Attune™ Cytometric Software

Image Processing Workflow

Pub. No. MAN0028531 Rev. A.0

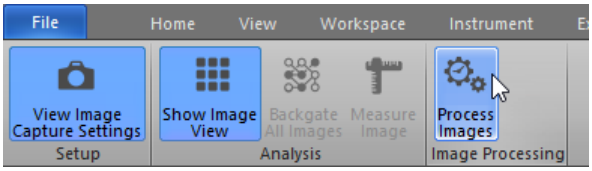
WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Note: For safety and biohazard guidelines, see the "Safety" appendix in the following product documentation: *Attune™ CytPix™ Flow Cytometer User Guide* (Pub. No. MAN0019440). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

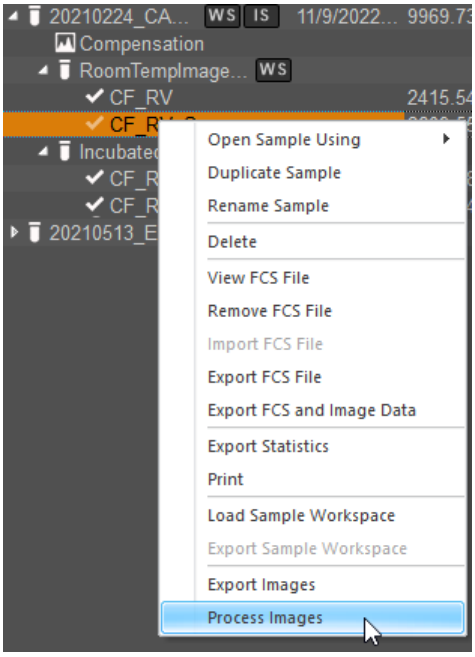
Process images

1. On the **Image Settings** ribbon tab, click **Process Images** to open the **Process Images dialog**.

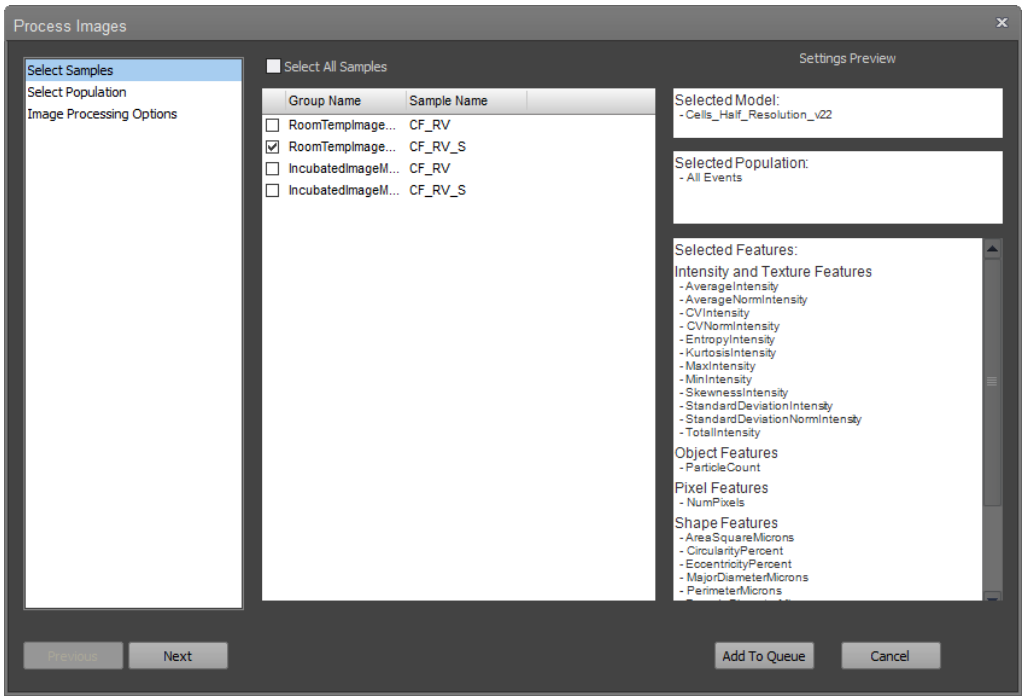
   Note: The **Image Processing** controls are visible only for Attune™ CytPix™ experiments. You can process images only for samples in the active experiment.



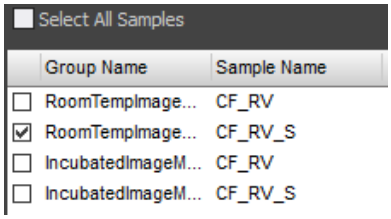
   Alternatively, select **Process Images** in the **Experiment Explorer** context menu.



2. On the **Select Samples** screen, select the samples for which to process images. To select or deselect all samples in the experiment, select or deselect the **Select All Samples** option above the sample selection list.



Note: If the Process Images dialog is opened from the Experiment Explorer, the samples are automatically selected based on the Sample, Group, or Experiment that was selected in the Experiment Explorer to open the dialog.



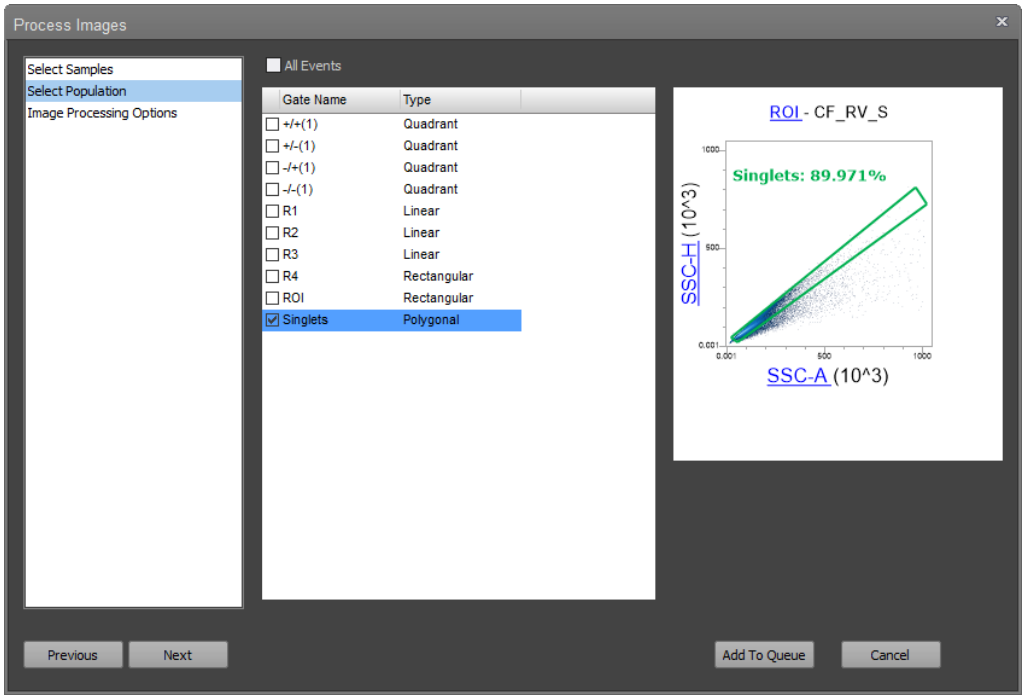
3. Click **Next** to go to **Select Population** screen to filter the images to be processed based on one or more populations (gates).

   Alternatively, click **Add To Queue** to add the samples to the image processing queue without selecting a population to filter the images.

   Note: When **Add to Queue** is clicked, all selected samples are added to a processing queue and are processed serially in the order they were added. You can manage the sample queue using the Attune™ Image Processing Dashboard (see “Attune™ Image Processing Dashboard” on page 5). Image processing proceeds even if you close the Attune™ Cytometric Software.

