

Job Aid

BD FACSDiscover™ S8 Cell Sorter: Performing spectral re-unmixing

This job aid contains instructions for how to perform spectral re-unmixing for your experiment in BD FACSCorus™ Software.

You can record multiple single-stain controls for every fluorochrome in your experiment and then select the confirmed recordings for your fluorochromes to generate the spectral matrix that you want to use for your experiment. This creates a new spectral unmixing matrix that is referred to as the Latest Matrix, and it will be applied to all samples acquired and recorded after it is created.

For additional information, see the *BD FACSDiscover™ S8 Cell Sorter with BD CellView™ and BD SpectralFX™ Technology user's guide*.



Before you begin

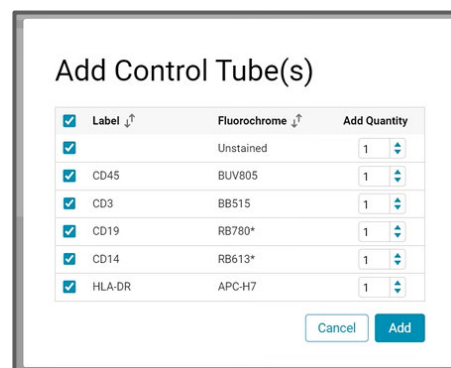
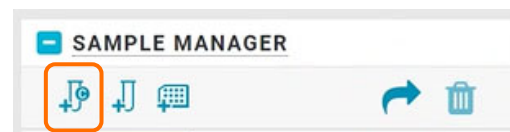
- Start up the system and run a daily or extended fluidics startup procedure.
- Add and design an experiment, adjust your scatter and spectral gains, and if imaging, adjust the Region of Analysis (ROA) for your sample. **NOTE** You can also start with an existing experiment that already contains an unmixing matrix.
- Run your initial single stain controls, calculate an unmixing matrix, and record unmixing data on a fully stained sample.

Generating the new unmixing matrix

1. Set up the re-unmixing controls.
 - a. In the Sample Manager panel, click the **Add Control Tube(s)** icon.
 - b. Uncheck all of the markers and then click the controls that need to be re-run.

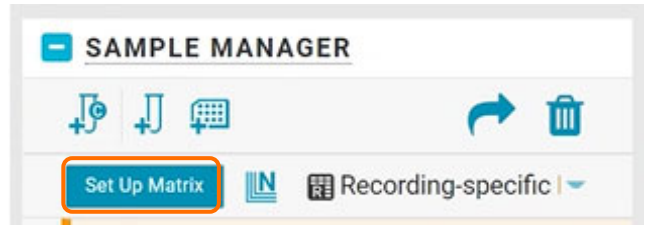
NOTE If you do not uncheck all Labels, a second tube will be created for all fluorochromes.

- c. Click **Add**.
- d. Rename the controls by clicking the pencil icon.

