

# FIJI Intermediate Workshop Notes



**Notes Prepared by Dr Sarah Creed and Dr Kirstin Elgass**

**Presented by Dr Kirstin Elgass and Dr Sarah Creed**

Monash Micro Imaging at MHTP 1ST EDITION - April 2018



---

# Table of Contents

Fiji Intermediate	1.1
PART 1: INTRODUCTION TO FIJI INTERMEDIATE	1.2
PART 2: MULTIPLE DIMENSIONS	1.3
PART 3: MASKS AND FILTERS	1.4
PART 4: IMAGE CORRECTION	1.5
PART 5: IMAGE ALIGNMENT	1.6
PART 6: SIMPLE IMAGE MEASUREMENTS	1.7
PART 7: MEASURING MOVEMENT IN LIVE CELLS	1.8
PART 8: MORPHOLOGY MEASUREMENTS	1.9
PART 9: CO-LOCALISATION ANALYSIS	1.10
Acknowledgements	1.11
Use and Distribution of Workshop Notes	1.12
Demonstration Images	1.13

# PART 1: INTRODUCTION TO FIJI INTERMEDIATE

## Requirements for undertaking FIJI Intermediate

This workshop assumes that you are familiar with the basic features of FIJI/ImageJ and now want to apply those functions to multi-dimmensional images and image analysis

## Analysis Tools in FIJI

FIJI is an open-source software, which means everyone can use it but also means everyone can add features and expand FIJI's capabilities. Researchers who developed a specific image analysis pipeline for their project and want to make it available to other researchers can add it to FIJI as a so-called plugin. For this reason there are a wide range of analysis tools available in FIJI, and in some cases multiple tools which have the same outcome.

These workshops are not able to cover every tool available in FIJI, but here we will be focusing on the most common types of image analysis and where possible will demonstrate different methods available. For some analysis tools which have multiple plugins we may feel that one is more accurate or appropriate than the other, and in these circumstances we will only focus on the more suitable analysis tool.

## Plugins Required From Separate Downloads

Some plugins are pre-installed in FIJI, others you will have to download and install yourself. We will be using both types of plugins in this workshop. The following plugins will need to be downloaded and installed.

**Drift Correction/Image Registration:** Google 'StackReg FIJI' and 'TurboReg FIJI' and download both plugins. They will appear as zipfiles. Unzip both files and then move the .jar file into the plugins folder of your FIJI software.

**Deconvolution:** Download *Iterative\_Deconvolve\_3D.class* from [http://imagej.net/Iterative\\_Deconvolve\\_3D](http://imagej.net/Iterative_Deconvolve_3D)

**Note:** There's also an *Iterative\_Deconvolve\_3D.java* available. The.java file does not work and you need to make sure you download the .class file instead.

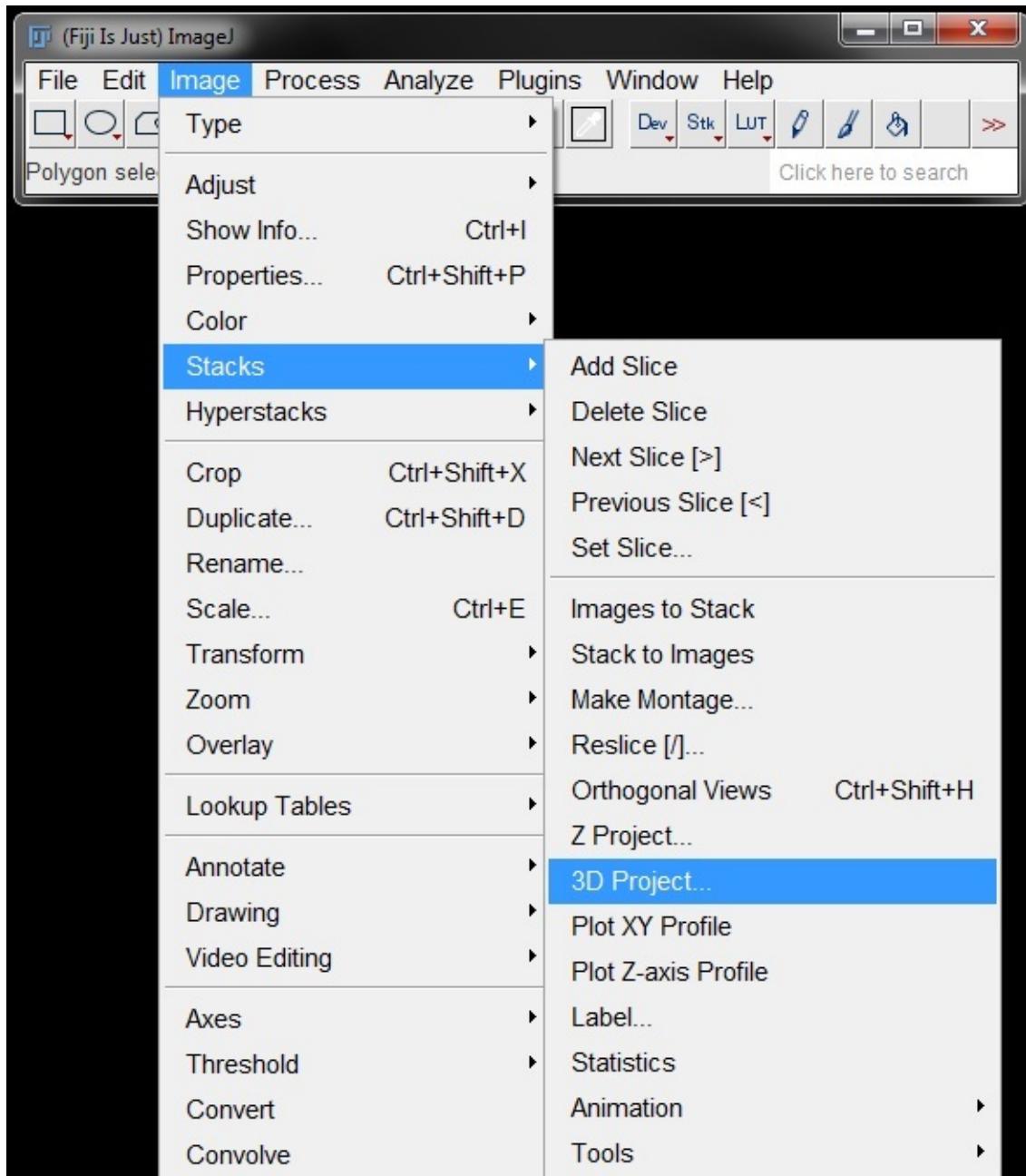
## PART 2: MULTIPLE DIMENSIONS

Our images often contain more than just a single flat picture. We can have multiple colour channels, introduction of the z-plane (3D), time series, or any combination of the above. In all of these case your data may be displayed in an image stack. In FIJI Basics we went through how to navigate and alter an image stack. Those instructions can be easily applied to images with a single multiple dimension of any kind (eg: z-stacks only), but when we add in more than 1 extra dimension (eg: z-stacks AND time series) we also need expand on our toolset to allow us to present the data in the most accurate and effective way possible. Here we will show you more tools for working with your multi-dimensional (3D, time series, 4D) image stacks.

In this section we use *Spheroid.tif* for 3D and *MovieStack.tif* for time series. *HyperStack.tif* will also be used for demonstration.

### 3D Projections - Method 1

When working with z-stacks, you can create a 3D projection of the stack by going to **Image -> Stacks -> 3D Project**.



For most 3D projections you can leave the settings in the **3D Projection** window as default.

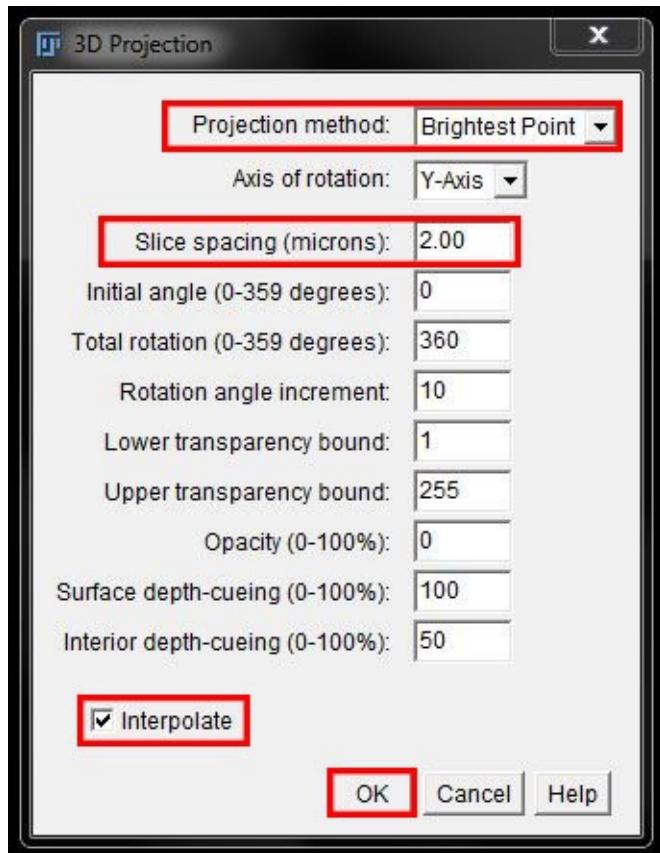
The **Projection Method** should be Highest Point by default. If it isn't, change it from the drop down menu.

If the image is calibrated the **Slice spacing** should be populated correctly from the metadata, but sometimes will need to be entered manually. Here we need to enter a step size of 2 microns.

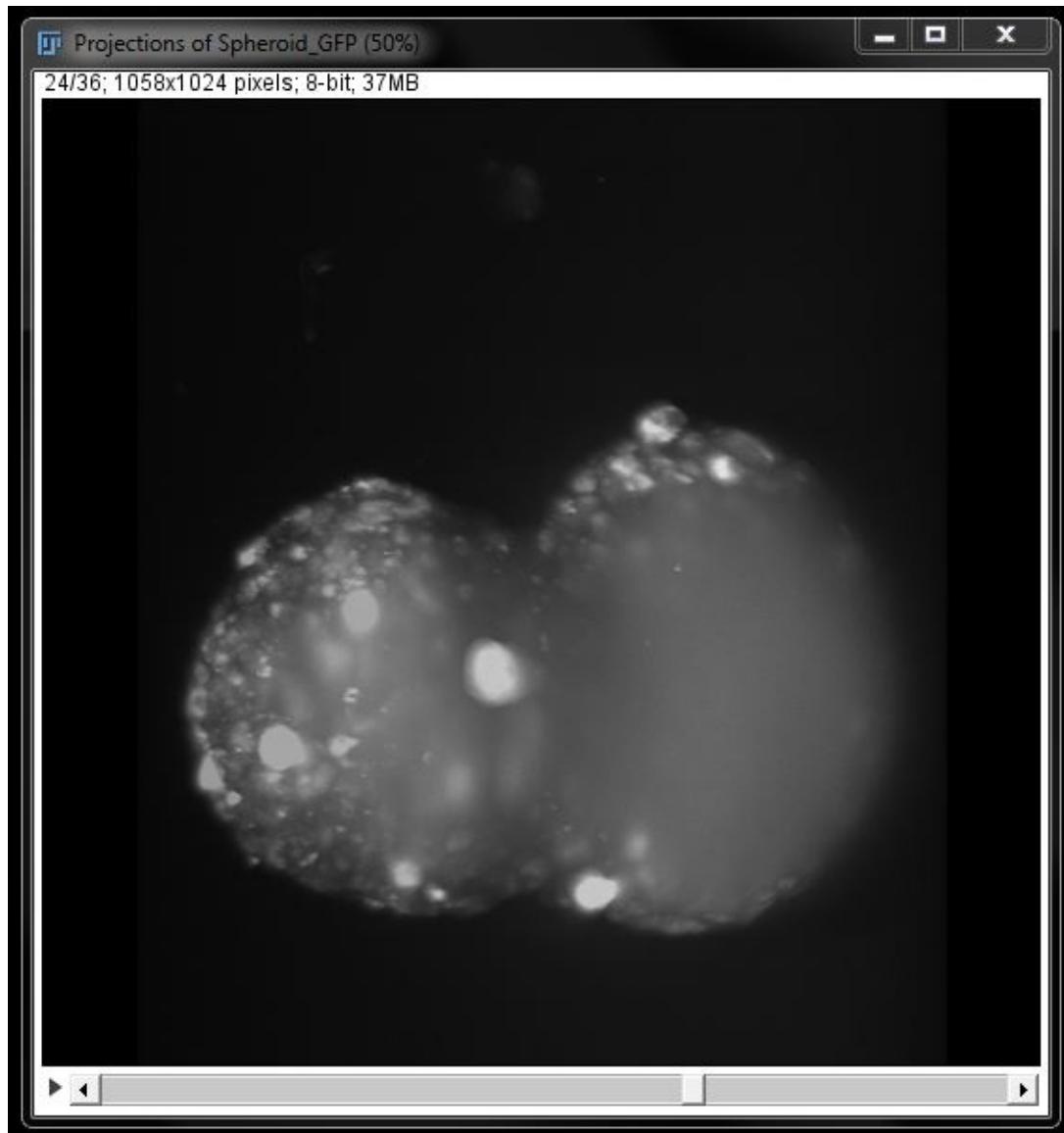
**Note:** The slice spacing is the z-step size used during image capture, not the calibration size.

If you check the box next to **Interpolate**, FIJI will 'guess' the image information between slices and fill in the blanks. While this helps to create a smooth projection it is adding information that is not in the original image. For quantitation this should never be used, however, for presentation of your 3D projection you can use it to create a nicer image.

Select **OK** to create the projection.

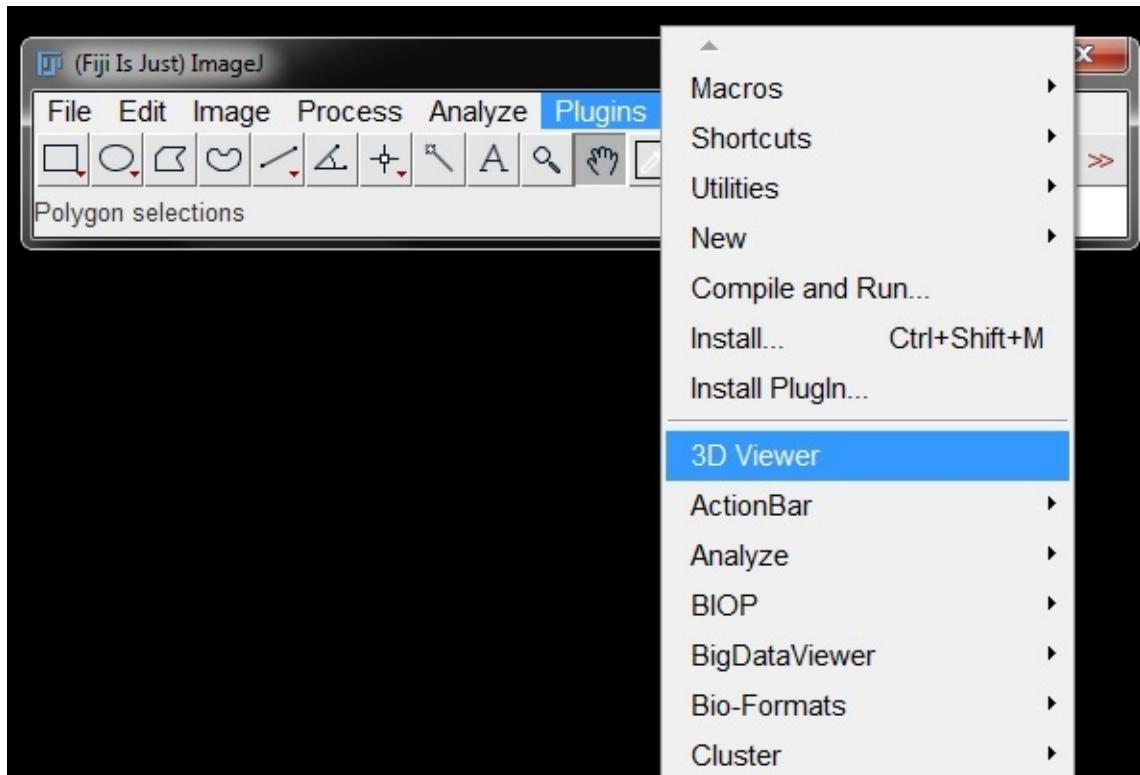


The resulting projection will have a slider at the bottom (similar to a stack) which you can use to rotate the 3D image.



