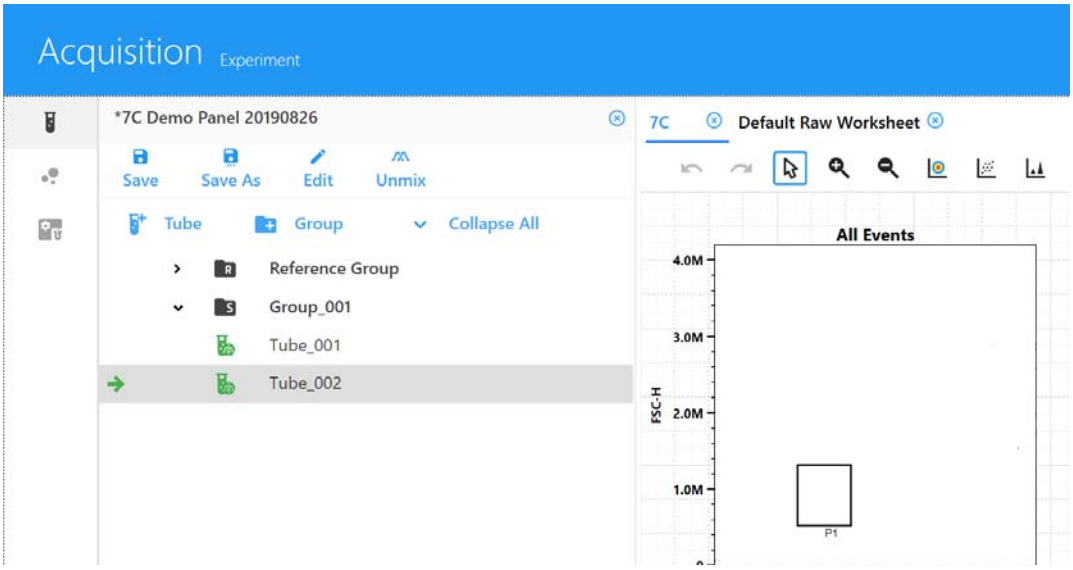
combination of signatures by looking at the ratio of the worst overlapping combination to the best overlapping combination.



10 Click Live Unmixing.

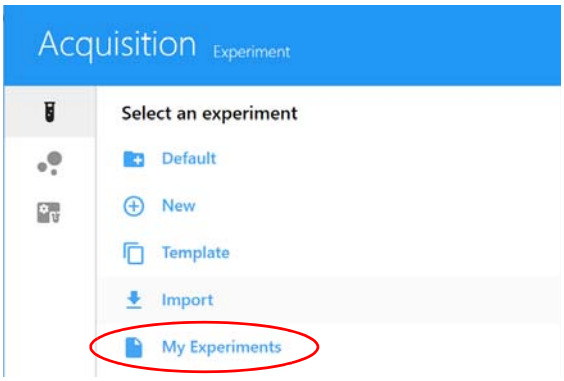


11 The wizard closes and the experiment reappears. The reference group now has the unmixed icon to the left of the tube(s). Select an unmixed worksheet to view the unmixed data.



12 Select the sample tube you wish to acquire. The green arrow indicates the tube is selected. Click Start, then Record.

Use My Experiments to open experiments you ran if you wish to review the data or acquire more samples.



FCS files are stored in the Export folder by default, or the folder you set as the default. See "Storage Preferences" on page 88 for information. FCS files for live unmixed data are saved as both raw data and unmixed data.

Alternately, you can click My Experiments, select the experiments you want to export, right-click and select **Export**. This will export the entire experiment as a ZIP file with all of the FCS files and worksheet templates contained inside. This experiment can be imported into other instances of SpectroFlo software, or unzipped to access the FCS files for analysis using other analysis software.

Post-Acquisition Unmixing

Samples can be acquired as raw data and then unmixed after acquisition is complete. This can be done through two methods:

- post-acquisition unmixing in the Acquisition module (see below)
- post-acquisition unmixing in the Extra Tools module (see page 67)

