FIJI (FIJI is just ImageJ)

Contents

Notations: Words enclosed in <> denote FIJI menu commands

Useful Links:

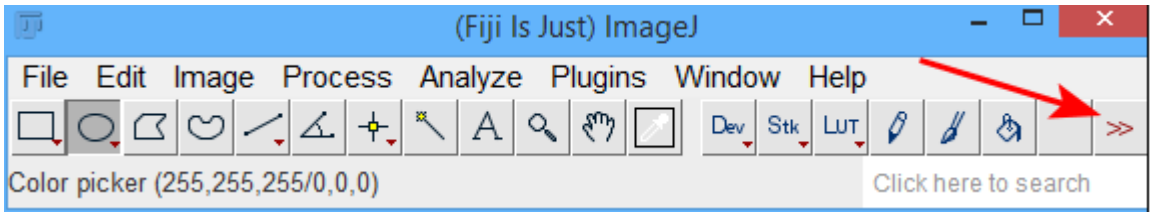
- FIJI website: http://fiji.sc/
- ImageJ Website: https://imagej.nih.gov/ij/
- FILM website (macro Toolsets): http://www.imperial.ac.uk/medicine/facility-for-imaging-by-light-microscopy/equipment/software---fiji/

FILM Macro Toolsets

FILM has a selection of macros in a series of toolsets that enhance the performance of FIJI Some of these are referred to in this guide.

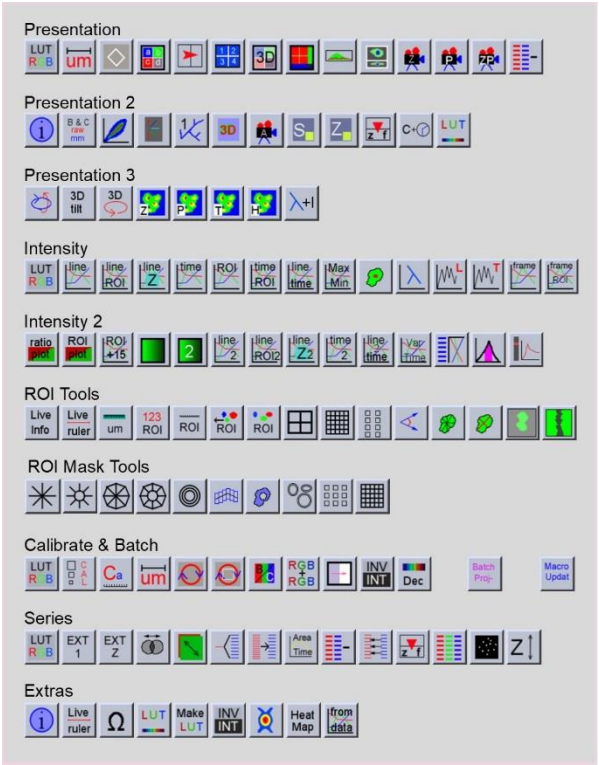
Installation:

- Download the Macros.zip file from the FILM FIJI website: http://www.imperial.ac.uk/medicine/facility-for-imaging-by-light-microscopy/equipment/software---fiji/
- Unzip the file and copy the contents to the macro/toolsets folder where you have installed FIJI
- The Macro Toolsets can then be selected from the drop-down menu by clicking on the **>>** on the tool bar.



There are 10 toolsets:

- **Presentation**, **Presentation 2** and **Presentation 3** which mainly have tools for Image display
- **Intensity** and **Intensity 2** for looking at intensities of images, particularly in Z and time Image series
- **Roi_Tools** for manipulating Regions of Interest
- **Roi_Mask** for generating arrays of Regions of Interest
- **Calibrate & Batch** which has macros to do with calibration and colour RGB image correction and analysis.
- **Series** for manipulating Z and time series images
- **Extras**



For a better description of what each macro does - see the macro description document on the FILM website

Opening an Image

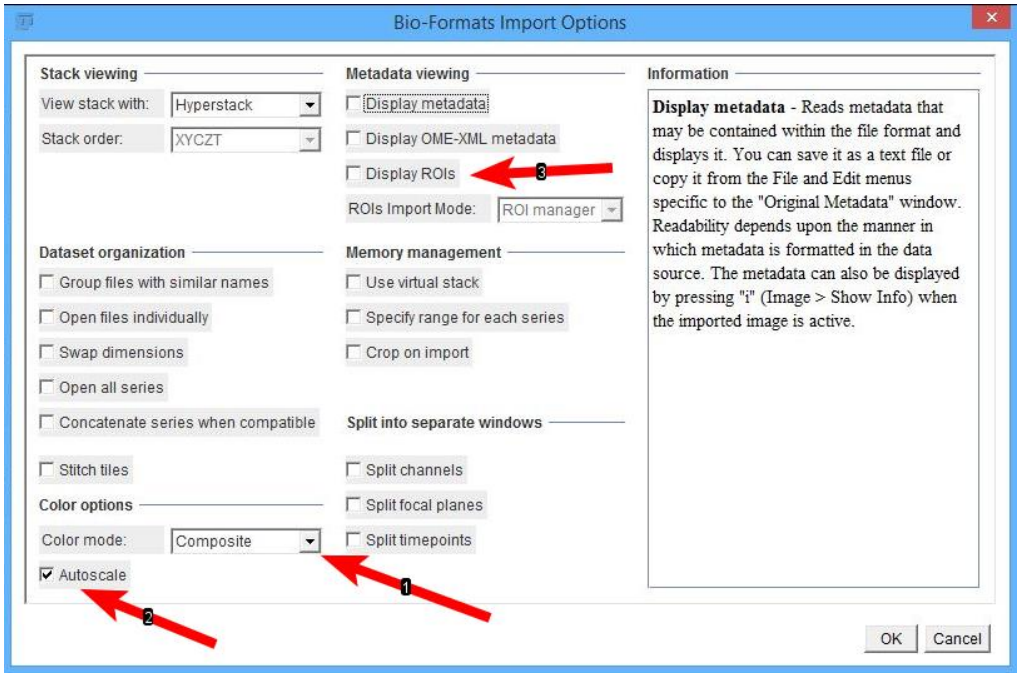
The two main types of image

- Un-calibrated exported formats (eg: tif, .jpg, .png, .avi) although some .tif images can still be calibrated.
- Raw data formats from microscopes or other instruments (eg: .czi (Ziess), .lif (Leica), .nd2 (Nikon)

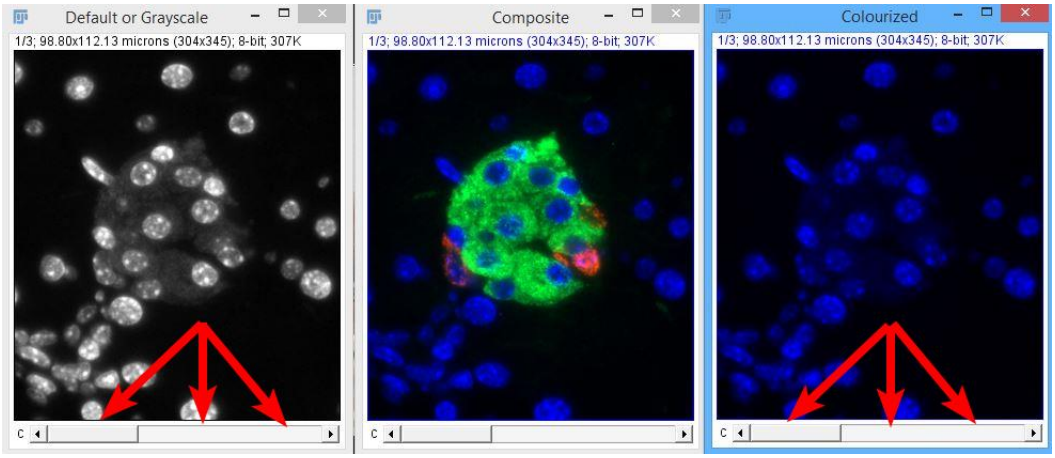
Images can be opened by either dragging them onto FIJI or using the menu commands <File><Open>.

- The first type of image will just open.
- The second type will invoke the Bio-Formats importer, which has the capability of reading the calibration and more from the raw data images. This is the preferred type of format to use when collecting images.

<u>The Bio-Formats Importer</u>



Usually the default settings are ok, however there are two settings that will alter the way the image is displayed. The Color mode (arrow 1) will determine whether the image is displayed in colour or greyscale and whether the channels are overlaid or displayed by channel.



In a multi channel image the Default and Colourized settings will display the first channel. Other channels can be selected on the channels bar "C" at the foot of the image. Using the Composite mode will display the mages overlaid.
The "Autoscale" check box (arrow 2), if ticked, will set the displayed brightness and contrast for each channel optimised for viewing the signal (Important - this does NOT change the data, it only changes the way the data is displayed).
Raw date RGB colour images - Use "Composite" and it is best to leave the "Autoscale" un-ticked as these images will load as 3 channel images and using the autoscale could change the colour balance
Any ROI's stored with the raw data can also be loaded (arrow 3).

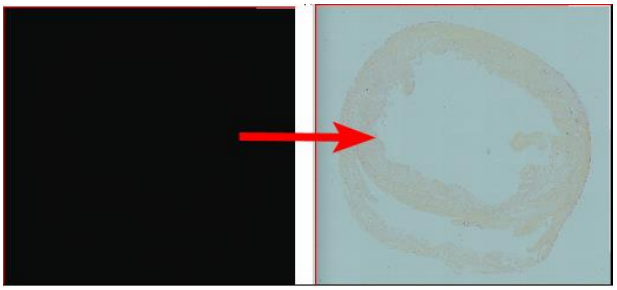
Opening a 12-Bit RGB Raw Data Image

Some colour cameras will save the image in a 3 channel 12-Bit format. Images can be opened by either dragging them onto FIJI or using the menu commands <File><Open> as usual.

When the Bio-Fomats dialogue opens, select “Composite” and uncheck the “Autoscale” check box

If the image is large then a preview window opens displaying multiple versions, selecting the first version will open the full data range (NB each of subsequent image version are reduced by a factor of 3 on the previous version and when loaded are not calibrated properly).

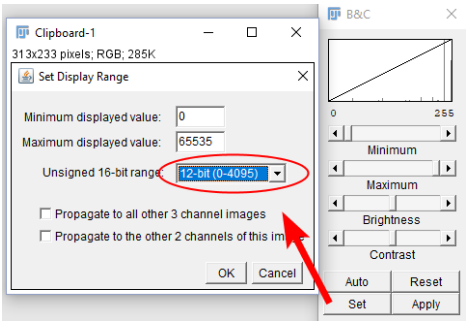
The image should open as a three channel image



If the image display looks wrong (ie all black or white), then the reason is that the display Bit range is set wrong. To change the display settings, select the brightness and contrast control. (<Image> <Adjust> <Brightness & Contrast>).

Click on Set and change the display range to 12-Bit.

The image should then be displayed correctly

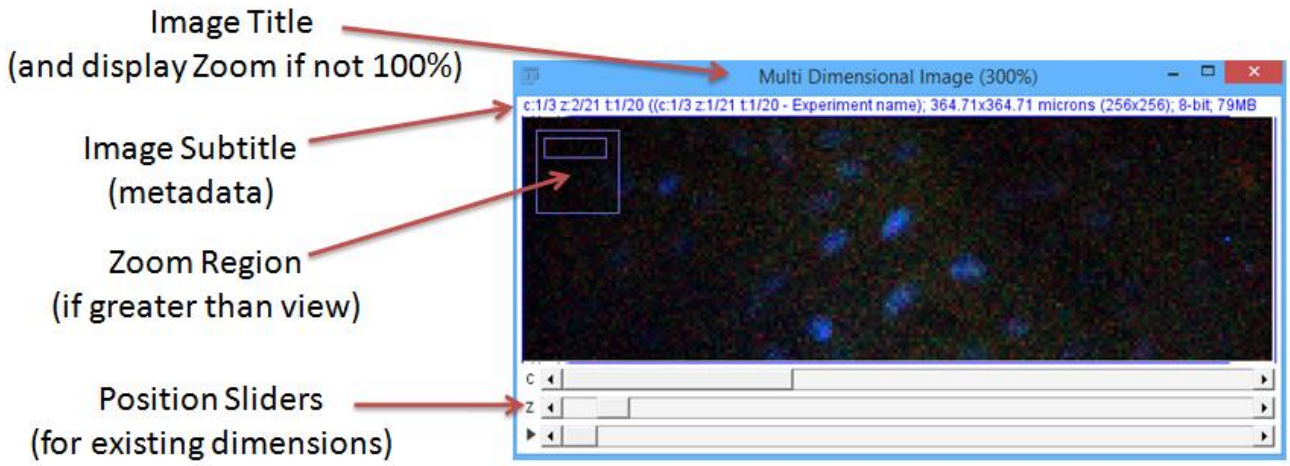


To create a single channel 8-Bit RGB version, run <Image> <Type> <RGB Colour>

NB Once you have set the bit scaling to 12-Bit it will remain so until you change it again (NB you will need to change this back to auto when opening other types of image)

Image Types, Subtitle and Footer Bars

Once an image is loaded, information about the image is displayed in the Subtitle bar (metadata), and if relevant, the footer bars will provide controls for multidimensional images. The <Image><Properties> commands will also display image information.



The Subtitle Bar (metadata) can display:

- the image dimensions,
- other experimental or dimension information
- image size and dimensions (calibration)
- image type and bit depth
- the size of the uncompressed data.

The Position Sliders (at the foot of the image) for control of:

- C - channel selection
- Z - z slice selection
- ► - time or frame selection. Note: clicking on the arrow will start the auto-play through the time series. Right clicking on the arrow will bring up the auto-play controls.