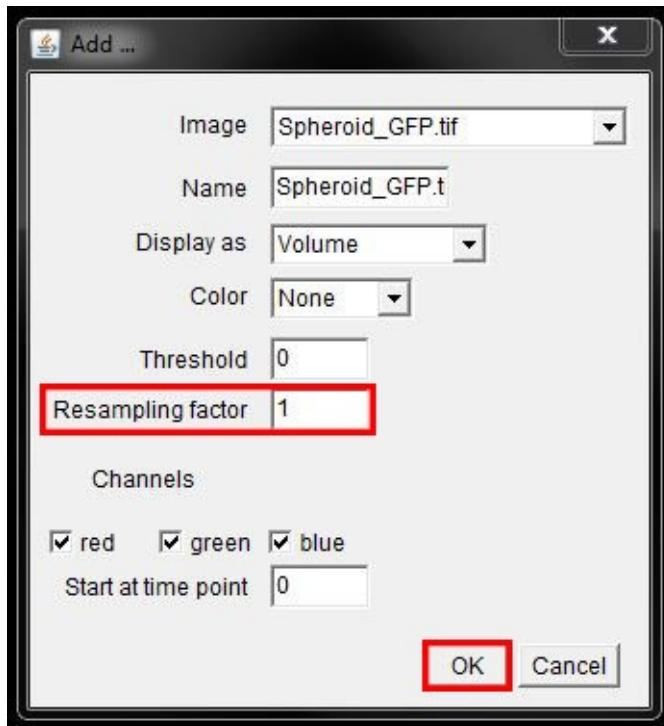
## 3D Projections - Method 2

You can also create a volume view of your z-stack using a different 3D projection tool. Find this tool under **Plugins -> 3D Viewer**.

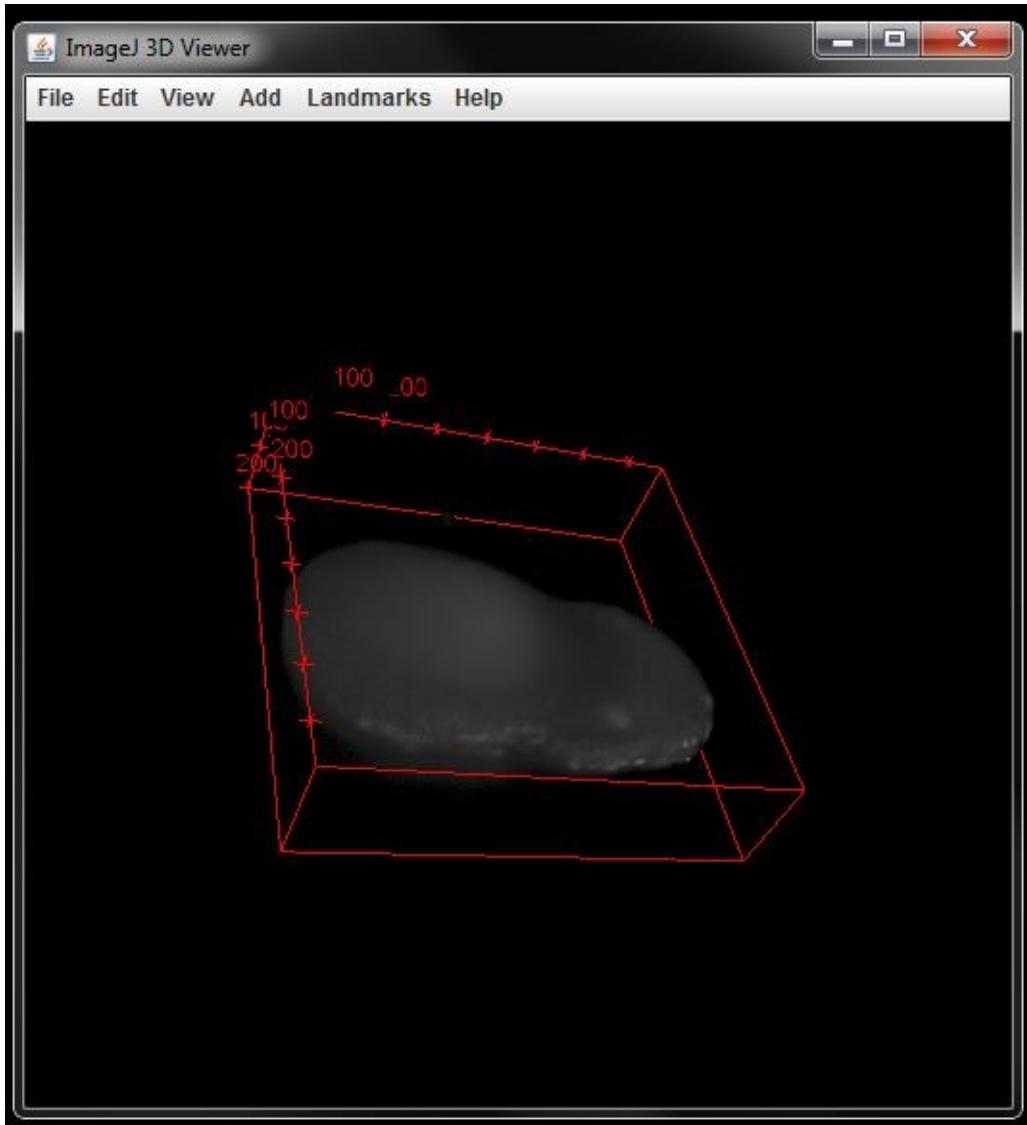


Select the image you want to project from the first drop down menu. You can give the projection a new name if you want to, or leave the default. Leave **Volume** (default setting) as the display type but ensure you change the **Resampling factor** from the default of 2 back to 1 so that no data is cut out of your image.

**Note:** There is now section in these settings to alter the z-step size. You must have a calibrated image, or have calibrated the z-step size in elsewhere first.



Select **OK** to create your 3D volume.

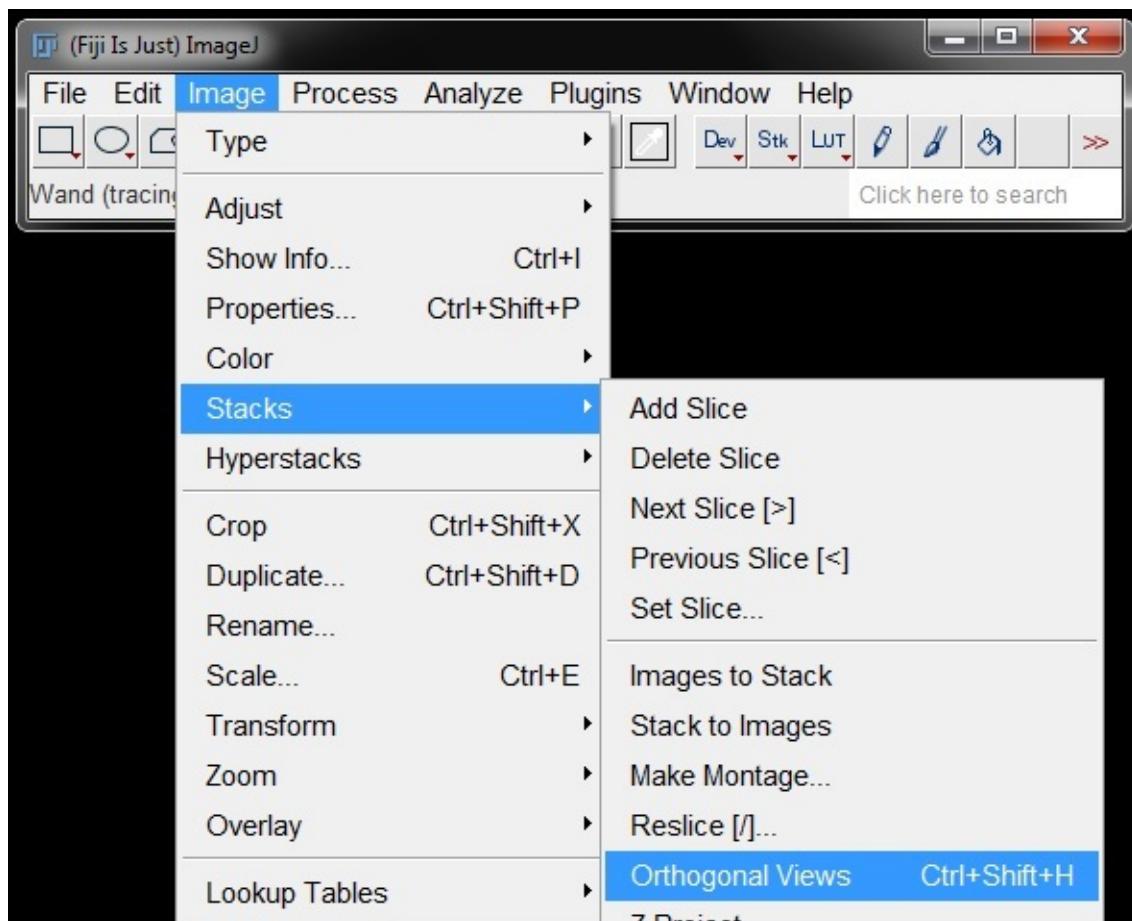


You can click and drag on the image to navigate around the volume.

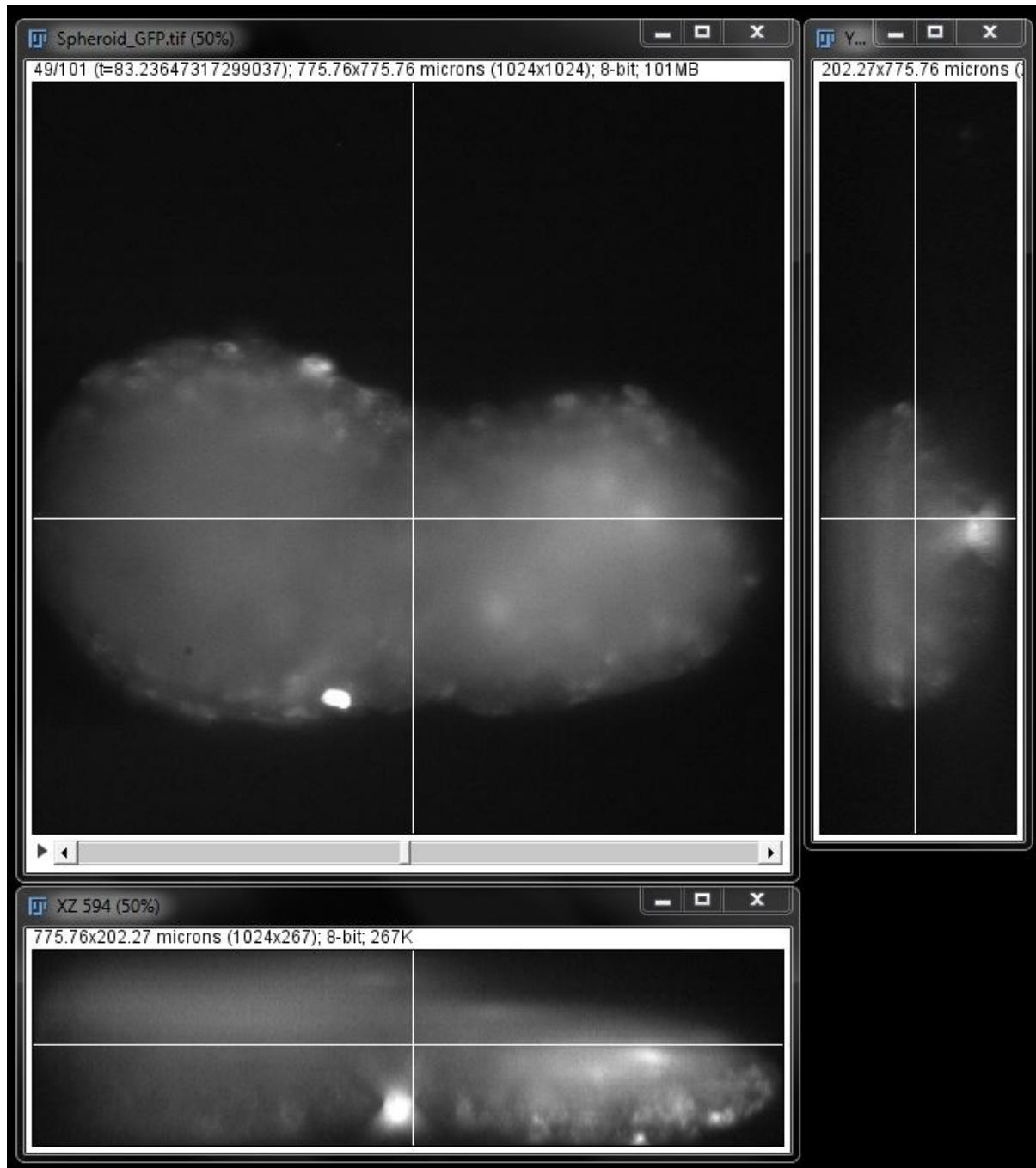
## Orthogonal Views

An orthogonal projection is a view created in the YZ or XZ dimension of an image stack. An orthogonal projection allows you to visualise depth information one slice at a time in your sample.

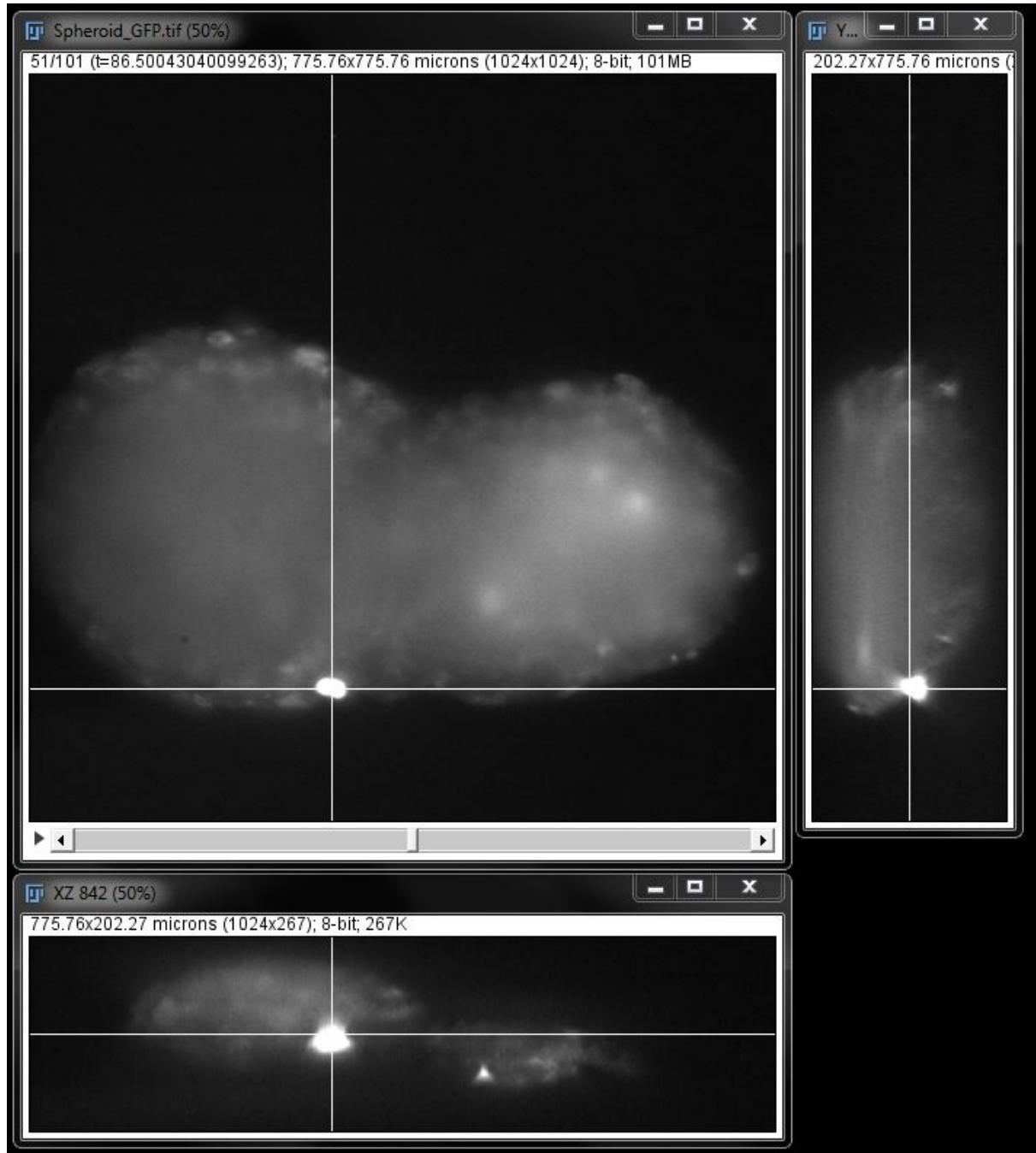
To generate orthogonal slices select your z-stack and go to **Image -> Stack -> Orthogonal View** (or use shortcut **Ctrl+Shift+H**).



Two windows will open to the right of and below the original stack. These windows show the orthogonal projections in YZ and XZ respectively.



To change the view seen in each window, move the cross hair in the original stack to change positions and scroll through the stack to change planes.