4. On the **Select Population** screen, select one or more populations (gates) to process only the events in the selected gates. By default, **All Events** is selected. The preview plot shows the selected population. When no gate is selected, the preview plot does not show any plot.

   Note: The software uses the selected gate to process all samples. If the gate does not exist on the selected workspace, the software tries the next level up in the gating hierarchy (Sample > Group > Experiment). If the software finds no gate, it reverts to **All Events** for that sample.

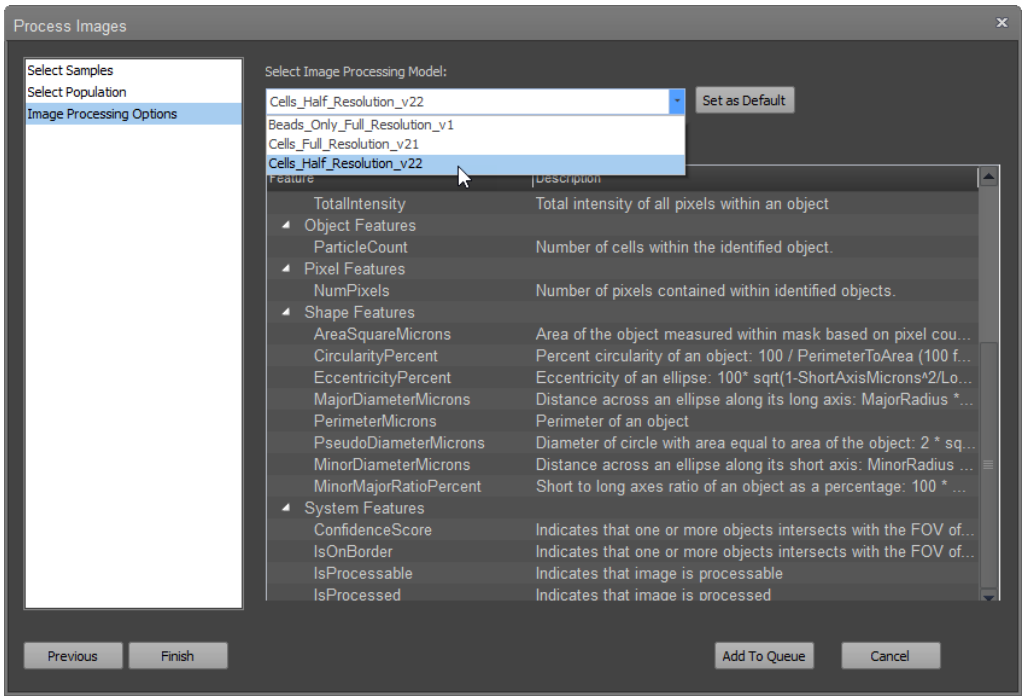


5. Click **Next** to go to **Image Processing Options** to select an image processing model.

   Alternatively, click **Add to Queue** to add the samples to the image processing queue without selecting an image processing model.

6. On the **Image Processing Options** screen, select an image processing model. For a list and description of image processing models, see “Image processing parameters” on page 9.

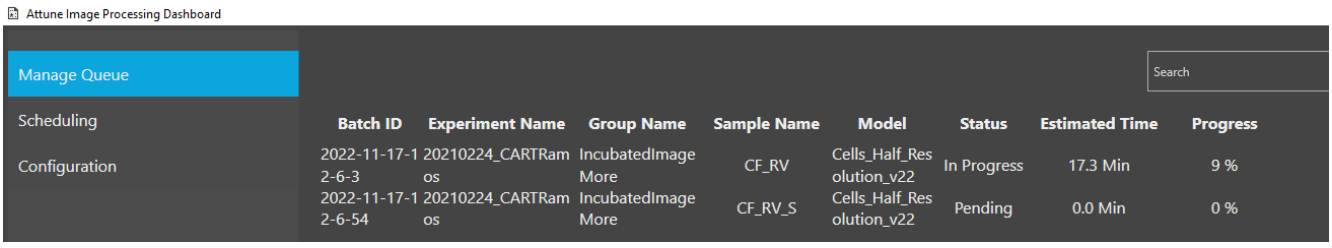
IMPORTANT! Regardless of the size of the captured images in the experiment, images are processed at either full resolution (248 × 248 pixels) or at half resolution (124 × 124 pixels). Processing the images at half resolution decreases data footprint by 4-fold and improves the processing times by >4-fold. However, this comes with a potential trade off in accuracy.



7. *(Optional)* Click **Set As Default** to set the selected image processing model as the defaul for subsequent image processing operations.

8. Click **Add To Queue** or **Finish** to start image processing.

   Samples in the processing queue are processed serially in the order they were added. You can view the status of the image processing job in the Attune™ Image Processing Dashboard, which also enables you to manage the sample queue (see “Attune™ Image Processing Dashboard” on page 5).



Note: If the selected samples already have image processing data, the software displays a warning dialog that enables you to overwrite the existing data, to skip the current sample, or to cancel image processing (see “Reprocess images” on page 8).

Note: Image processing proceeds even if you close the Attune™ Cytometric Software.

Note: Image processing is paused during sample acquisition.

Attune™ Image Processing Dashboard

The Attune™ Image Processing Dashboard is automatically opened when you power on the computer that runs the Attune™ Cytometric Software and it functions independently of the Attune™ Software.

The Attune™ Image Processing Dashboard enables you to:

- View image processing queue, status, and progress (page 6)
- Reprioritize the image processing queue (page 6)
- Cancel image processing requests (page 6)
- Pause or resume image processing (page 6)
- Schedule image processing

Figure 1 Attune™ Image Processing Dashboard

① Manage Queue tab
② Image processing queue
③ Move request up or down buttons
④ Delete button
⑤ Resume processing button
⑥ Pause processing button
⑦ Close dashboard button

View image processing queue, status, and progress

You can view the image processing queue, status, and progress in the **Manage Queue** tab of the Attune™ Image Processing Dashboard:

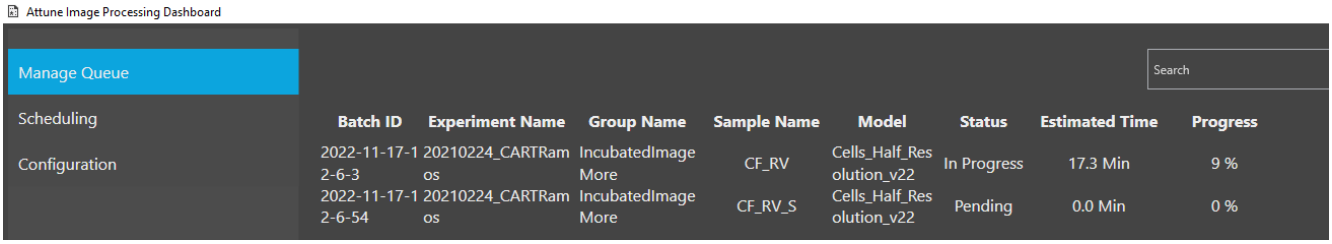


Figure 2 Image processing queue

- **Batch ID:** Unique identification assigned to the image processing job based on the date and time the sample was added to the processing queue.
- **Experiment Name:** Name of the experiment in Experiment Explorer from which the sample was selected for image processing.
- **Group Name:** Group name in Experiment Explorer from which the sample was selected for image processing.
- **Sample Name:** Name of the sample in Experiment Explorer that was selected for image processing.
- **Model:** Image processing model selected in Process Images wizard.
- **Status:** Image processing status for the sample (In Progress, Pending, or Paused).
- **Estimated Time:** Estimated time for the completion of image processing for the sample.
- **Progress:** Percentage of the image processing job that is completed.

Note: Image processing is paused during sample acquisition.

Reprioritize the image processing queue

To move a processing request up or down in the image processing queue, select the request, then use the **Move to Top**, **Move Up**, **Move Down**, and **Move to Bottom** buttons to move it to the desired position in the queue.

