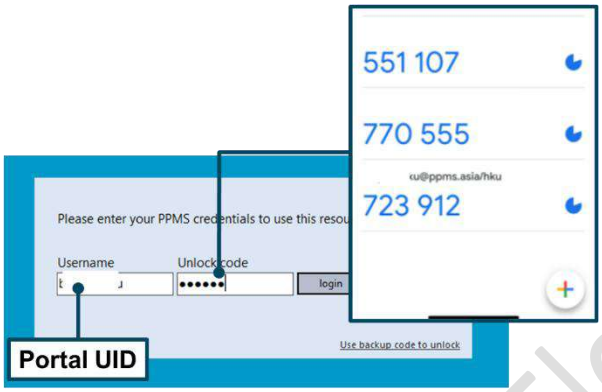
BD FACSDiscover S8 with CellView Standard Operation Protocol

Basic Operation

Log-in

1. Tracker Login



2 . Log in the Windows

Press Ctrl+Alt+Delete

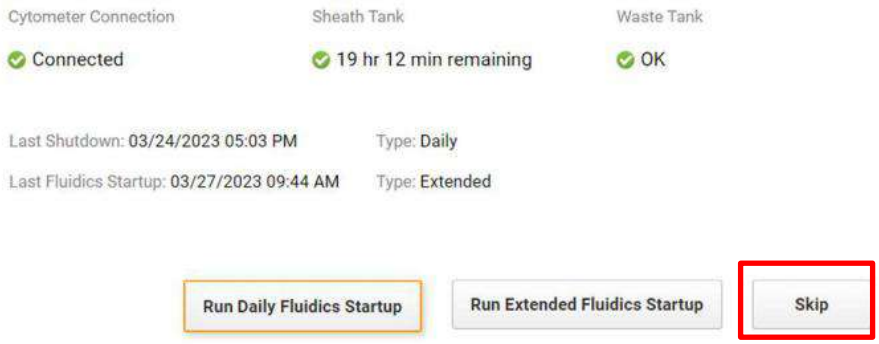
Account: **User**

Password: **S8user2025**

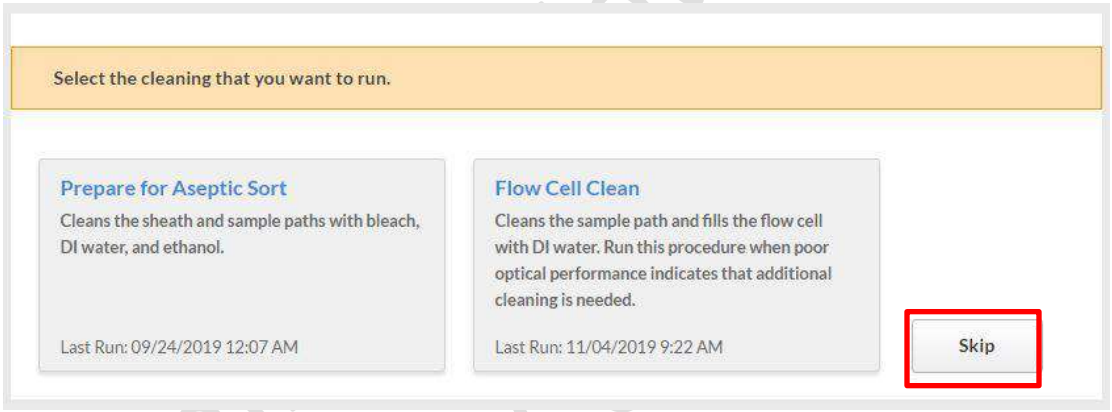
3. Log in BD FACSChorus with your username and password
(Password should be at least 8 characters with Upper case, Lower case, number and symbol)

Drop Delay (optional):

1. Click **Skip** to skip the Fluidics Startup

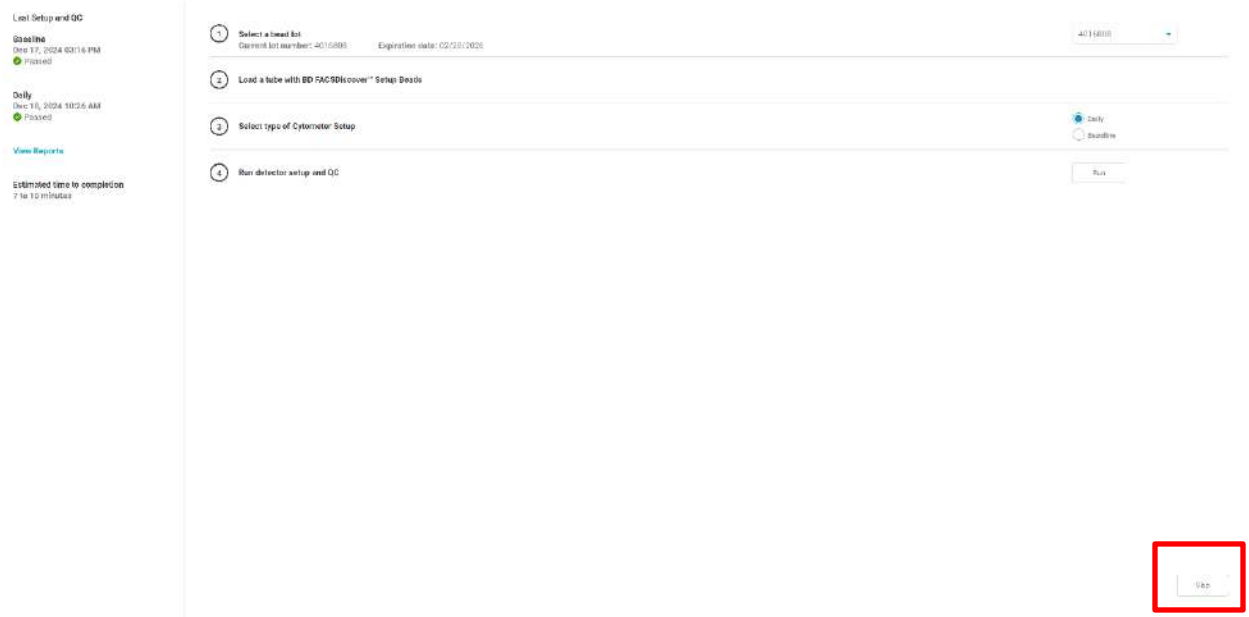


2. Click **Skip** to skip the cleaning

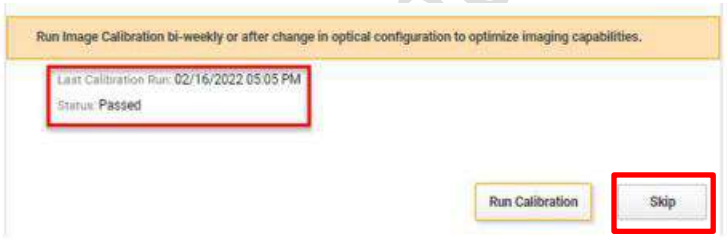


3. Click **Continue** to proceed

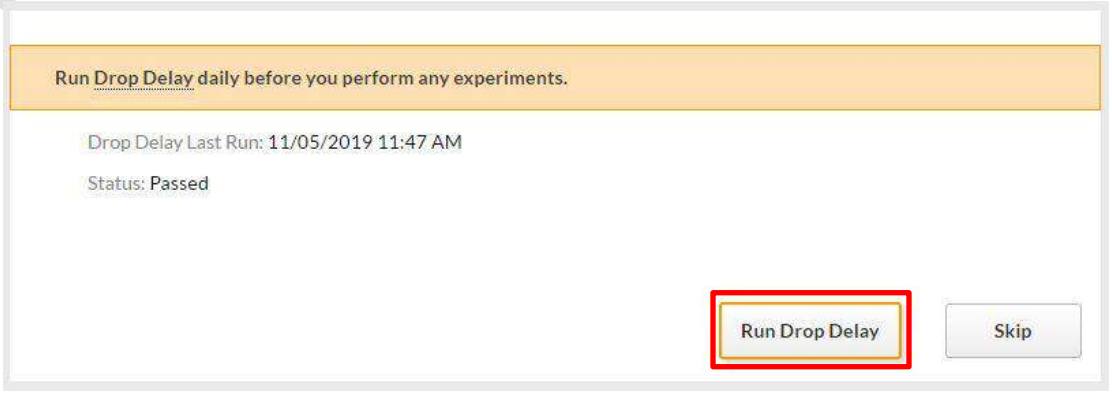
4. Click **Skip** to skip the CS&T



5. Click **Skip** to skip the Image Calibration



6. (Optional) Accudrop delay detection if you do target sorting
   - Find a tube of Accudrop RUO Beads from the fridge (500 uL PBS + 1 drop), vortex before use.
   - Double check the sort chamber door is closed tight.
   - Load the tube and click **Run Drop Delay** to run Drop Delay.



7. Click **Skip** if you don't want to perform Drop Delay.

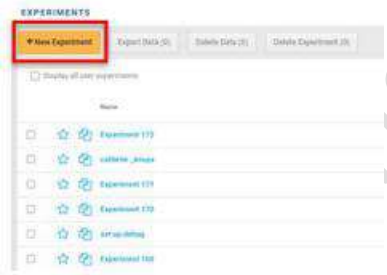
Perform Experiment:

Navigate to the **Experiments** page.

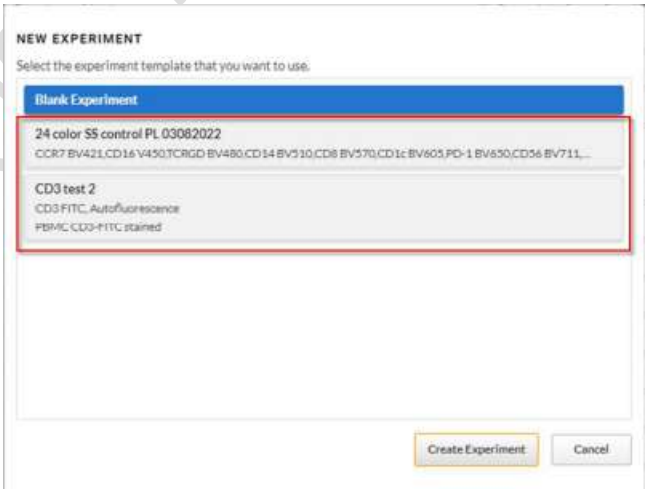
1. Click **Experiments**

2. Create a New Experiment:

- Click **+ New Experiment**



- Select a template or start with a blank experiment.



