5

Acquisition

Raw vs Unmixed Data

SpectroFlo software saves flow cytometry data in the FCS 3.1 format. Data is saved in both raw and unmixed formats. Raw data contains all the fluorescence information from each detector (ie, V1, V2, V3, etc). Each detector channel is designated by its excitation laser and position in the array. For example, B3 is the third channel of the blue laser detector array.

Unmixed data contains all the fluorescence information from each fluorescent tag in the experiment. To unmix data, single stained controls (or reference controls) for each of the fluorescent tags (as well as an unstained control) are required. During unmixing a mathematical algorithm is used for the decomposition of the fluorescent components in the sample using the reference controls. Parameters in unmixed data will display as the fluorescent tag name along with their associated labels.

The Acquisition module provides the tools that allow you to create an experiment. An experiment is a set of tubes, instrument settings, acquisition criteria (stopping rule), fluorescent tags, labels, and worksheets designed for the acquisition of samples. See "About Experiments" on page 18.

New and saved experiments can be created or accessed in the Experiments tab of the Acquisition module.

Unmixing and Compensation

Raw FCS files can be spectrally unmixed in the following ways:

- Reference group from the experiment – Reference controls collected as FCS files within the experiment can be used to unmix using the Unmixing wizard in the Acquisition module.
- Reference controls run during QC & Setup – Reference controls run during QC & Setup can be used to unmix using the Unmixing wizard in the Acquisition module.
- Unmixing from the Extra Tools module – FCS files collected from different experiments can be unmixed in the Extra Tools module. FCS files can be imported and unmixed in this module.

Raw FCS files can also be compensated with the conventional method using the Virtual Filters tab in the Extra Tools module. Detector channels can be binned together to simulate the analysis of the data as if it were acquired using a filter. See "Virtual Filters" on page 70 for more information.

Setting Up an Experiment

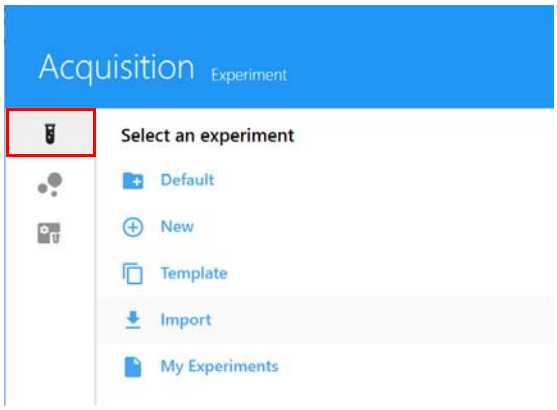
An experiment can be saved as a template or created for one time use. Setting up the experiment in SpectroFlo software involves:

1. (Optional) Providing a name and description for the experiment. A default name is provided.
2. Specifying the fluorescent tags used in the experiment.
3. Defining the reference group with associated reference tags, labels, and lot numbers, as needed.
4. Adding labels and lot numbers to each of the fluorescent tags.
5. Adding custom keywords. Custom keywords can be defined in the Library.
6. Selecting an acquisition worksheet—either new or a template.
7. Defining acquisition criteria (stopping rule based on events, time, or volume).

Acquisition Experiment Overview

The Acquisition module provides the necessary elements for data collection within the experiment.

Click the Experiment icon in the far left pane to open a template, the default, or a new experiment using the wizard.



Experiment Display

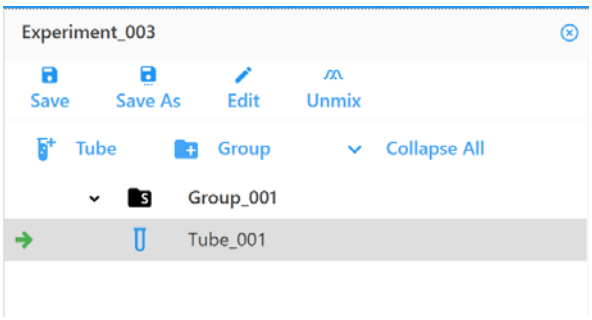
The experiment display in the Acquisition module includes the following panes. To show, hide, or undock (float) these panes from the experiment panel, click the corresponding icons in the top-right corner of the pane.

Group-Tube List and Hierarchy

The samples are listed in the upper left of the screen. Samples can be organized into groups. Use the ⊕ Tube and ⊕ Group icons to add tubes and groups. If running in plate mode, click Add Plate to add plates. Click Plate View to display a graphic image of the 96-well plate instead of the list of groups.

Click Save to save changes to the experiment, or click Save As to save an experiment template. Click Edit to edit the experiment. Worksheets can be applied to the experiment, groups, or individual tubes.

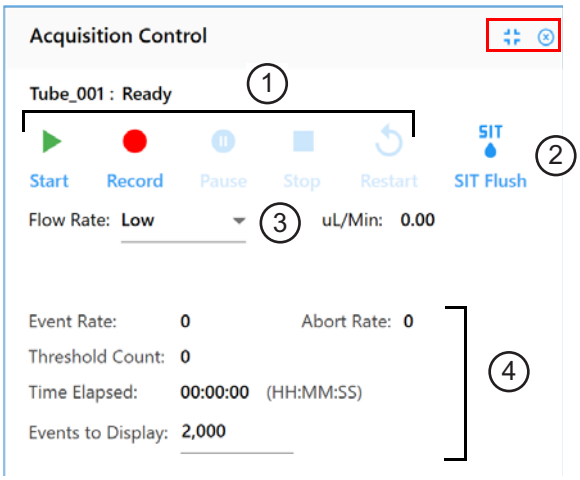
Tube mode



Acquisition Control

The Acquisition Control pane allows you to start, stop, and pause acquisition, record data, and restart acquisition counters. The acquisition controls are enabled when a tube is present on the SIP or when in plate mode. To show, hide, or undock (float) this pane from the experiment panel, use the dock/undock and hide icons in the top-right corner.

For information on the Loader Acquisition Controls when in plate mode, see "Loader Acquisition Controls" on page 96.



The following table describes the controls in the Acquisition Control pane.

