LAS AF 2D Analysis

LAS AF 2D Analysis User Guide

Living up to Life

LAS AF 2D Analysis User Guide

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Contents

Image Analysis

LAS AF 2D Analysis is an optional, licensed software module for image processing and analysis in quantitative microscopy. It comes in the following product variants:

- **2D Analysis**: The standard module

- **2D Analysis Multi Channel Extension**: Extends the functionality of 2D Analysis, allowing you to process multi-channel images

- **2D Analysis Classifier**: Extends the functionality of 2D Analysis or 2D Analysis Multi Channel Extension, allowing you to classify measured objects according to user-defined criteria

LAS AF 2D Analysis allows you to:

- Measure parameters (e.g. size, shape, position, orientation, intensity) for individual features (e.g. cells, fibres, nodules, particulates, pores)

- Measure the area percent, total area, perimeter and other parameters that are summed for the entire Field of View (e.g. bone sections, reflective minerals, tissue sections)

- Analyse multiple images and accumulate their data

- Show a list of selected parameters for all features measured

- Calculate a range of statistics

- Create histograms to display the distribution of sizes and shapes

- Export data to Excel to create user-defined reports

The combination of digital camera and microscope control makes **2D Analysis** the best application for automatic measurement in a diverse range of imaging tasks, including:

- Analysis of the size distribution of porosity
- Characterisation of the shape of a population of features
- Measurement of particulate dimensions
- Counting powders from pharmaceutical preparations
- Analysis of fibre cross-section

Starting the Image Analysis module

LAS AF 2D Analysis is part of the **Analysis** Workflow.
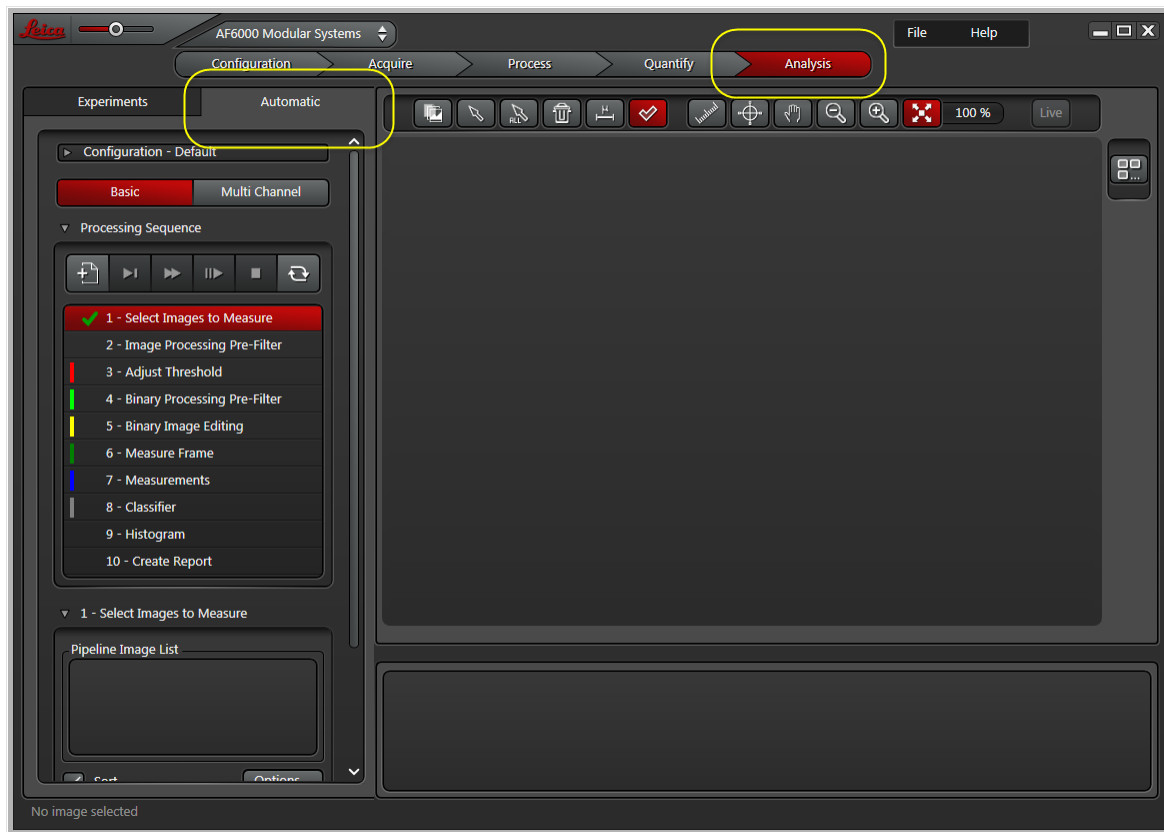
To start using 2D Analysis:

1. Click on the **Analysis** Workflow.

2. Display the **Experiments** tab and [open]19 any Experiments and images that you want to process.
   Note: Images used in the same Processing Sequence must be of the same resolution, dimensions, file type and bit depth.

3. Display the **Automatic** tab.

4. Set up the LAS AF Display Window using the tools (see below).

(Continued on next page)

LAS AF Display Window tools

Use the LAS AF Display Window tools to control how images are displayed in the LAS AF Display Window.

1. **Comparison View**: When enabled, the Viewer is split into two panes with the original image on the left and the processed image on the right.

2. **Lock View**: If you zoom in sufficiently on the LAS AF Display Window, scroll bars are automatically displayed. Enable **Lock View** to automatically synchronise both displays in Comparison View mode: scrolling in one view is reflected in the other view.

3. **Original/Result from previous step**: When Comparison View is enabled, toggling this option displays either the processed image from the previous operation or the original (unprocessed) image. Use this option to quickly compare processing steps.

4. **Hide Background Image**: Hides (masks) or reveals the underlying processed image to show only those areas that have been modified. Hiding the background image displays only the masked pixels.

5. **Show Binary Masks**: Toggles between showing Binary Mask and Binary Mask Outline. Highlights the processed areas (those falling within the threshold values) in your chosen color (the default is red). Use this option to quickly show the effects of binary editing. You can select different highlight colors for the following steps: Adjust Threshold, Binary Processing Pre-Filter, Binary Image Editing and Measure Frame.

Methods of working

LAS AF 2D Analysis works on images that have already been captured or imported (in **Acquire***); 2D Analysis itself cannot capture original images. You can use individual images, or part of an image sequence captured with another module. Use the [Processing Sequence][]13 control panel to decide how to handle your different images.

You can work with single images, image series, multi-channel images and z-stack (vertical spatial) image series.

The image detail to be processed must be different in either contrast or color from the background, and should be evenly illuminated (use shading correction).

The way you use **LAS AF 2D Analysis** depends on the type of image being processed and the features to be measured:

LAS AF 2D Analysis

Use LAS AF 2D Analysis to analyse a single type of object that can be differentiated by intensity or colour.

Using the Pre-processing and threshold tools, objects to be measured can be detected and the measurement results displayed. Typical applications range from simple counting to measurement of area, shape factor, intensity etc.
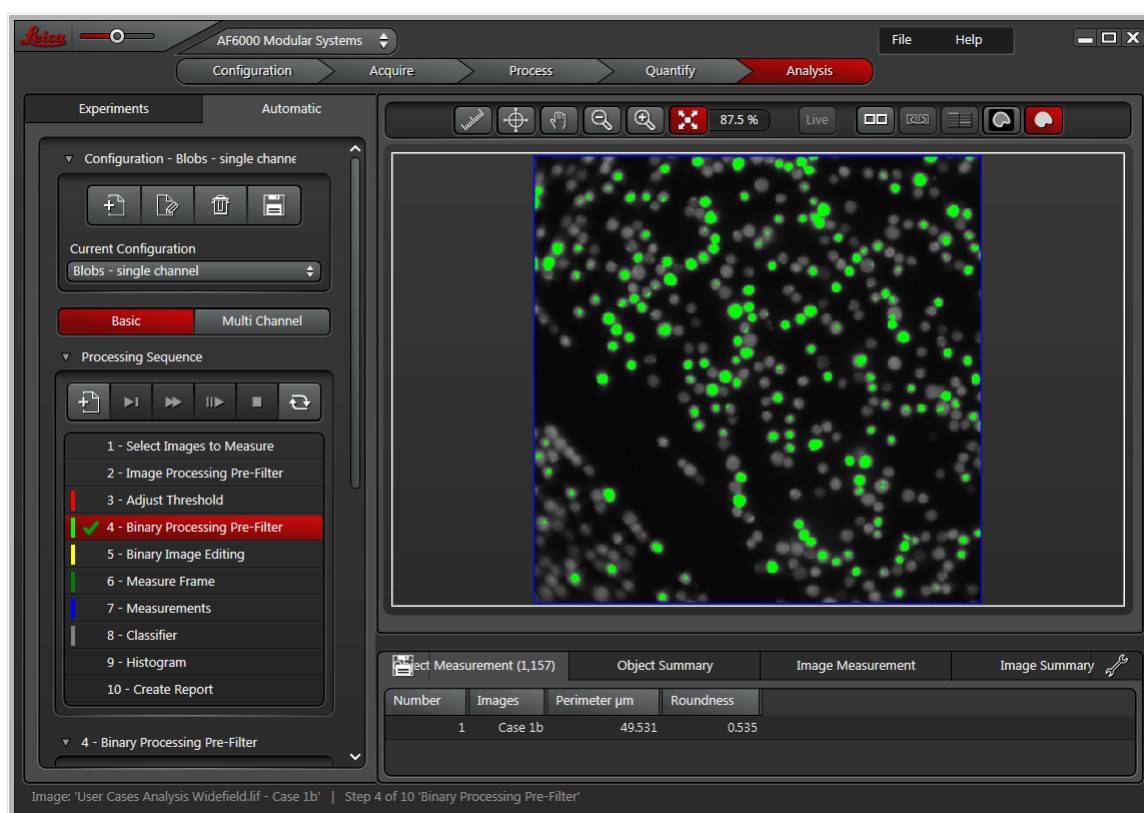
(Continued on next page)

LAS AF 2D Analysis Multi Channel Extension

Use the 2D Analysis Multi Channel Extension to analyse images with a number of different channels, such as different fluorescence labels. A typical requirement would be to define the cells that are labelled in one channel and count the number of spots within each of those cells. Using the powerful tools in the module you can define a threshold in one channel to detect the cells, and define a threshold in a second channel to detect the spots. Then with the Binary Operations tool the masks can be combined using logical operators to count only the spots that are located within cell boundaries.
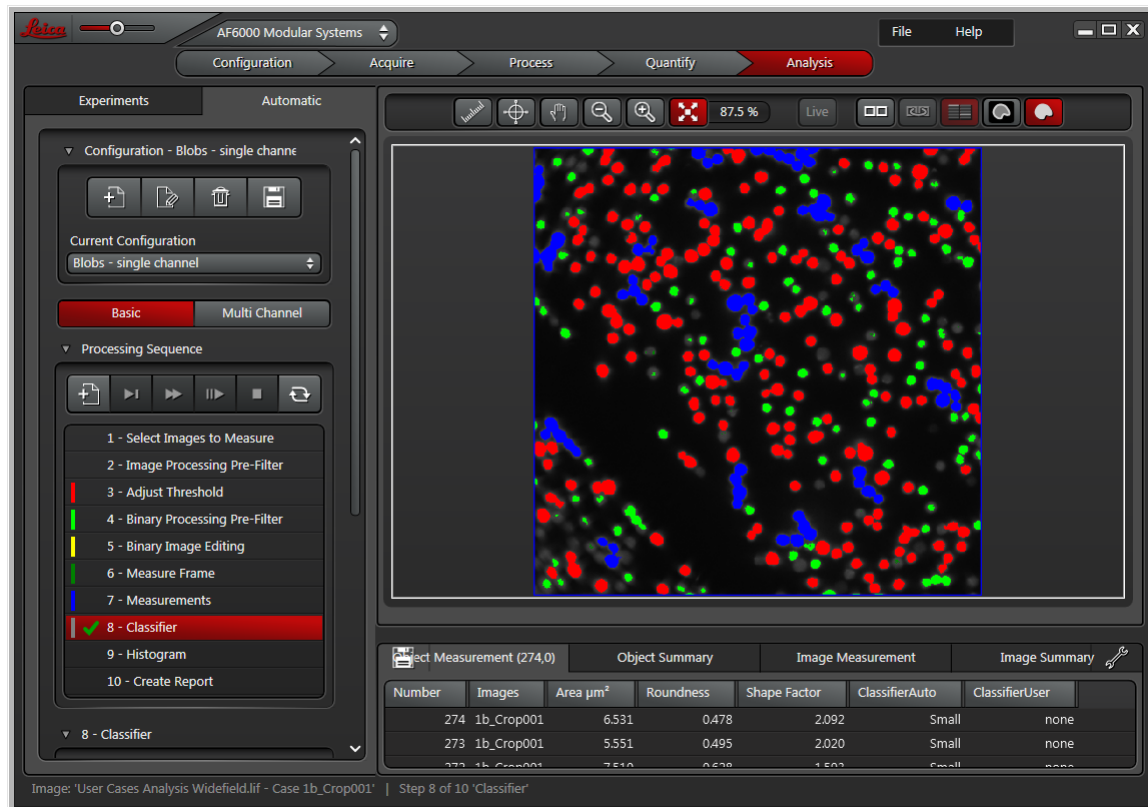
(Continued on next page)

LAS AF 2D Analysis Classifier

Use the 2D Analysis Classifier to recognise patterns in complex data sets, and to group objects according to various measured parameters.

A skilled human operator may, by eye, be able to group features in an image into different classes. However, there may be no simple mathematical rule to separate features into classes. For instance, it might not be enough to find all features greater than a certain area; In such cases, the 2D Analysis Classifier can help you make informed decisions based on multiple measurement parameters.

Working with Configurations

The **Configurations** panel is available at the top of the **Automatic** tab, during every step of the Image Analysis process. It allows you to save the current settings for most tools and panels as a named Configuration. You can then use the same measurement settings for different image sequences.
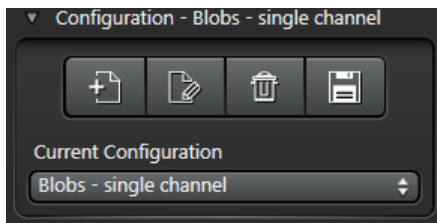
Selecting an existing Configuration

To select an existing (saved) Configuration:

1. Display the **Current Configuration** drop-down menu.
2. Click to select a named Configuration.

Managing configurations with the toolbar

1. **Create New Configuration**
   Saves all the current settings to a Configuration with your chosen name.

2. **Edit Selected Configuration**
   Renames the existing Configuration file.

3. **Delete Selected Configuration**
   Removes the current Configuration from the drop-down list.

4. **Save Selected Configuration**
   Saves any class and properties changes you have made to the current Configuration.

Processing Sequence

The **Processing Sequence** applies your chosen tools and settings automatically to groups or sequences of images.

If you are working with multi-channel images and have purchased and installed the **LAS AF 2D Analysis Multi Channel Extension**, you can set up parallel sequences for each channel.
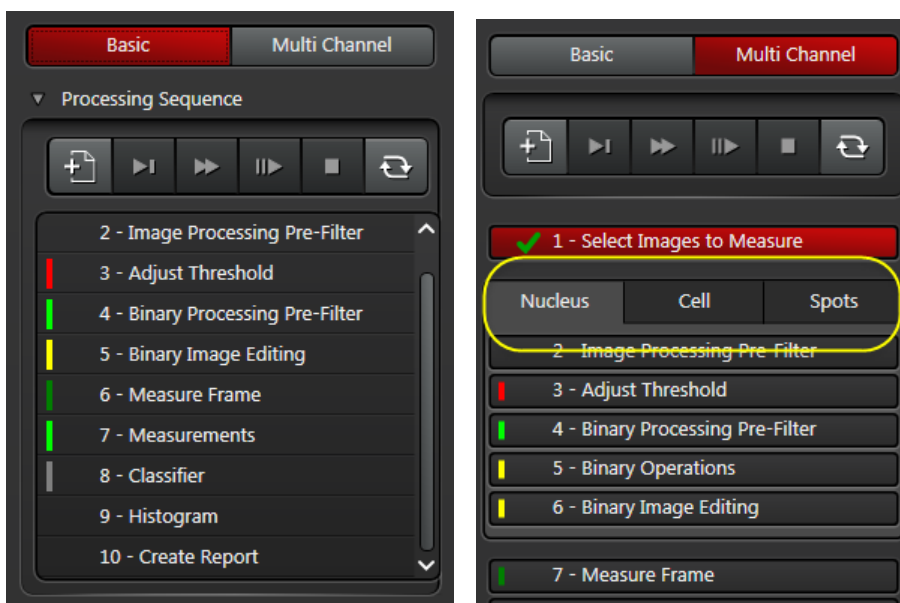

The colored bars next to each item indicate the color used to highlight different features in the LAS AF Display Window (color is specified on the appropriate control panel).

Basic procedure

LAS AF 2D Analysis is designed to make each step as simple as possible. The **Processing Sequence** control panel guides you through the acquisition, detection and measurement steps; once defined, you can use these steps again and again.

1. Work through the analysis steps, in order, on a single image (you can click on a step in the **Processing Sequence** control panel, or click the **Next Measurement Step** button in the toolbar (see below).

   The settings for each step are remembered when you subsequently run the sequence (note that some images in a sequence may require [different settings](#) [17]).

2. If necessary, you can program a pause at any step in the processing sequence, so that you can refine an image – to delete or separate features for example – before continuing.

3. Optionally, choose to generate and display a comprehensive report when processing is complete.

4. Save the Processing Sequence as a **[Configuration](#)** [12], so that you can run it again at a later date.

5. Automatically process all the images using the saved settings.

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Sequence steps

Generally, you follow the order of steps in the Processing Sequence panel, but you can jump between steps without losing settings.

- **Select Images to Measure** [18]: Choose the image folders, groups and sequences

- **Image Processing Pre-Filter** [26]: Improve and clarify features to measure

- **Adjust Threshold** [29]: Isolate features and create a binary output image prior to measurement

- **Binary Processing Pre-Filter** [36]: Fill holes and fissures in features

- **Binary Operations (Multi-Channel only)** [42]: If you have the 2D Analysis Multi Channel Extension, filter measurement results by creating masks, using logical operations on selected channels

- **Binary Image Editing** [44]: Add, remove, select and deselect features, draw and fill shapes, and group objects

- **Measure Frame** [50]: Create a frame to contain the features to be measured

- **Reference Mask** [53]: If you have the 2D Analysis Multi Channel Extension, choose an Analysis Channel (in a multi-channel image) to act as a reference for which measurement will be derived from other channels

- **Measurements** [54]: Select and configure the results to display in the Grid and Report [86]; count and measure the selected features

- **Classifier** [63]: Train LAS AF 2D Analysis Classifier to automatically classify objects according to a combination of measurement parameters (e.g. Roundness, Area)

- **Histogram** [77]: Display the measurement results graphically

- **Create Report** [86]: Configure, display and print the Report data and images.

Processing Sequence toolbar
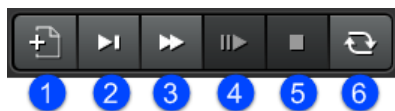
1. **Start a New Measurement** [15]: Clear all existing sequence information.

2. **Next Measurement Step**: Stop the automatic sequence so that you can proceed one step at a time. Click the **Run** button to resume automatic processing.

3. **Start Running Measurement Steps**: Start the current sequence.

4. **Continue from Present Measurement Step**: Resume the automated sequence (process all images using the current settings).

5. **Stop running Measurement Step**: Interrupt the current sequence (without processing all the files). Click **Yes** to confirm.

6. **Update**: Refresh the processing sequence if you make any changes to individual images.

(Continued on next page)

Starting a new Measurement

To start a new Processing Sequence:

1. Click the **Start a New Measurement** button. Note that this will delete all existing measurements.

2. Click **Yes** to confirm.
3. Click **Select Images to Measure** (step 1 in the **Processing Sequence** panel).
4. If working with multiple images:
    o   Append all the images that you want to process so they appear in the Pipeline Image List. See Select Images to Measure [18] .
    o   Select a typical image from the **Pipeline Image List**.
5. Apply the processing tools to the image. As you work, a green tick mark appears to the left of the current step to indicate normal processing with the current settings.
    You can also Pause (green pause icon) and Skip (red cross icon) steps.

6. If using a multi-channel image and you have the 2D Analysis Multi Channel Extension, repeat the steps for each channel. Steps are numbered accordingly. (In the example below, two channels have been set up [24] for measurement.)
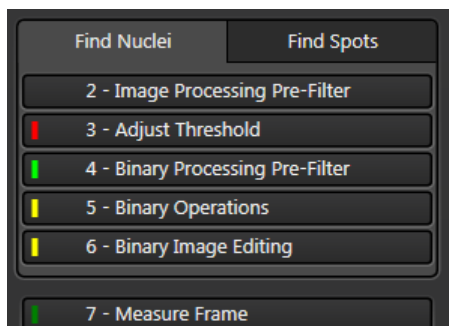
7. Configure the other steps in the sequence, such as **Measure Frame**, **Reference Mask** and **Histogram**.
8. If required, pause or skip one or more steps in the sequence (see below).
9. Save the sequence settings as a Configuration [12] .

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Pausing the sequence

Some images may require specific additional processing – for instance, you may need to remove artefacts on an image-by-image basis using the **Binary Edit** tool.

To pause an automatic image processing sequence at the same step for every image:

1. Click to the left of the step name in the **Processing Sequence** control panel to display the green Pause symbol (each time you click, the status toggles between Pause, Skip and Normal).
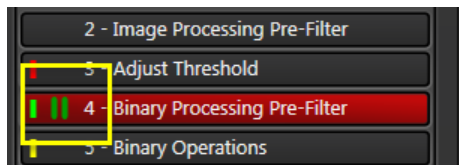
2. Run the measurement sequence: click the **Start Running Measurement Steps** button in the toolbar.

3. The sequence will pause at this step for the first image, allowing you to make changes.

4. Resume the sequence (click the **Continue from Current Measurement Step** button in the toolbar.

5. Each time the sequence pauses, make the necessary changes then click the **Continue from Current Measurement Step** button, until the measurement sequence is complete.

Skipping a step

You can skip any of the following steps:

- Image Processing Pre-Filter
- Adjust Threshold
- Binary Processing Pre-Filter
- Binary Operations (2D Analysis Multi Channel Extension)
- Binary Image Editing
- Classifier (2D Analysis Classifier)
- Create Report

To skip a step:

1. Click to the left of the step name in the Processing Sequence control panel to display the Skip symbol (a red cross); each time you click, the status toggles between Pause, Skip and Normal. The step text will be shown in italic.
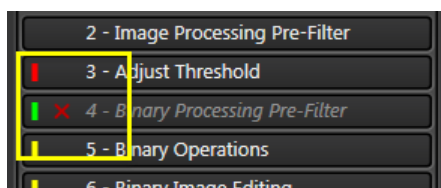
2. Run the measurement sequence: click the **Start Running Measurement Steps** button in the toolbar.

Images with Different Settings

You might need to apply different filters or settings to images in a sequence or group.

For example, consider a sequence containing images A, B, C and D.

1. Select image A and set up the filters and parameters.

2. Run the sequence. At this stage, the settings you made to image A will apply to **all** images in the sequence.

3. Now select image B and modify a setting (e.g. the Threshold).

4. Click the **Update**[13] button on the Processing Sequence toolbar to apply the changes to the Processing Sequence.

5. Run the sequence again.
   In this example, only the changed Threshold will apply to image B; all other settings (for all images in the sequence) are as defined for image A.

Notes

- If you need to change a setting for every image in a sequence, it is better to Pause[13] the processing sequence at this step.

- If you want to apply different settings to channels in a multi-channel image, you need to purchase and install the 2D Analysis Multi Channel Extension.