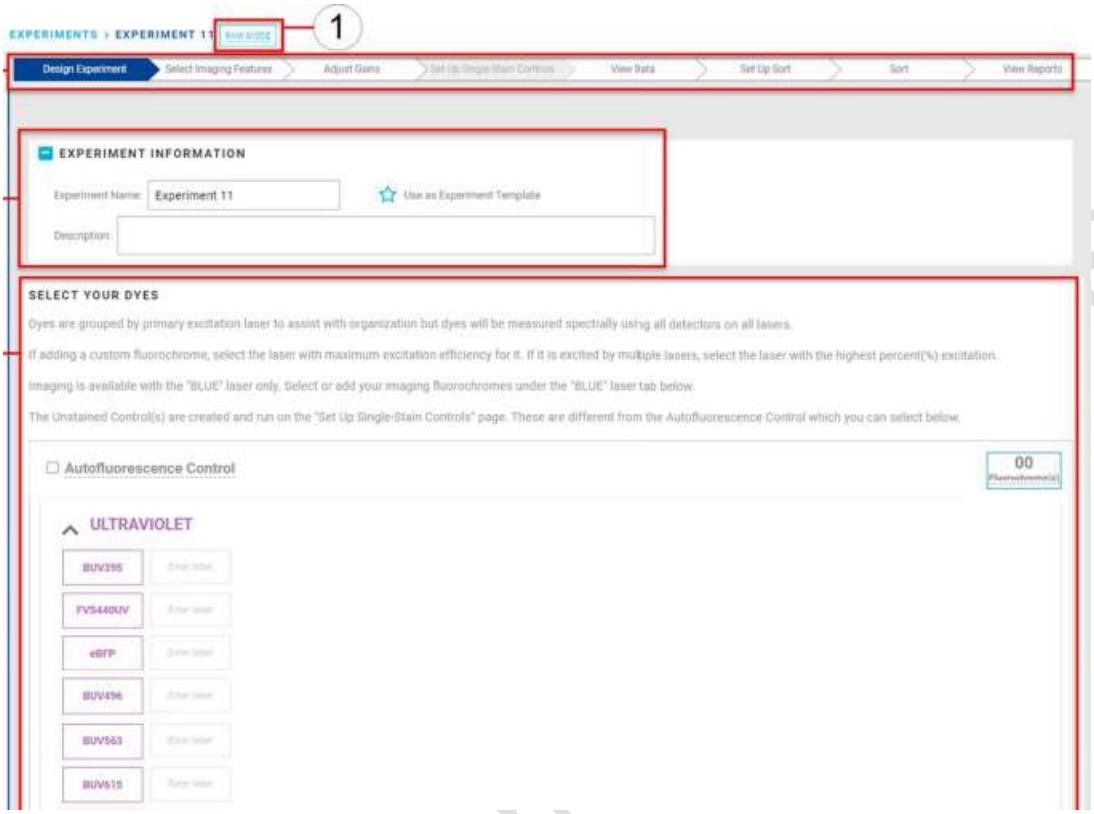
- Use template experiments

- Duplicate an experiment without data

- Name the experiment and provide an optional description.

3. Select Fluorochromes:

- In the **Design Experiment** page, fill in your experiment name and add description if needed, expand the laser rows and select the fluorochromes you will use in your experiment.

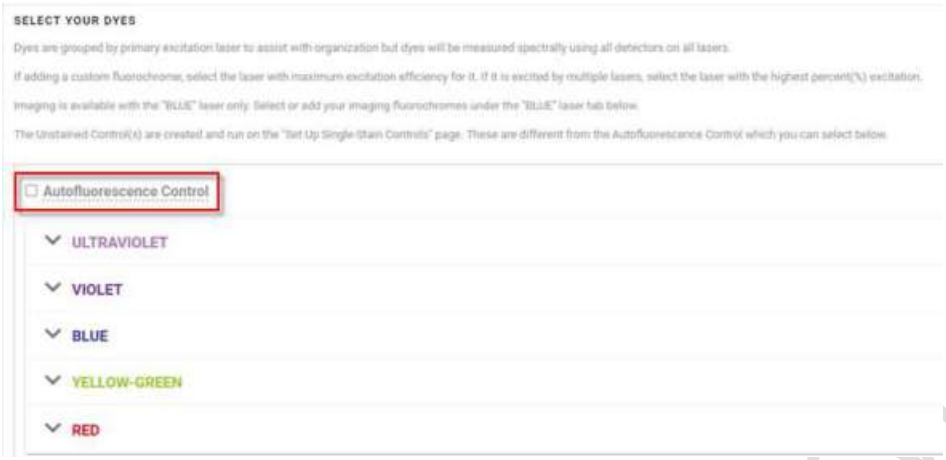


- Optionally, add labels to the fluorochromes for easier identification.

- If imaging is required, ensure the fluorochromes are selected under the **Blue laser row and the emission of a fluorochrome used for imaging must be within the following ranges of one the three imaging detectors:**

Image channel	Bandpass filter	Recommended dyes
ImagBlue 1 (535)	534/46 (511-557)	FITC, BB515
ImagBlue 2 (600)	600/60 (570-630)	PE, RB613, RB545
ImagBlue 3 (790)	788/225 (676-900)	PE-Cy7, RB780, RB744

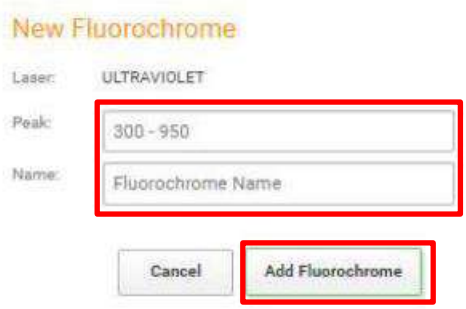
4. (Optional) To set up the removal of the intrinsic fluorescence of unstained particles from your sample, click **Autofluorescence Control**.



5. (Optional) To add a fluorochrome, do the following:

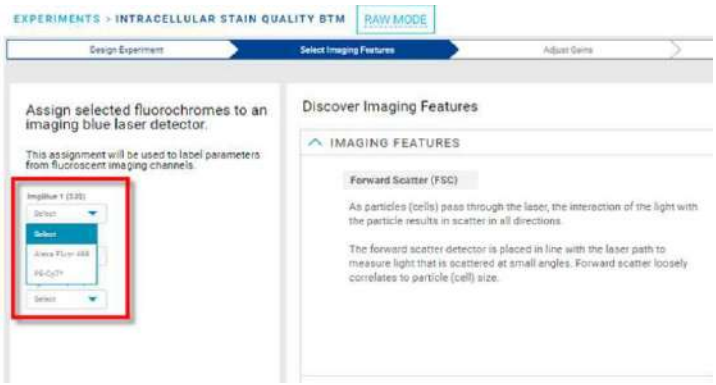
- Click **+**

- Fill in the **peak wavelength** and **Fluorochrome Name**, click **Add Fluorochrome**



6. Link Imaging Features (if applicable):

- Navigate to **Select Imaging Features** page



- Assign the selected fluorochromes to the appropriate imaging channels from ImgBlue 1, ImgBlue 2, or ImgBlue 3

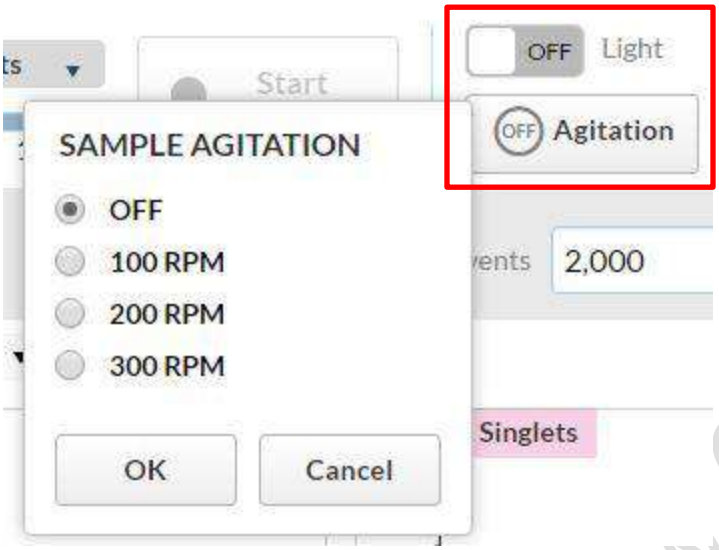
- Imaging features: displays the imaging features supported by the cell sorter (Please find the appendix for more details).

7. Adjust Gains:

- Load a fully-stained sample into the sample loading port.

- Click **Load Sample** in the acquisition dashboard to start acquiring data and adjust the **flow rate** as needed. (Note: Flow rate range from 1-50)

- (Optional) Turn on the agitation option and injection chamber light if needed.

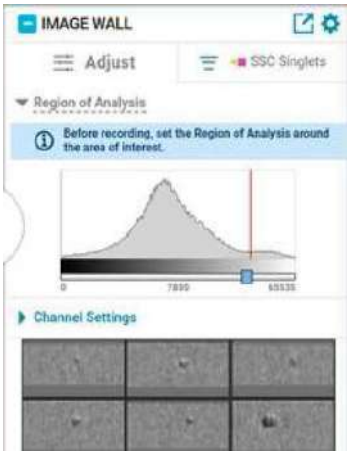


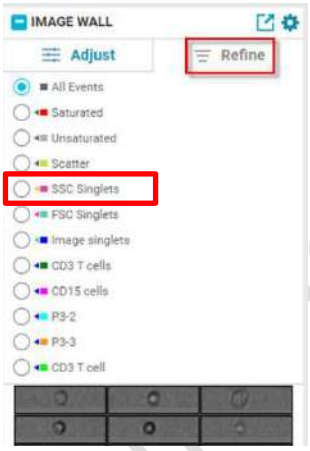
- Adjust the scatter and fluorescence gains to ensure the signals are on scale.

- **Region of Analysis (ROA) setting**

ROA is defined as the area of pixels that defines a single event, cell or particle.

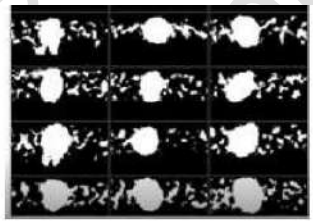
Set the **ROA** for imaging features by adjusting the ROA slider in the image wall from **SSC Singlets**. Note: for image features to be correctly calculated, **you must adjust the ROA first in the Adjust Gains page before you record your sample.** You can set it again on the View Data page as well when adjusting image wall settings. The ROA slider is not available for recorded data because adjustments cannot be made on recorded data.



