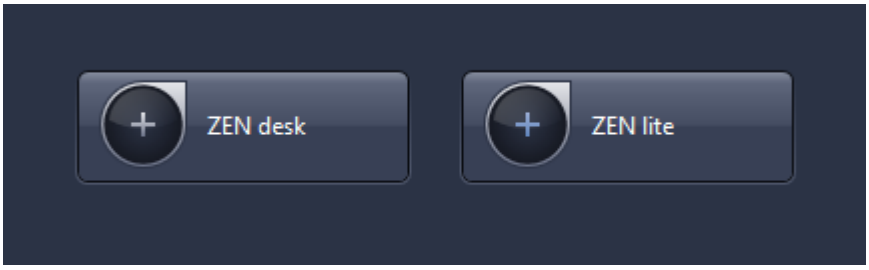
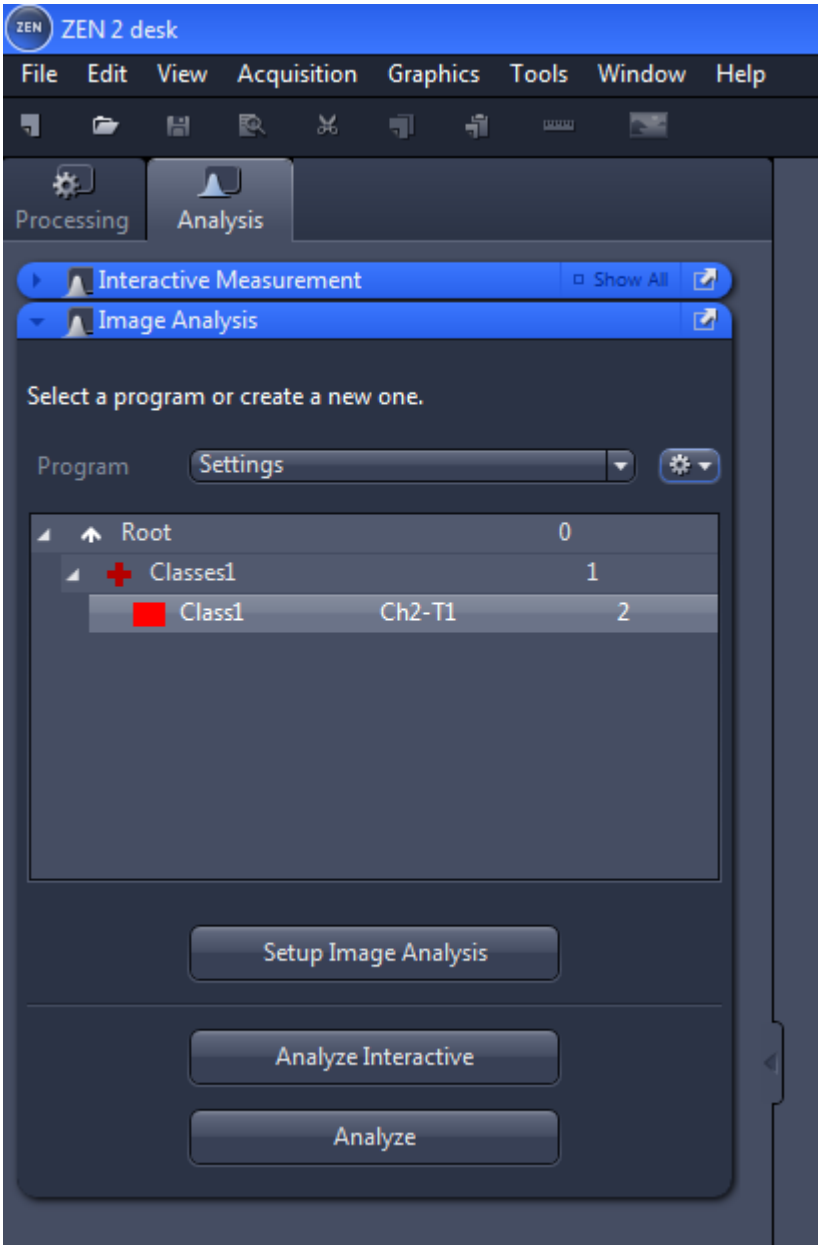
ZEN blue Image Analysis

How to use the Image Analysis Wizard to analyse fluorescent images

- Open ZEN blue.
- Select **Image processing/ ZEN desk**.



- Open the **Analysis** tab.
- Open the **Image Analysis wizard**.