Select Images to Measure

This panel allows you to select the images to be analysed in your Processing Sequence and set up some options.
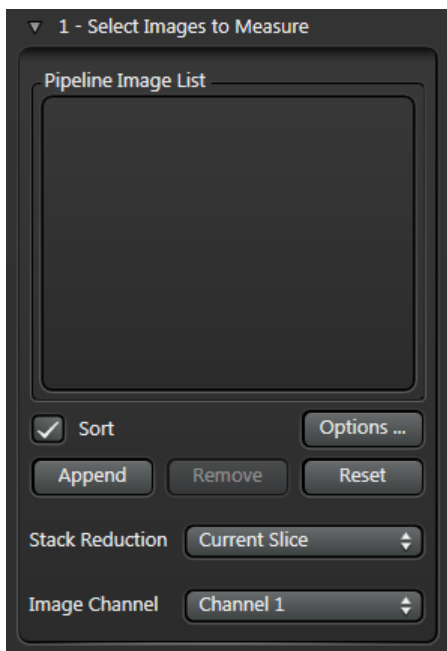
Note: Images used in the same Processing Sequence must be of the same resolution, dimensions, file type and bit depth.

Basic procedure

If you only want to process a single image, you do not need to **Append** it to the Pipeline Image List; 2D Analysis automatically processes the image currently displayed in the LAS AF Display Window.

1. Load[19] one or more images to the Pipeline Image List.
2. Sort[22] the images in the Pipeline Image List.
3. Click the **Options** button and set up any **Batch Process Settings**[21].
4. If the image series contains a Z stack, select a **Stack Reduction**[23] method from the drop-down menu.
5. If the image has multiple channels, select a **Channel** from the drop-down menu; this will be used for image analysis. (See here[24] if you want to work in **Multi Channel mode**.)

See also:

Loading images for analysis[19]

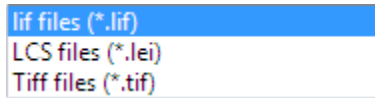Batch Process Settings (Options)[21]

Sorting and removing images[22]

Stack Reduction[23]
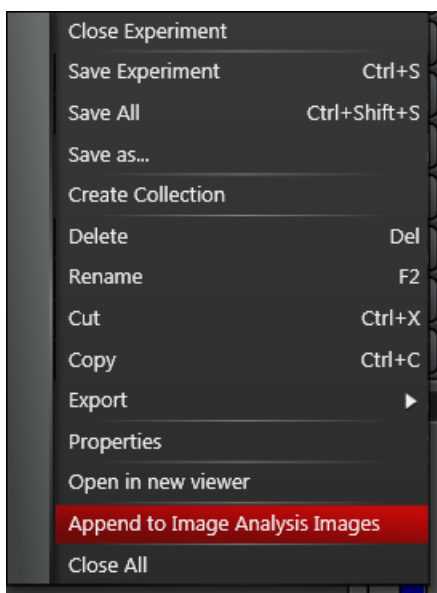
Multi Channel mode operations[24]
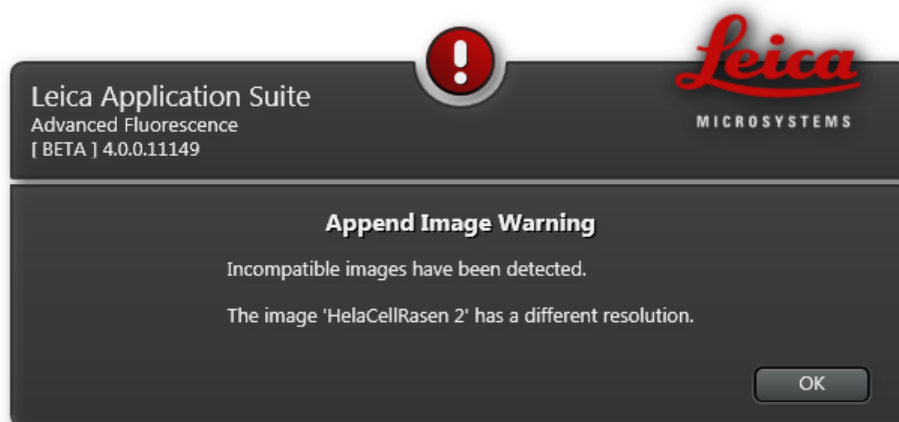
Loading images for analysis

To load images for analysis:

1. Switch to the **Experiments** tab.

2. Click **Open** and use the standard Windows file browser to load a lif, LCS or Tiff file:

lif files (*.lif)
LCS files (*.lei)
Tiff files (*.tif)

3. Repeat the previous step if you want to add more images into the Experiments tab.

4. You can load one or more images from the **Experiments** tab into your Image Analysis Processing Sequence:

   o To load just one image into Image Analysis, select it in the **Experiments** tab then display the **Automatic** tab; The image name will not appear in the Pipeline Image List, but the image will be displayed in the LAS AF Display Window. (You can click **Append** to add the image to the Pipeline panel, but it is not necessary.)

   o To load multiple images from the Experiments tab, right-click on each image in turn and choose **Append to Image Analysis images** from the drop-down menu, then display the **Automatic** tab.

Close Experiment	
Save Experiment	Ctrl+S
Save All	Ctrl+Shift+S
Save as...	
Create Collection	
Delete	Del
Rename	F2
Cut	Ctrl+X
Copy	Ctrl+C
Export	▶
Properties	
Open in new viewer	
Append to Image Analysis Images	
Close All	

   ▪ You will see an error message if an image does not have the same resolution, dimensions, file type or bit depth as the first image appended.

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Append Image Warning

Incompatible images have been detected.

The image 'HelaCellRasen 2' has a different resolution.

OK

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- All the image names will appear in the Pipeline Image List in the **Select Images to Measure** panel on the Automatic tab.
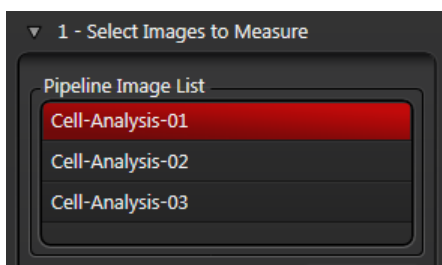
Batch Process Settings (Options)

You can make some global Image Analysis settings:

1. Click on the **Select Images to Measure** entry in the **Processing Sequence** panel.

2. Click on the **Options** button in the **Select Images to Measure** panel.

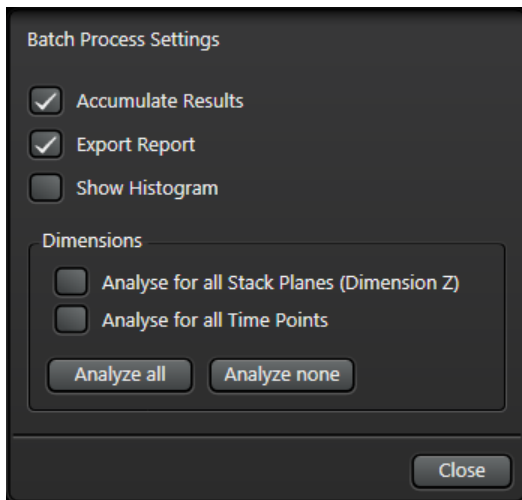3. Set up the options on the **Batch Process Settings** dialog.

4. Click **Close**.

The options are as follows:

- **Accumulate Results**: Produce a continuous list of results for all images.
  Disable this option to speed up processing if there is a large number of images in the sequence.

- **Export Report**: The results of the Processing Sequence will be loaded automatically to an Excel spreadsheet[86] when processing is complete.

- **Show Histogram**: Display the Histogram[77] during the Processing Sequence for each image (the **Show Histogram** option on the Histogram[77] panel must be enabled).

- **Analyse for all Stack Planes (Dimension Z)**: (Only available for images that have a number of Z planes) If enabled, each Z plane will be analysed. If disabled, the image analysed will depend on the Stack Reduction[23] setting.

- **Analyse for all Time Points**: (Only available for images that have a time series) If enabled, all images in a time series will be analysed.

- **Analyze All**: Enables all options in the Dimensions panel.

- **Analyze None**: Disables all options in the Dimensions panel.

Sorting and removing images

To remove a single image from the Pipeline Image List

1. Click to select the image.
2. Click the **Remove** button.

To remove all images from the Pipeline Image List

1. Click the **Reset** button.

To sort the images in numerical order

1. Enable the **Sort** check box.

Stack Reduction

If the image series contains a Z stack, this can be reduced to a single image for analysis based on the method chosen:

- **Current Slice**: Analyses the currently selected Z slice

- **Maximum Projection**: Creates a single 2D image from the Z stack.

- **Best Focus**: Analyses the Z slice that is calculated to have the best focus

Note: In the Options [21] panel, if **Analyse for all Stack Planes** is enabled, the Stack Reduction setting is ignored (as all Z planes will be analysed).

Multi Channel mode operations

If you have 2D Analysis Multi Channel Extension, as well as all the operations available with 2D Analysis, you can define Analysis Channels and map them to Acquisition Channels. You can then set up different analysis workflows for each channel. This applies to all steps from Image Processing Pre-Filter[26] to Binary Image Editing[44].
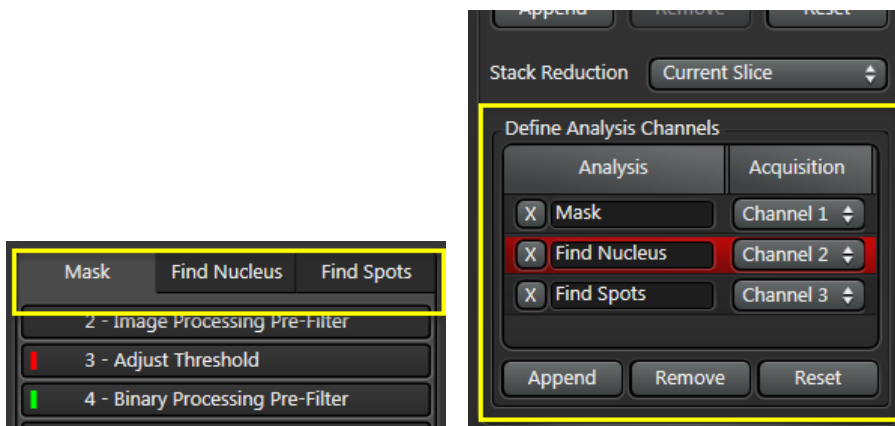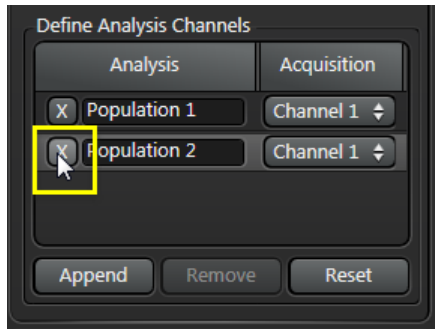
To define analysis channels

1. Click **Append** at the bottom of the **Define Analysis Channels** panel.
2. In the **Analysis** field, enter a descriptive name for the channel.
3. In the **Acquisition** field, select a channel to map from the drop-down menu.
4. Repeat to add more Analysis/Acquisition mappings.
5. This creates a separate processing stream for each image channel:

To remove an analysis channel

The simplest way to remove an analysis channel is to click on the cross icon next to its entry in the **Define Analysis Channel** panel.

You can also use the following method:

1. Click somewhere on the background of the channel's entry in the **Define Analysis Channel** panel. The entry will turn red to show that it is selected.
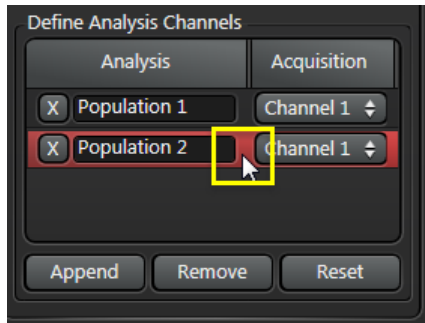
2. Click on **Remove**.

To reset (remove all) analysis channels

To remove all currently defined analysis channels, click **Reset** in the **Define Analysis Channel** panel.

See also:

Using a channel as a [Reference Mask](#)^{□ 53}

Image Processing Pre-Filter

The **Image Processing Pre-Filter** allows you to enhance feature recognition in color and monochrome images.

When Greyscale Pre-Filters are applied to an image, the original image is not affected at all – it remains intact on the hard drive. Applying a filter creates a Processed Greyscale Output Image in memory which is then passed on to other functions (such as Adjust Threshold[29]) for additional processing.
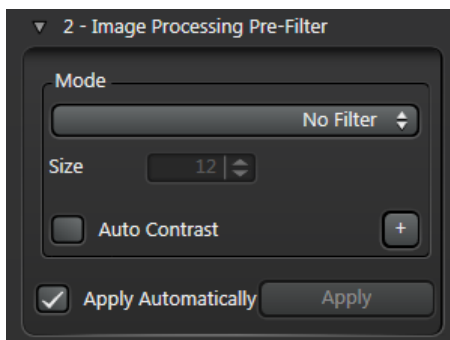
To use the Image Processing Pre-Filter

1. Click on the **Image Processing Pre-Filter** option in the **Processing Sequence** panel.

2. Select a pre-filter **Mode**[28] from the drop-down menu.

3. Set the structuring element matrix using the **Size** text box. Small increments are preferable, because there will be a point at which the image cannot be improved by making another 'pass'.

4. Decide whether to use **Auto Contrast**. This improves the contrast between features and background.

5. If you want to add extra filtering steps, click the plus button and set up the new filter as above.
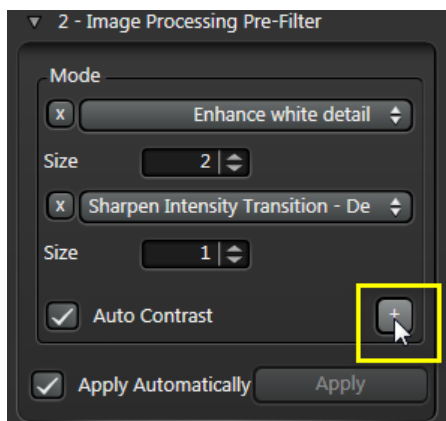
6. Decide whether pre-filtering results should be displayed interactively:

    o   **Apply Automatically** enabled: Each change in the settings is automatically reflected in the Output Image. This is the best setting for simple images.

    o   **Apply Automatically** disabled: Changes are only updated when you click **Apply**. This is better for more complex images that may take a little longer to process.

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To delete a filter

If there is more than one filter defined, click on the cross button ⊠ to the left of a filter to delete it.

See also

Image Processing Principles[91]

Examples of Pre-Filter usage[102]

Pre-Filter Modes

The Pre-Filters available using the **Mode** drop-down menu are as follows:

- **Smooth White Detail** brightens the Output Image by accentuating the lighter tones. This is an open filter – Erosion followed by Dilation.

- **Smooth Black Detail** darkens the Output Image by strengthening the darker tones. This is a close filter – Dilation followed by Erosion.

The illustration below shows original, **Smooth White** and **Smooth Black** images:
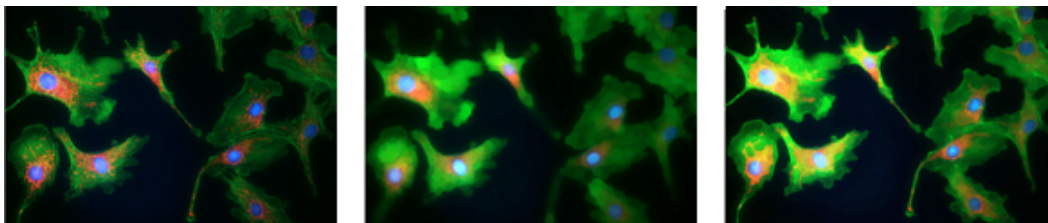

- **Enhance White Detail** accentuates the white – values approaching 255 – areas of the image.

- **Enhance Black Detail** improves the contrast between the black (values toward 0) and the lighter areas of the image.

- **Find White Detail Top Hat** detects and removes lighter detail from the image depending upon the size of the Structuring Element.

- **Find Black Detail Top Hat** detects and removes darker detail from the image depending upon the size of the Structuring Element.

The illustration below shows original, **Enhance White** and **Enhance Black** images, then **Find White** and **Find Black** top hat images:
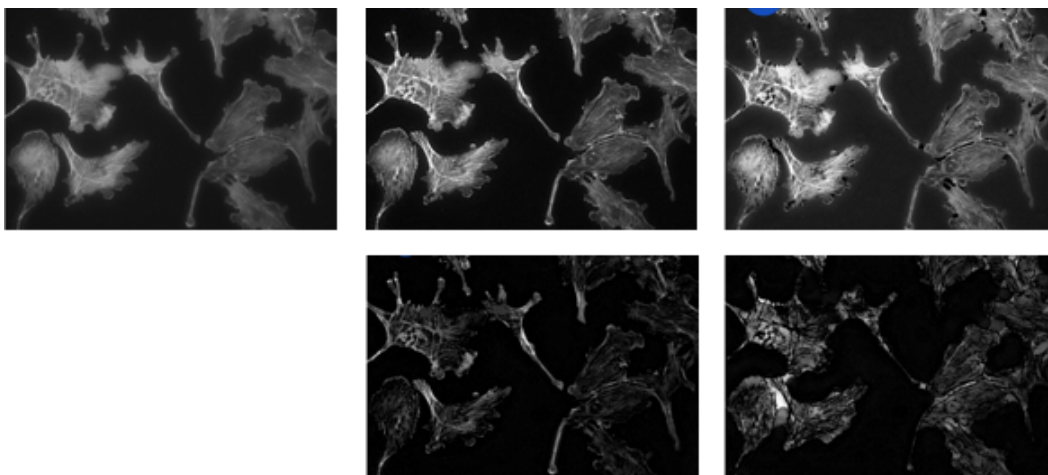
- **Sharpen Intensity Transition (Delineate)** removes intermediate grey tones to provide greater contrast between areas of the feature.

- **Noise Removal (Median)** filter removes artefacts down to pixel size from the image.

- **Sharpen Unsharp Mask** Sharpens the image without increasing the noise. A slightly blurred copy of the original image is created, which is subtracted from the original. This mask is effectively a high pass filter which, when combined with the original, increases the contrast at edges producing a sharper final result.
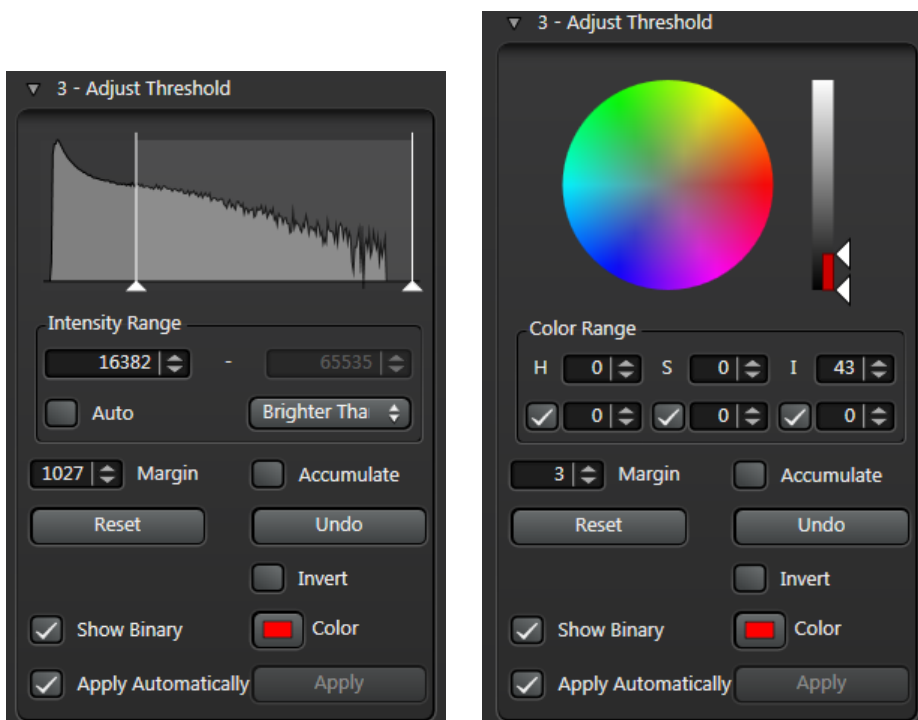
The illustration below shows original, **Sharpen Grey** and **Noise Removal** images:
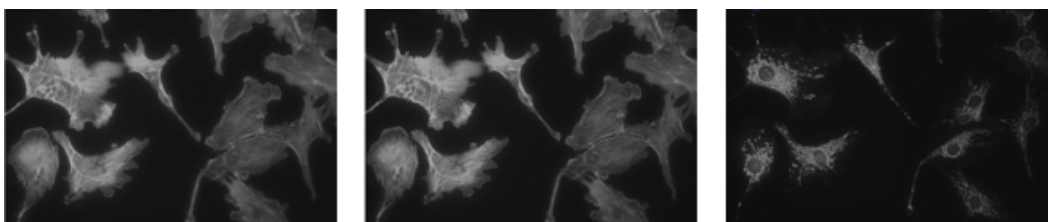
Adjust Threshold

Adjust Threshold allows you to select the features that you want to measure. LAS AF 2D Analysis recognises color and monochrome images, and displays appropriate tools during the Adjust Threshold step.

Both versions of the control panel have [common](#)□ 34 tools and buttons.

You can use the following methods to select features to be included in measurements:

- [Drawing a Region of Interest](#)□ 30 (color and monochrome images)
- [Using the Grey Range Selector](#)□ 31 (monochrome images only)
- [Using the Color Range Selector](#)□ 33 (color images only).

Note: You cannot add images that use different color modes to the same processing sequence.

Drawing a Region of Interest

LAS AF 2D Analysis recognises color and monochrome images, and displays the appropriate control panel during the Adjust Threshold step.

Note: You cannot add images that use different color modes to the same processing sequence.

To draw a Region of Interest (RoI):

1. Drag the pointer over a feature on the **LAS AF Display Window**. Any pixels within the drawn rectangle will be used to define the threshold.

2. To fine-tune your selection, use the appropriate greyscale or color controls:

    o [Using Intensity Range Selector](#) [31]

    o [Using the Color Range Selector](#) [33]

Using Intensity Range Selector

LAS AF 2D Analysis automatically detects monochrome images and displays the image histogram and **Intensity Range** selector in the **Adjust Threshold** panel.
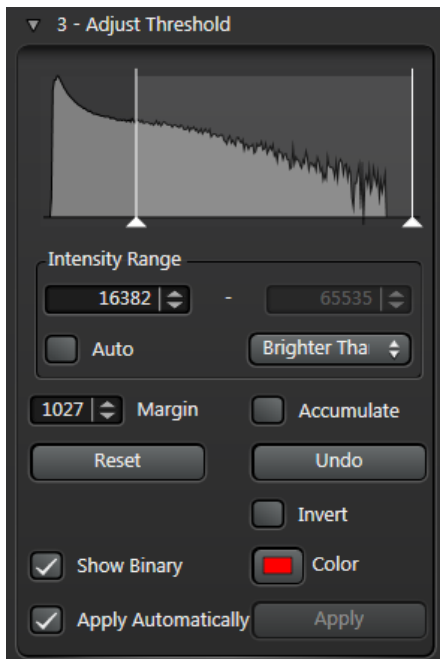
See also Common Tools [34] .

Using Auto mode

If the image has good contrast and well-defined features:

1. Click to enable **Auto** mode.

2. A new auto threshold will be computed for each image run through the workflow.

3. You can set an offset from the auto-calculated value by dragging the histogram sliders or entering a value in the Deviation field.

