

**BD** CellView™ Lens Plugin  
for FlowJo™ v10 Software  
User's Guide

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# 1

## Introduction

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This chapter covers the following topics:

- [Overview \(page 6\)](#)
- [Installing the BD CellView™ Lens plugin \(page 8\)](#)

## Overview

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The BD CellView™ Lens plugin for FlowJo™ v10 provides the complete set of tools that allows you to visually explore all the image data captured and then exported from the BD FACSDiscover™ S8 cell sorter. After the FCS and imaging data are exported from BD FACSCorus™, use the BD CellView™ Image Extractor or BD CellView™ Lens plugin to convert the exported image event data from Chorwave format (CVW) to TIFF (Tagged Image File Format). Further analysis of the extracted imaging data (TIFF) is performed by using the plugin. You can also use imaging data in other formats (jpg and png) from other cytometers or data sources to analyze them by using the plugin.

To know more about exporting data from BD FACSCorus™, see the Exporting and deleting data section in *BD FACSDiscover™ S8 Cell Sorter with BD CellView™ Image Technology User's Guide*.

To know more about BD CellView™ Image Extractor, see the Extracting images section in *BD FACSDiscover™ S8 Cell Sorter with BD CellView™ Image Technology User's Guide*.

With the BD CellView™ Lens plugin for FlowJo™ v10, you can:

- Convert the exported image event data from Chorwave format (CVW) to TIFF (Tagged Image File Format).
- Use the interactive Images Browser to display customized views of cell images for specific populations and also connect to FlowJo™ software's graph window to allow mouse-over image display. Images can be sorted by any sample parameter and snapshots taken and displayed in FlowJo™ software's layout editor automatically.
- Use the Image Filters tool to enhance the image for any single channel image by adding a sequence of filters, such as color, smoothing, and thresholds, and additionally compose an overlay channel that combines multiple channels into a single image.

To start using the BD CellView™ Lens plugin, first, load your FCS sample file into a FlowJo™ workspace by using FCS\_With\_Images\_Loader, a workplace plugin, and then create sub-populations using gating or clustering tools, if necessary. After selecting a population, save the file and then choose the BD CellView™ Lens plugin from the Workspace –Plugins menu to create a plugin node for that population. You can add a plugin node to another population using the Workspace –Plugins menu or by dragging an existing plugin node to the population.

## Best practices




For optimal performance of the BD CellView™ Lens plugin, we recommend:

- Running the plugin with the FCS and image files on a local drive rather than an external one.
- Storing the FCS, index sort CSV, and image files (.tiff or .cvw) in the same mapped folder.

The recommended hierarchy of files and sub-folders containing extracted TIFF images within a successfully mapped parent folder is shown in the following images.

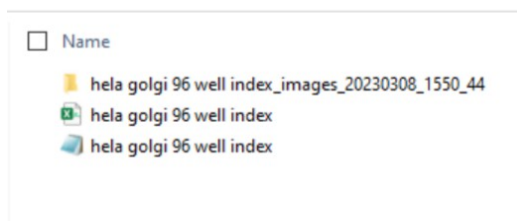
Recommended folder structure for a non-index sort experiment.

**Note:** Make sure that a non-index sort experiment mapped folder contains the image file (.cvw) or the extracted images folder and an FCS file.

	Cell cycle sample_images_20230322_1...	03/22/2023 10:23 AM	File folder	
	Cell cycle sample.cvw	03/20/2023 2:34 PM	Image Extractor C...	888,386 KB
	Cell cycle sample.fcs	03/20/2023 2:34 PM	FCS File	33,636 KB

Recommended folder structure for an index sort experiment.

**Note:** Make sure that an index sort experiment mapped folder contains a CSV file along with the image file (.cvw) or the extracted images folder and FCS file.



## Installing the BD CellView™ Lens plugin

To install BD CellView™ Lens plugin on your workstation:

**Note:** The following procedure works for Mac® and Windows® workstations.

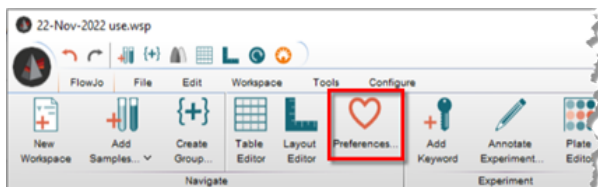
1. Go to <https://www.flowjo.com/exchange/#/> and search for the plugin.
2. Download the BD CellView™ Lens plugin.jar file on to your local workstation.
3. Copy the plugin.jar (BD\_CellView\_v2.0.jar) file to the FlowJo™ plugins folder in your workstation.

**Note:** If you start FlowJo™ software after performing step 3, you do not have to perform the following steps. You can start using the plugin by mapping the image files to events. See [Mapping the image files to events \(page 12\)](#).

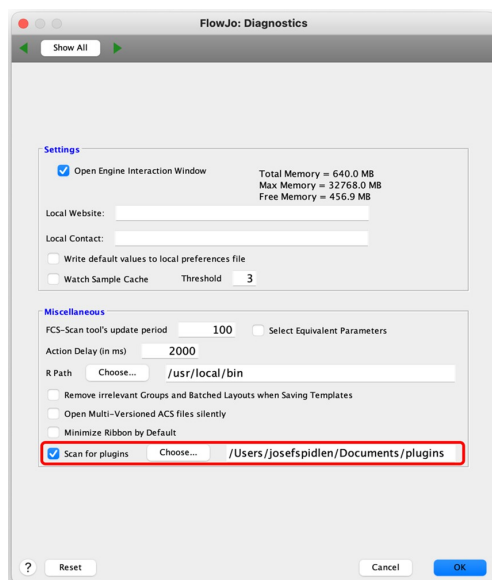
**Note:** If you have started FlowJo™ software, perform the following steps to continue installing the plugin.

**Note:** version 1.2.3 and later were updated to connect with FlowJo version 10.10. If you have an earlier version of FlowJo, please download one of the previous versions from the same page

4. Open FlowJo™ software and go to **FlowJo > Preferences > Diagnostics**.



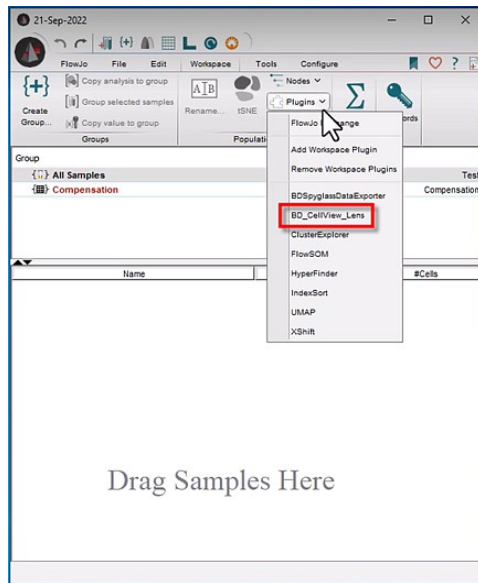
In the FlowJo: Diagnostics window, click to select the **Scan for plugins** option, then click **Choose**, and then select the location of the plugins folder.



5. Click **OK**.

6. Restart FlowJo™.

The BD CellView™ Lens plugin is available for you to use under **Workspace > Plugins** menu as BD\_CellView\_Lens.



You can start using the plugin by mapping the image files to events. See [Mapping the image files to events \(page 12\)](#).



# 2

## **Using the BD CellView™ Lens plugin**

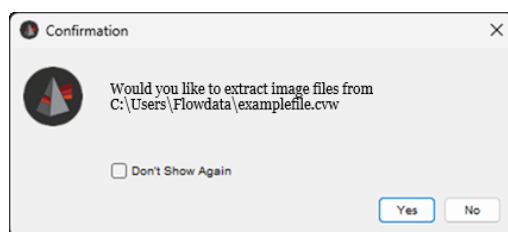
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This chapter covers the following topics:

- [Mapping the image files to events \(page 12\)](#)
- [Using the images browser \(page 23\)](#)
- [Using the Image Filters tool \(page 33\)](#)
- [Using the Index Sort Plate Viewer \(page 31\)](#)
- [Using the Event to image plot functionality \(page 45\)](#)
- [Using the Snapshot to layout functionality \(page 47\)](#)

## Extraction and Mapping

The CellView Lens can either extract images from .cvw or map extracted .tiff files to the current analysis. When the first plugin node is created for a sample's population, the new plugin node will prompt you whether you would like to extract the images if the Chorwave file (CVW) is found in the same location as the FCS you are running the analysis on.

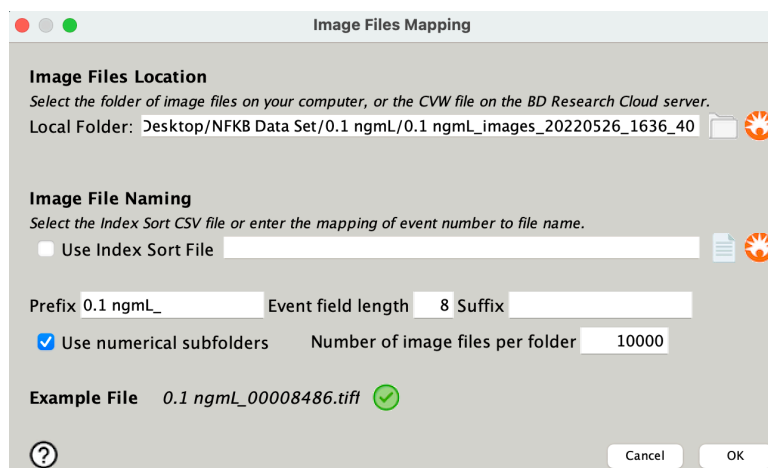


The first time you agree, the application will display a message noting that the software to do TIFF extraction is being installed. A CellViewExt folder will appear in your plugin folder with a pair of operating system dependent subfolders. Within the folder named for your OS the file doTiffextraction.exe will appear. This will only happen once, and then the software is ready to go.

The CellView Lens plugin will create a folder structure matching the Chorus structure and populate it with TIFFs. On completion an Image Explorer window will pop up.

If no CVW file is detected the plugin will attempt to find the image folder and file name mapping based on the location of the FCS file. Typically the image files are organized into numerical subfolders (e.g. '00000000', '00010000', etc) where 10,000 images files are located in each subfolder.

After mapping the image files to events, you can either confirm or change the root folder of image files and the name mapping information. If the file name prefix, suffix, and event number length are correct, the plugin displays a confirmation that an image file is correctly found. If not, you can enter the prefix and suffix values manually until the image file name is confirmed.



If you want to analyze images from the BD® Research Cloud, click the BD Logo icon.

If the folder and prefix are correct, a green check mark will appear next to the example file, indicating you are ready to proceed.



## Mapping the image files to events

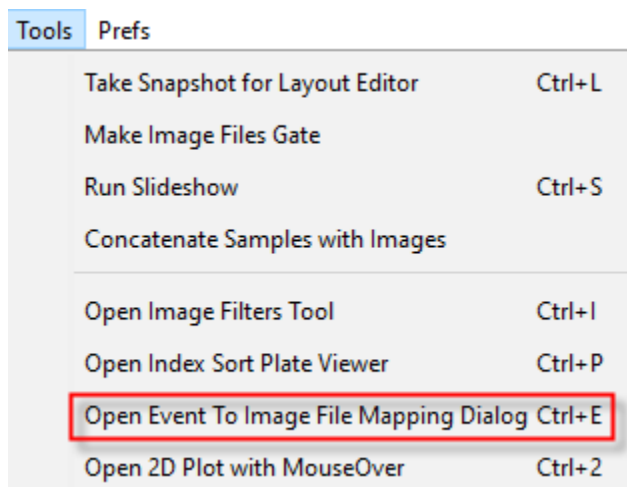
When the first plugin node is created for a sample's population, the new plugin node must determine the root folder where image files are located and be able to map an event number to an image file name. When first created, the plugin node will attempt to find the image folder and file name mapping based on the location of the FCS file. Typically the image files are organized into numerical subfolders (e.g. '00000000', '00010000', etc) where 10,000 images files are located in each subfolder.

**Note:** If your images are organized such that all image files are located in a single folder, make sure the **Use numerical subfolders** checkbox is not selected.

**Note:** The plugin nodes must be created under the root sample population for the mapping to be successful.

After mapping the image files to events, you can either confirm or change the root folder of image files and the name mapping information. If the file name prefix, suffix, and event number length are correct, the plugin displays a confirmation that an image file is correctly found. If not, you can enter the prefix and suffix values manually until the image file name is confirmed.

You can also perform the mapping procedure by navigating to the images browser menu bar and by clicking **Tools > Open Event To Image File Mapping Dialog**. See [Mapping the image files to events using the images browser menu \(page 19\)](#).