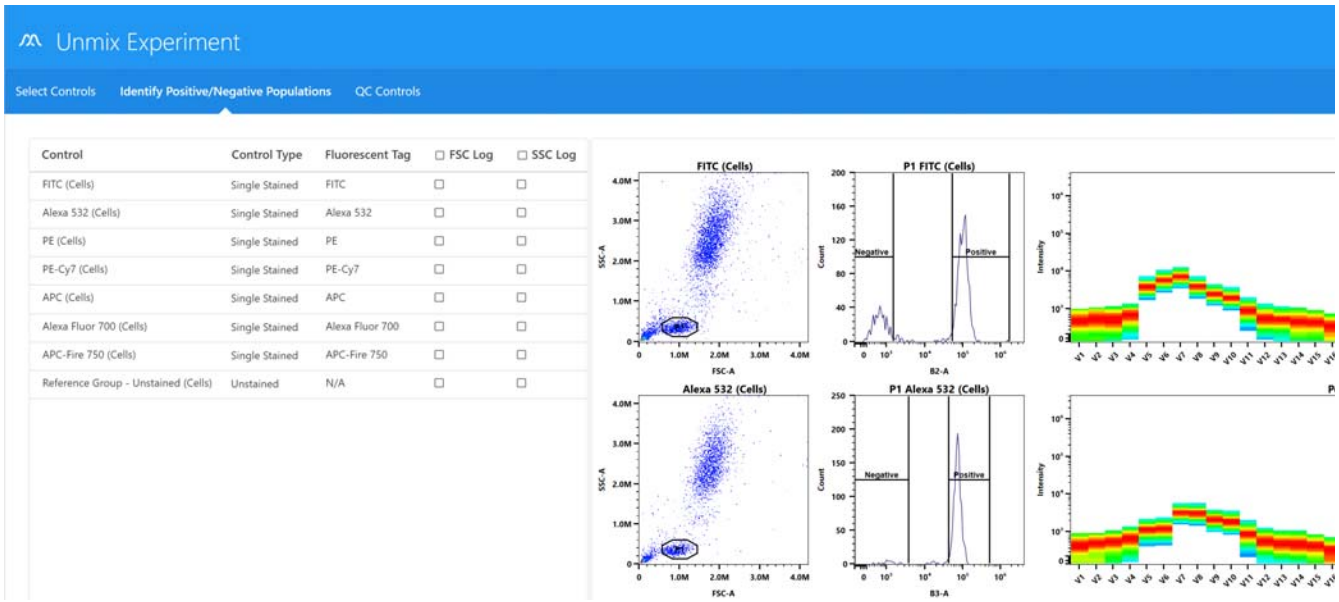
Post-Acquisition Unmixing in the Acquisition Module

The unmixing wizard in the Acquisition module limits reference controls to those coming from the reference group in the experiment or reference controls run in QC & Setup.

To perform post-acquisition unmixing in the Acquisition module, perform the same workflow as live unmixing except the following:

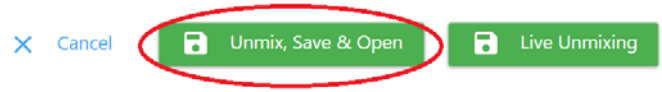
1. Acquire all reference control tubes and sample tubes prior to selecting the **Unmix** button in the upper-left pane.
2. You may find it more efficient to view the data in columns—adjust the gates in the FSC vs SSC plots first, then adjust the histogram gates. Finally, examine the spectra plots.
   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. Move the negative interval gate to include the negative population.

c. Move the interval gate on the spectrum plot on the right to select the channel that exhibits the brightest fluorescence intensity. This channel is the peak emission channel for the fluorescent tag.

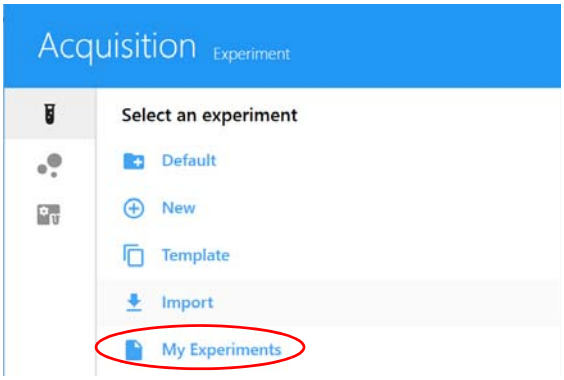


3 Click Unmix, Save & Open.

A new experiment opens with a new unmixed worksheet.



Use My Experiments to open experiments you ran, if you wish to review the data or acquire more samples.



FCS files are stored in the Export folder by default, or the folder you set as the default. See "Storage Preferences" on page 88 for information. FCS files for post-acquisition unmixed data are saved as unmixed data only.

Post Acquisition Unmixing in the Extra Tools Module

When performing post-acquisition unmixing in the Extra Tools module, you can pick and choose which FCS files to unmix (for example, controls coming from different experiments, reference controls run during QC & Setup, or single-stained controls that were not run as part of the reference group).

FCS files can be designated into three categories:

- Single Stained
- Unstained
- Sample

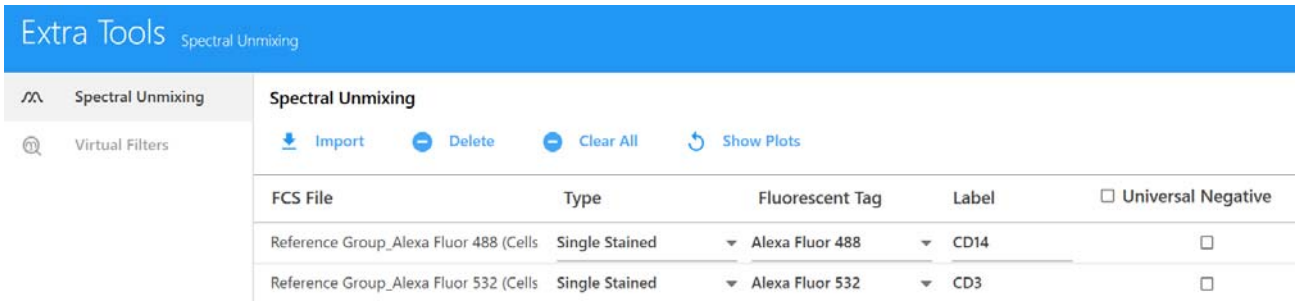
■ **NOTE:** There must be at least one single-stained FCS file and one unstained FCS file in the file list. Otherwise, unmixing cannot be performed.