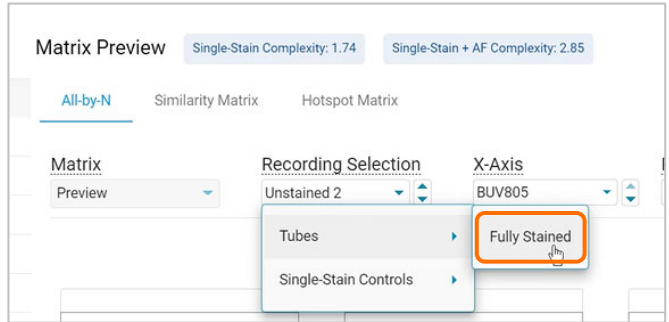


Generating the new unmixing matrix, continued

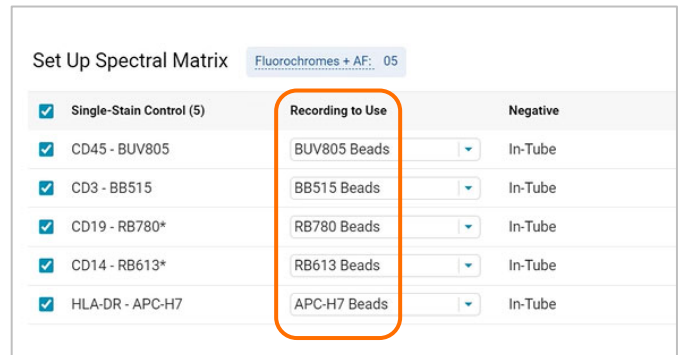
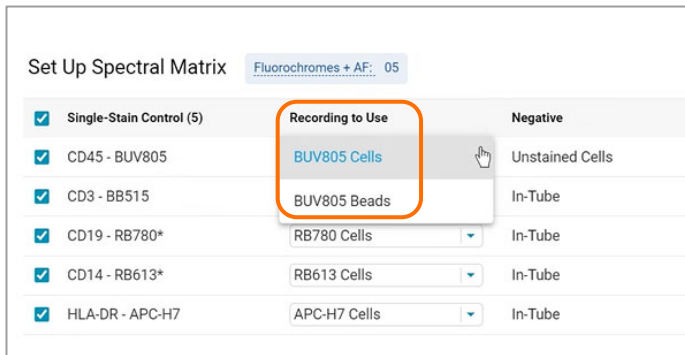
- Click **Set up Matrix** in the sample manager.



- Change the **Recording Selection** to your fully stained sample.



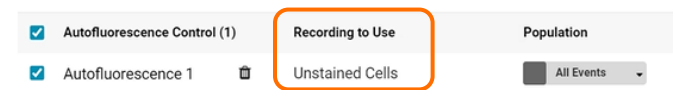
- Change the **Recording to Use** to the newly recorded single stain controls and preview the changes in the plot(s) by selecting each fluorochrome for the x-axis.



- (Optional) Add an autofluorescence control(s).
 - In the bottom left of the window, click **+Add Autofluorescence to use for Spectral Matrix**.



- Check the box for **Autofluorescence 1**.
- Select an unstained control sample as the recording and population to use.



TIP In order to add multiple autofluorescence controls, gate the population(s) in the unstained tube.

- (Optional) Preview the changes to the unmixing matrix by selecting each fluorochrome for the x-axis display.
- Click the **Generate Spectral Matrix** button and confirm that a matrix was generated with a timestamp.

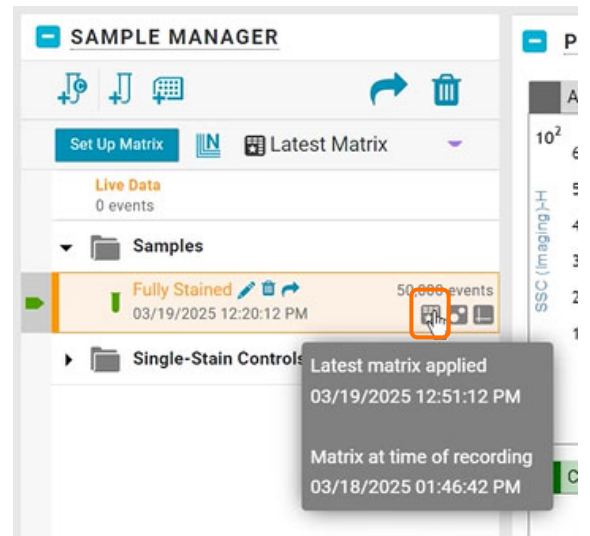
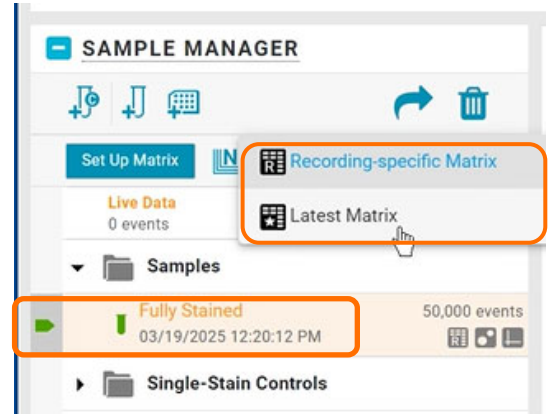


Viewing the data with the new unmixing matrix

1. Click the **View Data** page.
2. In the Sample Manager, click the Fully Stained sample to view the data.
3. Click the drop-down by Recording-specific and select **Latest Matrix**.