Cancel image processing requests

To cancel an image processing request, select the request in the image processing queue, then click **Delete**.

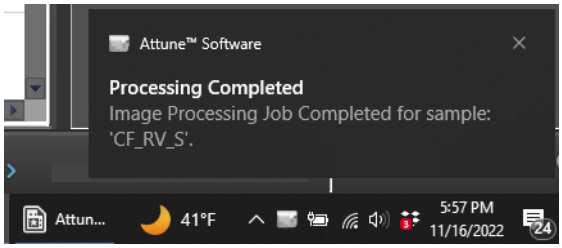
Pause or resume image processing

1. To pause an ongoing image processing of a sample, select the sample, then click **Pause**.
2. To resume image processing, click **Resume**.

Completion of image processing

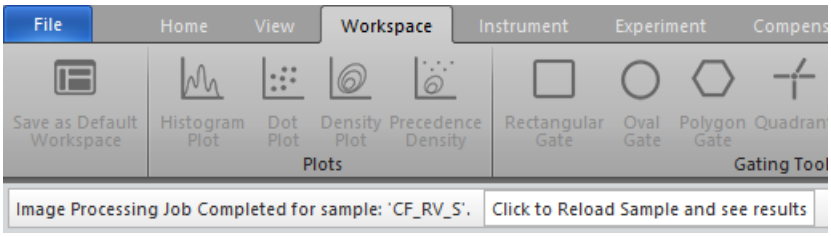
When the Attune™ Cytometric Software is running and image processing is completed for any sample in the processing queue that is specific to the currently logged-in user, the software provides the following notifications:

- System tray displays the **Processing Complete** notification.



Note: Windows notifications must be enabled to see system tray notifications.

- Message bar displays the **Image Processing Job Complete** notification with an option to reload the sample and see the results.



Note: Clearing the message by clicking **X** or **Reload Sample and see results** button hides the message bar.

- If the Attune™ Software is running in the SAE ("Security, Auditing, and Electronic Signature") mode and the experiment was signed, the message bar displays a warning that indicates that changes to the experiment will invalidate any existing signatures.

When image processing is completed for a sample, the icon next to the sample in the Experiment Explorer is updated to indicate that the sample has both FCS and image processing data.

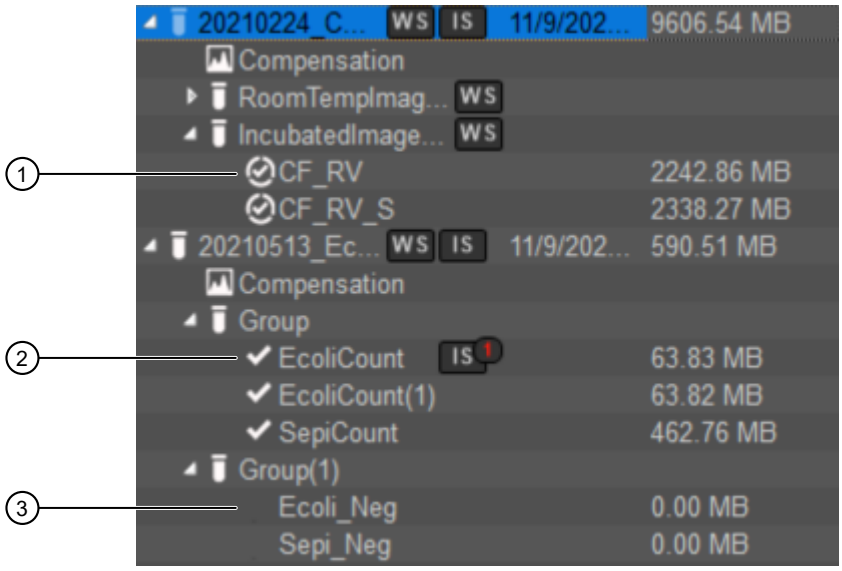


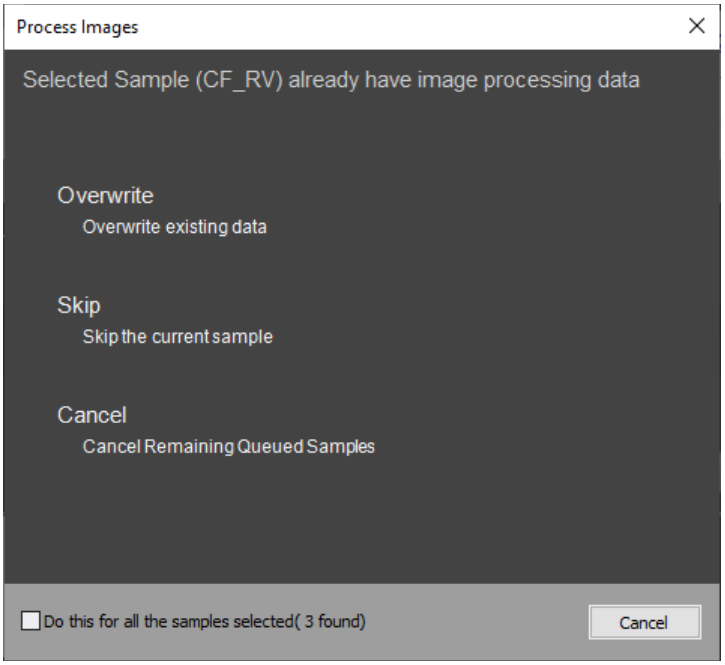
Figure 3 Experiment Explorer containing samples with and without image processing data

① Sample with FCS and Image Processing data
② Sample with FCS data only
③ Sample with no data

Reprocess images

You can reprocess images in a sample to allow a different processing model or algorithm, or to use other populations or features to extract cell image information. When samples are reprocessed, any ongoing processing is canceled and the new processing request is added to the queue.

1. Select the samples, populations, and image processing options as described in Process images. When you start a processing request for a sample that has already been processed, the software displays a dialog that states that the selected sample already has image processing data. The software displays the dialog for each sample that meets this condition.



2. To process the selected samples and overwrite the existing image processing data, select **Overwrite**.

   Note: If you have selected multiple samples with existing image processing data, you can apply the **Overwrite** option to all samples that meet this condition by selecting **Do this for all the samples selected** checkbox. When the last sample that meets this condition is confirmed, the image processing dialog is closed.

   The selected samples that already have image processing data are added to the queue and processed along with any samples that do not have existing data.

   IMPORTANT! When image processing completes for each sample, any existing results will be overwritten.

3. To skip the samples with existing image processing data and process only the samples that do not have existing data, select **Skip**.

   Note: If you have selected multiple samples with existing image processing data, you can apply the **Skip** option to all samples that meet this condition by selecting **Do this for all the samples selected** checkbox. When the last sample that meets this condition is confirmed, the image processing dialog is closed.

   The selected samples that already have image processing data are not added to the queue and only those samples that do not have existing data are processed.

4. To cancel the processing request without adding the selected samples to the image processing queue, select **Cancel**.

   The image processing dialog remains open, allowing you to update the sample selection, if desired.