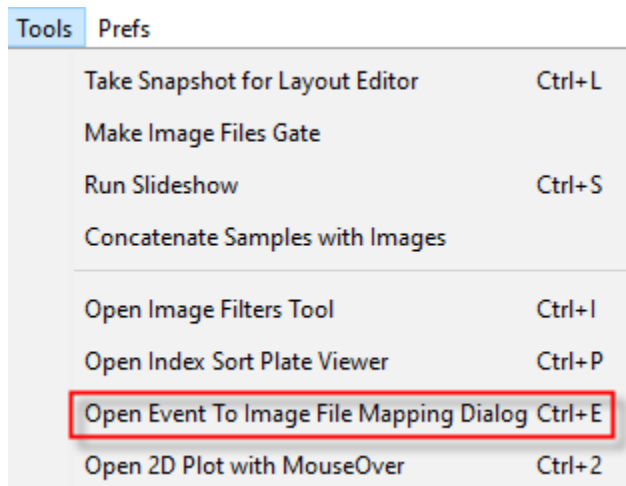
## Mapping the image files to events using the images browser menu

The first time you create a BD CellView™ Lens plugin node for a sample file, you will be automatically prompted to map the image files to events.

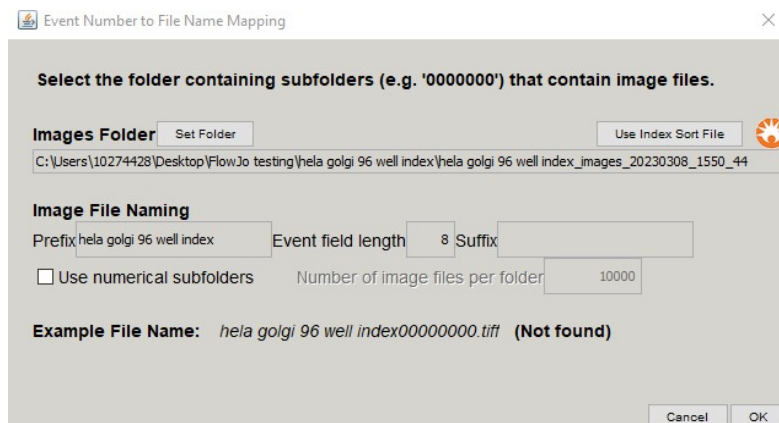
### Procedure

To map the image files to events using the images browser menu:

1. In the Images browser menu bar, go to **Tools > Open Event To Image File Mapping Dialog**.



The Event Number to File Mapping dialog displays.



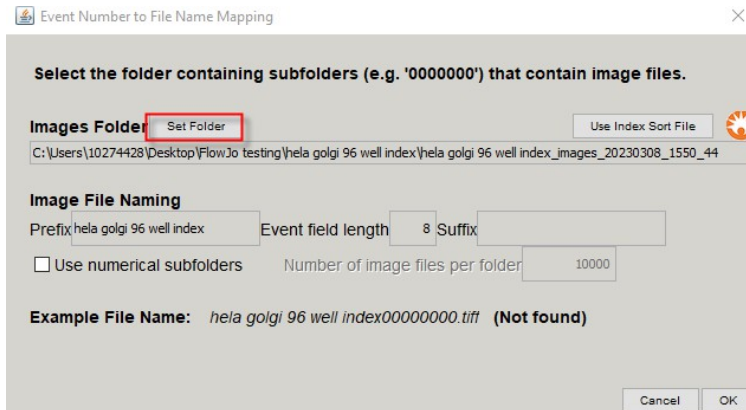
2. For non-index sort experiments, select or clear the **User numerical subfolders** checkbox depending on how the image files were extracted.

**Note:** If your images are organized after extraction such that all image files are located in a single folder, make sure the **Use numerical subfolders** checkbox is not selected.

For index sort experiments, you must clear the **User numerical subfolders** checkbox if it is selected.

## 3. Do one of the following:

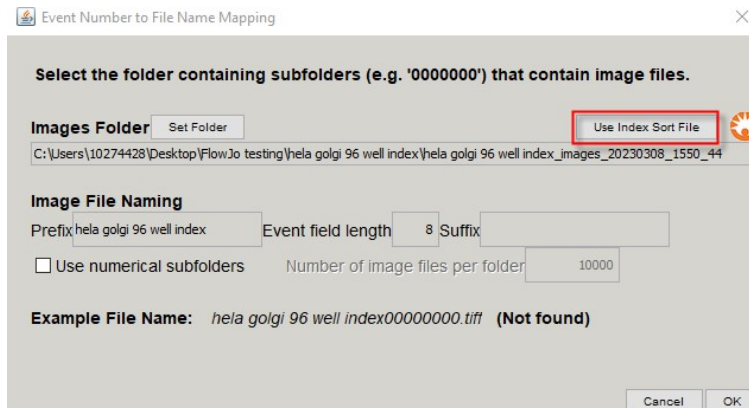
- a. If you want to analyze the data for a non-index sort experiment, click **Set Folder** and go to step 4.



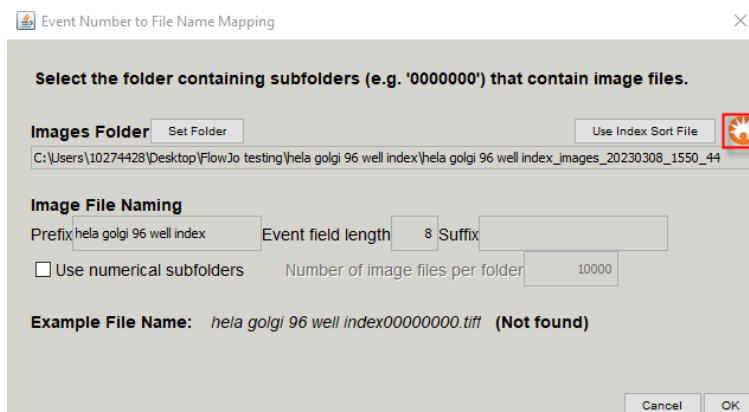
- b. If you want to analyze the data for an index sort experiment, click **Use Index Sort File** and go to step 5.

**Note:** Make sure that the **User numerical subfolders** checkbox is not selected.

**Note:** For the **Use Index Sort File** button to display in the Event Number to File Mapping dialog, the plugin node must be created under the root sample population.



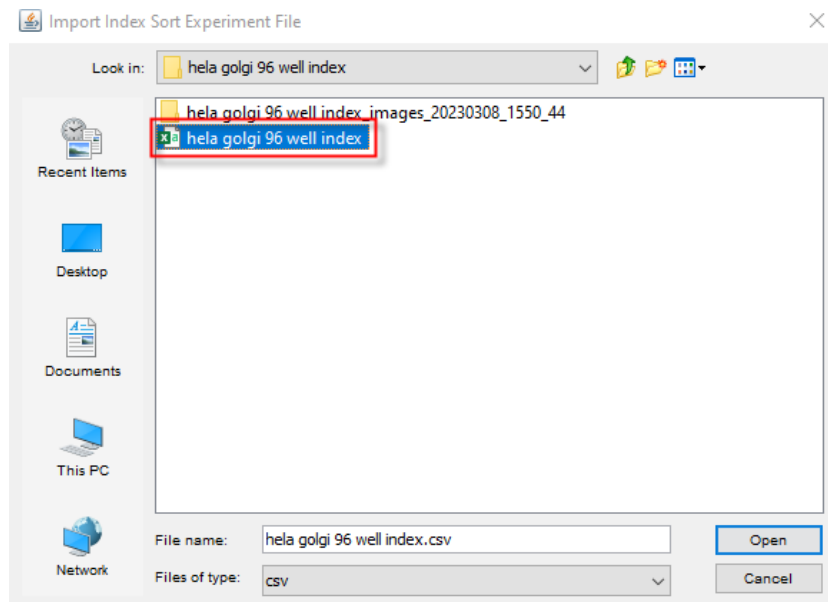
- c. If you want to analyze images from the BD® Research Cloud, click the BD Logo icon, select the images and go to step 6.



4. Browse the workstation to select the root folder where the extracted TIFF image files are located.

If your images are organized such that all image files are located in a single folder, make sure the **Use numerical subfolders** checkbox is not selected.

5. [Optional] To analyze the data for an index sort experiment, you must select the index sort CSV file and click **Open**.



The folder containing the index sort data is mapped now.

**Note:** To ensure successful mapping of the data for an index sort experiment, make sure that the plugin node is created under the root sample population.

6. Click **OK**.

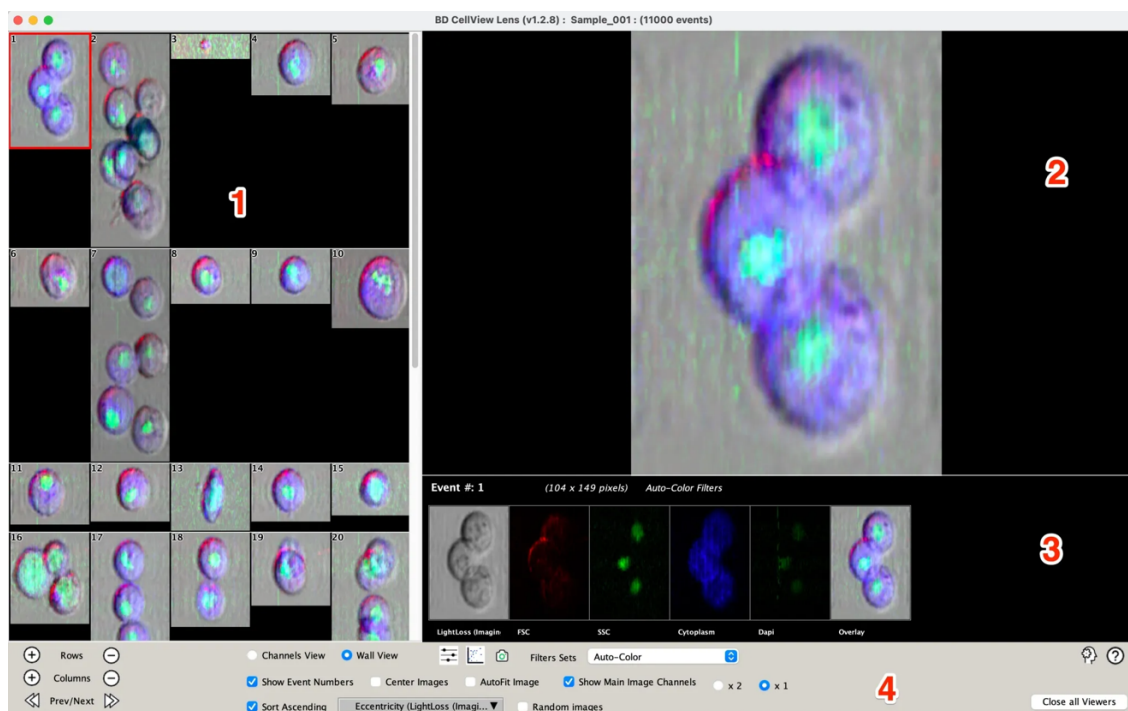
The BD CellView™ Lens plugin opens with the Images Browser displaying a wall of images. See [Using the images browser \(page 23\)](#).

After mapping the image files to events, you can either confirm or change the root folder of image files and the name mapping information. If the file name prefix, suffix, and event number length are correct, the plugin displays a confirmation that an image file is correctly found. If not, you can enter the prefix and suffix values manually until the image file name is confirmed.

## Using the images browser

Once the file name mapping is complete, the Images Browser opens to display a wall of images, and one of the single cell images highlighted in the high-resolution display panel. This panel shows the raw image in a higher resolution using linear interpolation of the pixels, as well as the image for each channel of the image data. You can click on an image in the image wall to change the highlighted single channel cell image, or click on one of the channels, of the particular image, located below the high-resolution display panel, to show that channel in the higher resolution display.