Description of the subtitle bar in this example:

- the "C 1/3" denotes that the image has 3 channels and channel 1 is currently selected.
- the "Z 2/21" denotes that this is a Z-stack image with 21 slices and slice 2 is currently selected.
- the "t 11/20" denotes that this is also a time series with 20 time points and that frame 11 is currently selected

Note- if none of the above are displayed then the image is either a single channel or RGB image.

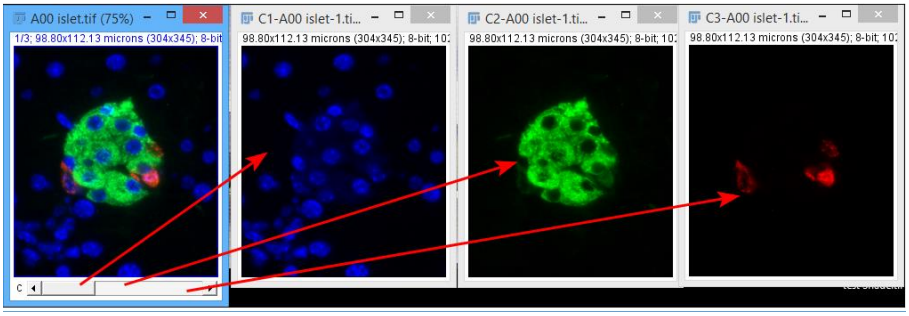
- the previous information is repeated in brackets with information about the experiment dimensions when included
- next the image size and calibration are displayed (364.71 x 364.71 microns) and then the number of pixels in brackets256 x 256(pixels**)**
    - If the size is displayed in "**Pixels"** then the image is un-calibrated
    - If the size is displayed in **"inches"** then it is still probably **not** calibrated, but the values have been imported from the "standard printer output" of 72 dots per inch (DPI) when the image was originally saved).
- next the bit depth is given 8-bit (256 grey levels)
    - 8-bit (256 grey levels) per channel - Tiff and raw data formats
    - 16-bit (65535 grey levels) per channel - Tiff and raw data formats
    - 24-bit (RGB colour - 3 x 8-bit) no channels- Colour image formats TIFF, GIF, JPEG, PNG, BMP, AVI. There are no channel sliders at the bottom of the

image although it can be converted to a Red, Green and Blue, 3 channel image for processing and analysis. <Image><Color><make Composite>

- Finally, the uncompressed image size is displayed (79 MB)

Multi Channel Images

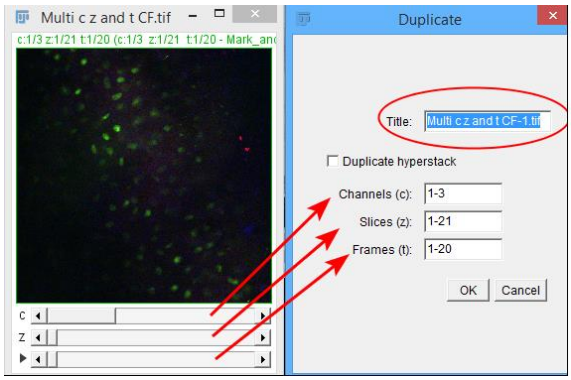
Multichannel images can be split into the separate channels using the commands <Image><Color><Split Channels> and re-merged using <Image><Color><Merge>



Z - Stacks and time series can also be separated in a similar way using <Image><Stacks><Stack to Images> and <Image><Stacks><Images to stack>

Selected channels/slices/frames can be extracted from multidimensional images using the duplicate command <Image> <Duplicate>.

This creates a new "copy" of the selection, the "Title" box allows naming of the new image



Duplicate also works on a selected region eg. using the rectangle tool on the FIJI menu bar. Only the selected region is duplicated, and the calibration is retained. This can be more useful than using the Crop command <Image><Crop>which will destroy the original image

.

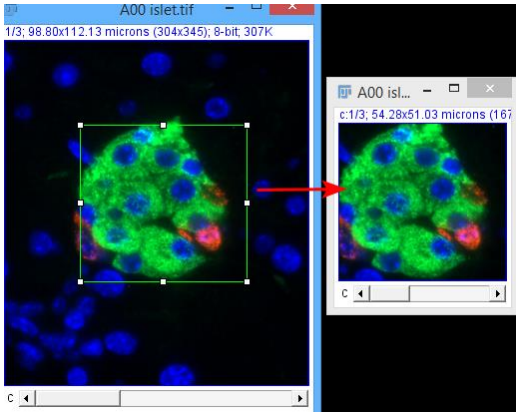
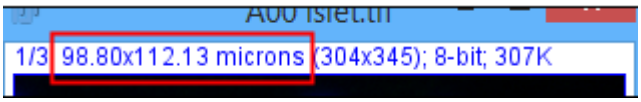


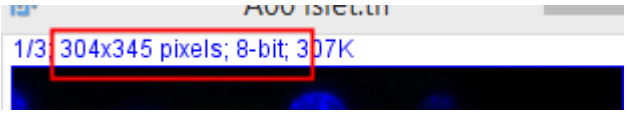
Image Calibration

Instrument raw data files are usually calibrated when opened in FIJI as displayed in the subtitle bar and the metadata (<Image><Properties>)

Calibrated
- dimensions displayed in microns



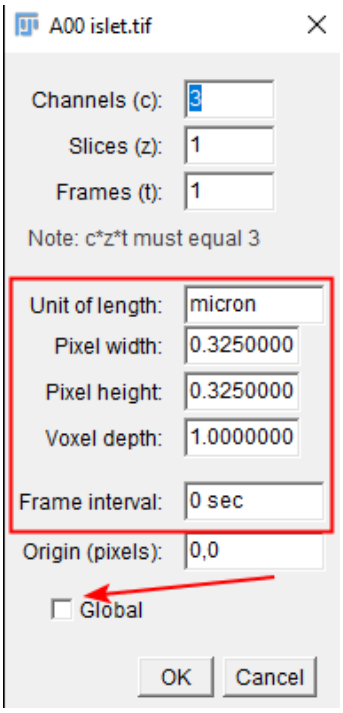
Un-Calibrated
- dimensions displayed in Pixels



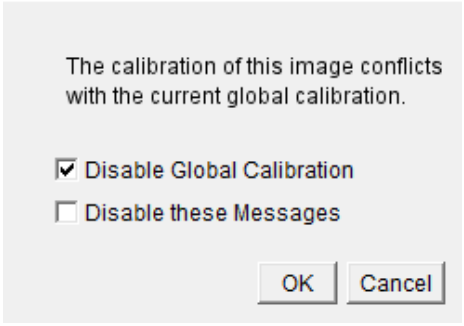
Calibrate an Image:

by entering the pixel size

- if the pixel size is known then this can be entered directly in the <Image><Properties> window
- the units can be entered eg mm, micron and also um is recognised as micron
- the frame interval can also be added if it is a time series

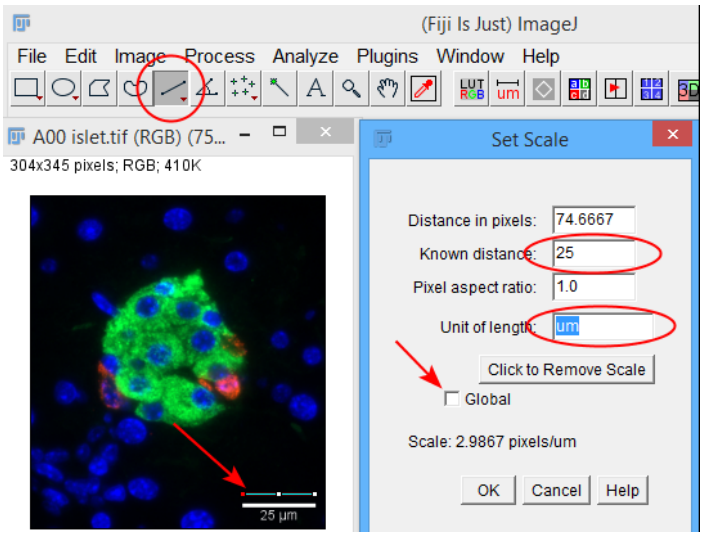


NB. Ticking "Global" will then cause **all open** images to have their calibration changed to this calibration. Any images opened thereafter will open a dialogue to confirm or disable this auto calibration



by direct measurement

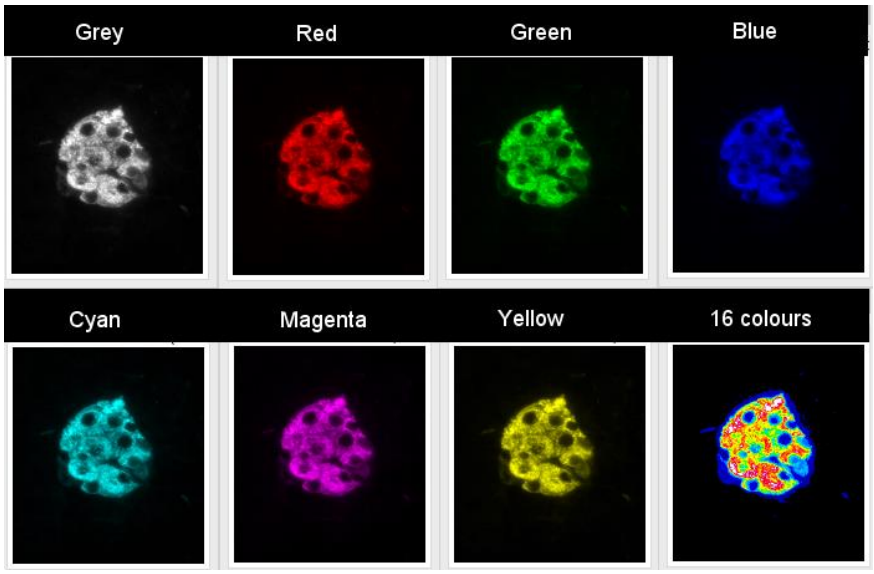
- if the image or image set has an image with a scale bar printed on it then this can be used to calibrate the pixel size
- open the image with the scale and select the line tool from the menu
- draw a line the length of the scale bar (tip. holding the shift key down will restrict the movement to the horizontal)
- run <Analyse><Set Scale>
- enter the known distance and the units - click OK
- propagate to other images as above
- if ticking global - see above



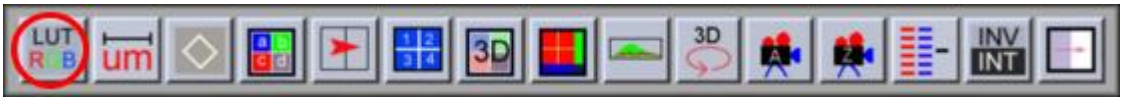
Look-Up Tables

Single and multi-channel greyscale images are assigned a "false" colour LUT (Look-Up Table) for each channel eg red, green, blue, cyan, yellow, magenta, grey, multi-colour .... to make visualisation easier, particularly when displayed as a composite image.

This LUT can be changed without changing the intensity information by selecting the channel and then using <Image><Lookup Tables> or by using the Lookup Tables toolset selected from the drop-down list when clicking on the **>>** on the menu bar

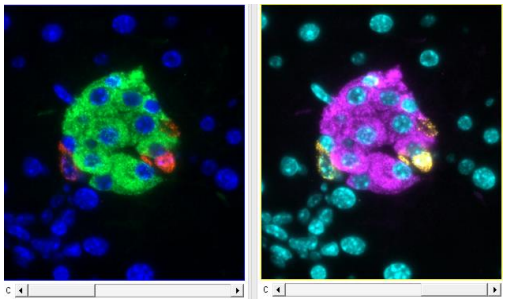


Alternatively, for multi-channel images, use the LUT macro tool from the FILM Presentation toolset - this allows changing all channel LUT's in one process.

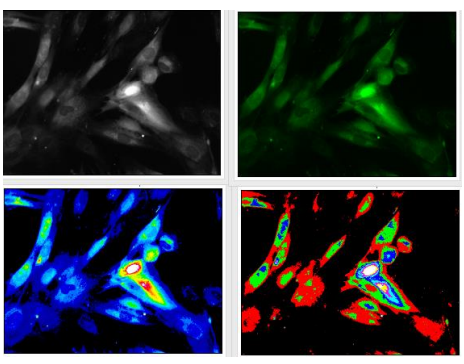


Using Red, Green and Blue in a composite image can cause difficulties for those with colour blindness, so sometimes it is better to use Magenta, Yellow and Cyan.

In multichannel images where signals overlay, the colours from each channel mix creating visually a new colour displaying this co-localization (eg red and green > Yellow)



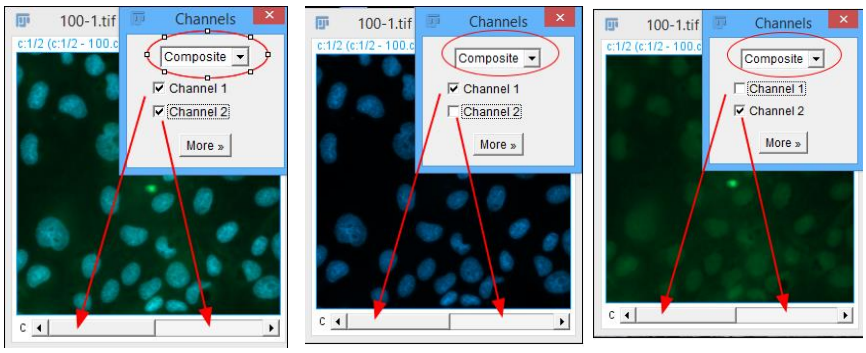
The Multi-colour LUT's are where different colours are assigned to different intensities in the same channel and are useful for emphasising slight changes in intensity or creating a "Heat Map".