(Continued on next page)

Using the Intensity Selector

If you need greater control when setting the threshold:

1. Select a thresholding method using the drop-down menu.

   o **Darker than** selects all features at or below the entered intensity value

   o **Brighter than** selects all features at or above the entered value

   o **Range** automatically sets two values (Blacker than + 1 and Whiter than – 1) as a centre band.
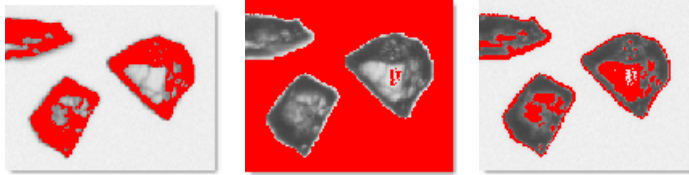
2. Fine tune the selection using any of the following methods:

   o Drag the histogram sliders

   o Enter values in the Intensity Range fields

3. Use any of the common tools 34 to optimise your feature selection and display.

Using the Color Range Selector

2D Analysis automatically detects color images and displays the **Color Wheel** and **Color Range** selector in the **Adjust Threshold** panel.

Use the **Color Wheel** to adjust and experiment with the Hue and Saturation values. *H=Hue, S=Saturation* and *I=Intensity.*

- **Hue** is measured along the Wheel's circumference; **Saturation** is measured along the Wheel's radius.

- The section of the Wheel outlined in black shows the current range of the Hue and Saturation settings used to select features for measurement.

- Drag the black outline of the *Wheel* segment to alter the **Hue** and **Saturation**; you can drag the radii (to alter the **Hue** boundaries), or the inner and outer arcs (to alter the **Saturation** boundaries); the actual values are displayed in the **Color Range** windows

- Drag the **Intensity Bar Threshold** pointers to alter the **Intensity** boundaries

- Fine-tune all three parameters using the Up/Down arrows to the right of the **Color Range** windows. Each pair of windows represents the span of values for that parameter

- You can disable one or two (but not all three) HSI check boxes while adjusting the other parameters. For example, it is often simpler to ignore the color saturation, since you are interested in the predominant color.

See also [Common Tools](#)⌐ 34 .

The following Threshold tools are common to both greyscale and color images.

Margin

Use the **Margin** up/down arrows to adjust the spread of selected threshold values.

For example, with Tolerance set to 5, a threshold value of 12 on the image would actually include all values in the range 7 to 17 (i.e. 12 ± 5).

Accumulate

The **Accumulate** tool adds the values of successive drawn regions to extend the Threshold range, including more features.

Reset and Undo

- **Reset**: Clears all Threshold values
- **Undo**: Removes the last pass.

Invert

Providing the background is reasonably uniform and has color content distinct from the 'target' features, the **Invert** tool can make selection much simpler.

Specimens that have complex detail can be time consuming to select. The seeds in *i*mage (A) display a wide color range, detailed textures and highlights. Drawing several **Regions of Interest** may still fail to pick up all of the variations - image (B)*.*

Providing the background is reasonably uniform and has color content distinct from the 'target' features, the *Invert* tool can make it much easier to select features. The principle is to select the background (not the features) and then to invert the selection so that the features are selected and the background is ignored.

1. Click the **Reset** button the clear any previous selections.
   Image C: Click and drag to draw a *Region of Interest* over an area of the background. If Interactive is disabled, click the **Apply** button.
   Image D: The background selected - red in this example.

2. Click to enable the **Invert** check box.
   Image E: The features are selected and the background deselected.

Note: If the background is very uniform, enable the *Invert* check box *before* drawing the **Region of Interest** on the background and the features will be selected directly.
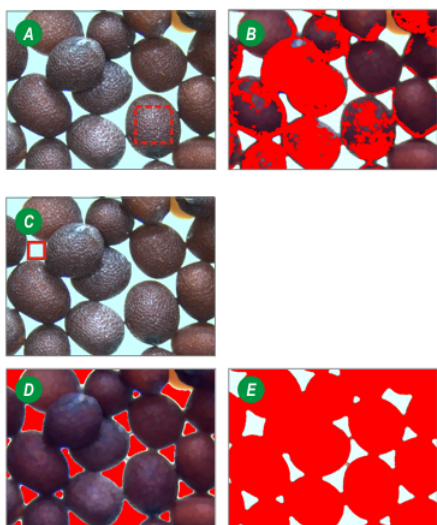

(Continued on next page)

Show Binary

When the **Show Binary** checkbox is enabled, features selected for measurement are displayed in the right-hand side of the **LAS AF Display Window**. You can change the default highlight color (see below).

Color

You can change the color used to display the selected pixels in the **Binary Output Image**. For example, if the features you are trying to measure are red, you might want to change the highlight Color to a bright blue so the features are easy to distinguish.

1. Click on the **Color** button.
2. Use the standard Windows color dialog to choose a new highlight color.
3. Click **OK**.

The illustration below shows the effect of changing the binary from red to green.
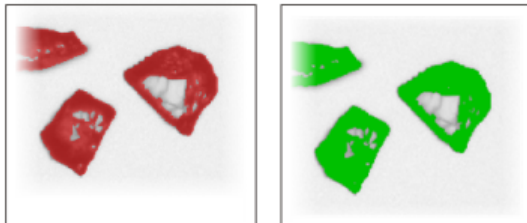
Don't forget, you can right-click in the **LAS AF Display Window** to temporarily disable the highlighted features, to help you see whether you have set your threshold correctly.

Interactive

- **Interactive** enabled: Each change in the settings is automatically reflected in the Output Image. This is the best setting for simple images.
- **Interactive** disabled: Changes are only updated when you click **Apply**. This is better for more complex images that may take a little longer to process.

Binary Processing Pre-Filter

The **Binary Processing Pre-Filter** uses the same techniques as the [Image Processing Pre-Filter][26] for improving and modifying images. However, instead of working with values ranging from 0 to 255, only binary pixel values of 1 and 0 are used.
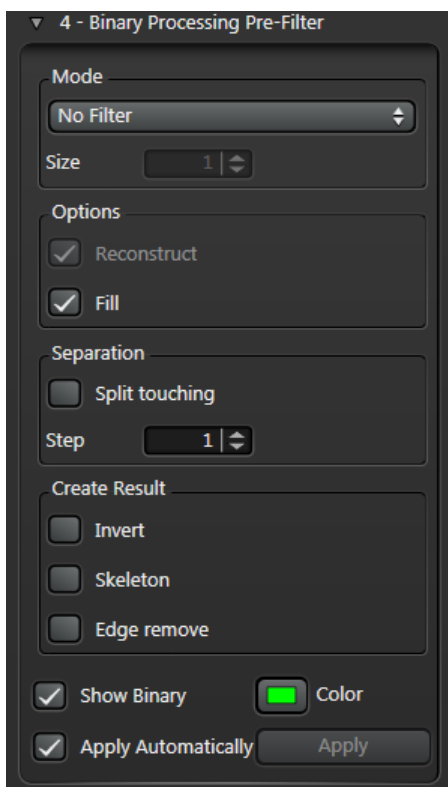
Usually, the image presented to the **Binary Processing Pre-Filter** has passed through the [Image Processing Pre-Filter][26] and [Adjust Threshold][29] stages.

If you need to learn about the binary processing principles in detail, see [this topic][110] .

To use the Binary Processing Pre-Filter

1. Click on the **Binary Processing Pre-Filter** option in the **Processing Sequence** panel.

2. Select an active filter from the **Mode**[37] drop-down menu.

3. Enable or disable tools in the **Options**[39] panel.

4. Set up the **Split touching**[40] tool, which helps to separate overlapping or touching features.

5. Decide which of the **Create Result**[41] tools to use; these allow you to modify the Binary Input Image in very specific ways.

6. Use **Show Binary** to preview the effects of binary editing. (You can also right-click in the **LAS AF Display Window** to temporarily disable the highlighted features.)

7. You can change the **Color** used to display the selected pixels in the Binary Output Image.

8. Decide whether pre-filtering results should be displayed interactively:

   o **Apply Automatically** enabled: Each change in the settings is automatically reflected in the Output Image. This is the best setting for simple images.

   o **Apply Automatically** disabled: Changes are only updated when you click **Apply**. This is better for more complex images that may take a little longer to process.

2D Analysis has the following Pre-Filter modes:

Discard Detail - Open

The **Discard Detail - Open** filter performs a sequence of erosion followed by the same amount of dilation. This cleans the image by removing small objects.

We will use an image of diamond chips as an example.

- The darker areas have been enhanced with the Image Processing Pre-Filter[28] > **Smooth Black Detail**.

- Applying **Adjust Threshold** has selected most of the darker areas but also included some mid-grey tones that are not required.
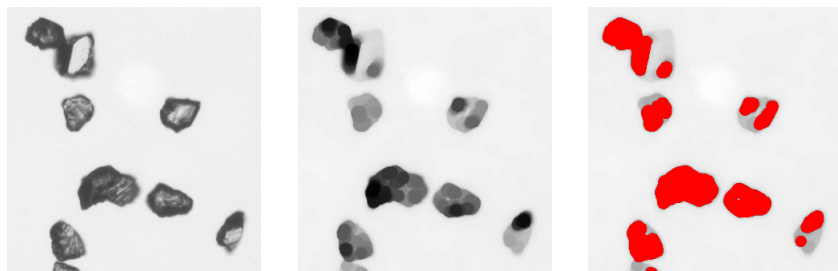

1. Using Binary Pre-Filter in **Discard Detail - Open** mode, we gradually increase the Size of the Structuring Element to shrink the selected areas, starting with the lighter tones, until only those required for use in measurements are highlighted.

   The selected feature areas are highlighted in green, shown below with Size settings of 10, 20, then 30 (this retains only the very dark areas, which was the aim of using the Discard Detail filter):
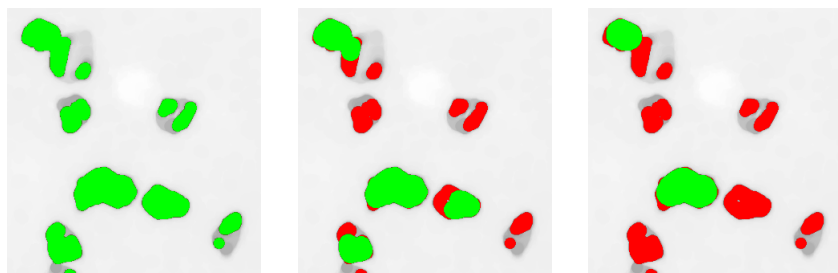
2. A quick Measurements > Number (Count) check reveals that only the required features are included.
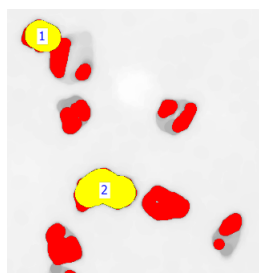
However, a part of the image has been rejected – possibly because two diamond chips at the top left were overlapping; this needs to be included without drawing in any other lighter features. See Reconstruct[39].

Combine Detail - Close

The **Combine Detail - Close** filter performs a sequence of dilation followed by the same amount of erosion. This removes fissures in features, leaving them well defined and ready for measurement.

1. Select the **Combine Detail - Close** filter from the **Mode** drop-down menu.

2. Gradually increase the Structuring Element[110] **Size**.

(Continued on next page)

Open and Close

The **Open and Close** filter performs a sequence of dilation followed by the same amount of erosion. This removes unwanted small objects, along with fissures in features, leaving them well defined and ready for measurement.

1. Select the **Open and Close** filter from the **Mode** drop-down menu.
2. Gradually increase the Structuring Element **Size**.

Grow Objects/Outlines without Merging

The **Grow Objects without Merging** and **Grow Outlines without Merging** filters increase the object size/ outline by the specified amount while keeping the objects separate.
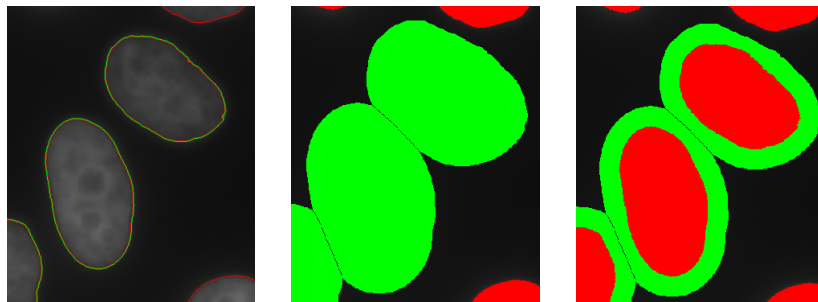
Reconstruct

The **Reconstruct** tool targets small, closely-related areas to be included in measurements.
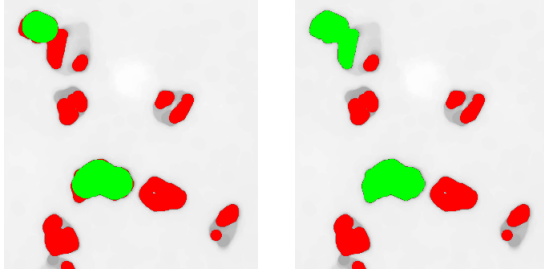
In the example above, the previously excluded area at the top left of the image is included; while this would not affect a Number (Count) measurement, it would be significant if Area was being measured.

Fill

The **Fill** tool can improve the Binary Input Mask for features that have been left with holes and fissures after passing through the Image Processing Pre-Filter 26 and Adjust Threshold 29 stages. This fills the holes well but leaves some fissures open.
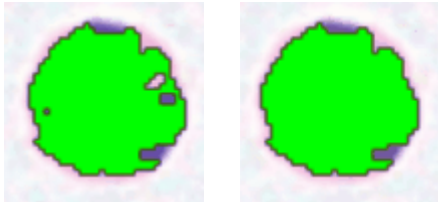

It is usually better use the Fill tool, rather than the **Threshold** 33 **Hue** and **Intensity** (which creates noise on the background).

Separation (Split touching)

The **Split touching** tool allows you to separate two or more discrete features that appear coalesced (either because the Threshold has been set incorrectly, or because they are actually overlapping):

1. Enable the **Split touching** tool in the **Separation** panel.
2. Gradually increase the **Step** value until a line appears, indicating that the features have been detected and separated.
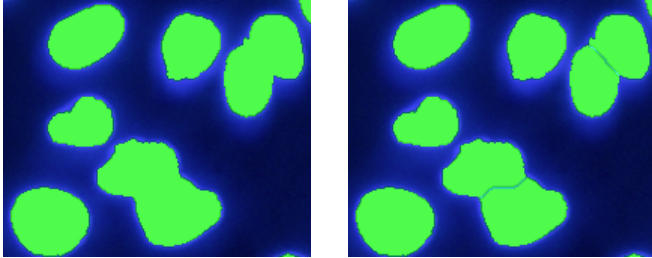
Invert tool

The illustration below shows part of a section through soft tissue.

1. The original image.

2. The image has been passed through the **Smooth White** greyscale filter and the **Threshold** filter to select the predominantly white area of the image. The selected areas are shown colored green.

3. Enabling the **Invert** check box deselects the previously selected areas – now displayed in a different color – and selects everything else. It is essentially a 'swap' tool.
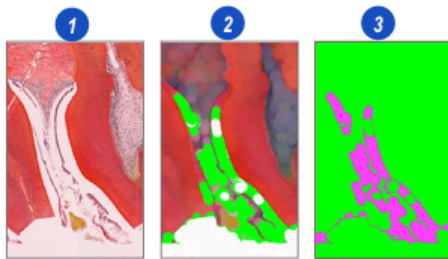
### Skeleton tool

Enabling the Skeleton tool erodes selected features to the point where only a 'backbone', just 1 pixel wide remains.

The illustration below shows wool fibres 'traced' using the Skeleton tool.
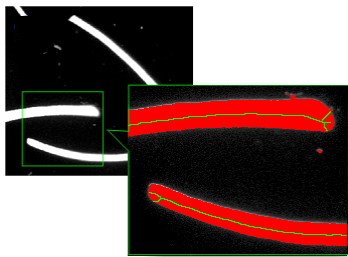

Edge Remove tool

The Edge Remove tool excludes from measurement any objects that are not completely within the image boundary. Such objects are colored red in the Binary Output Image.
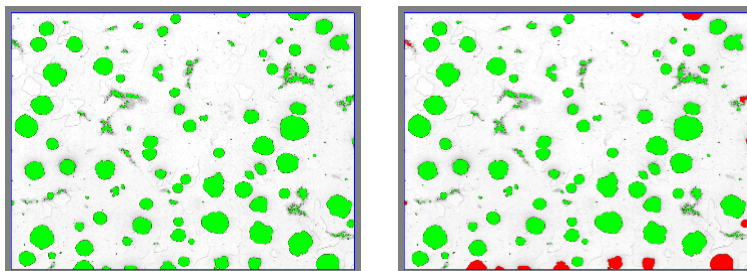
Binary Operations (Multi-Channel only)

Measurement results can be filtered using a binary mask. When processing multi-channel images, you can create a complex mask by combining the mask created in the [previous step](#)[36] with a mask from another channel, using logical operations on selected channels. You can combine multiple masks (using the **Append** button).
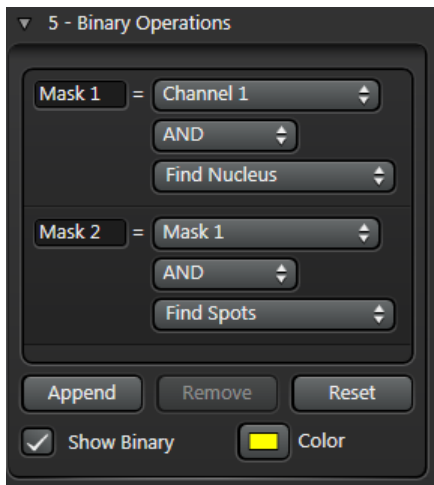
To create a simple mask

1. Click on **Append** in the Binary Operations panel.
2. Enter a name for the mask (or accept the default).
3. Select the channel to use as the mask, from the drop-down menu.
4. Select the binary operation to use on the channels. Choose from AND, OR, XOR, AND NOT.
5. Select the channel that is to be filtered using the mask.

To create a complex mask

1. Click on **Append** in the Binary Operations panel.
2. Enter a name for the mask (or accept the default).
3. Select an existing mask as the first stage in the filter.
4. Select the binary operation to use. Choose from AND, OR, XOR, AND NOT.
5. Select another channel that is to be filtered using the masks.

(Continued on next page)

To remove a mask

1. Click somewhere on the background of the mask's entry in the Binary Operations panel. The entry will turn red to show that it is selected.
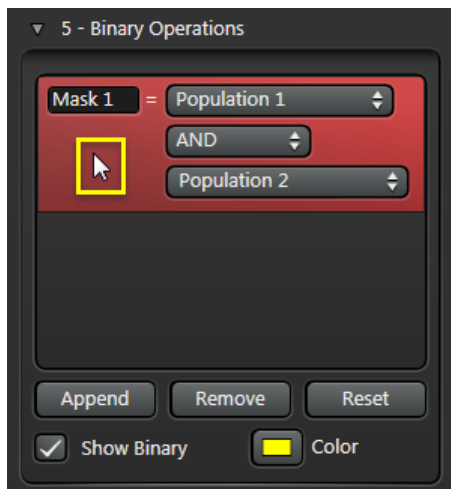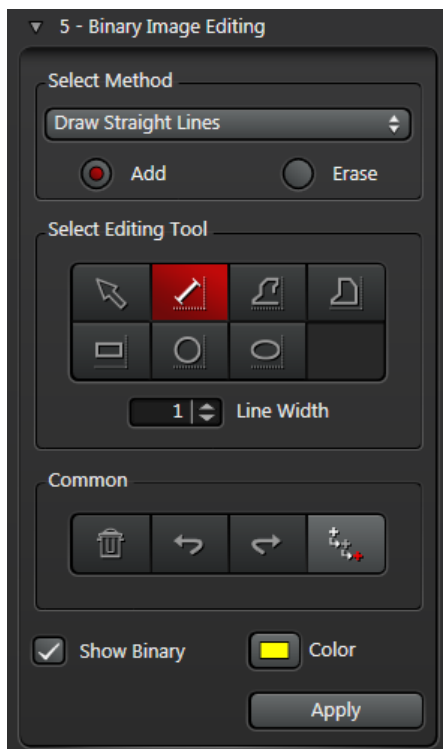

2. Click on **Remove**.

To reset (remove all) masks

To remove all currently defined masks, click **Reset** in the **Binary Operations** panel.

Binary Image Editing

Binary Image Editing tools allow you to specify which features from the **Binary Processing Pre-Filter** and **Adjust Threshold** steps are included in your measurements.

Binary Editing uses three separate images:

- The original image, which remains unchanged.

- The Binary Input Image, which is the result of the thresholding and binary processing steps, showing the features of interest.

- The Binary Output Image, which represents the edited image still in binary format.

Basic procedure

The basic procedure for Binary Image Editing is as follows:

1. In the **Processing Sequence** control panel, click on **Binary Image Editing**. The **Binary Image Editing** panel appears.

2. Use the **Select Method** pane to control the way in which the editing is performed.

3. Use the **Select Editing Tool** panel to add or erase features from your measurements. The tools available depend on which **Method** you choose.

4. Use the **Common** controls to undo and redo actions, delete all drawn regions, and control the cursor color.

5. Use the **Show Binary** check box to see the effect of your drawn shapes and selections before applying your changes.

6. You can change the **Color** used to display the selected pixels in the Binary Output Image.

7. Click **Apply** to merge all the drawings and selections with the Binary Mask.

(Continued on next page)

Common tools

1. **Delete All Drawings**: Remove any drawn shapes
2. **Next Undo**
3. **Next Redo**
4. **Toggle Cursor** color between red, black or white to suit the image.

Draw Straight Lines

You can draw straight lines on a Binary Image Mask to join or separate features that were not perfectly detected in previous processing steps. This method works in one of two modes:

- **Add mode**: Use this to join features that may have been cut as part of the Binary Processing Pre-Filter[36] step, or to include fibres that were not detected

- **Erase mode**: Use this to cut features that may have been joined as part of the Binary Processing Pre-Filter [36] step, or to ignore fibres that were detected

To draw a line:

1. Enable **Show Binary** to display the selected features in the current binary mask color.

2. Choose the **Draw Straight Lines** method from the drop-down menu.

   The **Line** tool is selected automatically.

3. Click to enable the **Add** or **Erase** radio button.

4. Set the **Line Width**.

5. Drag the cursor to draw a line.

   o **Add** mode: The line is shown in red until you click **Apply**, then it becomes part of the Binary Output Image

   o **Erase** mode: As the line affects the mask, it is shown in the mask color set in the Binary Processing Pre-Filter[36] step

6. If necessary, use the **Undo** and **Redo** buttons to review any lines you have drawn.

7. When you have finished drawing lines, click on the **Apply** button.
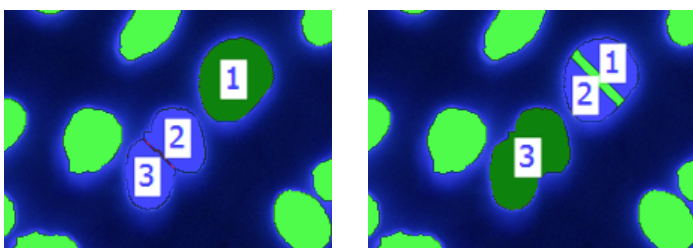
Example: Add mode
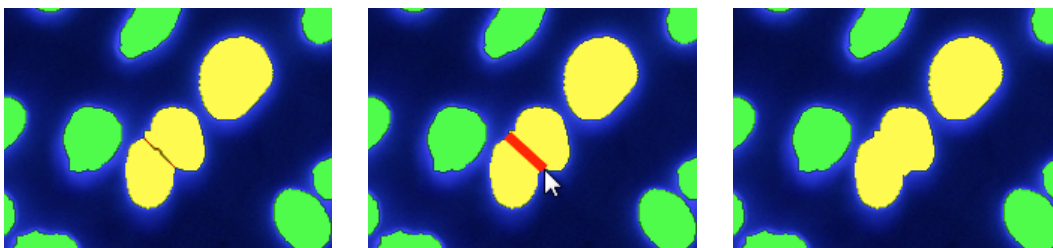
Example: Erase mode
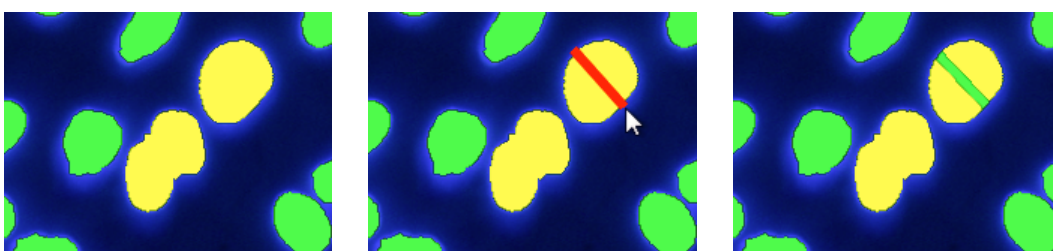
Processed image, before and after drawn lines

The illustrations below show the results of processing an image before and after the Add and Erase lines used in the examples above.

