Job Aid

BD FACSDiscover™ S8 Cell Sorter: Recording and analyzing data

This job aid contains instructions for how to record and analyze sample data using imaging in BD FACSChorus™ Software. For additional information, see the *BD FACSDiscover™ S8 Cell Sorter with BD CellView™ Image Technology and BD SpectralFX™ Technology User's Guide*.

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

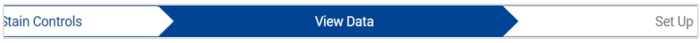
Before you begin

- 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 Region of Analysis (ROA) for your sample.
- Perform spectral unmixing by recording data for single-stained controls, if applicable.

Working with the View Data tab

Preparing the experiment

1. Click the **View Data** tab.



2. Load your sample tube.

   TIP Set the flow rate to 1 to conserve sample.

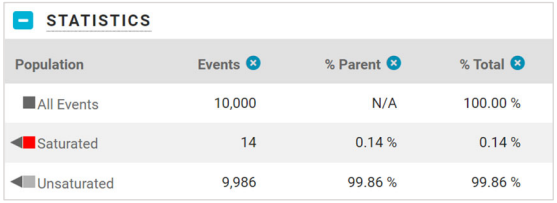
3. Clear the **Doublet Discrimination** checkbox, if needed.

4. Verify that the spectral or imaging detectors are not saturated in the spectral plot.

5. Verify that your population of interest is onscale in the LightLoss (Violet)-H vs. SSC (Imaging)-H plot.

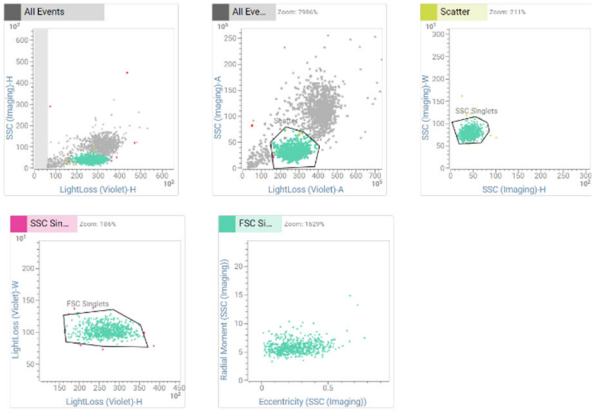
6. Adjust the plot zoom, scatter gains, and threshold appropriately. Keep the saturated events to a minimum.

   TIP In the Statistics panel, verify that most events are Unsaturated.



Preparing the experiment, continued

7. (Optional) To conserve the sample, click **Pause** in the dashboard.



8. In the Plots panel, adjust the Scatter, SSC Singlets, and FSC Singlets gates to encompass the population of interest.

   The SSC Singlets and FSC Singlets gates are only available if the Doublet Discrimination checkbox is selected.

   NOTE The Eccentricity (SSC (Imaging)) vs. Radial Moment (SSC (Imaging)) plot can be used for additional doublet discrimination. This plot will be removed when the Doublet Discrimination checkbox is cleared.

9. Adjust the Region of Analysis and Pixel Threshold for the detectors of interest in the Image Wall.

   NOTE The Region of Analysis (ROA) setting can affect the fluorescent data of your sample. You must set the ROA properly for the current particle type before recording.

   - Adjust the Region of Analysis slider until the white area in the images completely encompasses the events of interest while minimizing background pixels.

     TIP To adjust the slider in small increments, click the slider then press the arrow keys.

   - Adjust the Pixel Threshold slider for the imaging detectors until the white area in the images completely encompasses the area of interest while minimizing background pixels.

10. Set recording criteria:

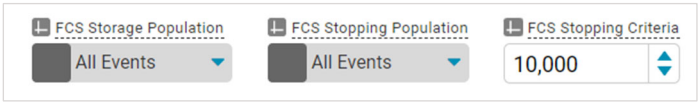
    a. Under FCS Storage Population, select **All Events**.

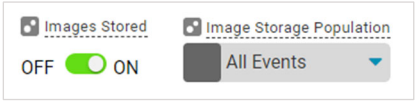
       NOTE Selecting children of All Events will only save that population to the FCS file.

    a. Under FCS Stopping Population and FCS Stopping Criteria, select the population and the number of events to record.

    b. Verify that the Images Stored switch is toggled on to save images.

    c. Under Image Storage Population, select the population for image storage.