Image processing parameters

Feature	Description	PnR[1]
Intensity and Texture features		
AverageIntensity	Average intensity of all pixels within an object	2^{10}
AverageNormIntensity	100 × AverageIntensity / (0.5 × $2^{(BitsPerPixel - 1)}$)	
CVIntensity	100 × StandardDeviationIntensity / AverageIntensity	
CVNormIntensity	100 × StandardDeviationNormIntensity / AverageNormIntensity	
EntropyIntensity	Entropy of intensity distribution of all pixels within an object	
KurtosisIntensity	Kurtosis of intensity distribution of all pixels within an object[2]	
MaxIntensity	Maximum intensity of all pixels within an object	2^{10}
MinIntensity	Minimum intensity of all pixels within an object	2^{10}
SkewnessIntensity	Skewness of intensity distribution of all pixels within an object[3]	
StandardDeviationIntensity	Standard deviation of intensity of all pixels within an object	2^{10}
StandardDeviationNormIntensity	100 × StandardDeviationIntensity / (0.5 × $2^{(BitsPerPixel - 1)}$)	
TotalIntensity	Total intensity of all pixels within an object	
Object features		
ParticleCount	Number of cells within the identified object	2^3
Pixel features		
NumPixels	Number of pixels contained within identified objects	2^{16}
Shape features		
AreaSquareMicrons	Area of the object measured within mask based on pixel count = NumPixels × (PixelSize.MicronsX × PixelSize.MicronsY)	2^{13}
CircularityPercent	Percent circularity of an object = 100 / PerimeterToArea (100 for a circular object)	2^9
EccentricityPercent	Eccentricity of an ellipse = 100 × Sqrt(1 – ShortAxisMicrons^2 / LongAxisMicrons^2)	0 to 100
MajorDiameterMicrons	Distance across an ellipse along its long axis = MajorRadius × 2 × PixelSize.MicronsX	2^7
MinorDiameterMicrons	Distance across an ellipse along its short axis = MajorRadius × 2 × PixelSize.MicronsX	2^7
PerimeterMicrons	Perimeter of an object	2^{15}
PseudoDiameterMicrons	Diameter of a circle with an area equal to the area of the object = 2 × Sqrt(Object.Area in µm^2/ pi)	2^{15}
MinorMajorRatioPercent	Short to long axes ratio of an object as a percentage = 100 × ShortAxisMicrons / LongAxisMicrons	0 to 100
System features		
ConfidenceScore	Indicates that one or more objects intersects with the FOV of the image	
IsOnBorder	Indicates that one or more objects intersects with the FOV of the image	
IsProcessable	Indicates that the image is processable	
IsProcessed	Indicates that the image was processed. This is generated when the image processing FCS file is loaded and merged with the raw FCS file data.	

[1] $PnR is the range for the selected parameter n.
[2] Kurtosis of intensity measures the peakedness of the distribution of all pixels within an object. See “Kurtosis” on page 10.
[3] Skewness of intensity measures the degree of asymetry in the pixel data of an object. See “Skewness” on page 10.

Kurtosis

Kurtosis is measures how peaked a histogram is and it is based on the size of a distribution's tails. The kurtosis of a normal distribution is 0. Distributions with short tails compared to a normal distribution have negative kurtosis (platykurtic) and distributions with relatively long tails have positive kurtosis (leptokurtic).

In the context of image processing, kurtosis describes whether distribution of gray tones is more spread-out (flat) or more concentrated around the mean (peaked).

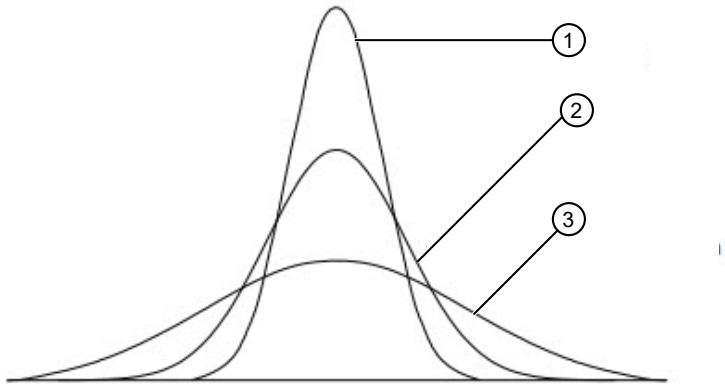


Figure 4 Kurtosis

① Positive kurtosis (leptokurtic)
② Normal distribution (mesokurtic)
③ Negative kurtosis (platykurtic)

Skewness

Skewness measures the degree of asymmetry exhibited by the data. If skewness equals zero, the histogramis symmetric about the mean.

In the context of image processing, skewness indicates the imbalance between the number of pixels that are darker or brighter than the mean.

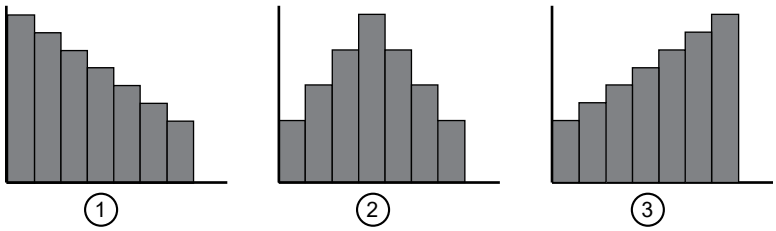


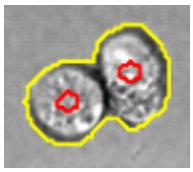
Figure 5 Skewness

① Positively skewed histogram
② Symmetric distribution histogram
③ Negatively skewed histogram

Customize Images

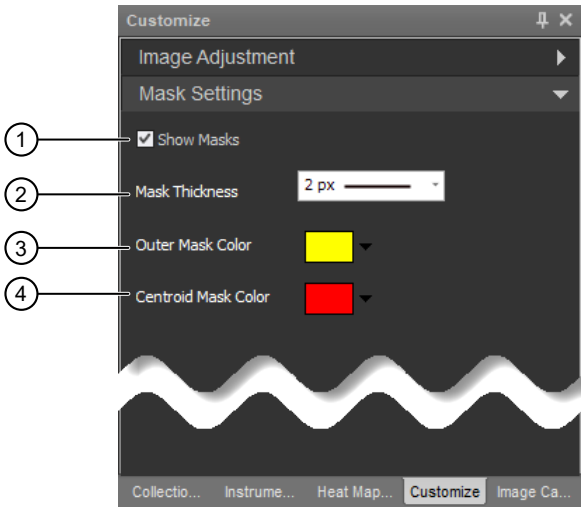
Change mask settings

When image processing is completed, the software also returns the image masks that were generated by the image processing.



- Image masks are binary representations of an image where an object is identified by the presence of a signal versus its absence. They provide a visual confirmation as to how the image processing “saw” the cell or the particle.
- The pixel coordinates of the mask contour are returned as outputs in the image processing results.
- The results include both the outer masks and the centroid masks (number of spots or cells detected in the Field of View). In the example above, the outer masks are depicted in yellow and the centroid masks in red.

1. In **Image View**, select an image, then click the **Customize** tab in the left panel to show the **Mask Settings** controls.



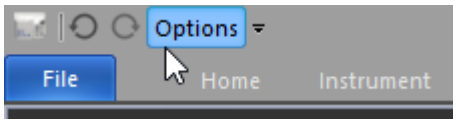
① Show Masks option
② Mask Thickness dropdown
③ Outer Mask Color dropdown
④ Centroid Mask Color dropdown