NOTE The selected matrix applies to all recorded samples.

4. Verify that the latest matrix has been applied by hovering over the icon in the Fully Stained sample.

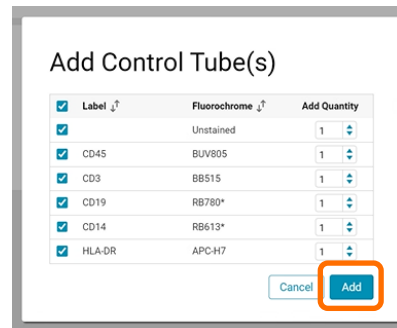


Tips and troubleshooting

- **CAUTION** The newest spectral unmixing matrix, also known as the Latest Matrix, will be applied to all samples acquired and recorded after it is created.
- Experimental data that has been unmixed can be viewed with either the Recording-specific Matrix (the matrix at the time of recording) or the Latest Matrix.
- Statistics exported from the experiment will be calculated using the matrix that is currently selected.
- The Chorus Experiment File (CEF) will contain only the Latest Matrix.

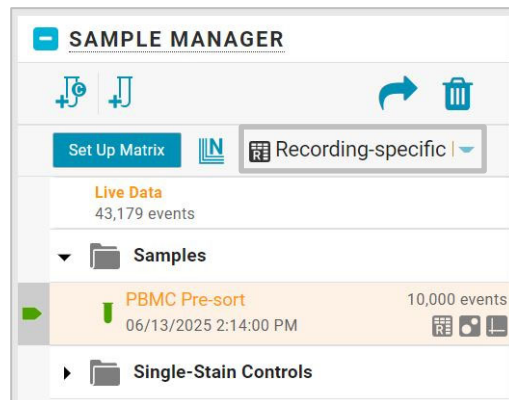
Re-recording controls

- Fluorochrome and autofluorescence controls can be re-recorded as many times as needed. Additional controls can be added by clicking on the add control tubes icon in the sample manager, then selecting the checkboxes and number of tubes of each to add to the experiment.



Re-unmixing after experimental data is recorded

- The spectral unmixing matrix can be recalculated and the new matrix applied to previously recorded data files. To change the spectral unmixing matrix, you must modify the existing fluorochrome controls or record new fluorochrome controls, and then confirm the changes.
- The newest spectral unmixing matrix is referred to as the Latest Matrix, and it will be applied to all samples acquired and recorded after it is created.
- Experimental data that has been unmixed can be viewed with either the Recording-specific Matrix (the matrix at the time of recording) or the Latest Matrix.

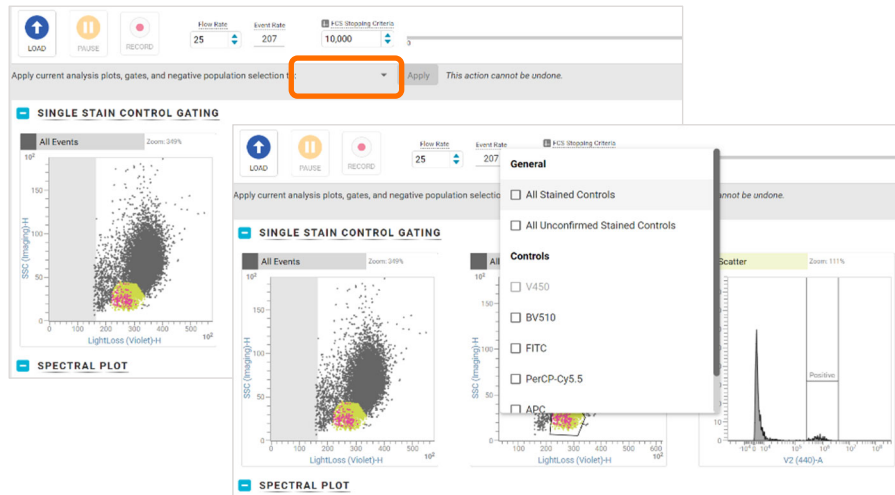


- Experimental data that has not been unmixed (acquired in Raw Mode) can be viewed with the latest matrix only.
- Any sorts performed will use the Latest Matrix for unmixing.
- Statistics exported from the experiment will be calculated using the matrix that is currently selected.
- The Chorus Experiment File (CEF) will contain only the Latest Matrix.