### Images Browser user interface



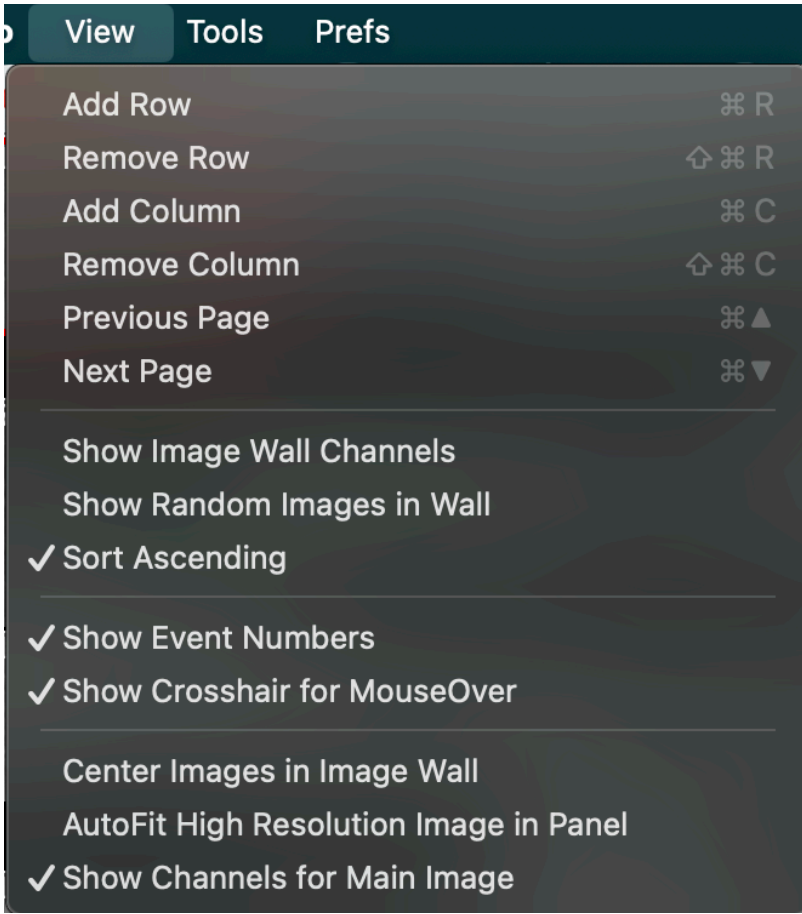
No.	Interface component	What you can do
1	Image wall	Displays FCS event images in wall view. The default view consists of ten rows and five columns.
2	High resolution display panel	Displays the raw image of a selected single image from the image wall in a higher resolution using linear interpolation of the pixels.
3	Channels of selected image	Displays the selected single image in a number of channels. The number of channels displayed depend on the number of image fluorescence channels you had selected for your experiment in BD FACSCorus™ and are interpreted from the extracted TIFF image files.
4	Image display controls	Displays the controls to customize the display of images in the image browser.  Allows you to view the extracted images for an index sort experiment by using the Index Sort Image Viewer window.  For more information, see <a href="#">Image display controls user interface (page 28)</a> .

Images browser menu bar

The images browser includes a menu that allows you to perform certain key functions.

**Note:** Depending on your workstation's Operating System (Mac® or Windows®), the location of the menu bar will vary. For a Mac® system, the menu bar displays at the top-left corner of your screen detached from the images browser. For Windows®, it displays attached to the images browser and at the top-left corner of the images browser window.

The images browser menu bar consists of the following tabs:

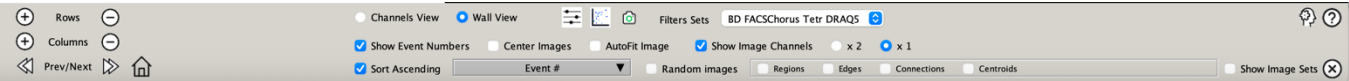
No.	Menu bar component	What you can do
1	View	<div></div> <p>Allows you to change the display of the images browser. Keyboard shortcuts are provided as well.</p> <p><b>Note:</b> The keyboard shortcuts vary depending on your workstation's Operating System.</p> <p>See <a href="#">Image display controls user interface (page 28)</a>.</p>

No.	Menu bar component	What you can do
2	Tools	<div data-bbox="613 237 1328 1024"> </div> <p>Allows you to access the following tools and the corresponding shortcuts:</p> <ul style="list-style-type: none"> <li>• Snapshot to Layout. See <a href="#">Using the Snapshot to layout functionality (page 47)</a>.</li> <li>• Make Image Files Gate - Allows you to create two subpopulations: one for events with image files, one for events that have no image files.</li> </ul> <p><b>Note:</b> The feature is only available when the plugin node is created on the root sample population.</p> <ul style="list-style-type: none"> <li>• Run Slideshow - Allows you to view through each image in an all open images browser. Click once to start the slideshow. Click it again for the slideshow to stop.</li> <li>• Concatenate Samples with Images - Allows you to create a new FCS file that is a concatenation of all sample files that also have a BD CellView™ Lens plugin plugin node somewhere in its gating hierarchy. The new FCS file will include all parameters for every file, and will remember how to map its image files to event numbers.</li> <li>• Image filter tool - See <a href="#">Image filters tool preview (page 42)</a>.</li> <li>• Index Sort Plate Viewer - See <a href="#">Using the Index Sort Plate Viewer (page 31)</a>.</li> <li>• Event to image file mapping dialog - See <a href="#">Mapping the image files to events (page 12)</a>.</li> <li>• Event to image plot (Open 2D Plot with MouseOver) - See <a href="#">Using the Event to image plot functionality (page 45)</a>.</li> </ul> <p><b>Note:</b> The keyboard shortcuts vary depending on your workstation's Operating System.</p>


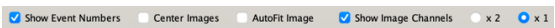


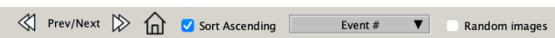

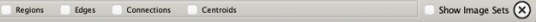
No.	Menu bar component	What you can do
3	Prefs	<div><div>Prefs</div><div><div>✓ Show Missing Image Files Warning</div><div>Show Placeholders for Missing Image Files</div><div>✓ Auto Accept File Mappings</div><div>Save Image Wall Prefs</div></div></div> <p>Allows you to change the default behavior of the images browser as follows:</p> <ul style="list-style-type: none"><li>• Show Missing Image Files Warning – If selected, a warning message displays in the image wall if there are enough missing image files that the wall could not be filled</li><li>• Show Placeholders for Missing Image Files – If selected, a blank rectangle is drawn for each image file that is missing (rather than skipping the file)</li><li>• Show BD Research Cloud GUI – If selected, display the BD® Research Cloud icon in the Images Browser to upload the image files for the current population, and display the same icon in the Event to Image File Mapping dialog to download image files from BD® Research Cloud. See <a href="#">Image display controls user interface (page 28)</a>.</li></ul>

Image display controls user interface



No.	Interface component	Description
1	<div><div><div><div>+</div><div>Rows</div><div>−</div></div><div><div>+</div><div>Columns</div><div>−</div></div></div></div>	Allows you to add or remove rows and columns to the image wall.
2	<div><div><div><div><input type="radio"/></div><div>Channels View</div></div><div><div><input checked="" type="radio"/></div><div>Wall View</div></div></div></div>	Channel view shows all of the imaging channels for each cell while Wall view shows the selected channel only for a larger number of cells (selected by default).

No.	Interface component	Description
3		<p>Allows you to access the following tools:</p> <ol style="list-style-type: none"> <li>1. Image filters – Allows you to add image processing filters for each channel of the image data. For more information, see <a href="#">Using the Image Filters tool (page 33)</a>.</li> <li>2. Event to image plot – Allows you to open a FlowJo™ graph window to enable mouse-over and cell location capabilities. For more information, see <a href="#">Using the Event to image plot functionality (page 45)</a>.</li> <li>3. Snapshot to Layout – Allows you to update FlowJo's layout editor with a snapshot image of the current view of the images browser for reports and publication. For more information, see <a href="#">Using the Snapshot to layout functionality (page 47)</a>.</li> <li>4. BD® Research Cloud – Allows you to upload all the image files for the currently viewed population to the BD® Research Cloud server. Upon clicking the icon, follow the prompts for the location (organization and project or workflow) to upload the files.</li> <li>5. Index Sort Plate Viewer – Allows you to open the index sort plate viewer dialog (that opens on top of the images browser) to view the extracted images for an index sort experiment in the images browser. For more information, see <a href="#">Using the Index Sort Plate Viewer (page 31)</a>.</li> </ol>
4		<p>By using the functions listed below, you can:</p> <ul style="list-style-type: none"> <li>• Show Event Numbers – Show the event numbers for each image on the image wall (selected by default).</li> <li>• Center Images – Center the images on the image wall.</li> <li>• Autofit Image – Fit the high resolution image display to the size of the main window (selected by default).</li> <li>• Show Image Channels – Show or hide the channels of the selected image in the high resolution interpolated display, and if selected, set its size to either 2x or 1x.</li> </ul>

No.	Interface component	Description
5	 <p>The screenshot shows a toolbar with the following elements from left to right: a double left arrow, the text 'Prev/Next', a double right arrow, a house icon, a checked checkbox labeled 'Sort Ascending', a dropdown menu labeled 'Event #' with a downward arrow, and an unchecked checkbox labeled 'Random images'.</p>	<p>By using the functions listed below, you can:</p> <ul style="list-style-type: none"> <li>• Prev/Next – Move to next or previous page of images in the image wall.</li> <li>• Sort Ascending – Sort images in ascending order of event numbers (selected by default).</li> <li>• Drop-down menu – Sort images based on specific sample parameters such as Event # (default option) and other comparable parameters depending on the experiment.</li> <li>• Random images – Display images in a random order.</li> <li>• Home – Reset image wall</li> </ul>
6	 <p>The screenshot shows two circular icons side-by-side. The first icon contains a gear inside a head silhouette, representing machine learning. The second icon contains a question mark, representing instructions or help.</p>	<ul style="list-style-type: none"> <li>• Open the machine learning tool kit</li> <li>• Show instruction on how to use the CellView Lens Image Browser window</li> </ul>
7	 <p>The screenshot shows a toolbar with the following elements from left to right: four unchecked checkboxes labeled 'Regions', 'Edges', 'Connections', and 'Centroids', followed by a checked checkbox labeled 'Show Image Sets' with a close button (X) to its right.</p>	<ul style="list-style-type: none"> <li>• Regions – display the region mask is the image filter “remove background is enable”</li> <li>• Edge – Draw the outer edge pixels of each region</li> <li>• Connections – Draw a line between each region</li> <li>• Centroids – Show the center of each region</li> <li>• Show current Image sets</li> </ul>

## Using the Index Sort Plate Viewer

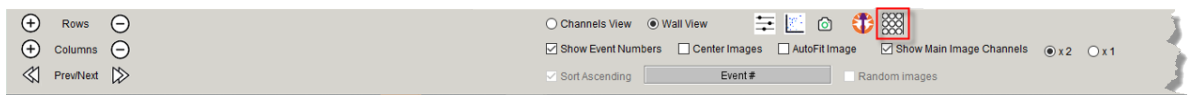
For analyzing index sort experiment data, use the Index Sort Plate Viewer to display a wall of images on the images browser with one of the single cell images highlighted in the high resolution display panel.

The Index Sort Plate Viewer allows you to control the set of images displayed in the image browser by selecting individual wells of the plate, or by selecting sorted populations defined by the wells.

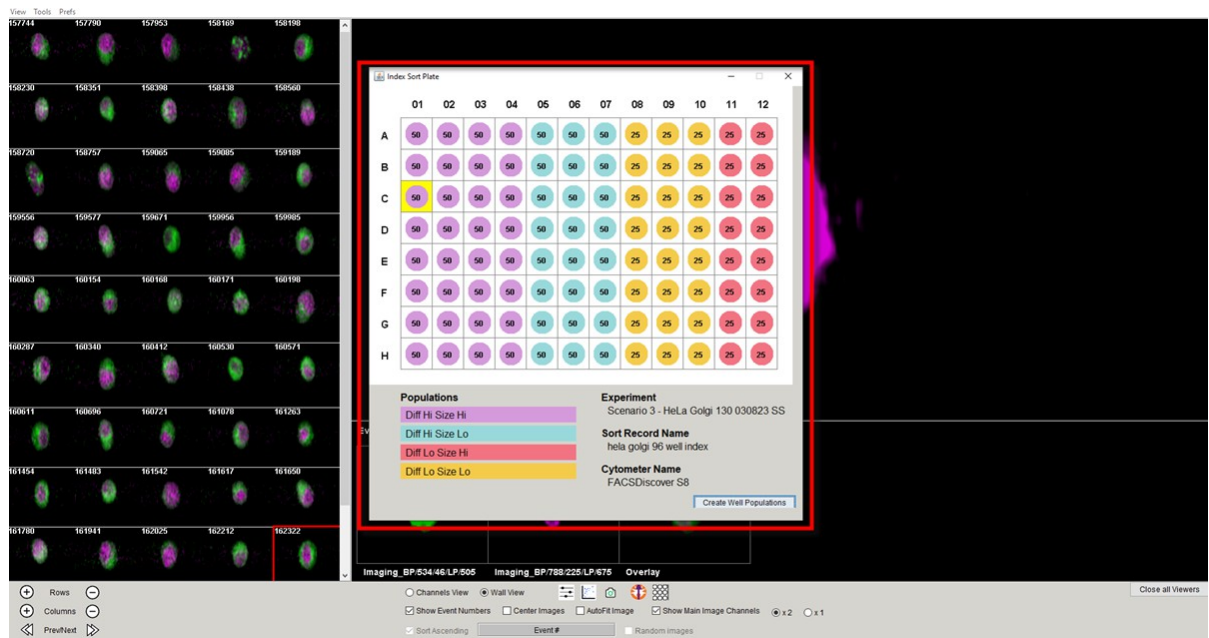
You can click on a single well to display all the images for that particular well, or you can click on a Population name to display the images for events in that population.