Figure 6 Customize panel Mask Settings controls

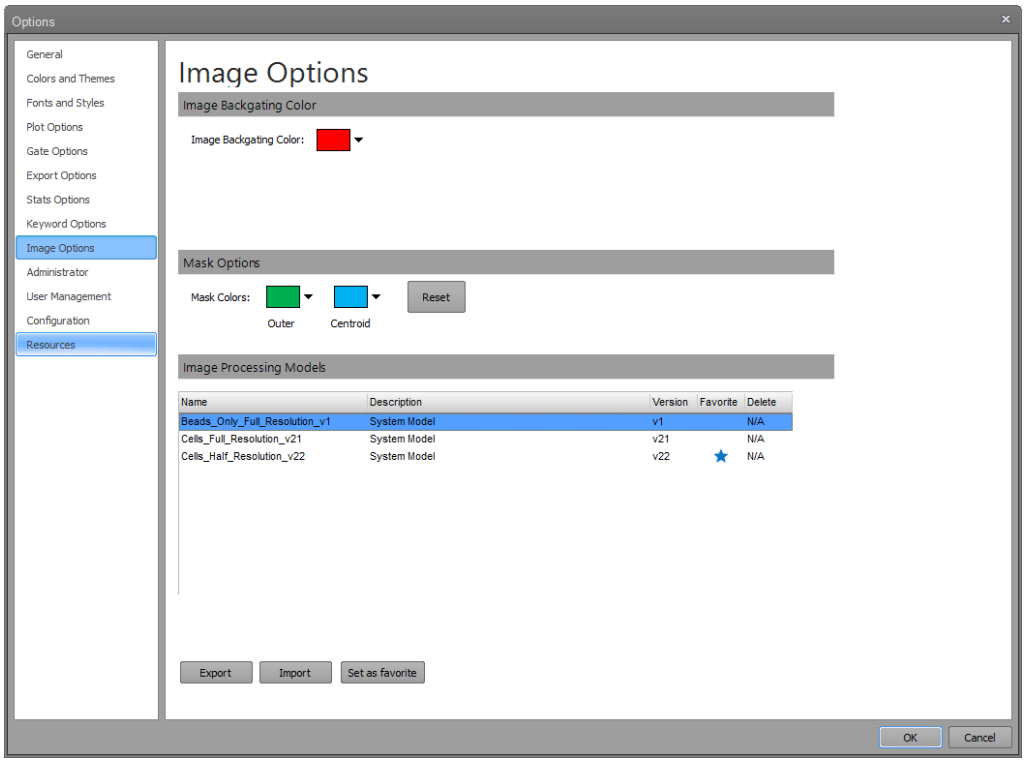
2. To show or hide the image masks, select or deselect **Show Masks**.
3. To change the line thickness of the mask contour, select the desired thickness in pixels from the **Mask Thickness** dropdown.
4. To change the color of the outer or the centroid mask, select the desired color from the **Outer Mask Color** or the **Centroid Mask Color** dropdown.

Change default mask colors

1. On the **Quick Access** toolbar, click **Options** to open the **Options** dialog.



2. In the **Options** dialog, click the **Image Options** tab, then under **Mask Options** select the desired **Mask Colors** for the **Outer** and **Centroid** masks.



3. To reset the default mask colors, click **Reset**.

4. Click **OK** to close the **Options** dialog.

Image scaling

The Attune™ CytPix™ Flow Cytometer captures images with 10 bits of dynamic range (0 to 1023) and stores them as 16-bit greyscale TIFF image files (maximum intensity of 65536). Because computer monitors can only display RGB between 0 to 255 per channel, the range that a single 8-bit byte can offer (which enables 16,777,216 colors – 256^3 colors and another 256 levels of opacity), images greater than 8 bits have to be scaled down to 8 bits per channel.

By default, the Attune™ Cytometric Software scales down the images such that the minimum intensity in each image becomes 0 and the maximum intensity becomes 255. This automatic scaling can result in visual artifacts, such as black images or images in which the background appears differenty in each image.

The Attune™ Cytometric Software enables you to manually scale the captured images, so that all images have the same minimum and maximum value (that is, they are normalized to a fixed intensity range). Setting a fixed-range normalization value enables images to be compared, where image scaling is identical.

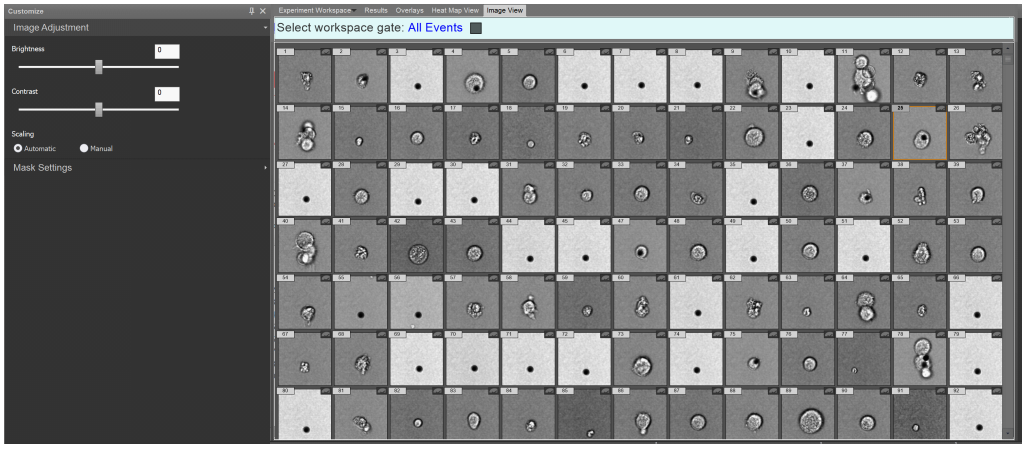


Figure 7 Automatic scaling

Automatic scaling (default) sets the minimum and maximum intensity within each image to 0 and 255, respectively.

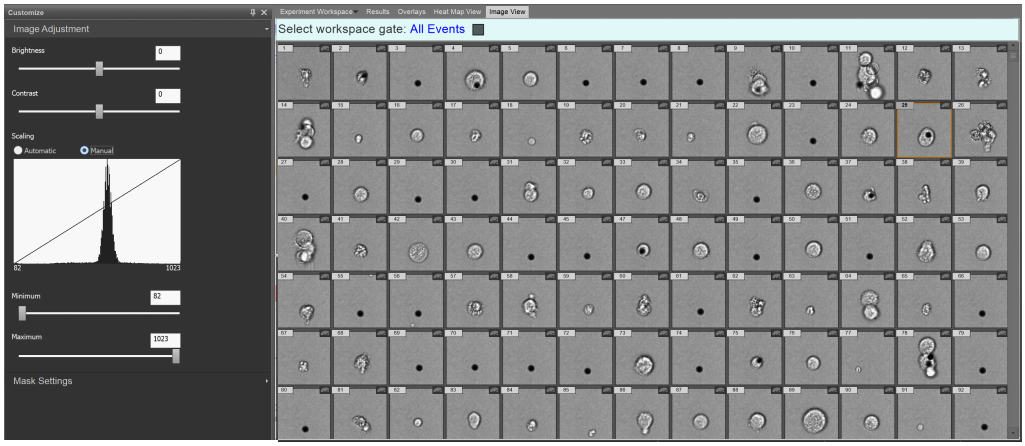
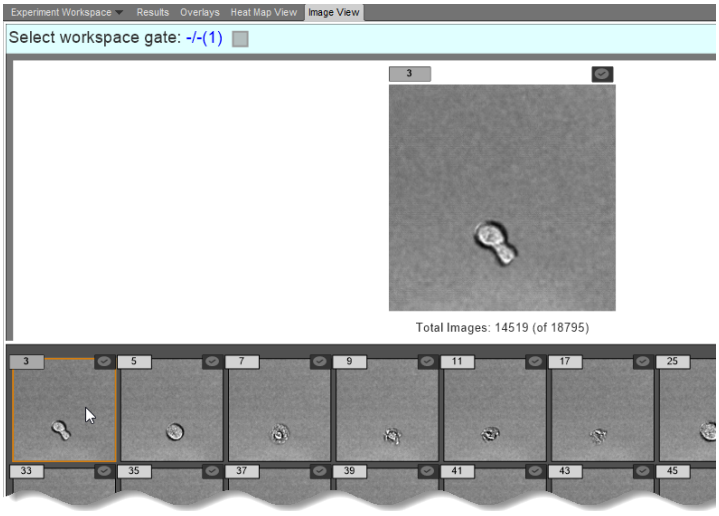


Figure 8 Manual scaling

Manual scaling enables you to set the minimum and maximum intensity for each image such that the specified minimum and maximum values are scaled to 0 and 255, respectively, which normalizes the images to a fixed intensity range.

