Job Aid

BD FACSDiscover™ A8 Cell Analyzer: Performing spectral re-unmixing

This job aid contains instructions for how to perform spectral re-unmixing for your experiment in BD FACSChorus™ 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™ A8 Cell Analyzer with BD CellView™ and BD SpectralFX™ Technology user's guide*.

Start up system → Set up experiment → **Establish settings** → Record and analyze → Manage data → Shut down system

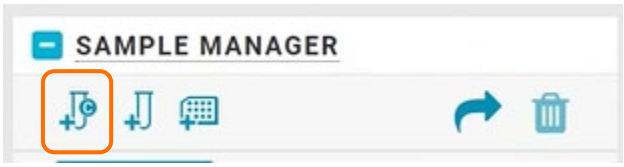
Before you begin

- 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 unmixed data on a fully stained sample.

Setting up and recording the re-unmixing controls

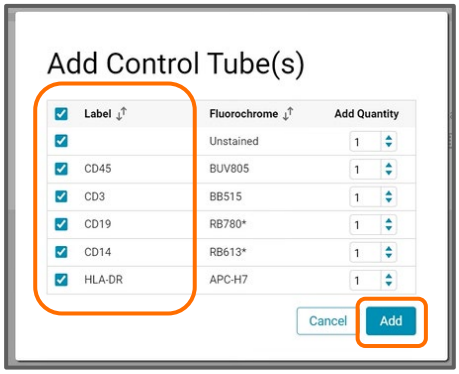
1. On the Set up Single Stain Controls page, in the Sample Manager panel, click **Add Control Tube(s)**.

   Alternatively, click the **Add plate/tube rack** icon if you are using the loader.



2. Leave all the checkboxes selected if you want to add all of the controls and change the quantity as needed.

3. Click **Add**.



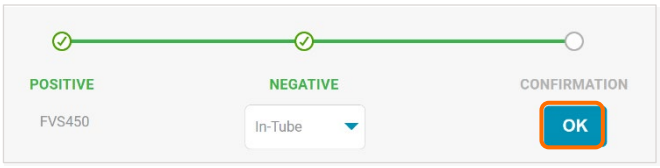
Setting up and recording the re-unmixing controls, continued

4. Record the data for each single-stained control needed for re-unmixing.

   NOTE For an imaging experiment, before acquiring your single-color controls, make sure the ROA has been set up for the specific particle in your sample using the Adjust Gains page.

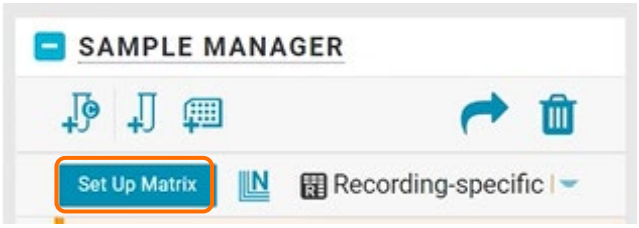
5. Adjust the scatter and interval gates as needed.

6. Confirm each control.

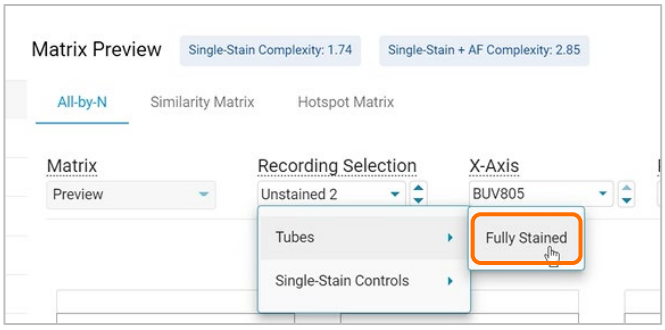


Generating the new unmixing matrix

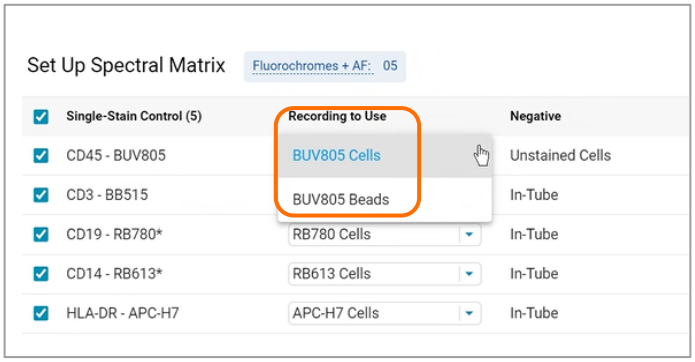
1. Click the **Set up Matrix** button to open the Set Up Spectral Matrix page.

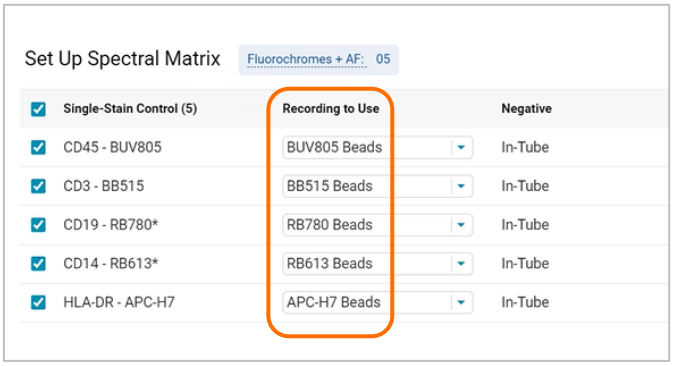


2. Change the **Recording Selection** to your fully stained sample.



3. 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.





