Job Aid

BD FACSDiscover™ Family: 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 or BD FACSDiscover™ S8 Cell Sorter user's guides*.

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. Set up the re-unmixing controls.

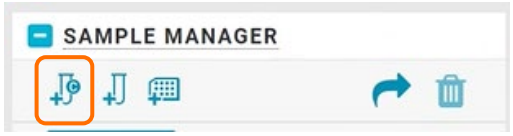
   a. In the Sample Manager panel, click the **Add Control Tube(s)** icon.

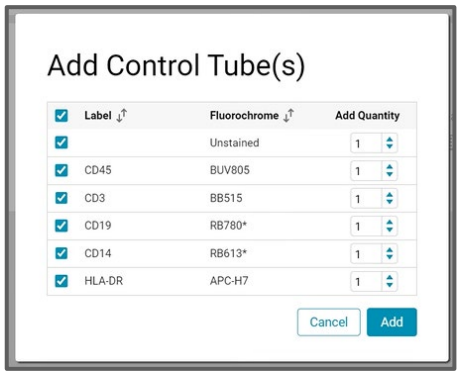
   b. Uncheck all of the markers and then click the controls that need to be re-run.

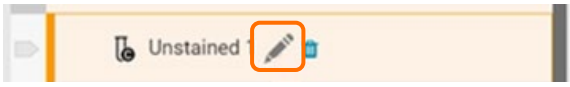
   NOTE If you do not uncheck all Labels, a second tube will be created for all fluorochromes.

   c. Click **Add**.

   d. Rename the controls by clicking the pencil icon.







Setting up and recording the re-unmixing controls, continued

2. 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.

   NOTE Control tubes can be acquired in any order.

3. Adjust the scatter and interval gates as needed.

4. (Optional) Create additional gates.

   TIP To add multiple autofluorescence controls, gate the population(s) in the unstained cells tube.

   a. View the data for the unstained cells.

   b. Select the scatter gate.

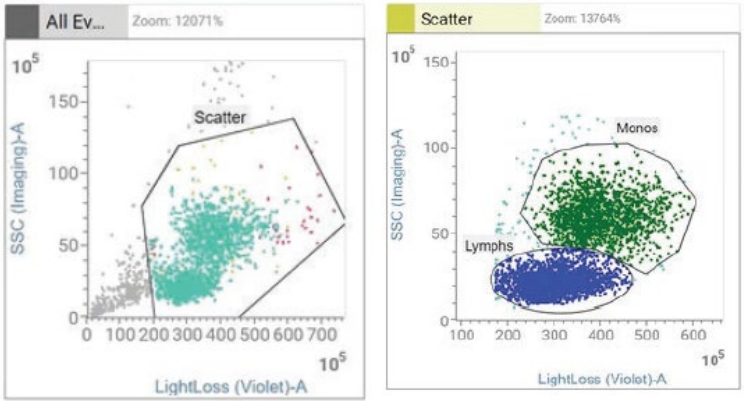
   c. Add a dot plot.

   d. Adjust the plot scaling to view the cell populations of interest.

   e. Create gates for the populations of interest.

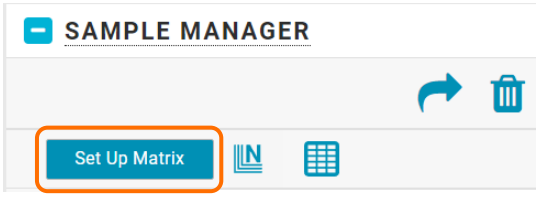
   f. Name the gates.

   These additional gates can be selected as additional autofluorescence controls.