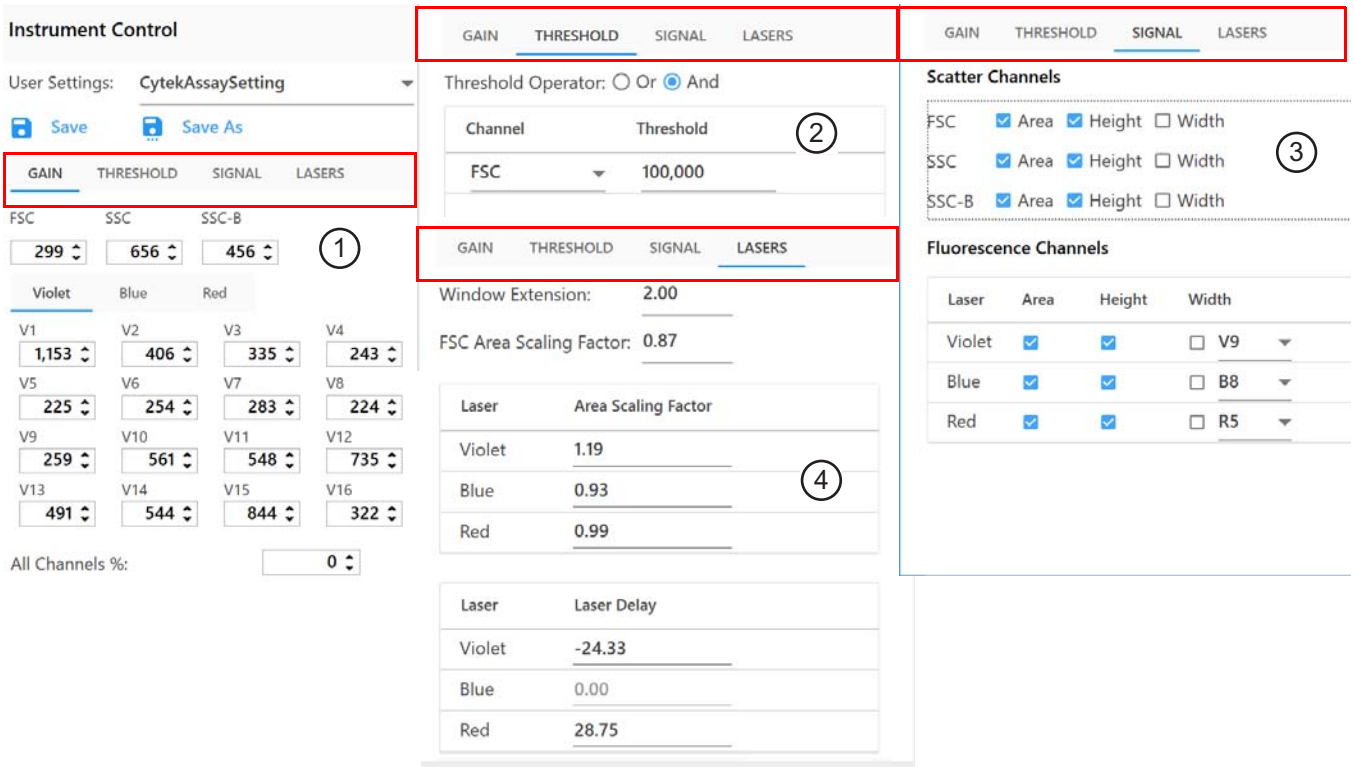
No.	Control	Description
1	Start/Record/Pause/ Stop/Restart	Start and Record are enabled when a tube is present on the SIP. Select Start to start acquisition Select Record to record data. Record can also start acquisition. Select Pause to pause recording. While paused you can adjust the flow rate. Select Record again to continue. Select Stop to stop acquisition. Select Restart to restart the acquisition counters. All events and results displayed are refreshed. Stop and Restart are enabled once Start is selected. Stop and Pause are enabled once Record is selected.

No.	Control	Description
2	SIT Flush	Select to perform a SIT Flush
3	Flow Rate	Select Low (15 µL/min), Medium (30 µL/min), or High (60 µL/min). The exact flow rate is displayed.
4	Event Rate, Abort Rate, Threshold Count, Time Elapsed	Displays the real-time counts during acquisition.
5	Events to Display	Enter the number of events to display during acquisition.

Instrument Control

The Instrument Control pane consists of the Gain, Threshold, Signal, and Lasers tabs for use in adjusting the instrument.

User Settings allow you to select CytekAssaySetting, Default, or any saved user settings for the experiment. We recommend using CytekAssaySetting as a starting point. This setting provides the optimal resolution for each channel, accommodates bright signals, and minimizes spread.