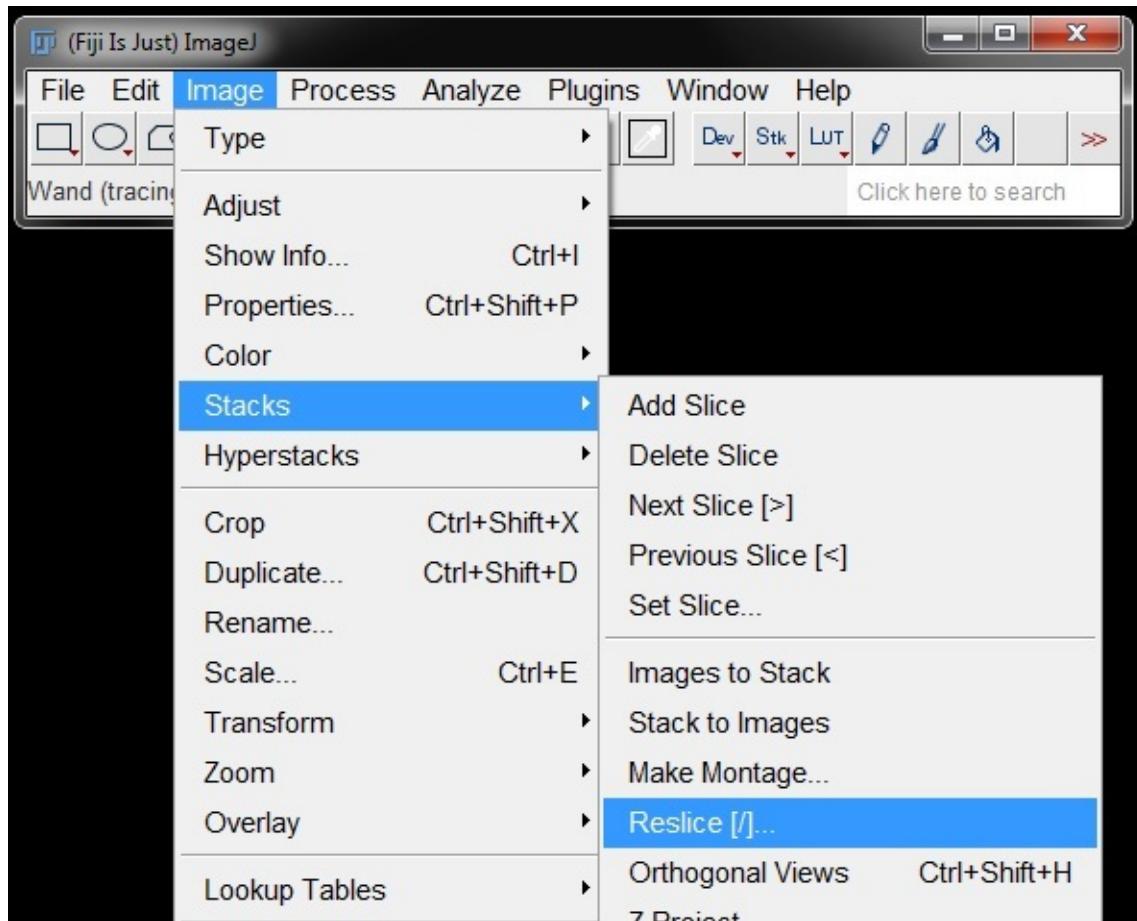


Each of these projections can be saved as tiff images for use later.

## Reslice Z

The orthogonal projection only lets you see one slice at a time and it has the overlay lines in the way. For this reason it can be useful to generate a new stack of orthogonal views instead. This is easily achieved by reslicing the stack along a different axis.

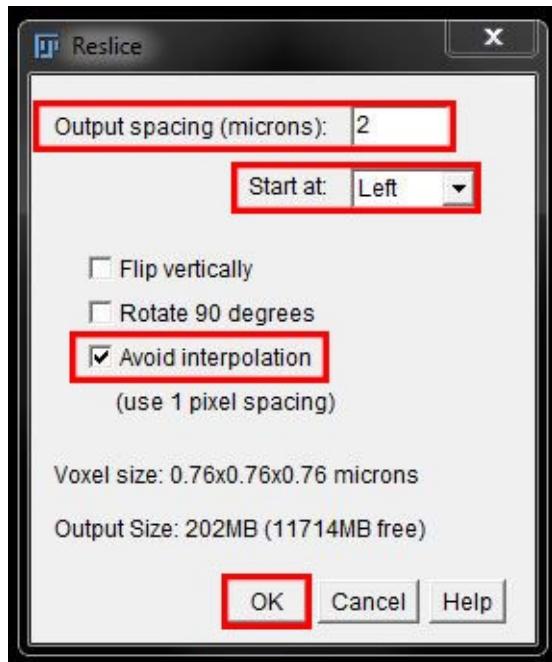
To generate an orthogonal stack go to **Image -> Stacks -> Reslice**.



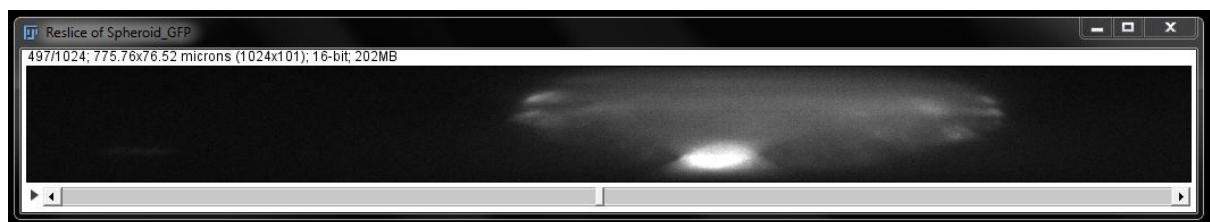
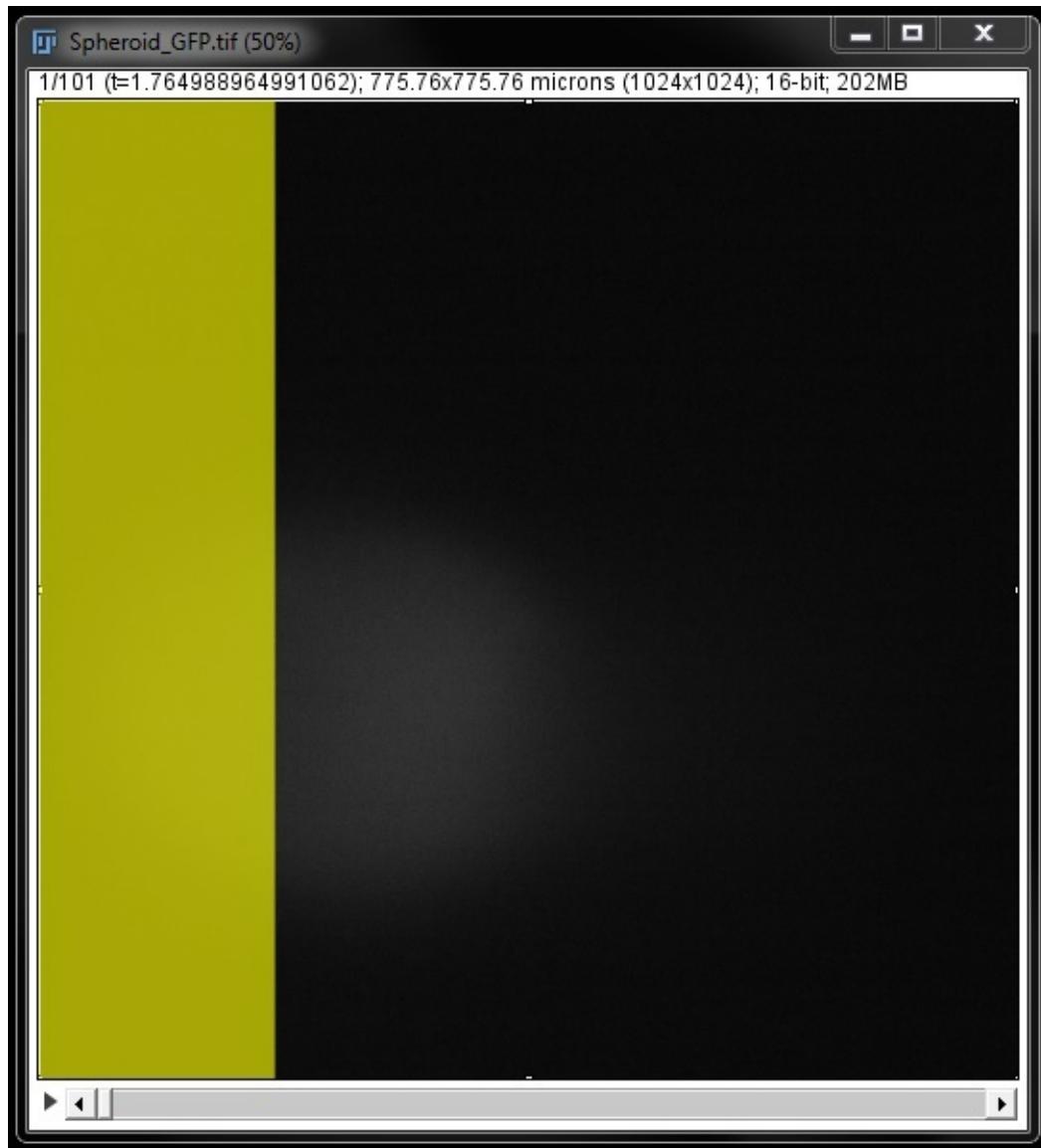
In the window that opens you can set the distance between slices, if not already calibrated (again enter 2 here), and choose how the stack should be resliced (top, bottom, left, right) from the drop down menu.

There is a tick box to select that says **Avoid Interpolation**. This should usually be ticked. As with the 3D projection, while interpolation will make the end result look better, it does this by adding extra data that wasn't in the original image.

Select **OK** to generate the resliced stack.



The program will then 'scan' through the image from the selected direction and generate the new stack.



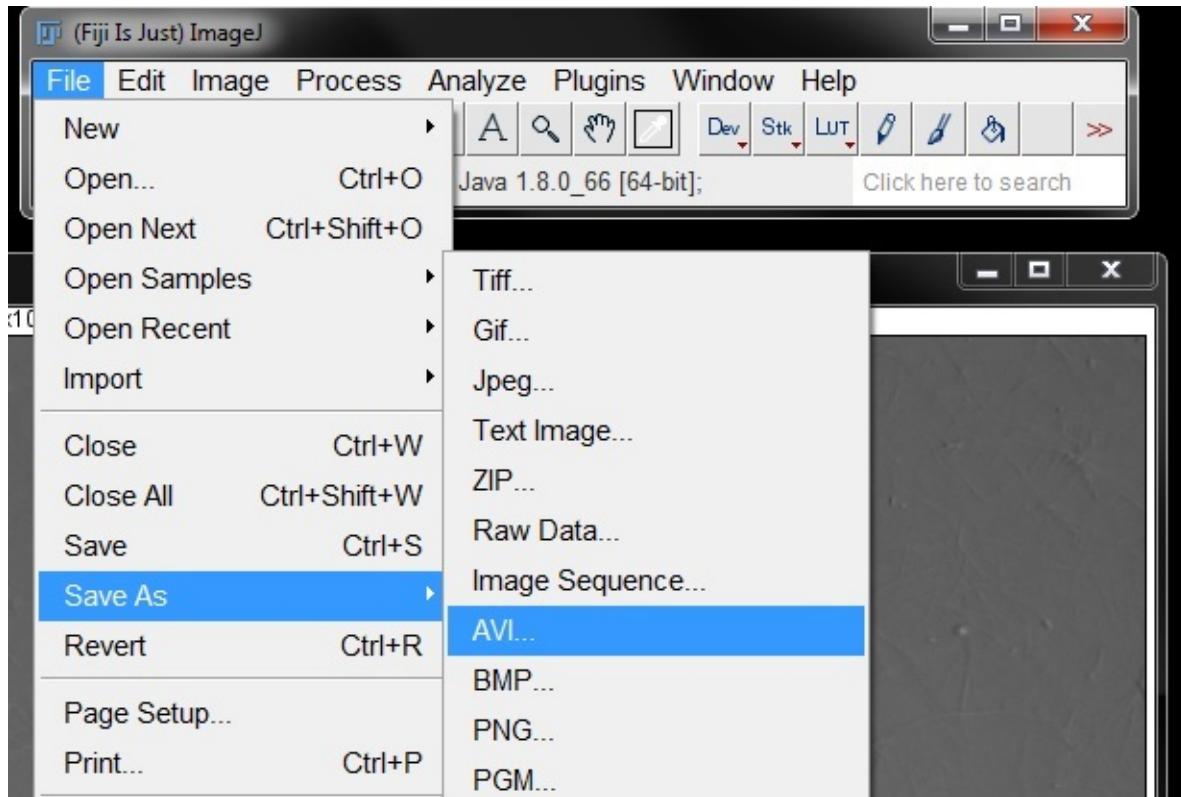
## Times Series and Saving Movies

Time series will also open as a stack that you can work with in a similar manner to colour or z-stacks.

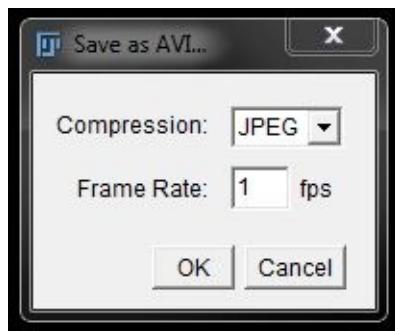


Here, the slider or play button move through the time points, rather than the channels or z-slices.

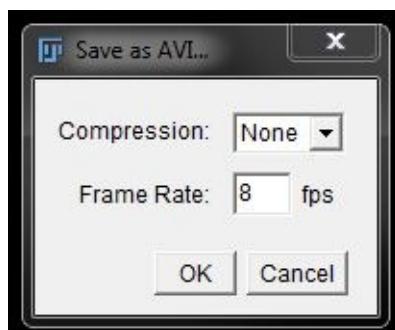
You can save your time series as a movie using FIJI by selecting **File -> Save As**, and choosing **AVI** as the file type.



A window with saving options will open, which will allow you to set specific aspects of the movie.



Where possible you should create your movie without compression (choose 'None' from the drop down menu under **Compression**). The ideal frame rate will be dependent on your images and time interval (ie: the fewer frames in the movie, the slower the frame rate should be). Sometimes a trial and error of different frame rates will be needed to generate a movie that plays at an appropriate speed. For this example we can use a frame rate of 8 frames per second (fps).



Click **OK** to create and save the movie. A movie file will be generated.

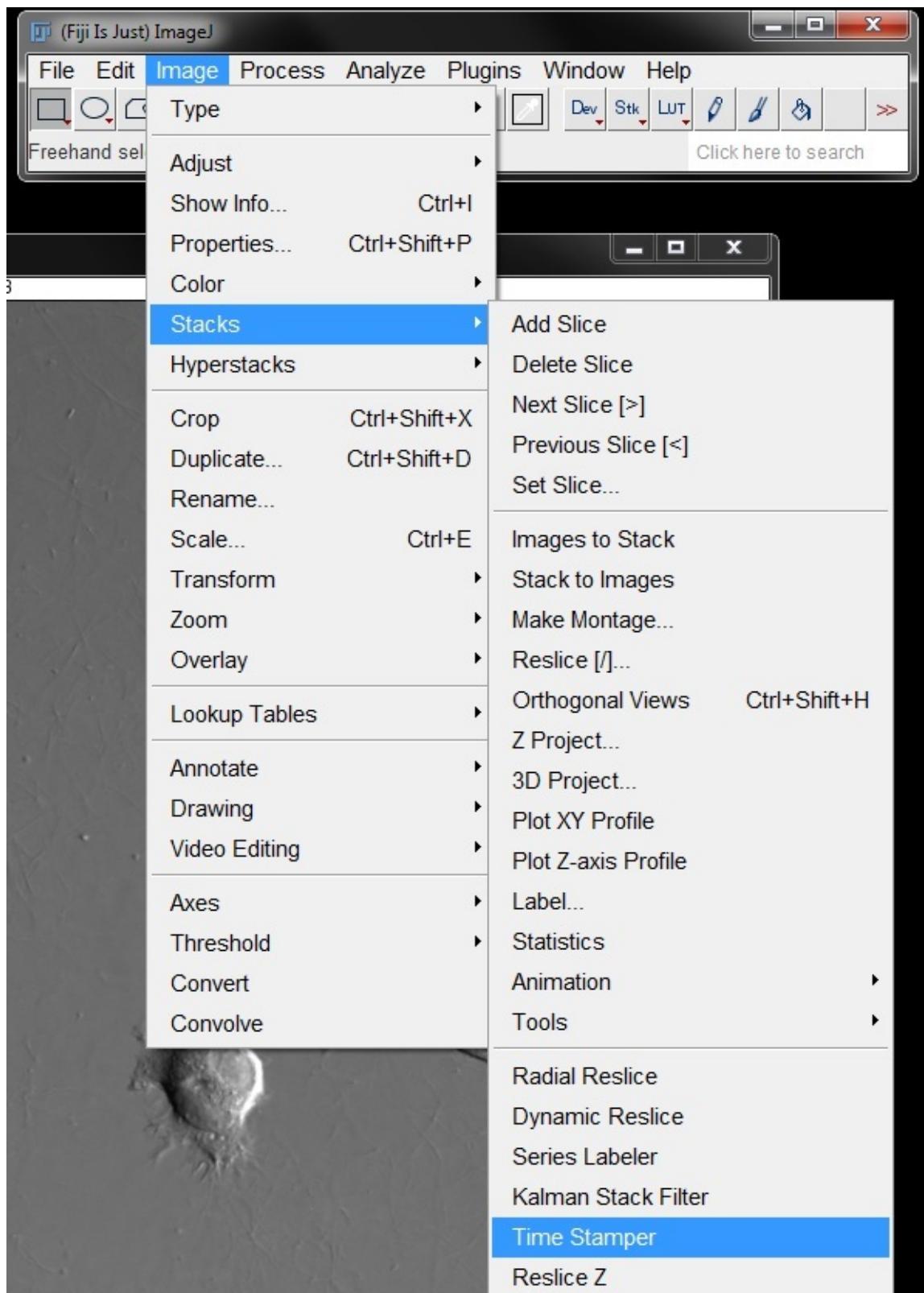


The movie will not open and play automatically after saving. You can open and play the movie file in programs such as QuickTime, Windows Media Player or VCL Media Player to check the frame rate. Repeat the process if a faster or slower rate is required.