In addition, raw FCS files can also be conventionally compensated in this module through the Virtual Filters tab. This function can simulate the presence of filters and can compensate data using conventional compensation methods (see "Virtual Filters" on page 70).

To Unmix Raw Data Files:

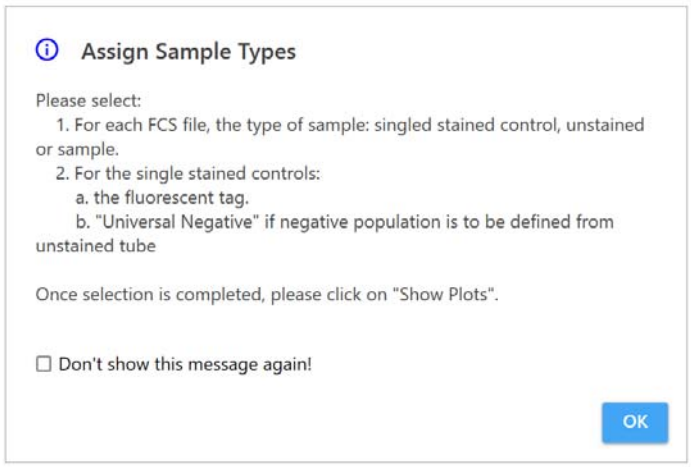
1 Select Spectral Unmixing from the Extra Tools module.