To view the Index Sort Plate Viewer:

1. From the image controls display panel, click the Index Sort Plate Viewer icon.

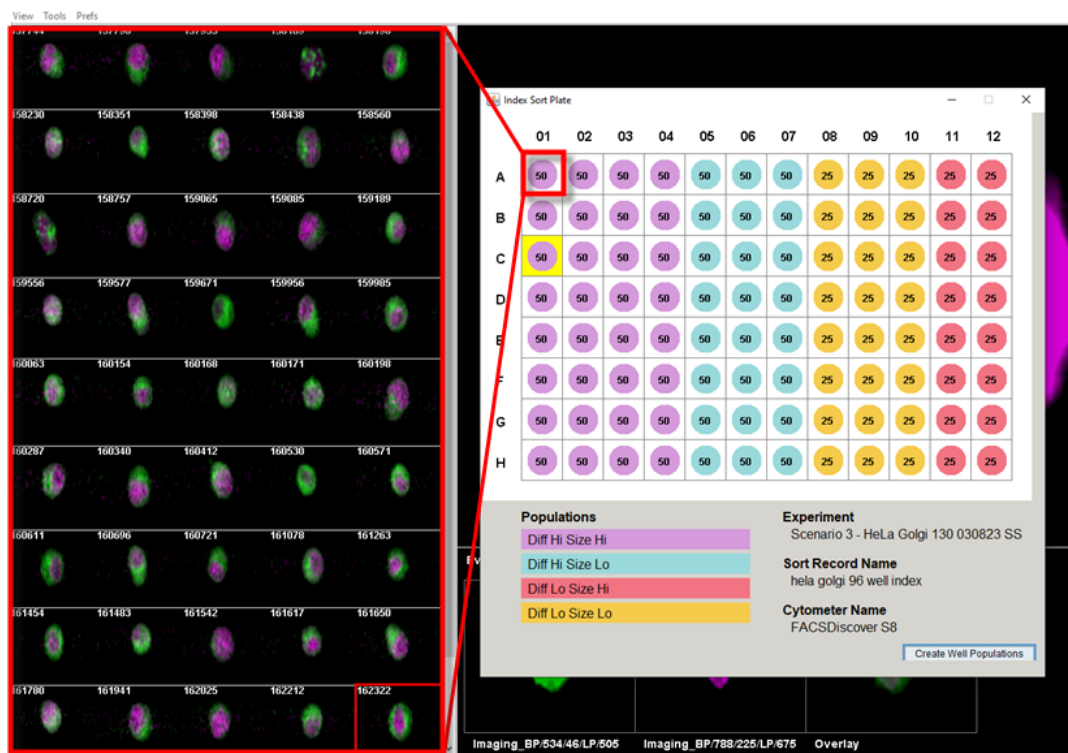


The Index Sort Plate Viewer dialog displays on top of the images browser.



After opening the Index Sort Plate Viewer dialog, a wall of images display on the images browser.

The number of images on display depends on the number of cells index sorted into that well.

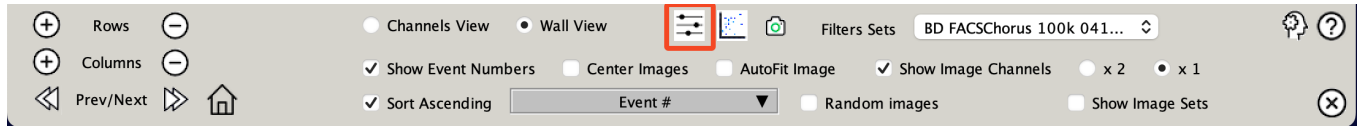


- Click on a single well to display all the images for that particular well, or you can click on a Population name to display the images for events in that population.

## Using the Image Filters tool

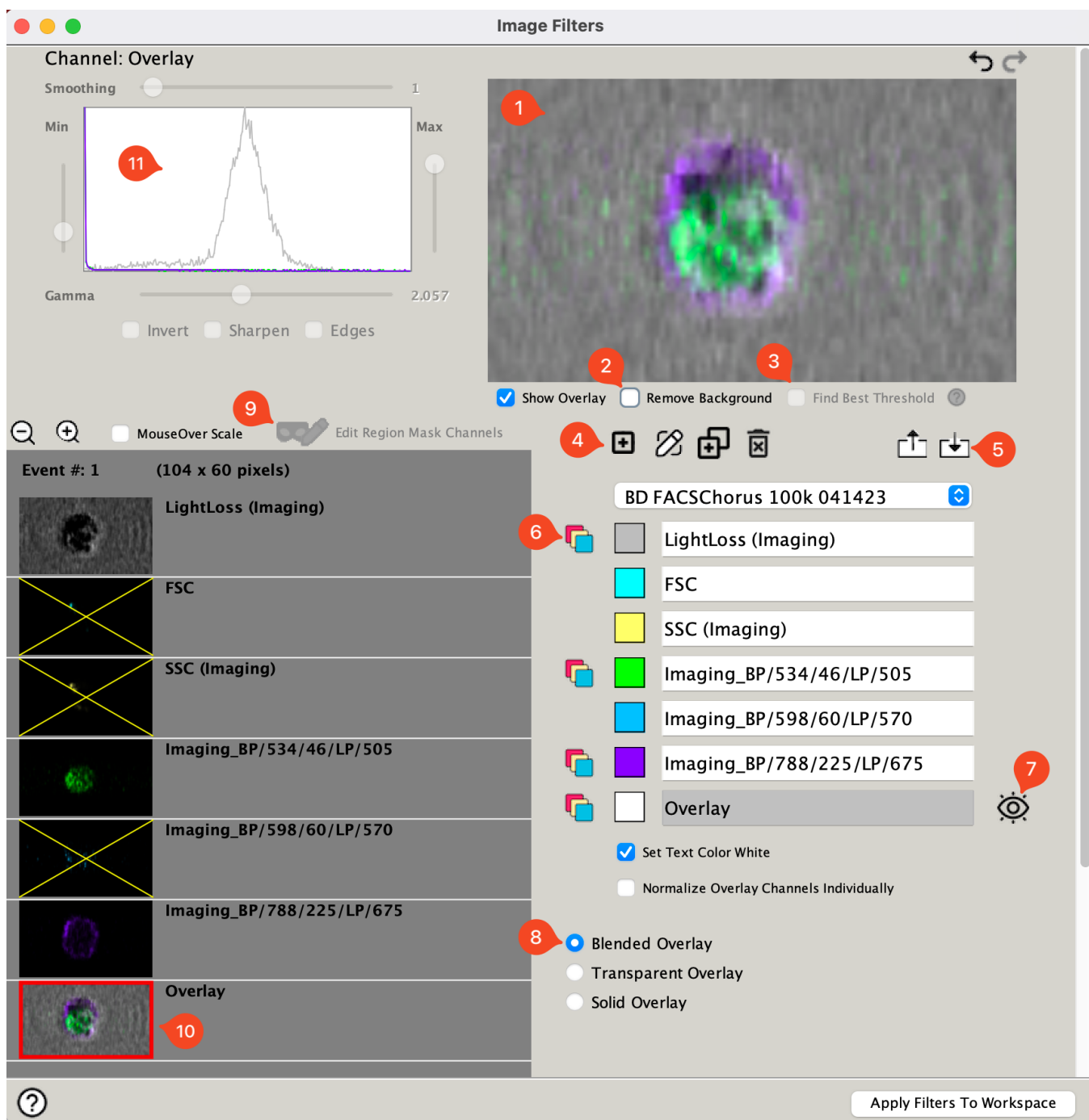
The image filters tool allows you to refine the display of images in each channel by adding different color options, image processing filters, and by controlling the composition of the Overlay channel.

To open the image filters tool, click the **Image Filters tool** button in the images browser display controls panel. The image filters tool displays as a separate window.



You can also open the image filters tool by double-clicking an image on the image wall or by navigating to the images browser menu bar and by clicking **Tools > Open Image Filter Tool**. See [Images browser menu bar \(page 25\)](#).

## Image filters tool user interface

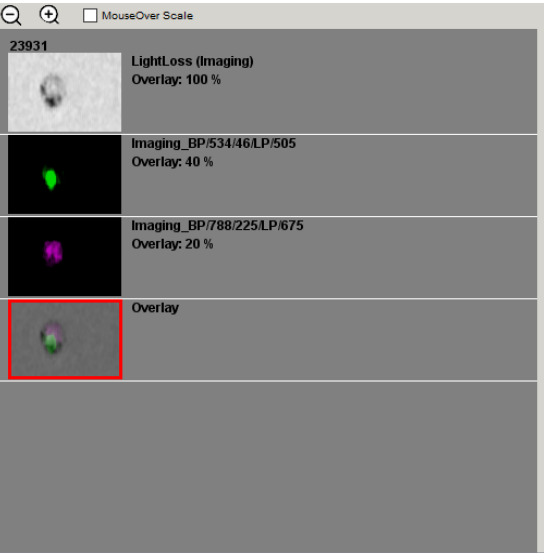


**Note:** Hovering over different sections of the Image Filter tool window displays a tool tip with a description for the feature.



No.	Interface component	What you can do
1	Image Preview	<p>For the selected channel, a high resolution interpolated image of a cell event image representing the selected channel (or the overlay channel) displays.</p> <p>The high resolution image is updated as you adjust the sliders and the filters in the channel adjustments panel.</p> <p>Click the <b>Show Overlay</b> checkbox to apply the overlay channel while adjusting the current channel.</p> <p><b>Note:</b> The Show Overlay checkbox is not selected by default.</p>
2	Remove Background	Using the Region Mask Channel(s) (see No. 9), contiguous edges will be identified and any signal outside that region will be removed as background.
3	Find Best Threshold	If Remove Background is selected checking this box will use an algorithm to identify the optimal boundary.
4	Create, rename, duplicate, or delete filter set	Organizational tools for filter sets
5	Save or load filter sets	Particularly well crafted filter sets can be saved as an external file and imported into other workspaces.
6	Channel controls	Clicking to the left of the color box will make a channel active and turn on the blue/yellow/box icon. Active channels will be used to create the overlay image and can be viewed in the image wall. Clicking on the colored box will change the highlight color of the channel.
7	Displayed channel indicator	The eye icon next to a channel displays which channel will be displayed in the image wall (if you are in channel view, all active channels will be displayed). Clicking to the right of any channel will move the eye to that channel. Only one channel can be the displayed channel at a time.
8	Overlay options	Select Blended, Transparent, or Solid to control how the colors are mixed from multiple channels.
9	Edit Region Mask Channels	<p>If Remove Background is checked on clicking this button will show a mask icon for each channel used to determine the image mask, meaning the edge of the identified cell(s) that will be kept after background removal. Control-clicking on any channel will toggle it on/off.</p> <p>If edit region is selected, the Show Overlay button becomes the Show Region Mask button, which allows you to display the area identified as 'cell' vs. noise that will be removed.</p>

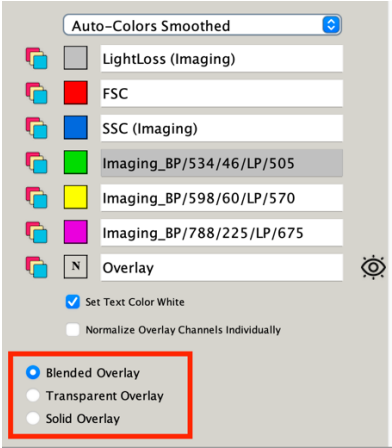
11	Channel adjustments	<p>Allows you to adjust the following channel filters by using the respective slider bars:</p> <ul style="list-style-type: none"><li>• Smoothing</li><li>• Minimum (Min)</li><li>• Maximum (Max)</li><li>• Gamma</li></ul> <p>Also allows you add or remove the following filters by selecting or clearing the corresponding checkboxes:</p> <ul style="list-style-type: none"><li>• Invert</li><li>• Sharpen</li><li>• Edges</li></ul> <p>For the selected channel, the image filter tool displays a histogram showing the distribution of pixel values in an image for that channel.</p> <p>The histogram is updated as you adjust the sliders and the filters above.</p> <p>You can also undo and redo all channel adjustments by using the respective icons.</p>
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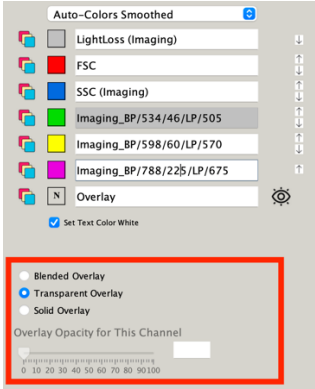

No.	Interface component	What you can do
10	<p>Image filters panel - Channel images</p> 	<ul style="list-style-type: none"> <li>• The red box indicates which channel is being edited currently. Double clicking on a channel toggles it on/off.</li> <li>• Use the positive and negative magnifying glass icons to increase or decrease the size of the cell event images.</li> <li>• Select the MouseOver Scale check box, the size that the image is adjusted to here will also be displayed in the 2D plot window that opens from the Event to plot button in the Images Browser.</li> <li>• View individual cell event images across all channels</li> </ul> <p>For more information, see <a href="#">Image filters tool - channel images user interface (page 39)</a></p>
11	Channel adjustments	<p>Allows you to adjust the following channel filters by using the respective slider bars:</p> <ul style="list-style-type: none"> <li>• Smoothing</li> <li>• Minimum (Min)</li> <li>• Maximum (Max)</li> <li>• Gamma</li> </ul> <p>Also allows you add or remove the following filters by selecting or clearing the corresponding checkboxes:</p> <ul style="list-style-type: none"> <li>• Invert</li> <li>• Sharpen</li> <li>• Edges</li> </ul> <p>For the selected channel, the image filter tool displays a histogram showing the distribution of pixel values in an image for that channel.</p> <p>The histogram is updated as you adjust the sliders and the filters above.</p> <p>You can also undo and redo all channel adjustments by using the respective icons.</p>

## Using Overlays

In addition to the individual images for each channel, the BD CellView™ Lens plugin creates an additional Overlay channel that combines the enabled channel images to make a single channel image. The images are overlaid in the order shown in the channel list, which can be rearranged using the up and down arrow buttons located next to the channel names, which appears when Transparent or Solid overlays are selected. To visualize the overlays before implementing the overlay settings, you can use the image filters tool preview feature. See [Image filters tool preview \(page 42\)](#).