Recording sample data

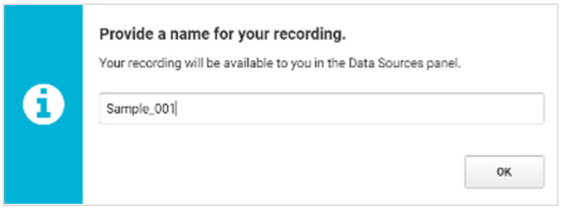
1. Click **Record** in the dashboard.

   When the target is reached, acquisition stops.

2. Enter a name for your recorded file and click **OK**.

   The new data file will appear in the Data Sources panel.

   NOTE The Data Sources panel displays all previously recorded data files, including Single Stain Control data, if applicable. To view a recorded data file, select it. To view live acquisition, select **Live Data**.



Analyzing sample data

1. Hover over the **Add Plot** ( ) icon and select a plot type from the menu to create any additional plots.

2. Click the plot axes and from the plot parameters dialog, select parameter and scaling options.

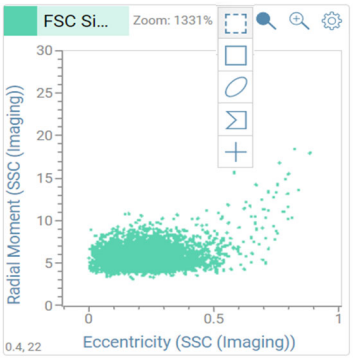
   NOTE Click the **Plot Properties** ( ) icon on the top right of the plot window to delete the plot or change the parent gate or the plot type.

   TIP Drag plots to reorder them in the Plots panel.

3. Add sort gates.

   Hover over a plot, click the **Add New Gate** ( ) icon, and select a gate type.

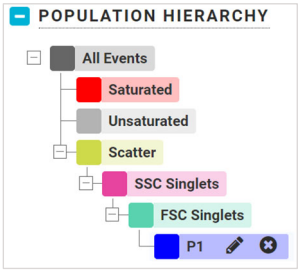
   - For rectangular and oval gates, drag an area on the plot to encompass the population of interest.
   - For polygon gates, click multiple times to create vertices around the population of interest.
   - For quadrant gates, click the plot to place the center of the quadrant gate.



4. In the Population Hierarchy panel:

   - Click the **Edit Population** ( ) icon to rename the population.
   - Click the **Delete** ( ) icon to delete the population.
   - Click the **Color** ( ) swatch to change the color of the population.

   TIP Drag gates to other locations in the hierarchy, if needed.



Analyzing sample images

1. Adjust the channel settings in the image wall for each detector of interest, as needed.

   NOTE The following adjustments can be made at any time before or after recording data. Only ROA and Pixel Threshold must be set correctly before recording.

   a. Select the checkbox for the channel of interest and clear other channels.

   b. Click **Histogram** to open the histogram panel.

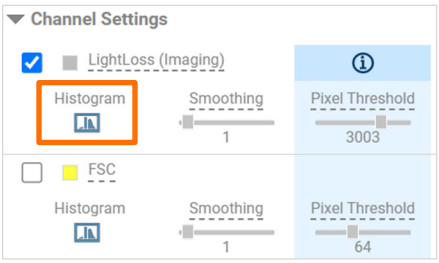
      i. Adjust the Minimum (red) bar and Maximum (blue) bar around the signal peak to optimize the brightness resolution of the images.

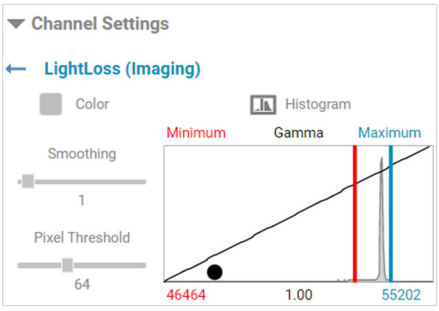
      ii. Adjust the Gamma (black circle) as needed to more easily visualize differences in signal intensity between images.

   c. Adjust the Smoothing slider to reduce blur in the images.

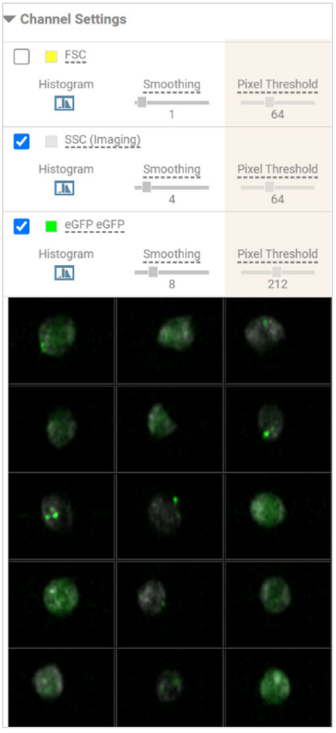
   d. Select the color swatch to adjust the color.

   e. Repeat steps a through d for all other imaging channels of interest.