2 Click Import to import raw FCS files for unmixing.

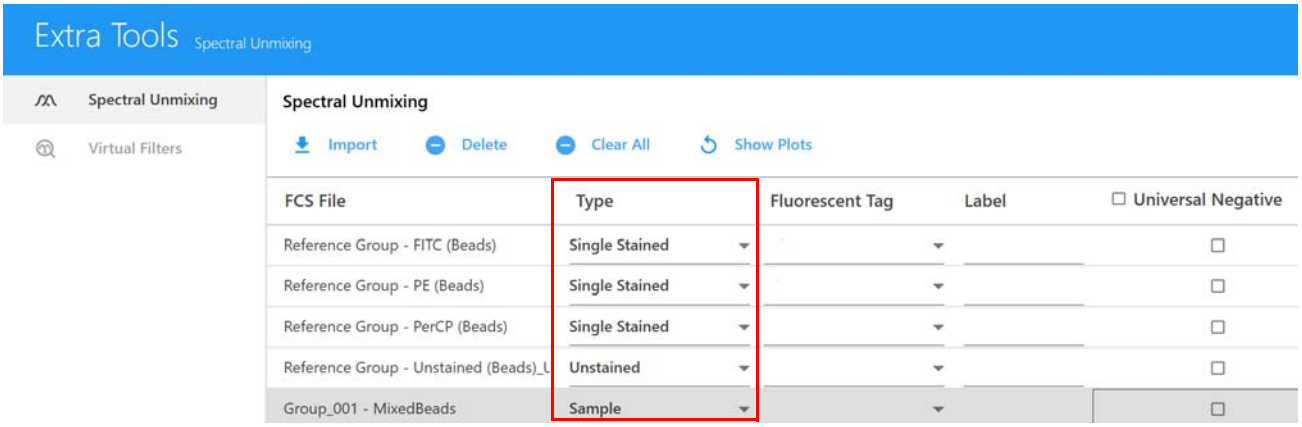


3 Select the files. Select multiple files using either the Shift or Ctrl key. Click **Open**.

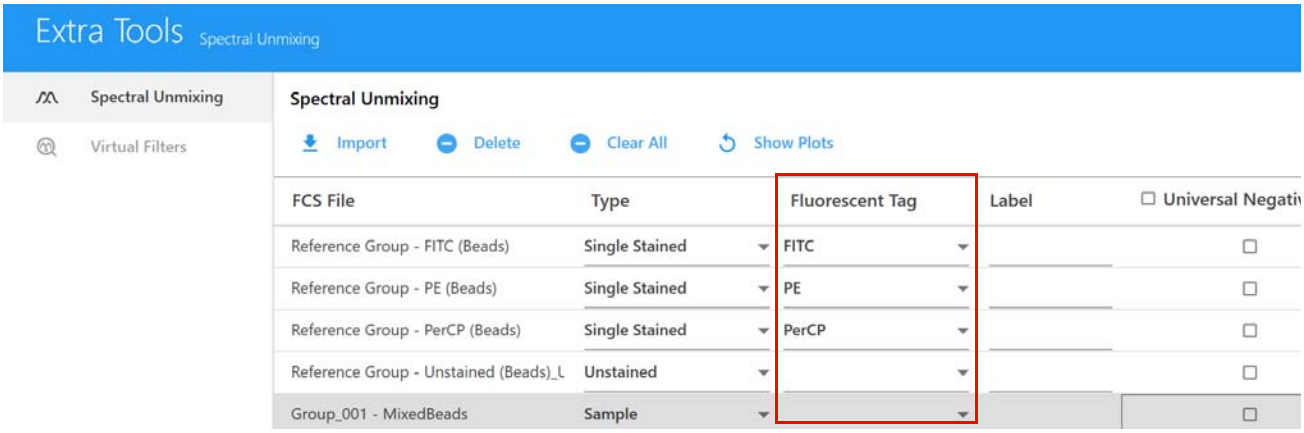
4 Upon importing, a dialog box on how to assign sample types appears. Read the instructions and click OK.



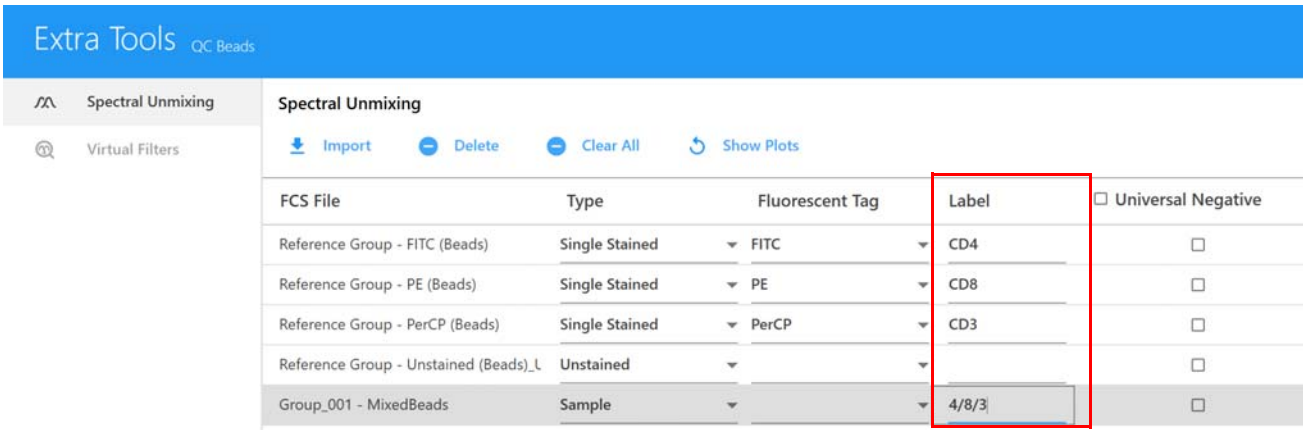
5 Once FCS files have been imported, select the sample type for each FCS file as Single Stained, Unstained, or Sample. The software will automatically designate the type based upon the file name. You can manually modify these if the automatic designation is incorrect.



6 FCS files designated as single-stained will require a fluorescent tag designation to specify what reference spectrum will be provided for unmixing.



7 Enter a label for each single-stained control and sample.



8 Select Universal Negative for single-stained FCS files that do not contain a negative population, and the unstained control will be used for the negative population. In the bottom left of the screen, check whether Auto Fluorescence will be used as a fluorescent tag.