When composing the Overlay channel, you can choose the following options for how the layers are constructed:

Overlay	What you can do
Blended	<div><p>In a <b>blended overlay</b>, the color of an individual pixel in an image is determined by adding the color of that pixel from each enabled channel. For example, if the color for the pixel in channel 1 is red and the color in channel 2 is green, the resulting pixel is colored yellow. Note: The order of the channels does not affect the final pixel color. A blended overlay implements a technique called ‘additive blending’, where each pixel in the overlay is constructed by adding the weighted pixel RGB values for each channel color.</p></div>

Overlay	What you can do
Transparent	<p>A transparent overlay is constructed by stacking the channel images on top of each other, where the amount shown for a specific channel is determined by its opacity setting, between 0 and 100%.</p> <p>If using transparent layers, you can set the opacity for each channel using the slider bar. You should adjust the opacity for each channel as it is overlaid, so that each channel shows through in the Overlay channel. For each channel as the opacity number is decreased or increased, the opacity increases or decreases, and less and more of the other channels will be seen through the current channel in the overlay, respectively. To help ensure that each layer is displayed and not blocked by a following layer, it is recommended that the first layer is set to 100%, and subsequent layers set 70% or lower, and a layer's opacity is not greater than the one before (for example: 100%, 70%, 50%, 30%, etc.)</p> 
Solid	<p>Use the slider bar to set the Pixel Overlay Threshold to determine whether a pixel from the channel image is included in the overlay image or not. The intensity of each pixel in the image is normalized to a percentage of the intensity range, and is compared to the threshold value.</p> <p>For example, you can specify that the top %50 brightest pixels for Channel X are used when constructing the Overlay.</p> 

## Image filters tool preview

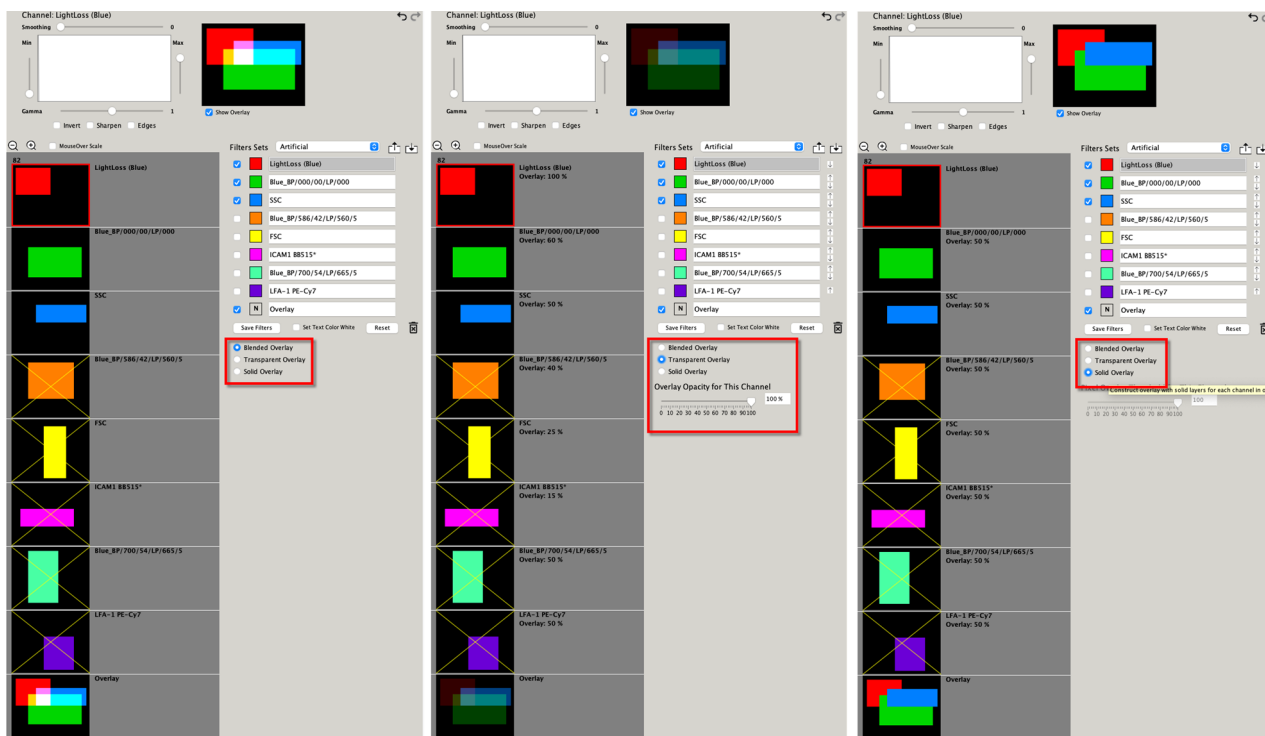
**Normalization** is required to scale the range of color per channel. By default the normalization is global across all channels. Just above the Overlay selections there is a checkbox that allows you to normalize each channel individually so that you might emphasize colors that are not brightly expressed, but an increase in signal is significant.

To help visualize the different types of overlays and how they are composed, the Image Filters Tool can be opened to display a preview of artificial channel images composed of overlapping rectangles for each channel.

To open the image filters preview tool, press the [Shift] key and double-click on an image in the image wall.

Using the image filters preview tool, you can select or clear channels and set their colors to illustrate how pixel colors are blended, how transparent layers are overlaid, or how solid layers are composed.

The following images are examples of the image filters preview tool with a single channel with the same color selections and with differing overlay variations:



## Region masks

Region masks are used primarily to help identify background and remove it. Edges are detected using any parameter or combination of parameters except for light loss. The edge detection tool identifies a continuous perimeter of pixels with color vs. uncolored. Because the region is contiguous two cells touching will be identified as one region. However, more than one region can be identified per event. Multiple regions will be identified via differing color.

Regions are created by checking the Remove Background box, item No. 2 in Figure 1. To remove the background the cell edges must be identified and regions are created. The Find Best Threshold checkbox will automatically detect the regions on a per cell basis. Leaving that off allows the user to use the Edit Region Mask Channels button, item No. 2 in Figure 1, to select which channels will be used for detecting regions and the channel editing sliders to adjust sensitivity manually.

## Saving filters sets

Once you have added the image filters and composed the Overlay channel, you can save these settings with a name as a filters set to your workspace by clicking on the Save Filters button. You can overwrite existing filters sets, or create a new filters set by adjusting the channel and filter settings and entering a new name.

## Restoring filters sets

To restore the tool to a previously saved filters set, choose an existing filters set from the drop-down menu.

## Resetting filters sets

If you want to start by creating a new filter set, use the Reset button to remove all filters and colors from all channels.

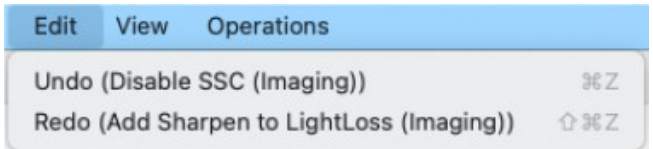
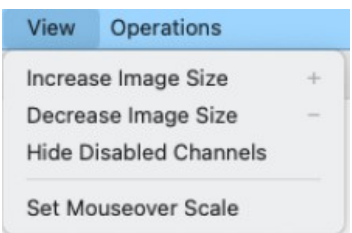
**Note:** When the first BD\_CellView\_Lens node is created for a sample with image files, the plugin will automatically create a filters set named 'BD FACSCorus' to use as a starting point for your custom Filters Set. This filters set is the same as the one setup on the instrument at the time of acquisition.

## Image filters menu bar

The image filters window includes a menu that allows you to perform certain key functions.

**Note:** Depending on your workstation's Operating System (Mac® or Windows®), the location of the menu bar will vary. For a Mac® system, the menu bar displays at the top-left corner of your screen detached from the images browser. For Windows®, it displays attached to the images browser and at the top-left corner of the images browser window.

The images browser menu bar consists of the following items:

No.	Menu bar component	What you can do
1	Edit	 <p>Allows you to undo or redo the last action you performed on the image filters window.</p> <p>The exact name of the last actions you performed is displayed next to Undo and Redo depending on the order in which you performed them.</p>
2	View	 <p>Allows you to:</p> <ul style="list-style-type: none"> <li>• Increase or decrease the image size by clicking the buttons or by clicking the corresponding shortcut keys (+ or -).</li> <li>• Hide the disabled (not selected) channels</li> <li>• Set the mouse-over scale for the plots</li> </ul>

No.	Menu bar component	What you can do
3	Operations	<div><div><div>Operations</div><div><div>New Image Filters⌘ N</div><div>Rename Image Filters⌘ R</div><div>Duplicate Image Filters⌘ D</div><div>Load Filters From File⌘ L</div><div>Save Filters To File⌘ S</div><div>✓ Set Text Color White</div><div>Apply Filters⌘ A</div></div></div></div> <p>Allows you to:</p> <ul style="list-style-type: none"><li>• New Image Filters</li><li>• Rename Image Filters</li><li>• Duplicate Image Filters</li><li>• Load filter sets from file</li><li>• Save filter sets to file</li><li>• Set text color to white (selected by default)</li><li>• Apply filter sets</li></ul>

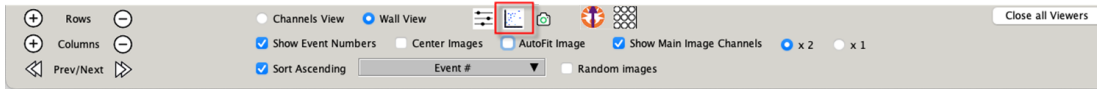


## Using the Event to image plot functionality

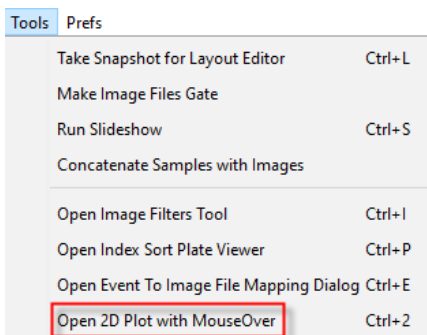
While viewing the images for a cell population in the images browser, you can open a FlowJo™ graph window for that population to show a 2 dimensional (2D) plot, where individual cell images are displayed as you move the mouse over the dots in the graph area.

**Note:** For an index sort experiment, only index sort cell images are displayed.

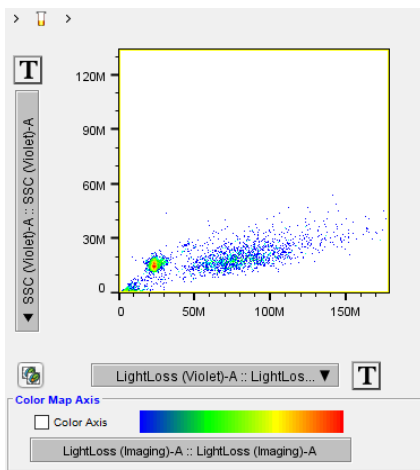
Click the **Event to image plot** button to use a graph window that is already open for the population selected in FlowJo™. If a graph window is not already open, a new graph window opens.