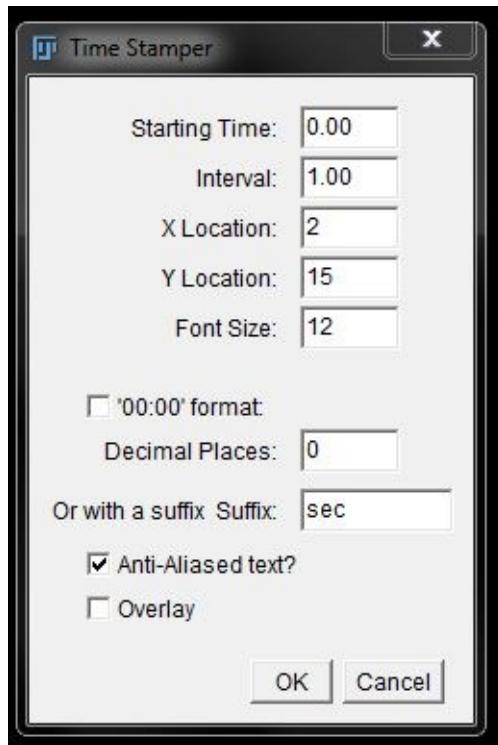


## Adding a Time Stamp and Scale Bar to Movies

Before saving your movie you can embed a time stamp, which helps the viewer understand the time interval and total time of the movie as they are watching it. To add a time stamp, select your time series stack and go to **Image -> Stack -> Time Stamper**.



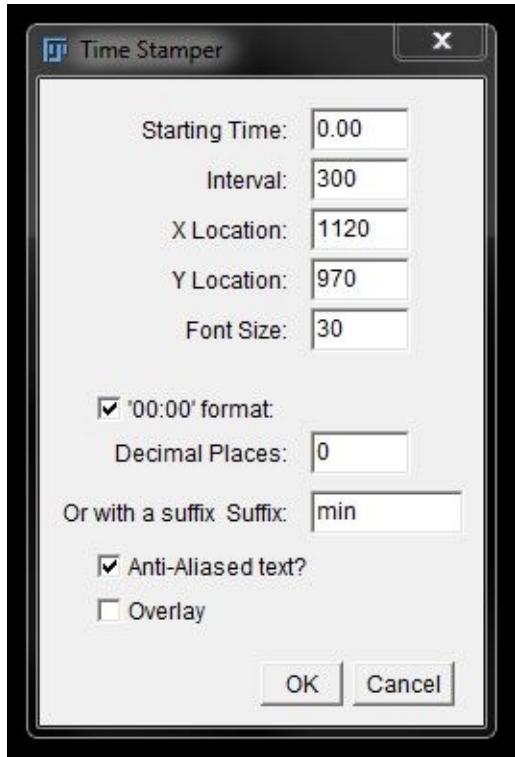
In the **Time Stamper** window you will need to enter the time frame between images and **Interval** and the format you want the time stamp to be in. You can also specify the **X Location** and **Y Location** of the time stamp in the image (given in pixels).



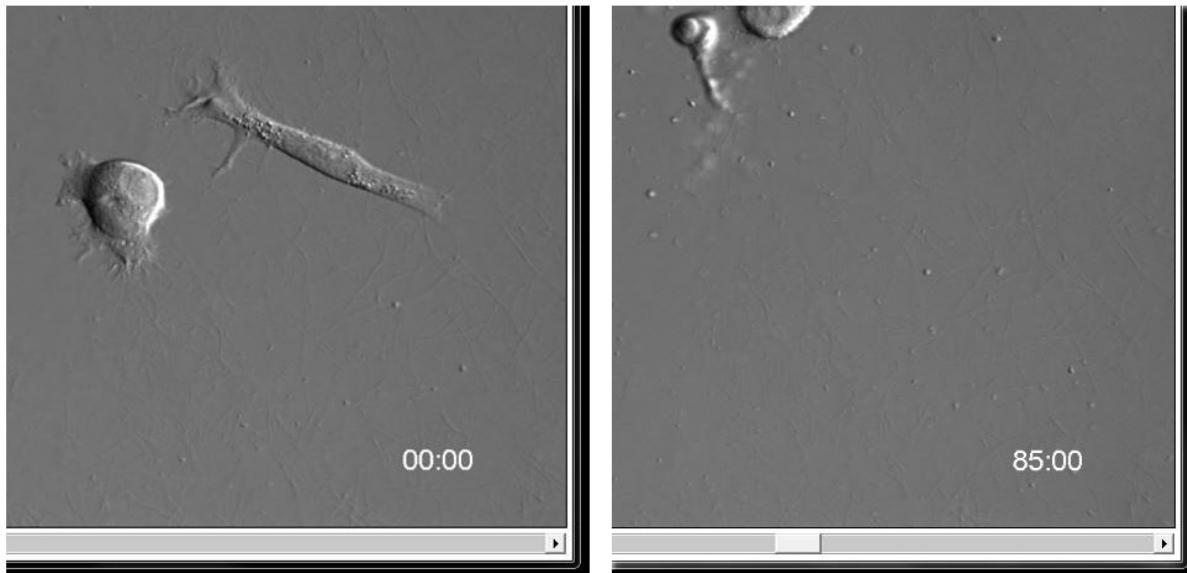
In this example, the time interval is 5mins (enter 5.00 for minutes or 300 for seconds). How you enter the interval will also determine the time stamp format. If using the time format - '00:00' with the interval entered in minutes, you will see HR:MIN in the time stamp. If the interval was entered in seconds, you will see MIN:SEC in the time stamp. If using a suffix, the interval and suffix must have matching formats (ie: don't enter 300 as your interval and then use mins for the suffix).

Pixels for an approximate location can be found by hovering the mouse over the area of the image you wish to use. Pixel co-ordinates will be displayed in the information bar. Enter the pixel co-ordinates you want for placement in the **X Location** and **Y Location** boxes.

**Note:** The pixel co-ordinates become the starting point for your time stamp, not the centre point.

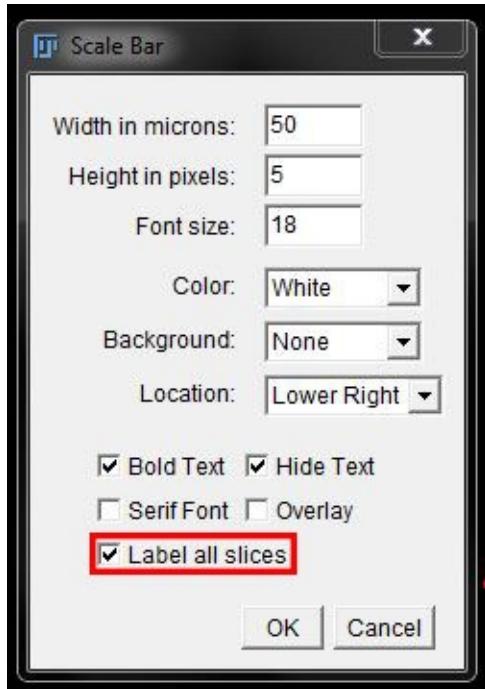


Select **OK** to insert the time stamp into the stack. The timer will automatically run as you scroll through the stack.



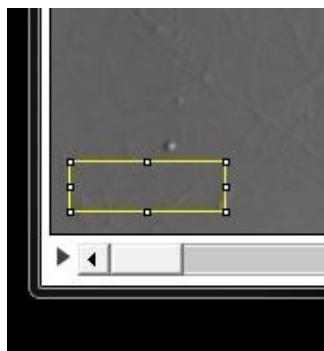
You can now save your time series as a movie, using the same method as above, and the time stamp will remain embedded.

To add a scale bar to your movies, follow the same method as taught in the FIJI Basics workshop for single images. Go to **Analyze -> Tools -> Scale Bar** and set desired parameters. For movies you must ensure you check the box next **Label all slices** before clicking **OK** to insert the scale bar. This will ensure all frames of the movie contain the scale, not just the first frame.

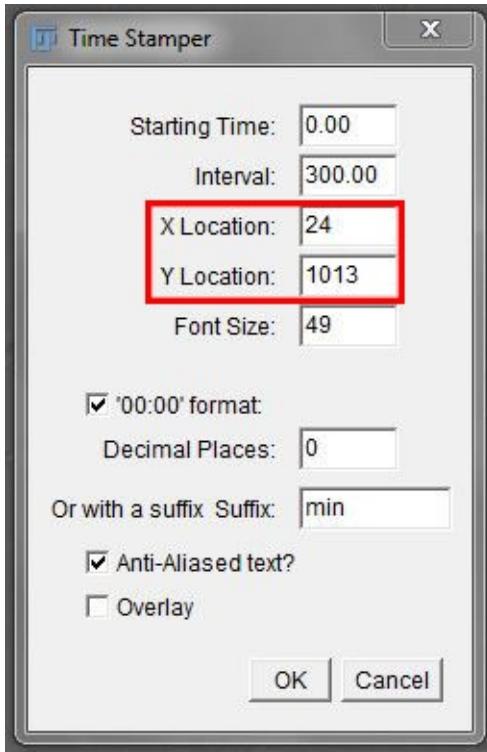


You can also use ROIs for placement of the time stamp and scale bar in movies to ensure accurate positioning in your desired location.

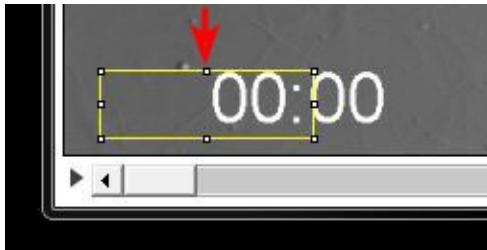
For time stamps, use a rectangular ROI and outline the area you want to place the time stamp in.



Follow the instructions to insert a time stamp as described above, only now the **X Location** and **Y Location** boxes will be automatically filled with co-ordinates from the ROI. Do not change these. Enter your other values as required and click **OK** to insert the time stamp.

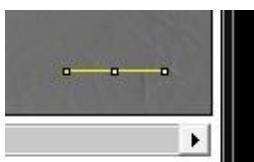


**Note:** The *middle* of the ROI box is the start of the time stamp position.

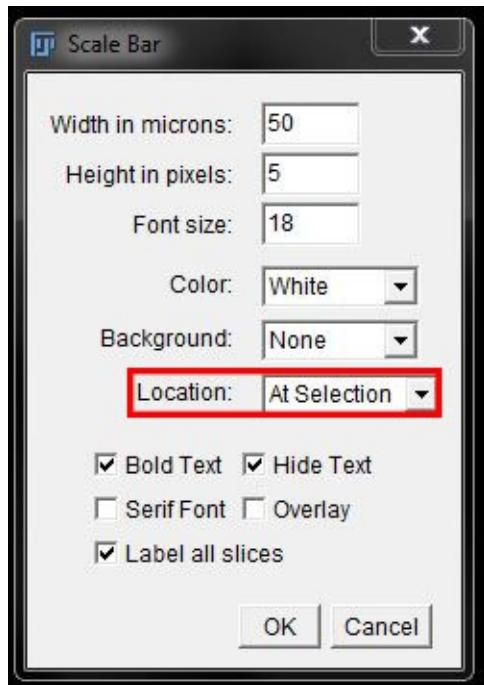


Once you have inserted your time stamp, click anywhere on the image to remove the ROI. Save your movie file as normal.

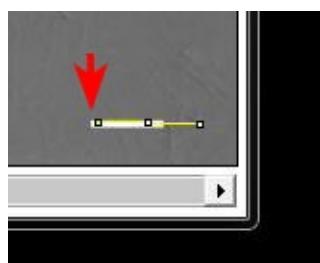
For scale bar placement, use the line ROI tool and draw a line in the area you want to place the scale bar.



Follow the steps to insert a scale bar as normal. This time in the options, the length of the ROI will be detected and used as the scale bar length - you can change this back to your desire length. To use the ROI co-ordinates for placement, chose 'At Selection' from the drop down menu under **Location**. Again, ensure **Label all slices** is checked before clicking **OK** to insert the scale bar.



**Note:** For scale bars, the start of the ROI line is also the start of the scale bar.



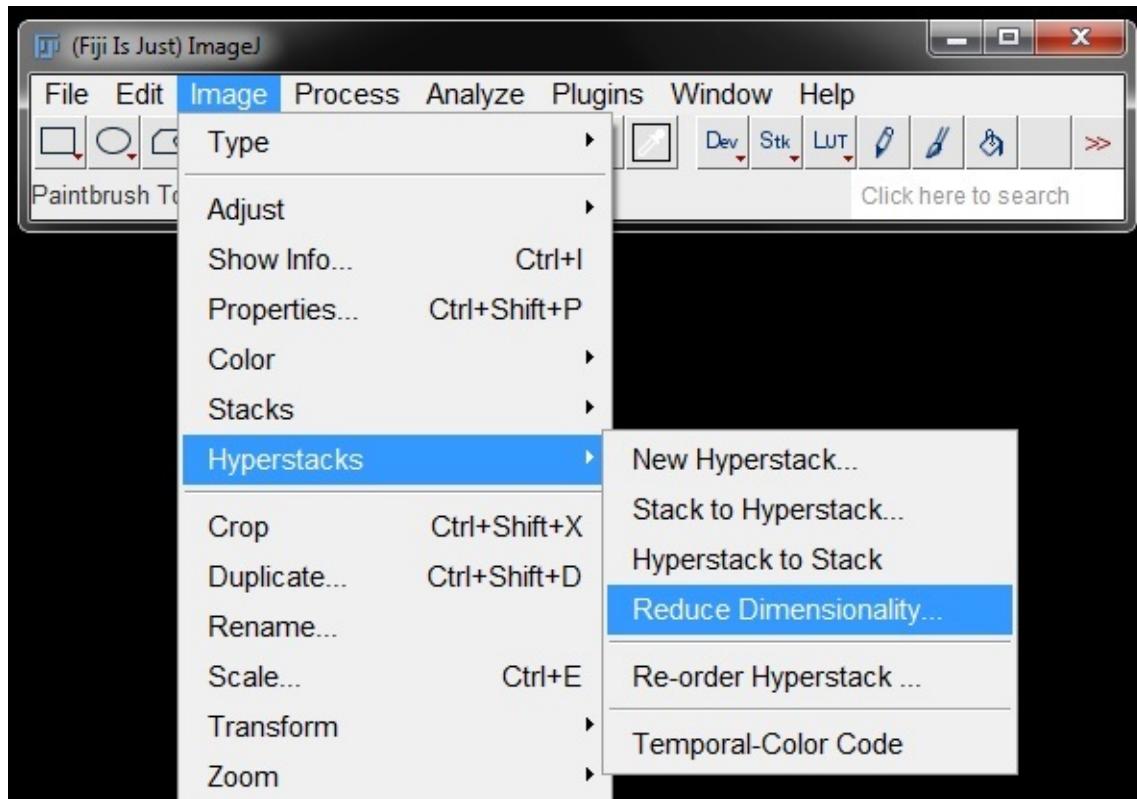
## Hyperstacks

Often images will be captured with a 4th or 5th dimension; that is they will have a combination of multiple channels, z-sections and time. For these images the data will be presented as a hyperstack.

The dimensions are presented within a single window, with each dimension represented individually and having its own slider. You can scroll through each dimension independently using these sliders.



Hyperstacks have their own specific tools found under **Image -> Hyperstack**. Most of these tools can be used to reduce the dimensionality in a specific way.