Channels Tool and Brightness & Contrast

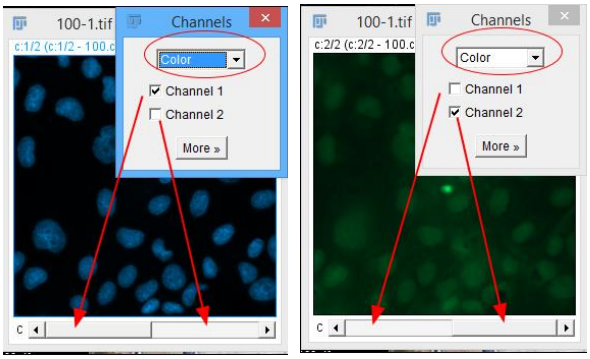
Once opened there are two basic image controls for changing the way the data appears

The Channels tool (opened by: <Image><Color><Channels Tool>) allows control of multi channel images and can be used to create a final format for importing into reports (word, PowerPoint, etc.).

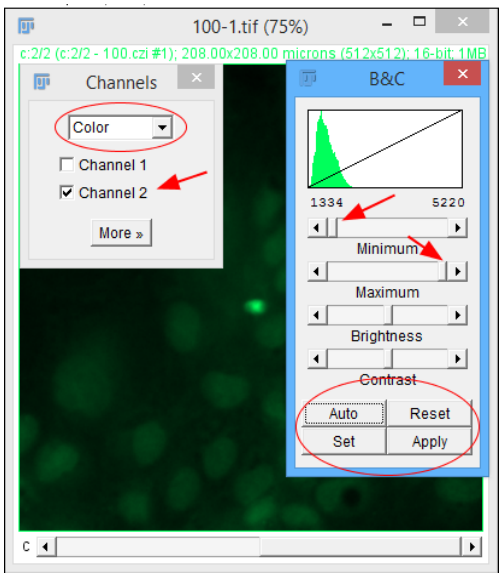


In Composite mode the channels to **display** can be selected from the tick boxes. note - in this mode, the tick boxes do not make the channel active

In Color mode only one channel can be ticked at any one time. This not only then displays the ticked channel but also makes the channel active. this is useful for editing brightness & contrast and testing threshold etc



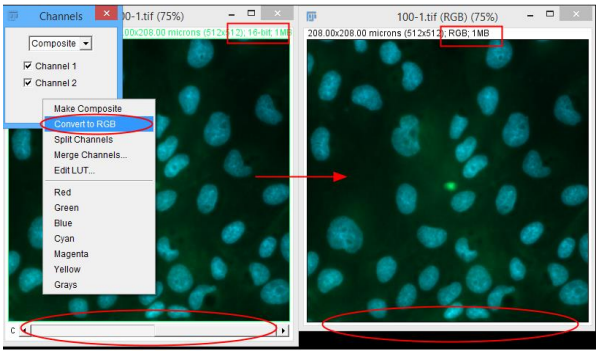
The Brightness and contrast tool (<Image><Adjust><Brightness and Contrast>) allows changing the way images are displayed. This can be useful for images that are not very bright and captured with a high bit depth (16-bit). **IMPORTANT - when changing the display settings for an image, ensure that they altered in a way that does not bias the result, particularly when comparing multiple images.**

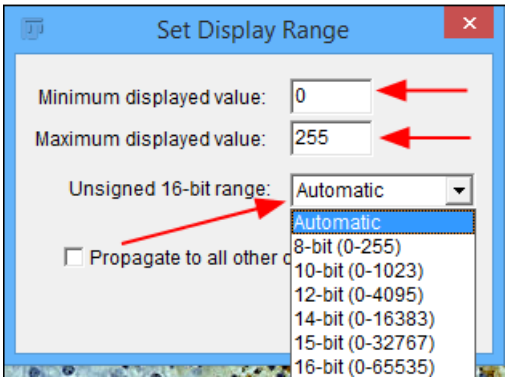


For a single channel image or a colour RGB image just select the image. For a multichannel image, select the channel to change either by selecting in the "channels tool" window (in Color mode) or by using the channels bar at the foot of the image. Adjust the sliders or use the Auto button (the auto button gives settings for a "best fit" display but is highly dependent on the data). The Reset button will set the display back to its min/max setting - which will depend on the current range setting (see the Set button)

Important note. Adjusting the sliders and pressing "Auto" does **not** change your data - it only changes the way the data is displayed - However, clicking the "Apply" button **will** change your data.

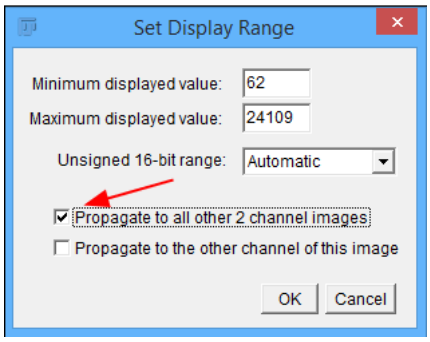
The resulting displayed image with its new display settings will be required for publication. The easiest method for this is to convert (flatten) the image into an RGB format using the "more" "Convert to RGB" buttons in the "Channels Tool" window. This will create a new image which no longer has the original data information but is useful for display purposes





The **Set button** will display the current range setting and can be changed from the drop-down arrow. There are also two boxes to input fixed min and max display values. The "Automatic" range will set the min max values to those of the image, all the other ranges are fixed on the bit depth. NB. - do not select a bit depth smaller than your images' bit depth or the data will be changed.

It is important that if you change the settings for one image then you should change the settings for all images by the same amount. This can be done manually for each image by entering the values or you can open all images to be changed. select one of the images and alter the brightness and contrast optimally for that image. then select the Set button and tick the propagate to all other (similar) images. This will change the display settings for all other open similar images



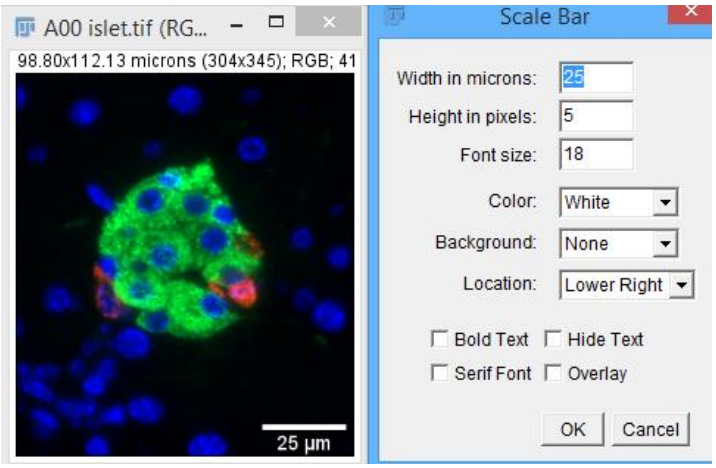
Adding a Scale Bar

Once an image is calibrated, it is possible to add a scale bar using the menu commands <Analyse><Tools><Scale Bar>.

As the scale bar is usually required for publication it is better to convert the image into the RGB format to allow the colour function to work. <Image><Type><RGB Color> or use the "more" "Convert to RGB" buttons in the "Channels Tool" window.

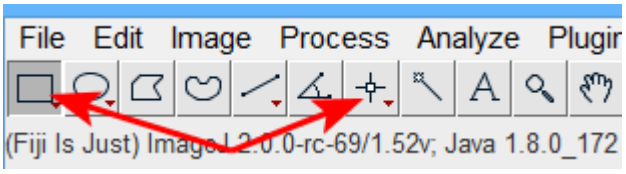
There are options for:

- the width, which is automatically calculated on the image allowing any specified width to be used.
- for the thickness of the bar (Height in pixels)
- the colour and background
- text font: type and size, and whether to display it
- the location



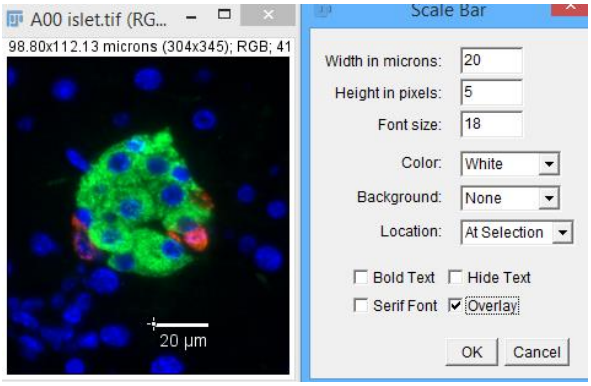
Before clicking the "OK" button, the position and appearance can be viewed by changing any of the settings

The location has 5 options: Upper Right, Upper Left, Lower Right, Lower Left and At Selection. The first 4 options are at predetermined locations, the last allows the user to position the scale bar. This position has to be selected before running Scale Bar and can be best set by the Rectangle or Point Tool

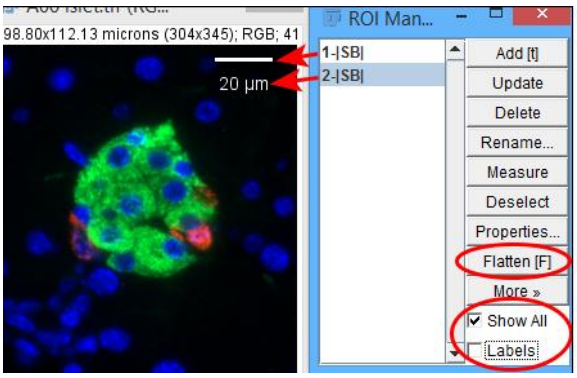


A further option is to add the scale bar to the "Overlay".

If the overlay is then copied to the ROI Manager <Image><Overlay><To ROI manager> the final position can be adjusted using the ROI manager. This must be followed by the "Flatten" command or the overlay will not appear in publication.

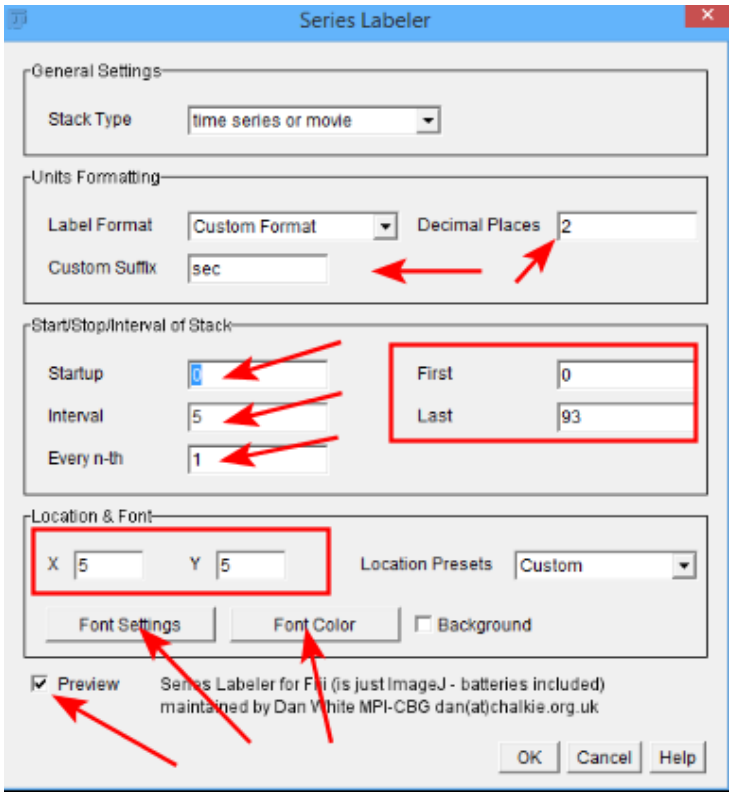


- select each ROI in turn and re-position
- check the "Show All" box and make sure the "Labels is un-checked
- then click "Flatten"

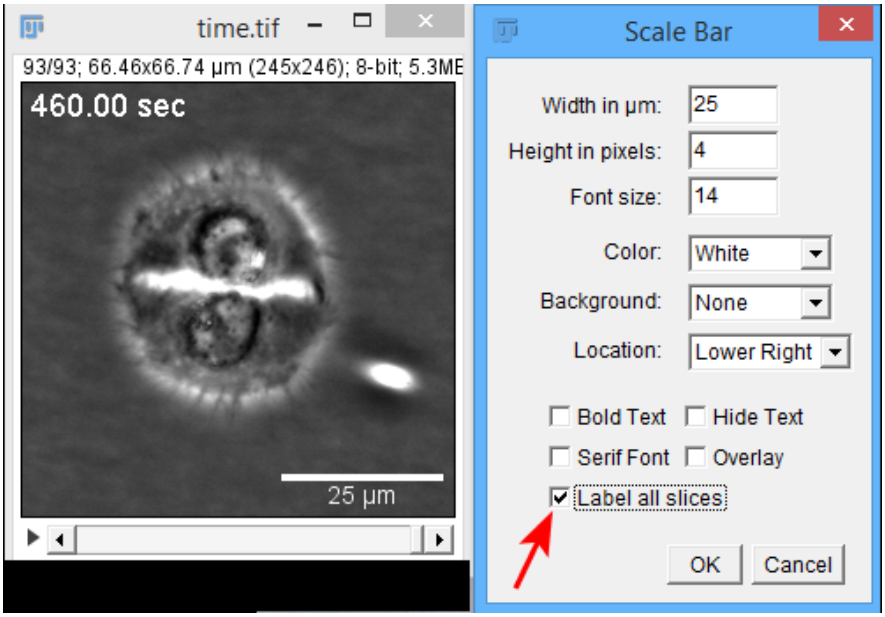


Adding "Time" and a Scale Bar to a Time Series

- open time series
- adjust Brightness and contrast as necessary
- as this is usually for presentation purposes, it is best to convert the image to RGB format <Image> <Type> <RGB Color>.
- run "Series Labeler" <Image> <Stacks> <Series Labeler>
- enter the values units, decimal places, start time, interval, step size for the image
- use the preview button and the location and font buttons to set the required display