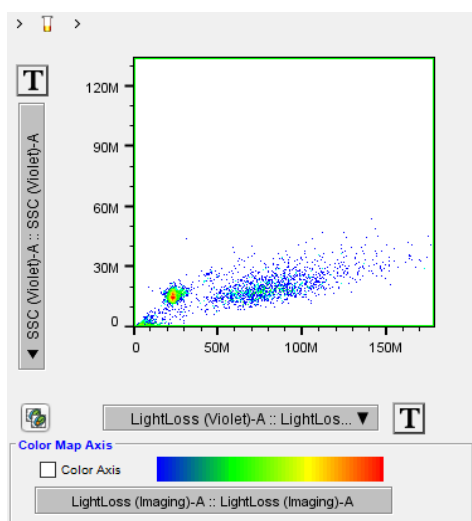
You can also use this feature by navigating to the images browser menu bar and by clicking **Tools > Open 2D Plot with MouseOver**. See [Images browser menu bar \(page 25\)](#).



As the plugin retrieves information for the image plot, graph window displays a yellow border.



On moving the mouse into the graph area, the border turns green indicating the mouse-over feature is working.



You can change the x or y axis parameters and the plugin updates to continue with the mouse-over feature.

In the image above, note that only one image is shown, although there may be many cells at that location in the plot. In addition to showing a cell image using your mouse, you can also show the location of any cell that is displayed in the image wall of the Images Browser by clicking on the image in the image wall.

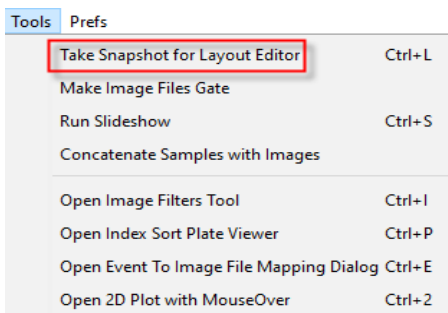
## Using the Snapshot to layout functionality

If you want to take pictures of the cell images that are displaying in the Images Browser for use in a report for publication, you can use the Snapshot to layout button in the Image display control panel.

Click the **Snapshot to layout** button to capture the cell images that are visible in the Wall View or Channels View, and automatically places them in FlowJo™'s layout editor.



You can also use this feature by navigating to the images browser menu bar and by clicking **Tools > Take Snapshot for Layout Editor**. See [Images browser menu bar](#) (page 25).



The plugin will create a new layout with the population's name or use an existing layout with that name. If you want to capture larger images from the image wall (consisting of multiple images), select an image on the

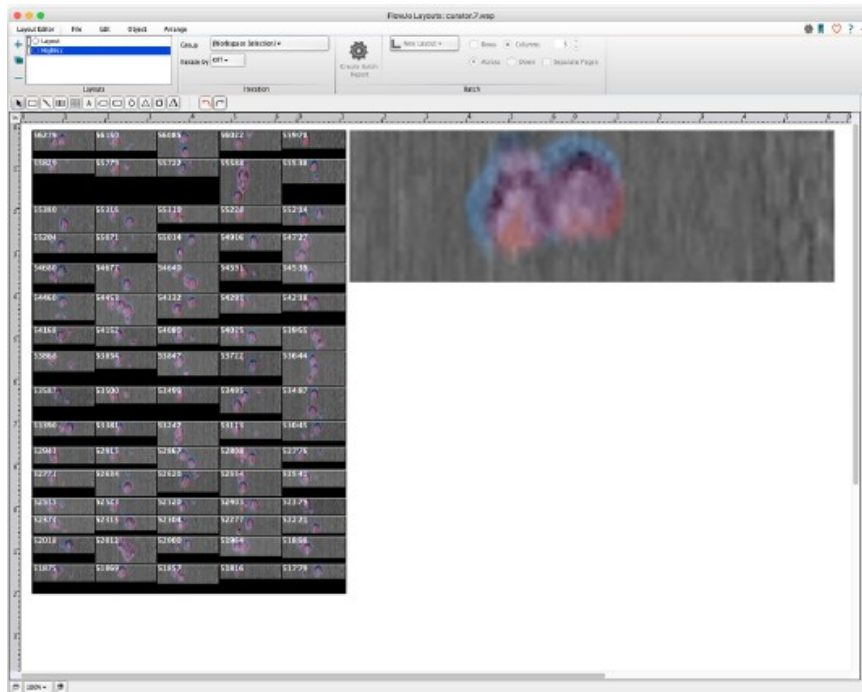


image wall and then press the [Shift] key while clicking the **Snapshot to layout** button to include the other images on the image wall as necessary.

## Bd CellView Lens Image Sets

Image sets are a means to curate interesting populations visually within the CellView Lens tool.

If there is a characteristic pattern within your cell images that you would like to study further that does not correlate with an easily identifiable threshold on one or few parameters, image sets can be used to select those cells in a straight-forward matter.

### Creating an Image Set

Within the CellView Lens plugin, there is a checkbox for Show Image Sets:

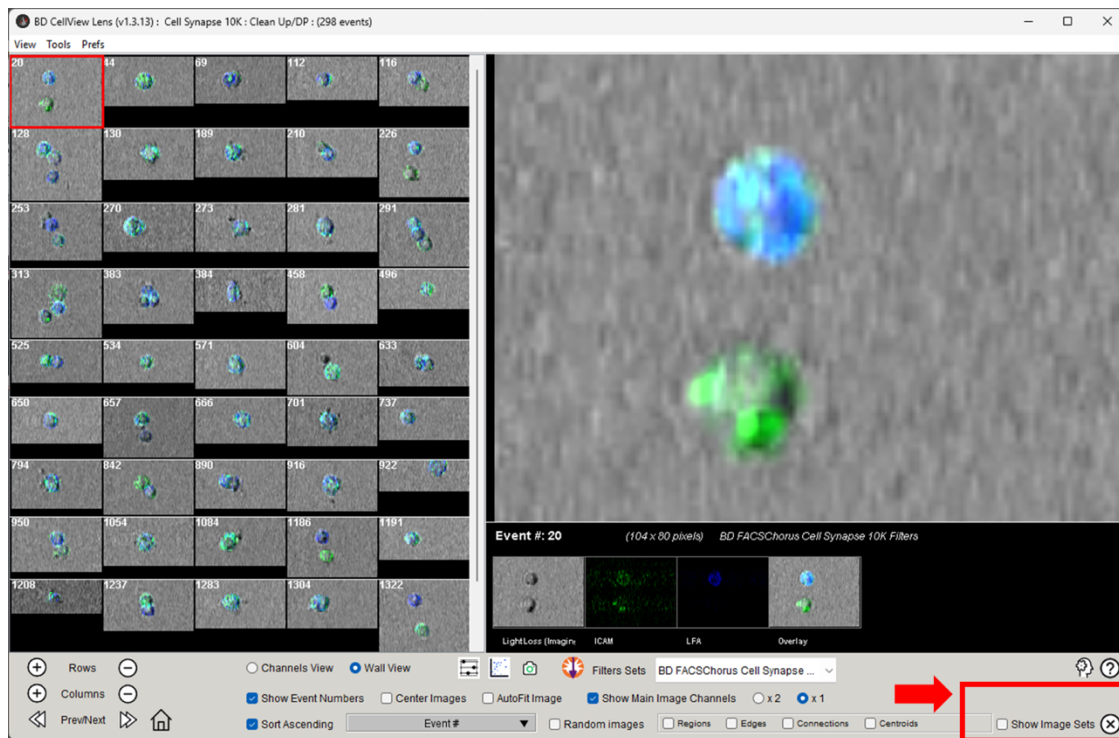


Figure 1: Show Image Sets

Checking this box opens a window that replaces the individual cell highlight window with a gallery that you can populate by clicking on images from the image wall. To modify an image set, you must click the lock icon to unlock it for editing. Then, simply click on images in the wall to add them. In Figure 2, Show Image Sets is checked, the lock icon is unlocked, and images that include both a cell expressing the green fluorescent probe indicating ICAM, and one expressing the blue fluorescent tag indicating LFA have been selected and added to the image wall. Selected cells are highlighted with the green border. The image wall on the left can be scrolled to see and select more cells within the chosen population. Selected cells appear on the right.

### Delete an Image from an Image Set

You can delete an image from the image wall by selecting it and on a PC pressing the Delete Key on your Keyboard, and on a Mac holding down the *fn* key while clicking Delete on your keyboard.

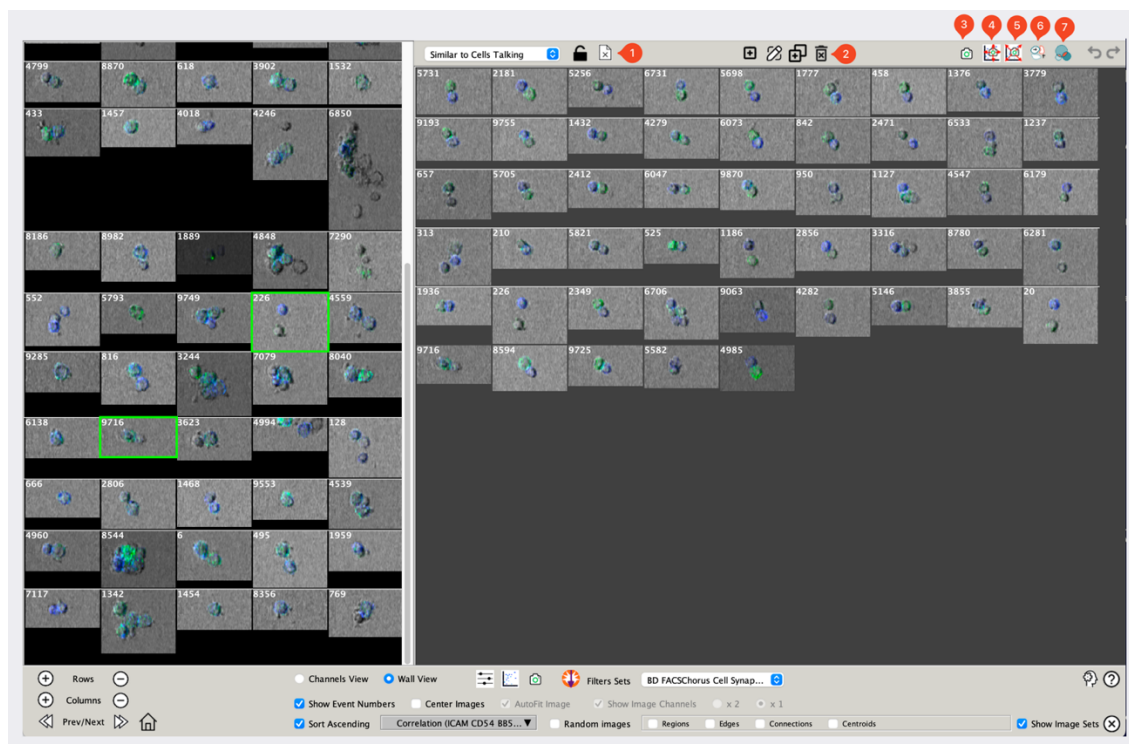


Figure 2: Image Set Controls

No.	Tool
1	Image set name, unlock, and remove all images from the set
2	Make a new image set, edit the name, duplicate a set, and delete the entire set
3	Add the images in the current image set to the layout editor
4	Create a 2D plot with image callouts, aligned around edges of the plot
5	Create a 2D plot with image callouts, aligned in quadrants
6	Find images similar to this set using selected measured parameters and a K-nearest neighbors algorithm
7	Create a population of these cells in the FlowJo hierarchy

## Image Set Tools

Figure 2 calls out a set of tools that can be applied to image sets. Call out 2 points to the tools for creating new image sets, duplicating or deleting them, and renaming them. You can make as many image sets as you need and select a set to view from the dropdown menu identified in call out 1. Also in call out 1 is the unlock icon. To prevent accidental modification, image sets are locked by default. To modify the selected image set, click the unlock icon.

Call outs 3-5 are a variety of ways to represent an image set in the layout editor. Call out 3 will create a graphic of the wall of images in the image set. *If a 2D plot has been created for these images*, as shown in Figure 3 (click the icon, mouse over the plot that appears until the edges turn green, select parameters for viewing on the axis), call out 4 will create a graph in the layout editor of the selected 2D plot with the images in the set's position indicated, and the images placed along the border of the plot. Call out 5 will do the same, but arrange the images in quadrants. Examples of these plots are shown in Figure 4.