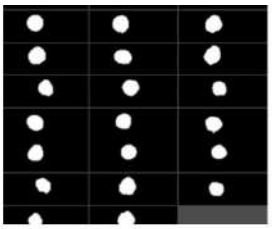


On clicking the Region of Analysis slider, the color of the images on the image wall appears as binary colors: the events (particles) of interest appear as white and the background as black as follows:

Region of Analysis set too high so that the complete outline of the particles of interest is not visible



Region of Analysis set too low so that a lot of background pixels are visible outside of the particles of interest

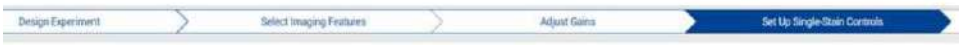


Optimally adjusted Region of Analysis so that only the particles of interest are visible

- Unload the tube

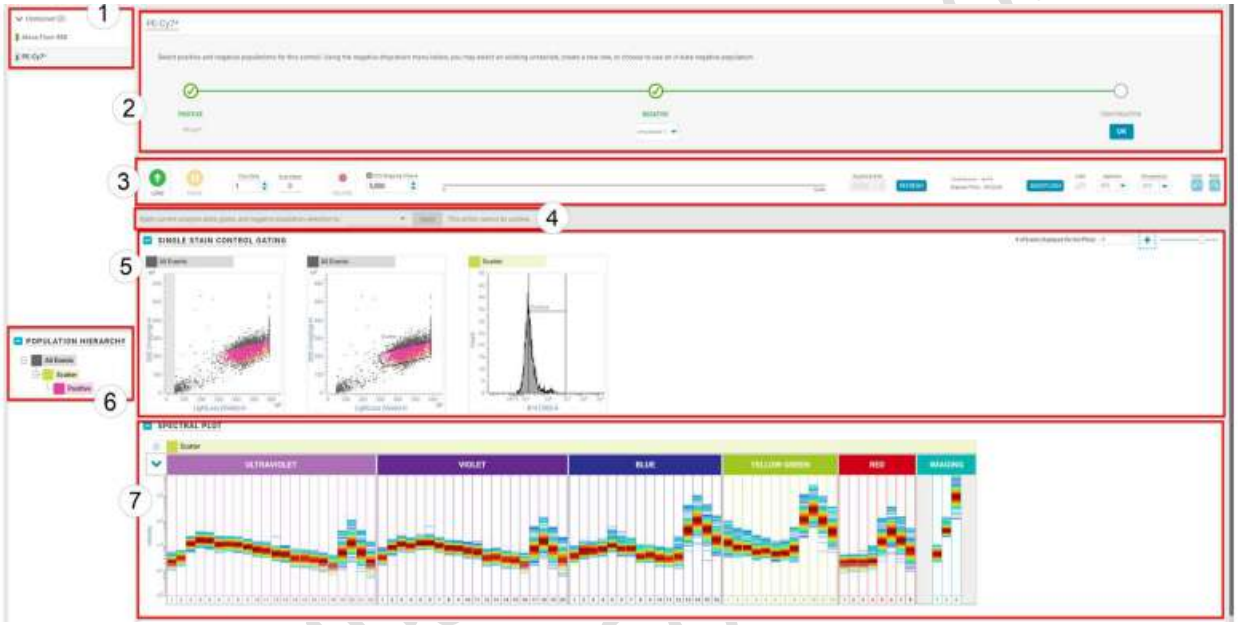
8. Set Up Single-Stain Controls:

- Navigate to the **Set Up Single-Stain Controls** page.

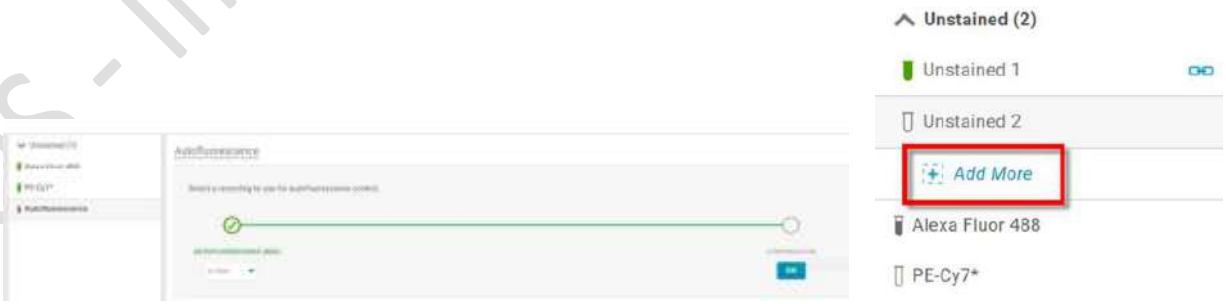


- Load and record single-stain control tubes for each fluorochrome.

- Confirm the positive and negative populations for each control.



- If using autofluorescence, load and record the autofluorescence control. Additionally, if it is preferred to have a separate tube as an unstained control, as opposed to gating on the unstained portion of your positive control tubes, you may add one or more unstained control tubes as necessary.



Adjust gains as necessary to place population on scale in both scatter and spectral plots. In the acquisition dashboard, click **Record**. Default FSC stopping cell event number is 10,000 of all events, and the FSC Stopping Criteria must be greater than 100 events.

After positive and negative populations have been selected and validated (green check marks), click **OK** under Confirmation to complete setting up the single-stain control for the fluorochrome. A successful spectral unmixing is indicated by the removal of the **Raw Data** icon at the top of the BD FACSChorus® software screen.

9. View Data

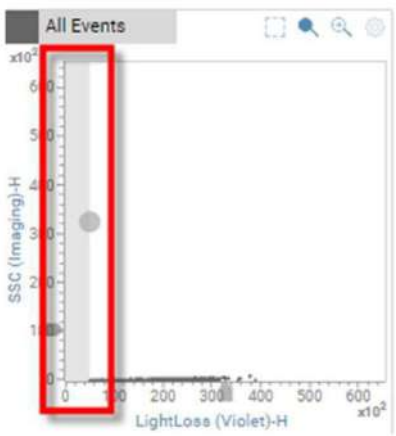
(1) Load the Sample

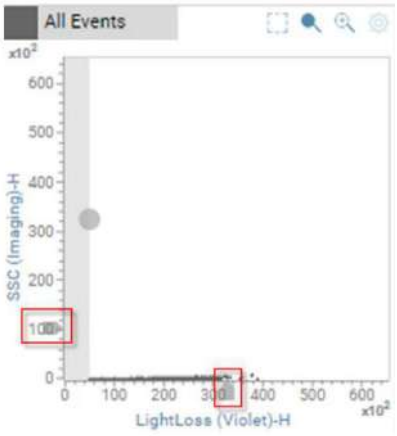
- Load the sample tube into the sample loading port and close the sample input door.

- Click **Load** in the acquisition dashboard to start acquiring data.

(2) Adjust Gains and Gates:

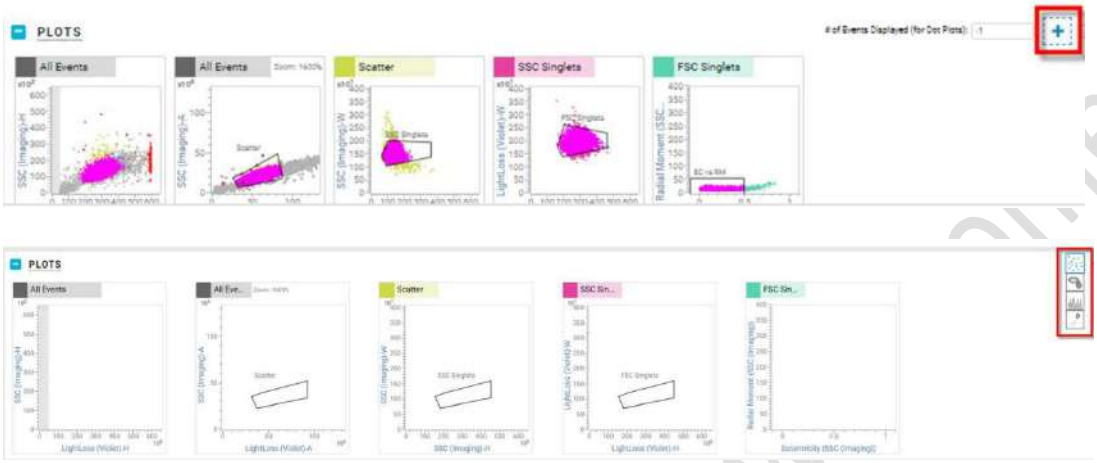
- Set up the acquisition threshold by hovering over the y-axis of the first default plot to make the threshold marker (gray dot) display. Move the marker horizontally to drag a gray shaded area along the x-axis. This movement adjusts the acquisition threshold of the trigger channel and removes the low-end debris from the plot. Adjust the scatter gains by moving the sliders along the axes as needed.





- Use the **Doublet Discrimination** feature to eliminate doublets if necessary.

- Add plots by clicking the **+** icon. A dropdown menu displays under the + with 4 different plot types: dot, contour, histogram and density. Select the plot type as needed.



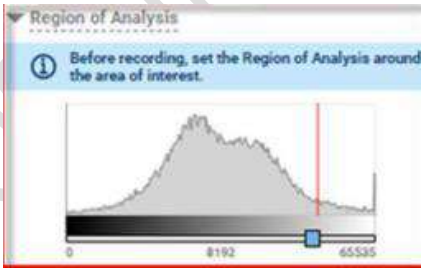
- Create gates to define the populations of interest using the scatter plots. Hover over a plot to make the plot tools display. To create a gate, click the **Add New Gate** icon (Square icon with dotted lines), select a gate type from the list and add it to the plot.

(3) Define x- and y- axes in the plots by selecting plot parameters

In a plot, click on either axes to open the Plot Parameters dialog box. From the grid displaying the available parameters and imaging features for your experiment, select the necessary parameters to define the X and Y axes of your plots. Imaging features could only be shown when you select the fluorophores under the imaging channels.

(4) Adjust **Region of Analysis** and **Pixel Threshold**

Adjust the **Region of Analysis (ROA)** and **Pixel Threshold** in the image wall to refine the imaging features. **For image features to be correctly calculated, you must adjust the ROA and Pixel Threshold before recording your samples, other image adjustments can be done at any time.**

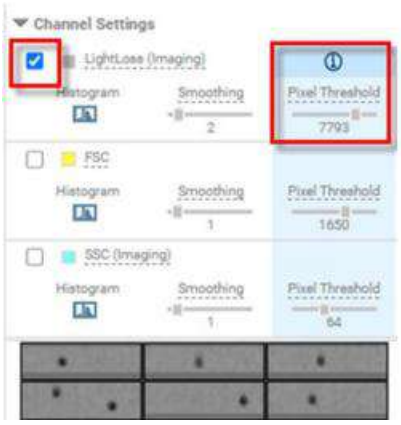


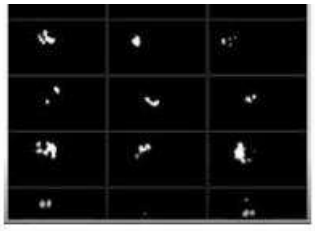
- For ROA adjustment, please refer to P10 from this SOP.

- For Pixel Threshold adjustment, please follow the below steps:

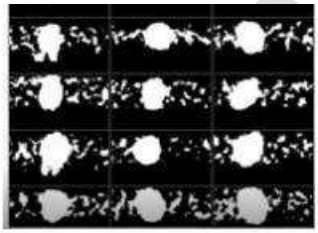
i. The Pixel Threshold is defined as a user-defined brightness threshold above which a pixel will be counted. It is necessary to calculate the Size imaging feature. All other imaging features are dependent

on the ROA for calculation. In the Channel Setting menu, select a channel by clicking the box to the left of it and then click the Pixel Threshold slider bar for that channel, you can only adjust the Pixel Threshold for one channel at a time.