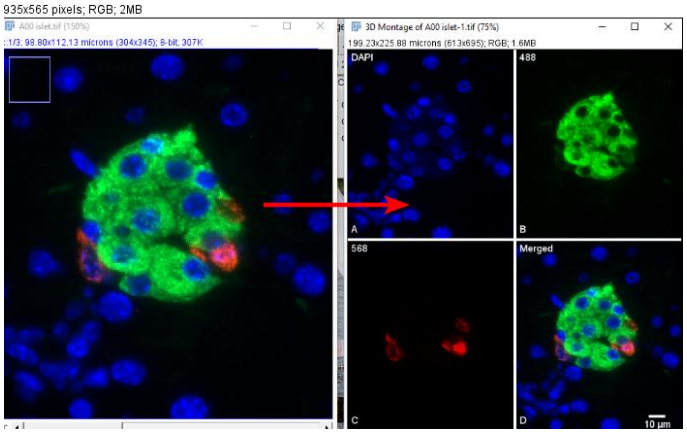
- add a scale bar as previously described <Analyse> <Tools> <Scale Bar> but make sure the "Label all slices" checkbox is ticked before clicking "OK"

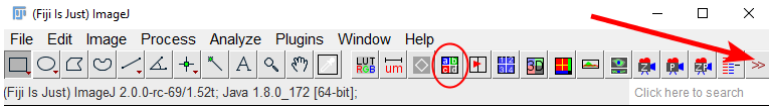


- the image can then be saved in AVI video format <File> <SaveAs> <AVI>

Creating an Image Gallery from a Multi-Channel Image

(using the "Annotated Gallery" Macro from the FILM "Presentation" macro toolset see website: http://www.imperial.ac.uk/medicine/facility-for-imaging-by-light-microscopy/equipment/software---fiji/)

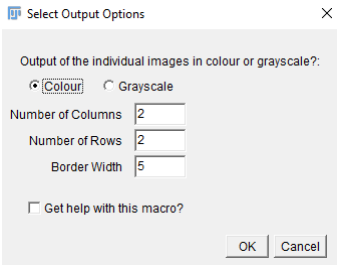




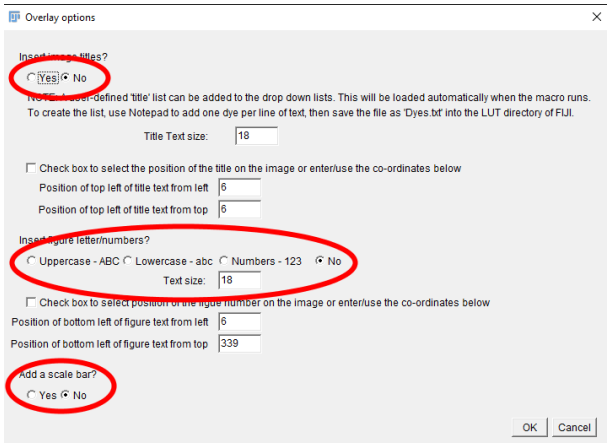
Method:

- Open Fiji
- Select the Presentation Toolset (>> arrowed above)
- Open the multichannel image and run the "Annotated Gallery" macro tool (circled above)
- Select which channels to display in the merged panel in the channel tools window - click OK

- Select output formant. Individual channel images van be displayed in colour or greyscale, and the number of columns and rows can be changed

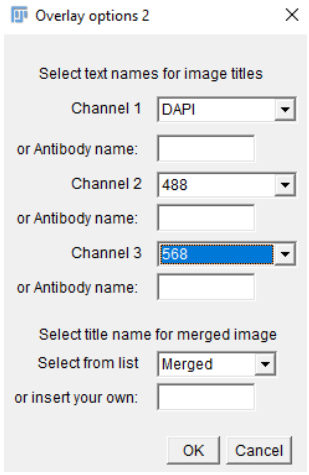


- The next window allows selection of overlays: channel titles, panel numbering and adding a scale bar to the last image

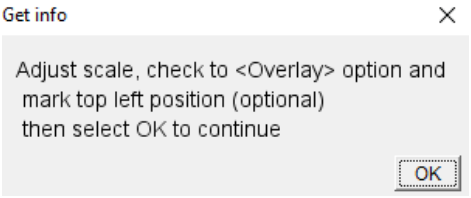


NOTE: A user-defined 'title' list can be added automatically to the drop-down lists. This will be loaded automatically when the macro runs - To create the list, use Notepad to add one dye per line of text, then save the file as 'Dyes.txt' into the LUT directory of FIJI.

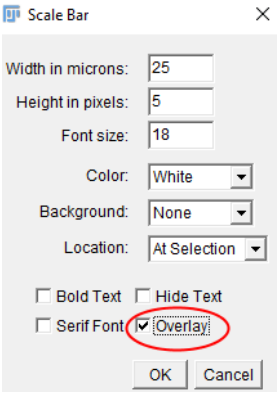
- If the title option is selected, then a dialogue will open allowing selection or addition of panel names. The user text box if used will have preference over the dropdown selection



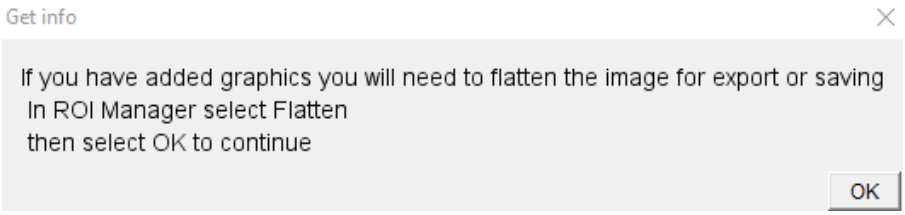
- If adding a scale bar option is selected, then a message will appear suggesting using the add to overlay option which will allow editing of the position in the final window



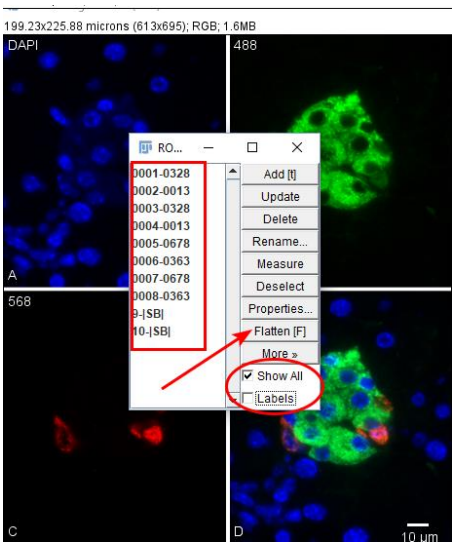
- Change the size, thickness, font and check to “Overlay"



- The final dialogue reminds the user to flatten the overlay onto the image using the ROI manager "Flatten" button before saving



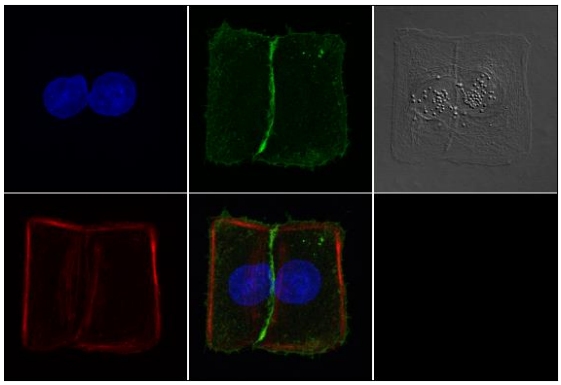
- Selecting the ROI's in the ROI manager allows the user to position and change colour of the various elements.
- NB. Make sure the "show all" is ticked and that the "labels" is un-ticked before flattening.



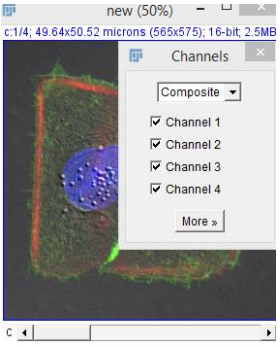
Further Options using the Annotated Gallery Macro Tool

(using the "Annotated Gallery" macro from the FILM "Presentation" macro toolset)

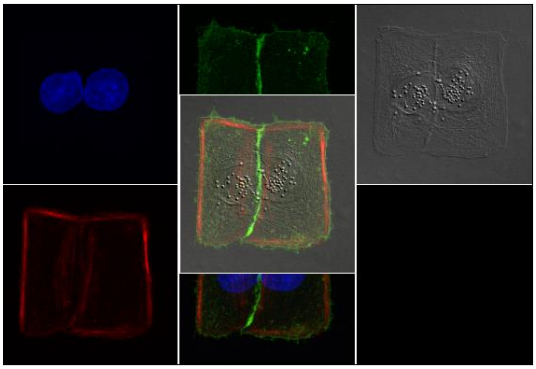
Method to add further overlay images particularly if the final gallery has an uneven number of images



- Create the gallery as described above.
- Select the original multichannel image and change to composite mode if not already in that mode
- Open the channels tools window <Image><Colour><Channel tools>
- Select/Deselect the channels you require for the additional overlay image

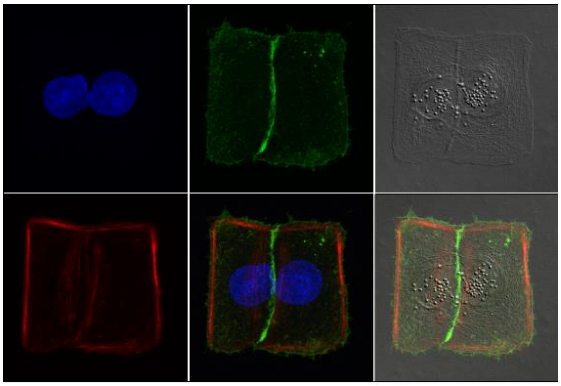


- Then using the "more" option and "convert to RGB" to create the new overlay image
- Select this overlay image and copy it (<Edit><Copy> menu commands)
- Select the gallery image and paste (<Edit><Paste> menu commands): – the overlay image will be pasted into the middle of the gallery image.



- **<u>Now be careful</u>** - click only inside the pasted image and you will be able to drag/move it to its required final position (the arrow keys should also work for fine movement).

- Clicking outside the pasted image will fuse it with the background gallery image but behind any graphics you have added. (you will not be able to move it again after doing this).
- You can copy/add further graphics either using the ROI manager or menu commands
- Remember to flatten the graphics using the Flatten command in the ROI Manager if there is an overlay

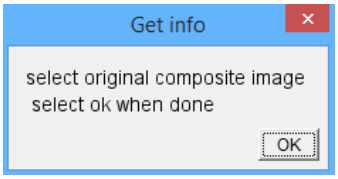


Adding further Annotations to the Image Gallery

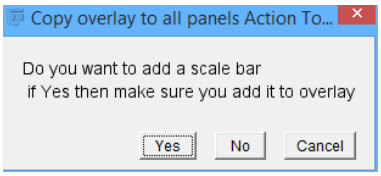
(using the "Copy Overlay" macro from the FILM "Presentation" macro toolset)

Method

- Create the gallery as described above, flatten and close the ROI manager.
- Select the Presentation Toolset (>>) and run the "Copy overlay" macro tool (circled above).
- It asks the user to select the original multichannel image.

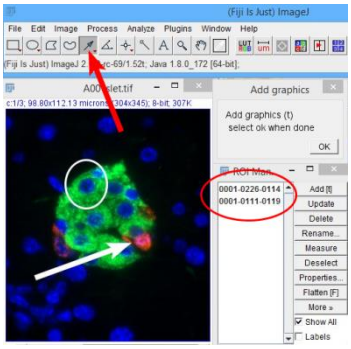


It gives the option to add a Scale Bar to each gallery panel. If you select Yes, then make sure to check the "add to overlay" box in the scale bar window when adding

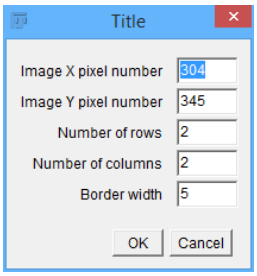


Add the graphics dialogue will appear

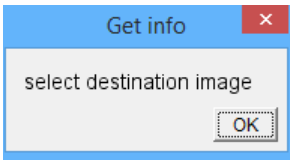
- draw on boxes, arrows and other required graphics
- after drawing each graphic press t to add the graphic to the ROI manager



The format dialogue will come up (check it matches the original gallery image format). If you changed the original format you will need to change it here as well.

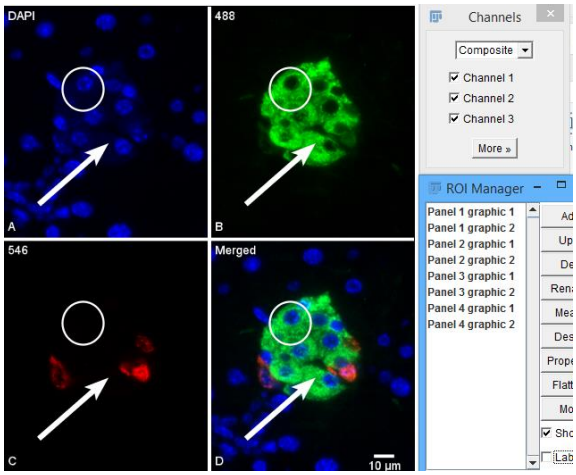


The dialogue to select the destination (gallery) image will appear



The overlay is copied to all images and the objects are listed in the ROI manager

- Selecting the ROI's in the ROI manager allows the user to position and change colour of the various elements.
- Finally flatten the overlay onto the image using the "Flatten" button in the ROI manager. NB. Make sure the "show all" is ticked and that the "labels" is un-ticked before flattening.