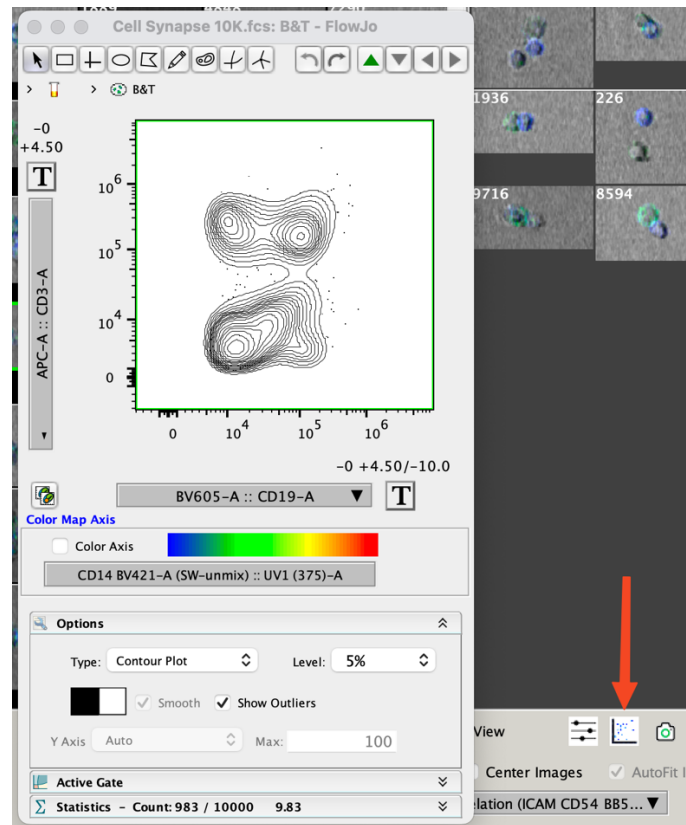


Figure 3 Initiating a 2D plot for mouseover of images

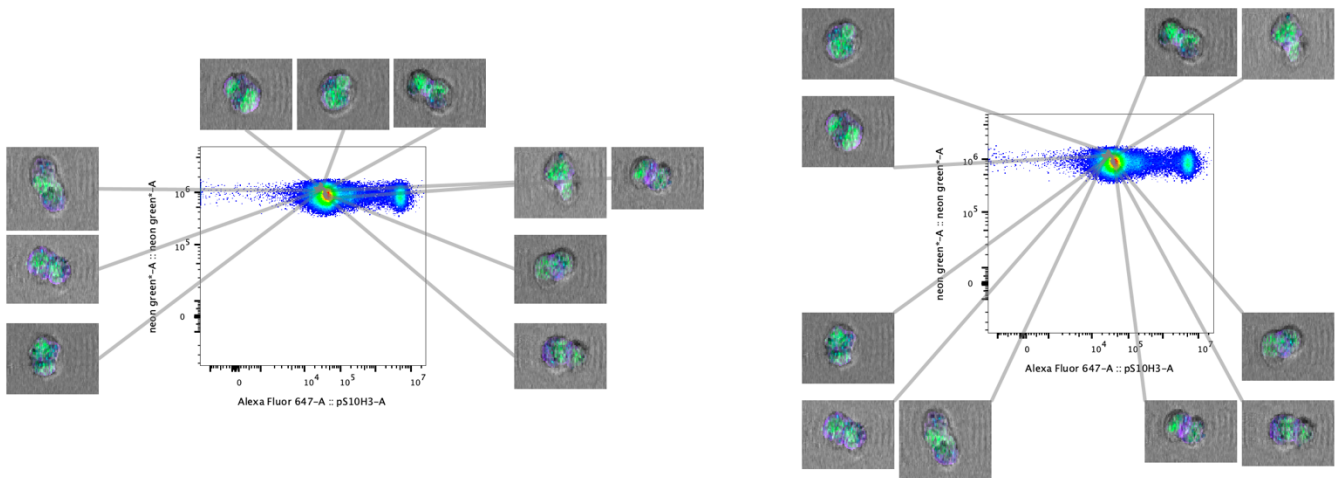
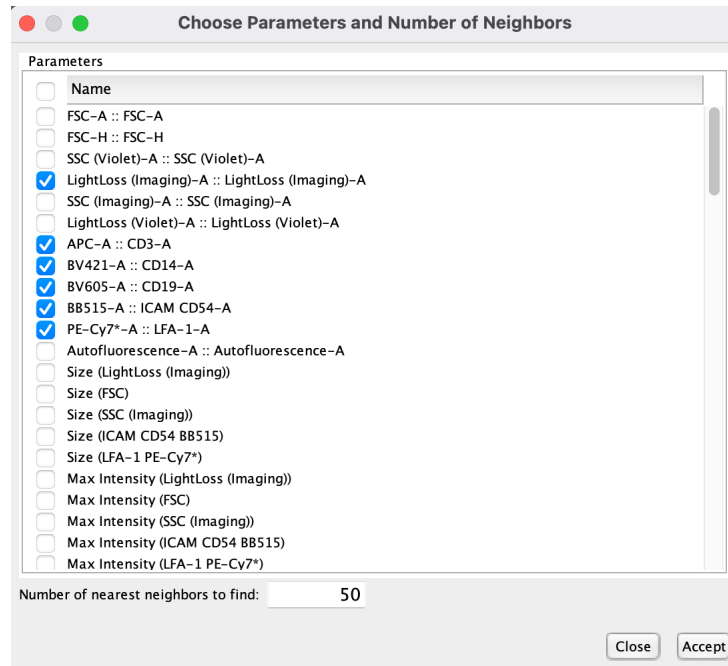


Figure 4 Image sets shown in the layout editor with a 2D plot for reference. The left plot shows images arranged along the edges of the 2D plot, the right plot shows images arranged by quadrant.

Call out 6 in Figure 2 is the button for finding similar images using a Nearest Neighbors Search tool, operating on the numeric parameters, both measured and derived. On clicking this button the window shown in Figure 5 will appear. Select any parameters that might contain information that may be useful for determining if a cell is similar to the set of cells selected in your image set. For example, if you wanted to find round cells that were CD3+ CD19-, you would select the fluorescent markers for CD3 and CD19, and the eccentricity of side scatter. The algorithm will rank all of the events in the selected population from most similar to least and return the top 50 (by default). You may change the number of nearest neighbors to find if you want more. Click Accept that the algorithm will run, producing a new image set called 'Similar to...' whatever the original hand-curated set was called.



*Figure 5 Parameter selection for finding similar cells*

Once you have identified a set of interesting cells you can click call out 7 to create a population in the workspace hierarchy from this image set. You will get these events isolated, as well as a not-gate of the rest of the events in the population. Further work can be done on this population, like any other population in FlowJo, including using Hyperfinder to recapitulate this set of cells using traditional gating to see which parameters distinguish these cells.



## BD CellView Lens Nearest Neighbor Search

The Nearest Neighbor Search in the CellView Lens plugin identifies similar cells based on numeric parameters and can create a population of those cells.

If you have used [image sets](#) to visually identify cells of interest, you may want to extend this set by including the most similar cells based on their measured or derived parameters. To use this tool, click the Find Similar Images button as shown in Figure 1.

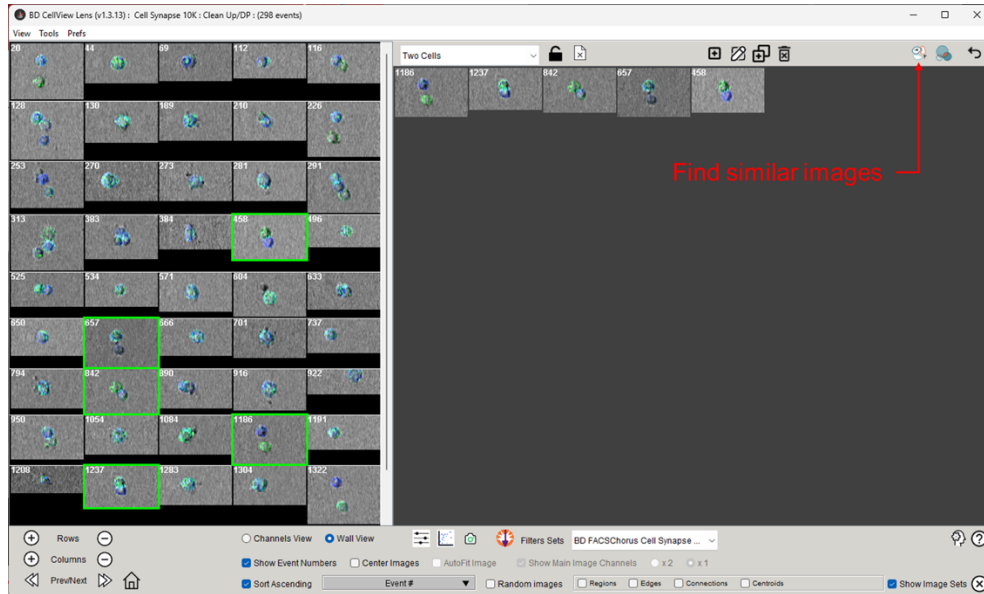


Figure 1: Find similar images

The dialogue box shown in figure 2 will appear, allowing you to select which parameters to use in identifying cells via K-nearest neighbors. Essentially, the algorithm will calculate the difference in parameter intensities between your selected cells and every other cell in the current population, using every parameter you choose, and then rank them from smallest difference to largest difference. The most similar cells are the 'nearest neighbors'. You can enter a number of nearest neighbors to include in you image set via the text entry box at the bottom of the dialogue.



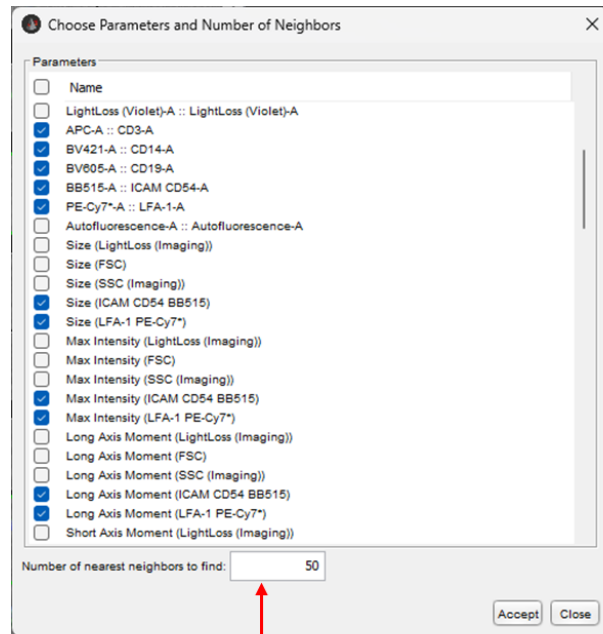


Figure 2: Nearest Neighbors dialogue

Importantly, K-nearest neighbors (KNN) does not automatically determine a threshold for what constitutes 'nearest'. The algorithm simply returns the most similar images so you may want to start with a smaller number of similar images, or curate the set afterwards. Any image that you do not want in the image set can be removed by clicking on it and pressing the delete key on your keyboard.

## Parameter Selection

As visible in Figure 2, both fluorescent parameters and image derived parameters can be used to identify nearest neighbors. Keep in mind that this tool will only use the numeric value of each of the image derived parameters in doing this calculation, so a limitation of this tool is that it will not use specific spatial information in determining similarity. One big long cell will have a very similar eccentricity measure compared to two smaller cells abutting. Thus, providing the algorithm more parameters to work with improves its ability to identify similarity.

The algorithm attempts to select all of the fluorescent parameters by default. This will likely include the raw image channels, ImgB1 – ImgB3, as well as the second set of unmixed parameters that include the SW-unmix suffix. It is almost always worth deselecting these.

## Output

The algorithm will work quickly if all of the images are already on your local computer. A larger number of requested similar images will take more time. Once it completes you will see your image set update with the number of request images, and a new image set appear called 'Similar to...' whatever your original set was.



Figure 3: Output of the KNN search

If you click the Create population button next to the KNN button, FlowJo will create two populations in your hierarchy, the similar images and the NOT of that population.

▼ Cell Synapse 10K.fcs		10000
% Similar to Two Cells		
▼ 🧹 Clean Up	77	7716
▼ 🧹 DP	3.9	298
🔍 BD_CellView_Lens of DP		Ready
🧹 Similar to Two Cells.Pop0	100	9950
🧹 Similar to Two Cells.Pop1	0.5	50

Figure 4: Output populations in the FlowJo workspace

These populations can be plotted, gated on, clustered, or refined in any way that any population in FlowJo can be interrogated. The not of the population is created so that one by use it as the counter example for training a classifier to distinguish between the two and identify the cells of interest. Because the NOT population is always created from the root population, it will tend to be overly general. You may want to apply any clean up gating you performed to the NOT population if you plan to use it further.

In Figure 5, some options for further analysis are shown. Observe that if the clean up and double positive gates (DP) are dragged from the root level to the NOT gate, the 248 cells that were not among the 50 nearest neighbors are returned, giving you a refined set to train against if you prefer. Also, Hyperfinder was run on the Similar population. Observe that a series of 6 polygon gates with the prefix HyFi recovered 47 of the 50 'similar' cells, using the parameters noted in each gate name. You can see that a mixture of fluorescent and image derived parameters were used to achieve this. These gates could be used to find more of the same type of cell on another file in this experiment, or opened on the BD FACSCorus™ Software and used to sort the identified cell type. Last, a CellView Lens node was dropped on the gated NOT population, indicating that you can look at images of these specific cells as well.