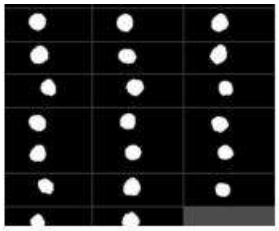




Pixel Threshold set too high so that the complete outline of the particles of interest are not visible

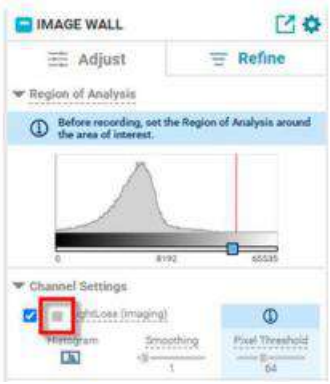


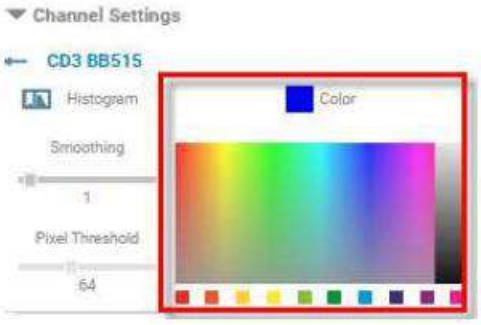
Pixel Threshold set too low so that a lot of background pixels are visible outside of the particles of interest



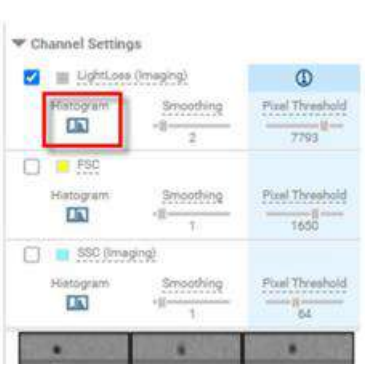
Optimally adjusted Pixel Threshold so that only the particles of interests are visible

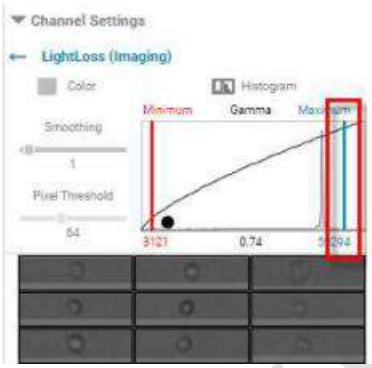
ii. To change the color of a channel, select the channel and click the Color Box to expand the color palette, and select a color you want.



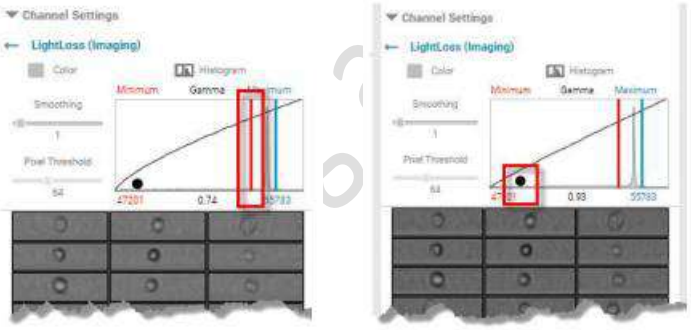


iii. To change the appearance of images in the selected channel, click **Histogram** to open the Histogram panel. In the Histogram panel, adjusting the Maximum (blue bar) so that it is close to the top of the histogram peak usually results in the best image appearance.

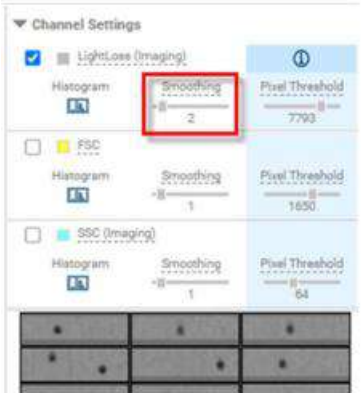




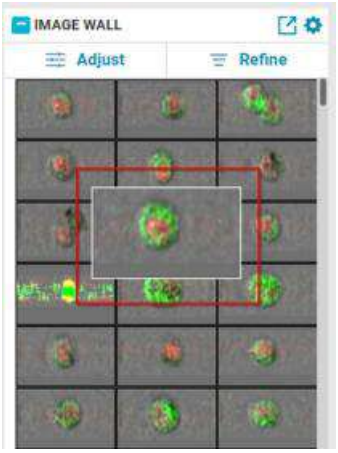
iv. Adjusting the Minimum (red bar) to somewhere lower than the histogram peak can help to minimize background. Adjusting the Gamma control (black dot) to different positions can help to improve the image.



v. Adjust the Smoothing slider slowly (right and left) to reduce blur in the images and to improve the image appearance.



vi. View the images of the events in the image wall to verify the gating and imaging settings.



(5) Record Data:

- Click **Record** in the acquisition dashboard to save the data. Turn OFF the image stored icon if imaging data are not required.

- File size from S8:

Event number	FSC file size	Imaging CVW file size	Extracted TIFF file size
10,000	~ 17 MB	~ 1.5 GB	~ 3 GB

Data format	Description
FCS	Flow cytometry standard data
Images	Imaging data (CellView or CVW)
CSV	Index sort data

- Provide a name for the recording when prompted.

- The recorded data will be available in the **Data Sources** panel.

10. Sorting Cells

(1) Set Up Sort:

- Navigate to the **Set Up Sort** page.

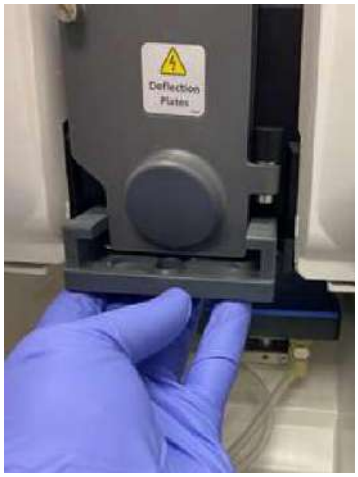
- Select the format (e.g., 2-way, 4-way, or 6-way sorting).

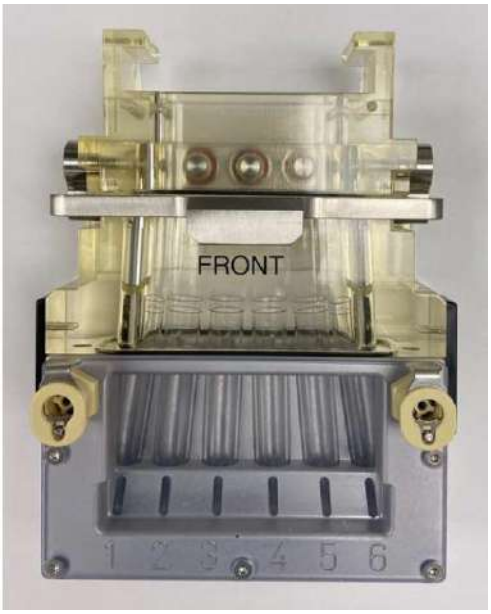
Nozzle size (um)	Sort way
85	2-way, 4-way, or 6-way
100	2-way or 4-way
130	2-way

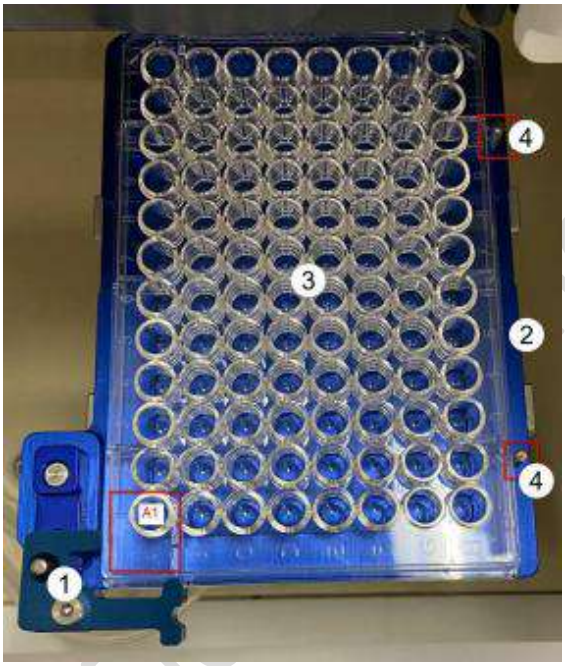
- Select the sort mode (Purity, Yield, or Single-cell mode)

- Choose the collection device (1.5, 2.0, and 5.0 mL-tubes, 6/24/48/96/384 plates, or slides).

Note: Slash shield will be used for plate or slide mode.





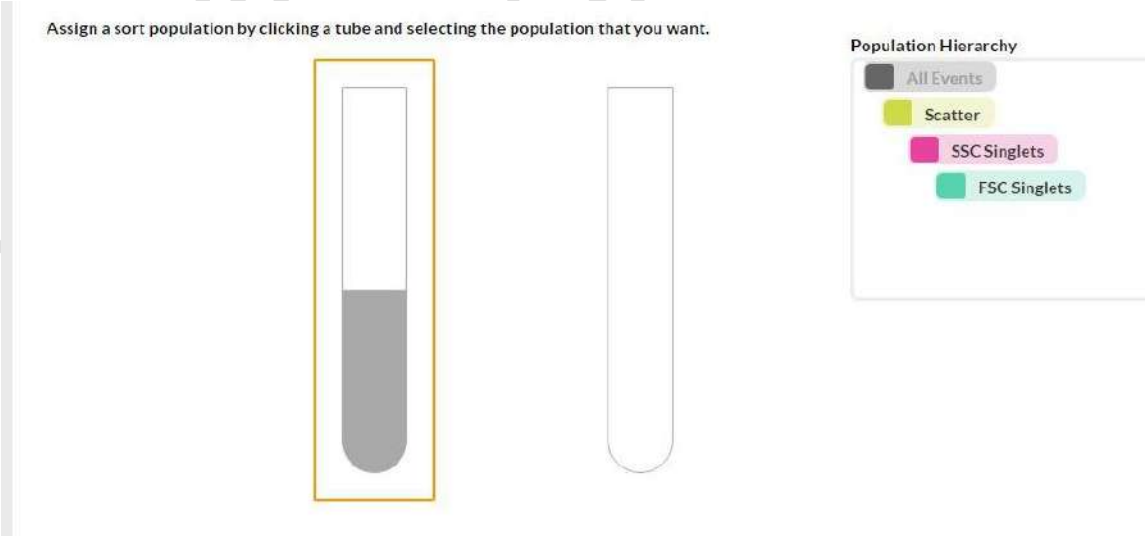


(A1 is on the bottom left)

(2) Define Sort Gates:

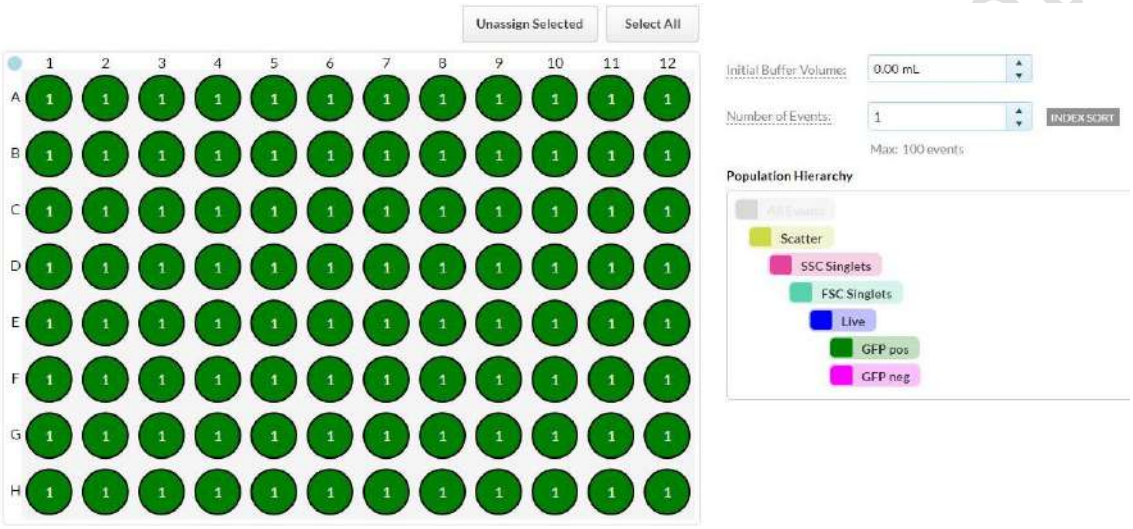
- Use the scatter and fluorescence plots to define the gates for the populations you want to sort.