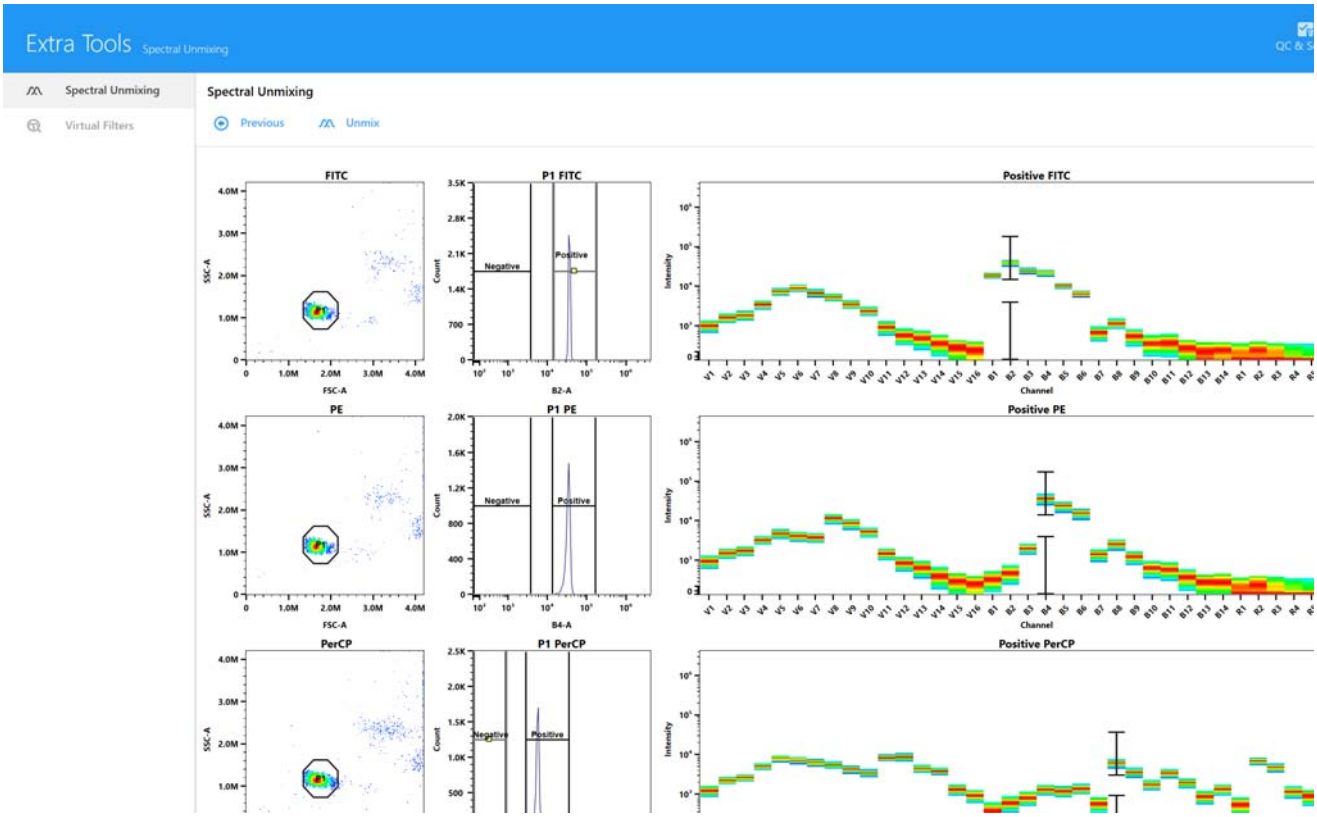
9 Click Show Plots to display the data in the FSC vs SSC plot, peak emission channel histogram, and spectrum plots.

10 The positive and negative populations need to be identified through the appropriate placement of the existing gates. Click OK to adjust the gates.

a. Move the polygon gate in the FSC vs SSC plot to include the singlet population.

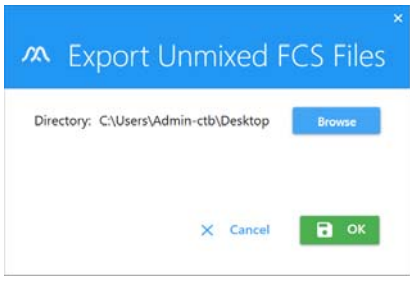
b. Move the interval gate in the histogram labeled *Positive* to include the positively stained population. Move the interval gate in the histogram labeled *Negative* to include the negative population. Do not adjust the negative gate when using the Universal Negative.

c. Move the interval gate on the spectrum plot on the right to select the channel that exhibits the brightest fluorescence intensity. This channel is the peak emission channel for the fluorescent tag.



11 Click Unmix.

12 Select the directory to which the unmixed FCS files are exported or leave the default. Click OK.

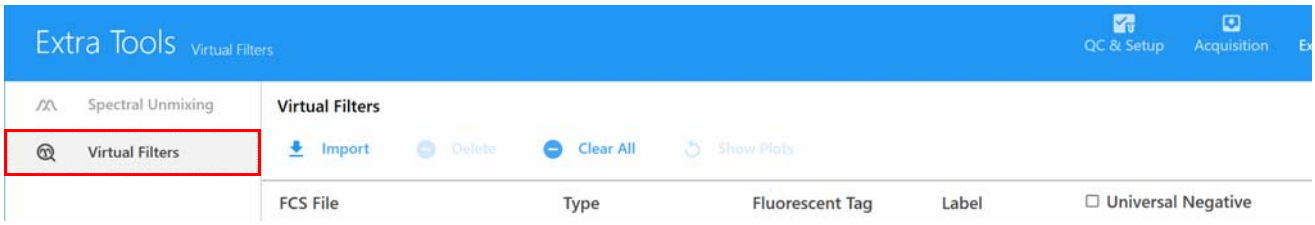


These FCS files can then be imported to an experiment for analysis or analyzed using third-party software.

Virtual Filters

The Virtual Filters option in the Extra Tools module allows you to compensate raw FCS data using conventional compensation methods.