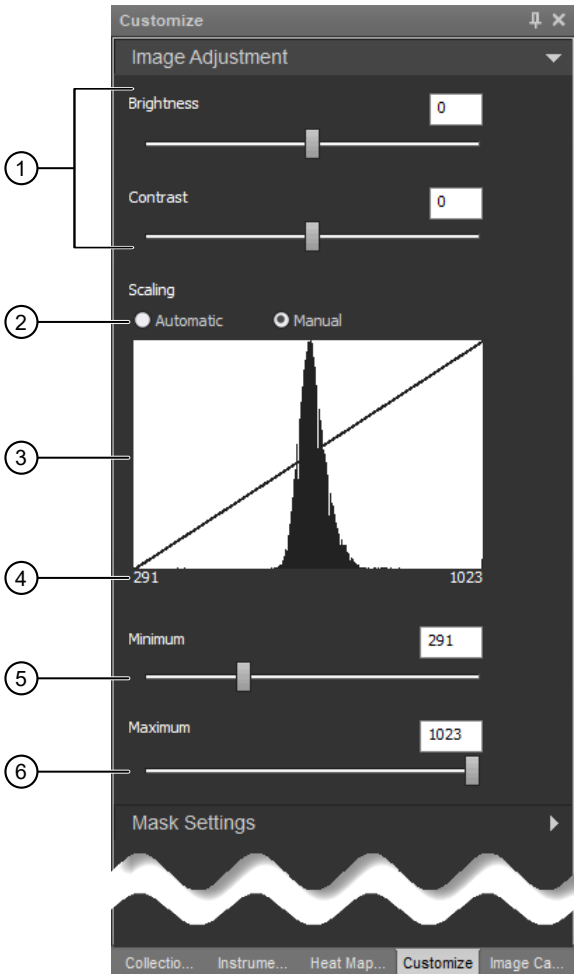
Manually adjust image scaling

1. In **Image View**, select the image to use for manual scaling.



2. In the **Customize** panel, select **Manual** for **Scaling** under **Image Adjustment**.

   The software displays the histogram for the active image that was selected in **Image View**, and the **Minimum** and **Maximum** values reflect the raw intensity values for the selected image.



① Brightness and Contrast sliders
② Scaling selection (Automatic or Manual)
③ Image histogram
④ Minimum and maximum raw intensity values
⑤ Minimum intensity slider
⑥ Maximum intensity slider

Figure 9 Customize panel Image Adjustment controls

3. To adjust image scaling, move the **Minimum** and **Maximum** sliders until the you are satisfied with the images displayed in **Image View**. Alternatively, type in the desired **Minimum** and **Maximum** intensity value.

   Note: Image scaling is applied to **all** images in the Image View and gallery, as well as the cell images in the Experiment Workspace. During acquisition, the image scaling is only applied to newly acquired images; the existing preview images remain unchanged until the acquisition completes.

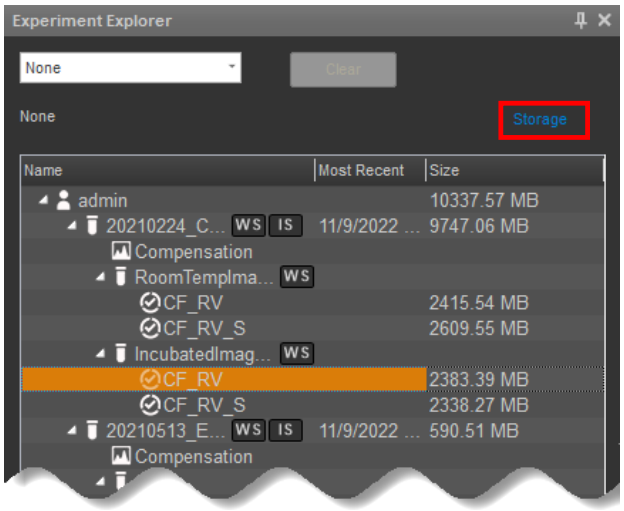
Data management

There are many types of data associated with the Attune™ instrument and software, including tube and plate experiment data, as well as instrument and user management data. Addition of imaging to the flow cytometry workflow also increases data storage requirements greatly. To ensure that there is sufficient disk space to acquire experiment data, export and clean up experiment data.

For more information about how to manage and back up data in the Attune™ Cytometric Software, see "Appendix B: Data Management in Attune™ Cytometric Software" in the *Attune™ Cytometric Software User Guide*.

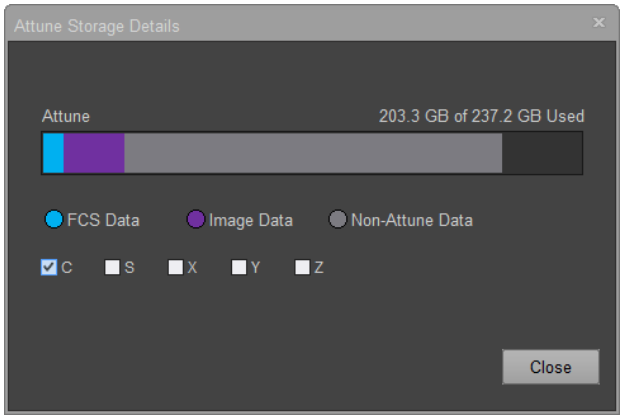
Disk usage

To view the data storage details on the instrument, click the **Storage** hyperlink at the top of the **Experiment Explorer**, which opens the **Attune Storage Details** window.

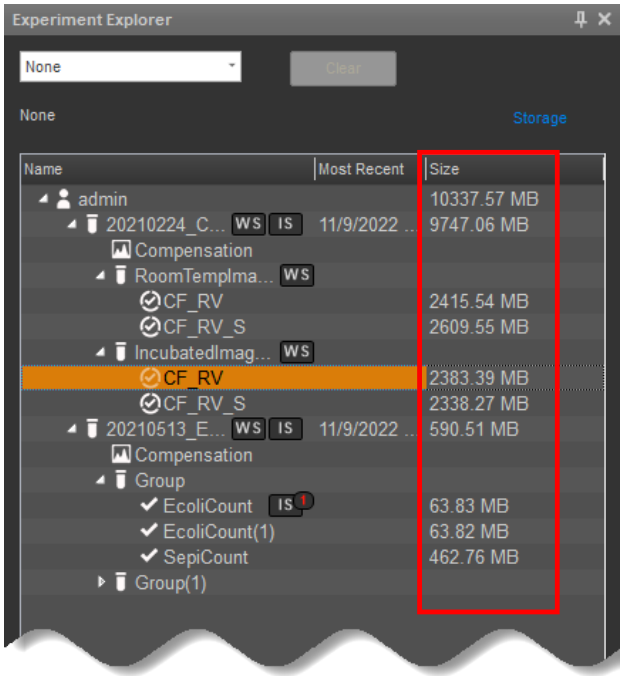


The **Attune Storage Details** displays the total used space and the total combined space broken into:

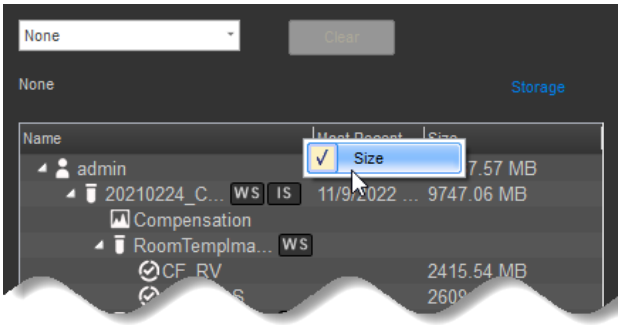
- FCS Data
- Image Data
- Non-Attune Data



The size details for Experiments, Groups, and Samples are shown in the **Size** column in **Experiment Explorer**, which are calculated on sign in and when any changes are made to an experiment (data added or removed).

