

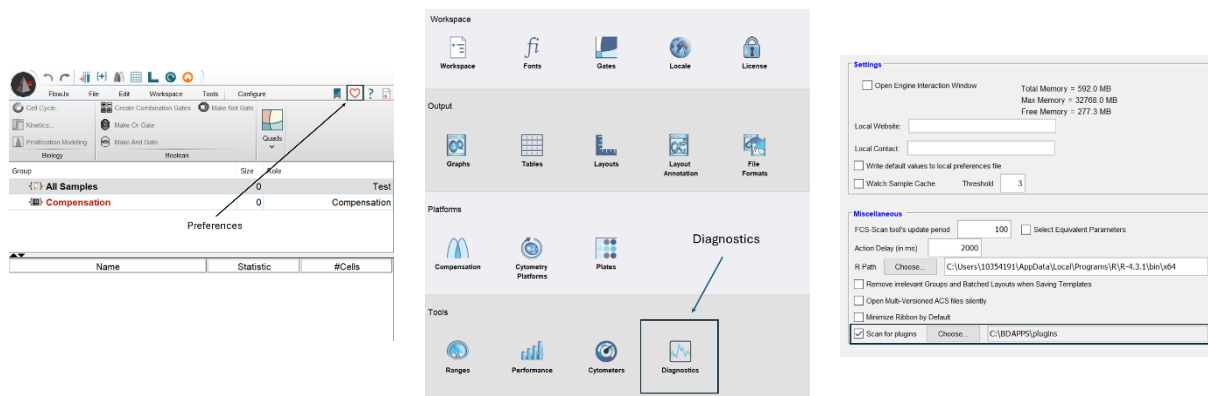
## SingletSeeker v1.2.5 Plugin

### Introduction

The SingletSeeker plugin automatically identifies singlets, multiplets, and debris, simplifying the user's workflow and taking the guesswork out of gating singlets. The algorithm is built on a popular density-based clustering algorithm known as Density-Based Spatial Clustering on Applications with Noise (DBSCAN). This algorithm identifies high-density regions of an n-dimensional space by automatically determining a density threshold and discarding anything below that threshold as noise. Because multiplets often vary greatly in size (doublets, triplets, etc.), they appear as low-density data compared to singlets or other uniformly sized events, like debris. As a result, the SingletSeeker plugin will discard multiplets as noise and keep singlets and debris. Singlets are then distinguished from debris based on size parameters. Overall, this plugin will facilitate high-fidelity classification of singlets, simplifying your workflow and ensuring more reproducible data processing and analysis. The plugin has been optimized for parameters acquired by the BD FACSDiscover™ imaging cytometer, but can also be used on datasets containing forward scatter area and height measurements.

### Download and Installation

1. Place the plugin .jar file in your Plugins folder.
2. Navigate to Preferences > Diagnostics and select the “Scan for Plugins” checkbox to point FlowJo to your Plugins folder (Figure 1).
3. Click OK.
4. Restart FlowJo.



**Figure 1** – FlowJo Preferences navigation for Plugins folder.

### Requirements

- The plugin makes use of five different potential feature sets (Table 1). Which feature set the user chooses to use depends on what parameters they have available and their preference for performance.

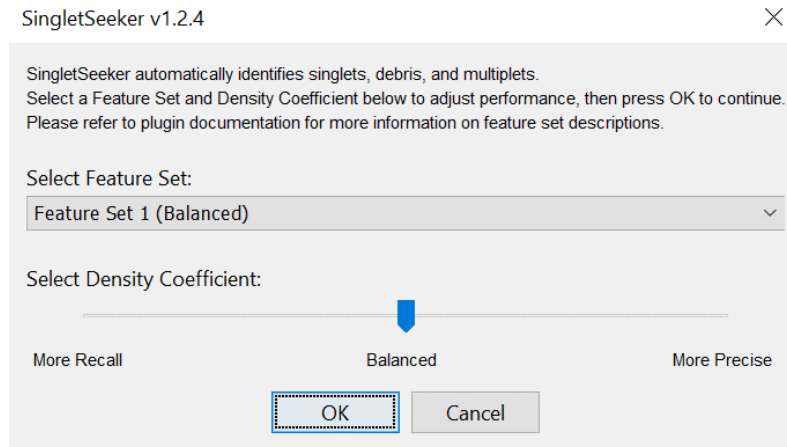
**Table 1** –Feature sets used by plugin.