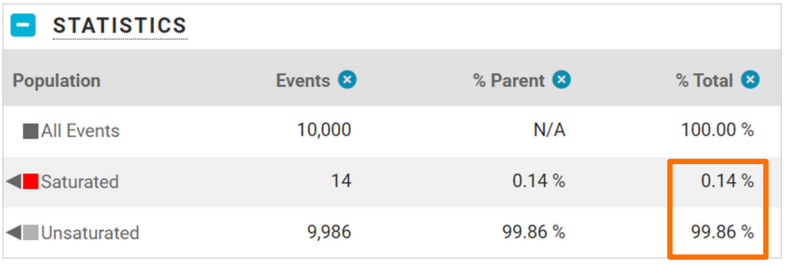
2. Select the checkboxes for channels of interest to visualize cells appropriately.



Tips and considerations for working with the View Data tab

Minimizing saturated events

- Use the Statistics panel on the View Data page to determine what percentage of events are saturating. A majority of events should belong to the Unsaturated population.



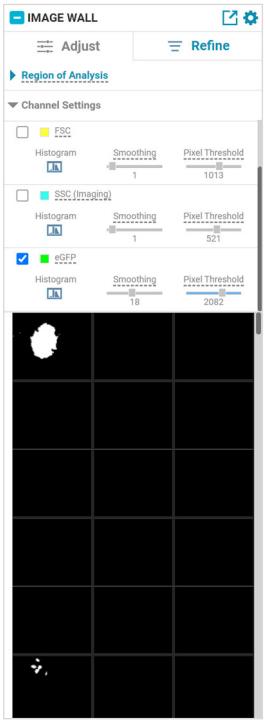
STATISTICS

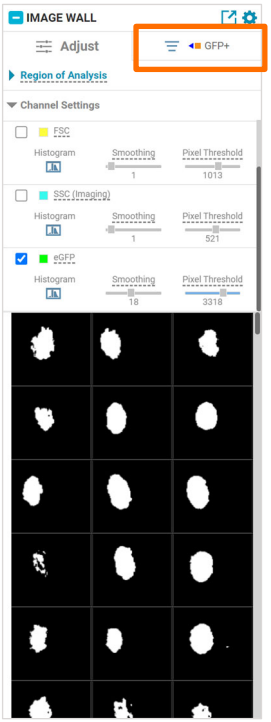
Population	Events	% Parent	% Total
All Events	10,000	N/A	100.00 %
Saturated	14	0.14 %	0.14 %
Unsaturated	9,986	99.86 %	99.86 %

- If too many events are Saturated, use the spectral plot to determine which fluorescent channels are saturating. Click the **Plot Properties** (⚙) icon to display the Saturated population on the spectral plot.
- Saturated detectors could include scatter parameters, which are not included in the spectral plot. To check the scatter parameters, you need to manually create plots containing FSC-H, SSC (Violet)-H, and LightLoss (Imaging)-H. SSC (Imaging)-H and LightLoss (Violet)-H are automatically displayed in the first default plot.
- If SSC (Imaging) gain is adjusted to minimize saturation, the Region of Analysis setting will be impacted. Verify that Region of Analysis is set correctly after adjusting the SSC (Imaging) gain.

Adjusting Pixel Threshold for rare populations

- When imaging cells with rare positive populations, it might be easier to adjust the Pixel Threshold for the fluorescent channel of interest if you first display the positive population on the image wall. On the View Data tab, create a plot to display data from the channel of interest, and gate the positive population. Select the population in the Refine tab of the image wall before adjusting the Pixel Threshold for that channel.
- Both ROA and Pixel Threshold can be adjusted on either paused or live acquisition of the sample.