- Click on the target tube (selected tube will be highlighted by orange box) and then **click the targeted population** on the population hierarchy respectively.



- For plate mode, click **Select All** to select all wells and then **click the targeted population** on the population hierarchy to assign the population to selected wells.

(Note: Drag over the wells to select multi-wells OR Hold **Ctrl** on the keyboard and click on specific wells to multi-select)



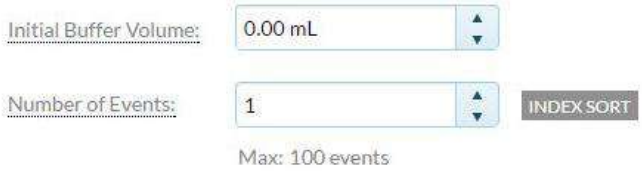
- Confirm the gates and ensure the populations are correctly identified.

- (Optional) **Check the Enable index sort** if you would like to perform index sorting from plate mode. (Note: target cell number will be set to 1-100 if index sort is enabled)

- Key in the volume collection buffer (Initial Buffer Volume)

Sort collection device	Maximum initial buffer volume
1.5-mL tube	0.75 mL
2.0-mL tube	1 mL
5.0-mL tube	2.5 mL
6-well plate	0.63 mL
24-well plate	0.2 mL
48-well plate	0.13 mL
96-well/96-well PCR	0.07 mL
384-well plate	0.03 mL
Slide	No initial volume

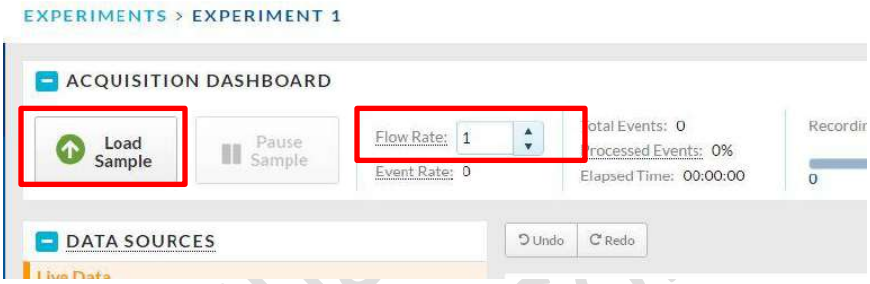
- Key in the target cell number (Number of Events). The maximum cell number will change depends on your initial buffer volume.



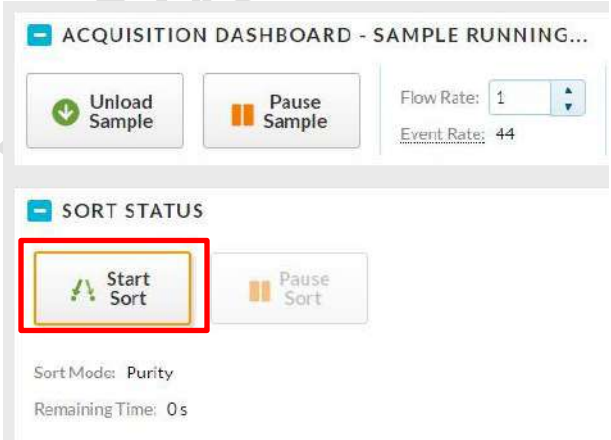
(3) Start Sorting:

- Load the collection device (tubes, plates, or slides) into the sort collection chamber.

i. Load your sample tube on the sample loading stage.
ii. On the Acquisition dashboard, click **Load Sample** and **adjust the flow rate** as needed. (Note: Flow rate range from 1-50, set below 20 for sorting experiment)



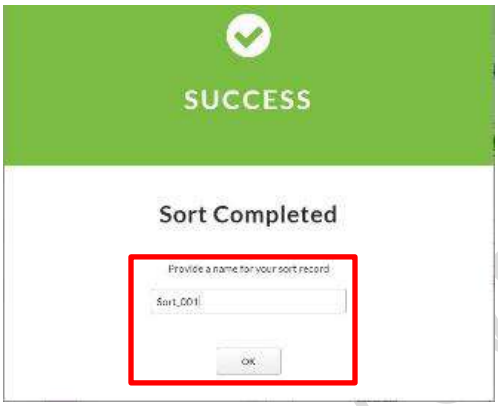
iii. (Optional) Turn on the agitation option and injection chamber light if needed.
iv. Click **Start Sort** to start the sorting.



v. You can terminate the sort at any point by clicking **Stop Sort**. You can also **Pause** the sort and resume at any time.
vi. Once the target cell number is reached, sort will be stopped automatically and sample will be unloaded.

vii. Open the lower chamber door and remove the sort collection device and your collection tube or plate.

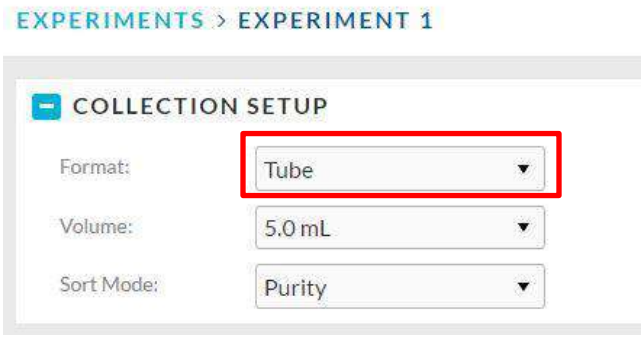
viii. **Name** the sort and click **OK**. View the corresponding sort report.



<u>To retract plate ACDU,</u>

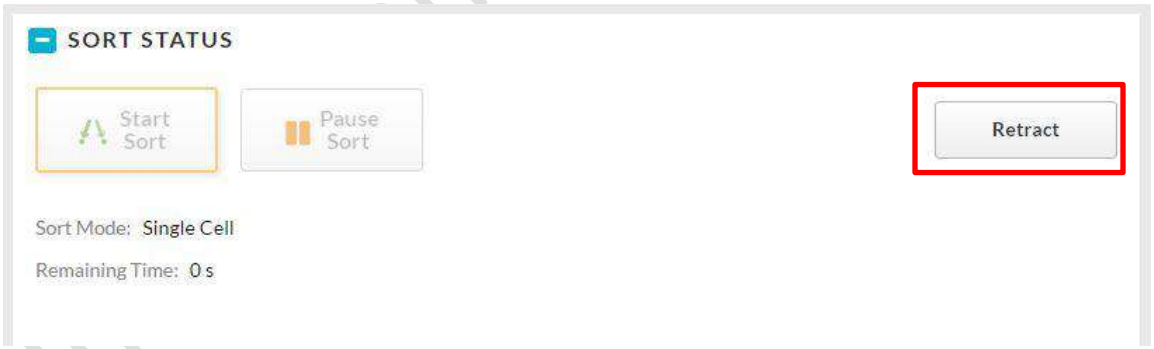
- Click the **Set Up Sort** tab.

- Change Format to **Tube.**



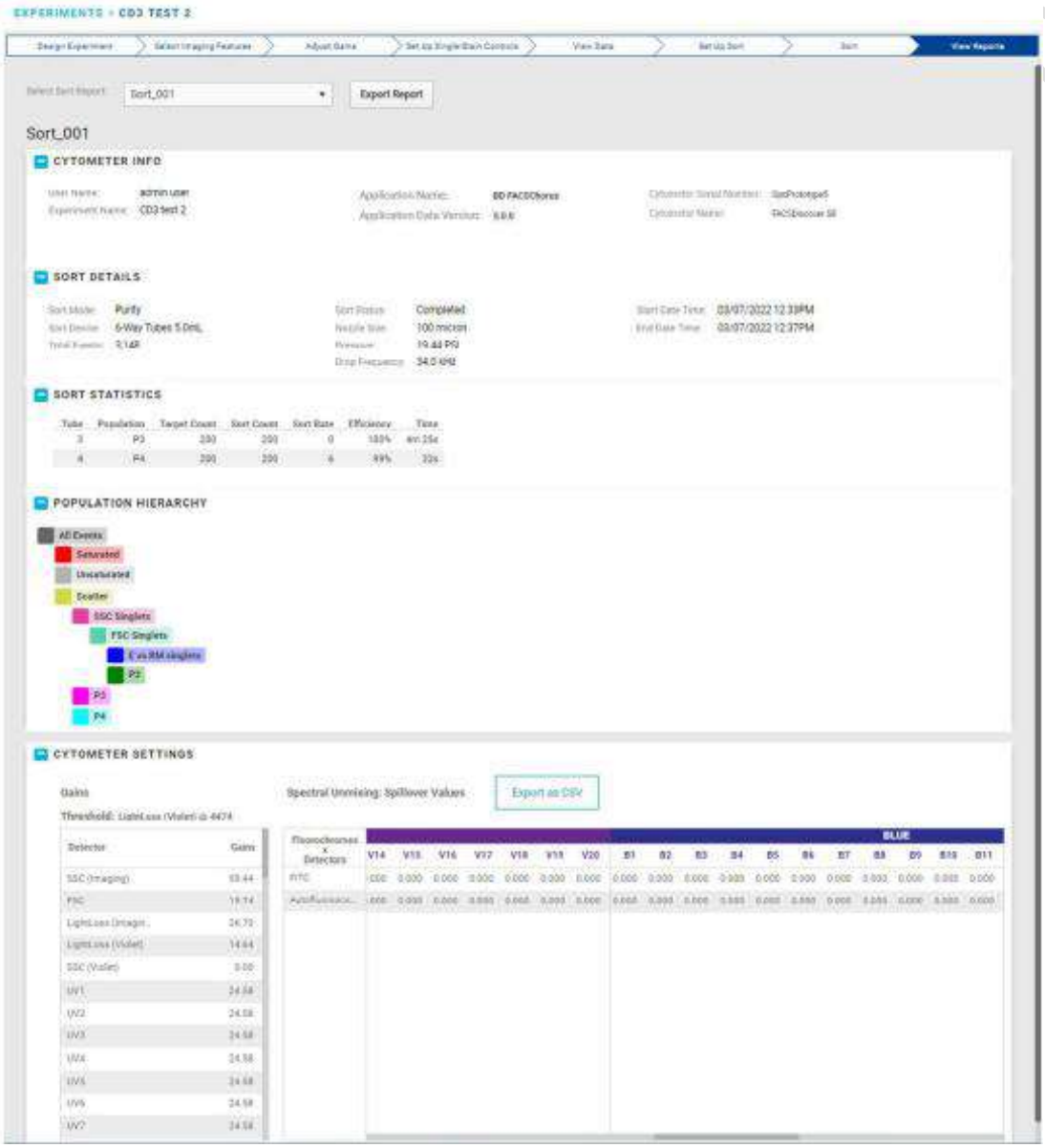
- Click **Sort** tab.