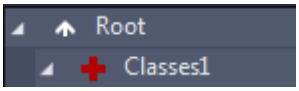


- Create a new program (click on the cog and select new, name the program, then save it).
- In the left-hand area you will see 7 steps which will guide you through the wizard.

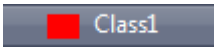
Step 1: Classes

This step establishes the hierarchy of your objects of interest, and how they can be grouped by their fluorochromes.

Classes = groups



= Everything (all objects) of colour A



= each individual object of colour A

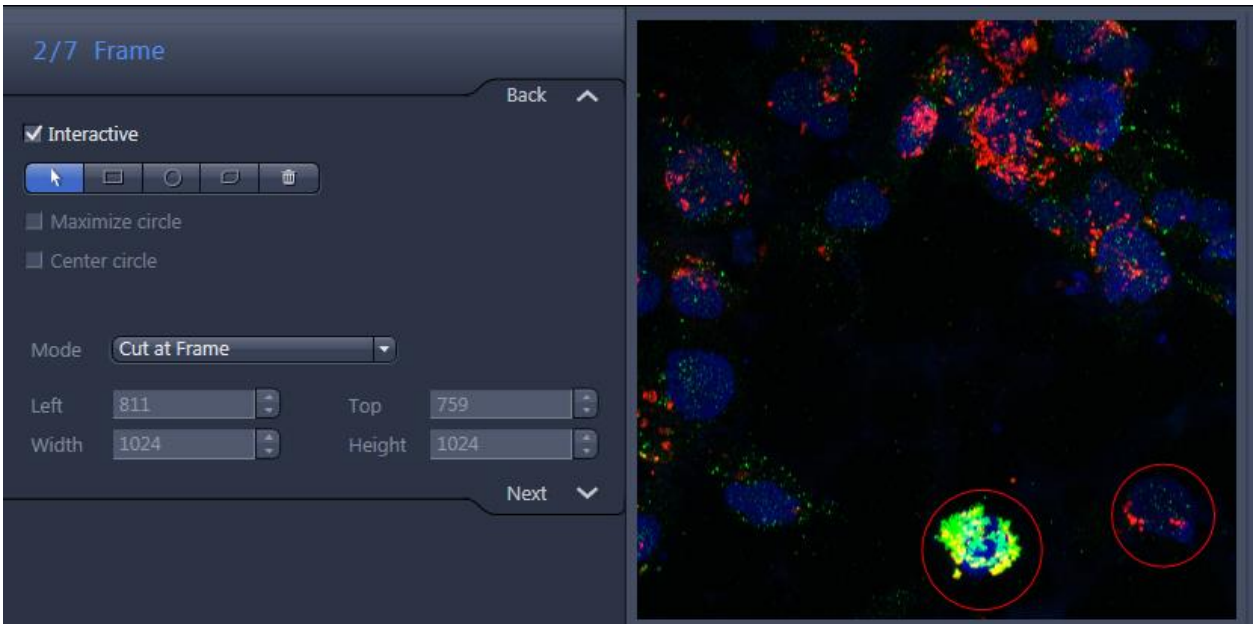
You can select a pseudocolour for highlighting objects within a class (ie the colour of the dye might be red, but to make it easier to see what red objects have been identified by the program you might want to select white as the pseudocolour for highlighting selected objects).
You should be able to create sub-classes by highlighting the 'parent class' and then clicking 'add class'.
Creating sub-classes can be useful for analysing whether one dye is present within the same area as another dye eg whether a dye is within a nucleus. The 'parent' class effectively becomes an ROI.

Step 2: Measurement frame

This step enables you to identify your regions of interest (ROI).

- Interactive box: tick this if you want the program to pause here and require an interaction every time you run it.
- Select your ROI / ROIs: eg a square frame around the whole image eg a circle frame around every cell.
- Select the mode:

Inside and touching – all objects that are lying completely inside the frame, are touching it are measured.
Inside only – only objects that are lying completely inside the frame are measured.
Cut at frame – all objects that are lying completely within the measurement frame are measured, and objects that are intersected by the frame are measured precisely up to the frame.
You can tick the 'maximize' box if you have drawn just one circle for your ROI and you want this to be centred and fill the screen.



Step 3: Automatic segmentation

This step enables you to set thresholds which automatically determine which objects are selected for analysis.

- Generally avoid using 'smooth' and 'sharpen'!
- **Smooth** – can enlarge or soften the edge of the threshold area.
- **Sharpen** – can reduce the edge of the threshold area.
- Tick the execute box.
- Generally always tick the interactive box.
- Set the **Minimum Area** for object segmentation using the slider – this scale is in pixels (range = 1-1,000)
- Use one of the following tools to set the threshold for fluorescence:

1) **Setting fluorescence threshold by clicking on the image**

- Select the colour (dye) of interest in the class and click on the image to tell the program how to 'find' objects of that colour.