Select Exisiting Regions

You can draw regions of interest on an image mask to include or exclude features that were missed or included incorrectly in previous processing steps. This method works in one of two modes:

- **Accept mode**: Enclosed features in selected regions will be included in the measurements
- **Reject mode**: Enclosed features in selected regions will be excluded from the measurements
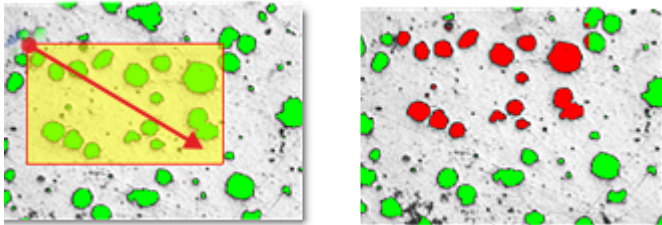
To select an existing region:

1. Enable **Show Binary** to display the selected features in the current binary mask color.
2. Choose the **Select Existing Regions** method from the drop-down menu.
3. Click to enable the **Accept** or **Reject** radio button.
4. Click to activate a drawing tool to create the RoI:
   - **Fill Area**
   - **Rectangle**
   - **Circle**
   - **Ellipse**
5. Drag the cursor to draw the RoI.

   Fill Area tool: Left-click at each point on the shape, and right-click on the final point to complete the filled area.
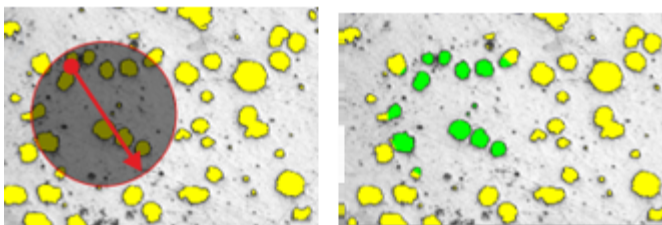
   The drawn region is filled with a semi-transparent color to make it easier to identify the selected features.
6. If necessary, use the *Undo* and Redo buttons to review any regions you have drawn.
7. When you have finished drawing regions, click on the **Apply** button.

Example: Accept mode

Example: Reject mode

Draw New Shapes

You can draw lines and shapes on an Binary Image Mask to add or erase regions that were not perfectly detected in previous processing steps. This method works in one of two modes:

- **Add** mode: Use this to edit features that may have been missed as part of the <u>Binary Processing Pre-Filter</u>^{□ 36} step

- **Erase** mode: Use this to exclude features that may have been included as part of the <u>Binary Processing Pre-Filter</u>^{□ 36} step

To draw a shape:

1. Enable **Show Binary** to display the selected features in the current binary mask color.

2. Choose the *Draw New Shapes* method from the drop-down menu.

3. Click to activate a drawing tool:

   o **Line***:* Use this in the same way as <u>Draw Straight Lines</u>^{□ 46}, for example to separate features that may have been joined incorrectly

   o **Freehand Line***:* Use this to trace and join features that are not closed shapes, such as fibres; left-click at each point on the shape, and right-click on the final point to complete it

   o **Filled Area**: Left-click at each point on the shape, and right-click on the final point to complete it

   o **Rectangle**

   o **Circle**

   o **Ellipse**

4. Click to enable the **Add** or **Erase** radio button.

5. Set the **Line Width**.

6. Draw a shape:

   o **Add** mode: The shape is shown in red until you have finished drawing, then it becomes part of the Binary Output Image

   o **Erase** mode: As the line affects the mask, it is shown in the mask color set in the <u>Binary Processing Pre-Filter</u>^{□ 36} step

7. If necessary, use the **Undo** and **Redo** buttons to review any shapes you have drawn.

8. When you have finished drawing shapes, click on the **Apply** button.

The **Click to Select Regions** method lets you quickly include or exclude features that have already been detected.

- **Keep** mode: Use this when your image has many detected features, but you only want to include a few; All features are deselected to begin with; simply click on those you wish to keep

- **Delete** mode: Use this when your image has many detected features, but you only want to delete (reject) a few; All features are included to begin with; simply click on those you wish to delete

To select an existing region:

1. Enable **Show Binary** to display the selected features in the current binary mask color.
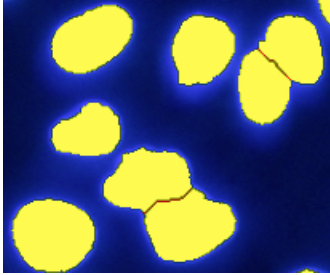
2. Choose the **Click to Select Regions** method from the drop-down menu. The **Selection** tool is enabled (you cannot select any other tools).

3. Click to enable the **Keep** or **Delete** radio button.

4. Click on features to keep or delete them, depending on the setting in the previous step.

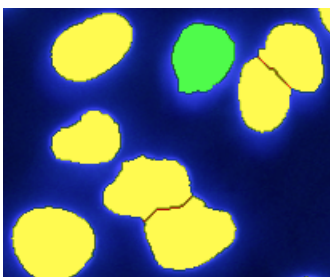5. If necessary, use the *Undo* and Redo buttons to review your changes.

Example: Keep mode

When you click on a feature, it is marked as 'keep'; all others are marked as 'deleted'. You can click to select multiple regions.
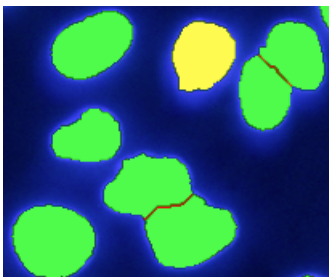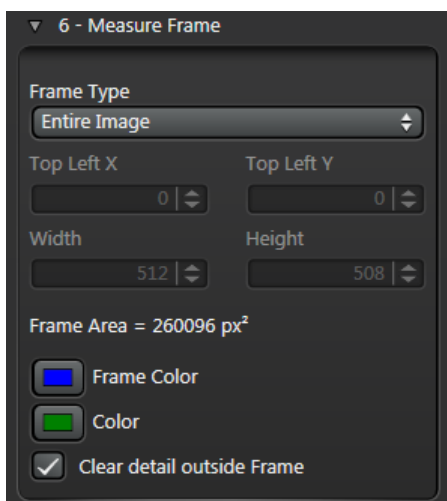

Example: Delete mode

When you click on a feature, it is marked as 'deleted'; all others are marked as 'keep'. You can click to select multiple regions.

Measure Frame

A **Measure Frame** determines the area of an image that will be used for analysis and measurement. It can be the entire image or, more usually, a representative area.

- An **Image Measurement** processes all binary pixels inside the Measure Frame and none outside it.

- For an **Object Measurement**, the Measure Frame determines which features to include or exclude. A valid feature will have all visible pixels processed, even if they fall outside the Measure Frame, while a feature rejected by the Measure Frame will not be considered at all. (**Important**: see **Clear detail outside frame**[50] below.)

The main function of the Measure Frame is to avoid measurement errors, especially with Object Measurements and objects cut by the edge of the field of view. When acquiring an image set by automatically stepping a motorised stage, the images will be overlapped so that every feature appears in full in at least one image. The Measure Frame is set to correspond to the overlap and ensures that incomplete features are rejected, and that every feature is measured only once.

Creating a Measure Frame

To create a Measure Frame:

1. Click on the **Measure Frame** option in the **Processing Sequence** panel.

2. Select a **Frame Type**[52] from the drop-down menu and set the size if appropriate.

3. Set the **Frame Color** and Binary **Color**.

4. Enable the **Clear detail outside frame** check box.
   Note: This function should be used for visualisation only, and should normally be deselected before proceeding. In particular, it is NOT recommended with Feature Measurements, since it actually modifies the binary. (In the illustration below, the red part of the feature at the top of the frame would be incorrectly measured).
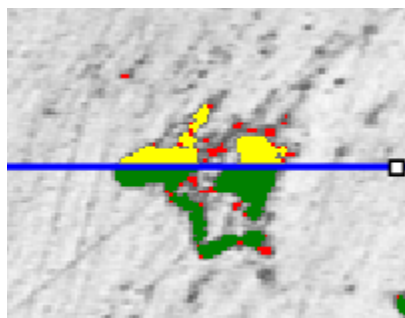

Measure Frame features

The illustration below shows Measure Frame features:

1. The edge of the image.

2. A typical **Measure Frame**.

3. The Guard Region.

4. An object that is completely within the Measure Frame, and is included in the results.

5. An object that lies partly outside the Measure Frame but, since its bottom right-hand pixel is inside the Measure Frame, it *is* included.

6. An object that is mostly within the Measure Frame but, since its bottom right-hand pixel is either touching or outside the frame boundary, it is **not** included.
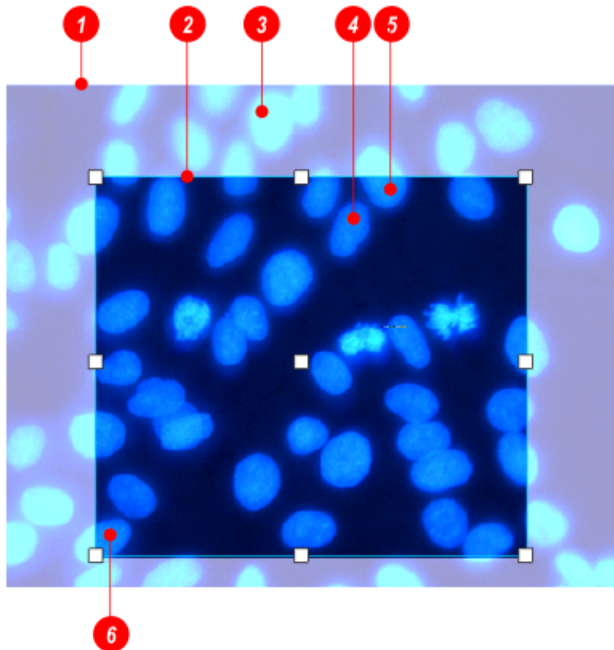
Rules for including/excluding objects

- Any object that has its bottom right-hand pixel lying within (not on) the Measure Frame boundary is included in the analysis, even if any of its other pixels lie in the Guard Region – outside the Measure Frame boundary.

- Field measurements are performed within the Measure Frame; any pixels outside the Measure Frame are not included.

Measure Frame Types

Entire Image

The frame boundary coincides with the edges of the image.

Adjust to Max Particle

To create a frame based on a user-defined particle size:

1. Enter a value (µm) in the **Max Particle Diameter** field.

The *Guard Region* is based on:

- 100% of the particle diameter at the top
- 50% of the particle diameter on the sides
- 0% of the particle diameter at the bottom.

Typical Size

This option will suit a wide variety of microscope images. Based on an 'average' particle size, it creates a Guard Region with:

- 100% of average particle diameter at the top
- 50% of average particle diameter on the sides
- 0% at the bottom.

Manually Define

This option allows you to create a frame of specific dimensions and position it anywhere on the image.

1. Select **Manually Define** from the **Frame Type** drop down menu.
2. Enter the **Top Left X/Y** coordinates.
3. Enter the **Bottom Right X/Y** coordinates.
4. (Alternatively, drag the Measure Frame handles to the required size.)
5. Reposition the Measure Frame by dragging its centre handle.

Reference Mask (Multi Channel Extension)

If you are using the 2D Analysis Multi Channel Extension, you can assign an Analysis Channel (in a multi-channel image) to act as a **Reference Mask** for which measurements will be derived from other channels, such as Count, Intensity, Area.
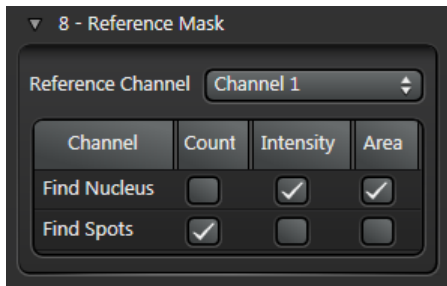

To define a channel as a Reference Mask:

1. Set up the [Analysis Channels](#)[24] for your image.

2. In the **Reference Mask** step of the Processing Sequence (in Multi Channel mode), select the channel you want to use as the **Reference Channel** from the drop-down menu.

3. Enable the options for **Count**, **Intensity** and **Area** in the other image channels.

Measurements

Use the Measurements panel to filter your measurement results. This is useful if you want to reduce the number of measurements included in your results (for example, to exclude obvious outliers).

You can also define how measurements are labelled on the binary output image.
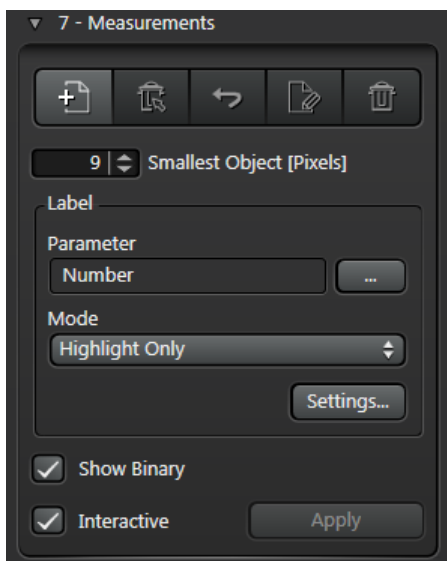
Basic method

To control how your measurements are filtered and displayed on the LAS AF Display Window and Results Grid[60]:

1. Before using the Measurements panel, use the Table Tools[60] icon on the Results Grid to include all the object parameters that you want to measure.
   Note: If you are using the 2D Analysis Classifier, it only works with measurements that are Measured (whether or not they are Visible on the Results Grid).

2. Create and edit measurement filters[55]. Active filter items appear in the parameter filter list, below the toolbar.

3. Fine tune[56] the range of results to be included by editing the filter limits. Most parameters can accept lower and upper limits; only features falling within these limits are found and labelled.

4. Exclude small particles and noise from your results using the **Smallest Object (Pixels)** tool.

5. Use the Label[57] pane to define which parameter is labelled, and how labels are formatted.

6. Enable the **Show Binary** check box to see the effect of your drawn shapes and selections before applying your changes.

7. Use the **Interactive** check box to specify whether changes you make to the output image are updated in real time as you work (Interactive enabled), or only when you click the **Apply** button (Interactive disabled).

Creating and editing measurement filters

When creating a filter, the list of available parameters depends on which parameters are enabled in the Measure column in the Results Grid; see Table tools[60].

To create a new filter:

1. Click on the **New Filter** button in the Filter Toolbar[56].

2. On the **Select Measurement Parameters** dialog, expand a parameter group if necessary and enable one or more **Parameters**.
   Note: Classifier parameters are only available if you have installed and licensed the 2D Analysis Classifier[63].
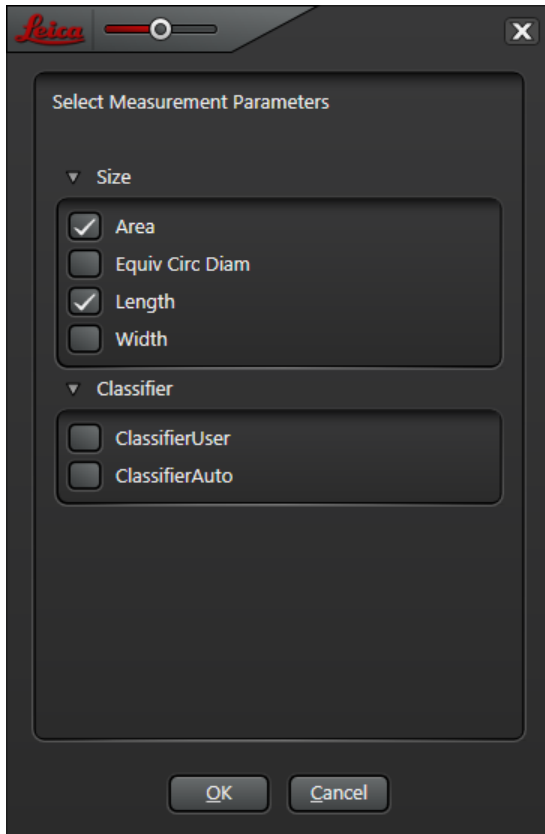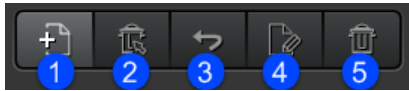

3. Click **OK**.

The chosen **Parameter** names appear in the **Filter List** with the default lower and upper values. These values are based upon the image in the **LAS AF Display Window**.
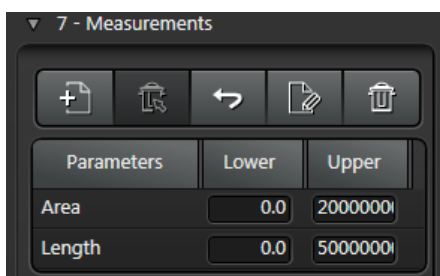
(Continued on next page)

Filter Toolbar

The **Filter** toolbar on the **Measurements** control panel has the following tools:

1. **New**: Adds a new measurement filter to the list.

2. **Delete**: Deletes the currently selected filter from the list

3. **Reset Defaults**: Resets all filter limits to their default values

4. **Reset Measured**: Resets filter limits to lower and upper limits actually measured on the image.

5. **Clear All**: Removes all currently defined filters from the list.

Setting filter limits manually

To edit filter limits manually:

1. Click in a **Lower** or **Upper** field in the filter list.

2. Enter the required limit for this parameter.

3. Repeat until you have set all the upper and lower limits.

You can choose which parameter is labelled on the LAS AF Display Window, and how labels are formatted.
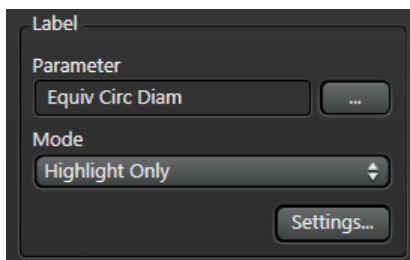
Choosing which parameter is labelled

You can choose which [parameter](#)[113] is labelled on the binary output image.

1. In the **Label** pane of the **Measurements** panel, click on the browse button to the right of the **Parameter** field. This will display the **Select Measurement Parameter** dialog.
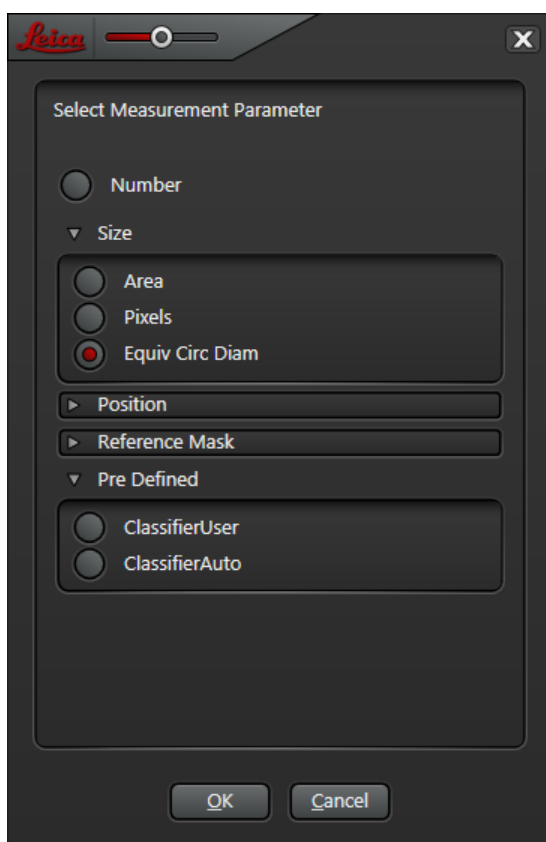

- o Remember, the list of parameters from which you can choose depends on which parameters are enabled in the Measure column when using the [Table tools](#)[60] icon in the Results Grid.

- o **Note**: The Pre Defined Classifier parameters are only available if you have installed and licensed the [2D Analysis Classifier](#)[63].

2. If necessary, expand a parameter group (e.g. **Size**) by clicking the arrow.

3. Click to enable all the parameters you want to display (e.g. **Length**).

4. Click **OK**.

(Continued on next page)

Labelling Mode

To specify which features on the image are labelled:

1. Select a **Mode** from the drop-down menu in the **Label** pane.
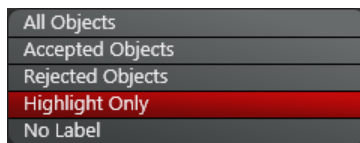
- o **All Features**: All the features highlighted on the Binary Output Image are labelled regardless of whether they fall inside limits or not.

- o **Accepted Features**: Features are labelled that fall within specified limits. For example, if the Area parameters are set to find image features no smaller than 50px2 and no greater than 150px2, then only those features that fall within the parameters are labelled. All others are ignored.

- o **Rejected Features**: Use this option to label features that fall outside the set parameters. Using the example above, features smaller than 50px2 and greater than 150px2 would be labelled.

- o **None**: Turns off labelling.

Label Control

To change the way **Labels** and results are displayed on the **Measurements Binary Output Image**:

1. On the **Label** panel, click on the **Settings** button to display the **Label Control** dialog.

2. Use the **Position** drop-down menu to set the location of the label with respect to a feature.

- o **Centroid** displays the label in the centre of the object.

- o **Below-Centre** displays the label below and on the centre line of the object.

- o **Below-Left** displays the label below and to the left of the feature.

3. Specify whether the parameter **Name** and **Units** should be displayed:

- o Enable the Name check box to display the Parameter – Length, Number, Diameter etc – alongside the feature and result.

- o Enable the Unit check box to display the measurement units – pixels (px), millimetres (mm) – next to the feature.

- o **Note**: To set the number of digits following the decimal place on the display, change the
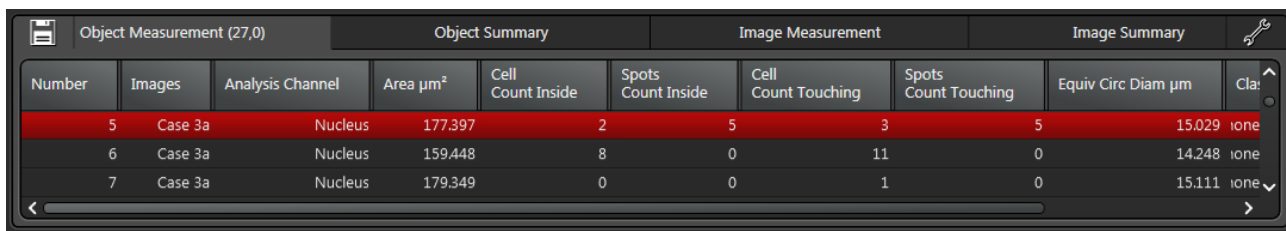
Measurement Display value on the main **Preferences > Image** dialog.

4. Click on the **Color** swatch to display a standard color selector, and specify the label color.

5. Click on the **Font** button to display a standard font selector dialog, and specify the label color.

6. Click on the **Background** swatch to display a standard color selector dialog, and specify a background color for the label text boxes.

7. Use the **Outline** box to display an outline around the selected features. A value of '0' turns off outlining.
   Note: The **Outline** color is the same as the **Label Font** color.