- Click **Retract** under **Sort Status** section



11. View Sort Report

(1) Click the **View Reports** tab



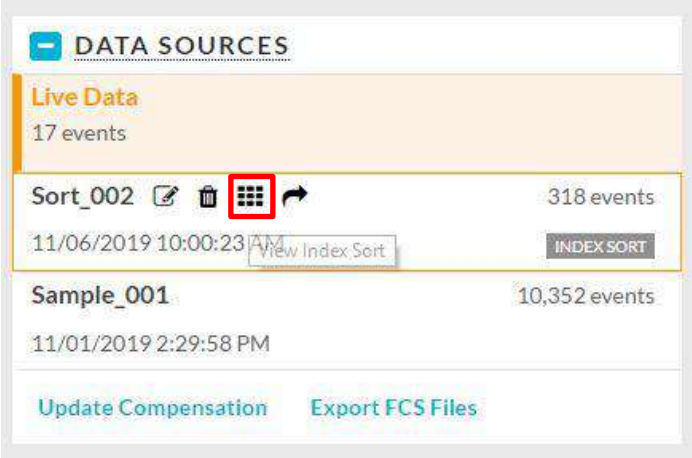
(2) Select the sort report to view from the pull-down list.

(3) Click **Export Report** to save the .pdf file of the sort report under the following location: D drive > Users > Department > Personal folder.

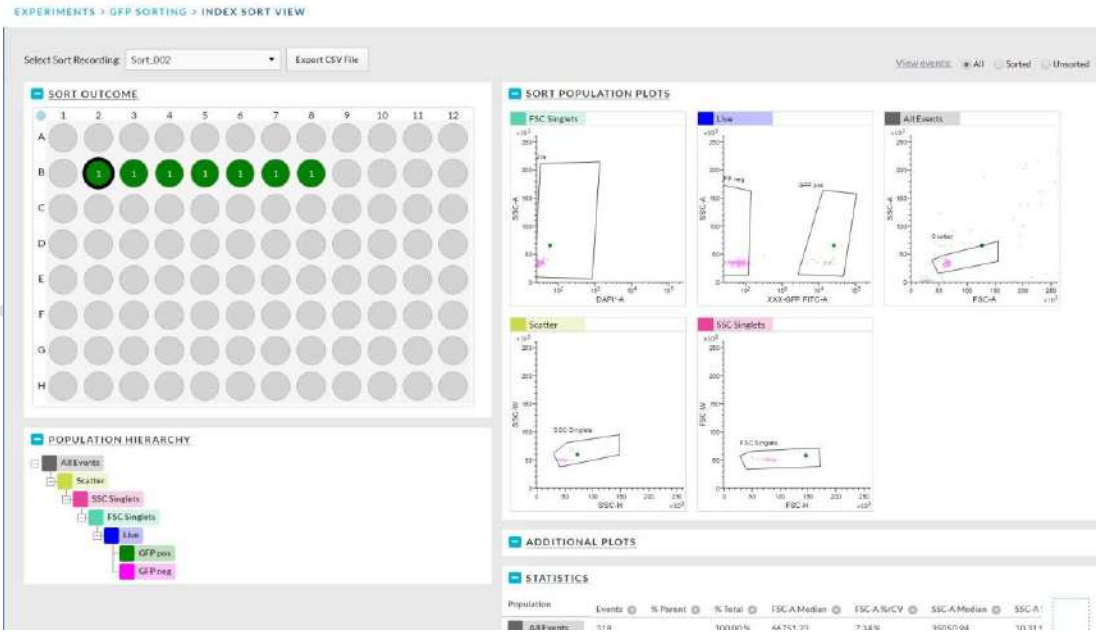
12. View Index Sort Report (if index sort is performed)

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

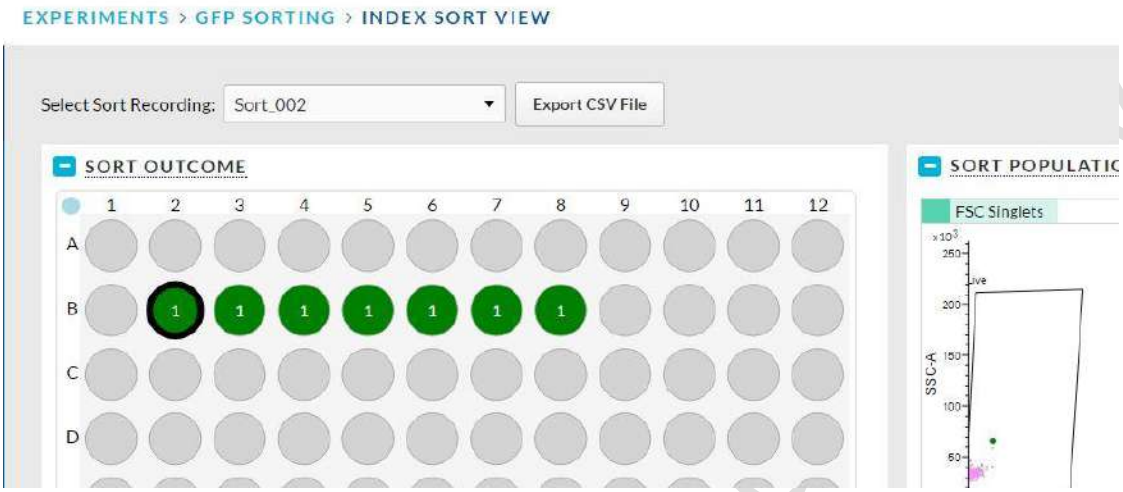
(2) Hover over the index sort file under the **Data Sources** section and **click the grid icon** to open the index sort view window.



(3) **Click on the well** (e.g. B2) to view the single cell data shown on the plots. Press **Ctrl+P** on keyboard to save .pdf file of the report under the following location:
D drive > Users > Department > Personal folder.



(4) Click the Experiment Name on the top to leave the index sort view page.



13. Export Data

(1) Export data from Experiments page:

The Export Data dialog displays with a list of files for the selected experiments.

Data format	Description
FCS	Flow cytometry standard data
Images	Imaging data (CellView or CVW)
CSV	Index sort data

When your selections are complete, click Export Selected and save to D drive > Users > Department > Personal folder.

(2) Exporting data from Data Sources panel in the View Data page

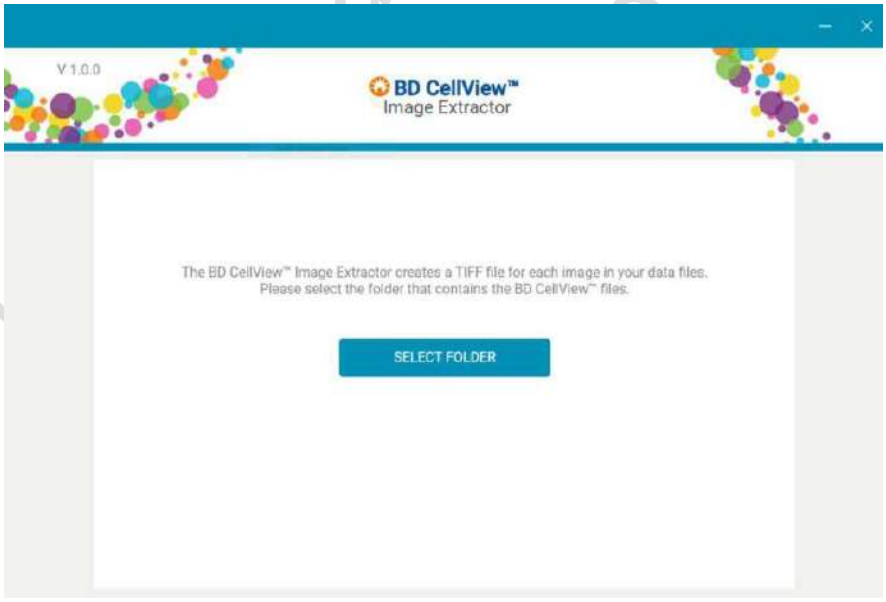
Click the Export Recording icon ① next to the data source panel heading or click the Export Recording icon ② for a recorded sample.

When your selections are complete, click Export Selected and save to D drive > Users > Department > Personal folder.

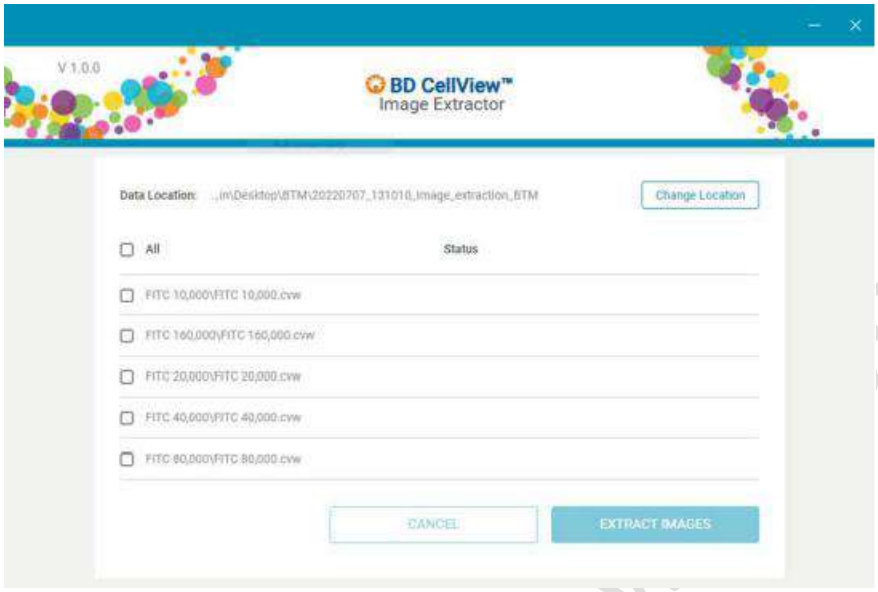
14. Extract images

The BD CellView™ Image Extractor allows you to convert the exported image event data from CellView format (CVW) to Tagged Image File Format (TIFF) for analyzing imaging data in other software applications.

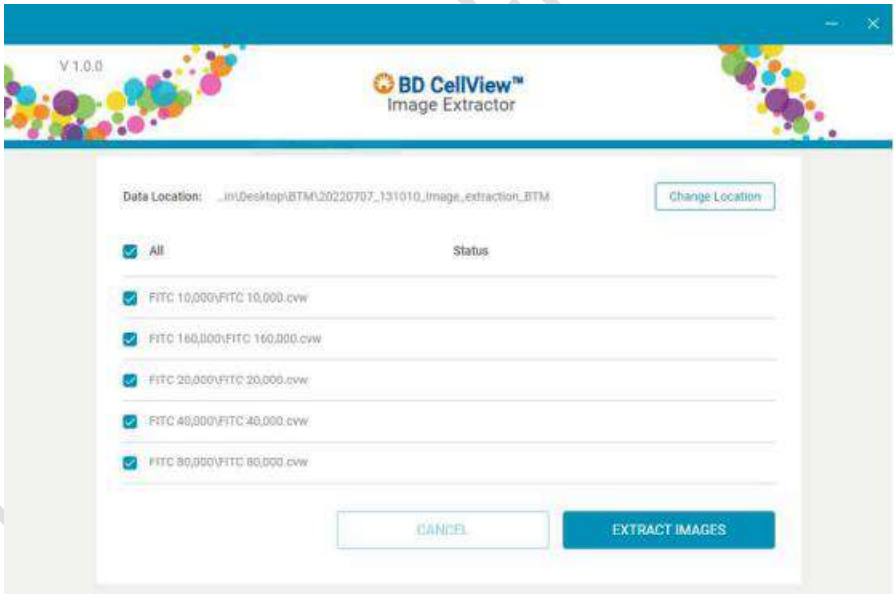
(1) Open the BD CellView™ Image Extractor from the desktop



(2) Click **Select Folder** and browse the workstation to select the folder which contains the CVW image event data that you want to convert. After selection, a list of CVW files displays along with the location of the selected folder.