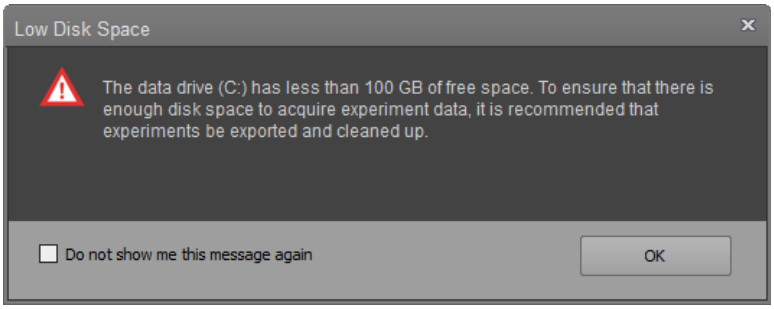


- The size data are displayed for all samples that contain data and include the size of the FCS file, the image data, and all metadata for the sample.
- The size data are displayed for the Experiment and include the summed size of all Sample data and any Experiment metadata.
- To sort the size data in ascending or descending order, click the **Size column header**.
- To hide or show the size column, right-click the **Experiment Explorer heading row**, then deselect or select **Size**.



Data storage warnings

By default, when activating experiments, the Attune™ Cytometric Software displays the **Low Disk Space** warning if the available disk space is less than 100 GB on the primary data (image data and experiment data) drives.

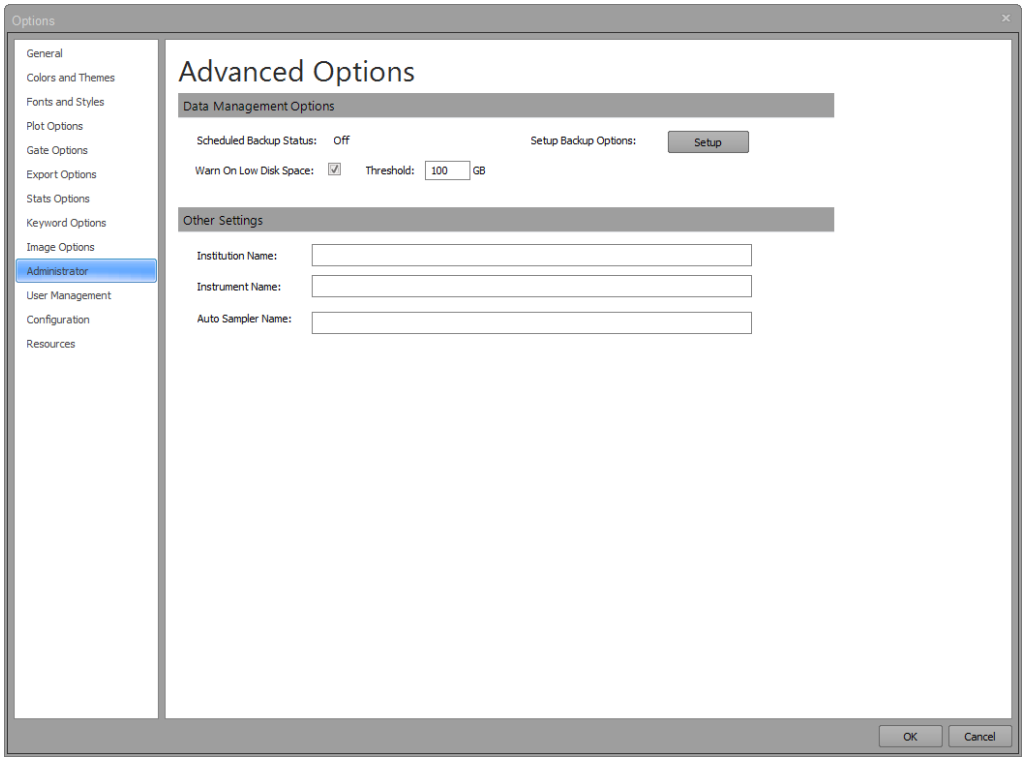


- To ignore and suppress the warning for the length of the session, select **Do not show me this message again**, then click **OK**.
- To disable the warning or to change the low disk threshold, see “Change low disk space warning options”.

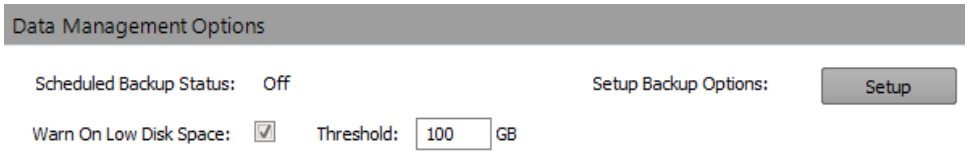
Change low disk space warning options

Users with Administrator or System Administrator accounts can change backup and low disk space warning options in the **Administrator** tab of the **Options** dialog.

1. On the **Quick Access** toolbar, click **Options** to open the Options dialog, then select the **Administrator** tab.



2. To disable the Low Disk Space warning, deselect **Warn On Low Disk Space**.



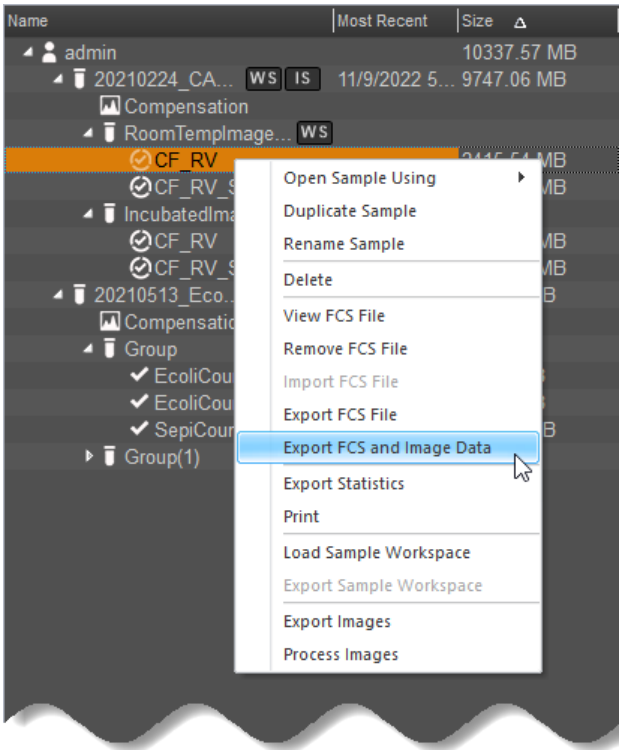
3. To change the threshold of available disk space on the primary disk drives below which the Low Disk Space warning is displayed, enter the desired value (in GB) in the **Threshold** field.

   Note: By default, the threshold is set to 100 GB. You can enter a threshold value of between 1 GB and 1000 GB.

Data management - Export data

Export FCS and Image Data

To export FCS files and imaging data together for a Sample, right-click the Sample in Experiment Explorer, then select **Export FCS and Image Data**.