Feature Set	Features	Feature Descriptions	Performance
1	LightLoss (Violet)-A	BD FACSDiscover™ S8 parameter, 0°-angle scatter measurement using the violet laser, area of intensity curve	Balanced performance between singlet precision and recall.
	Long Axis Moment (SSC (Imaging))	BD FACSDiscover™ S8 parameter, measures the moment of inertia around the long axis of an event, derived from side scatter using the blue laser	
	Radial Moment (FSC)	BD FACSDiscover™ S8 parameter, measures the average distance of pixels from the centroid or relative center of the forward scatter image.	
	Radial Moment (LightLoss (Imaging))	BD FACSDiscover™ S8 parameter, measures the average distance of pixels from the centroid or relative center of the LightLoss image.	
	Radial Moment (SSC (Imaging))	BD FACSDiscover™ S8 parameter, measures the average distance of pixels from the centroid or relative center of the side scatter image.	
2	LightLoss (Violet)-A	BD FACSDiscover™ S8 parameter, 0°-angle scatter measurement using the violet laser, area of intensity curve	Best singlet precision
	LightLoss (Violet)-H	BD FACSDiscover™ S8 parameter, 0°-angle scatter measurement using the violet laser, height of intensity curve	
3	LightLoss (Violet)-A	See above	Best singlet recall
	LightLoss (Violet)-W	BD FACSDiscover™ S8 parameter, 0°-angle scatter measurement using the violet laser, width of intensity curve	

	Size (FSC)	BD FACSDiscover™ S8 parameter, measures the number of pixels in the image, which are brighter than a user-defined Pixel Threshold in the forward scatter image.	
	FSC-A	Measurement of light scattered at low-angle in the direction of the blue laser, area of intensity curve	
	Radial Moment (LightLoss (Imaging))	See above	
4	LightLoss (Violet)-A	See above	
	Short Axis Moment (LightLoss (Imaging))	BD FACSDiscover™ S8 parameter, measures the moment of inertia around the short axis of an event, derived from LightLoss using the blue laser	
	SSC (Imaging)-A	BD FACSDiscover™ S8 parameter, 90°-angle scatter measurement using the blue laser, area of intensity curve	
	LightLoss (Imaging)-A	BD FACSDiscover™ S8 parameter, 0°-angle scatter measurement using the blue laser, area of intensity curve	
	SSC (Violet)-A	BD FACSDiscover™ S8 parameter, BD FACSDiscover S8™ parameter, 90°-angle scatter measurement using the violet laser, area of intensity curve	
5	FSC-A	See above.	Use this feature set if you do not have access to the other features obtained from the BD FACSDiscover™ S8.
	FSC-H	Measurement of light scattered at low-angle in the direction of the blue laser, height of intensity curve	