(3) Click the checkboxes to select the CVW files individually or click **All** to select all of them.



(4) When your selections are complete, click **Extract Images.**

(5) Accessing extracted images

Go to the location of the converted TIFF image event data file by clicking the Folder icon next to each CVW event image data that were converted.

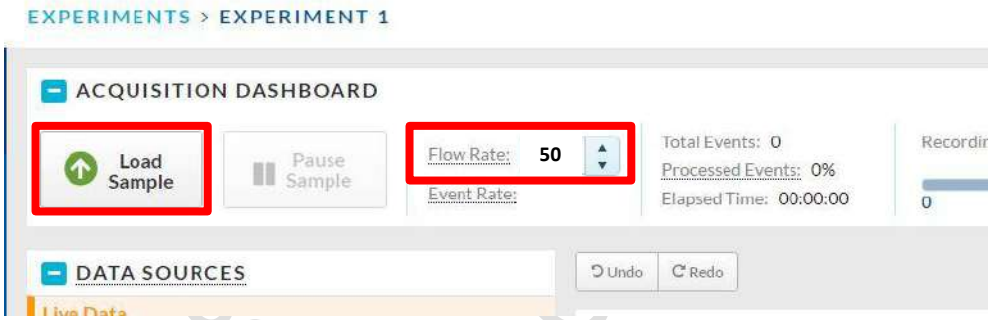
In the folder, the TIFF image files will be broken down into sub-folders, each containing the TIFF image files for 10,000 FCS events. The default labeling for the sub-folders is “00000000” for the first 10,000 FCS events, “00010,000” for the second 10,000 FCS events, and so on.

15. System Cleaning

(1) Click the **View Data** tab

(2) Load a tube of 2 mL Cleaning Solution 1 on the sample loading stage.

(3) On the Acquisition dashboard, click **Load Sample** and adjust the **flow rate to 50** and run for 5 minutes.

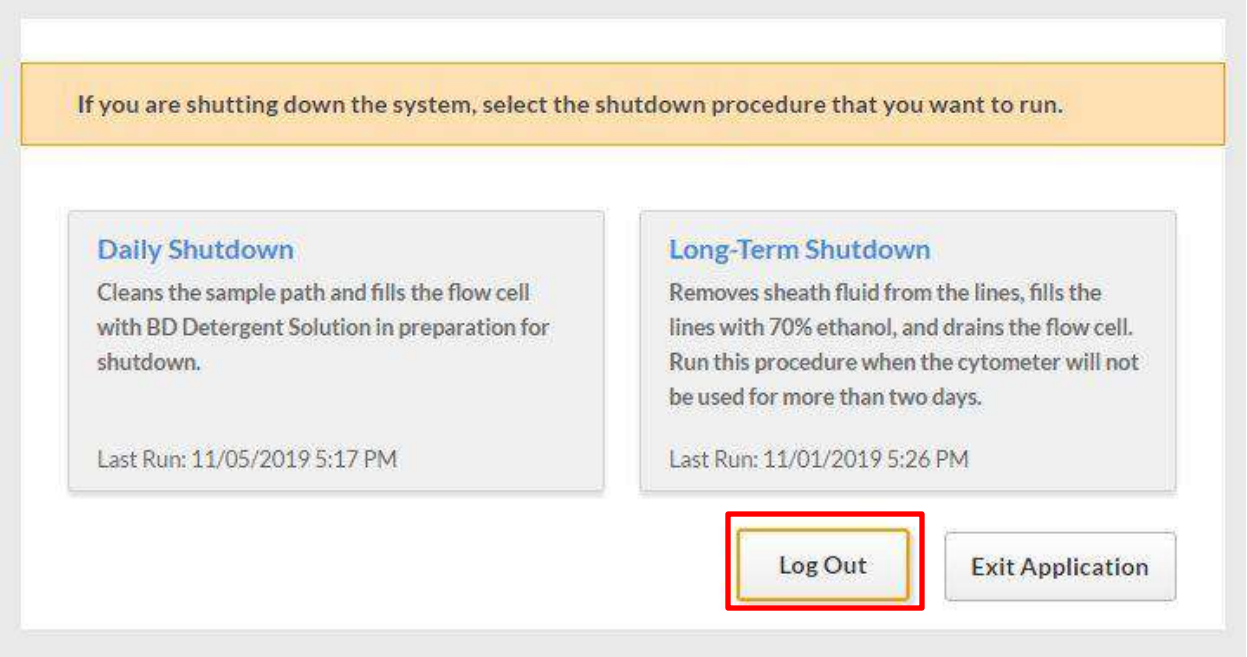


(4) Click **Unload sample** to unload the tube and repeat step 2 and 3 with Cleaning Solution 2 and 3. Note: if PI is used from your sample, please clean the system with solution 2 for 10 mins.

Log-out

1. Click **Log out** on the Left panel.

2. Click **Log out**.



3. Log out Tracker before leaving

Appendix: Imaging features from BD FACSDiscover™ S8 (from manual P117-123)

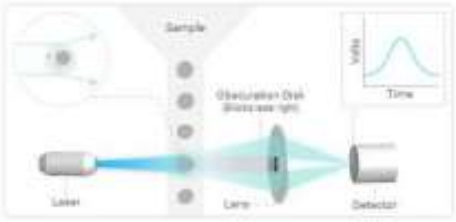
Scatter parameters

The following table describes the scatter parameters that are supported by the BD FACSDiscover™ S8 cell sorter.

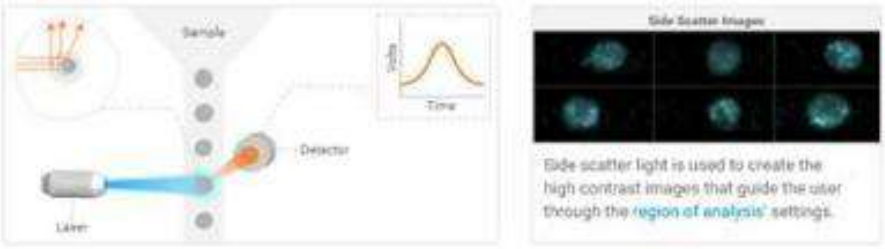
Imaging feature	Definition	Usage example
Forward Scatter (FSC)	As particles (cells) pass through the laser, the interaction of the light with the particle results in scatter in all directions. The forward scatter detector is placed in line with the light path to measure light that is scattered at small angles. Forward scatter loosely correlates to particle (cell) size.	Cell morphology and label free sorting
Side Scatter (SSC)	As particles (cells) pass through the laser, the interaction of the light with the particle results in scatter in all directions. The side scatter detector measures light that is scattered perpendicular (90°) to the laser path. Side scatter is loosely correlated to optical density or complexity of the particle.	Create high contrast images that guide you to set up the Region of Analysis
Light Loss (Violet)	Light loss can be measured from the violet laser in addition to the blue laser, but it does not generate images. Only the blue laser can be used to generate light loss images. Violet laser can be used to differentiate size and serves as an alternative to forward scatter to separate classic whole blood sub-populations.	Visual reference and sample quality control
Light Loss (Imaging)	Light loss that generates images can be measured from the blue (imaging) laser. As particles (cells) pass through the laser, the interaction of the light with the particle results in scatter in all directions. Light loss is a measure of light (photons) lost from the laser due to scattering and absorption of light by a particle (cell). Light loss images are similar to brightfield microscopy.	Visual reference and sample quality control

Representative images for scatter parameters

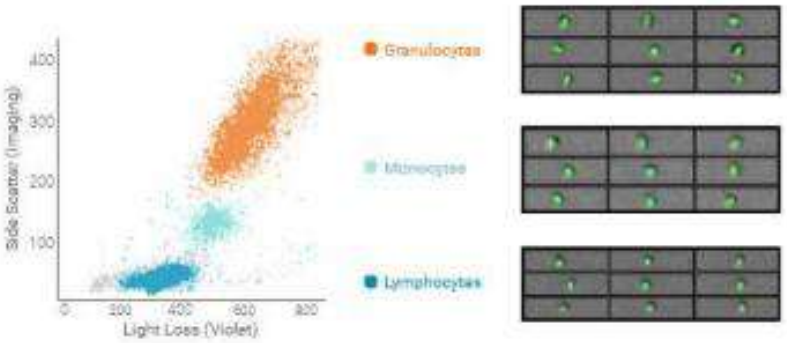
Forward Scatter (FSC)



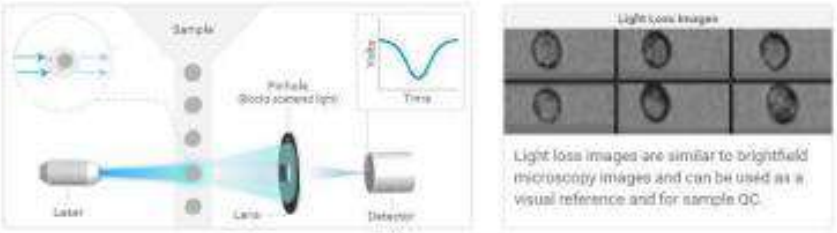
Side Scatter (SSC)



Light Loss (Violet)



Light Loss (Imaging)



Imaging features

The following table describes the imaging features that are supported by the BD FACSDiscover™ S8 cell sorter.

The Region of Analysis (ROA) adjustment is necessary to calculate all the imaging features that are described in the following table except for the Size parameter, which is only calculated by the Pixel Threshold adjustment.

The Region of Analysis (ROA) is defined as the area of pixels that defines a single event, cell, or particle.

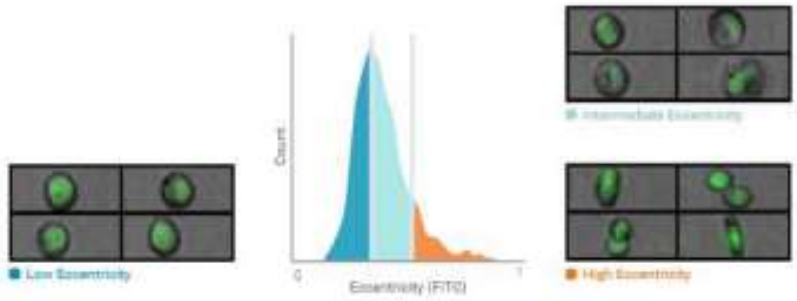
Note: ROA is sample-dependent and must be set first in the Adjust Gains page before recording your sample. You can set it again on the View Data page as well when adjusting image wall settings.