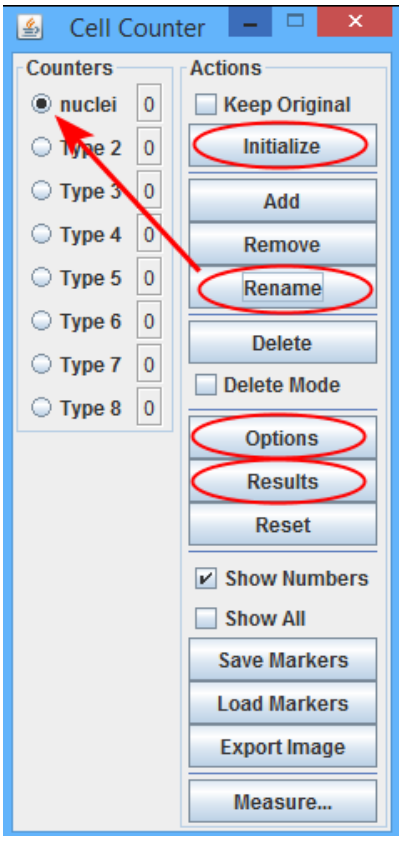


Counting Objects (manual counting)

Using the Cell Counter Plugin

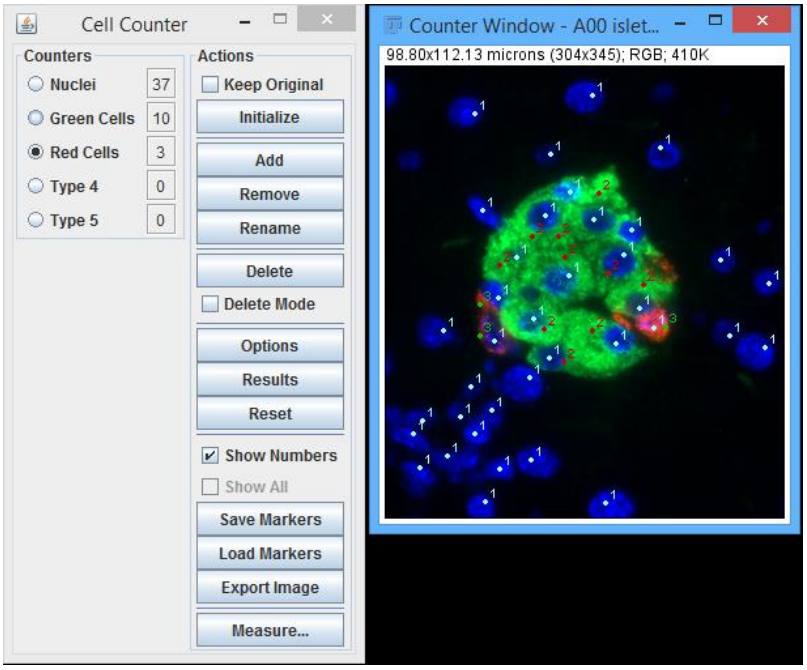
setup:

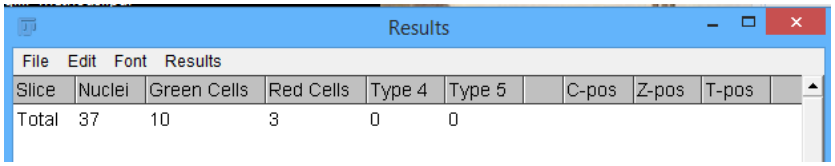
- open the image in FIJI
- run the Cell counter Plugin: <Plugins><Analyse><Cell Counter><Cell Counter>
- click "Initialize"
- select a counter and click "Rename" to enter the object name
- select other counters as necessary and repeat
- the "Options" button allows changing the colour of each counter type
- the "Remove" button can be used to remove unwanted counter types and use "Add" to add more counter types



counting:

- select the counter type
- then every time you click on the image the counter will increment
- the "Delete" will remove the last added mark
- if required, change to a different counter type and repeat
- the "Reset" button clears all measurements
- when finished, click "Results" to get the output



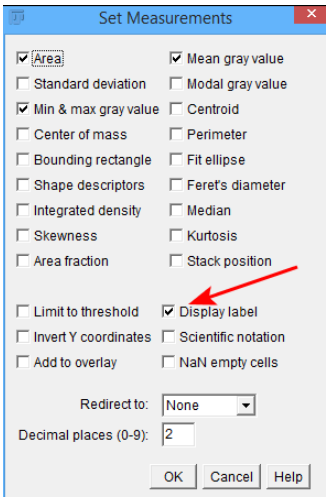


NB. If counting on multi-dimensional images, the channel, Z-slice and time point can all be selected for each counter type and the results will display the counts in each of these positions

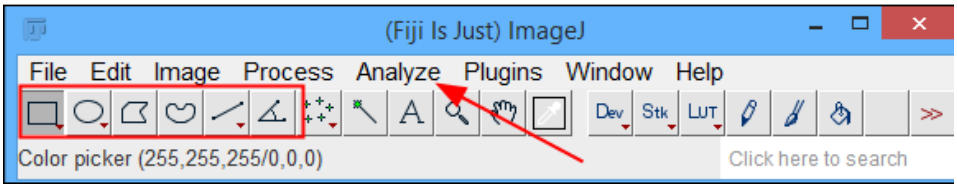
Measuring Images

Area and Line

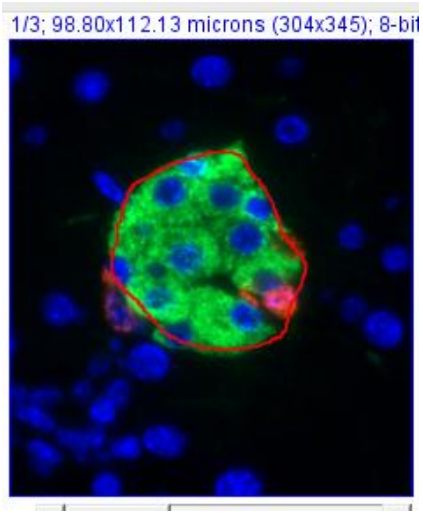
- the types of measurements to be displayed can be selected <Analyse><Set Measurements>
  - tick the check boxes of the required measurements. eg Area etc. eg the Ferets diameter will give the longest dimension and the angle of an area. More details can be found by pressing the Help button. (note that even if ticked only measurements appropriate to the tool will be displayed)
  - it is useful to tick "Display label" checkbox as this will associate the measurement with the image
  - if the images are not calibrated, the results will be in pixels (square pixels) otherwise they will be displayed in the image units- see section on image calibration

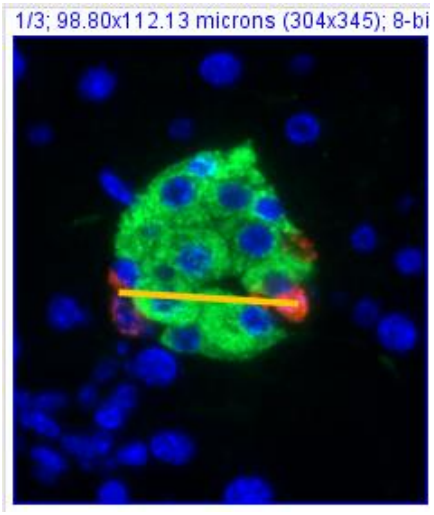


- select the required measuring tool from the FIJI menu bar (rectangle, oval, polygon or freehand for areas and the line tool for lengths and angles



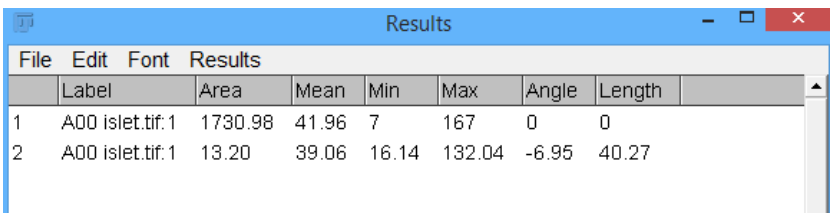
- draw areas or lines on the image





- select measure <Analyse><Measure> and the results table will be displayed

- If you want to keep the measurements, they can be saved in spreadsheet format using the "File" "SaveAs" from the results window. The file extension can be .TXT for test format, .CSV for comma separated variable or .XLS for Excel. All formats will open in spreadsheets.

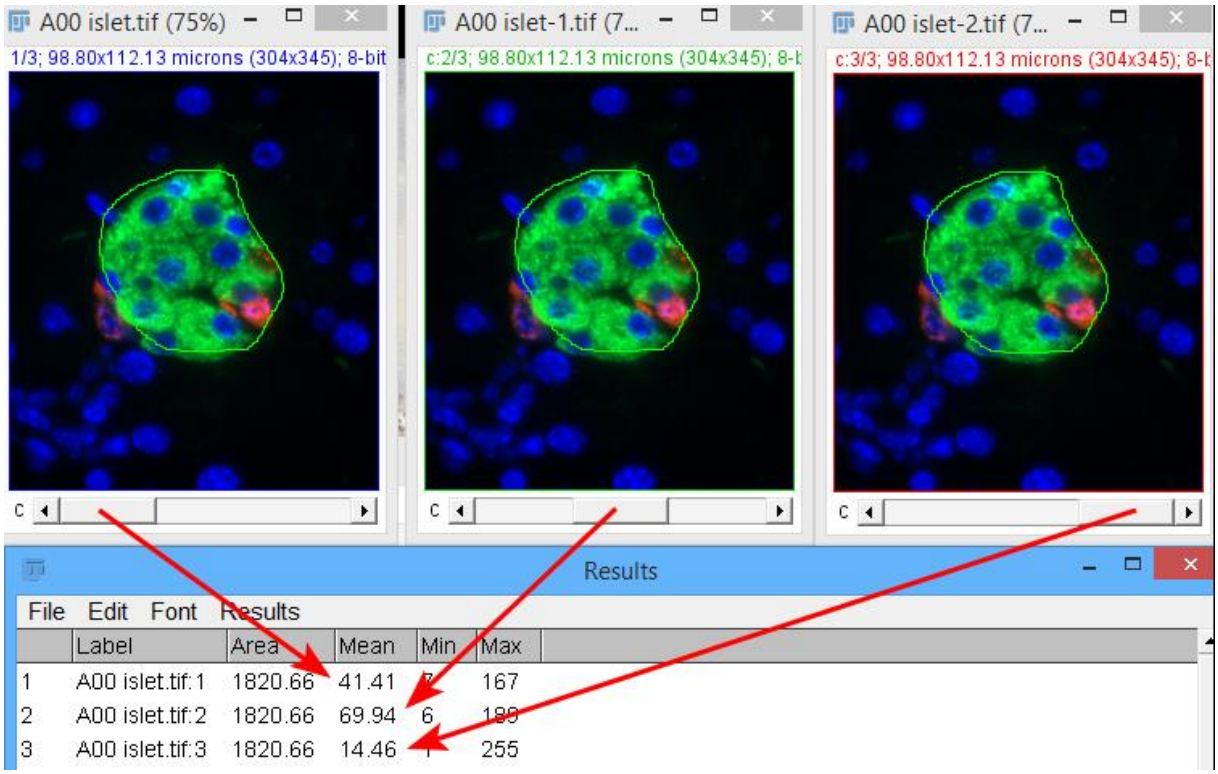


	Label	Area	Mean	Min	Max	Angle	Length
1	A00 islet.tif:1	1730.98	41.96	7	167	0	0
2	A00 islet.tif:1	13.20	39.06	16.14	132.04	-6.95	40.27

Intensity
(Mean, Min and Max)

The intensity can be measured if the "Mean Gray Value" (average intensity) and Min and Max gray value" checkboxes are ticked in the "Set Measurements" window (<Analyse><Set Measurements>). The intensity units are arbitrary, and the range is dependent on the bit depth of the image (8-bit = 256 gray levels, 16-bit = 65535 gray levels).

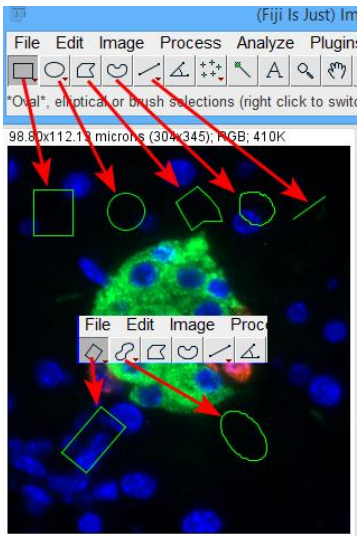
Intensity can be measured for the whole image (<Analyse><Measure> with nothing selected) or for discrete ROI's as above. If the image is multi-dimensional, then only the active channel/slice/frame will be measured. Select the required image from the sliders at the foot of the image before running <Analyse><Measure>



Alternatively run the split channel command and measure images separately

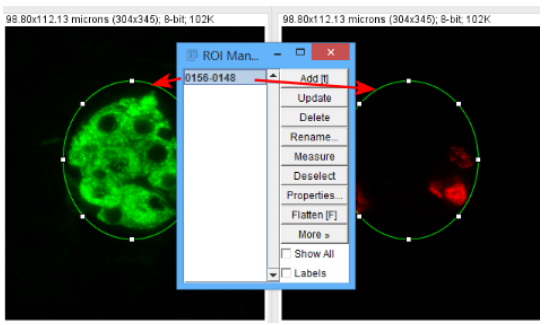
Drawing and Storing Regions of Interest (ROI's)

The drawn lines and shapes can be stored in the ROI (Region of Interest) manager by pressing "t" after each is complete. The manager will open automatically after the first is added.