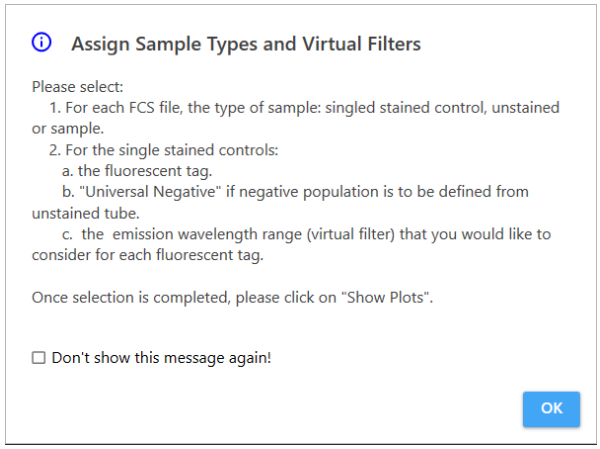
1 Click the **Virtual Filters** tab in the Extra Tools module.



2 Click **Import** to import raw FCS files for virtual filter analysis.

These FCS files can be single-stained reference controls, unstained controls, and/or sample files. However, you must include an unstained control FCS file.

3 Upon importing, a dialog box on how to assign sample types appears. Read the instructions and click **OK**.

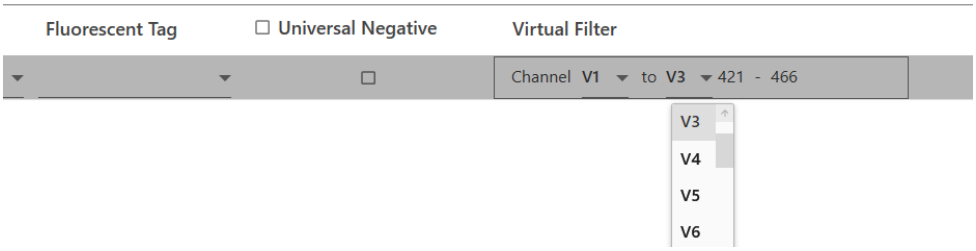


4 Once FCS files have been imported, the sample type for each FCS file needs to be designated as Single Stained, Unstained, or Sample. The software will automatically designate the type based upon the file name. You can manually modify these if the automatic designation is incorrect.

5 FCS files designated as single stained require a fluorescent tag designation. Select the fluorescent tag for each single-stained sample.

If there is no negative population in the single-stained FCS file(s), select **Universal Negative**, and the unstained control will be used for the negative population.

The virtual filter is automatically assigned by the software based upon the fluorescent tag designation. (Optional) To increase the bandwidth of the virtual filter, use the channel pull-down menus to select the desired range. See the following table for wavelength ranges.



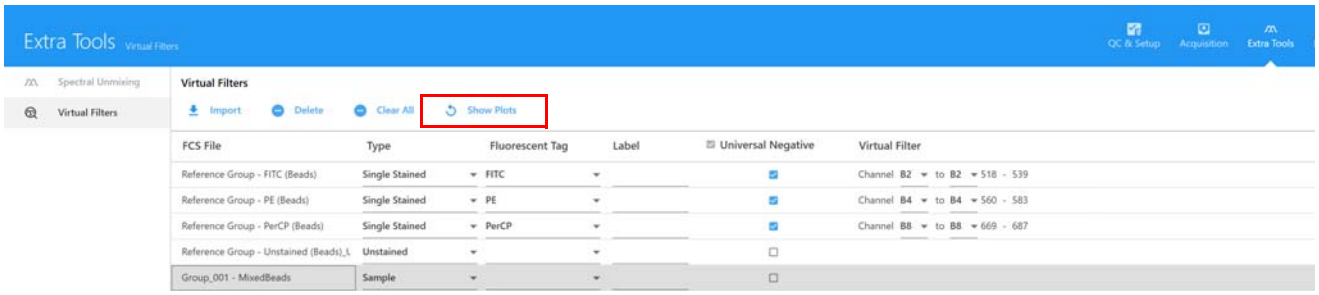
The following table shows the system's filter bandwidths.

Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Ultraviolet	UV1	373	15	365	380
	UV2	388	15	380	395
	UV3	428	15	420	435
	UV4	443	15	436	451
	UV5	458	15	451	466
	UV6	473	15	466	481
	UV7	514	28	500	528
	UV8	542	28	528	556
	UV9	582	31	566	597
	UV10	613	31	597	628
	UV11	664	27	651	678
	UV12	692	28	678	706
	UV13	720	29	706	735
	UV14	750	30	735	765
	UV15	780	30	765	795
	UV16	812	34	795	829
Violet	V1	428	15	420	435
	V2	443	15	436	451
	V3	458	15	451	466
	V4	473	15	466	481
	V5	508	20	498	518
	V6	525	17	516	533
	V7	542	17	533	550
	V8	581	19	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

Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Blue	B1	508	20	498	518
	B2	525	17	516	533
	B3	542	17	533	550
	B4	581	19	571	590
	B5	598	20	588	608
	B6	615	20	605	625
	B7	661	17	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
Yellow Green	YG1	577	20	567	587
	YG2	598	20	588	608
	YG3	615	20	605	625
	YG4	661	17	653	670
	YG5	679	18	670	688
	YG6	697	19	688	707
	YG7	720	29	706	735
	YG8	750	30	735	765
	YG9	780	30	765	795
	YG10	812	34	795	829
Red	R1	661	17	653	670
	R2	679	18	670	688
	R3	697	19	688	707
	R4	717	20	707	727
	R5	738	21	728	749
	R6	760	23	749	772
	R7	783	23	772	795
	R8	812	34	795	829

6 (Optional) Select a label for the single-stained controls and samples.