Results Grid

The Results Grid is where all your measurements are displayed.

| Number	Images	Analysis Channel	Area µm²	Cell Count Inside	Spots Count Inside	Cell Count Touching	Spots Count Touching	Equiv Circ Diam µm	Clas
5	Case 3a	Nucleus	177.397	2	5	3	5	15.029	ione
6	Case 3a	Nucleus	159.448	8	0	11	0	14.248	ione
7	Case 3a	Nucleus	179.349	0	0	1	0	15.111	ione

The position of the Results Grid depends on whether you are using single or dual monitors:

- Single monitor: The Results Grid is positioned below the LAS AF Display Window

- Dual monitors: The Results Grid is positioned to the left of the LAS AF Display Window. This allows you to have all your results displayed on one monitor, and have the image displayed on the other monitor

Configuring the Results tables

You can control which features and measurements appear in the results tables, and which features are included in your measurement calculations.

Note: If you are using the 2D Analysis Classifier, it only works with measurements that are Measured (whether or not they are Visible on the Results Grid)
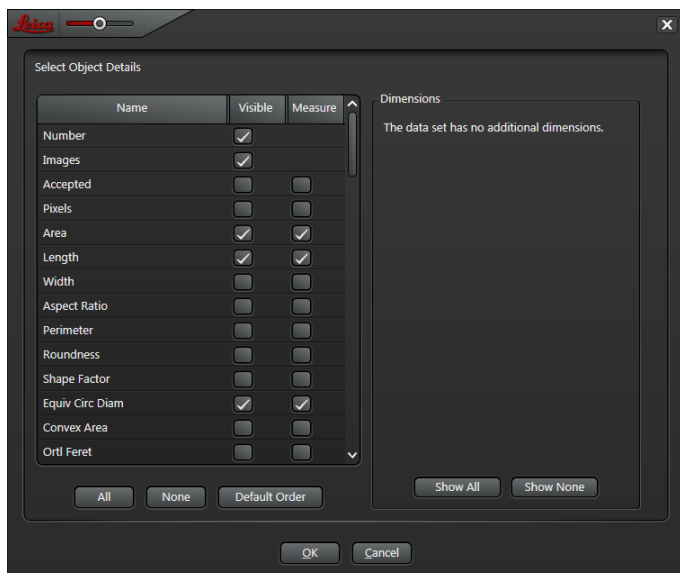
1. Click on a tab to display the Results table that you want to configure (e.g. **Object Details**).

2. Click on the Table tools ![icon] icon at the right of the Results Grid header.
   The appropriate dialog will be displayed.

   - This allows you to specify which results are displayed on the current Grid tab, and which results are included in your measurements. For example, if the Object Details tab is visible, the **Select Object Details** dialog will be displayed.

   - Click to enable the **Visible** check box for each item you want to display on the Results Grid.

   - Click to enable the **Measure** check box for each item you want to include in your measurements

   - You can also enable **All** or **None**, or restore the column order and width for all results tables to their **Default Order**.

3. If the data set has additional dimensions (e.g. for a time sequence or z-stack), they will be listed on the right-hand side of the dialog; you can choose to **Show All** or **Show None**.

4. Click **OK**.

Results tables

The following tabs are available on the Results Grid:

- **Object Measurements**: Measurements relate to individual object features, and include parameters such as size and length

- **Object Summary**: Statistical information about the measurements on the Object Measurements tab

- **Image Measurements**: Measurements relate to a complete Field of View, and include parameters such as Area Percent and Mean Chord. This represents the summed value for all objects within the Measure Frame, regardless of whether they are touching or separate (whereas Object data produces a separate value for each isolated feature that ends inside the Measure Frame).

- **Image Summary**: Statistical information about the measurements on the Image Measurements tab

Saving table data to the experiment

You can save all your measurement data to the Experiment:

1. Click on the **Save table to experiment** icon .

2. Enter a name for the result table and click on **OK**.

3. The table is listed in the Experiment tree.

Copying and exporting table data

You can:

Copy table data to the clipboard then paste it into the application of your choice

Export table data as an .xsls spreadsheet file

To do this:

1. Display the tab in the Results Grid from which you want to copy or export data.

2. Right-click on the table and select an option from the context menu:

   o **Copy** or **Export table content**: Copies or exports all the **visible** table data (i.e. data that is measured but not visible is not included)

   o **Copy** or **Export selected rows**: Copies or exports all the **visible** data in the currently selected rows (i.e. data that is measured but not visible is not included)

   o **Copy** or **Export all**: Copies or exports all the **visible and measured** table data

Resizing, displaying and hiding the Results Grid

You can resize the Results Grid to suit your workspace:

- Move the pointer over the border below (single monitor) or to the left of (dual monitors) the LAS AF Display Window and the Results Grid; it will change to a double-headed arrow, and you can drag the border to resize the Results Grid.

- Drag the border all the way down (single monitor) or to the left (dual monitors) to hide the Results Grid.
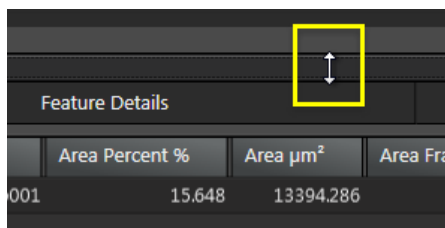
- To display the Results Grid if it is hidden: Move the pointer below (single monitor) or to the left of (dual monitors) the LAS AF Display Window until it changes to a double-headed arrow and drag upwards (single monitor) or to the right (dual monitors) to reveal the Results Grid.

Other Results Grid operations

- Results displayed in the grid are controlled by the Table Tools button; see <u>here</u> ⌐ 60.

- Click on an entry in the **Grid** to highlight the feature on the image; the label will be shown in a contrasting color.

- Click on a feature on the **Binary Output Image** to highlight the results for that feature in the **Grid**. If an image sequence is being measured, the appropriate image will be automatically displayed.

- Ctrl-click on rows to make multiple selections. Shift-click to make a contiguous selection.

- To change the sort order of a column in the **Grid**, click on the column header.

- Drag column headers to change the order in which columns are displayed in the Grid.

- Drag the vertical line between two column headers to change column width.

Classifier

The LAS AF 2D Analysis Classifier is an optional module designed to help recognise patterns in complex data sets, and to group objects according to various measured parameters.

Disclaimer: To achieve good results, the 2D Analysis Classifier relies on high-quality images, relevant training data and informed operation. These can be influenced by a) the optical, electronic and mechanical components used, b) the working conditions and sample preparation process and c) the individual and specific interpretation of any results produced. These are the responsibility of the user; Leica disclaims any liability in that context.

Before you start, we recommend that you read the [Classifier Overview](#) 65.

Basic procedure

To classify objects:

1. Set up your [Measurements](#) 54 so that the results you want to classify are included in the [Results Grid](#) 60 .
    The first time training is run, the Classifier is trained using those features that are **visible** in the [Results Grid](#) 60 . All subsequent training uses the same set of features, provided that they are currently being **measured**. They do not need still to be visible.
2. [Create](#) 68 a new Classifier settings file.
3. [Add](#) 70 classes to your classifier, and set up class colors and hotkeys.
4. [Manually classify](#) 72 some objects and train the Classifier.
5. [Run Auto Classification](#) 73 , to classify all objects based on the training data.
6. [Reclassify](#) 74 any objects that may have been incorrectly classified as a result of Auto Training.
7. [Save](#) 69 your Classifier settings file.
8. View classification results in the [Histogram](#) 77 .

(Continued on next page)

Classifier Settings toolbar

Use this to manage your named [Classifier settings files](#) 68 .
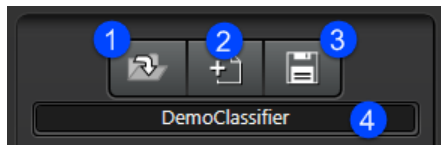
1. **Load Classifier**: Load an existing (previously saved) Classifier settings file.
2. **Create Classifier**: Create a new Classifier XML file, based on the current settings. Note that this will not be complete until you have trained the Classifier and saved the settings.
3. **Save Settings**: Saves the current settings and training data to disk.
4. Current Classifier settings file name.

Classifier Class toolbar

Use this to manage Classes and train the Classifier.

1. **Add Class** 70
2. **Check Details** 76
3. **Remove Class** 70
4. **Remove All Training Data** 75
5. **Train Classifier** 72

The Classifier is designed to help recognise patterns in complex data sets.

A skilled human operator may, by eye, be able to group features in an image into different classes. However, there may be no simple mathematical rule to separate features into classes. For instance, it might not be enough to find all features greater than a certain area; the operator may need to make a decision based on size, shape and intensity.

For samples where a simple threshold can be applied, it is probably not necessary to use the Classifier. In more complex cases, given the right training data, the Classifier can be an extremely powerful diagnostic tool.

Example

Consider using a single parameter, area, to define whether an object in sample is "good" or "bad". You might plot a histogram of the area and set a threshold to identify the population to which a sample belongs.
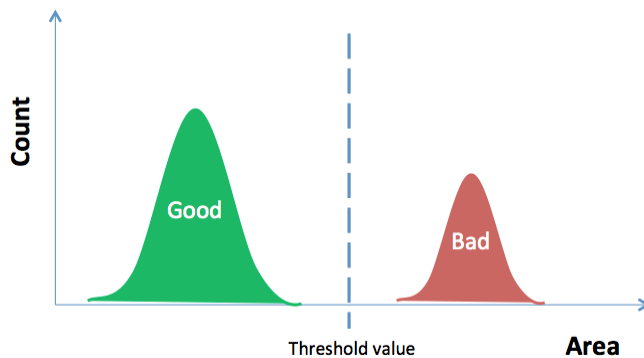
If classification is dependent on multiple features, the Classifier in effect tries to discern the boundary between the populations in some multi-dimensional space:
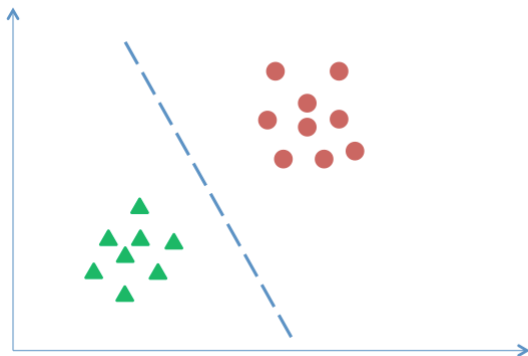

For biological samples (in particular) there may be no clear division between populations. There will be some overlap and uncertainty between classifications. Therefore, a human expert may still be required to judge the borderline cases:
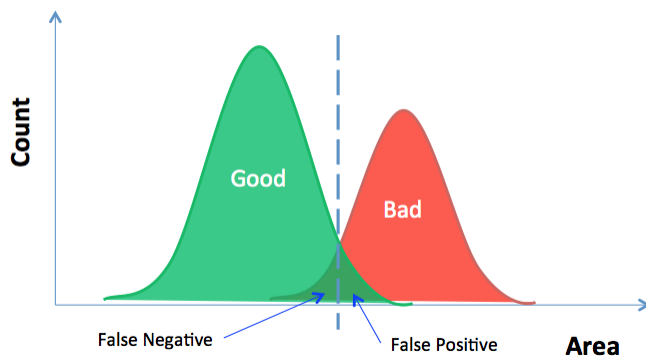
Giving more training data to the Classifier may improve its accuracy. However, including incorrect samples in the training data will give unpredictable outcomes. Depending on the nature of the samples, there is also a possibility of over-training the Classifier:
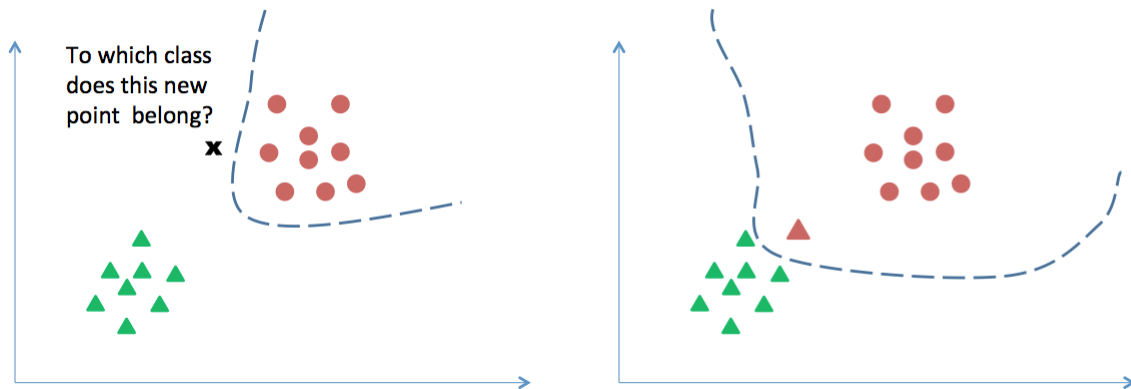
Measurement Feature Selection

When deciding how to train the Classifier, you measure features using the following types of parameter:

- Scale-sensitive (e.g. Area)
- Scale invariant (e.g. Roundness)
- Rotation-sensitive (e.g. Orientation)
- Rotation-insensitive (e.g. Joins)
- Position sensitive (e.g. X FCP)
- Channel sensitive (e.g. Intensity Variance)

The more parameters you give to the Classifier, the more training data it will require to make an accurate classification.

(Continued on next page)

An example

Take the following sample image, which contains triangles and squares of different colour and size:
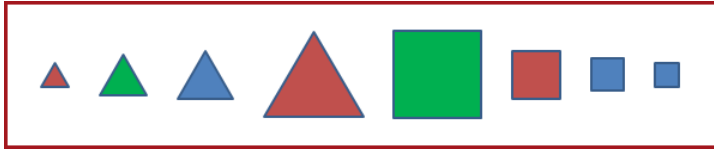
Grouping by Channel-specific parameters (e.g. red/green/blue mean)

Grouping by scale-invariant parameters (e.g. Roundness)

Grouping by scale-sensitive parameters (e.g. Area)

Training

- The first time training[72] is run, the Classifier is trained using those features that are visible in the Results Grid[60]. All subsequent training uses the same set of features, provided that they are currently being measured. They do not need still to be visible.

- A warning is raised and training will fail if any of the required parameters are no longer being measured. If extra features are set to be measured in the Results Grid[60], they will be ignored by the training.

- Similarly, auto classification[73] uses the same set of features that were during training. The features must be in the set of features selected to be measured in the Results Grid[60], but they do not need to be visible. Any additional features that are being measured will be ignored by the Classifier.

  For example, you might train the Classifier against feature size to distinguish between "good" and "bad', but only be interested in finally reporting the intensity of those blobs reported to be "good". In your final results, you might want to have Intensity results visible, while Area is measured but not visible.

- When a Classifier is reloaded[69], it attempts to set the features it requires to be measured. However, some features may still need to be enabled by hand. In particular, the operator will need to manually set up multi-channel analysis measurements. Similarly, if multi-channel features are used to train a Classifier but a single channel image is processed, the Classifier will not run.

- The Classifier is sensitive to the quality of the training data provided. If an object used in the training has been wrongly labelled by the user, this will cause unexpected results.

- When a Classifier runs it will always put every object into one of the available classes. If an object is reported as being of class "None/Unclassified" this is because the Classifier is not valid. Check[72] that the Classifier is properly trained. Check[76] that all the required features are set to be measured.

# Creating and managing Classifier settings files

You can create Classifier settings files, train the Classifier, and then save the settings file so that it contains your training data.

- Initially, when you create a Classifier, it is saved as an XML file in your chosen location.

- After you have trained the Classifier, when you save the Classifier settings file, two more files are created alongside the XML file:

    o   .svm holds the trained classifier

    o   .dat holds the training data

    o   .xml holds user settings: class names, colours, hotkeys

Until you have saved a Classifier settings file, you will not be able to load it; it is not valid without its associated .svm and .dat files.

Classifier Settings toolbar

Use this to manage your named Classifier files.
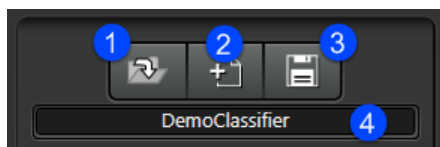
1. **Load Classifier**: Load an existing (previously saved) Classifier settings file.

- **Create Classifier**: Create a new Classifier XML file, using default settings. Note that this will not be complete until you have trained the Classifier and saved the settings.

2. **Save Settings**: Saves the current settings and training data into the

3. Current Classifier settings file name.

Creating a new Classifier settings file

To create a new Classifier:

1. Click on the **Create Classifier** button on the Classifier Toolbar.

2. Navigate to your chosen location and enter a name for the Classifier (it will be saved as an XML file).

3. Click **Save**.

A new file will be created. Initially, it will have two default Classes:

See [Creating and managing Classes](#) [70]

(Continued on next page)

Saving a Classifier file

To save the current Classifier settings:

1. Click on the **Save Classifier** button on the Classifier Toolbar.
2. If a Classifier file already exists with this name, click **Yes** to overwrite with the current settings

To save with a new name

1. Ctrl-click on the **Save Classifier** button.
2. Navigate to your chosen location and enter a name for the Classifier (it will be saved as an XML file).
3. Click **Save**.

Loading a Classifier file

To load an existing Classifier settings file:

1. Click on the **Load Classifier** button on the Classifier Toolbar.
2. Navigate to your chosen location and select the Classifier XML file.
3. Click **Open**.
4. If the classifier settings are not compatible with the current data, or the Classifier you are trying to load was not previously saved after training was carried out, a message will be displayed telling you to retrain the Classifier or load a different Classifier settings file. Click **OK** to continue.

Creating and managing Classes

You can add new Classes to your Classifier settings file. Note: If you have already trained the Classifier, this will invalidate the existing trained Classifier and you will need to retrain it. This may also invalidate your previous training data (you will be advised to delete the old training data before continuing).

To create a Class

To add new Classes to the current Classifier settings file

1. Click on the **Add Class** button .

2. If training data exists for this Classifier, a message will remind you that this action will invalidate any existing training data.

   o Click **Yes** if you want to continue.

   o If you do not want to overwrite existing training data, click **No**, and save the current Classifier settings file. You can then create a new Classifier settings file.

3. Enter a **Class Name**.

4. Click on the **Color** button and select a color for objects of this class.

5. If you want to change the Hotkey for a particular Class, click in the **Hotkey** field, then press the keyboard key that you want to assign to this Hotkey.

6. Repeat to create additional Classes (up to a maximum of nine).

7. Click on the **Save Settings** button .

To delete a Class

Note: You cannot have fewer than two Classes; The Classifier works with two or more Classes.

To delete a Class from the current Classifier settings file:

1. Click to select the Class you want to delete.
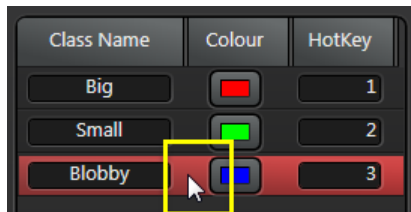
2. Click on the **Remove Class** button .

3. If training data exists for this Classifier, a message will remind you that this action will invalidate any existing training data.

   o Click **Yes** if you want to continue.

   o If you do not want to delete existing training data, click **No** and save the current Classifier settings file. You can then create a new Classifier settings file.

(Continued on next page)

Defining the Unclassified colour

When you train the Classifier and apply auto classification, all objects are put into one of the defined Classes. If there are some objects that you do not want to be classified and included in your results, you can select them and press the Unclassified hotkey (**0**). You cannot change the hotkey for this operation, but you can change the color assigned to Unclassified objects.

To define the color for Unclassified objects:

1. Click on the **Unclassified** Color button and select a new color (the default is grey).

Unclassifed   [ ]   Hotkey 0

Manual classification and training

Once you have created a Classifier settings file[68] and added some Classes[70], you can manually classify some objects to start the training process.

Note:

- You need at least one example of each Class
- You need at least 10 examples in total
- Generally more examples gives better results
- However, putting mis-classified examples into training data will cause unexpected results

To start the training process

1. Enable the **Show Binary** option.

2. Click on the **Show Manual Classification** button.

3. Select an object in the LAS AF Display Window or the Results Grid.

4. Press a hotkey to classify the object with an appropriate Class. The Classifier automatically toggles to Markup view.
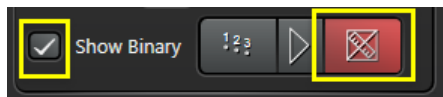
5. Repeat the process until you have enough training examples. If you have fewer than 10 items in an image you wish to use as training examples, click the **Train the Classifier** button.
   Training data will be saved but the Classifier will report that training failed. Now you can open a different image and mark up features in that.

   o   You can sort entries in the Results Grid, Shift-click to select objects with similar properties, and classify them all at once.)

6. Click the **Train the Classifier** button to feed this object Class data into the Classifier.

7. Click **OK** when you see the Training completed message.

Train the Classifier on remaining Classes of object

Repeat the steps above to train the Classifier on your other object Classes. Each time you press the Train Classifier button, you will see a message that training data already exists.

1. Click **Yes** to append your new data to the existing training data.

2. Click **OK** when you see the **Training completed** message.
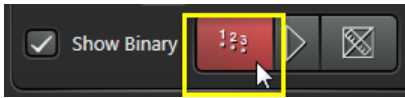
Save your training data

When you feel you have added enough training data:

1. Click on the **Save Settings** button to save your Classifier settings file.

Auto classification

Once you have manually classified some objects and trained the Classifier, you can auto-classify the remaining objects in your image:

1. Click on the **Show auto classification** button.

2. The 2D Analysis Classifier will classify all remaining unclassified objects according to your existing training data.

3. You can now switch between Manual and Auto classification results to see the difference. At this point, you can:

   o Add some more training data and train the Classifier[72] again, to refine your object classification

   o Edit any individual auto-classification results[74] if you think they are incorrect

Editing auto classification results

Once the auto classification has been performed, you might want to change the Class of some objects if you feel they have been incorrectly classified. This is particularly likely near Class boundaries.

Copy auto results

Before editing any of the auto-classified results:

1. Click on the **Copy auto results to manual** button.

This updates all your manually classified results (in the **ClassifierUser** column) so that they match the auto-classified results (in the **ClassifireAuto** column).

Editing auto training results

Depending on the complexity of your problem and the accuracy of your Classifier, you may wish to mark up more training data and retrain the Classifier, or simply to mark up corrections to the automatic results before outputting the results to a report.

To change any of the auto-classified results:

1. Select an object (click on its entry in the Results Grid, click on the object in the LAS AF Display Window).
2. Press the appropriate Class hotkey to manually reclassify the object (or press 0 to mark object as unclassified "none").
3. Click on the **Train the Classifier** button to update the object's Class data.
4. Click **Yes** to append the new training data.
5. Click **OK** when you see the **Training completed** message.
6. Click on the **Show auto classification** button.

Removing training data

Sometimes you might want to delete all the training data associated with a Classifier settings file.

This deletes the existing training data but does not delete the existing trained Classifier (the Classifier will continue to be applied until you retrain it).

1. Click on the **Remove all training data** button.

2. Click **Yes** to confirm.

At this point, you can the Classifier.

Checking details against training data

If you change the data displayed in the Results Grid (by adding parameters[☐ 60] to the list of those being measured), or if you load[☐ 69] a different Classifier settings file, there will be a mismatch in the training data.

To check for a mismatch:

1. Click on the **Check Details** button on the toolbar. If there is a mismatch, an information message will be displayed. This will tell you which columns in the Results Grid do not match the measurements in your current Classifier settings file.

2. Click **OK**.

3. Click on the Table tools[☐ 60] icon on the Results Grid and set up your measured and visible results so that they match what you are trying to classify.

Notes:

- The Classifier is trained using the [visible*] measurement parameters at the time you click the **Train the Classifier** button.

- Subsequently when the Classifier is applied it will try to use that set of features to make its auto classification. At auto classification time the parameters do not need to be visible, but **must** be selected to be "Measured".