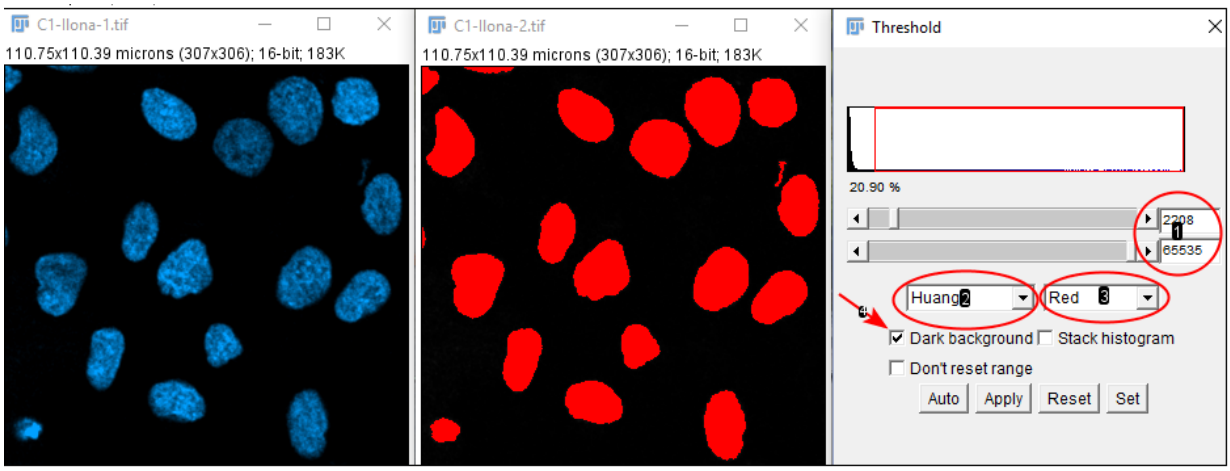
These ROI's can be measured using the Measure button on the manager

The ROI's can be transferred to other images by selecting the other image and then clicking on the ROI (NB the ROI's are calibrated so only useful for images with similar dimensions)



Using Greyscale Threshold

- objects can be selected by using their intensity<Image><Adjust><Threshold>



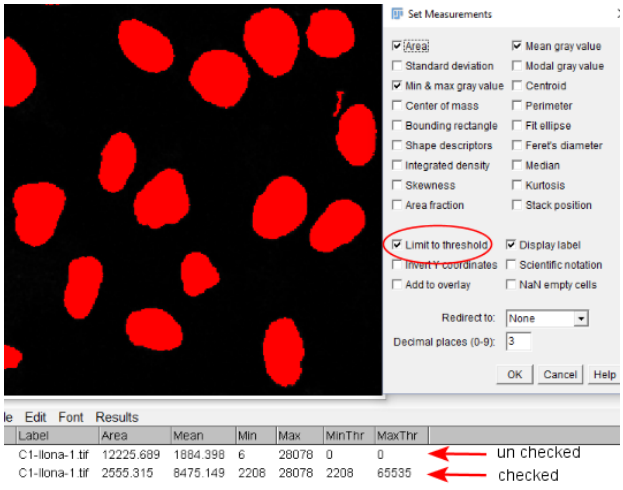
- only works on single channel greyscale images - there is a separate "colour threshold" for RGB images
- options for selecting light objects on a dark background or dark on a light background
- the intensity range can be entered manually (1) by using the "Set" button, or by auto detection algorithms (2) from the drop-down list and using the "Auto" button.
- the detected region can be displayed in Red or Black and White from the drop-down list (3).
- when thresholding a stack of images, the "Stack Histogram" checkbox will cause the algorithm to look at the whole stack and not just the current slice.
- the "Don't Reset Range" check box will use the current brightness and contrast setting, rather that the full intensity range, for the algorithm. This can cause confusion and must be used with care
- the "Apply" will convert the image to 8-bit Binary where background has an intensity value of 0 and the selected foreground has an intensity value of 255. Only use this to create a mask or if you don't need intensity measurements from the original image
- the "Reset" button returns the image back to un-thresholded view unless the "Apply" button has been pressed

Information on the auto algorithms and how they work can be found at:

https://imagej.net/Auto_Threshold

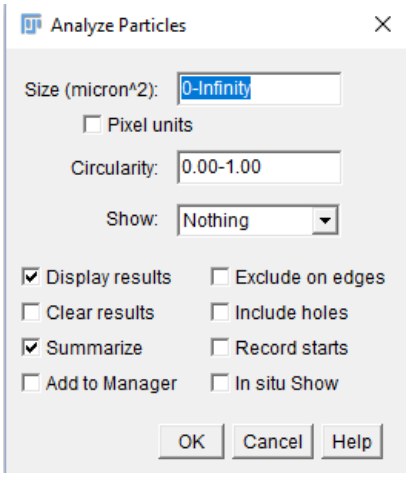
Measuring Thresholded Images

Using <Analyse><Measure> will only work if in the "Set Measurements" dialogue the "Limit to Threshold" is checked, otherwise it will measure the whole raw image



Using <Analyse><Analyse particles>can measure each thresholded object and output a "Summary" table and also a "Results" table listing the individual measurement for each object

There are a range of options for this command:



Filter measurements by:

- Size: Upper and lower area limits can be set in either the calibrated units (default) of by pixels
- Circularity: The roundness of an object from 0 (infinitely elongated polygon), to 1 (perfect circle).
- Exclude on edges - exclude objects touching the edges
- Include Holes - interior holes will be included

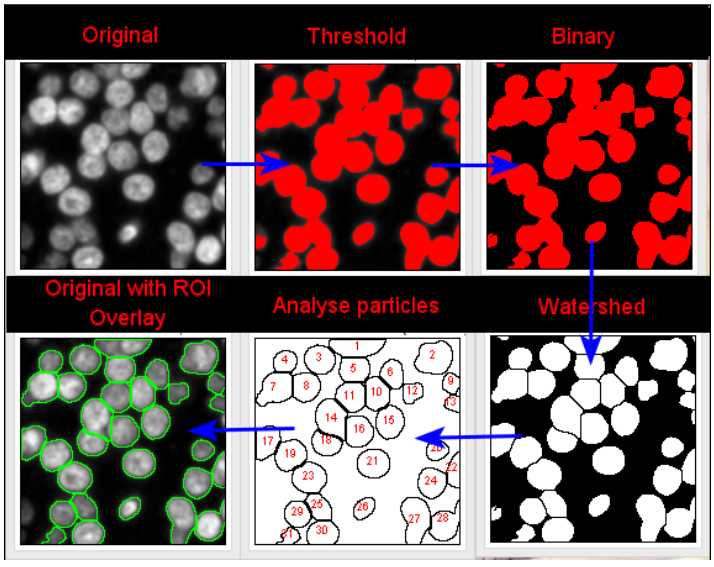
Output options:

- Display results - will generate the results table for the individual objects selected
- Clear results - will empty the previous results table
- Summarize - will generate a Summary table for all objects selected
- Add to Manager - will add each selected object as an ROI (Region of Interest) to the ROI manager. This can be useful for both display and region manipulation

the drop-down Show button can produce additional display outputs (masks overlays, etc)

Using Binary Watershed to Split Objects

Occasionally objects like nuclei can touch or merge together, making counting and measurements difficult. Binary Watershed segmentation is a way of automatically separating or cutting apart objects that touch.

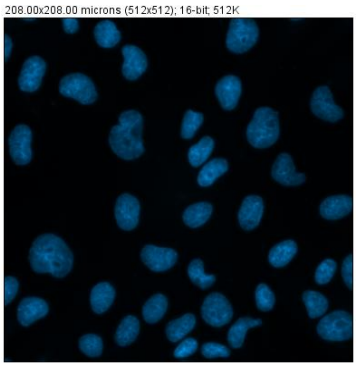


- threshold the image as above
- then use the “Apply” button to make the image binary
- next split touching objects by Watershed<Process><Binary><Watershed>
- run the <Analyse><Particles> to count and measure area on the watershed image
- if intensity measurements are required, create and use a copy of the original image. Use the "add to the ROI manager" to create an ROI mask. Select the original image, then in the ROI manager, click "Show all" to overlay the mask on the original Image and use the measure button in the ROI manager to measure the ROI's

Counting and Measuring by Threshold

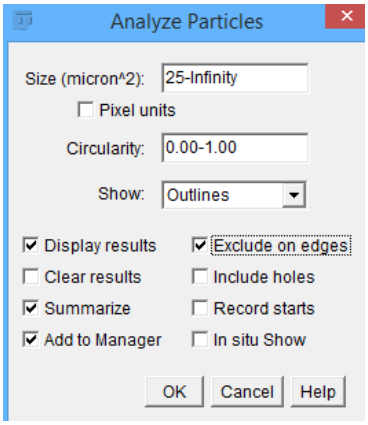
Example: Automatic Counting and Measuring of Nuclei

- open original image
- if multichannel then Split channels <Image><Colour><Split Channels>
- select the nuclei channel
- threshold the image <Image><Adjust><Threshold>
- adjust the levels to include nuclei as necessary (NB you do not need to click apply to just measure).
- run <Analyse><Analyse Particles>
- select options and OK

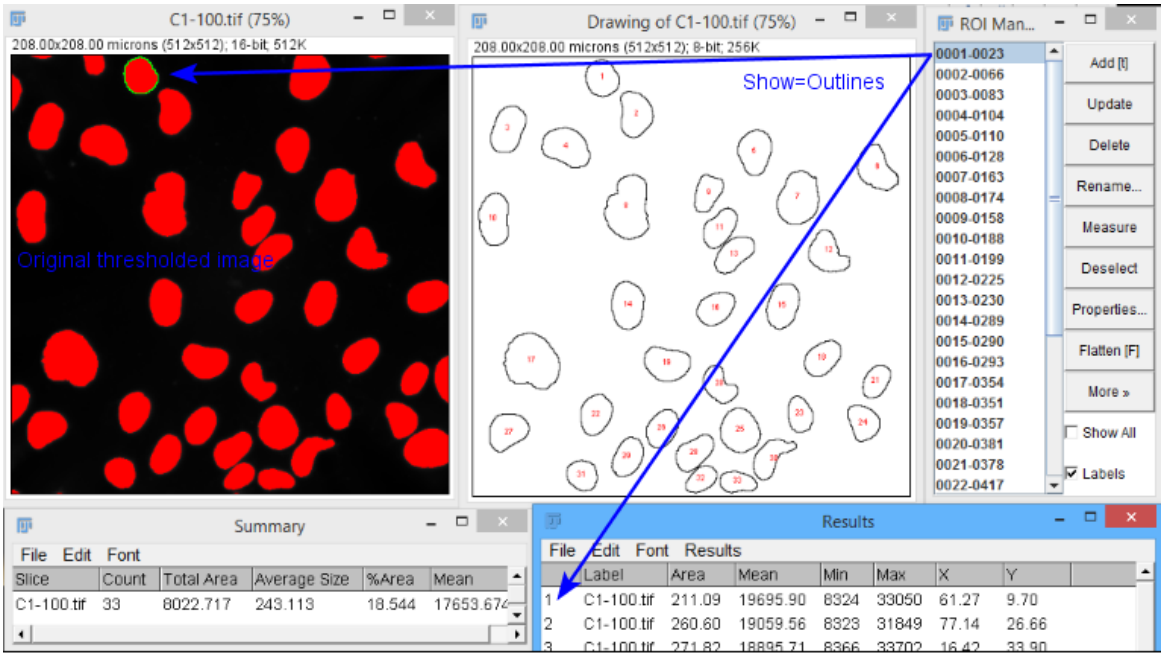


with the settings:

- size: 25sq um to infinity
- all shapes
- exclude on edges
- display results, summary and outlines
- add the ROI's to the manager



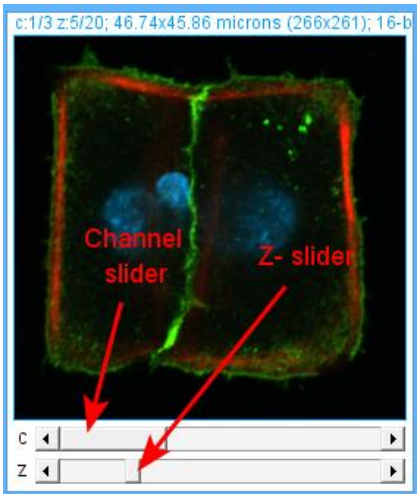
Output:



Z-Stacks Presentation

There are 5 basic methods of displaying a z-stack image:

- Saving as an AVI
- Montage
- Projection
- Orthogonal view
- 3D rendering

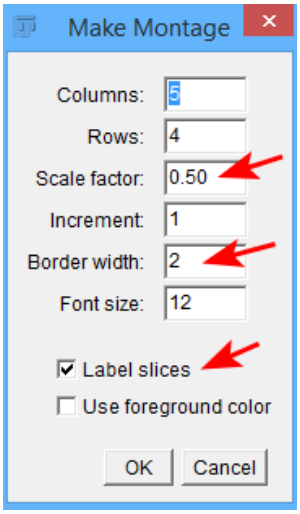


Saving as an AVI

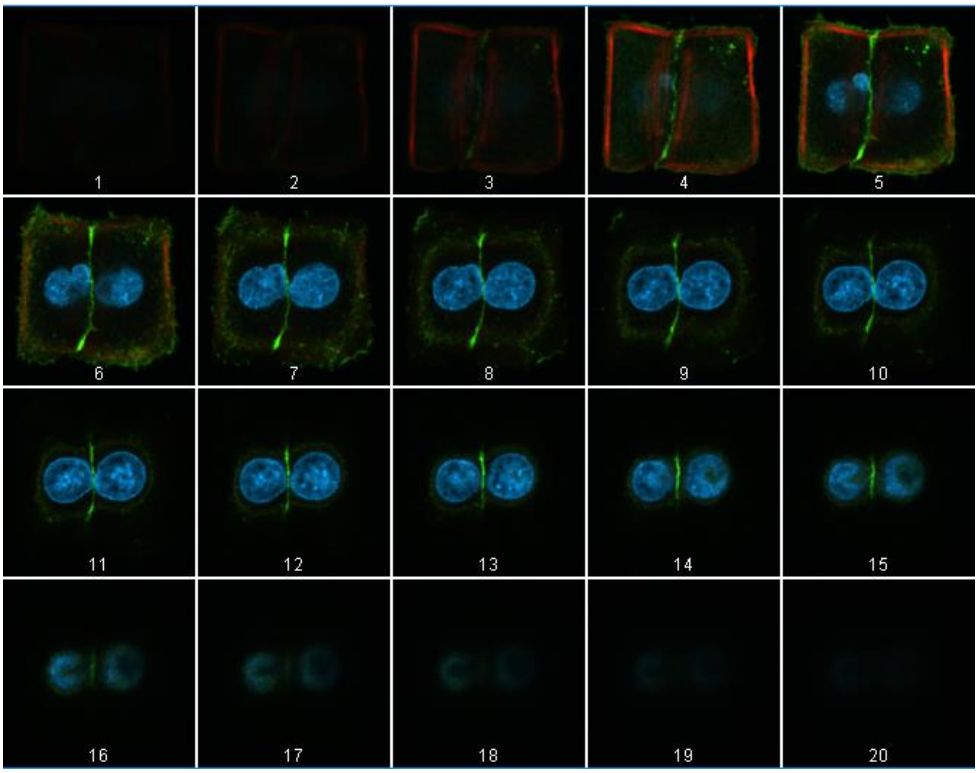
- the image can be saved in AVI video format <File> <SaveAs> <AVI>.