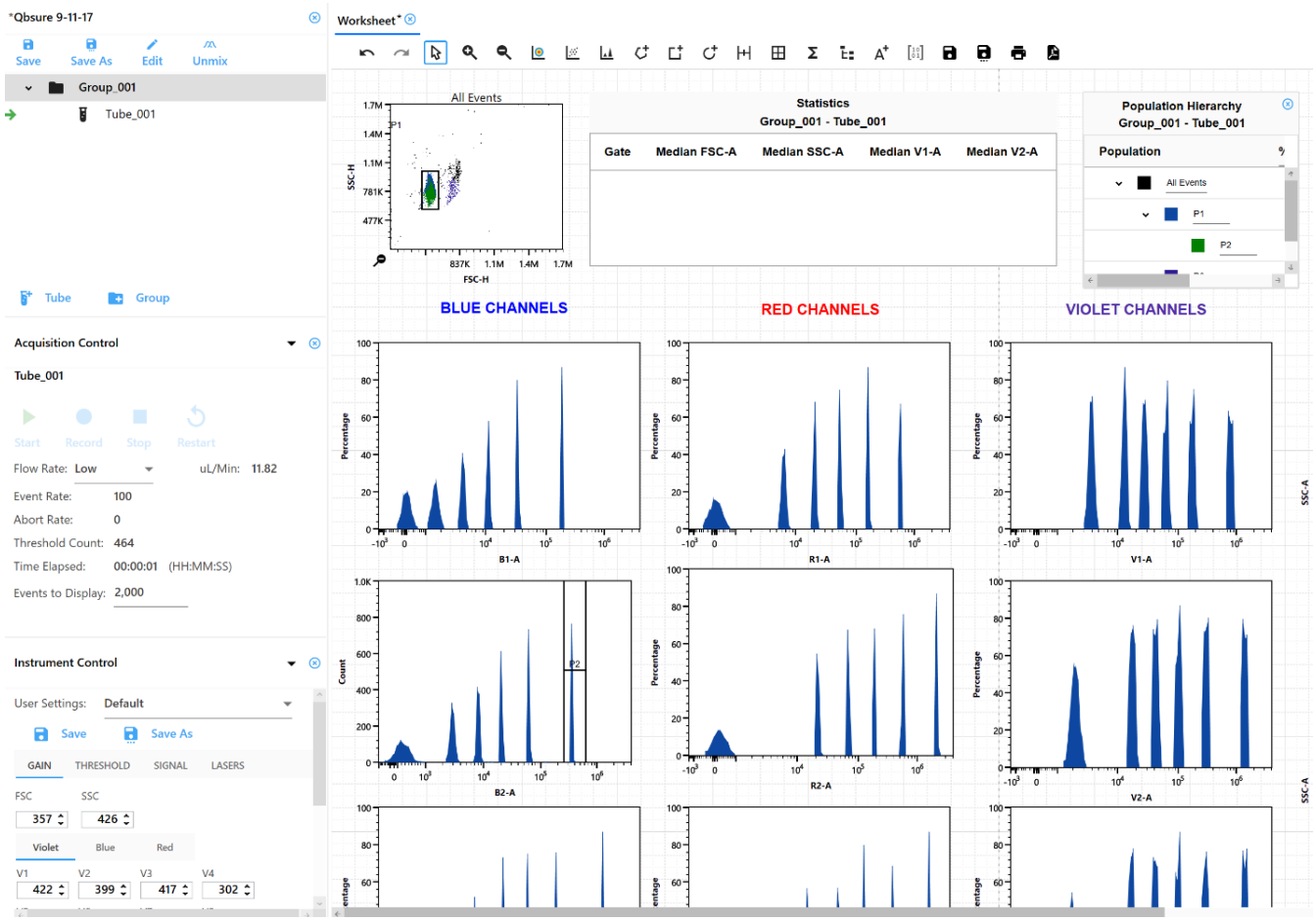


The following table describes the tabs in the Instrument Control pane.

No.	Tab	Description
1	Gain	Gains can be adjusted for all detector channels for all lasers using the gain spinboxes. FSC gain can be adjusted from 1–1,000. SSC and fluorescence detector gains can be adjusted from 10–10,000. To change the value that the gain increments, see "Acquisition Preferences" on page 81. Use All Channels % to increase/decrease all gains for a selected laser by the percentage you select.
2	Threshold	Use the Threshold tab to set the threshold parameter and minimum threshold channel value. Multiple parameters can be set as a threshold using either the AND or OR operator. Use OR when at least one parameter is available.
3	Signal	Use the Signal tab to select area, height, or width for each signal. Area and height can be selected for all channels. Width can be selected for only one channel per laser.
4	Lasers	Use the Lasers tab to set the area scaling factor and laser delay. These values are automatically set and updated in all user settings upon completion of the Daily QC. ■ **NOTE:** If you run large cells and the lowest FSC gain setting is not low enough to see your cells, lower the FSC area scaling factor (for example, 0.5).

Worksheet

The worksheet allows you to view the data in plots and create plots, statistics, population hierarchy, and gates. Click the worksheet icon in the far left pane to select, import, save, and print worksheets.



Worksheet Toolbar

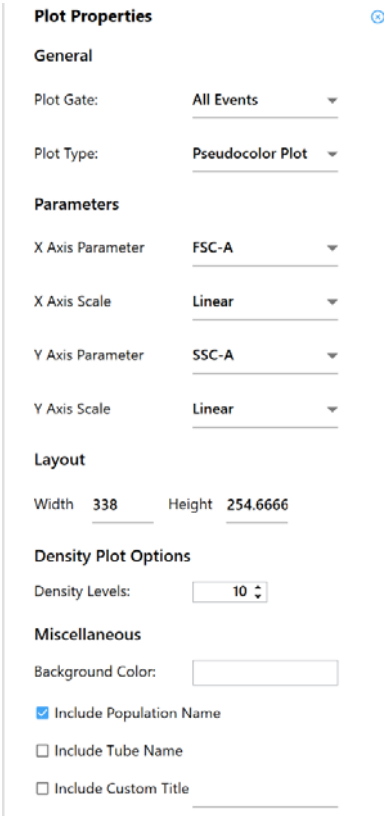
A toolbar at the top of the worksheet area allows you to undo/redo, zoom; create plots, gates, statistics, population hierarchy, annotations; and save, print, and save a PDF of the worksheet. Hover the cursor over an icon to see a description and keyboard shortcut.

Plots

Four plot types can be created in the worksheet:

- dot plots
- pseudocolor plots (density plots)
- histogram plots
- spectral plots

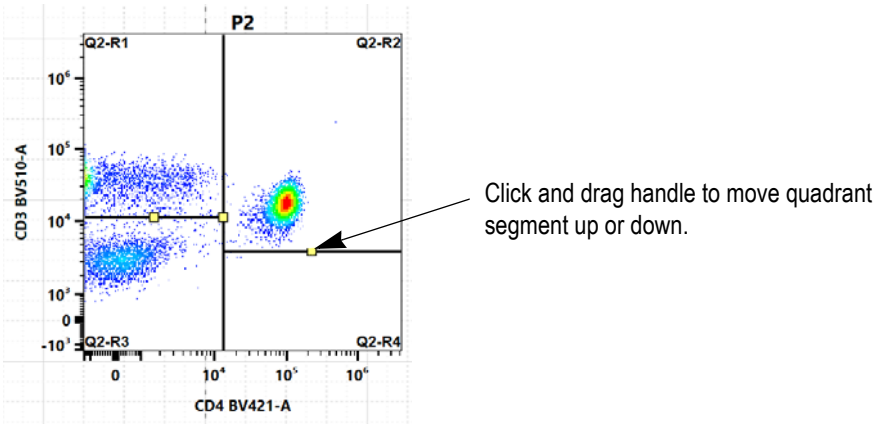
To change the properties of a plot, right-click the plot and select **Properties**. You can select the plot type, parameters, scale, background color, and labels.



Gates

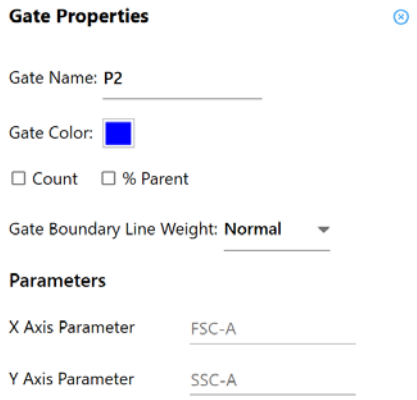
Gates types include:

- rectangle
- oval
- polygon
- interval
- quadrants and staged quadrants (select and drag an offset handle to move the quadrant segment up or down)



Gate Properties

Gates properties can be changed by right-clicking the gate. 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.



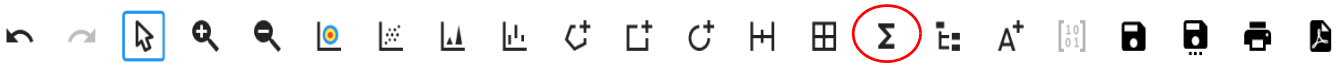
Logical Gates

Select multiple gates and right-click to open a menu to:

- Intersect the gates with the AND operator – events that are present in all of the selected gates are part of the intersected gate population.
- Join the gates with the OR operator – events that are present in at least one of the gates are part of the joined gate population.

Statistics

To create a statistics box, click the Statistics icon in the worksheet toolbar, then click in the worksheet area.