Tips and troubleshooting, continued

Applying gates to all controls

- To apply the current analysis plots, gates, and negative population selection to some or all controls, click the dropdown menu above the scatter plots to select the controls and then click **Apply**.



Apply current analysis plots, gates, and negative population selection to: All Unconfirme... **Apply** This action cannot be undone.

- Once overwritten, the default gate positions cannot be restored. However, plots, gates, and negative population selections can be further edited as needed.

Using the Matrix Preview panel



Preview panel options	Description	Examples																																				
All-by-N	<ul style="list-style-type: none"> Provides a visual overview of unmixing performance. The All-by-N window displays how a single unmixed parameter relates to every other unmixed parameter in your experiment. Each plot in the grid has the same unmixed parameter on the x-axis and a different unmixed parameter on the y-axis. 	<p>Matrix Preview Single-Stain Complexity: 1.86 Single-Stain + AF Complexity: 2.70</p> <p>All-by-N Similarity Matrix Hotspot Matrix</p> <p>Matrix: Preview Recording Selection: Tube_001 X-Axis: BUVR805 Plot Scale: Biexponential R-Value: <></p>																																				
Similarity Matrix	<ul style="list-style-type: none"> Provides the similarity scores for each pair of fluorochromes. The similarity score indicates how similar the spectral signatures (SOVs) of two fluorochromes are, ranging from 0 to 1. Nearly identical spectra will have a similarity of 1, while very different spectra will have a similarity of 0. 	<p>All-by-N Similarity Matrix Hotspot Matrix</p> <table border="1"> <thead> <tr> <th></th> <th>BUVR805</th> <th>BB515</th> <th>RB780*</th> <th>RB613*</th> <th>APC-H7</th> </tr> </thead> <tbody> <tr> <th>BUVR805</th> <td>-</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <th>BB515</th> <td>0.00</td> <td>-</td> <td></td> <td></td> <td></td> </tr> <tr> <th>RB780*</th> <td>0.09</td> <td>0.06</td> <td>-</td> <td></td> <td></td> </tr> <tr> <th>RB613*</th> <td>0.00</td> <td>0.06</td> <td>0.04</td> <td>-</td> <td></td> </tr> <tr> <th>APC-H7</th> <td>0.25</td> <td>0.00</td> <td>0.14</td> <td>0.00</td> <td>-</td> </tr> </tbody> </table>		BUVR805	BB515	RB780*	RB613*	APC-H7	BUVR805	-					BB515	0.00	-				RB780*	0.09	0.06	-			RB613*	0.00	0.06	0.04	-		APC-H7	0.25	0.00	0.14	0.00	-
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Hotspot Matrix	<ul style="list-style-type: none"> Predicts the impact of unmixing-dependent spread. Summarizes which spectral signatures in a spectral matrix will cause unmixing-dependent spreading (spread in an unmixed parameter that is caused by the unmixing matrix itself). 	<p>All-by-N Similarity Matrix Hotspot Matrix</p> <table border="1"> <thead> <tr> <th></th> <th>BUVR805</th> <th>BB515</th> <th>RB780*</th> <th>RB613*</th> <th>APC-H7</th> </tr> </thead> <tbody> <tr> <th>BUVR805</th> <td>1.03</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <th>BB515</th> <td>0.03</td> <td>1</td> <td></td> <td></td> <td></td> </tr> <tr> <th>RB780*</th> <td>0.25</td> <td>0.24</td> <td>1.01</td> <td></td> <td></td> </tr> <tr> <th>RB613*</th> <td>0.03</td> <td>0.25</td> <td>0.19</td> <td>1</td> <td></td> </tr> <tr> <th>APC-H7</th> <td>0.51</td> <td>0.09</td> <td>0.35</td> <td>0.04</td> <td>1.04</td> </tr> </tbody> </table>		BUVR805	BB515	RB780*	RB613*	APC-H7	BUVR805	1.03					BB515	0.03	1				RB780*	0.25	0.24	1.01			RB613*	0.03	0.25	0.19	1		APC-H7	0.51	0.09	0.35	0.04	1.04
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