Making a montage (gallery) view

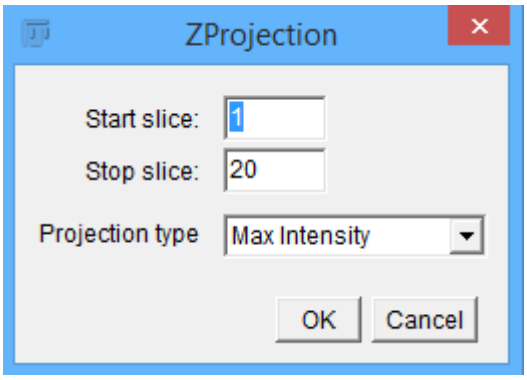
- open the z-stack image and adjust contrast and brightness as required
- run <Image> <Stacks> <Make Montage>
- adding a "Border" will separate the images
- set the scale factor and adjust columns and rows as required
- "Label Slices", if checked will label the montage panels with the slice labels. Slice labels (up to 60 characters) correspond to the image subtitle.



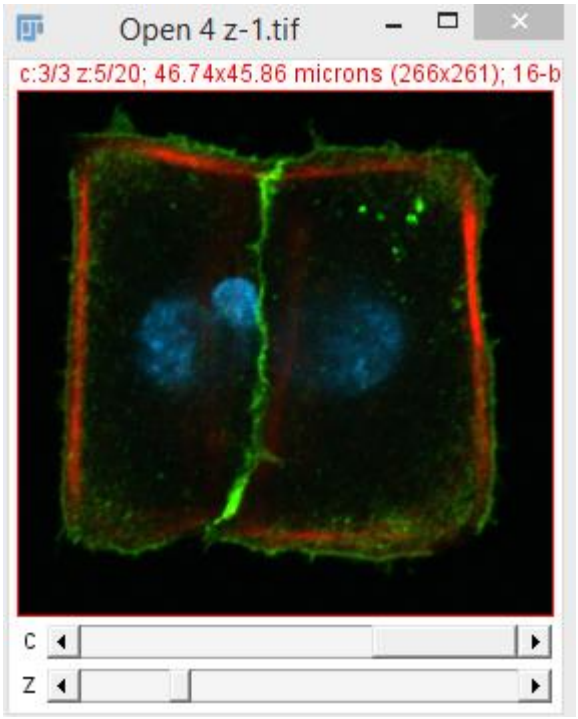
are



Making a projection view

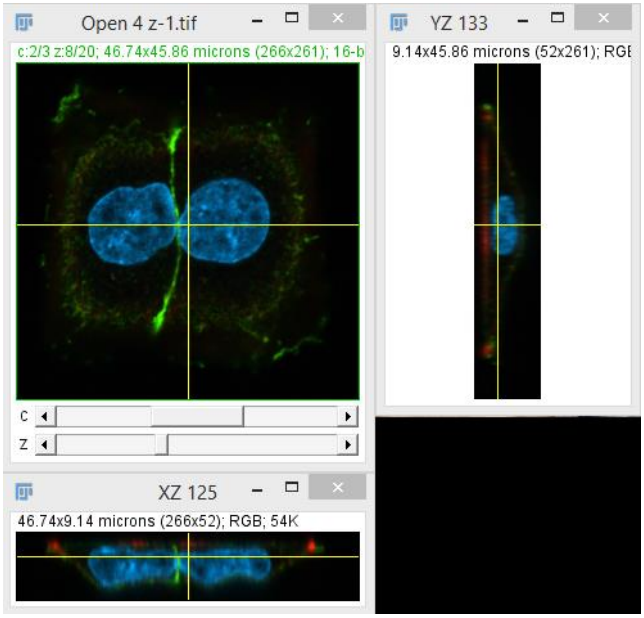


- open the z-stack image and adjust contrast and brightness as required
- run <Image> <Stacks> <Z project>
- select the projection method (usually Max Intensity) from the drop-down box:
  - ***Average Intensity*** projection creates an output image each of whose pixels contains the average intensity value over all images in the stack at the corresponding pixel location.
  - ***Maximum Intensity*** projection (MIP) creates an output image each of whose pixels contains the maximum intensity value over all images in the stack at the particular pixel location.
  - ***Sum Slices*** projection creates a real image that is the sum of the slices in the stack.
  - ***Standard Deviation*** projection creates a real image containing the standard deviation of the slices.
  - ***Median*** projection outputs an image wherein each pixel stores median intensity over all images in stack at corresponding pixel location.



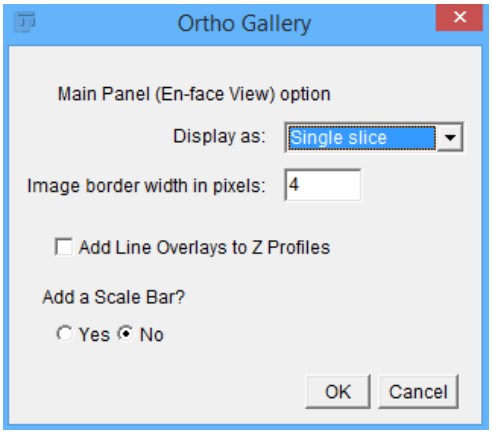
Making an orthogonal view

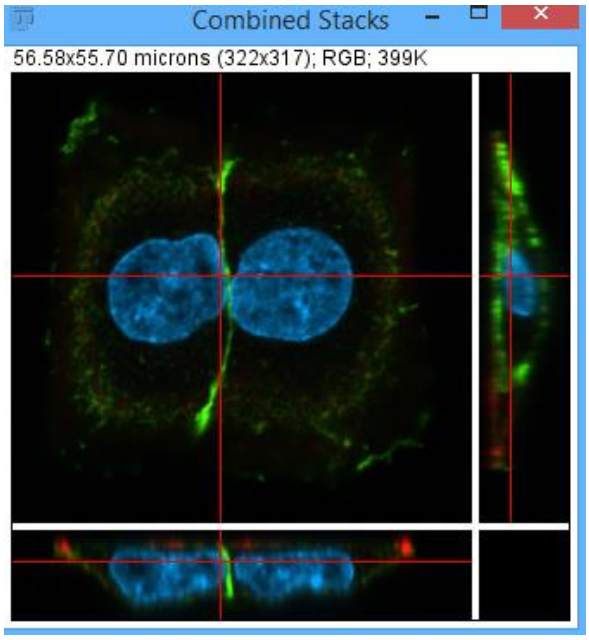
- run <Image> <Stacks> <Orthogonal Views>
- the two extra planar views are displayed in ‘sticky’ panels next to original image
- the intersection point of the three views follows the location of the mouse click and can be controlled by clicking and dragging in either the XY, XZ or YZ view. XY and XZ coordinates are displayed in the title of the projection panels. The mouse wheel changes the screen plane in all three views.
- the two planar views can be saved separately



Alternatively, a combined final image can be obtained using one of the FILM macros from the presentation set (OrthoView Gallery Tool).

- set the display option of the main image as either: the current **Single Slice**, **Maximum Intensity Projection** or **Average intensity projection**
- if "single slice" is selected then the cross lines can be added to the main image
- the "Border" width and a "Scale Bar" can also be added

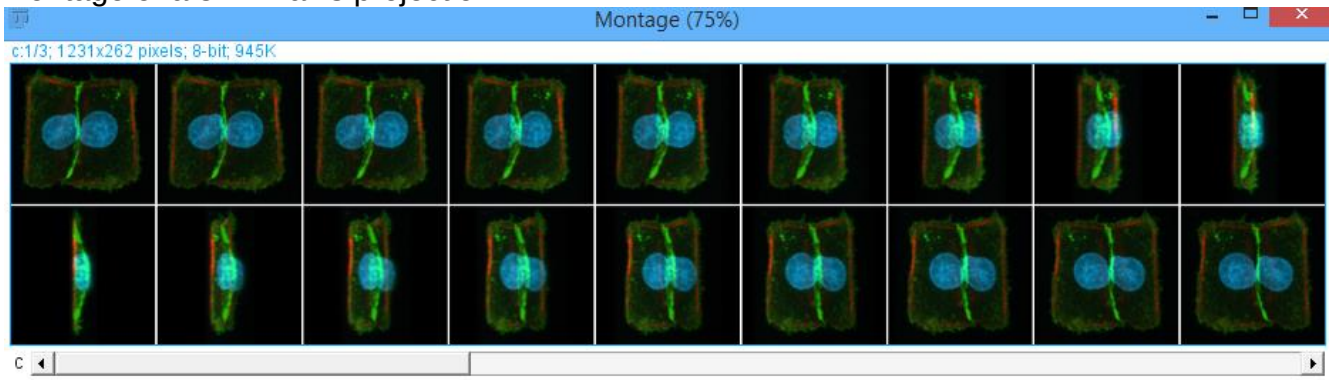




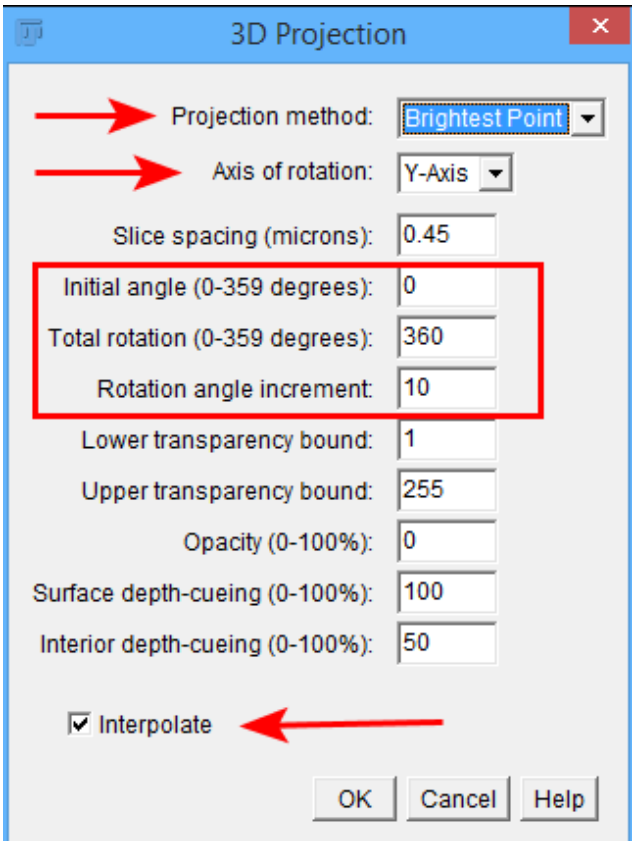
Making a 3D rendered image (using 3D projection)

Generates an animation sequence by projecting through a rotating 3D data set onto a plane. Each frame in the animation sequence is the result of projecting from a different viewing angle

Montage of a 3D Y- axis projection



- run <Image> <Stacks> <3D Project>.
- Select projection method - usually "Brightest Point" - see below
- set the "Axis of rotation" (Y- vertical, X horizontal and Z effectively rotates)
- set the "Initial Angle" - start angle
- Set the "Total Rotation". - final angle
- set the "Rotation Angle Increment",
- select interpolate - this will only have an effect if the slice spacing is above 1 - see below
- the resulting image can be saved in AVI video format <File> <SaveAs> <AVI>. or used to generate a montage



for other options - see below

- *Brightest Point* projection examines points along the rays, projecting the brightest point encountered along each ray.
- *Nearest Point* projection to produce an image of the surfaces visible from the current viewing angle.
- *Mean Value* projection, a modification of brightest-point projection, sums the values of all transparent points along each ray and projects their mean value. It produces images with softer edges and lower contrast,
- *Slice Interval* is the interval, in pixels, between the slices that make up the volume. ImageJ projects the volume onto the viewing plane at each
- *Lower and Upper Transparency Bound* parameters determine the transparency of structures in the volume. Projection calculations disregard points having values less than the lower threshold or greater than the upper threshold. Setting these thresholds permits making background points (those not belonging to any structure) invisible. By setting appropriate thresholds, you can strip away layers having reasonably uniform and unique intensity values and highlight (or make invisible) inner structures.