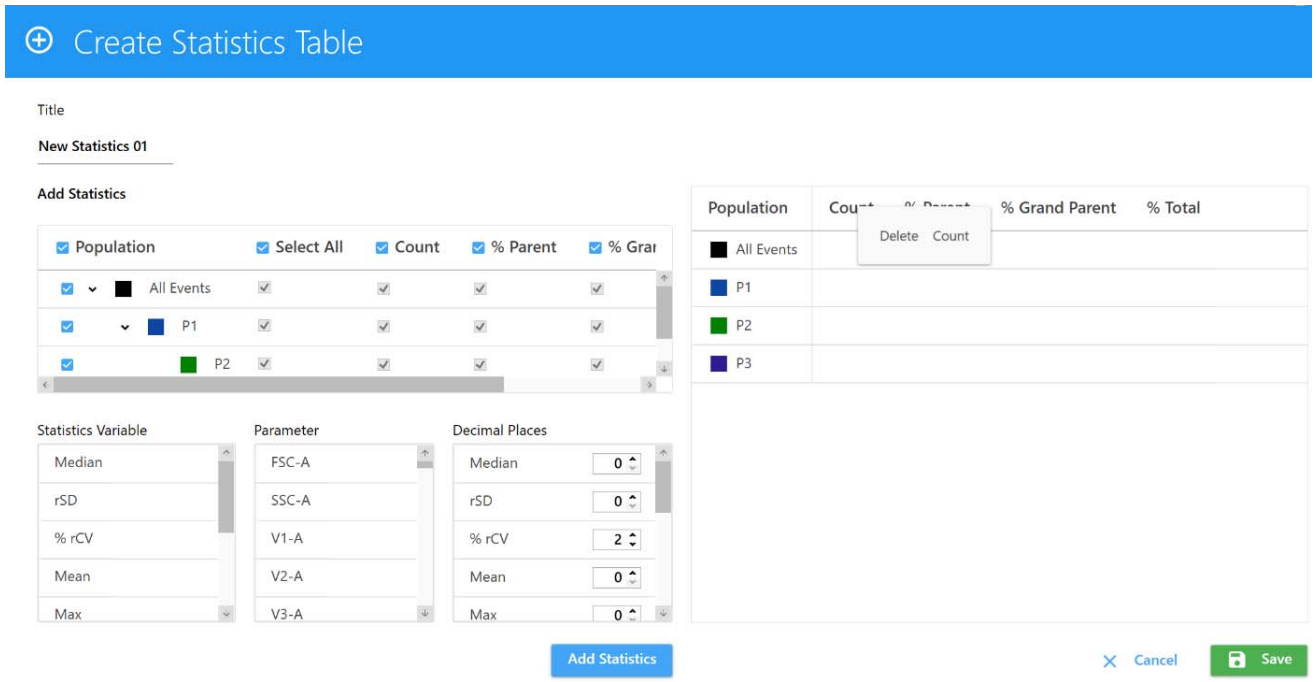
Select the population checkbox next to the populations that have stats to display. To add a statistic, select the statistic from the Statistics Variable list.

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.

To adjust the precision of the statistics, select the decimal place in the Decimal Places table. To remove a statistic, right-click the column header and select **Delete**.

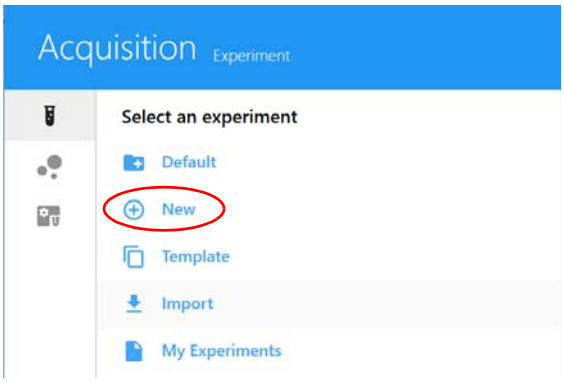
Once the statistics are complete, you can export the stats as a CSV file to the location you choose. Statistics output can be appended or overwritten if you export the stats into the same CSV file.



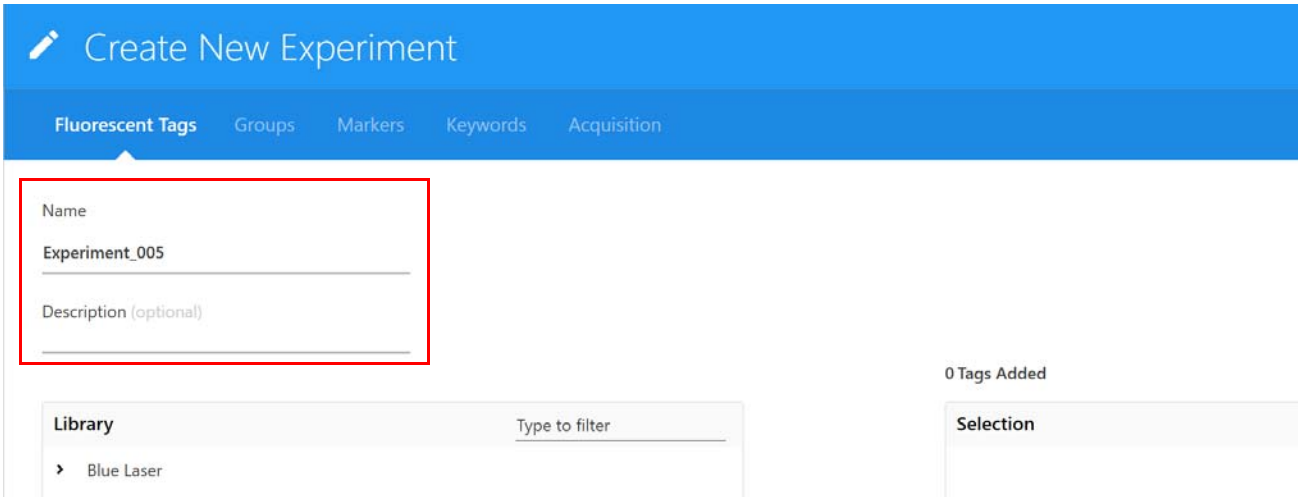
Creating a New Experiment

Selecting New in the Experiment menu opens the New Experiment wizard. The wizard walks you through the steps to create a new experiment.