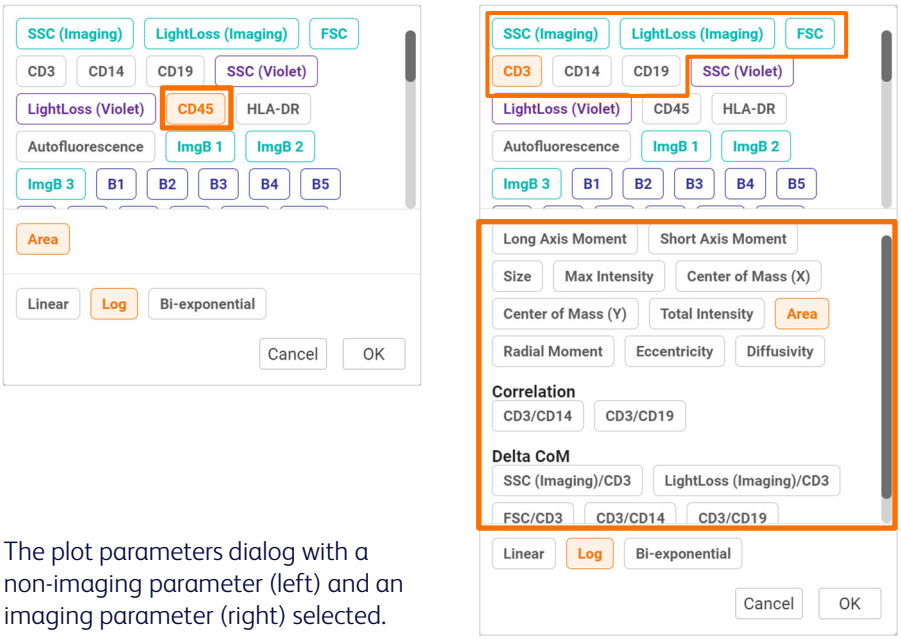
- Adjust the Pixel Threshold on the View Data page, after acquiring single-stain controls and before recording experimental data. Pixel Threshold only impacts the Size imaging feature, which is not used in the spectral unmixing calculation.

Data visualization on View Data tab

- For fluorescent parameters, plots display with log scaling by default. If unmixing has been performed, select biexponential scaling in the plot parameters dialog.
- To drill down: Click a gate to select a population of interest, then create a new plot. The plot will automatically display the data from the selected population.
- Change the number of dots to display on dot plots by entering a different number into the **# of Events Displayed (for Dot Plots)** field. Enter -1 to view all recorded events.

Using imaging features for analysis

- Imaging features are automatically enabled and calculated for every experiment.
- To generate a list of selectable image features in the plot parameters dialog, you must select an imaging channel parameter. These are displayed at the top of the parameters list, and include SSC (Imaging), LightLoss (Imaging), FSC, and any fluorochromes/labels that have been assigned to imaging channels in the Select Imaging Features tab.



The plot parameters dialog with a non-imaging parameter (left) and an imaging parameter (right) selected.

- Imaging features cannot be displayed in biexponential scaling. Depending on your experiment and the feature(s) used, you might find a linear or log scale to be more helpful in displaying image feature data.
- The Region of Analysis adjustment is necessary to calculate all the imaging features described in the following table, except for the Size parameter, which is calculated by the Pixel Threshold adjustment.
  - The Pixel Threshold is a user-defined brightness threshold above which a pixel will be counted.
  - The Region of Analysis is the area of pixels defining a single event, cell, or particle within the image.

Using imaging features for analysis, continued

Image feature	Definition	Usage example	Representative images (low → high)
Eccentricity	A ratio of the shortest to the longest axis (moment) within the Region of Analysis.	Doublet discrimination, cluster identification, cell morphology	
Radial Moment	The average distance of the pixels from the centroid within the Region of Analysis.	Doublet discrimination, cell-to-cell interactions (cellular synapse)	
Size	The number of pixels in the image which are brighter than a user-defined Pixel Threshold.	Label-free sorting, punctate fluorescence	
Max Intensity	The intensity of the brightest pixel in the image.	Punctate fluorescence, phagocytosis, cell cycle analysis	
Long Moment	The measurement of the longest axis (moment) within the Region of Analysis.	Cell morphology, cell-to-cell interaction, aggregates	
Short Moment	The measurement of the shortest axis (moment) within the Region of Analysis.	Cell morphology, cell-to-cell interaction, aggregates	
Center of Mass (X)	The position of the particle in the horizontal direction within an image.	Image quality control, antigen cellular location, phagocytosis	
Center of Mass (Y)	The position of the particle in the vertical direction within an image.	Image quality control, antigen cellular location, phagocytosis	
Total Intensity	The sum of the intensities of all pixels within the Region of Analysis.	Quantitative fluorescence measurements	
Diffusivity	The ratio of the total intensity to the maximum intensity.	Cell morphology, phagocytosis	
Delta Center of Mass	The distance between two fluorescent signal sources in any two imaging channels within the Region of Analysis.	Cell-to-cell interaction, phagocytosis	
Correlation	The degree to which the location of two imaging channels are the same within the Region of Analysis.	Translocation assay, cell-to-cell interaction	

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