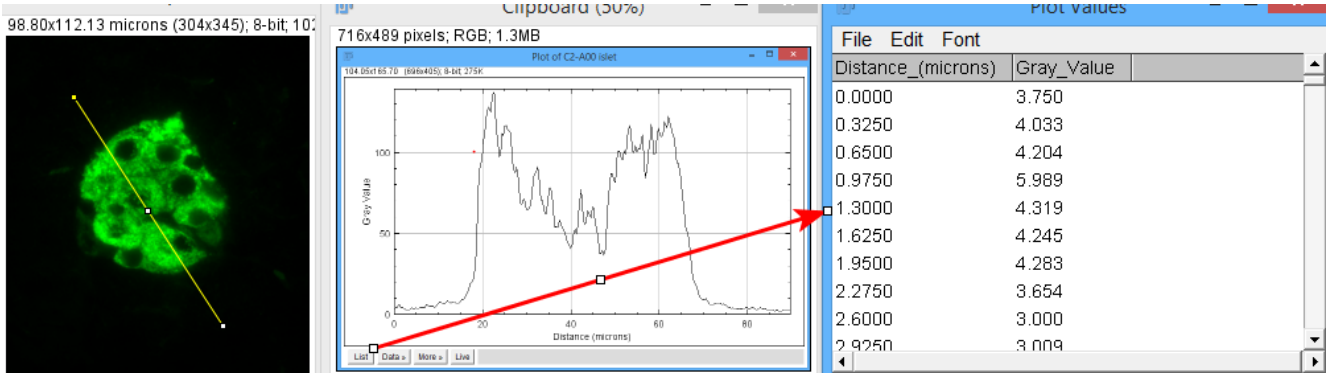
- *Surface Opacity* parameter permits the display of weighted combinations of nearest-point projection with either of the other two methods, often giving the observer the ability to view inner structures through translucent outer surfaces. To enable this feature, set *Surface Opacity* to a value greater than zero and select either *Mean Value* or *Brightest Point* projection.
- Depth cues can contribute to the three-dimensional quality of projection images by giving perspective to projected structures. The depth-cueing parameters determine whether projected points originating near the viewer appear brighter, while points further away are dimmed linearly with distance. The trade-off for this increased realism is that data points shown in a depth-cued image no longer possess accurate densitometric values.
- *Surface Depth-Cueing* works only on nearest-point projections and the nearest-point component of other projections with opacity turned on.
- *Interior Depth-Cueing* works only on brightest-point projections. For both kinds, depth-cueing is turned off when set to zero (i.e. 100% of intensity in back to 100% of intensity in front) and is on when set at 0<n 100 (i.e. (100-n)% of intensity in back to 100% intensity in front). Having independent depth-cueing for surface (nearest-point) and interior (brightest-point) allows for more visualization possibilities.
- Check *Interpolate* to generate a temporary z-scaled stack that is used to generate the projections. Z-scaling eliminates the gaps seen in projections of volumes with slice spacing greater than 1.0 pixels. This checkbox is ignored if the slice spacing is less than or equal to 1.0 pixels.

Intensity Analysis Plots

Line plots

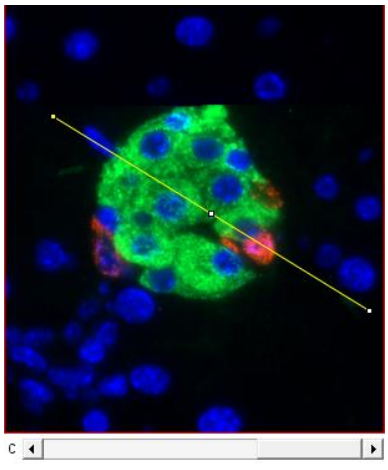
It is possible to generate an intensity line profile for a single channel image

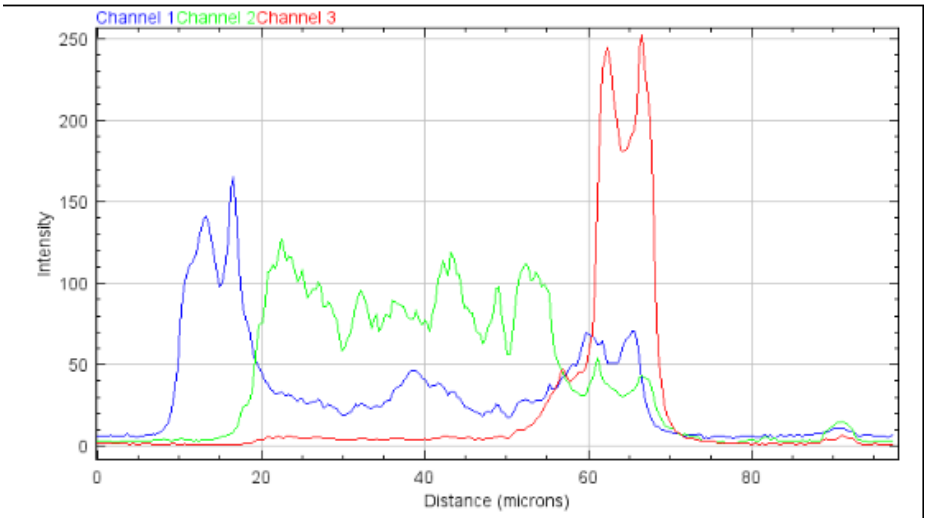
- draw a line using the line tool on the menu bar
- run <Analyse> <Plot Profile> will produce an intensity line plot
- clicking on "List" will generate the plot values in a table that can be exported to Excel



for multichannel images you can either spilt the channels <image> <Color> <Split Channels> and run the profile tool on each channel or you can use "Multi Channel Line Plot" macro in the "Intensity" Toolset from the FILM Website (see earlier FILM Macros Toolsets)

- draw a line using the line tool on the menu bar on the multichannel image
- run the Multi Channel Line Plot macro
- options will appear for the graph output
- it will then produce an intensity line plot for each channel using the LUT for each channel as the plot colour
- it will also generate a table with plot values that can be exported to Excel





Time series ROI Intensity Plots

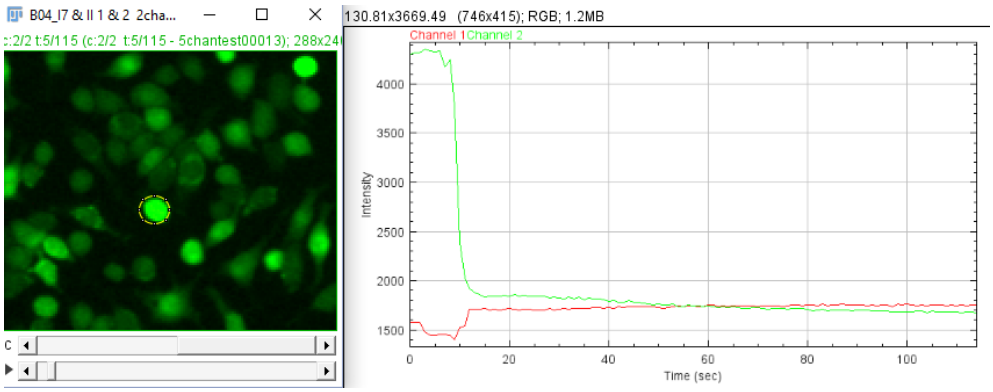
FILM has produced a number of macros for measuring intensity over time

for example:

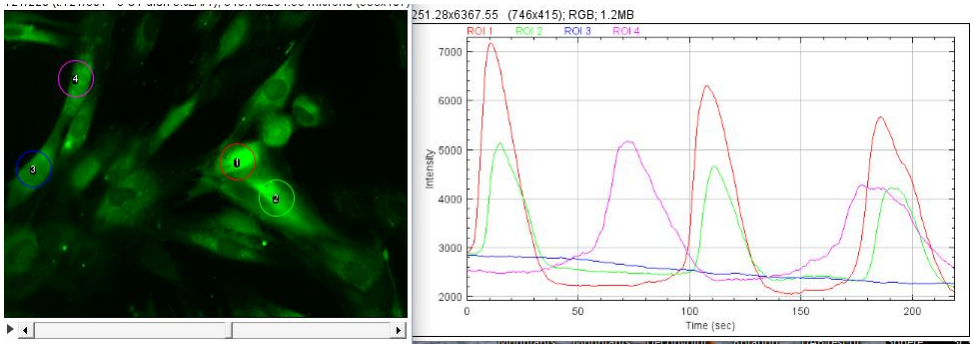
Each macro

- options will appear for the graph output
- an intensity line plot is created using the LUT for each channel as the plot colour
- a results table is generate with the plot values that can be exported to Excel

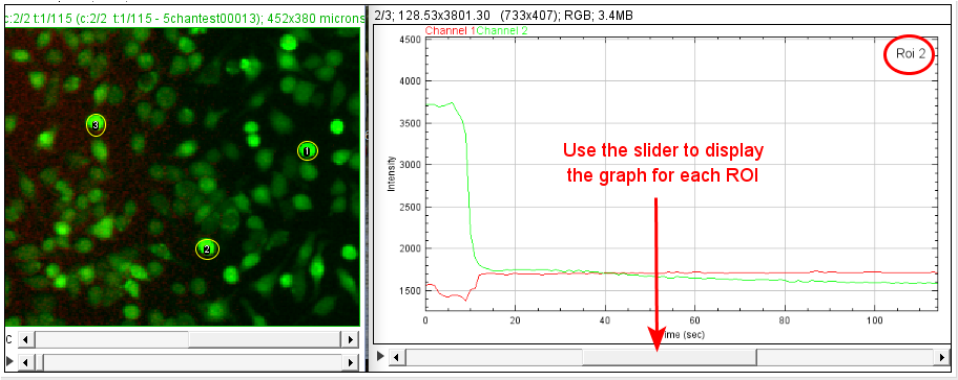
1. Multi Channel Single ROI's



2. Single Channel Multi ROI's

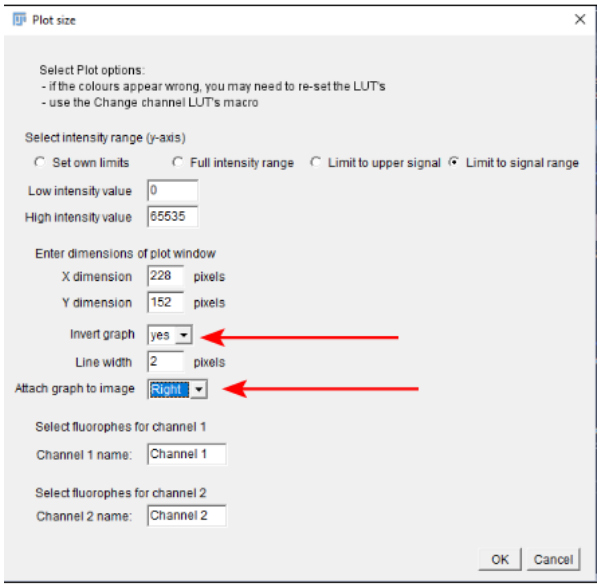


3. Multichannel Multi ROI's

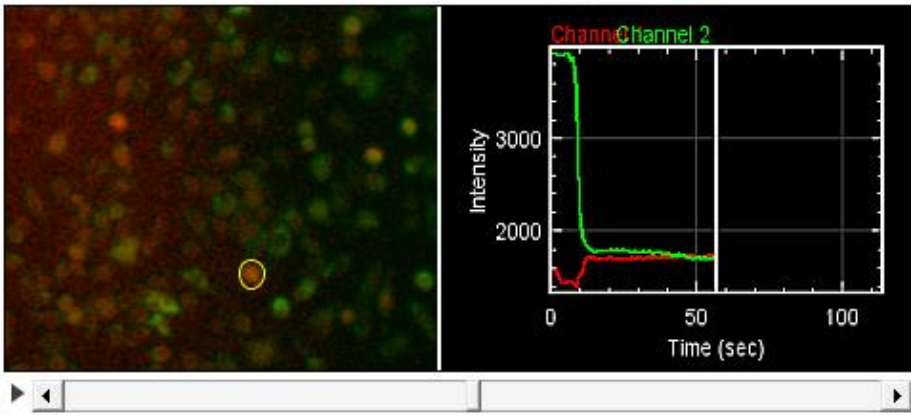


And to create plot movies

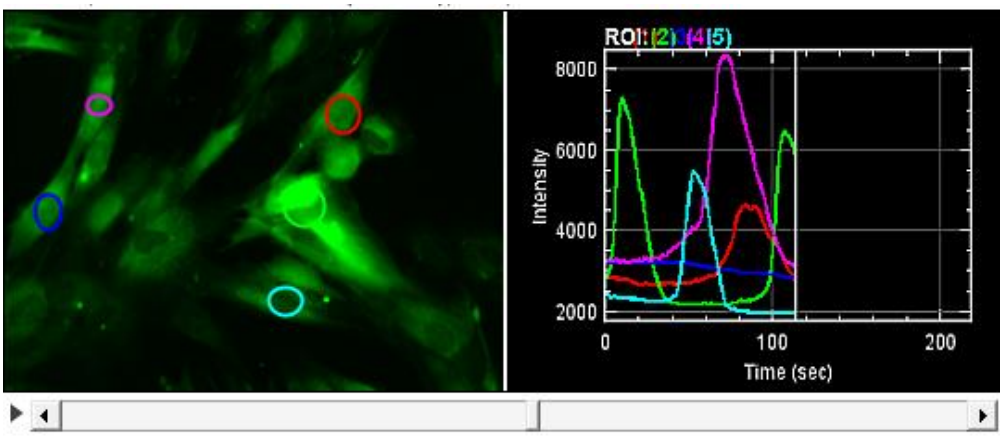
- onthe plot options appear, select whether you want to invert the colour of the plot widow and if you want to attach the plot to the image, select the position



4. Multichannel single ROI plot frame by frame



5. Multi ROI single channels plot frame by frame



- the resulting image can be saved in AVI video format <File> <SaveAs> <AVI>.