1 Click New in the Acquisition Experiment menu.



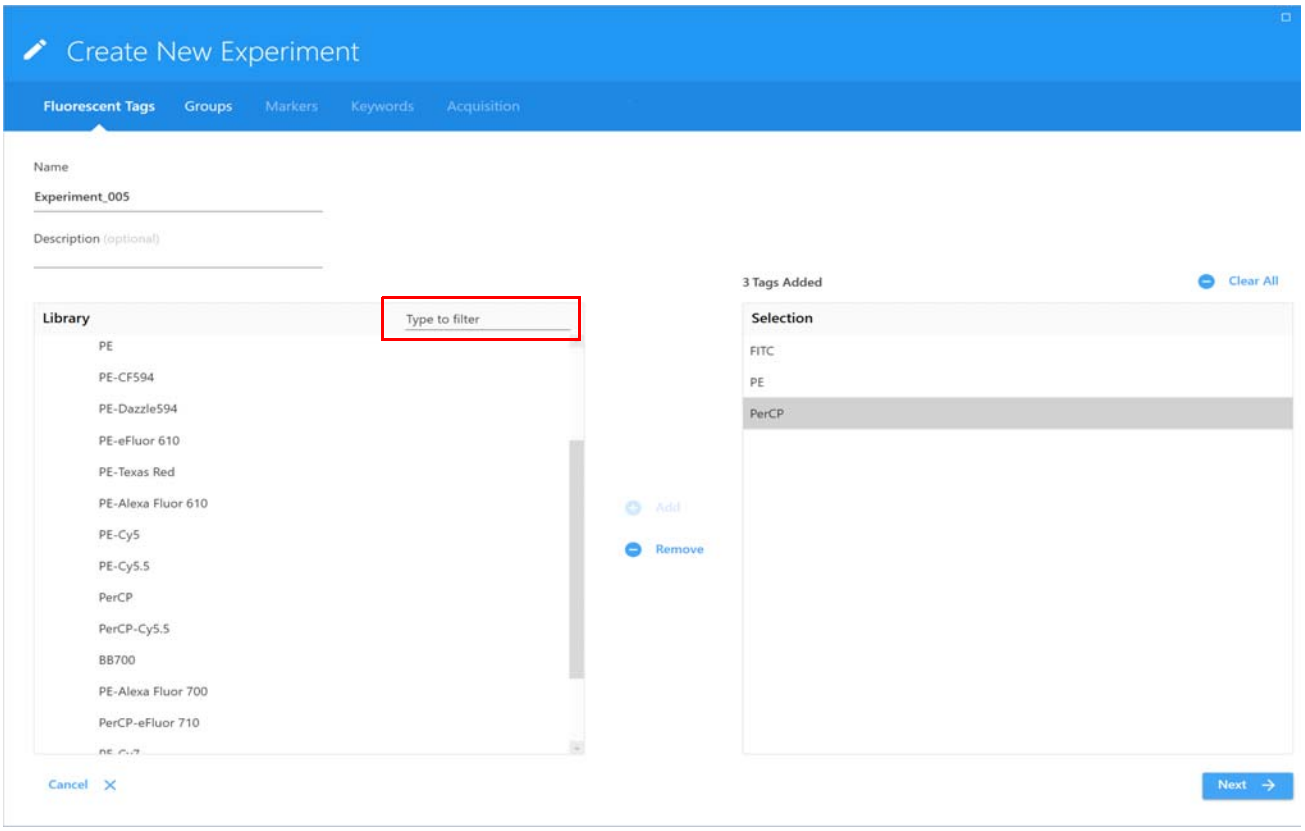
2 The Create New Experiment wizard opens. Specify a name for the experiment or use the default name. (Optional) Type in a description.



3 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. 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. See "Fluorescent Tags" on page 75.

You must select all fluorescent tags present in the experiment, as this will determine which reference controls are to be used during spectral unmixing.



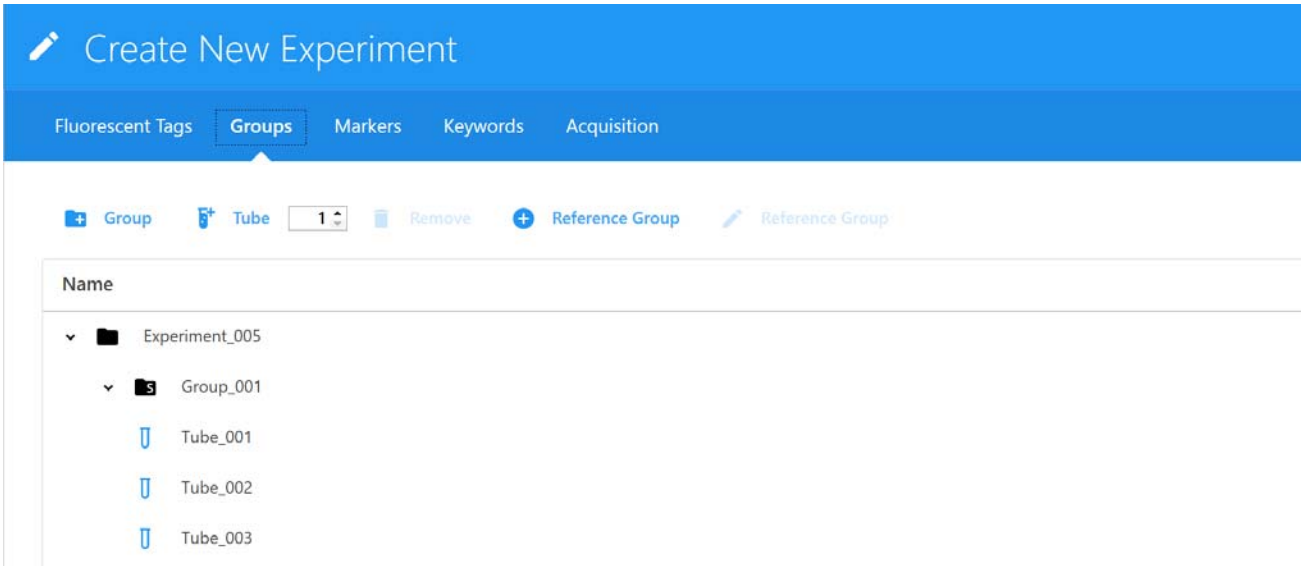
To remove individual fluorescent tags from the Selection list, click to select the tag, then click **Remove**. To remove all fluorescent tags, click **Clear All**.

4 Once all fluorescent tags have been chosen from the Library list, confirm the list in the selection pane, then click **Next**.

5 Create groups for your samples by selecting **Group**. Add tubes to the groups.

For information on creating groups when in plate mode, see "Creating Groups When Using the Loader" on page 99, then proceed to step 7.

■ **NOTE:** When running sticky samples, such as LNW samples, we recommend adding cleaning wells between samples to thoroughly clean the mixing probe. For example, add two wells, one with 10% bleach and the next with DI water. At the end of a plate, consider adding a group of four wells, two with 300 µL of 10% bleach and two with 300 µL of DI water. Program a long mix (15 seconds at 1500 rpm) to thoroughly clean the mixing probe.