- If a subset of parameters is selected to be measured, a warning will be shown.
  - o As long as at least one measurement is available from the training set of parameters, the Classifier will attempt to make a classification.
  - o However, if no measurements are available, all features will be marked as unclassified.

- Other measurements that were not part of the training set will be ignored by the Classifier. For example:
  - o You may wish to train a Classifier to recognise shapes (triangles, squares etc.) regardless of size. So you would use scale-invariant features for the training, such as Roundness.
  - o When applying the Classifier, you may wish to enable other measurements (such as Area) for your final report, so that you can then see the area of triangles compared to the area of squares.

Histogram

Use the **Histogram** control panel to display results graphically as a vertical or horizontal bar chart, or a pie chart.

Set up and save a Histogram settings file

Use the Histogram control panel[79] to create and save a Histogram settings file:
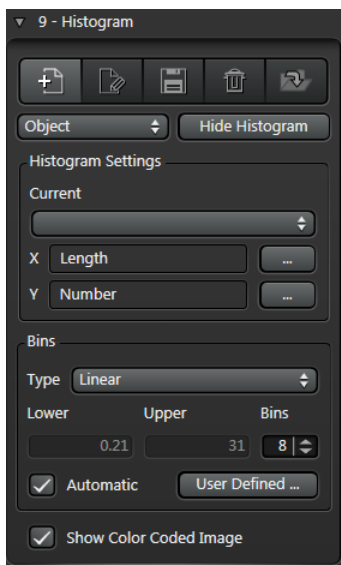
1. Click the **Create new Histogram** button and enter a name to create a new Histogram settings file.

2. Use the **Histogram Settings** panel to set the X and Y parameters for your histogram.

3. Use the **Bins** panel to set up bin type, bounds and appearance.

4. Enable **Show Color Coded Image** to preview how features will be allocated to Bins on the Histogram (colors are defined here[82])

(Continued on next page)

View and modify the histogram

Use the [Histogram window](#)[84] to view and modify the histogram:
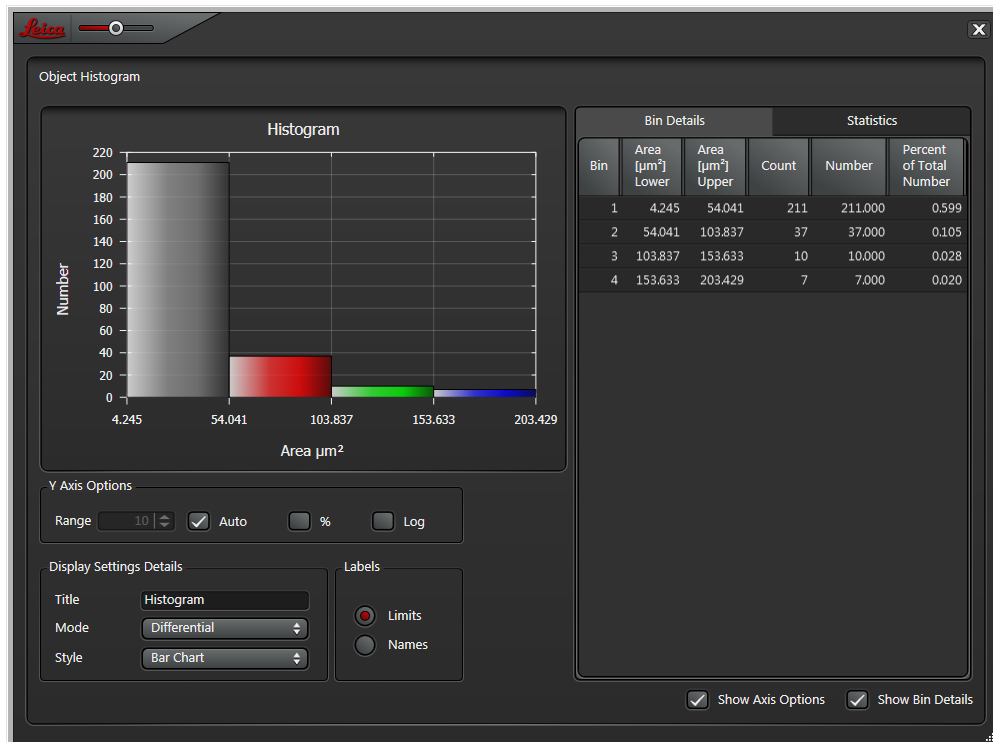
1. Click the **Show Histogram** button in the Histogram control panel to display your histogram.
2. Enable **Show Axis Options** to control the Y-axis range and Display Settings Details.
3. Enable **Show Bin Details** to view Bin Details and Statistics.

Use the Histogram control panel to create and display one or more histograms for the current experiment.
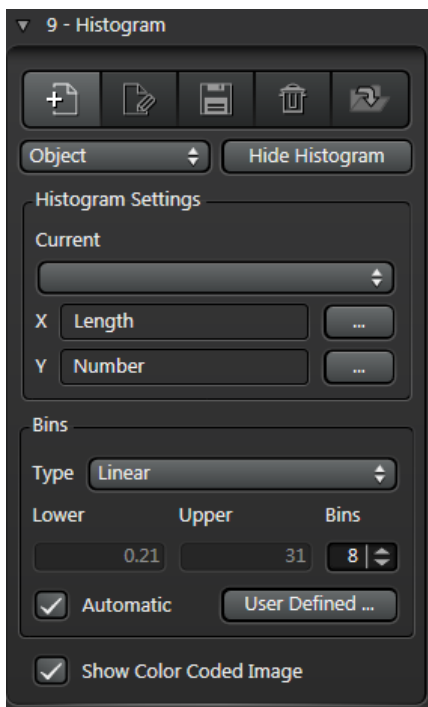
You can create multiple Histogram settings files, and switch between them for comparison.

Parameter types and automatic Bin allocation

Bear the following in mind:

- If you select any linear X parameter while the Bin allocation is set to **Automatic**, the Bin **Type** is set to **Linear** and is not immediately editable. You can make it editable by disabling Automatic Bin allocation.

- If you are using the 2D Analysis Classifier and/or the 2D Analysis Multi Channel Extension, and you select one of the Pre Defined X-axis parameters (ClassifierUser, ClassifierAuto or Analysis Channel), the Bin Type is set to Pre Defined and is not editable. The Bin allocation is also set to Automatic, and is not editable.

Histogram Toolbar

The Histogram control panel has the following tools:

1. **Create new histogram**: Allows you to create a new Histogram settings file. You can create multiple settings files to illustrate different aspects of the same experimental data.

2. **Edit current histogram**: Allows you to change the name of the currently selected Histogram settings file.

3. **Save selected Histogram Settings**: Allows you to save any changes you have made to the current Histogram settings file. Any subsequent alterations to the Histogram settings can be saved and loaded (retrieved) at a later date to replicate the Histogram display.

4. **Delete current histogram**: Deletes the current Histogram settings file.

5. **Load selected Histogram Settings**: Discards any changes made since you last saved the current Histogram settings file.

Object and Image Histograms

Switch between different Histogram types using the drop-down menu:

- **Object**: Includes object-level parameters such as size, shape, position, orientation, intensity

- **Image**: Includes parameters that are summed for the entire Field of View, such as area percent, total area and perimeter

Showing and hiding the Histogram

The button near the top of the **Histogram** control panel changes, depending whether the Histogram window 📄84 is currently displayed:

- If the Histogram is not displayed, click **Show Histogram**

- If the Histogram is displayed, click **Hide Histogram, or** click the cross icon in the top of the Histogram window

Histogram Settings

This panel shows the current Histogram settings file in use (if any), and the current X and Y-axis parameters.

Switching between Histogram settings files

To load a previously-saved Histogram settings file:

1. Select a file from the **Current** drop-down menu.

2. Any changes you have made will be lost.

Setting X axis parameter

You can specify which measurement parameter is used for the X axis of the Histogram (currently, the Y axis is always set to display **Number** of objects having the specified X axis parameter).

To set the X axis parameter:

1. Click on the Browse button to the right of the X field.

2. On the **Select Measurement Parameters** dialog, expand the required parameter group.

3. Select the parameter that will be used on the X axis and click **OK**.

(Continued on next page)

Setting up Histogram Bins

Set the Bin type

To configure the bounds for Histogram Bins:

1. Choose an option from the **Type** drop-down menu in the **Bins** pane:

   o **Linear** applies limits to each bin in a linear progression from the lower limit to the upper limit.

   o **Log** uses the same value range and bin count but applies logarithmic increments.

   o **User Defined** allows you to customise the bin limits, and the names and colors used on the histogram. See Bin Setup: User Defined Settings[⌐ 82].

   o **Pre Defined** allows you to display a histogram of your data according to any classifications[⌐ 63] that you have defined (one bin per classification type, with the number of each type shown in the respective bin).

Automatic Bin allocation

In Automatic mode, the upper and lower Bin limits are automatically set to the largest and smallest values measured in the current feature results (which could possibly come from more than one image).

This is the default setting, so that you are sure to see something in the histogram (if, for example, you don't know what values to expect).

Setting the Upper and Lower limits manually

Turn off **Automatic** mode if you know the size range you want, and don't want the limits to change with every measurement.

1. Disable the **Automatic** check box.

2. Enter the highest and lowest results to be displayed on the Histogram in the **Upper** and **Lower Limit** fields.

3. Set the number of **Bins**.

Note: Some combinations of Bin Type and Parameter do not permit you to change bin limits. See also Bin Setup: User Defined Settings[⌐ 82] .

Show Colour Coded Image

Enable this to preview how features will be allocated to Bins on the Histogram (colors are defined here[⌐ 82]).

Bin Setup: User Defined Settings

In the **Type** drop-down menu, **User Defined** allows you to manually define the limits for your classes, rather than having them imposed by the software. This is so you can have different sized classes corresponding to special requirements.

You have to select the **User Defined** mode for these settings to be applied. In Pre Defined, Linear or Logarithmic modes the class limits are automatically calculated.

Setting user-defined colors

You can override the default bin colors whenever the **Automatic** option is available (i.e. not greyed-out).

Note: You cannot disable Automatic mode for Pre Defined Bin types when you are using the 2D Analysis Classifier; the bin colors and limits are defined by your Classifier types).

1. Click the **Bins** button.

2. Do one of the following to change bin colors:

    o   Click **Auto range of Colors** to set the Bin colors to preset shades

    o   Click an individual **Color** swatch and select a color in the resulting dialog

    o   Click **Set all same color** and select a color for all the bins in the resulting dialog

3. Click **OK**.

(Continued on next page)

<u>**Setting user-defined limits**</u>

You can override the default bin limits whenever the **Automatic** option is disabled.

1. On the **Histogram** control panel, disable **Automatic** mode.
2. Select **User Defined** from the **Type** drop-down menu.
3. Click the **Bins** button.
4. Click in a **Limit** field and enter a new limit for the selected Bin.
5. Click **OK**.

<u>**Notes**</u>

- You can also change the upper and lower bin limits directly in the Bins area of the Histogram control panel whenever Automatic mode is disabled.
- You cannot disable Automatic mode for Pre Defined Bin types.

Displaying and controlling the Histogram

Once you have created a Histogram settings file, you can display the Histogram.
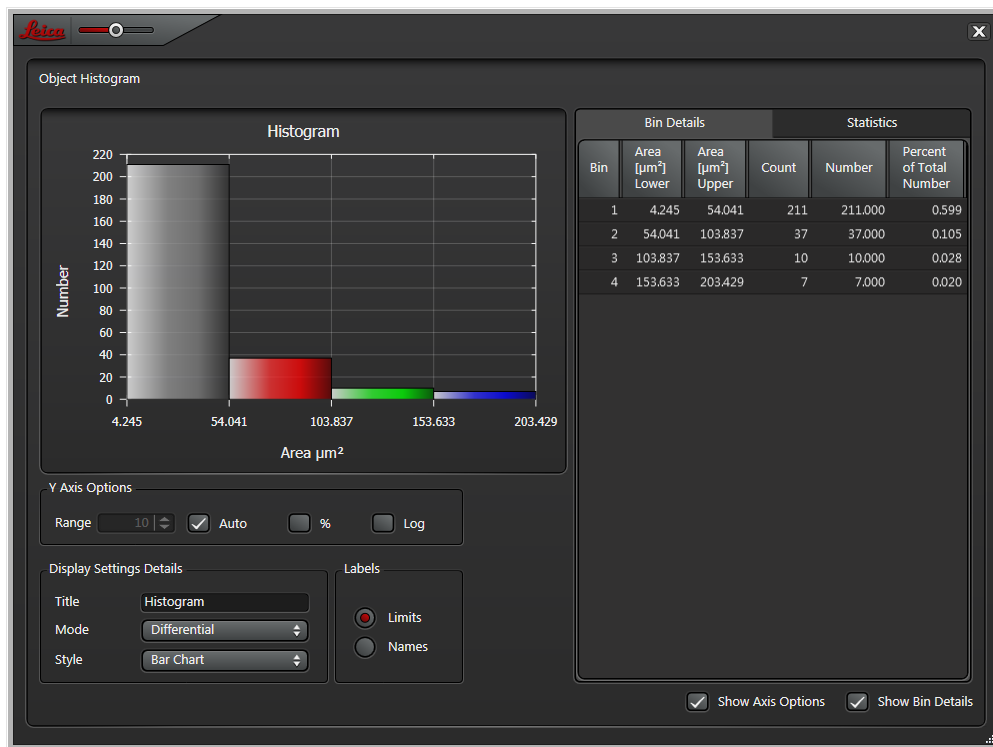
Showing Bin details and Statistics

Enable the **Show Bin Details** check box at the bottom of the **Histogram** window.

- **Bin Details** tab: displays a comprehensive breakdown of the results in each bin
- **Statistics** tab: Gives statistical information about the results (e.g. Standard Deviation and 2-S range).

Axis options

Enable the **Show Axis Options** check box at the bottom of the **Histogram** window.

Y Axis Options

- **Range**: If Auto is disabled, you can specify the Y axis range
- **Auto**: If enabled, optimum Y axis range will be calculated and displayed automatically
- **%**: Results displayed as a percentage of all measurements
- **Log**: Results displayed on a logarithmic scale

Display Setting Details

Title

You can enter a name for the currently displayed Histogram. This is a label that appears at the top of the Histogram display, and is used in the report. It is not the same as the name of the Histogram settings [79] file.

(Continued on next page)

Mode

1. Choose a display mode from the **Mode** drop-down menu in the **Display Settings** pane:

    o **Differential**: displays the results in each bin as a direct value against the Y-Axis scale.

    o **Cumulative+**: adds the results progressively across the Histogram to display an ascending ramp

    o **Cumulative-**: subtracts the results across the bins resulting in a declining ramp

Style

To change the way your results are displayed, choose an option from the **Style** drop-down menu in the **Display Settings** pane:

- **Bar Chart**: Standard bar chart with measurements along the Y axis and Bin Names or Limits along the X axis

- **Pie Chart**: Standard pie chart

- **Horizontal Bar Chart**: Bar chart, but with measurements along the X axis and Bin Names or Limits along the Y axis

Labels

- **Limits**: Display the range of results allocated to each bin along the X axis.

- **Names**: Display the Bin Name along the X axis.

Create Report

The **Create Report** step saves data generated by a Processing Sequence into a Microsoft Excel spreadsheet.
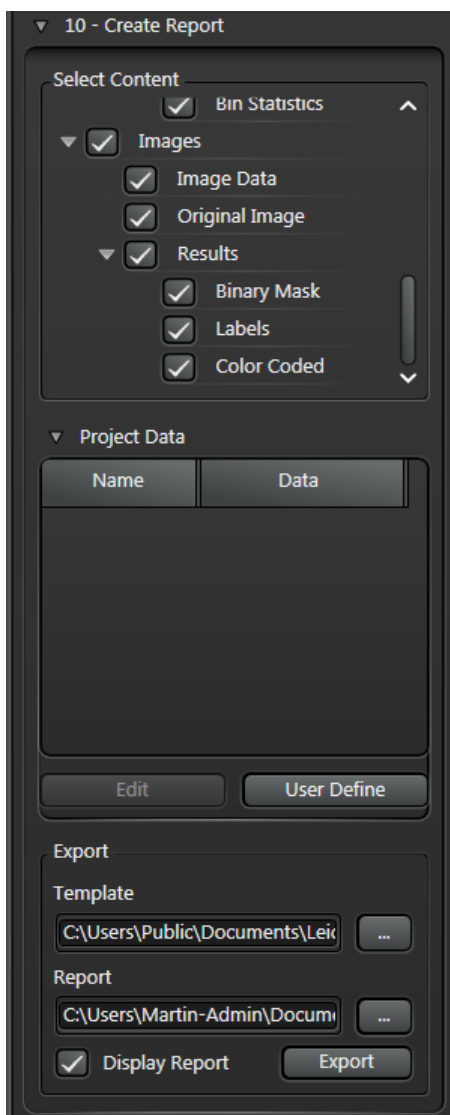
A standard Excel-compatible template is supplied with **LAS AF 2D Analysis**. If you want to develop your own template, open the standard template, save it with a different name, and work with that copy.

To set up and create a Report:

1. Click on the **Create Report** entry in the **Processing Sequence** panel.

2. In the **Create Report** control panel, enable check boxes in the **Select Content** panel to include images and other data items in the Report.

3. Use the **Project Data** [87] panel to customise the project-specific information to be saved with the report (e.g. Specimen, Technologist name).

4. If necessary, use the browse buttons in the **Export** panel to select a Report Template and specify the output file name.

5. If you want to view the report automatically after it has been generated, enable the **Display Report** check box.

6. Click **Export**.

By default, Report templates are stored in the following folder:

C:\Users\Public\Documents\Leica Microsystems\LAS AF\Measurement\ReportTemplates

Project Data

Any **Project Data** you enter as part of the Processing Sequence is included when you create a [Report](#)[86] .

Toolbar buttons

1. Create New Project Data Entry
2. Delete Selected Entry
3. Show/hide selected entry
4. Show all entries
5. Hide all entries
6. Move selected entry up
7. Move selected entry down

To edit a Data field quickly

If Project Data fields are already defined:

1. Click on the **Create Report** entry in the main menu.
2. In the **Project Data** control panel, enter relevant text in the **Data** text boxes.

To edit the keywords in a Data field

1. In the **Project Data** control panel, select a **Data** field and click on the **Edit** button.
2. Make your changes in the resulting dialog (the dialog header displays the name of the field being edited); you can use line returns.
3. If necessary, click **Next** or **Previous** to navigate between fields; the dialog title changes as you do this.
4. Click **OK** when you have finished editing.

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To manage Project Data field names

1. In the **Project Data** control panel, click the **User Define** button.
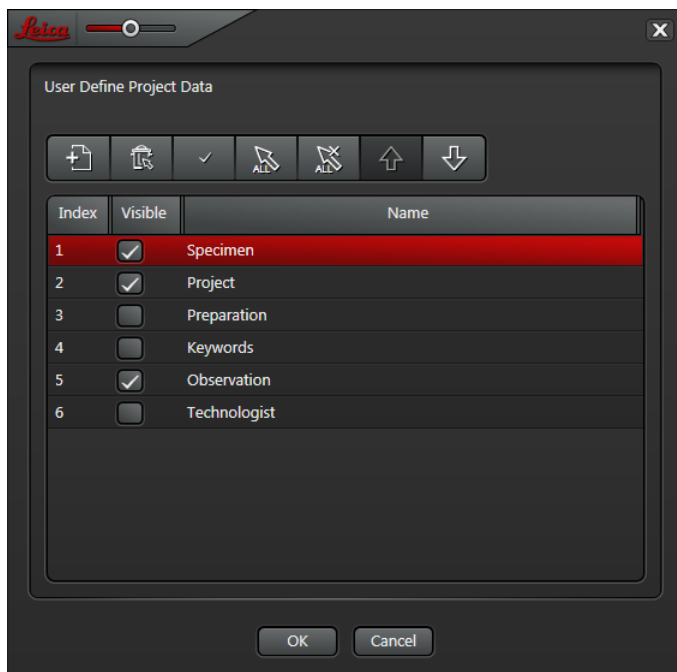
2. Use the **Tool Bar** buttons to make changes.
3. Click **OK**.

Default Report Contents

The default Image Analysis Report has a number of tabbed Excel sheets. Depending on which options you enabled using the Table tools⌐60 icon, some sheets may not contain any data.

- **Analysis Report**: A summary of the results; some cells refer to data contained on other sheets (such as the Feature Histogram).

- **Reference Data**: Reflects the information you entered on the Project Data⌐87 panel.

- **Feature Statistics**: Summary and full measurement statistics.

- **Feature Details**: Automatic measurement results (summary and full details).

- **Feature Histogram**: Histogram statistics, bin data and histogram chart.

- **Field Measurements**: Tables containing field measurements (summary and full details); Histogram statistics, bin data and histogram chart.

- **Images**: For each image specified in the **Select Content** pane of the **Create Report** panel, this sheet contains: Image data (name, pixel size, etc), source image, binary mask image, label image and color coding image.

Appendices

This section contains supplementary information about image processing, along with some examples.

At the heart of most image processing is a concept called a **Structuring Element**, shown in its simplest form in Illustration (**A**). It comprises a matrix of cells, each of which will be a binary value – either 1 or 0. The centre (red) cell is called the Origin.

In Illustration (**B**):

1. The original Greyscale Input Image, with each pixel represented as a value in the range 0 to 255 - black to white.

2. The Output Image which will be created using values determined by the Input Image and the Structuring Element.

3. The Structuring Element is used as an 'overlay' with the Origin (red) Cell positioned over a pixel on the Input Image. This is also colored red and called the Input Pixel.
   Those pixels either side of the Input Pixel are called Neighbours, shaded pink.

4. The value of the Neighbour pixels are used to create a new Output Pixel in the Output Image.
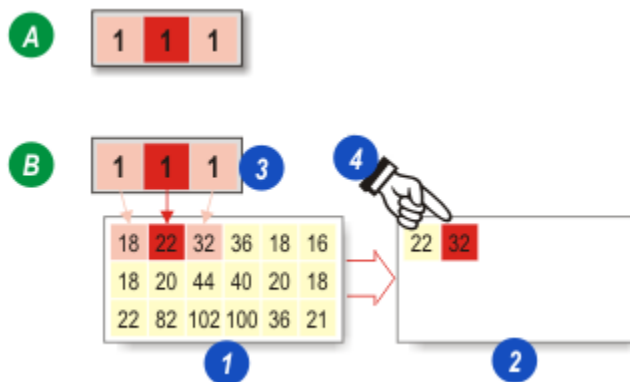
A Neighbour pixel is only tested if the corresponding cell in the Structuring Element is set (=1). In the example, both cells in the Structuring Element are set so the Neighbour pixels either side of the Input Pixel will be evaluated.

Brightening detail (Dilation)

In this process, the Neighbour pixel with the greatest value determines the value of the Output pixel. Since, in a greyscale image, the lighter pixels have the greatest values (closer to white at 255), the effect is to increase the area of lighter detail and reduce that of darker detail.

In the Illustration (B) the Structuring Element Origin (red) is positioned above an Input Pixel with the value 22. The Neighbour pixels values are 18 and 32. The intention is to brighten the image and so the Neighbour pixel with the greatest value is used for the new Output Pixel – in this case 32.

In Illustration (C) the Structuring Element has moved right to the next Input Pixel. Now the values in the Neighbour pixels are 22 and 36 and again the greatest is used for the Output Pixel.
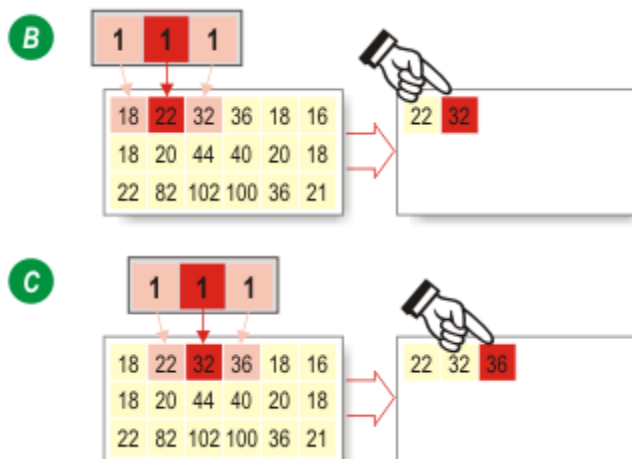
Darkening detail (Erosion)

To darken the image the same evaluation process is used but in this process the Neighbour pixel with the lowest value (closer to black at 0) is used as the Output Pixel. Illustrations (D) and (E) show the process.