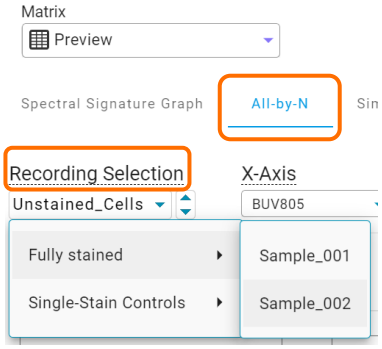


Generating the new unmixing matrix

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

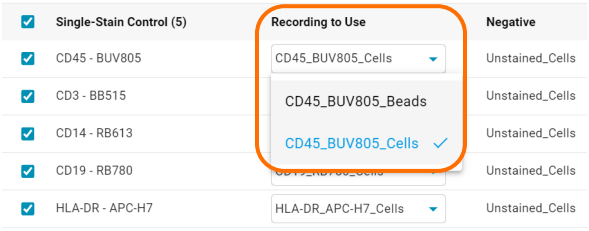


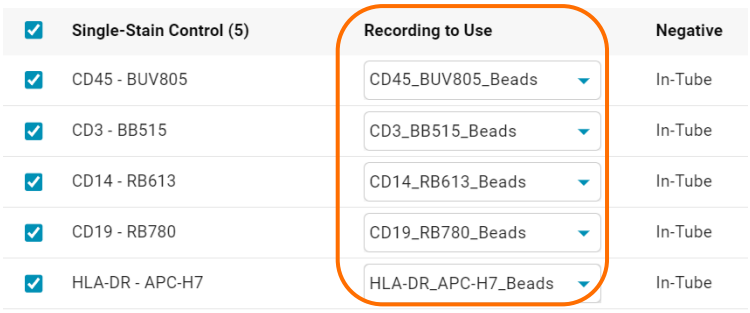
2. Click the **All-by-N** and change the **Recording Selection** to your fully stained sample.



Generating the new unmixing matrix, continued

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.





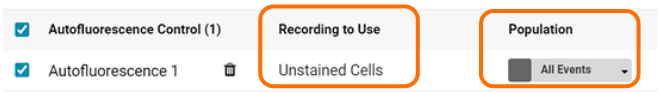
4. (Optional) Add an autofluorescence control(s).

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

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

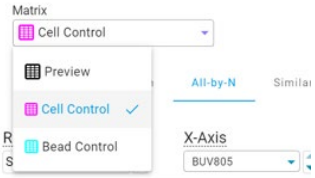
   c. Select an unstained control sample as the recording and population to use.

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



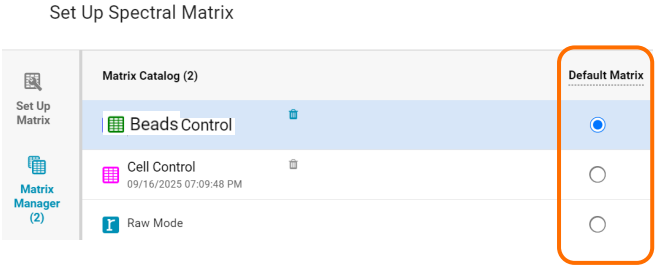
5. Name the matrix, click **Save Matrix,** and confirm that a matrix was generated with a timestamp.

6. Select each saved matrix and preview the data.



7. Click the **Matrix Manager** icon to select a default matrix.

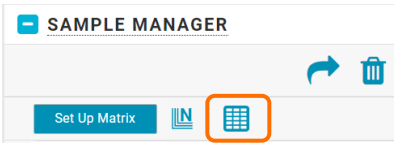
8. Select the matrix that you want as the default matrix.



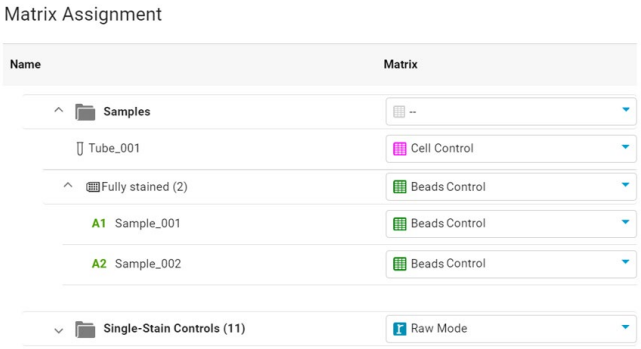
9. Close the **Set Up Spectral Matrix** window.

Applying the unmixing matrices

1. Click the **View Data** page.
2. Click the **Assign Matrix** icon.

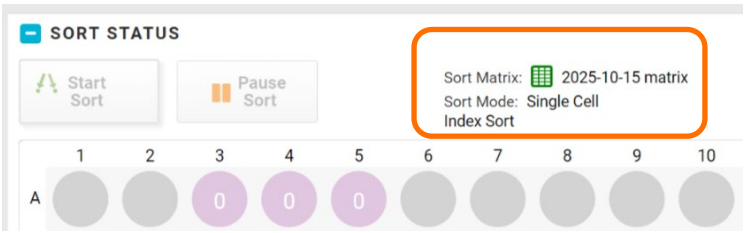


3. Select the preferred matrix for the recorded data.



4. Click **Assign** to apply the changes or click **Close** to discard changes.
5. Verify that the selected matrix has been applied by hovering over the icon for each of the recorded data.

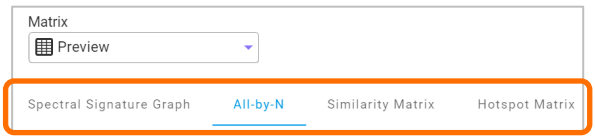
TIP For the BD FACSDiscover™ S8 Cell Sorter, the sort matrix is visible from the sort page.