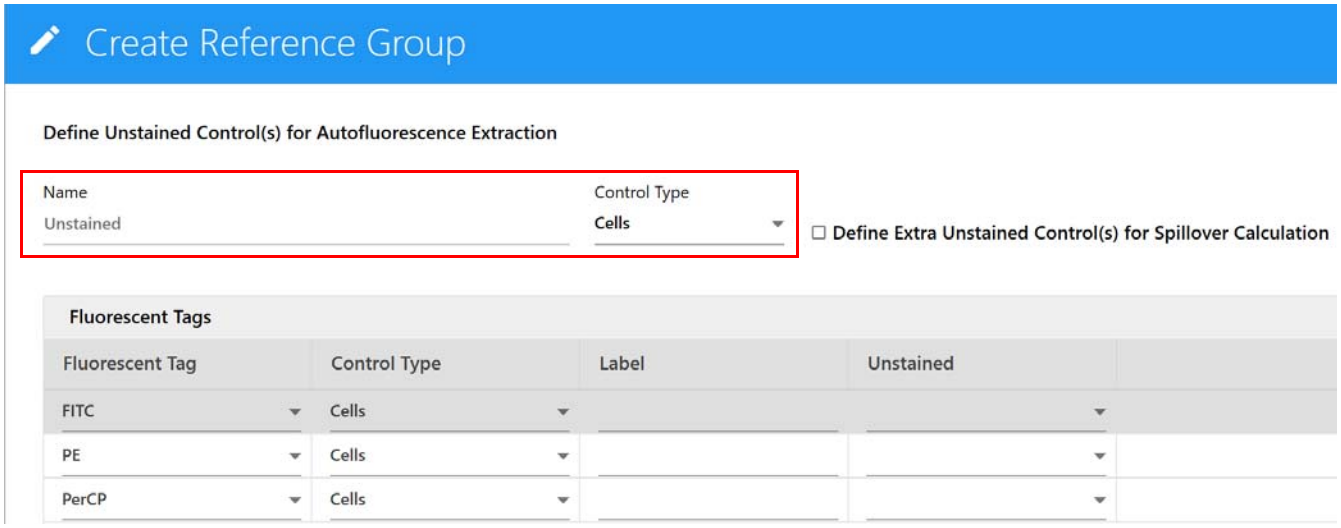
6 Select **Reference Group** if you are intending to unmix with all or some controls acquired in this experiment.

This creates a list of reference control tubes for each fluorescent tag specified as part of the experiment.

■ **NOTE:** If you plan to unmix the samples using only reference controls run in QC & Setup, step 6 is not necessary.

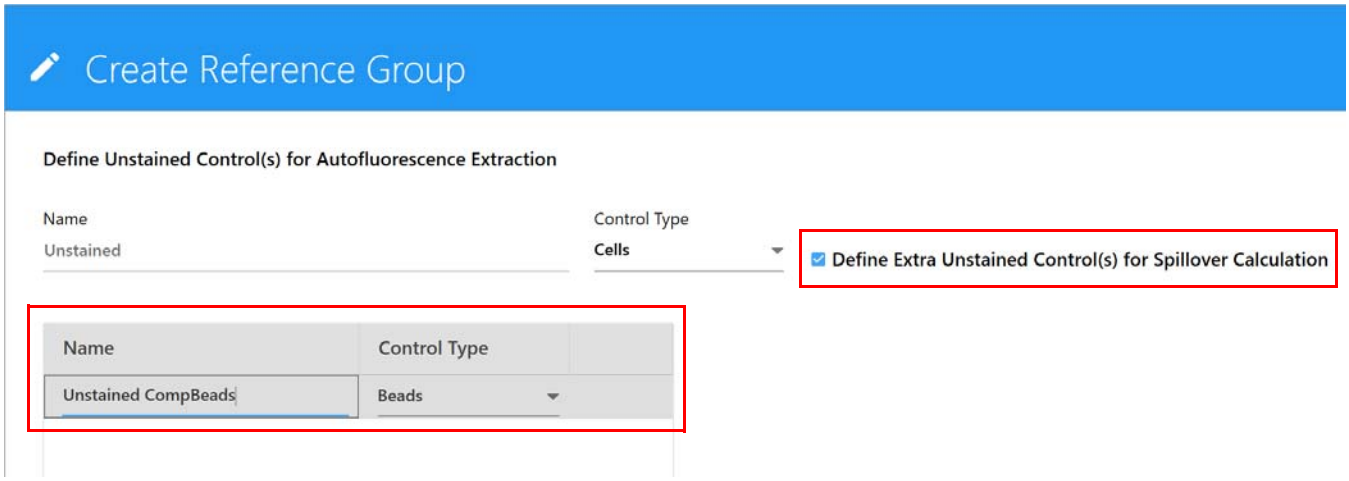
■ **NOTE:** To mix and match reference controls acquired in the experiment with reference controls run in QC & Setup, define the controls to acquire in the reference group, acquire the controls, then after selecting Unmix, select the remaining controls from the reference controls run in QC & Setup.

7 **IMPORTANT:** Define an unstained control for autofluorescence by selecting its control type (beads or cells). 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.



8 If applicable, select Define Extra Unstained 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 unstained control.

For example, if test samples are cells and the reference controls are beads, all with only positive peaks, you will need to run a separate tube of negative beads for the spillover calculation. An extra unstained control is not needed if your unstained autofluorescence control (and sample) is the same type as the reference controls.



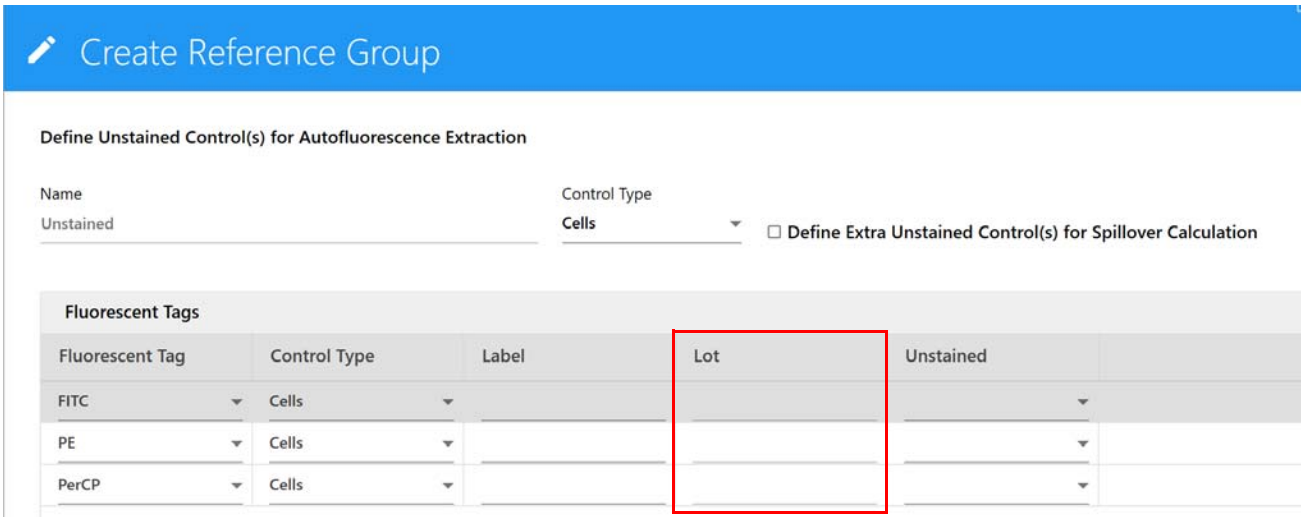
9 Select the control type (beads or cells) for the single-stained reference controls.