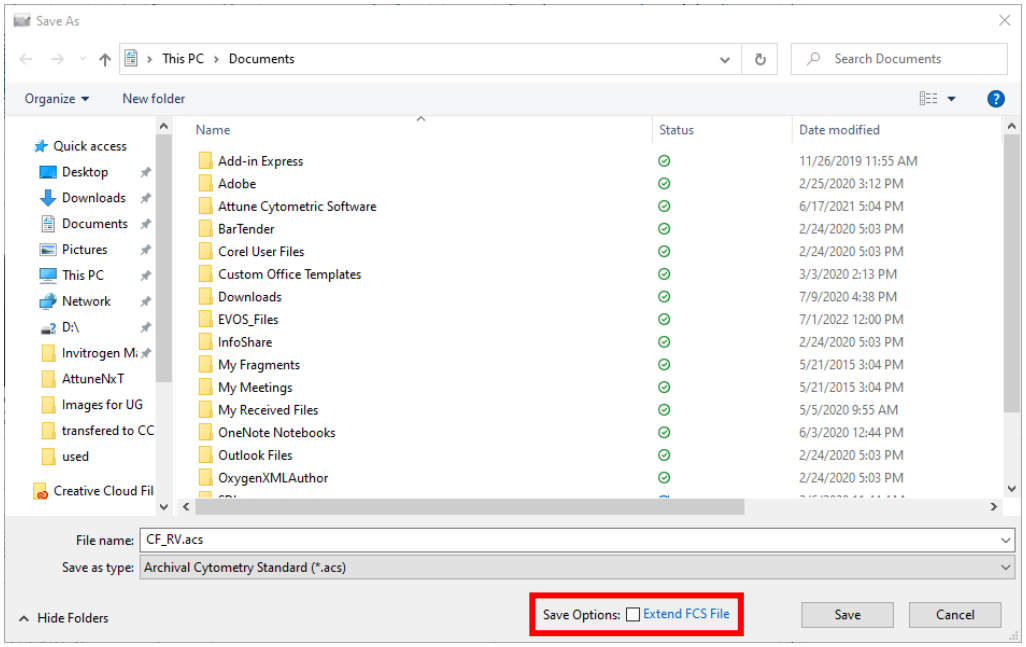
- By default, images, extended parameters (image processing data), and image mask are exported into a single zip file based on the ACS file format.
- Exported ACS files can be imported into a sample, which imports the FCS data and all imaging data.
- Exported ACS files can be opened in third party software that supports the ACS standard (such as FlowJo™ Software and FCS Express™ Software).

Export FCS files with extended data

When exporting sample data that has image processing data, the image processing results can be appended to the original FCS file, which creates a single FCS file that includes the image processing parameters.

1. To export FCS files or FCS files and imaging data together, right-click the sample, then select **Export FCS Files** or **Export FCS and Image Data**.

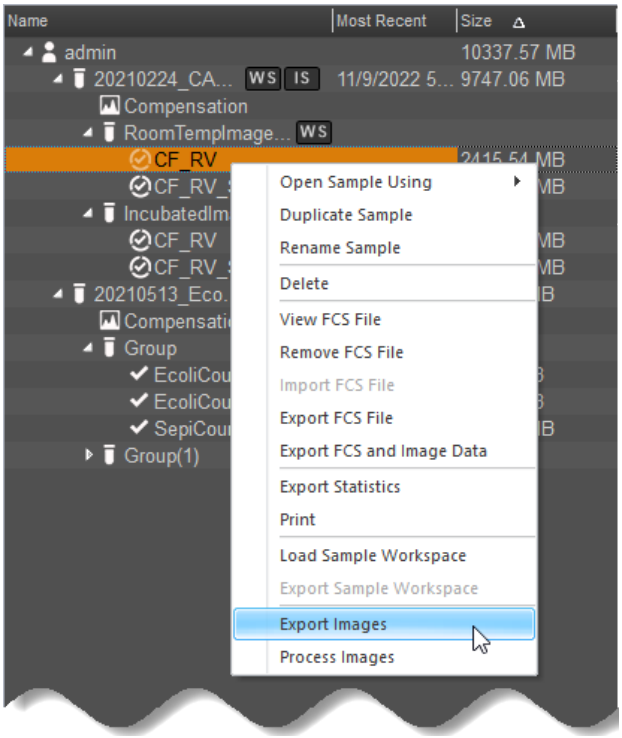
2. In the **Save As/Folder Selection** dialog, select **Extend FCS File** for **Save Options** to add the new data to the original FCS file.



When **Extend FCS File** option is selected, the original FCS file is appended with the image processing parameter data, and the $ORIGINALITY, $LAST_MODIFIER, and $LAST_MODIFIED keywords in the FCS file are updated to indicate that the data have been appended and modified.

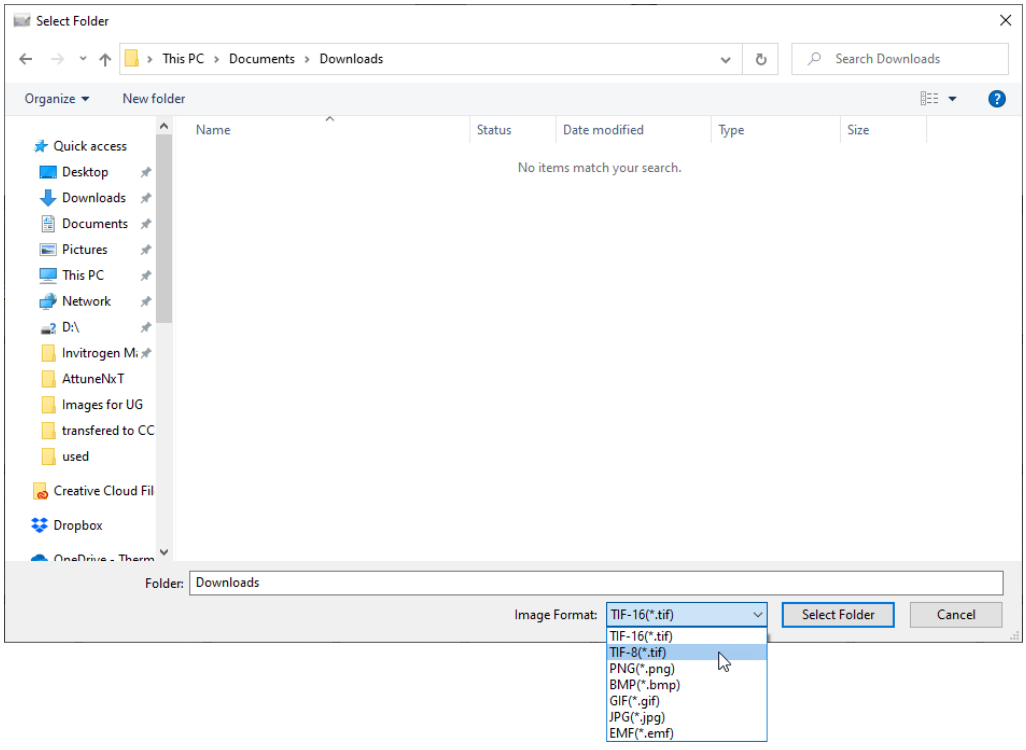
Export Images (from Experiment Explorer)

To export images for an Experiment, Group, or Sample, right-click the Experiment, Group, or Sample in Experiment Explorer, then select **Export Images**.



- When images are exported, all images for the selected sample(s) are exported into a single zip file.
- By default, images are exported as TIF-16 files, which is the native file format collected from the Attune™ CytPix™ Flow Cytometer.
- You can select to save the images as TIF-16, TIF-8, PNG, BMP, GIF, EMF, or JPG files in the **Save As/Folder Selection** dialog.

IMPORTANT! When saving in any format other than TIF-16, images are converted to 8-bit images, which results in loss of imge pixel data.



Save Images (from Image View)

To save images from the **Image View** and **Image Gallery**, right-click the image, then select **Save As**.

- By default, the selected image is saved as a TIF-8 file.
- You can select to save the images as TIF-16, TIF-8, PNG, BMP, GIF, EMF, or JPG files in the **Save As/Folder Selection** dialog.

  Note: TIF-16 is the native file format collected from the Attune™ CytPix™ Flow Cytometer. Saving images in any other format results in loss of image pixel data.
- If images have been processed and mask overlays are turned on, images saved from the gallery or image view have the mask data overlayed on the images.
- If multiple images are selected, selected images are saved as individual image files.

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Revision history: Pub. No. MAN0028531 A.0

Revision	Date	Description
A.0	15 February 2023	Initial release.

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