All of the greyscale Input Image pixels are tested until the Output Image is complete.

Dilation and Erosion
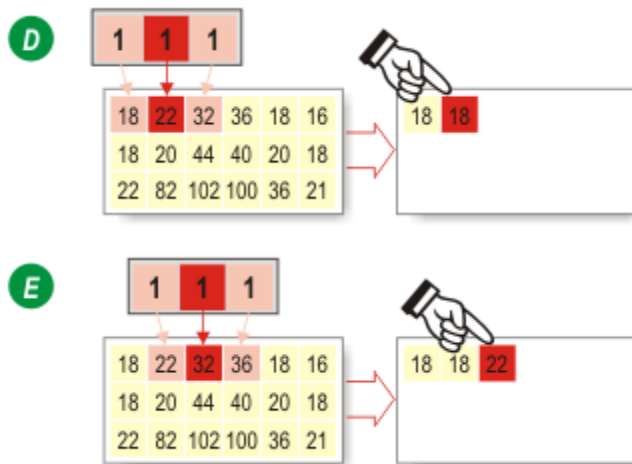
A simple example

Illustration (A) is a greyscale image – a small block of pale grey pixels each with a value of 120, surrounded by dark grey pixels with a value of 30.

A 2 x 3 Structuring Element (1) is to be applied to the image, first as a Dilation and then as an Erosion.

Dilation uses the Neighbour pixels with the greatest values so in Illustration (B) the pale grey block has 'grown' adding pixels to the top and sides.

The process is reversed by Erosion in Illustration (C). Erosion uses the smallest values found in the Neighbour pixels so the pale grey pixels (with a value of 120 closer to white) have been replaced by dark grey pixels (with a value of 30 closer to black).

The image shift – the pale grey block has moved upward by a row of pixels – is due to the design of the Structuring Element, and can be prevented by using a more appropriate matrix.
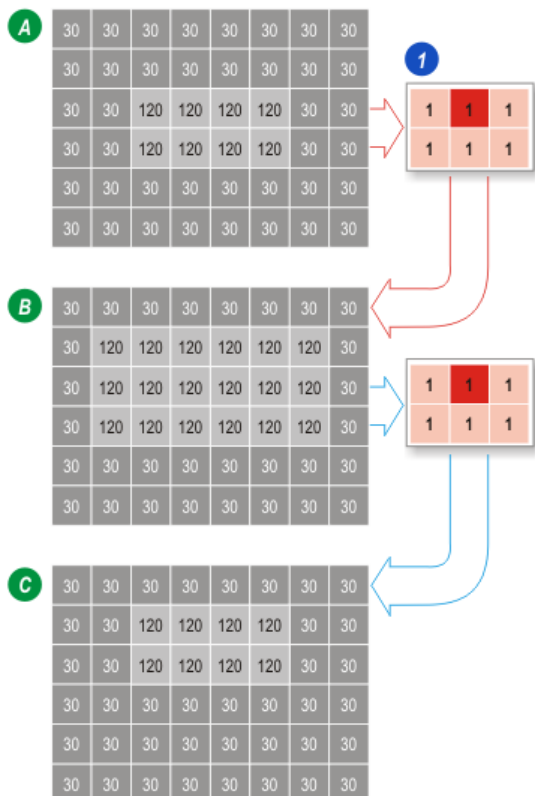
(Continued on next page)

A practical example

Greyscale image (1) shows a group of 8 black discs on a white background. The transition between black (0) and white (255) on an image is seldom that distinct – very often there will be several grey pixels around the boundary of a dark feature giving it a 'blurred' appearance. The blurring may be due to incorrect focussing, poor lighting or perhaps the feature itself is fuzzy.

The blurred boundary can make adjacent features appear to be touching each other and, if the intention was to count the features in an image, the discs in Figure (1) would be treated as 3 features – groups (A), (B) and (C) - instead of 8.

The boundaries between 2 adjacent discs may look like the pixels in Illustration (2), the black pixels of the discs blurring through shades of grey with white background pixels appearing at the extreme edges.

A Dilation (Illustration 3) uses the greatest Neighbour pixel values to create an Output Image so at the edges of the blurring, white (value of 255) would replace pale grey pixels (value say of 210). The 'new' white background pixels can be seen in the centre.

The amount of Dilation depends upon the Structuring Element matrix. Illustration (4) shows the effect of applying a Structuring Element with a greater number of cells testing a wider group of Neighbours. Here several grey pixels have been replaced with white pixels to create a larger gap between adjacent discs.
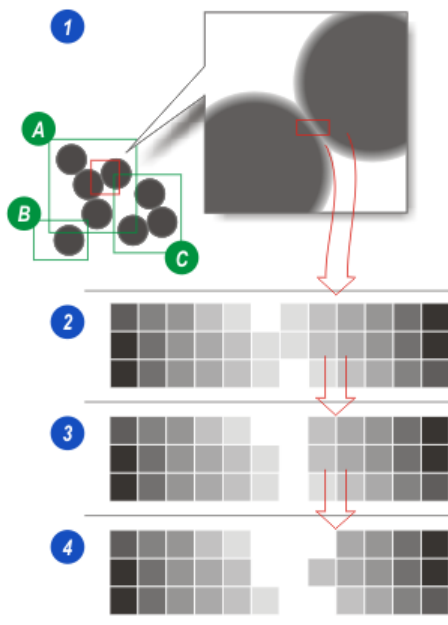
Erosion then Dilation

An Erosion followed by a Dilation is called an Opening Filter.

1. The same greyscale image but this time 8 white discs on a black background and apparently 'grouped' because of edge blurring. The task is the same – separate the discs without losing important dimensional data.
   Light features against a dark background are often better separated by using *Erosion* followed by *Dilation*.

2. The original 'blurred' image.

3. **After Erosion – Neighbour** pixels with the smallest value (nearer to black at 0) replace those with *greater* value (closer to white at 255) – the grey pixels around the boundaries of the discs have been replaced with black.

4. **After Dilation – Neighbour** pixels with the greatest value (closer to white at 255) replace those with closer to black (at 0). The effect is to 'grow' the white discs and, with the appropriate *Structuring Element*, to closely re-establish the discs' diameter without causing them to touch again.
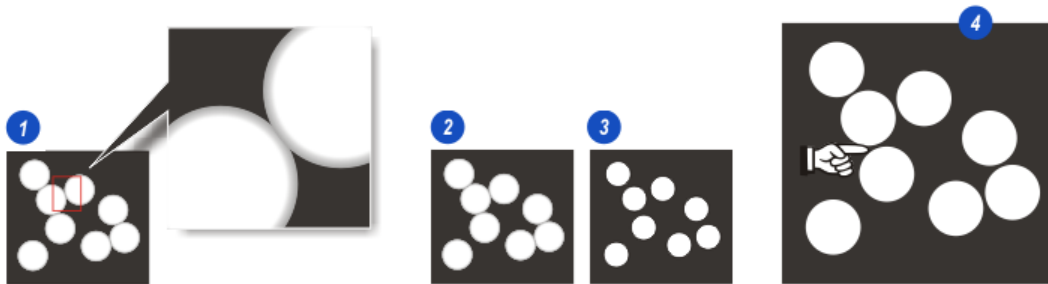
Dilation then Erosion

Dilation followed by Erosion is called a Closing Filter.

Illustration (5) is the original blurred image and (6) is the same image with successive Dilations until all of the grey pixels have been replaced with white – only distinct, separate black discs and white background remain – 8 features now instead of 3.

However, removing the grey pixels may have also reduced the actual size of the discs, which could be detrimental if areas or dimensions are to be measured. An Erosion will make the black discs grow – smallest value Neighbour pixels (those nearest black at 0) replace higher value pixels (those closest to white 255). Erosions continue until the black discs are close but not touching to complete the transition (7).
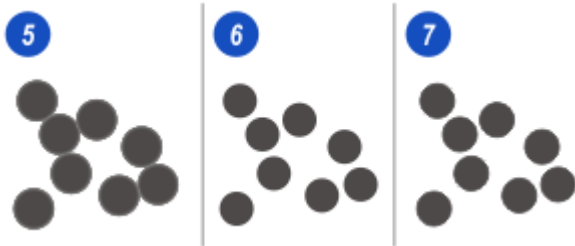
Open and Closed Filters

Open and Closed Filters have a wide range of applications in image processing, especially in noise removal.

1. A greyscale image with clumps of small features in the background which could be 'noise' or just insignificant parts of the image (unwanted artefacts that need to be removed before accurate measurements can be mad) e.

2. Successive Dilations have not only separated the black discs but also removed the 'noise' artefacts completely – simply because they are small.

3. Erosion enlarges the discs to their original size but because the 'noise' no longer exists there is nothing to enlarge.

4. Removing small artefacts in Image Analysis; The original Input Image.

5. The Output Image with the artefacts removed and the grey features restored to their original size.
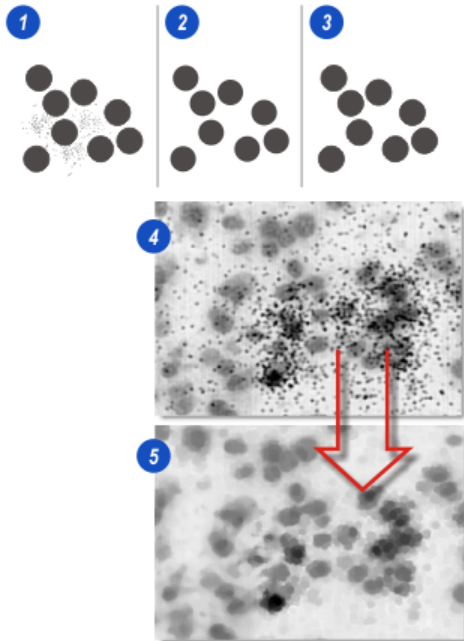
More About Structuring Elements

On the edge of the Input Image, the first Neighbour Cell of the Structuring Element will point to a non-existent pixel beyond the edge of the image. For greyscale images (Illustration A) and depending upon the process – Dilation or Erosion – these pixels are assumed to have values of 0 for Dilation and 255 for Erosion. This arrangement prevents border effects – fine, dark lines appearing around the output image.
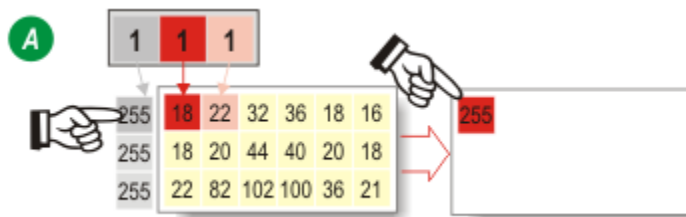
Almost any layout can be applied to a Structuring Element; (Illustration B) shows a common 3 x 3 cell configuration in which a set cell (=1) is an active Neighbour used to evaluate the Input Image pixel values, whereas a cell cleared (=0) will be ignored.
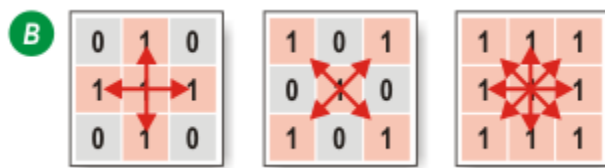
The Structuring Element matrix – how many cells it contains – generally reflects the size and shape of the features being detected within the Input image. Image Analysis uses a circular configuration which expands or contracts depending upon the size selected by the user (Illustration C).
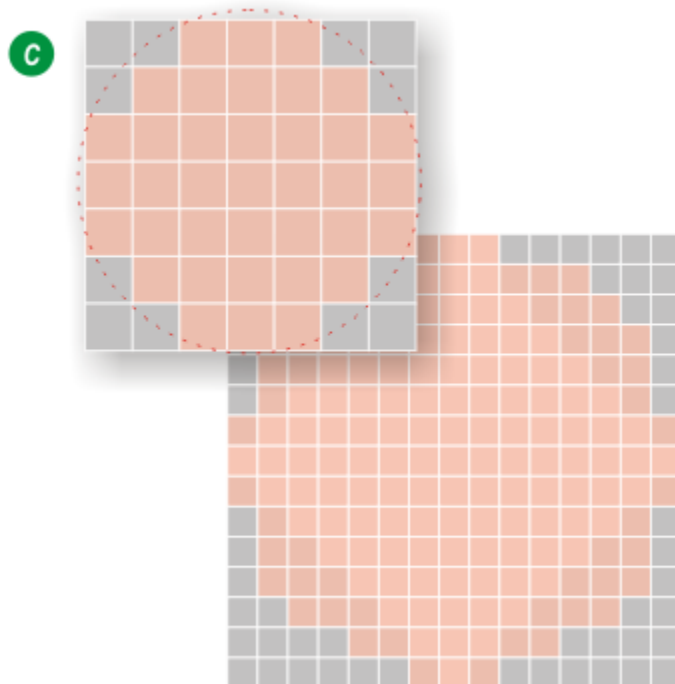
Median Filter

A Kernel with a 3 x 3 matrix – the Origin has 8 Neighbour Cells. The filter extracts the 9 pixels from the Input Image and calculates a Median value, that is a value at the mid-point when the pixels are arranged in order of value, not an average.
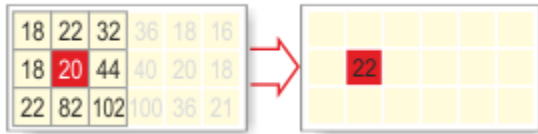
Image Analysis in action with the Median Noise Removal tool selected. (1) is the Input Image and (2) the Output Image.
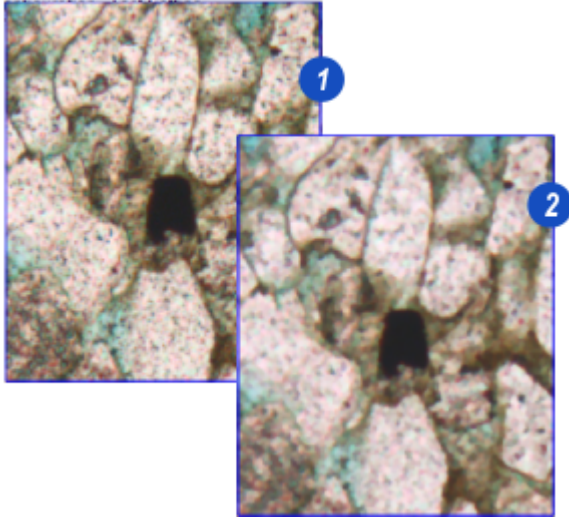
Tophat Filters

Some of the most useful morphological filters, Tophats, belong to a sub-class of operations called residues (since they produce something which is 'left over' from the original image).

Tophats can be defined as:

Smoothing > Difference > Tophat

The net effect is to enhance whatever was removed during the smoothing process, which will usually be the detail of the image.

The most common Tophats are constructed by using grey openings and closings as the smoothing operations. The Find black detail -Tophat is defined as:

Tophat = Closed Image - Original Image

The effect of a black Tophat is to pick out the small dark features in the image. The closing will remove the dark features up to a size defined by the cycles used, replacing them with the grey level of the lighter regions which surround them, and the subtraction of the original image will thus show the grey level difference between these 'filled in' pixels and their grey levels in the original image.
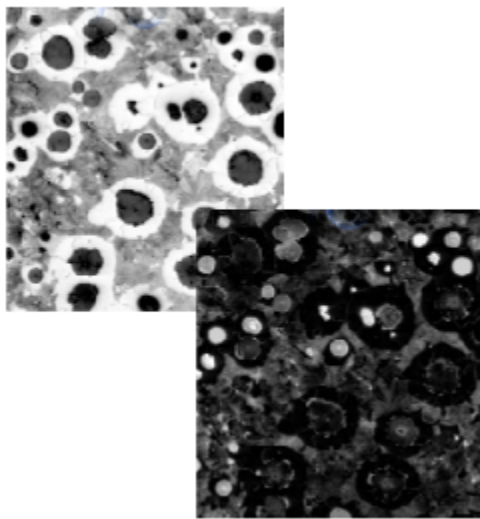
Unsharp Mask

An unsharp mask filter amplifies the high-frequency components of a signal.

The "unsharp" of the name derives from the fact that the technique uses a blurred, or unsharp, positive image to create a mask of the original image. The unsharp mask is then combined with the negative image, creating an image that is less blurry than the original. The resulting image, although clearer, may be a less accurate representation of the image's subject.
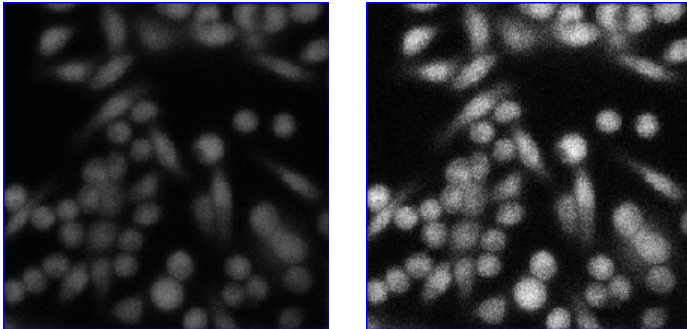
Examples of Pre-Filter usage

The following topics give some examples of pre-filter usage.

Example 1

The aim of this example is to measure the number of biological cells on an image.

The problem with the original image (A) is that the cells overlap, have a range of differing colors, have irregular outlines with varying contrast, and some have coalesced into indeterminate 'blobs'.

Skipping a Greyscale Pre-Filter and going directly to Adjust Threshold (Illustration B) results in a fuzzy Binary Output Image with scattered additional artefacts, all of which could be included in the measured count. Adjustments to intensity could improve the image slightly, but it would be a time-consuming process, not necessarily resulting in a better count. It is better to use a Pre-Filter to isolate individual cells, allowing each to be counted with acceptable accuracy.

Because the cell outlines and contrast are so variable, an alternative solution would be to count the 'nuclei' – they have a more-or-less consistent color and size – and where cells overlap the nucleus tends to show through and can be counted.
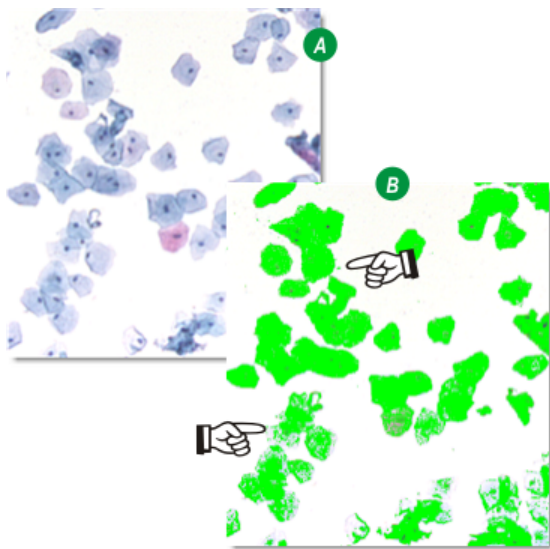
The **Enhance Black Detail** filter was chosen to improve the contrast between the nuclei and the surrounding cell tissue. Applying a structuring element of size 12, many of the intermediate greys have disappeared, the cell edges have become more clearly defined and the nuclei contrast is especially enhanced.

There are still areas of the image in which the cells have clumped together but these may be removed or more clearly defined during **Adjust Threshold** or with **Binary Image Edit**.
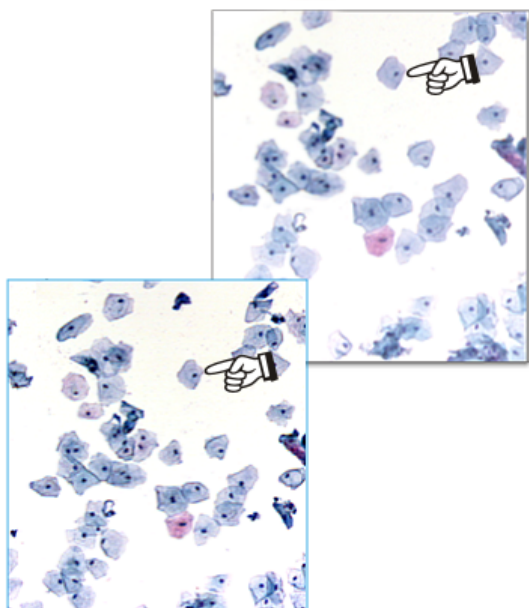
(Continued on next page)

The next step is to apply the Thresholds.

1. Check that **Show Binary** is enabled.

2. Check that **Accumulate** is enabled.

3. With adjustments to the upper and lower Intensity Thresholds, the resulting Binary Output Image highlighted the nuclei and some clumps of artefacts all shown in green the default color.

4. A first measurement for cell count yielded 86 but that included the clumps of artefacts as well. The feature highlighted in yellow is currently selected on the Results Grid.
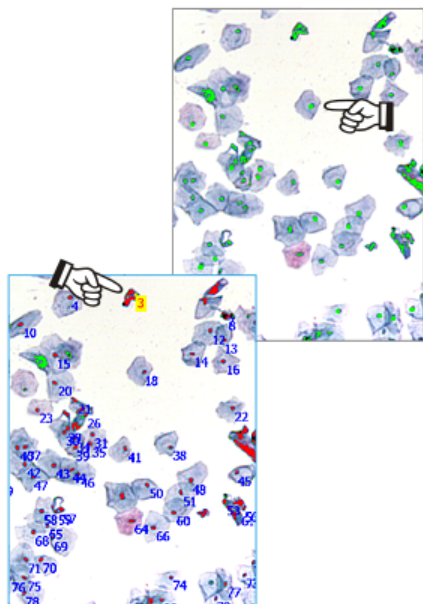
There are several methods for removing unwanted features – Reject and Delete Modes in Binary Edit for example, but in this example they were excluded by refining the upper and lower limits of the **Area Measurement** tool.

A first pass with a wide difference between the upper and lower limits produced an area value for all of the features, nuclei as well as unwanted artefacts. From this it could be seen that the nuclei areas fell between 10 and 50μm^2 whereas the unwanted artefacts were considerably larger.

The Area Parameter limits were then set to include the nuclei with the result that all of the unwanted artefacts were ignored and the count reduced to 80.

In this example a Measure Frame was used to select just a small part of the image since the features were evenly distributed across the entire image and the smaller Measure Frame would yield a good mean result. It also speeds some of the processing time.

(Continued on next page)

Once the correct filtering and thresholds have been established, the Measure Frame can be set to Entire Image and a further measurement made – in this case it resulted in a total count of 294 cells.
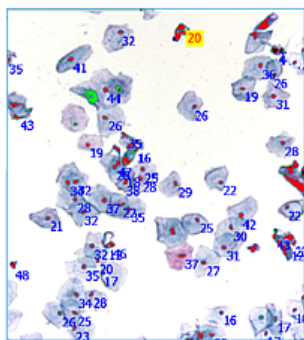
See also:

[Adjust Threshold](#) 29

[Results Grid](#) 60

[Measurement Tools](#) 54

[Area Parameter](#) 113

Example 2

In this example a piece of schist, a crystalline rock, is the original image. The specimen contains some iron ore which shows up as red-orange areas scattered randomly across the image. The test is to determine what percentage of the sample comprises iron ore.

In such a complex image there are likely to be many single, unconnected pixels that are close to the iron ore color range for which we will be searching. Leaving these pixels in place will slow the measurement process considerably without beneficially affecting the end result, so an overall 'tidy-up' to remove 'stray' pixels and consolidate larger groupings is a good starting point.