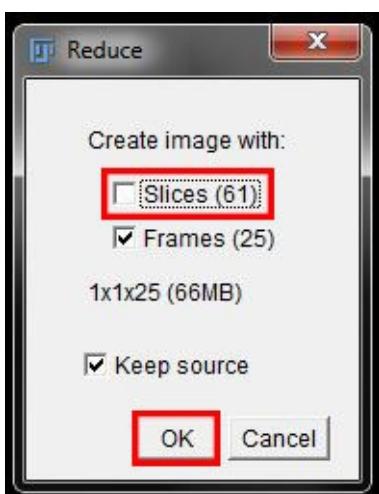


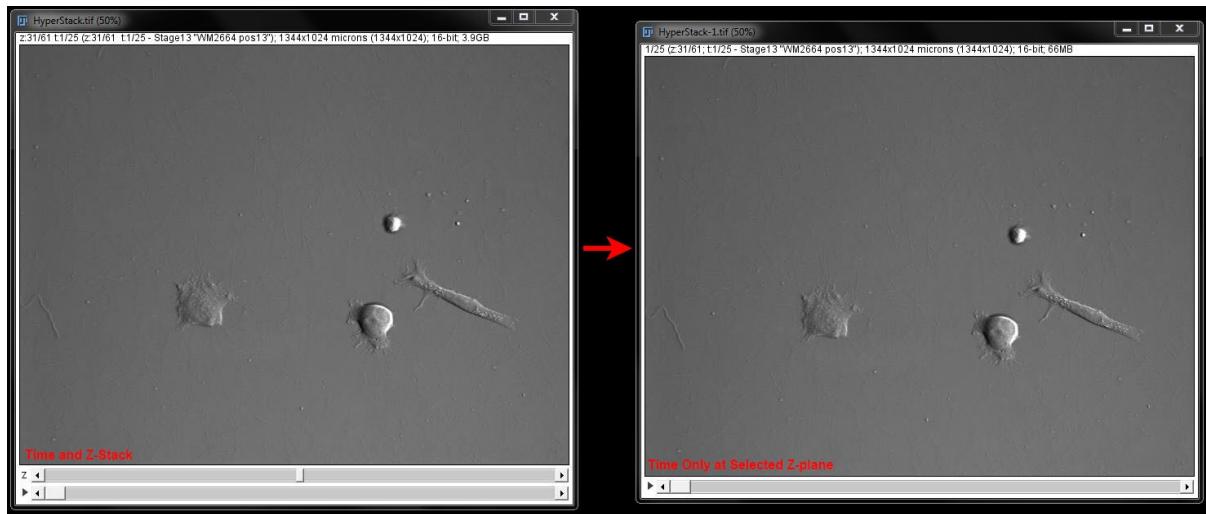
**Hyperstack to Stack** will combine all dimensions into a single stack with c\_z\_t frames (ie; here 1 colour \* 61 z-planes \* 25 time points = 1525 frames).



**Reduce Dimensionality** will allow you to pick one dimension to remove or keep in the hyperstack.

For example you can select time only by un-checking the box beside 61 slices, and a single stack of all time points at the current z-plane will be generated.





In a hyperstack some of the tools demonstrated in FIJI Basics for single dimension stacks can also be applied. And all can be used on a hyperstack following reduction in dimensions as shown here.

## PART 3: MASKS AND FILTERS

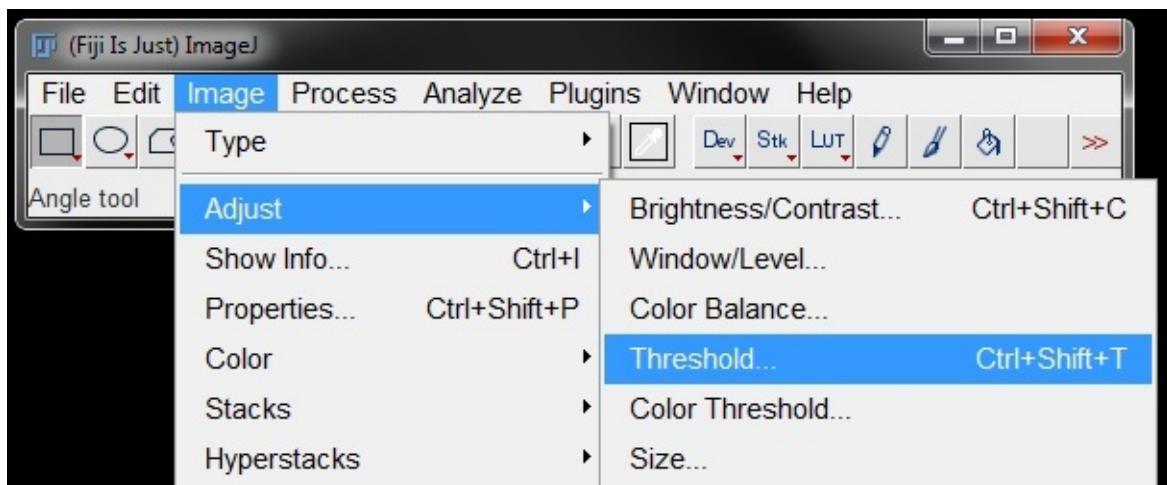
Creating thresholds and masks and applying filters forms the basis for a number of analysis methods. This section outlines the various options for masks and filters and how to apply them to your images prior to analysis.

The image *Nuclei-1.tif* and the image set '*RGBstackProjection*, *RGB-blue*, *RGB-green* and *RGB-red*' are used for demonstration purposes in this section.

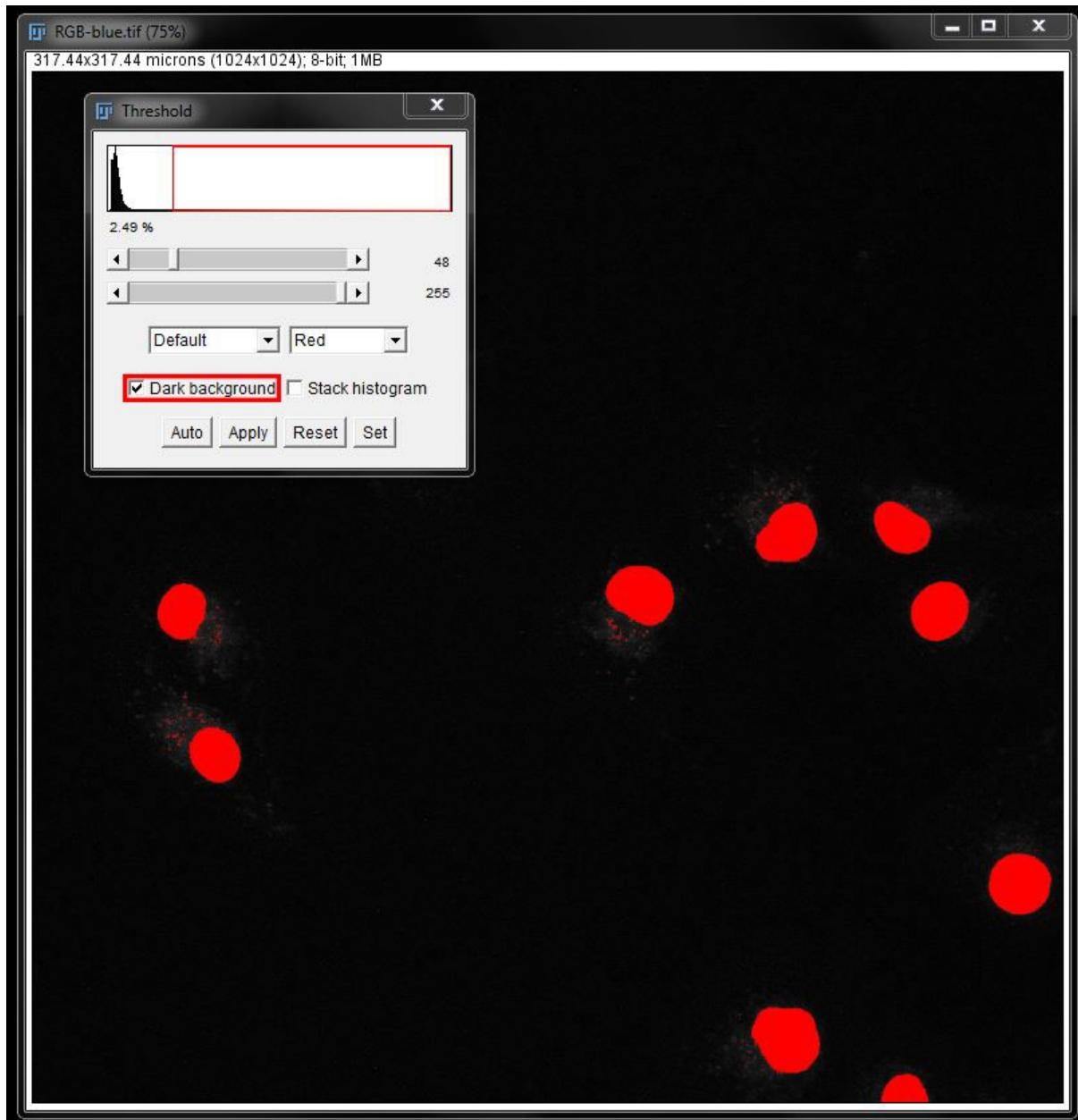
### Threshold Image

For a number of basic measurements an image first needs to have a threshold applied to it.

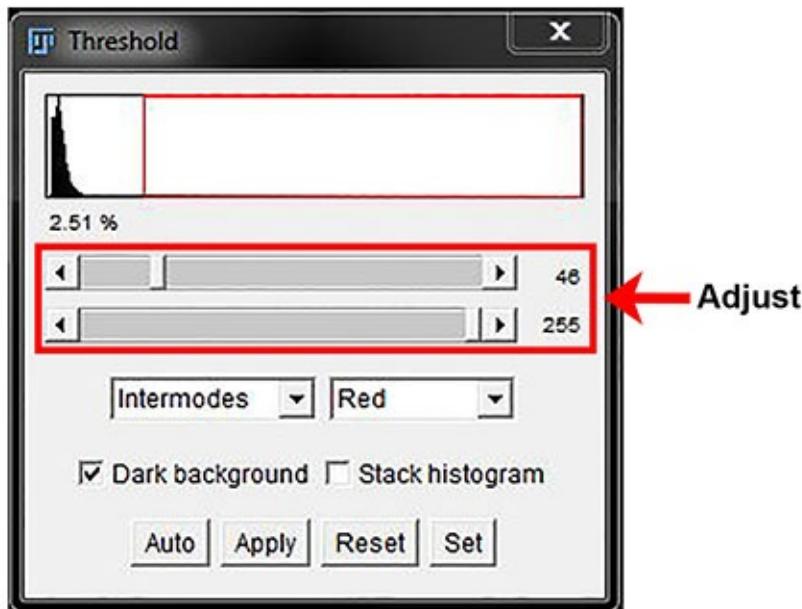
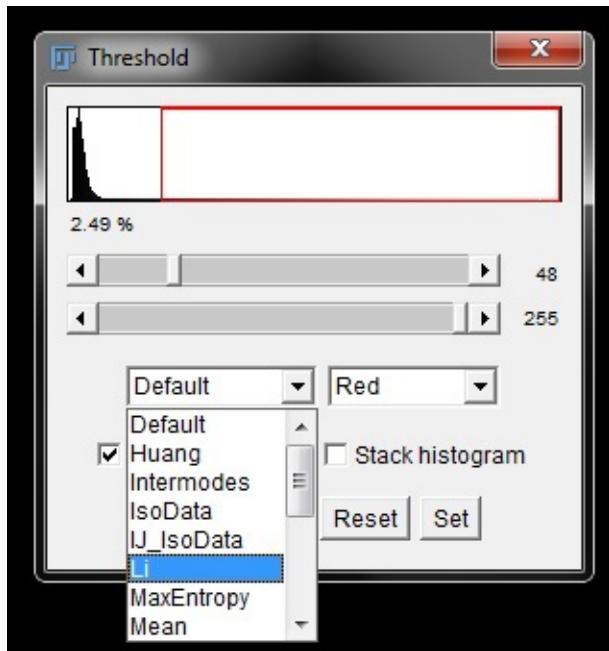
To apply a threshold, select your image and go to **Image > Adjust > Threshold** (or shortcut **Ctrl+Shift+T**).



A red overlay will be placed on the parts of the image that are selected by the threshold and a window will open where you can adjust the threshold on the image. In the window that opens, ensure the box beside **Dark background** is checked for fluorescence images.



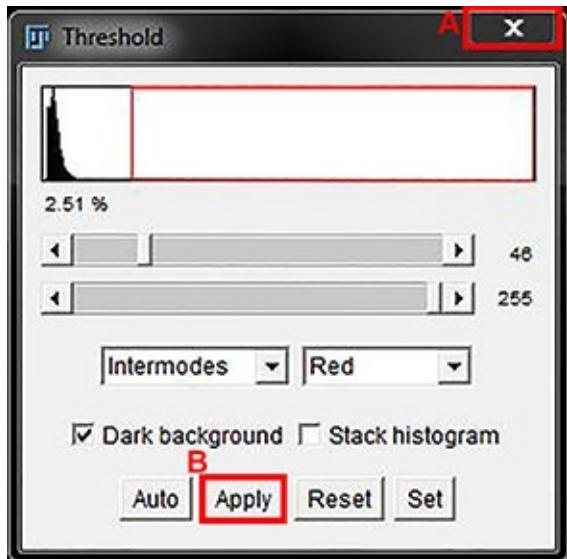
The drop down box on the left (the one that says 'Default' in the image above) has 16 different auto threshold algorithms to choose from. Choose the threshold that looks like the best fit for your data then further adjust by moving the sliders above. The top slider sets the bottom range of the threshold and the bottom slider adjusts the top range of the threshold. The red box on the histogram shows which parts of the histogram are being thresholded.



Rather than manually adjust, you can also select your algorithm and use the **Auto** button to allow the program to adjust the threshold levels for you.

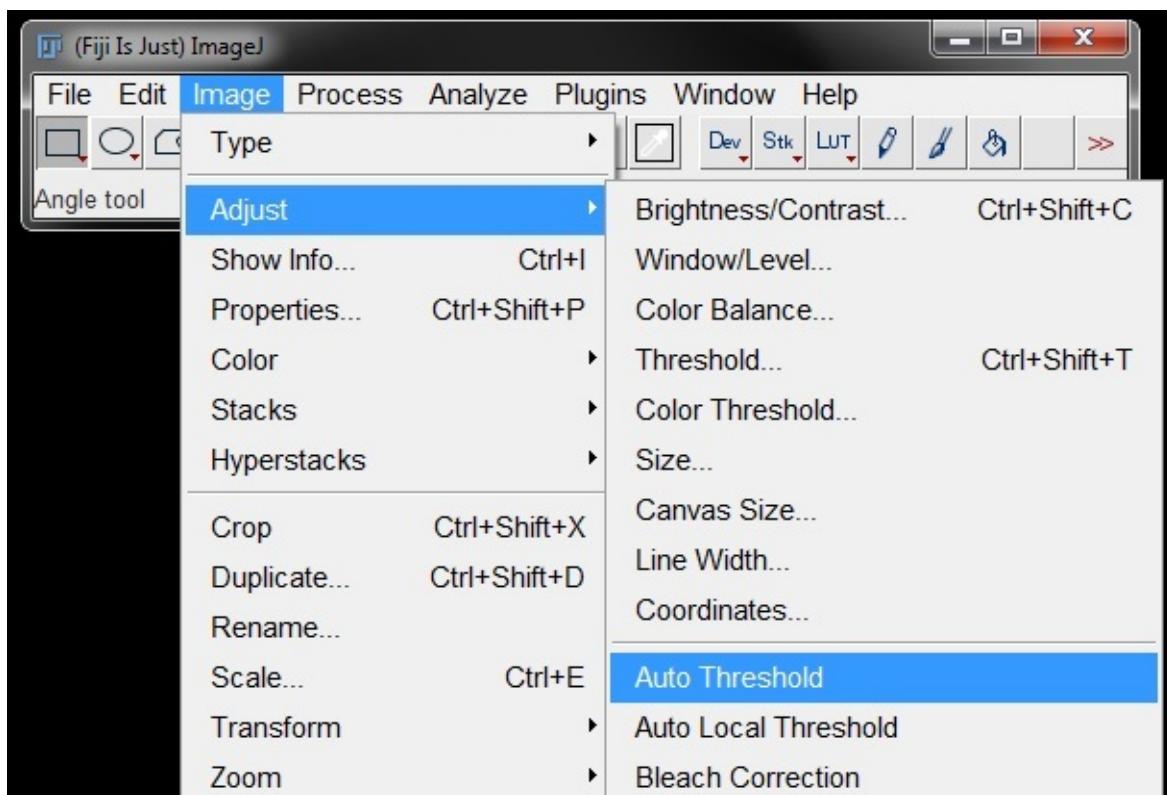
Reset will remove any overlayed threshold and allow you to begin again.

When you are happy with the selection (ie: the red covers the parts of the image you want selected well, without any additional background), close the threshold box to keep it as a threshold only (A in the image below), or click **Apply** (B in the image below) then close the threshold box to convert it to a mask. (**Note:** there will be more details on masks and binaries in the next sub-section).