7 Click **Show Plots** to display the plots.



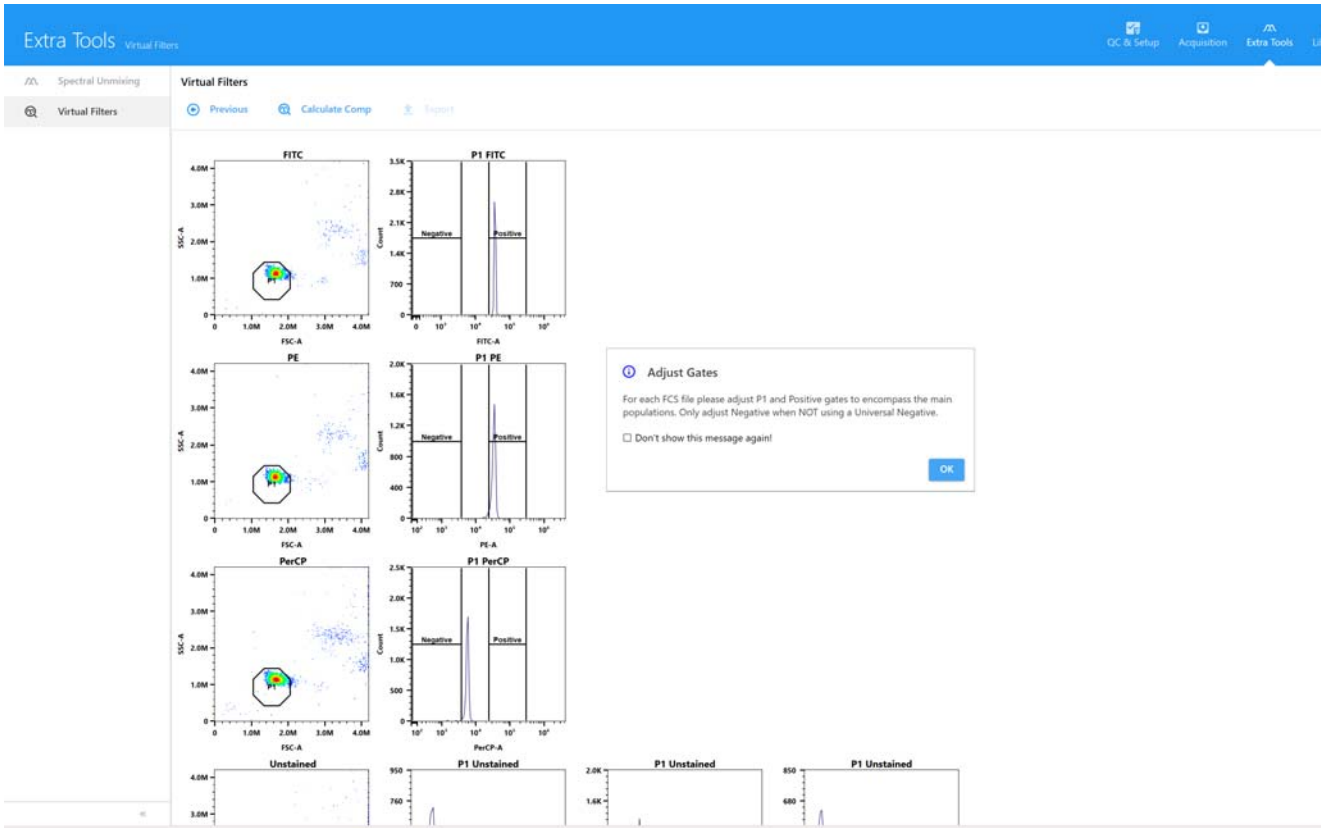
The data is displayed in the FSC vs SSC plot and fluorescent tag histogram plot.

8 The positive and negative populations need to be identified through the appropriate placement of the gates. Click **OK** to adjust the gates.