Generating the new unmixing matrix, continued

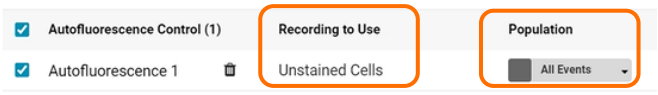
4. (Optional) Add an autofluorescence control(s).

   a. In the bottom left of the window, click **+Add Autofluorescence to use for Spectral Matrix**.

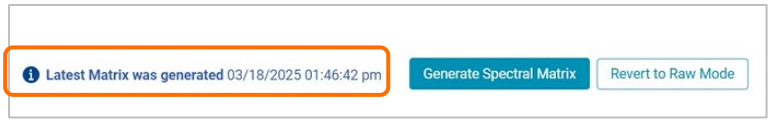
   b. Check the box for **Autofluorescence 1**.

   c. Select an unstained control sample as the recording and population to use.

   d. (Optional) Preview the changes to the unmixing matrix by selecting each fluorochrome for the x-axis display.



5. Click the **Generate Spectral Matrix** button and confirm that a matrix was generated with a timestamp.

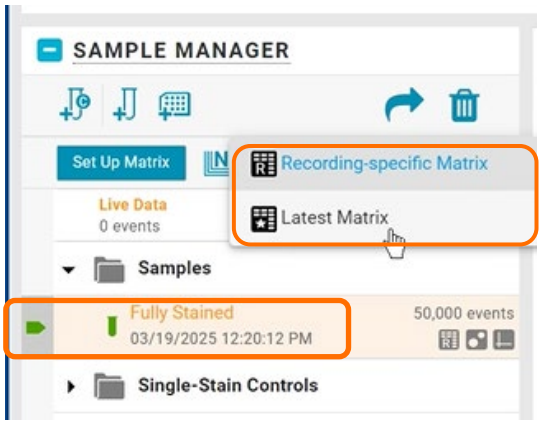


6. Close the Set Up Spectral Matrix page by clicking the **X** in the top right

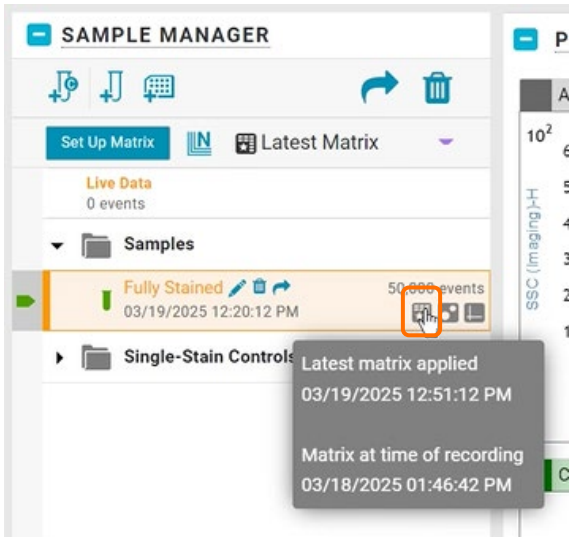
Viewing the data with the new unmixing matrix

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.



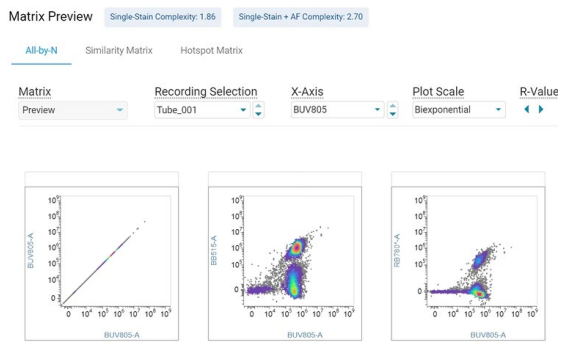
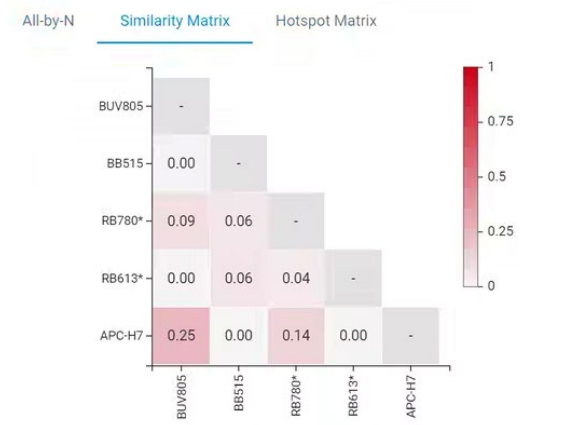
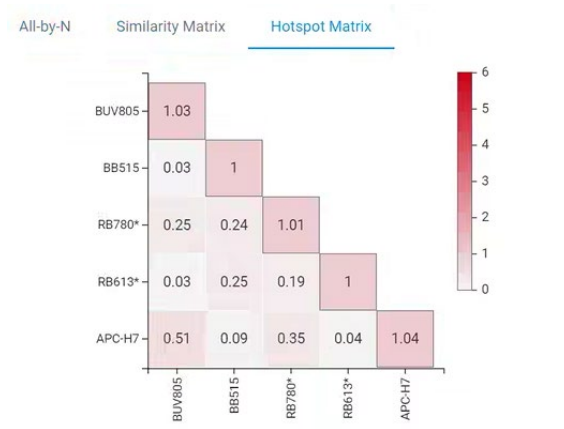
4. Verify that the latest matrix has been applied by hovering over the icon in the Fully Stained sample.



Tips and troubleshooting

- 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.
- 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.

Using the Matrix Preview panel

Preview panel options	Description	Examples
All-by-N	• 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.	
Similarity Matrix	• 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.	
Hotspot Matrix	• 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).	

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