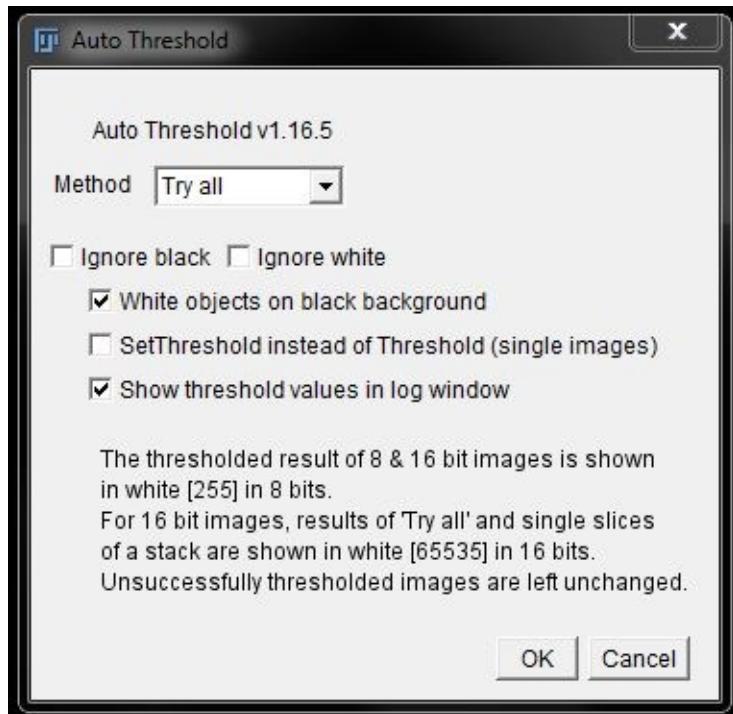


You can also automatically test the range of threshold algorithms on your image without scrolling through the dropdown list one at a time.

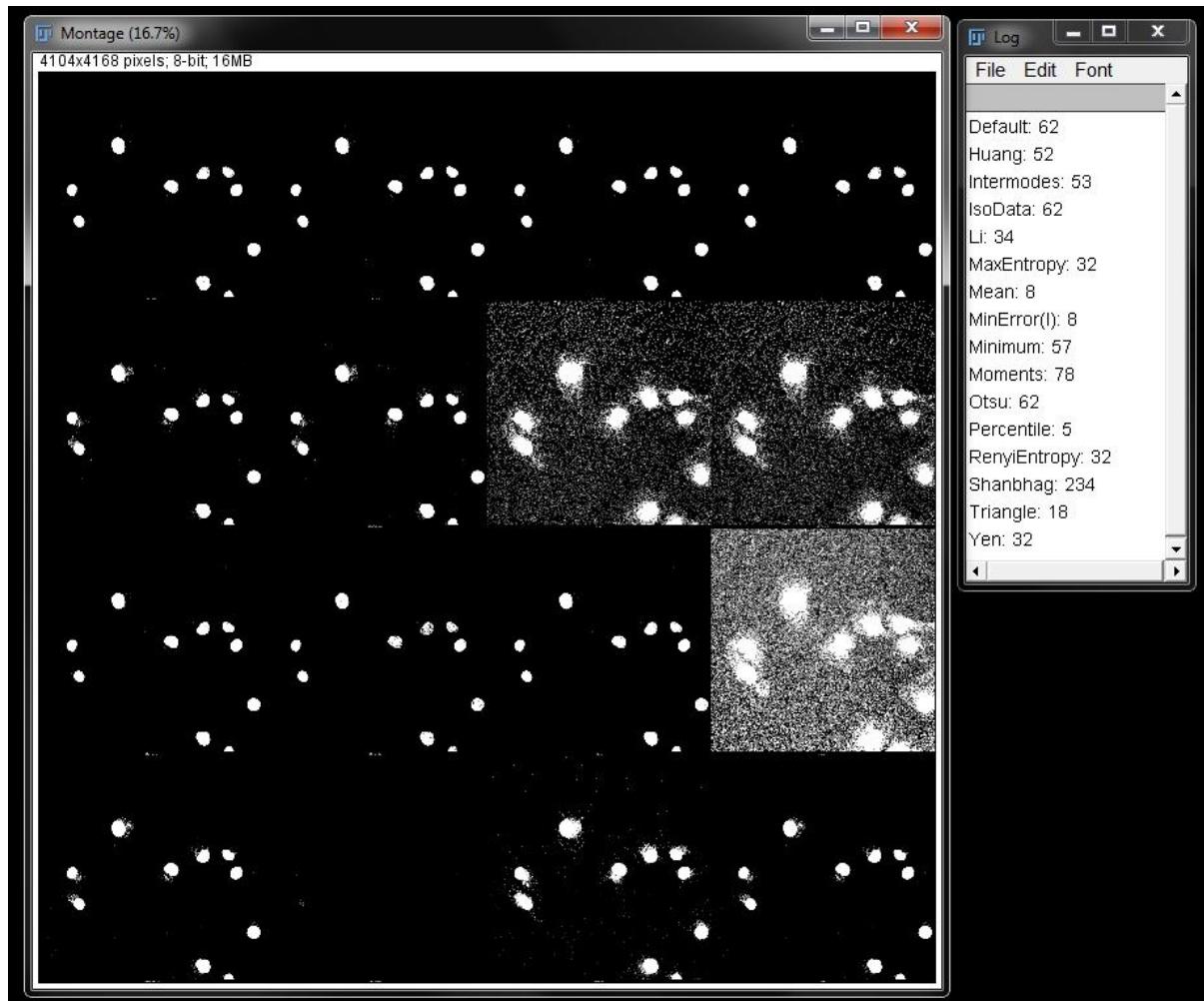
To use the auto-test for thresholds, go to **Image -> Adjust -> Auto Threshold**.



In the dialog box that opens up set the **Method to Try All**, make sure the **White objects on black background** (for fluorescence images) and **Show threshold values in log window** boxes are ticked. Press **OK**.



A montage will be generated showing what each auto threshold algorithm would produce on the image. The list in the log that was created will show the order of the algorithms used in the montage.

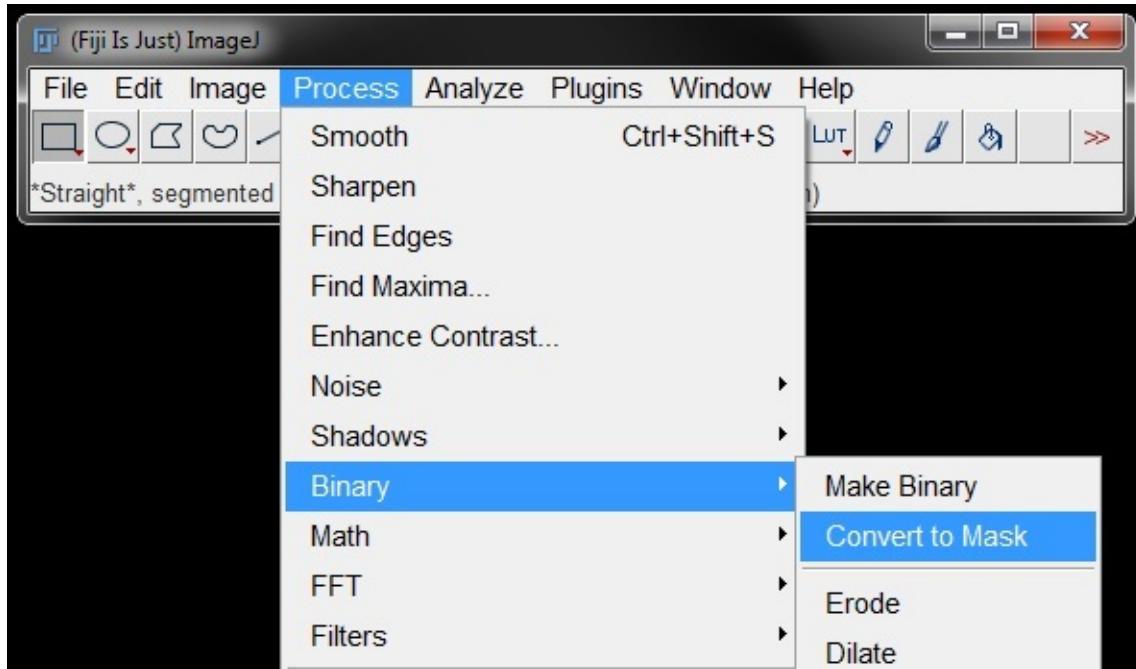


From this montage you can decide which algorithm may suit your data the best and apply it using the steps above.

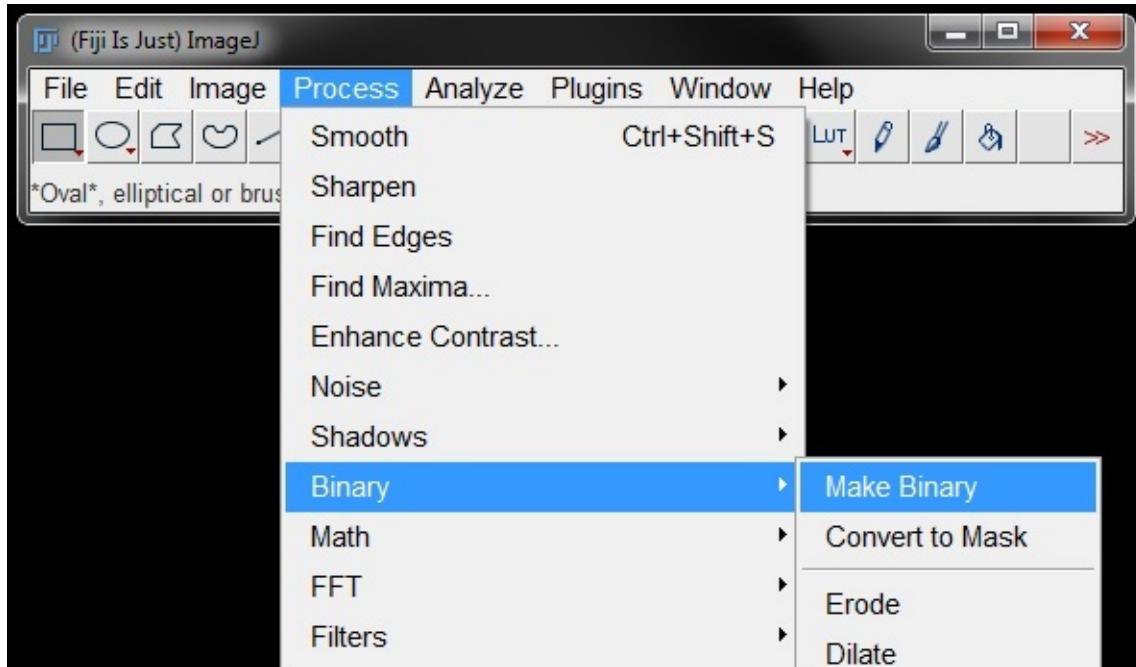
## Creating a Mask

If you need to convert your threshold to a mask, or binary, there are several options you can use. As shown above, if you select **Apply** in the threshold window it will convert the threshold to a mask.

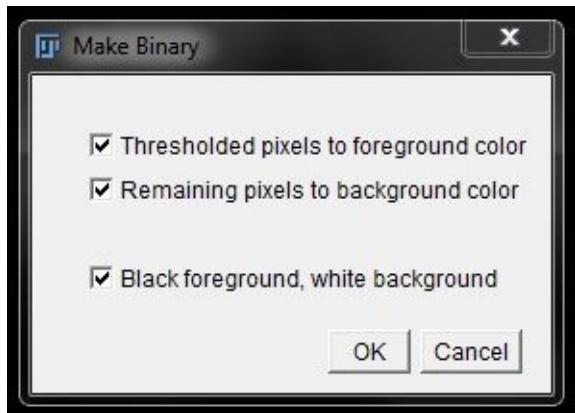
You can also go to **Process -> Binary -> Convert to Mask**.



To generate a binary go to **Process -> Binary -> Make Binary**.



For this option, a window will open with binary options, select your options and click **OK** to generate the binary image.

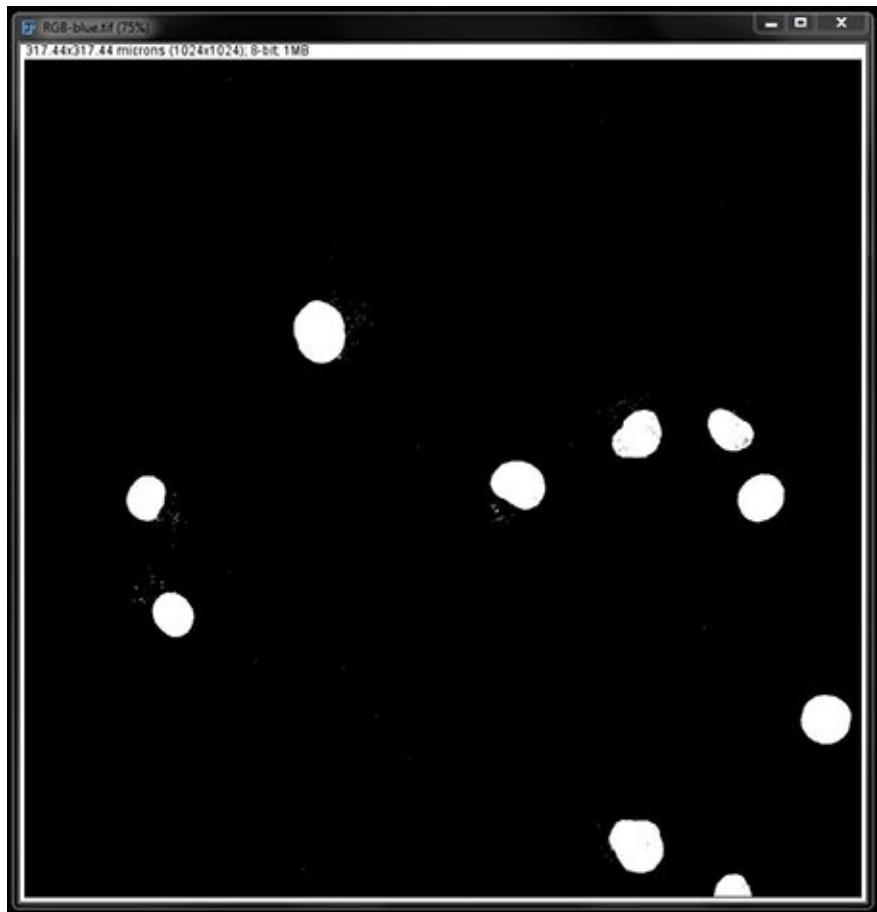


Generally masks and binaries will appear as shown in the images below. However, depending on your settings (both the general settings in FIJI, and those selected from the binary options above), the background and foreground colours can sometimes be reversed in the mask and binary. Macs will often produce reversed background and foreground colours also.

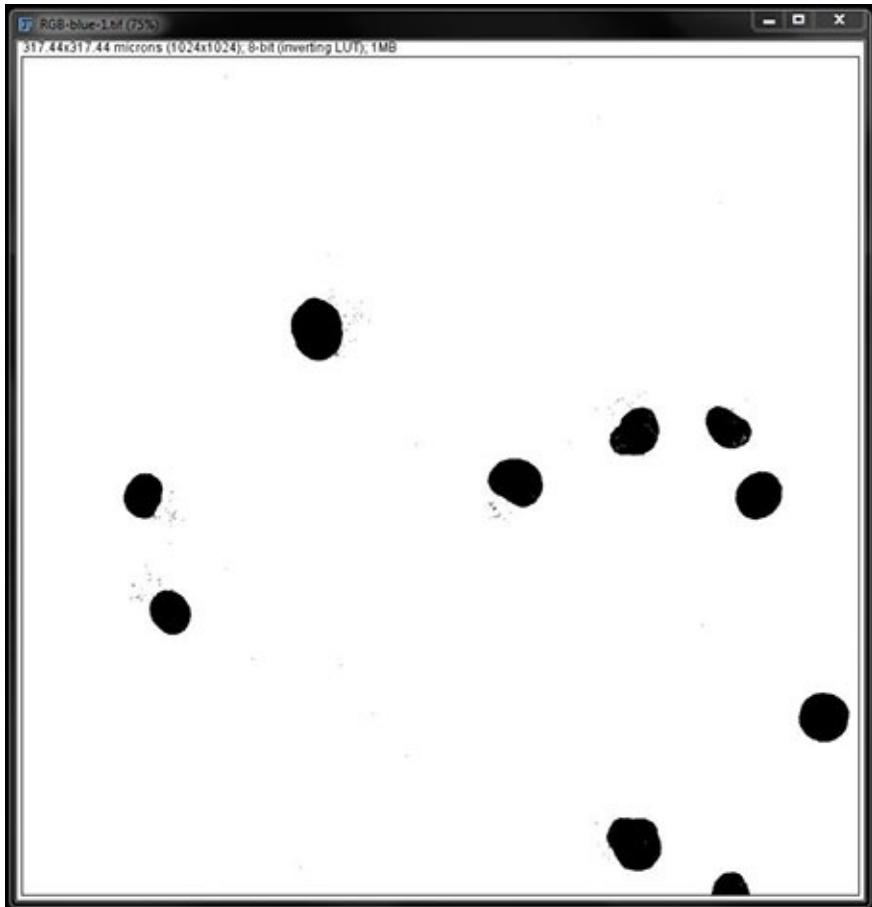
### Original Image:



### Mask:

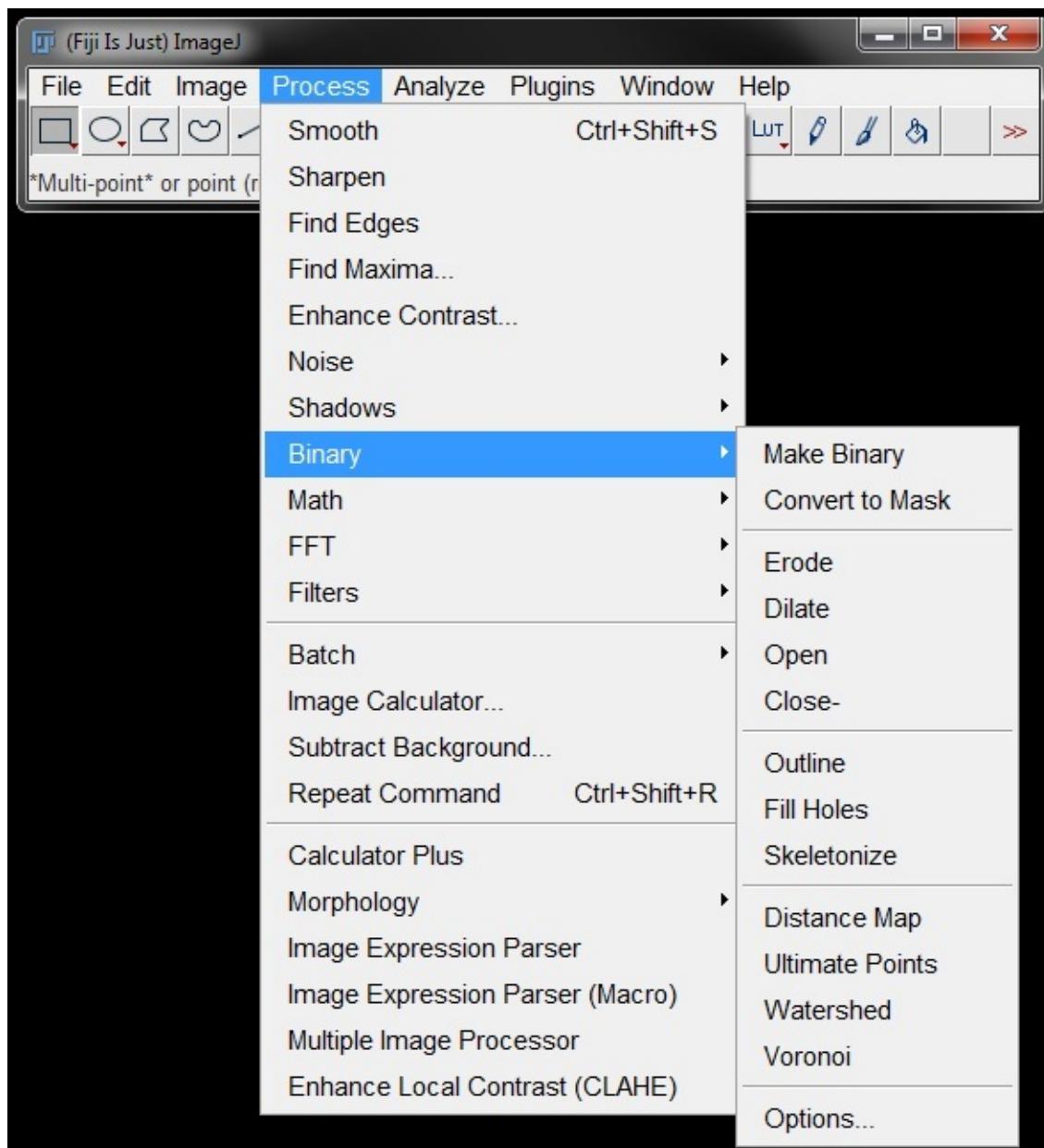


**Binary:**

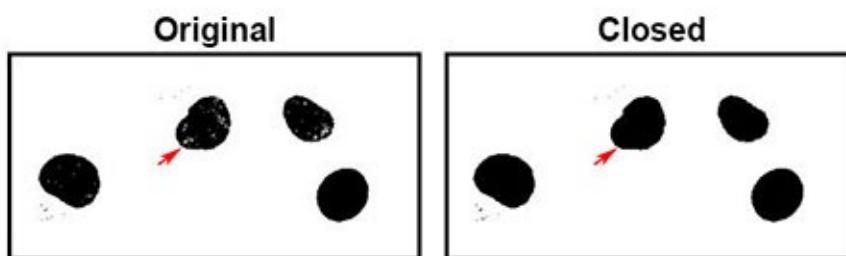


## Making Adjustments to Masks and Binaries

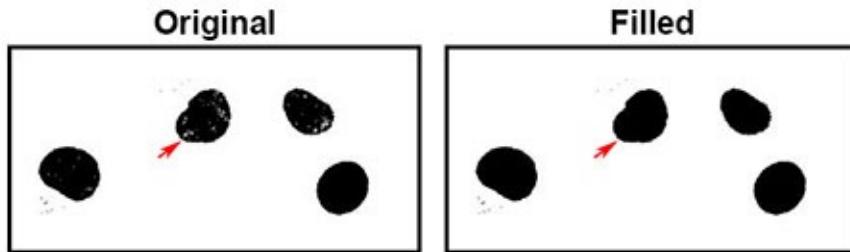
Sometimes adjustments need to be made to binaries/masks before analysis to ensure it is accurately representing the data. There are a number of tools available to help you get the best fit to your data. All of these tools can be found under **Process -> Binary**.



Small gaps left in the mask or binary after thresholding can easily be corrected for by using the **Close** tool.

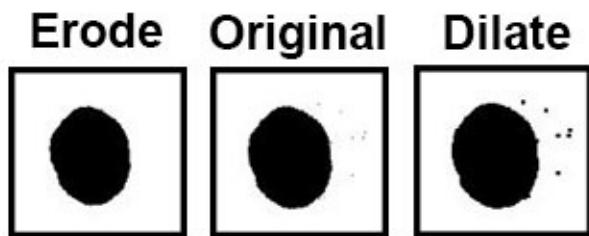


Similar to this is **Fill Holes**.

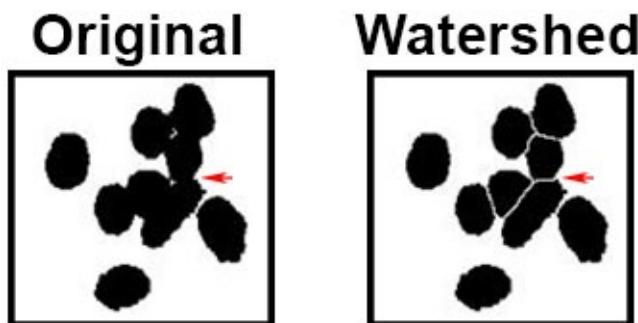


Sometimes you will need to add or remove a small layer from the edges of the mask/binary. This could be useful for things like membrane measurements or morphology analysis.

To add or remove from the edges of a mask or binary you can use the **Dilate** or **Erode** tools. **Dilate** will add a thin layer (1 pixel) around the edge of the existing mask/binary. **Erode** will remove a thin layer (1 pixel) from around the edge of the mask/binary.



Sometimes separate objects in your image will be touching. When you create a mask/binary of these they will appear as a single object. This can be a problem for some types of analysis, such as counting. But you can use the **Watershed** tool to detect and separate these objects so they can be counted as individual objects again.



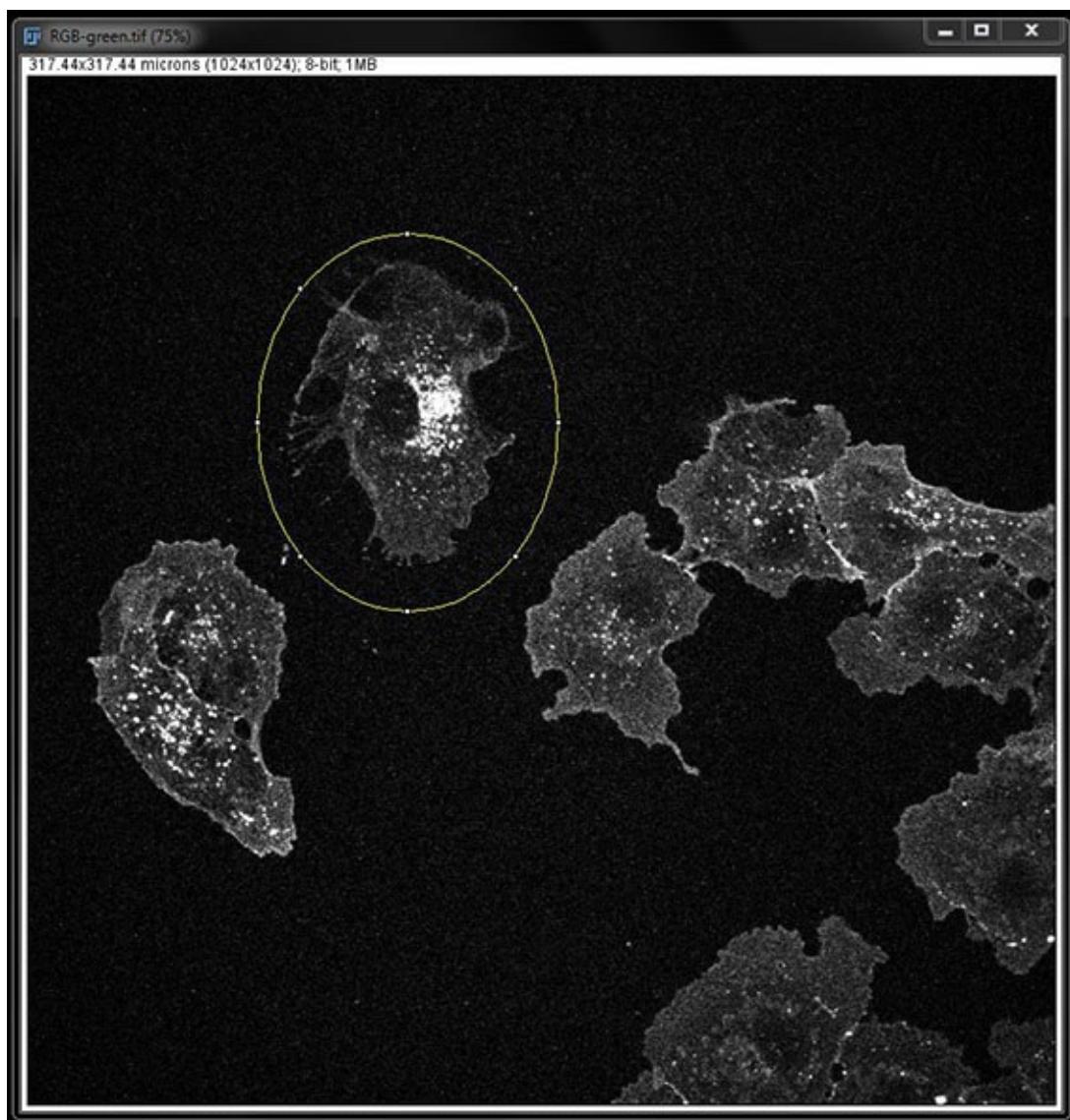
**Note:** It is important to remember that all of these adjustments will always work on the "black" part of the image so keep this in mind if your colours are reversed.

## Clear Inside/Outside

Clear inside and outside can also be useful for adjusting your masks or binaries before making measurements. Sometimes there can be small blemishes in your image that effect your measurements. Or you may want to focus your measurement on a single area of the image. You can crop the image to remove items before analysis, or you can use the clear functions.

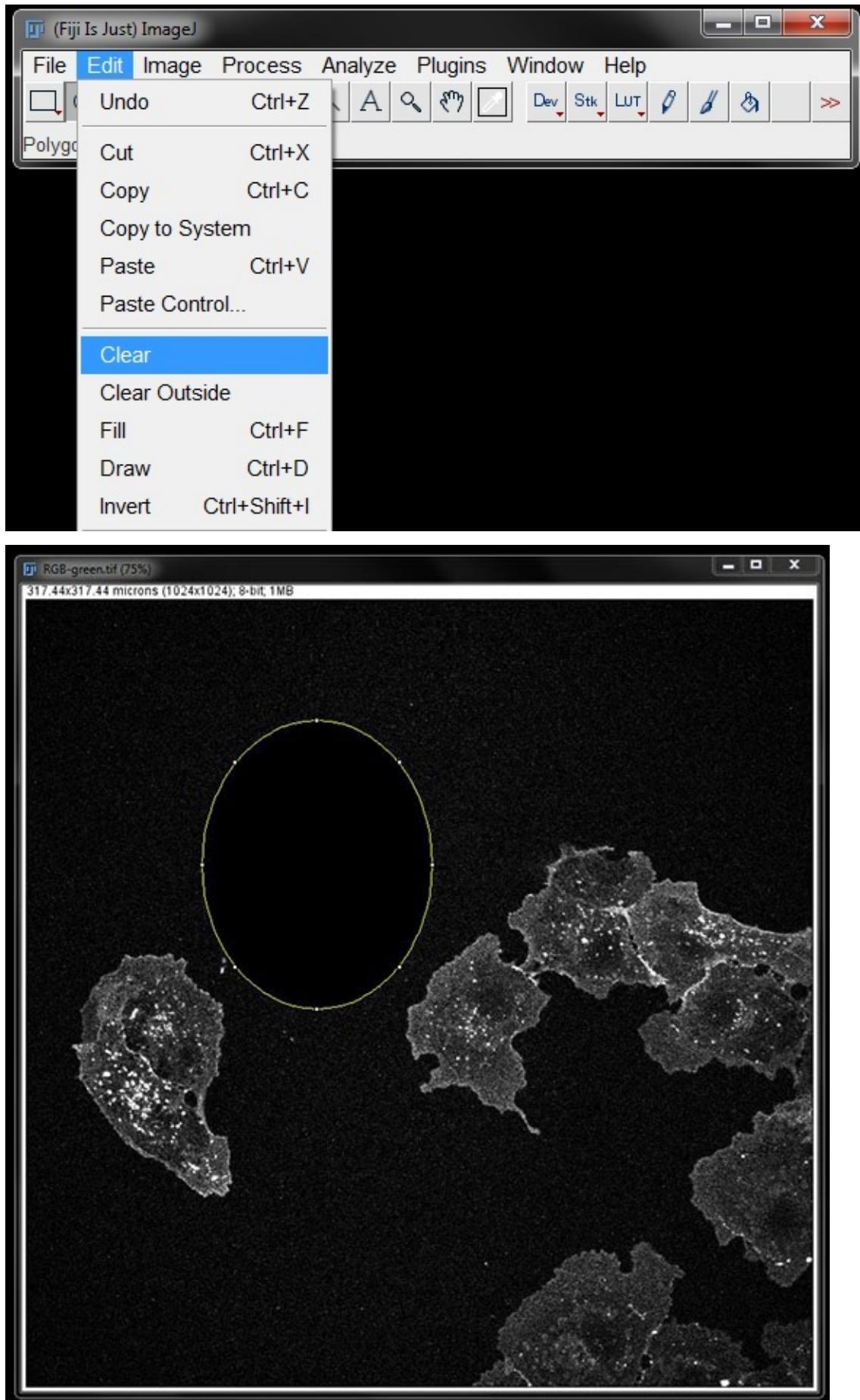
**Note:** We strongly advise against removing objects from images before analysis and publishing of results. **Removal of any image information that can alter the outcome can be considered image manipulation.** Use these functions with care and caution and always on a duplicated image so that the original remains unaltered.

To use the clear functions you first need to draw an ROI around the area you wish to clear, or keep.