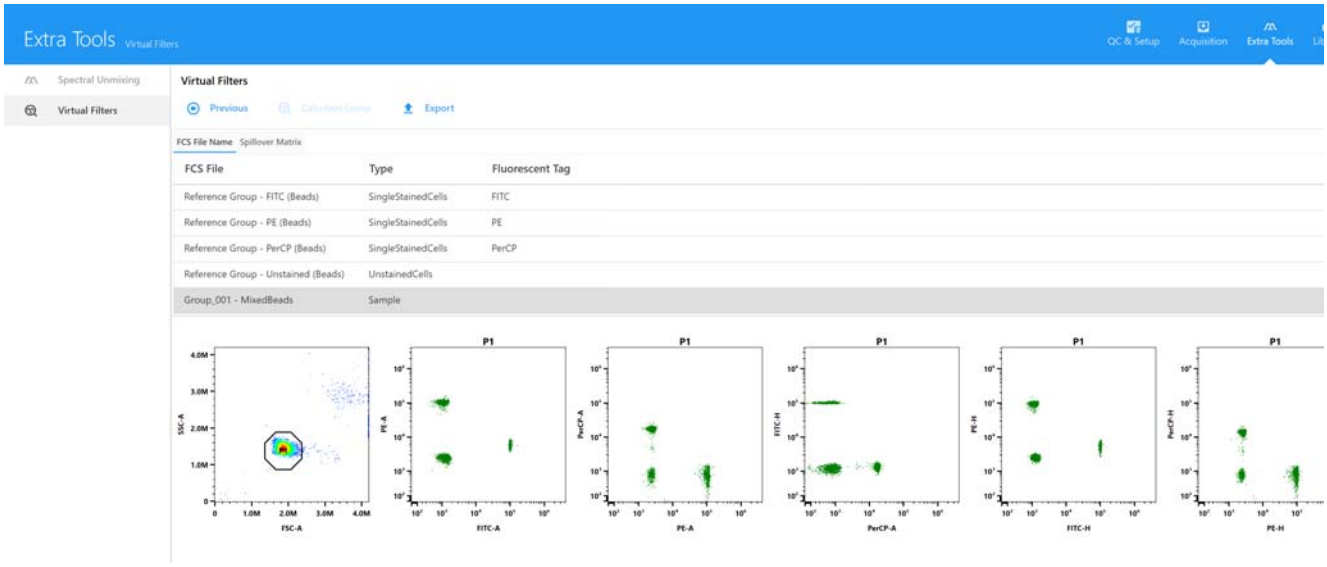
a. Move the polygon gate in the FSC vs SSC plot to include the singlet population. Hold down Ctrl to move all the polygon gates at once.

b. Move the interval gate in the histogram labeled *Positive* to include the positively stained population. Move the interval gate in the histogram labeled *Negative* to include the negative population. Do not adjust the negative gate when using the Universal Negative.

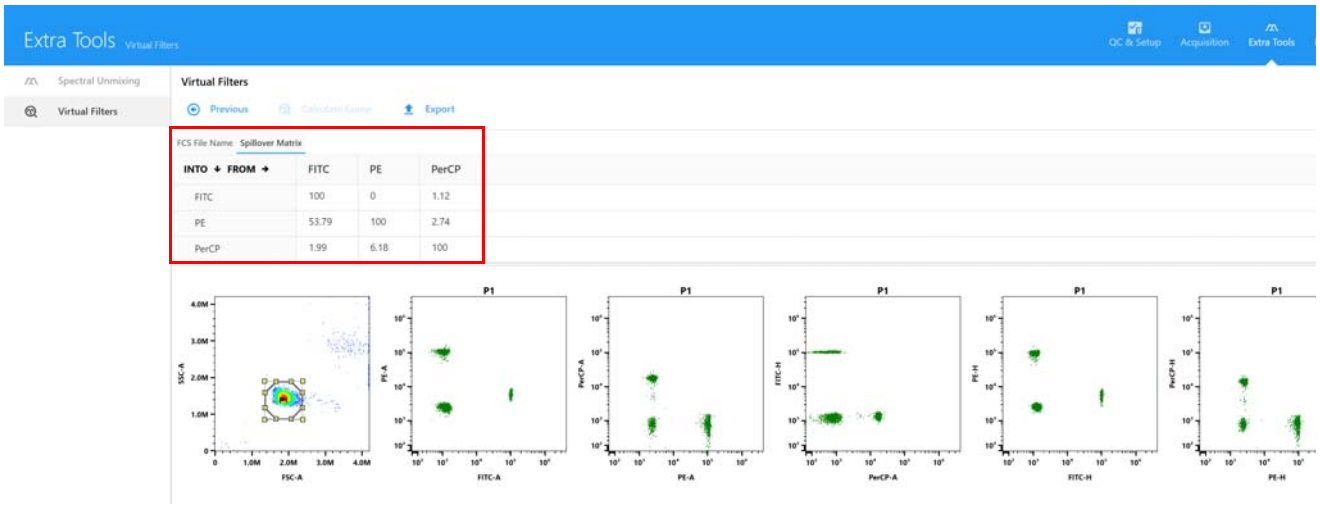
The histogram x-axes are labeled with the fluorescent tag instead of the channel/detector.



9 Click Calculate Comp once gates have been set correctly. The conventionally compensated data is displayed. To view the data for a specific FCS files, select the file.



The spillover matrix is also calculated. Click Spillover Matrix to view the spillover values.



10 Click Export and select the location where you wish to export the conventionally compensated data. The files are exported to a folder named Compensated followed by the current date and time. Files can then be imported back into an experiment to analyze in SpectroFlo software.