10 (Optional) Enter the label (for example, CD nomenclature) that is conjugated to the fluorescent tag.

11 If applicable, enter the lot number(s) of the reference controls.

■ **NOTE:** If you selected Label/Lot Specific Unmixing in the Acquisition Preferences (see page 82), the software will search the library and experiment reference groups for reference controls that

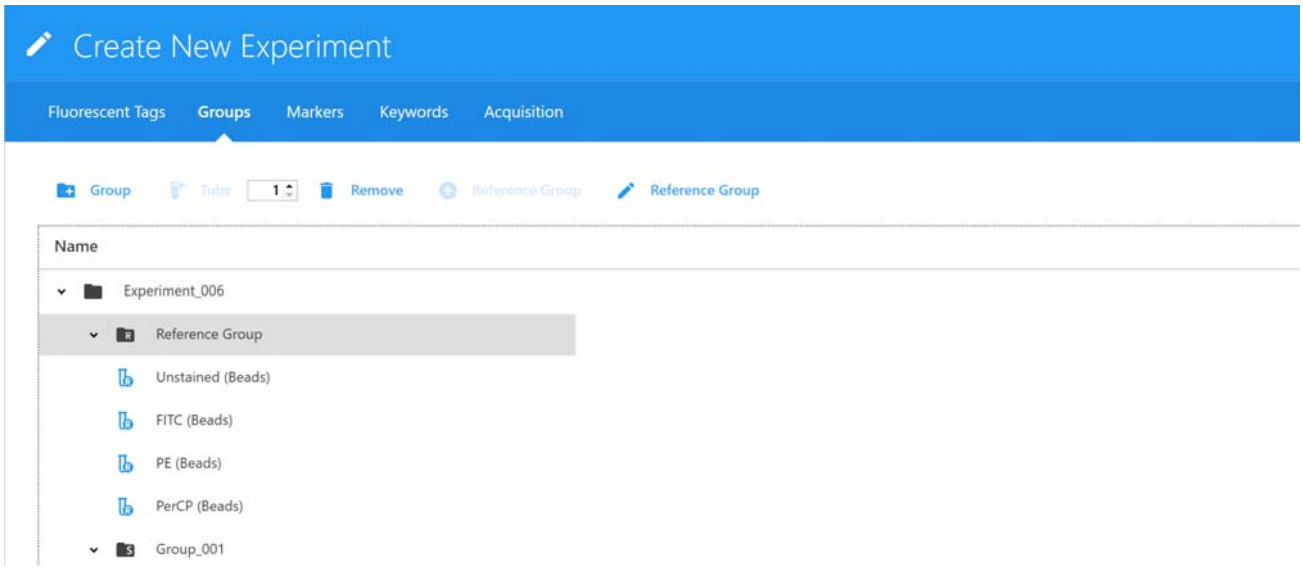
have the same fluorescent tag, label, and lot information in order to use the corresponding control for unmixing.



■ **NOTE:** Use the red trash can icon to delete an individual tube from the reference group. This may be necessary if you wish to mix and match references acquired in this experiment with reference controls run in QC & Setup. Any stored controls you plan to use should be deleted from the reference group.

12 Click Save.

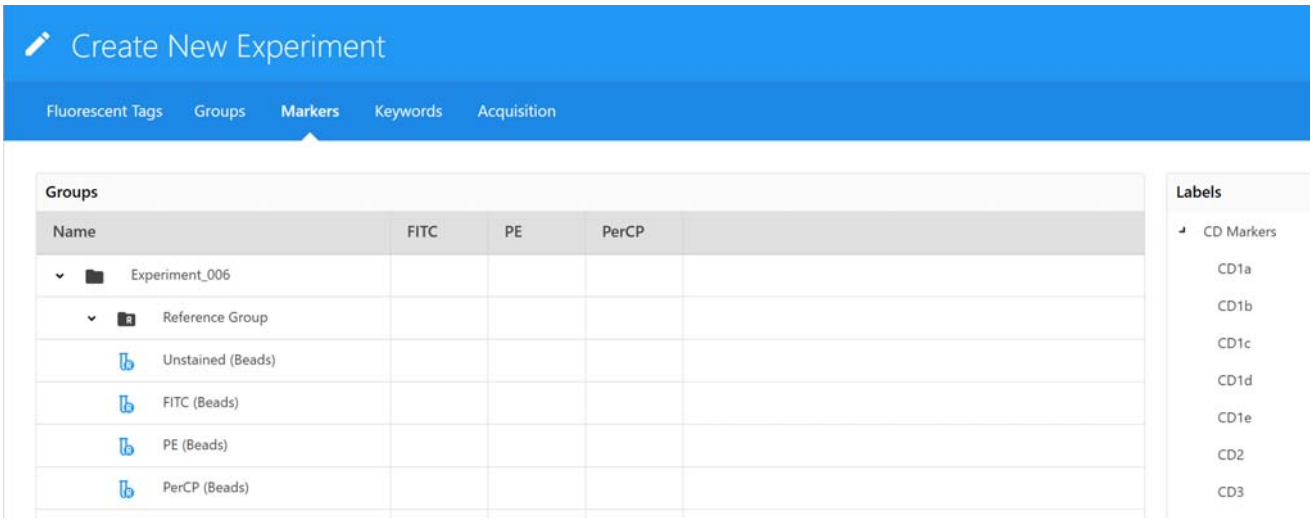
Once the reference group has been created, entries for each of the references will be displayed. Each of the reference group tubes/wells will have an icon (with the letter R) associated with it under the reference group.