The Pre-Filter chosen was Smooth White Detail with a structuring element of Size 3. Because Smooth White Detail is an open filter – erosion first and then dilation – some features disappear completely which is good for removing the stray pixels, but it can also start to reduce the area of the wanted features. Since this example will measure area, the filter has to be used sparingly.

The second image shows the result of the Smooth White Detail Pre-Filter.
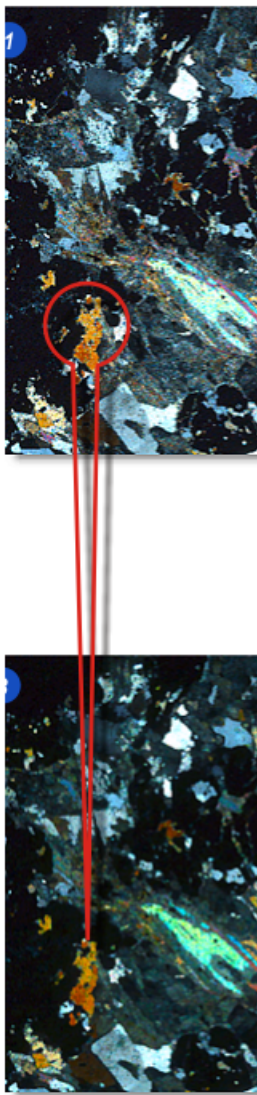
(Continued on next page)

It took only minor alterations to the Adjust Threshold [29] intensity and saturation to produce a clean, well-delineated Binary Output Image (1) which, when compared with the original (2) proved to have faithfully pin-pointed the traces of iron ore and ignored everything else.

However, there were some holes and fissures in the main accretions which looked as though they should be included, but trying to fill them with the Threshold controls also tended to draw unwanted artefacts into the Binary Image.
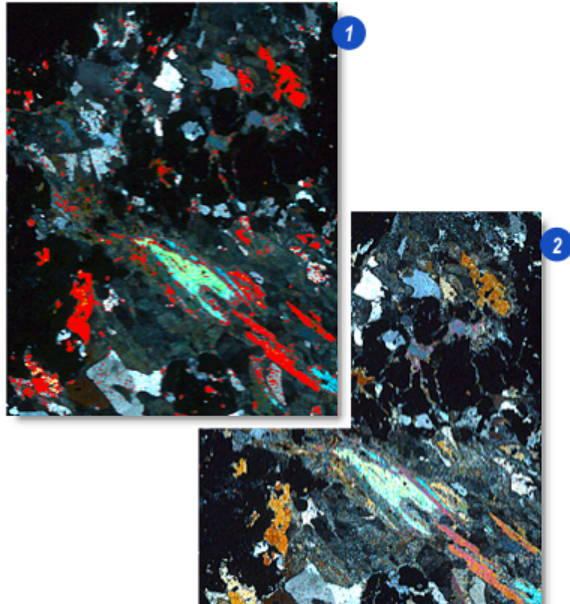
There is an easier way to fill holes: Adjust Threshold [29]

1. The **Combine Detail** filter, which is part of the Binary Processing Pre-Filter collection [36], can quickly, simply and precisely fill holes and fissures in a Binary Output Image.

2. The **Reconstruct** and **Fill** options were enabled.

3. The structuring element **Size** control was incremented in single steps until the holes in the major features were filled without gathering together some of the more separated features.

4. A first measurement yielded a total feature (iron ore) area of 152670µm^2 which was displayed on the Grid View.

5. This was within a manually defined Measure Frame of 2587631µm^2 giving a value of 5.9% iron ore.

To check that the measure frame represented an average part of the image, the frame was reset to Entire image and the area measurement was taken again. This yielded a feature area of 577912µm^2 within an image area of 9513571µm^2 - 6.0%.

See also:

Binary Processing Pre-Filters [36]

Grid View [60]

Example 3

The diamond chips in the original image (1) produce an acceptable Binary Output image if passed directly through the Adjust Threshold, but some fast processing with a Greyscale Pre-Filter reduces the amount of fine adjustments made to the Threshold and can improve measurement precision considerably.

LAS AF 2D Analysis is so fast that it is always beneficial to run an image through several greyscale filters to check for improvements.

In this example the image was processed with the Smooth Black Detail Pre-Filter that removed some of the intermediate grey values and increased the chip edge contrast (2).

However, it had to be used carefully since this was to be a feature count – how many chips in the image – and because several features overlap, the Smooth Black filter could have blended them together resulting in a single feature rather than two.
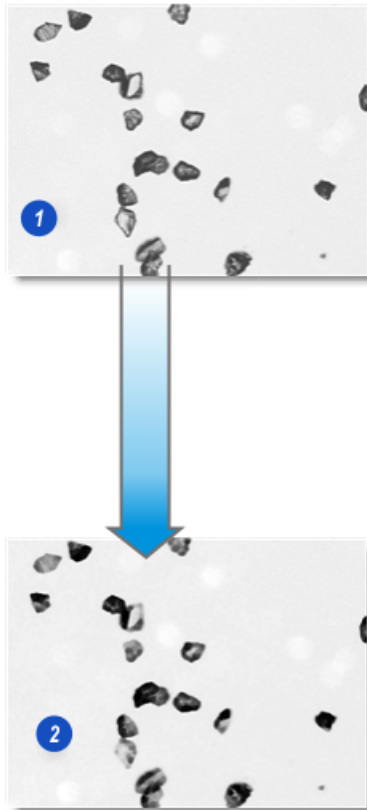
1. Diamond chips is a monochrome image with predominant greys closer to the black end of the scale, so the **Grey Range Selector** was set to **Blacker than** and minor adjustments made to the **Histogram** sliders.

2. The result is a very good **Binary Output Image** with small holes in just one of the chips.

In this example the holes in several of the diamond chips would not have made a difference to the final feature count, but since it was necessary to pass the image though a **Binary Pre-Filter** to make sure that there was good separation where the chips overlapped, the **Combine Detail** with the **Fill** option enabled was used to fill the holes with the *Size* setting at 6. Again, this was used sparingly because some of the chips are close together and could have been combined.

There were four chip pairs that were either overlapping or touching; They can be clearly seen on the original.

(Continued on next page)

With **Separation > Split Touching** enabled, two steps were sufficient to separate the overlaps, indicated by the red lines on the Output Image**.**
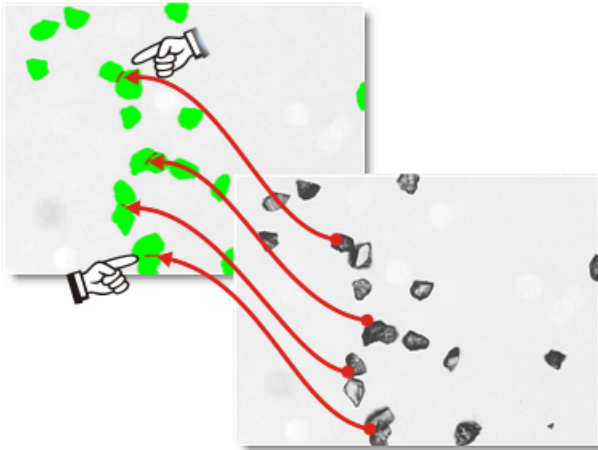
The final step before counting the diamond chips was to remove the fragment (bottom right) to prevent it being counted. This was achieved with **Binary Edit Delete Mode**. The fragment was not actually removed from the Binary Output Image but only highlighted and ignored in the count.

The count shows 18 chips in the image and shows that the **Cut Touching** tool is working well.
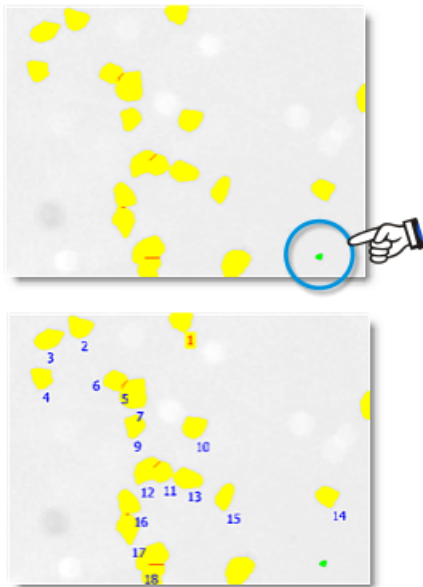
Binary Processing Principles

Illustration **(A)** shows a typical Binary Processing sequence:

1. The Greyscale Input Image comprising 8 touching disks; the task is to count the disks and measure their areas. Performing a **Number** (Count) and **Area** calculation on this image would yield just 3 features with diverse areas because some of the disks are touching.

2. The Output Image after a Greyscale Processing **Erosion** – the disks are no longer touching so a **Number** (Count) would yield 8 features. But some diameter has been lost so the *Area* calculation would be understated.

3. The Output Image after a Greyscale **Dilation**. The disks are still separate, and much of their original diameter has been restored. A count measurement would now be much more accurate.
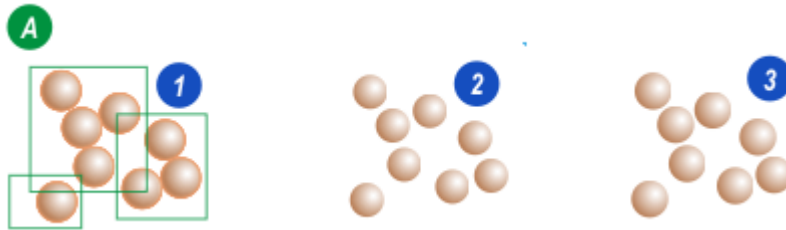
In Illustration **(B)**:

1. The image has passed though **Adjust Threshold** and those grey values falling within the threshold limits are colored red. The highlights on the disks have not fallen within the threshold limits and so remain white.

   The **Output Image** from the **Adjust Threshold** has converted a Greyscale Input with grey value ranging from 0 (black) to 255 (white), to a *Binary Output Image* in which those pixels selected are set to a binary value of '1' and those not selected cleared to a binary value of '0'.

2. An enlarged view of a disk. The string of binary digits below the illustration represents a single row of pixels (shown by the green line in the illustration). The selected (red) areas of the disk are set to '1' and the unselected highlight (white) areas cleared to '0'.
   There are 'holes' in the disks!s

3. For a proper **Area** measurement those 'holes' need to be selected and filled. **Binary Processing** can do that quickly and efficiently.
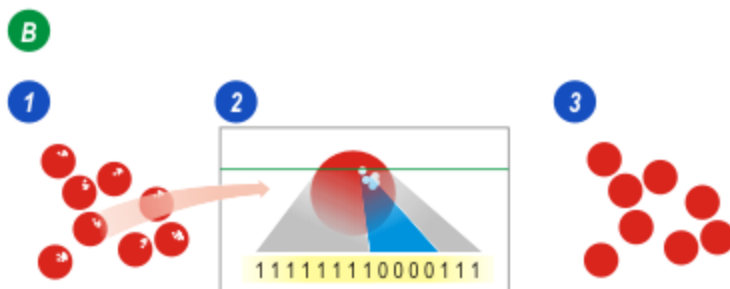
(Continued on next page)

Binary Dilation

The **Binary Pre-Filters** provide the tools for modifying the Binary Image in the same manner as Greyscale Pre-Filters.

In Illustration **(A)**:

- The Structuring Element **(1)** is an 'electronic overlay' with cells that are also set or cleared to binary values. There are three cells, all are set to have a binary value of 1.

- The middle cell **(2)** is called the Origin (colored red) and it 'looks' at a single pixel (also colored red) called the Input Pixel in the Binary Input Image.

- The process examines the pixels neighbouring the Input Pixel to determine whether the corresponding pixel **(3)** in the Binary Output Image **(4)** should be set or cleared. The Structuring Element settings determine whether a neighbour is tested (1) or ignored (0).

- The process described in this illustration is called a Dilation – if any of the neighbours are set (=1) then the Output Pixel is also set – which is the case in Illustration (A). A Dilation has the effect of increasing the number of selected pixels.

Illustration **(B)** shows a simplified form of dilation:

- The original object **(5)**.

- An imaginary 'roller' **(6)** tracing around the periphery of the object.

- The new outline of the object **(7)**.

In Illustration (C) the Structuring Element has moved one pixel to the right. Now the Input Pixel (red) and its neighbours are all cleared (=0) and so the pixel in the Binary Output Image is also cleared (1). The process continues for every pixel until a complete and new Binary Output Image has been created.

Binary **Dilation** would be a most suitable pre-filter for filling the highlight 'holes' in the example image **(D).**
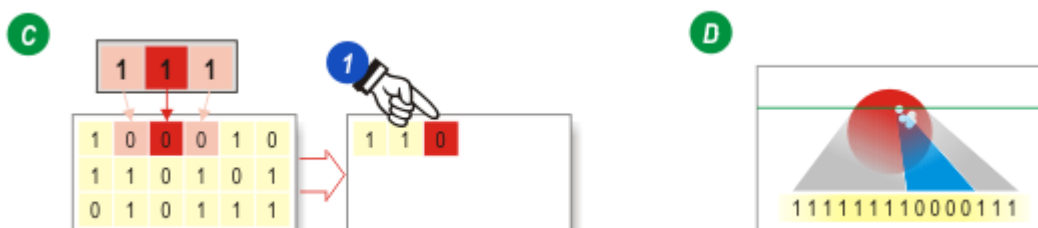
(Continued on next page)

Binary Erosion

1. The Structuring Element.

2. The Binary Input Image with pixels either set (=1) or cleared (=0).

3. The Binary Output Image. Set pixels are included in a measurement but cleared pixels are not.

The Erosion process examines the Input Pixel's neighbours and if any one is cleared (=0) then the Output Pixel is also cleared. This has the effect of removing selected pixels from the image.

In Illustration (A) a neighbouring pixel is cleared (=0) and so the Output Pixel (4) is also cleared.

The Structuring Element has moved four pixels to the right in Illustration (B) and now both of the neighbours are set (=1) so the Output Pixel (5) is also set.

To determine the value of pixels on the extreme edges of the Output Image, Illustration (C), the Input Pixel (colored red) is 'ghosted' (6) to become a neighbour. The illustration represents a Dilation so the Output Pixel is set, but if this were an Erosion the Output Pixel would be cleared because the right-hand neighbour is cleared.
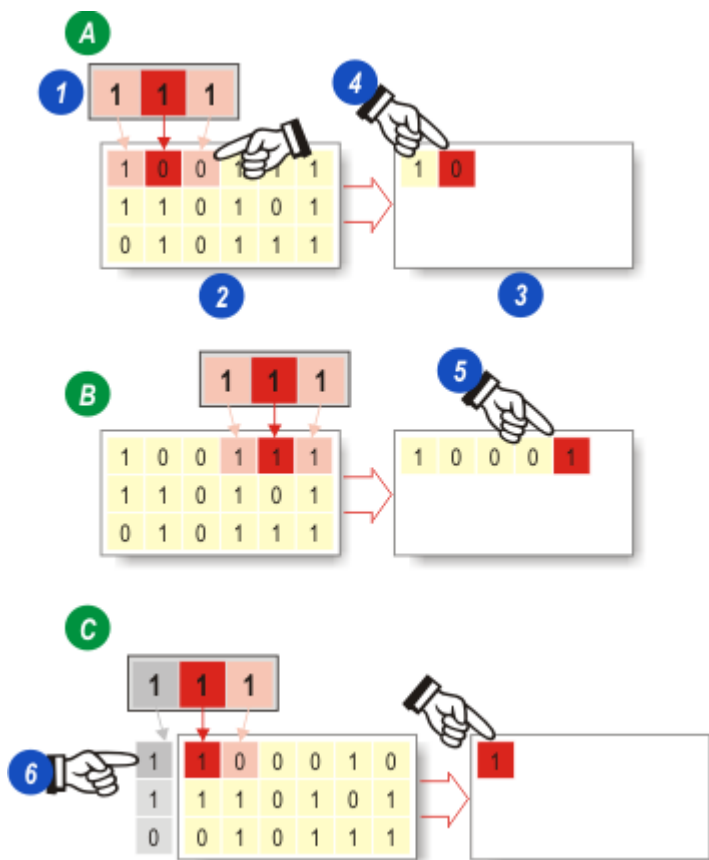
Erosion and Dilation can be used in combination to achieve specific results.

- Dilation followed by an Erosion is called a closing filter, and is used for filling 'holes' in an image.

- Erosion followed by Dilation is called an opening filter and is often used for removing small details such as noise and dust.

Measurement Parameter Descriptions

This section describes the different measurement parameters.

Number

- **Number**: Carries out a count of all the selected objects on the Binary Output Image.

Size

- **Area**: Measures the Area of every selected object.
- **Convex Area**: Derived from the mean Feret diameter approximating to the enclosing polygon.
- **Equivalent Circle Diameter**: The diameter of a circle that has the same area as the feature.
- **Length**: The greatest distance between parallel lines drawn through 2 points on a feature's boundary regardless of orientation. Also called Max Feret Diameter.
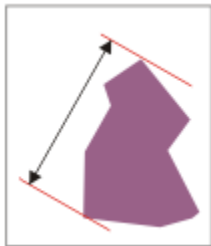
- **Width**: The shortest distance between parallel lines drawn through two points on a feature's boundary regardless of orientation.
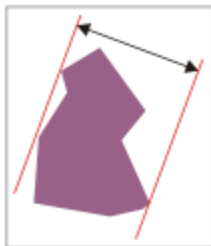
- **Orthogonal Feret**: The Feret diameter perpendicular to the Max Feret diameter or length.
- **Feret 0**: The greatest horizontal distance (width) measured in the horizontal direction.
- **Feret 90**: The greatest vertical distance (height) measured between parallel lines in the vertical direction. Feret 0 and Feret 90 are the equivalent of the width and height of a Bounding Box around the feature.

- **Curve Length**: The Curve Length can measure the actual length of an irregular feature – a piece of string for example - the curves of which may overlap.

(Continued on next page)

- **Curve Width**: Measures the mean or average width of a feature (the feature will probably not have a uniform thickness like it does in the illustration below).
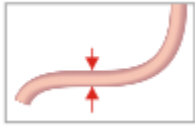
Shape

- **Roundness**: Roundness is defined as $4*pi*area/perimeter^2$. It is 1 for a perfect circle and drops to 0 as an object becomes less round.

- **Shape Factor**: 1/Roundness.

- **Curve Ratio**: The ratio of Curve Length to Curve Width; a good measurement for defining whether something is a fiber (i.e. long and thin).

- **Aspect Ratio**: Object length divided by object breadth or Feret Length/Feret Breadth.

- **Fullness Ratio**: The square root of the object area divided by the object's Convex Area. v(A/CA).

Boundary

- **Perimeter**: Distance around the boundary of the feature, given in the current caibration units. It includes all inlets and projections and compensates for the edge orientation.

- **Convex Perimeter**: Derived from the Feret diameter and approximates to the perimeter of the enclosing polygon.
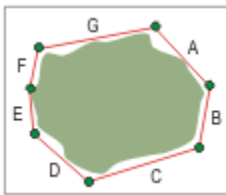
- **Vertical Projection**: The distance representing the shadow cast by a feature if light were impinging upon it from a vertical direction. Presented in the current calibration units.
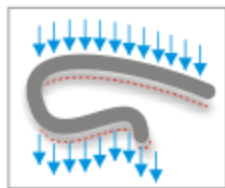
- **Horizontal Projection**: The distance representing the shadow cast by a feature if light were impinging upon it from a horizontal direction. Presented in the current calibration units.

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Position

Note: All the parameters on this page are in pixels.

FCP = Feature Count Point; represents the bottom rightmost pixel in the feature.
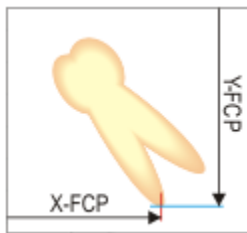
- **X-FCP**: measured from the left-hand edge of the image to the Feature Count Point
- **Y-FCP**: measured from the top edge of the image to the Feature Count Point

Centroid is the Centre of Mass of a feature. Its position is measured from the edges of the image.
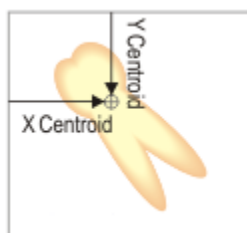
- **X Centroid**: The distance from the left hand edge
- **Y Centroid**: The distance from the feature's top edge.

The feature's position co-ordinates on the image:
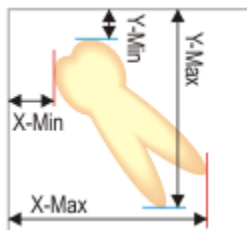
- **X-Min** and **X-Max**: The boundary pixel closest to and furthest from, the left hand edge of the image.
- **Y-Min** and **Y-Max**: The boundary pixel closest to and furthest from, the top edge of the image.

Orientation

Note: All the parameters on this page are in degrees, counting clockwise from vertical, which is 0°.

Derived Orientation is recommended over orientation for measurement of direction. It gives more accurate results than the traditional Orientation.

- **Orientation**: The angle of the Feret Length to the horizontal.
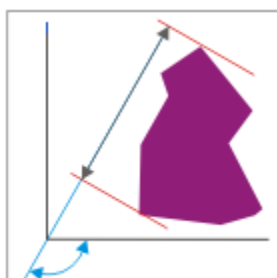
- **Orthogonal Orientation**: The angle perpendicular to the Orientation (Feret Length) - essentially the Orientation minus 90°).
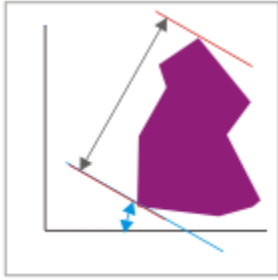
- **Derived Orientation**: The angle representing a line plotted through a range of pixel co-ordinates.
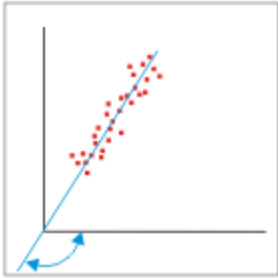
Topology
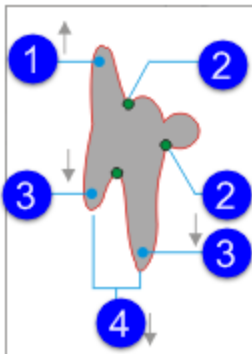
1. **Tops**: The count of upward-extending projections (limbs).
2. **Joins**: The number of points at which a projection (limb) joins the feature.
3. **Ends**: The number of downward-extending projections (limbs).
4. **Forks**: The number of downward projecting fork-shaped limbs.

(Continued on next page)

Intensity/RGB values

Basic intensity/RGB values range from 0 to 255. Other parameters are derived from these values. Intensity parameters apply to single-channel greyscale images and colour images with separate RGB channels); Red, Green and Blue parameters apply to single-channel colour images.

- **Intensity/RGB Mean**: Average brightness of the pixels in the feature; calculated from the sum of the value of all of the pixels in the feature (Integrated Intensity) divided by the number of pixels in the feature (Equivalent to Area).

- **Integrated Intensity/RGB**: Sum of the intensity values of all of the pixels within the feature.

- **Intensity/RGB Variance**: Variance (Standard Deviation²) of the values of the pixels in the feature. This can give an indication of surface texture; smooth objects have a lower value.

- **Intensity/RGB Maximum/Minimum**: The maximum and minimum brightness of the pixels in the feature.

- **Integrated Intensity/RGB (Processed)**: Sum of the intensity values of all of the pixels within the feature, after being passed through the Binary Processing Pre-Filter [36].

- **Intensity/RGB Average (Processed)**: Average brightness of the pixels in the feature, after being passed through the Binary Processing Pre-Filter [36].

- **Intensity/RGB Variance (Processed)**: Variance (Standard Deviation²) of the values of the pixels in the feature, after being passed through the Binary Processing Pre-Filter [36].

Classifier

The **ClassifierUser** and **ClassiifierAuto** parameters are only available if you have installed and licensed the 2D Analysis Classifier.

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