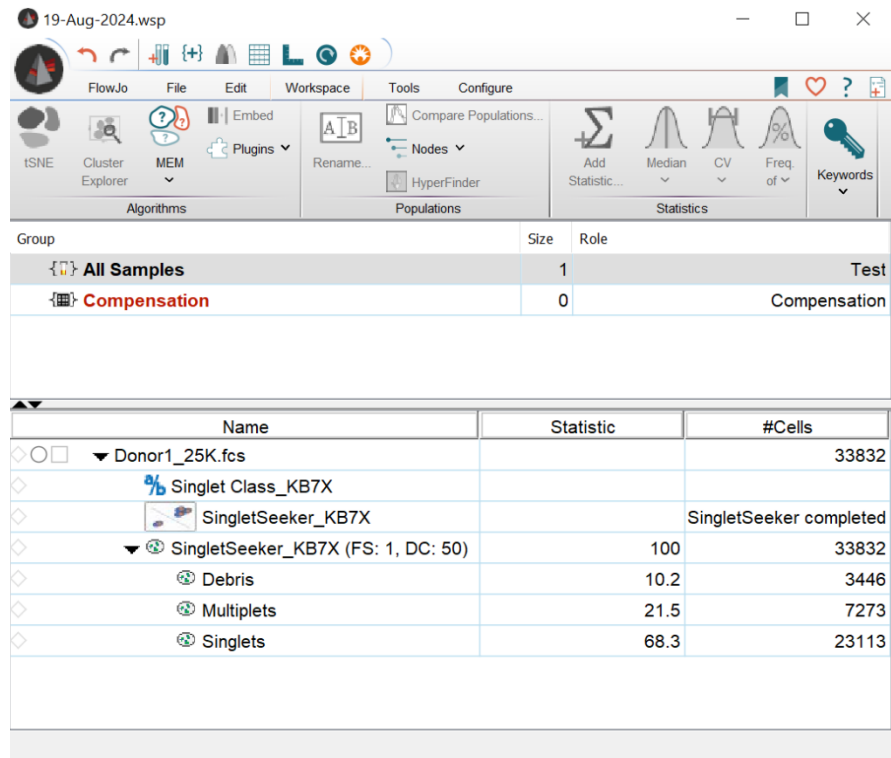
## Usage

1. Begin by saving your workspace.
2. Once saved, select your FCS file of choice in your workspace. Apply on the entire sample. This plugin will not run on subpopulations.
3. A simple graphical user interface (GUI) opens with a brief description of the plugin, a slider to select density coefficients, and a combo box of the feature sets (Figure 2).



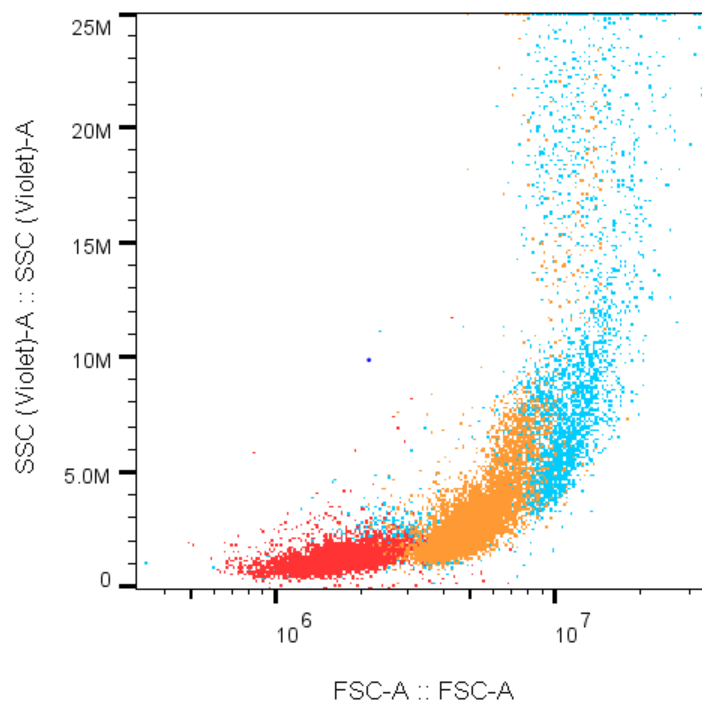
**Figure 2** – The SingletSeeker dialog box provides a brief description of the plugin’s functionality and provides a slider bar for the selection of the Density Coefficient.

4. Select the preferred/appropriate Feature Set for your data.
5. The “Balanced” Feature Set is selected by default.
6. Select the preferred Density Coefficient for your data.
7. The slider bar allows the user to tune the aggressiveness of the algorithm when searching for singlets.
  - a. Higher Density Coefficient values increase the threshold for an event to be considered high density. This means that the population returned as singlets will be more purely singlets and less likely to contain any multiplets.
  - b. Lower Density Coefficient values do the opposite, meaning the algorithm is more inclusive. This will return a greater number of cells in the final singlets population, but may be more contaminated with multiplets.
8. A Density Coefficient of 50 is set by default as generally the optimal balance between inclusion and exclusion.
9. Once the Feature Set and Density Coefficient have been selected, simply press OK to continue.
10. Three populations are then returned under a new parameter named “Singlet Class\_”, with a unique, 4-digit run ID appended to the end of each run. Three subpopulations will now exist as singlets, debris, and multiplets (Figure 3).
11. Each run also includes the feature set and density coefficient that were used in the analysis as FS and DC in the text of the plugin node.



**Figure 3** – The singlet, multiplet, and debris subpopulations can be found under the file the plugin was run on.

12. For demonstration, the classified events can be viewed in the Layout Editor to confirm results (Figure 4).



	Sample Name	Subset Name	Count
	Donor1_25K.fcs	Singlets	25201
	Donor1_25K.fcs	Debris	4532
	Donor1_25K.fcs	Multiplets	4099

**Figure 4** – In the FlowJo Layout Editor, singlet, multiplet, and debris subpopulations can be visualized with regard to the full event population.

#### Contact Us

- Please contact us at [flowjo@bd.com](mailto:flowjo@bd.com) for any questions or concerns regarding this plugin.