- "Pick behaviour" determines whether objects are added or removed when you click them. Tick the plus sign to add objects or the minus sign to remove objects.
- Neighbourhood = how many neighbouring pixels the program looks at when you click on a pixel.
- Increasing the neighbourhood value increases the number of pixels segmented per click.
- Increasing the tolerance value also increases the number of pixels segmented per click.
- Tick 'fill' to ensure that holes in segmented objects are filled and included within the object.

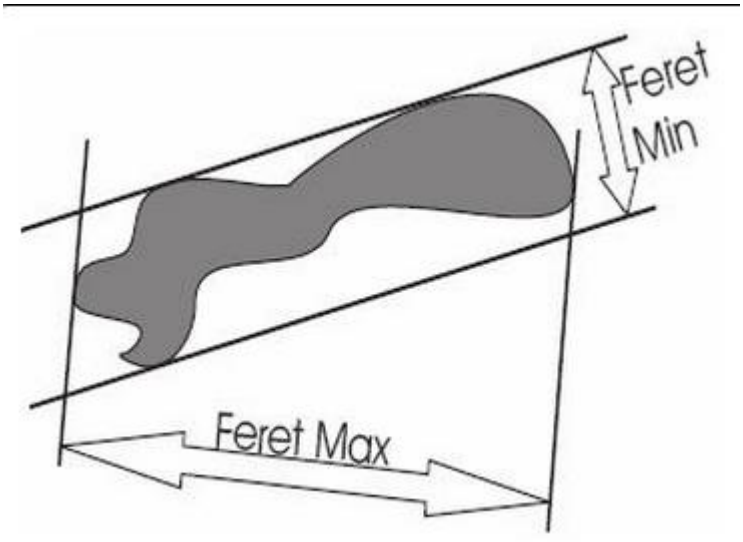
2) <u>Setting fluorescence threshold by adjusting the histogram</u>

- Drag the arrows underneath the histogram to increase or decrease the threshold for segmentation.
- After setting the fluorescence threshold you can separate objects that are touching eachother using a segmentation algorithm.
- By trial and error you can select an algorithm and move the sliding bar to see if this effectively selects your objects of interest.
- The numbers on the sliding bar do not indicate a linear scale.
- The advantage of selecting an algorithm is that, unlike other methods, an algorithm automatically adjusts the threshold for object identification relative to the average fluorescence intensity in the image (which may vary between batches of slides).
- **Watersheds** – separates objects of the same shape.
- **Morphology** – separates objects of different shape.

<u>Step 4: Condition</u>

This step places further conditions on what constitutes an object within each class.

- Select the class you want to place a condition on.
- Click edit.
- Select the condition from the menu:
  eg **Area** – as measured in pixels or μm^2.
  eg **Circularity**
  eg **Roundness** – which is related to circularity, and is basically the same thing.
  eg **Feret ratio** – this is useful for irregularly shaped cells. It is the ratio of the greatest width/ length of the cell to the smallest width/ length.



<u>Step 5: Interactive segmentation</u>

In this step you can manually separate objects or merge objects.

- Use the **draw** tool to add pixels to the objects.
- Use the **cut** tool to separate objects – drag the line and then right click to separate objects either side of that line.

<u>Step 6: Measurement features</u>

In this step you can select the features you want to measure for each class.
eg Perimeter – this is actually pointless because it is scale-dependent.

eg Count – to count the number of objects in a class or sub-class.
eg Area percent – to calculate the percentage of the total area filled by all the objects in that class.

Step 7: Measurement
This step provides an overview of the program.

- Click **Finish** to save the program.
- Select **Analyze** to perform automatic analysis without enabling the interactive steps.
- OR select **Analyze Interactive** to perform automatic analysis with the interactive steps.

How to use the Image Analysis Wizard to analyse brightfield colour images

This is the same as above, except in **Step 3: Automatic segmentation** you set the recognition of the colours based on the levels of **RGB** (red, green, and blue) in 'colour space', or on **HLS** (hue, saturation, and luminance).
(!) The RGB method is not very good at differentiating brown staining (which is an equal mix of RGB), especially dark brown (which could be any colour).

You can measure the intensity of red, blue, green, and grey (shadow/ absorbance) by selecting these in **Step 6: Measurement features.**