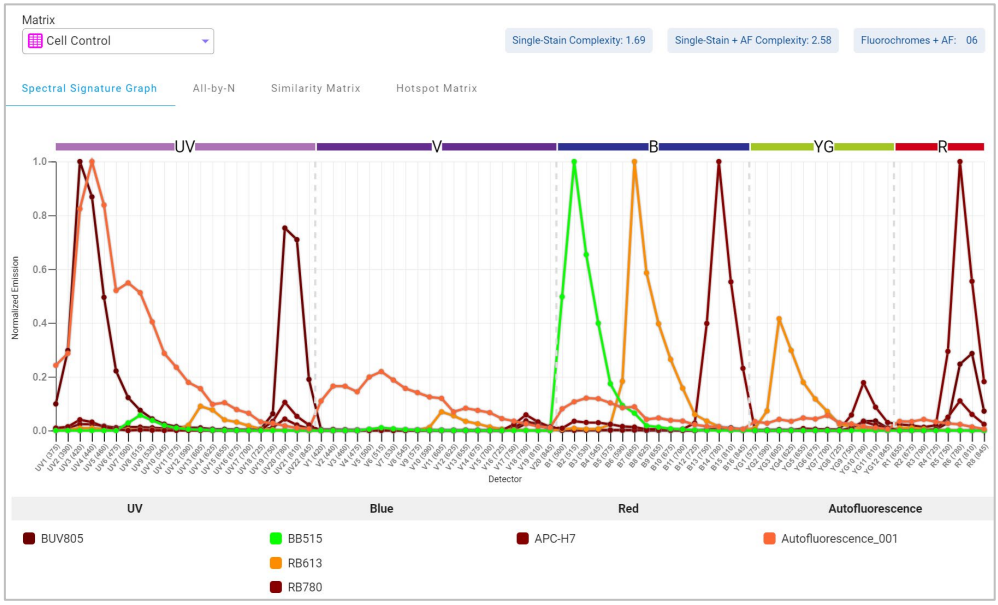


Tips and troubleshooting

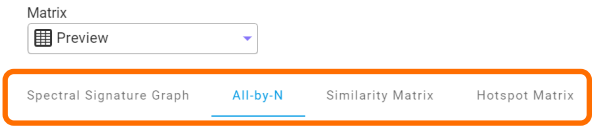
- For any new tubes added to the experiment, the selected default matrix will be used when data is recorded.
- Experimental data that has been unmixed can be viewed with a different saved matrix as needed.
- Statistics exported from the experiment will be calculated using the matrix that is currently selected.
- The Chorus Experiment File (CEF) will contain only the Latest Matrix.

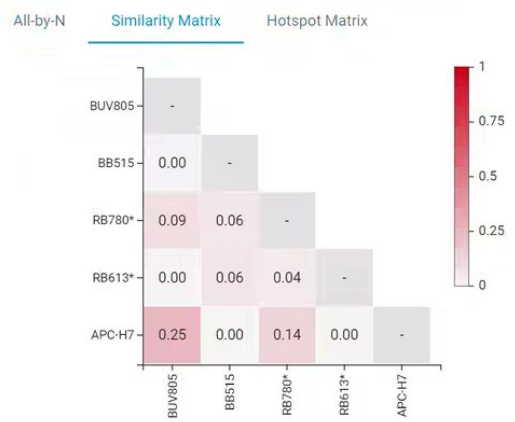
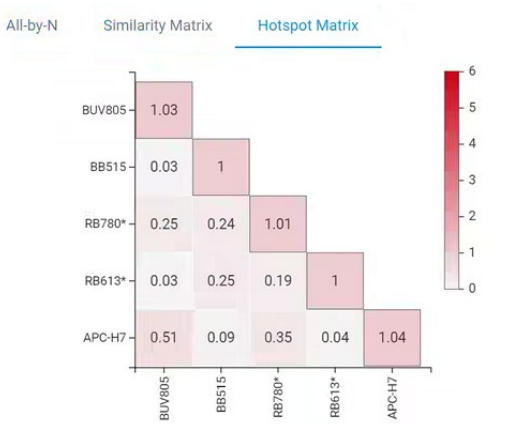
Using the Matrix Preview panel



Preview panel options	Description and examples
Spectral Signature Graph	• Displays the spectral signatures for each fluorochrome (and autofluorescence, if included) in the spectral matrix. • Displays the relative measured signal across all detectors for a given fluorochrome (or autofluorescence). • The graph helps identify and distinguish between different fluorochromes and autofluorescences based on their unique spectral properties. 
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. 

Using the Matrix Preview panel, continued



Preview panel options	Description	Examples
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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