Name	Statistic	#Cells
▼ Cell Synapse 10K fcs		10000
% Similar to Two Cells		
HyperFinder		Done: Final Recall=0.7222 ...
▼ ⌕ Clean Up	77	7716
▼ ⌕ DP	3.9	298
BD_CellView_Lens of DP		Ready
▼ ⌕ HyFi_APC-A vs Radial Moment (ICAM CD54 BB515)	39	3895
▼ ⌕ HyFi_Radial Moment (LFA-1 PE-Cy7*) vs Diffusivity (LFA-1 PE-Cy7*)	50	1952
▼ ⌕ HyFi_BV421-A vs Diffusivity (ICAM CD54 BB515)	79	1550
▼ ⌕ HyFi_Max Intensity (ICAM CD54 BB515) vs Eccentricity (LFA-1 PE-Cy7*)	44	682
▼ ⌕ HyFi_BV605-A vs Eccentricity (ICAM CD54 BB515)	6.9	47
HyFi_APC-A vs BV605-A	100	47
▼ ⌕ Similar to Two Cells Pop0	100	9950
▼ ⌕ Clean Up	77	7666
▼ ⌕ DP	3.2	248
BD_CellView_Lens of DP		Ready
⌕ Similar to Two Cells Pop1	0.5	50

Figure 5: Further analysis

## Using Regions in the BD CellView Lens

Regions can be useful in examining cell to cell interaction.

The CellView Lens can identify the edges of cells within an image and whether there are more than one. Regions can be created by turning on the Remove Background option within the Image Filter controls. Once calculated, regions can be viewed by clicking on the Regions box in the plugin window as shown in Figure 1. You will notice that the cell wall now shows the region masks as opposed to the cell images. Multiple colors in one image implies that there are more than one cell present.

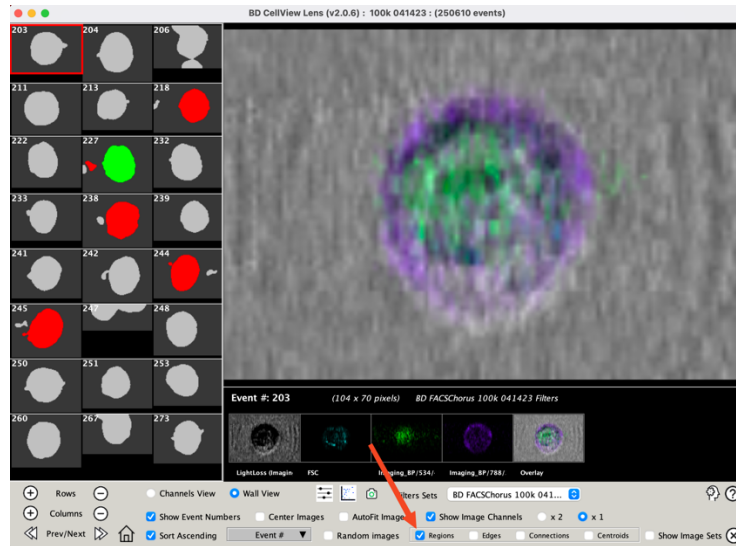


Figure 1 Regions check box

If the Edges and / or Connections boxes are checked the detected edges will be displayed and/or a line showing the minimum distance between the edges is drawn, as shown in Figure 2. If the Centroids box is checked the connection will be drawn between the Centroids of the identified regions.

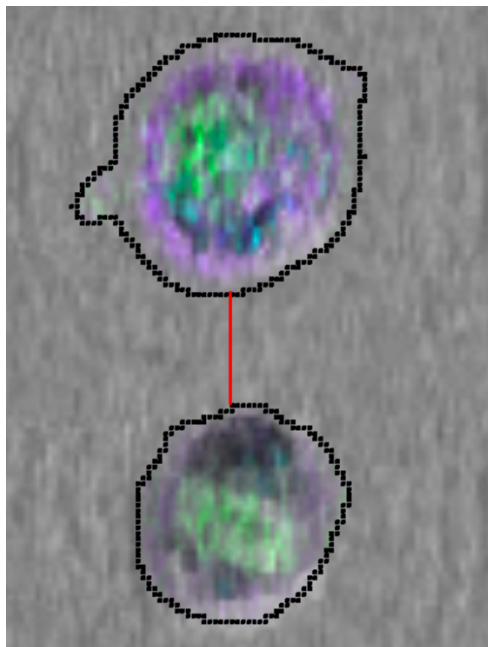


Figure 2 An image with edges and Connections turned on

Beyond making nice visualizations, regions, sub-regions, and the distance between sub-regions can be used to sort or identify interesting populations. A series of statistics related to regions can be calculated by:

1. Turning on Remove Background in the Image Filter tool
2. Creating a CellView Lens node **on the root population** in the FlowJo hierarchy
3. Opening that node and selecting Make Image Parameters from the Tools menu as shown in Figure 3

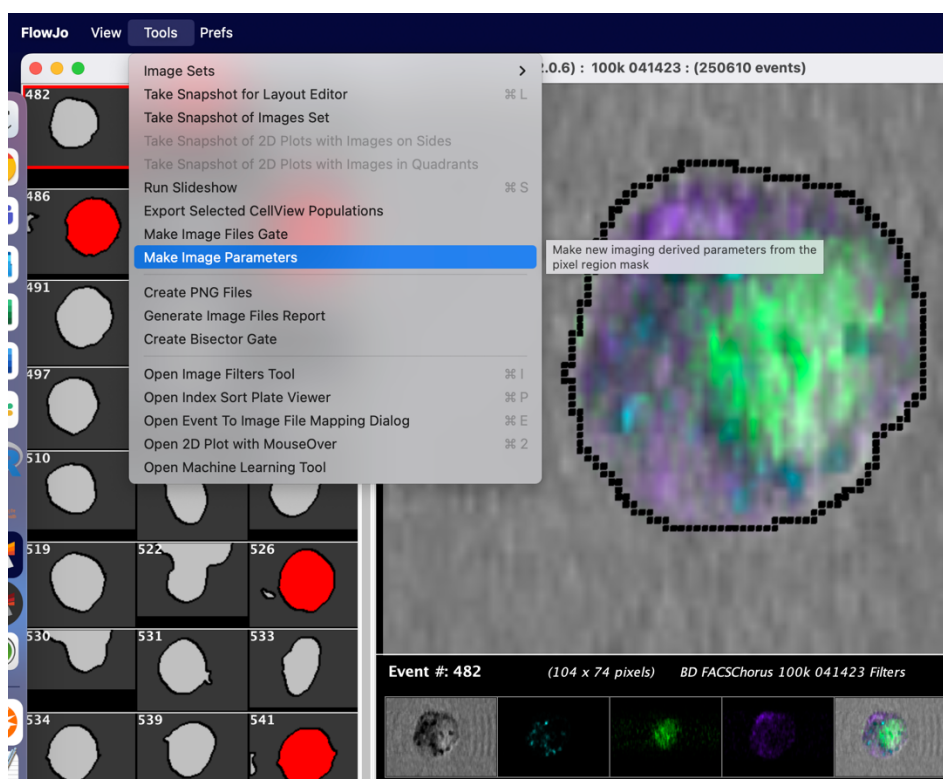


Figure 3 Make Image Parameters tool

The calculation will take a little time depending on how many cells with images are your experiment, but on completion a set of derived parameters will be created as shown in Figure 4.

Name	
100k 041423.fcs	
% Avg Distance Between Subregions	
% Images Files	
% Max Subregion Area	
% Min Subregion Area	
% Number of Subregions	
% Region Mask Area	
% Region Mask Circumference	
BD_CellView_Lens	

Figure 4 Derived parameters related to regions

These derived parameters are now available in the parameter list of any subset of this root population. They can be displayed on an axis and gated to separate events with the most sub-regions or the greatest distance between sub-regions. Figure 5 shows using the Number of Subregions as a parameter to observe that there are many cells with no regions (those are cells for which an image was not collected), many cells with one region, a few with two

and three, and a handful of cells with more. A gate has been drawn on the plot to isolate events that have more than one region for further study.

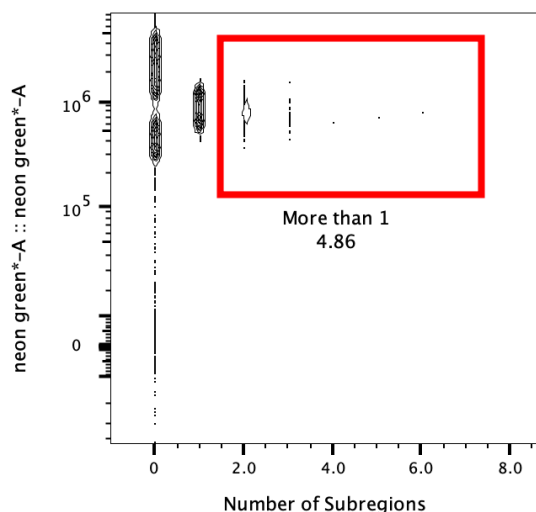


Figure 5 Graphing Number of Subregions and gating on it.

These statistics are also available in the CellView Lens plugin window as options for sorting. Figure 6 displays an example of using gates in FlowJo to identify a population of dividing cells, then sorting by the Number of regions so that the events with the fewest regions can be selected for to remove images that contain cell fragments.

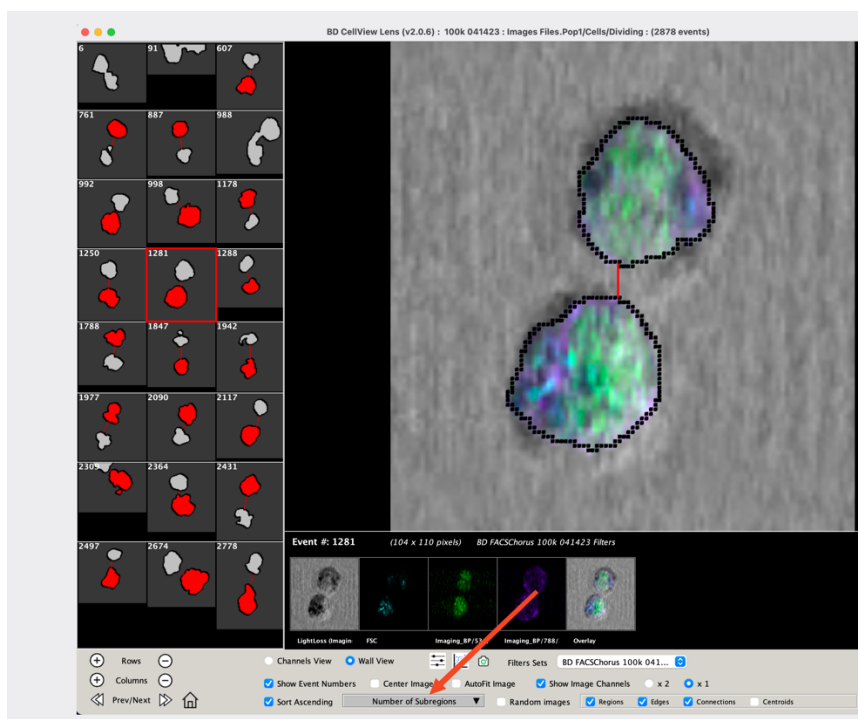


Figure 6 Sorting by Number of Regions

# 3

## Troubleshooting

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This chapter covers the following topic:

- [Troubleshooting the BD CellView™ Lens plugin \(page 50\)](#)

## Troubleshooting the BD CellView™ Lens plugin

Observation	Possible cause	Recommended solution
Images are not loading for index sort file.	The Use numerical subfolders checkbox is checked, but the image files are stored in one folder	Check the folder structure where the image files are stored and select the 'Use numerical subfolders' checkbox accordingly
	The index sort CSV file, FCS data file, and images folder are not found together in the same parent folder	Move or copy the index sort CSV file, FCS data file, and images folder so that they are all contained in the same folder, ideally with no other files or folders
	Image file names contain characters that corrupt the file path	Check the names of the image files for the '/' (slash) character. If found rename or re-export the files to omit the '/' (slash) character
	Images folder is incorrect	Confirm if the images folder is correct and if necessary, change the folder by clicking 'Set Folder' in the Event Number to File Name Mapping dialog
	The user does not have read permission for the image files	Confirm if all the image files have the correct permissions to be accessed by the user and the application.
	The index sort CSV file has incorrect format or is missing information	Inspect the index sort CSV file and confirm it contains columns for 'Well', 'Event', 'Sort Population', 'EventIndex'
Background is observed on overlay images which is not present on individual channels images.	One or more channels contains 'noisy' pixel values that vary over the entire image	<p>Use the Image Filters Tool to inspect individual channels for noisy data, determine if channel can be disabled.</p> <p>Adjust the Gamma filter value for channels with noisy data.</p> <p>Use the Transparent or Solid Overlay options.</p>



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