Imaging feature	Definition	Required settings adjustments	Usage example	Availability
Eccentricity	A ratio of the shortest to the longest axis (moment) within the Region of Analysis.	Region of Analysis	Doublet discrimination, cluster identification, cell morphology	All imaging channels
Radial Moment	The average distance of the pixels from the centroid within the Region of Analysis.	Region of Analysis	Doublet discrimination (with Eccentricity), cell-to-cell interactions (cellular synapse)	All imaging channels
Doublet Discrimination	Eccentricity and Radial Moment can be used together to identify doublets or clusters or both.	Region of Analysis	Image quality control	All imaging channels
Long Moment	The measurement of the longest axis (moment) within the Region of Analysis.	Region of Analysis	Cell morphology, cell-to-cell interaction, aggregates	All imaging channels
Short Moment	The measurement of the shortest axis (moment) within the Region of Analysis.	Region of Analysis	Cell morphology, cell-to-cell interaction, aggregates	All imaging channels
Size	The number of pixels in the image, which are brighter than a user-defined Pixel Threshold.	Region of Analysis followed by Pixel Threshold	Label-free sorting, punctate fluorescence	All imaging channels
Total Intensity	The sum of the intensities of all pixels within the Region of Analysis.	Region of Analysis	Quantitative fluorescence measurements	All imaging channels
Maximum Intensity	The intensity of the brightest pixel in the image. **Note:** It is not affected by the Region of Analysis.	Region of Analysis	Punctate fluorescence, phagocytosis assay, cell cycle analysis	All imaging channels

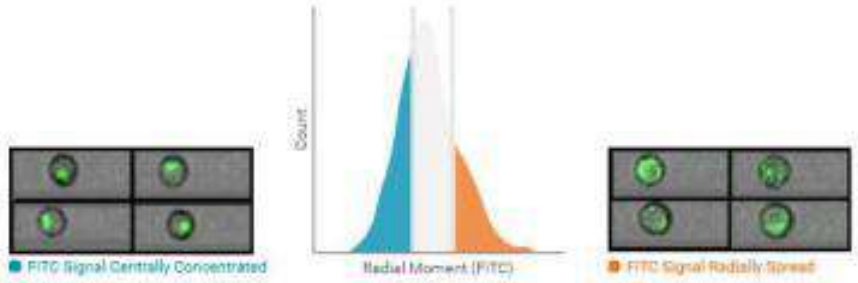
Imaging feature	Definition	Required settings adjustments	Usage example	Availability
Diffusivity	The ratio of the total intensity to the maximum intensity.	Region of Analysis	Cell morphology, phagocytosis assay	Any individual imaging channel
Correlation	The degree to which the location of two imaging channels are the same within the region of pixels (as defined by the Region of Analysis).	Region of Analysis	Translocation assay, cell-to-cell interaction	Any two imaging fluorescence channels
Delta Center of Mass	The distance between two fluorescent signal sources in any two imaging channels within a particle (as defined by the Region of Analysis).	Region of Analysis	Cell-to-cell interaction, phagocytosis	Any two imaging channels
Center of Mass (X)	The position of the particle (as defined by the Region of Analysis) in the horizontal direction within an image.	Region of Analysis	Image quality control, antigen cellular location, phagocytes	Any individual imaging channels
Center of Mass (Y)	The position of the particle (as defined by the Region of Analysis) in the vertical direction within an image.	Region of Analysis	Image quality control, antigen cellular location, phagocytosis	Any individual imaging channels

Representative images for imaging features

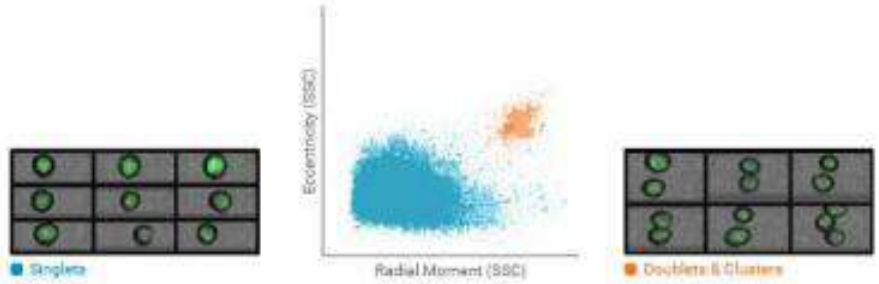
Eccentricity



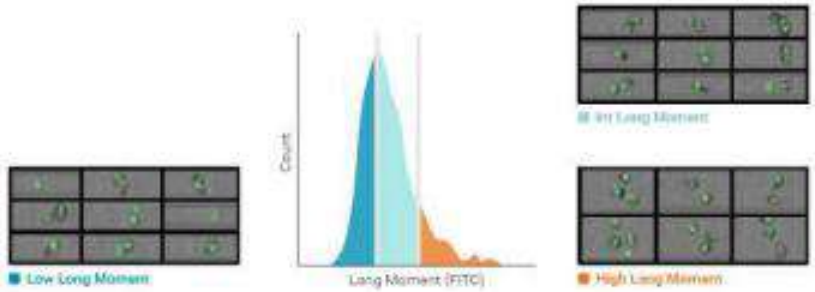
Radial Moment



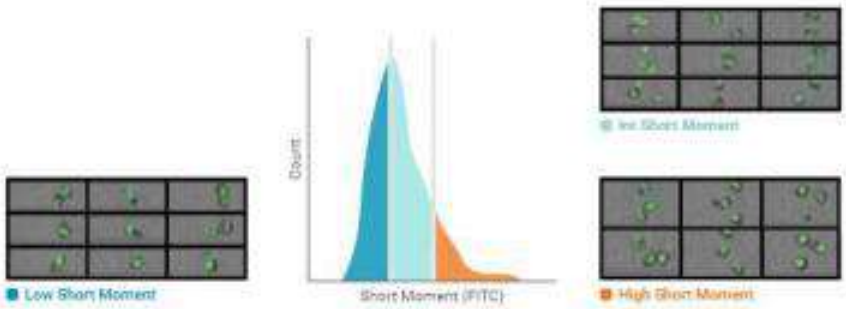
Doublet Discrimination



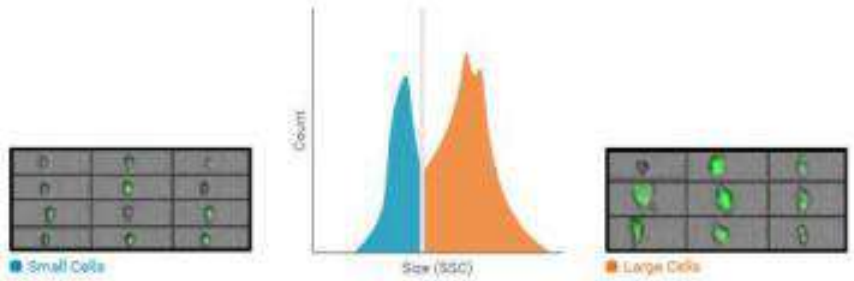
Long Moment



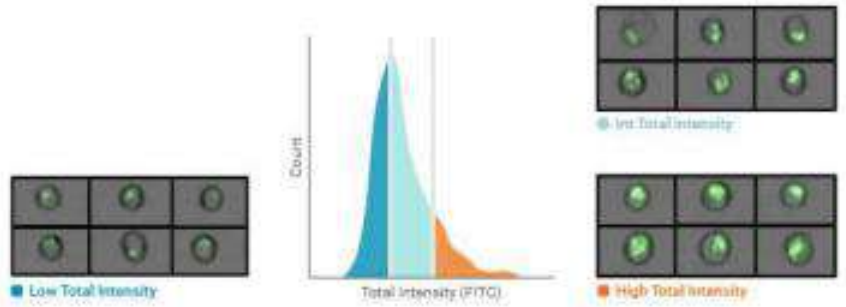
Short Moment



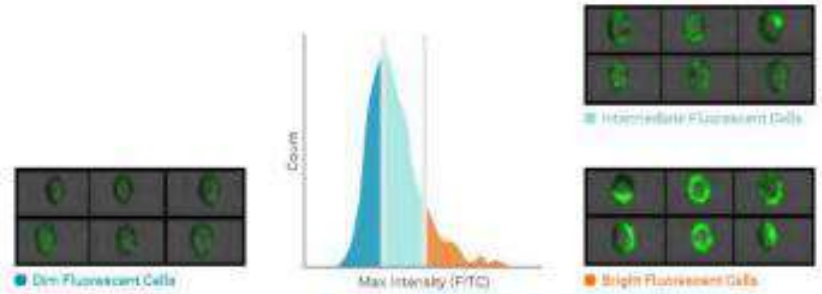
Size



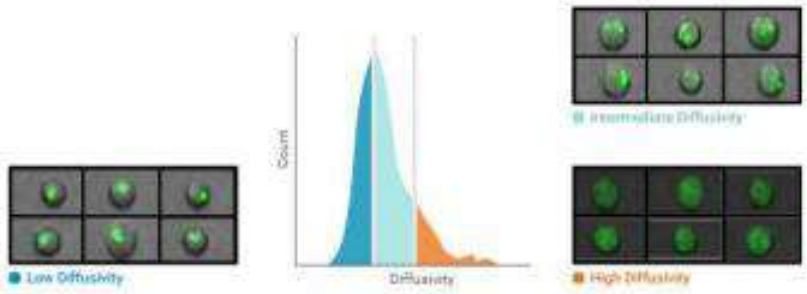
Total Intensity



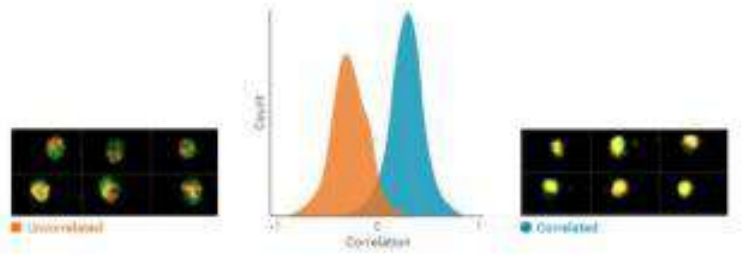
Maximum Intensity



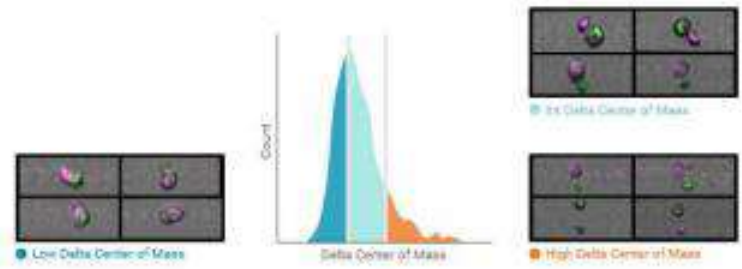
Diffusivity



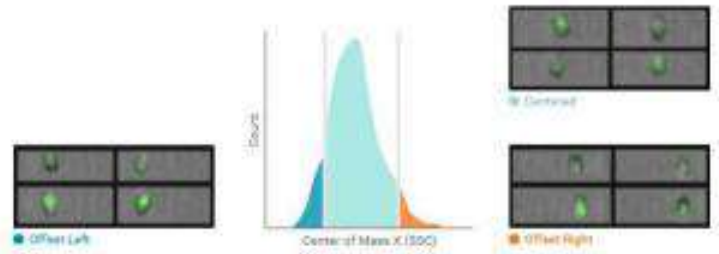
Correlation



Delta Center of Mass



Center of Mass (X)



Center of Mass (Y)