13 If necessary, continue adding tubes/wells, click Next when all tubes are created.

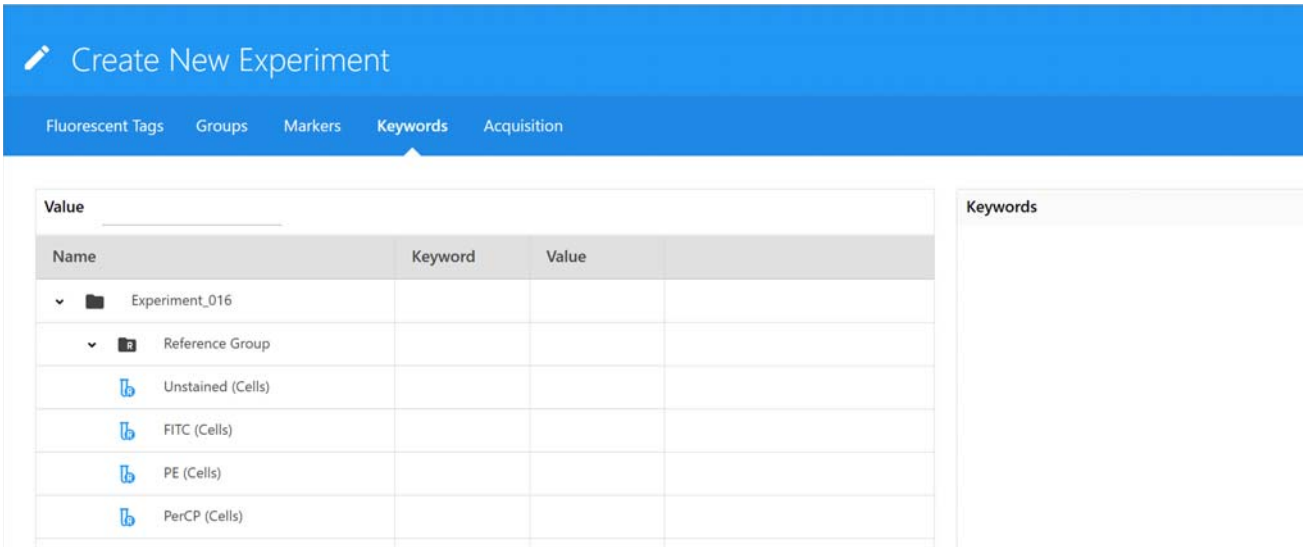
14 Add markers/labels to the remaining sample tubes before continuing. They can be chosen from the Labels list on the right, typed directly into the table, or copied and pasted. Labels can be added at the group or tube level and can be applied to multiple cells selected at once. Labels are required for reference controls if you selected Label/Lot Specific Unmixing in the Acquisition Preferences (see page 82). Click Next when all the tubes are labeled.

■ **NOTE:** If you selected **Label/Lot Specific Unmixing**, the software will search the library and experiment reference groups for reference controls that have the same fluorescent tag, label, and lot information in order to use the corresponding control for unmixing.



15 (Optional) Enter custom keywords and click **Next**.

Custom keywords can be added at the experiment, group, or tube level. You must define the custom keywords in the Library before you can add them to an experiment (see "Keywords" on page 78 for information). Drag and drop the keywords from the Keywords list on the right to the experiment, group, or tube and enter keywords values as needed. You can also copy and paste custom keywords across different tubes in this wizard.



16 Select the acquisition settings and worksheet(s). The worksheet menu lists all the worksheets for the given user.

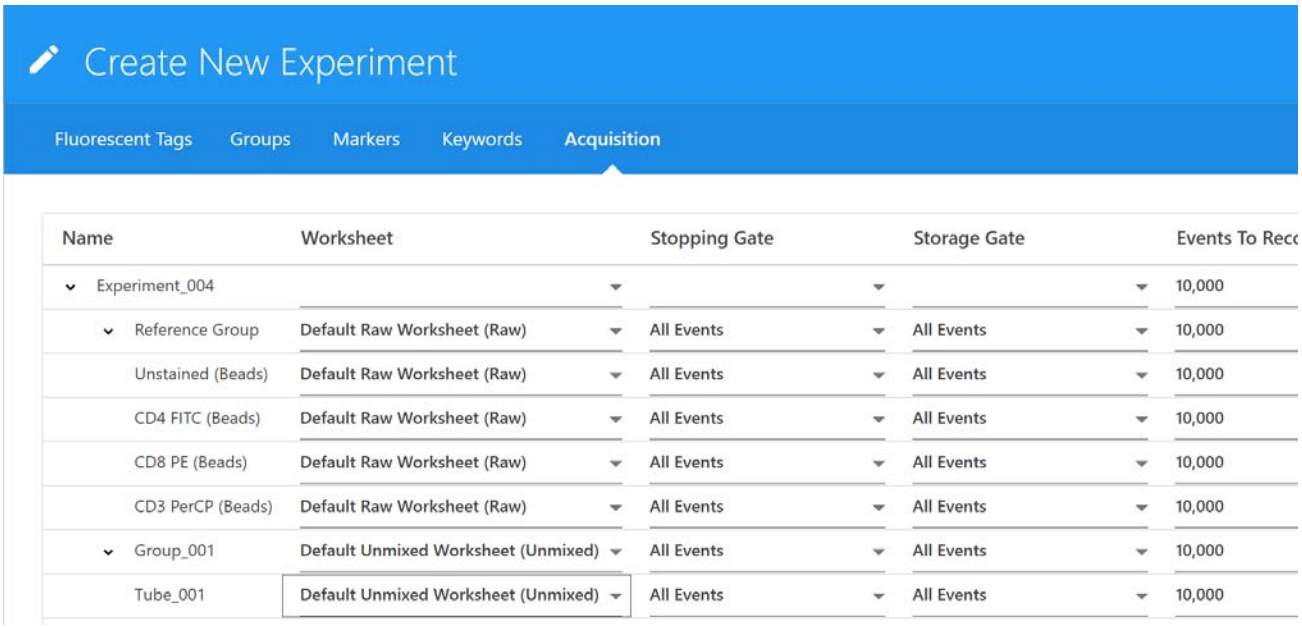
- Select the Default Raw Worksheet (Raw) for the Reference Group and for the sample groups if you plan to perform post-acquisition unmixing.
- Select the Default Unmixed Worksheet (Unmixed) or any user-created unmixed worksheet for your sample groups if you are performing live unmixing. Worksheets can be selected at the experiment, group, or tube level.

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 (time, volume, number of events). These criteria can be selected at the experiment, group, or individual level.

- 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:** If running in plate mode, when selecting worksheets and acquisition settings, select the top level (group or experiment) to apply the settings to all wells within the group or experiment.

■ **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.



17 If running in plate mode, define the Loader settings. See "Defining Loader Settings" on page 100.

18 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.

19 To acquire controls and samples and perform live unmixing, see "Live Unmixing" on page 60.

20 If running in Plate mode, calibrate the SIT. See "Calibrating the SIT" on page 103.