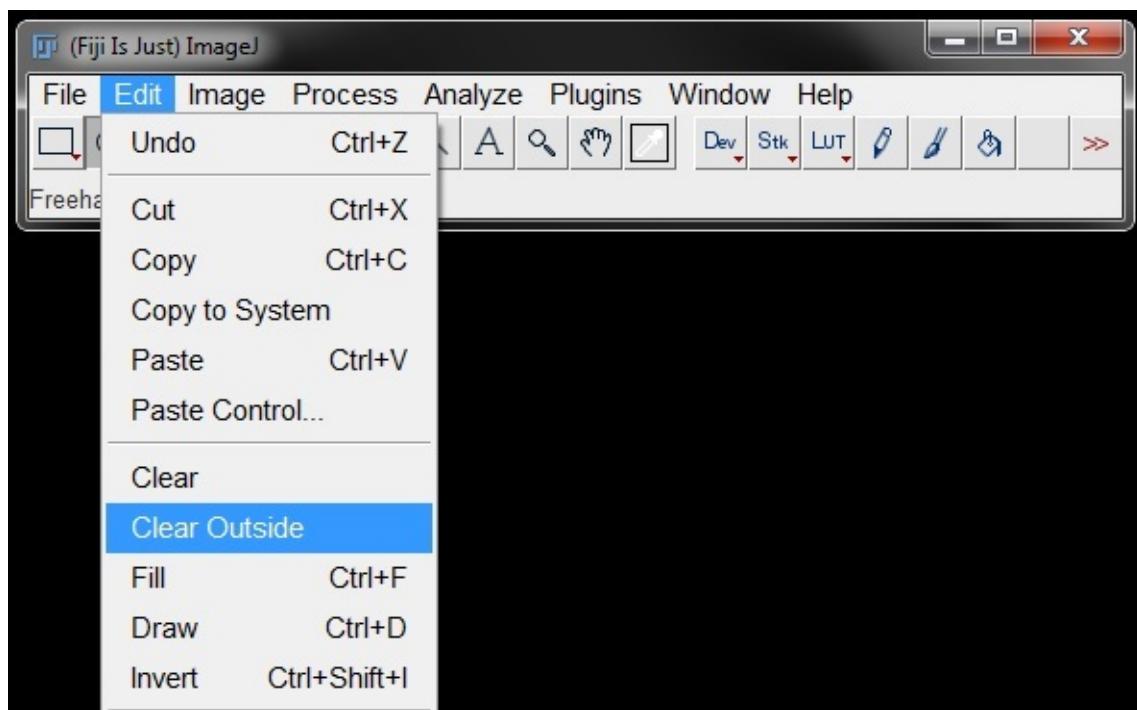


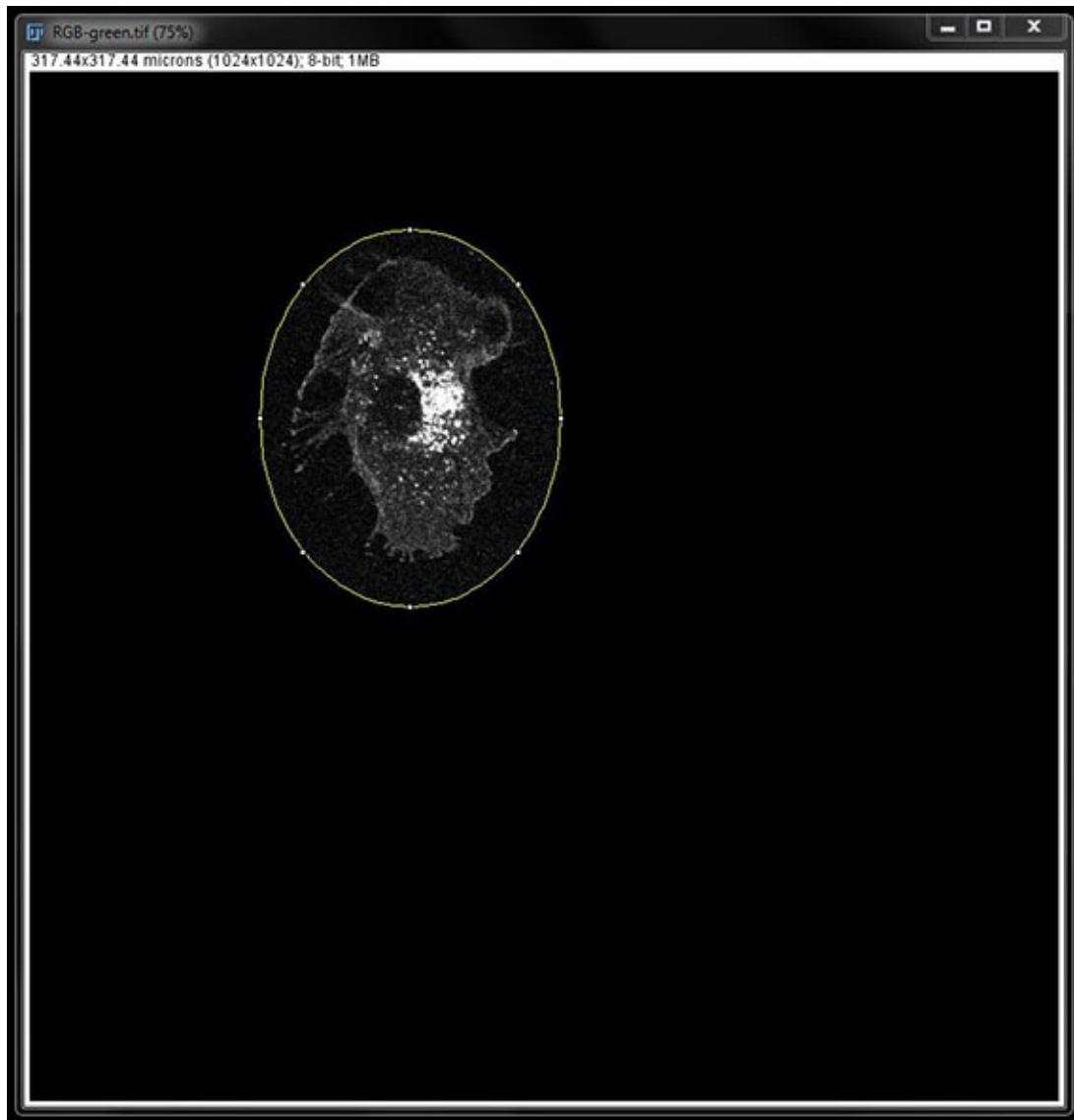
**Note:** In this example we are using cells for emphasis, but clear functions are usually applied to binaries/masks only to remove blemishes or mistakes from thresholding prior to analysis and we do not recommend removing cells from images in this manner.

Once you have your ROI, go to **Edit -> Clear** to clear the inside of the ROI.



Or use **Edit -> Clear Outside**, to clear the rest of the image outside the ROI.

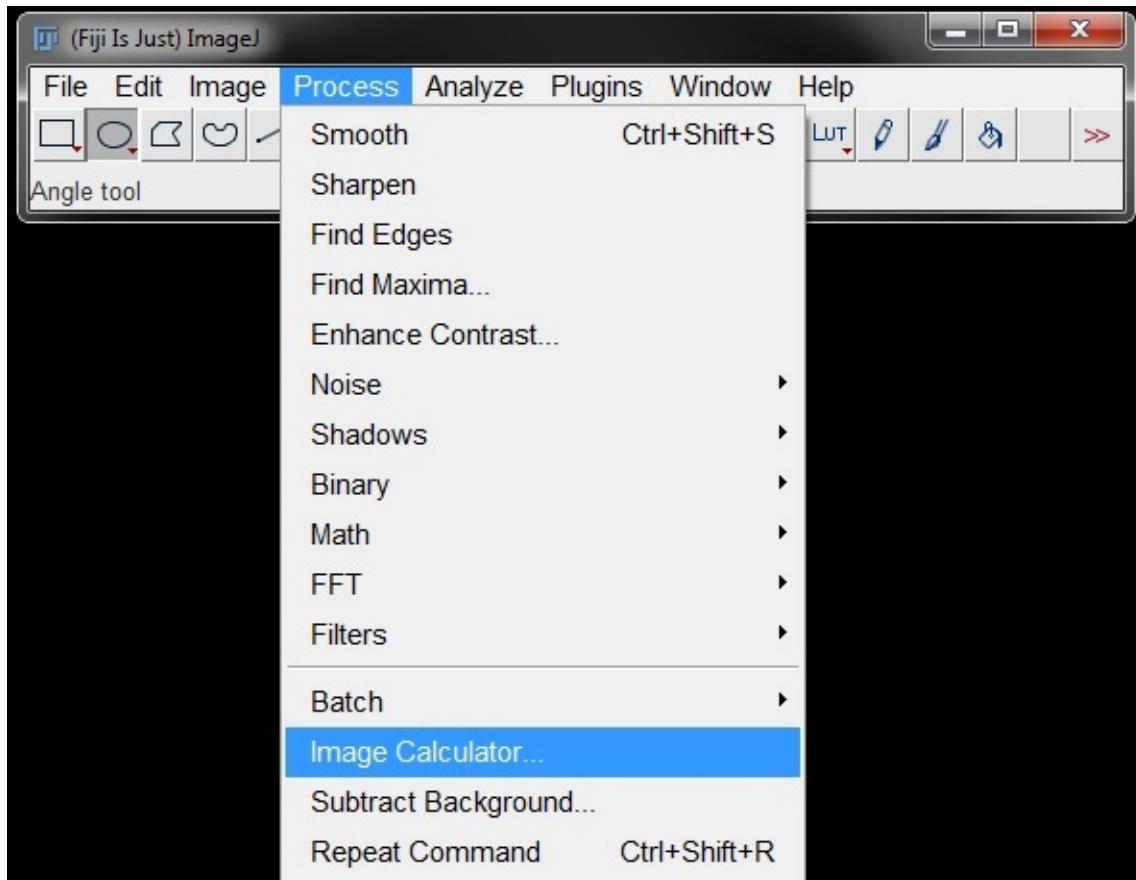




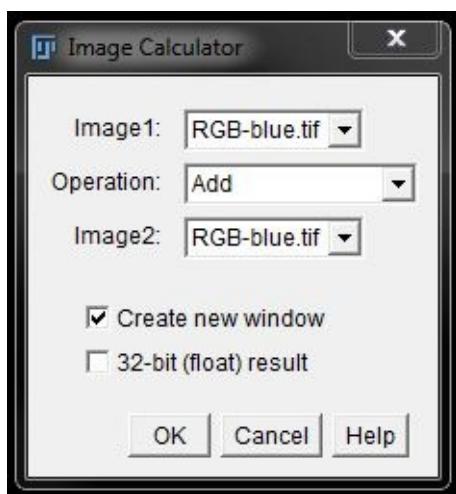
## Image Calculator

Image calculator is another way to select for specific areas where you want to apply your analysis. Image calculator performs arithmetic operations between two images. These can be your original images or stacks, an image/stack and a mask or two masks.

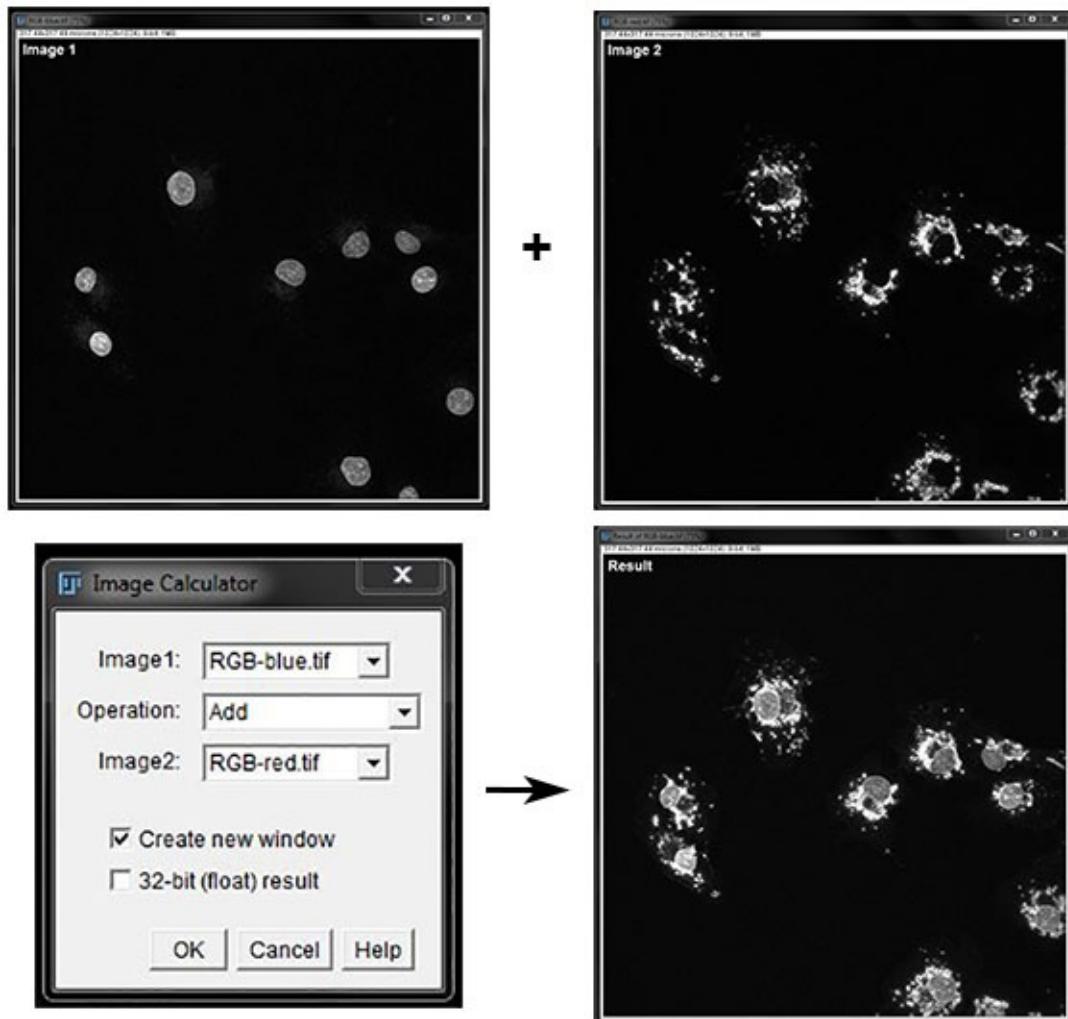
The **Image Calculator** can be found under **Process -> Image Calculator**.



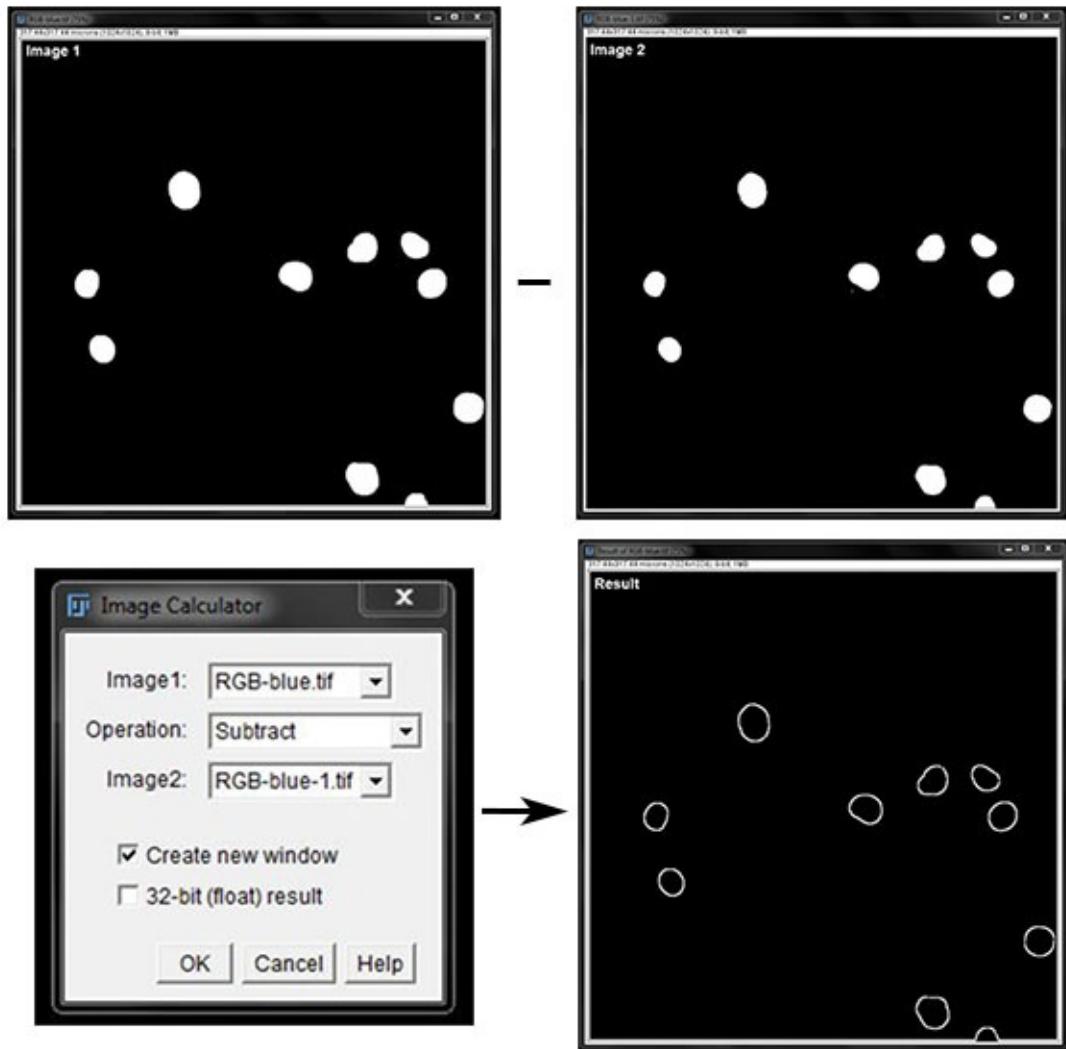
In the resulting window you can select image 1 and image 2 from the drop down menus and choose your operation from the list in the middle. Check the box next to **Create new window** to generate the calculation as a new image, otherwise it will override image 1.



In the image calculator **Add** creates a new image with the pixels from image 1 added to the pixels from image 2. This is demonstrated below, combining two images into a single image for measurements.



**Subtract** will remove the pixels in image 2 from image 1. This is demonstrated below with two masks of the same image. The mask in image 2 has been eroded and will be subtracted from the original to leave only the edge of the nuclei for analysis.



You can also use a mask to subtract an area of an image prior to analysis. For example we use the mask of the nucleus here to subtract pixels from the green channel so only cytoplasmic signal remains for analysis.

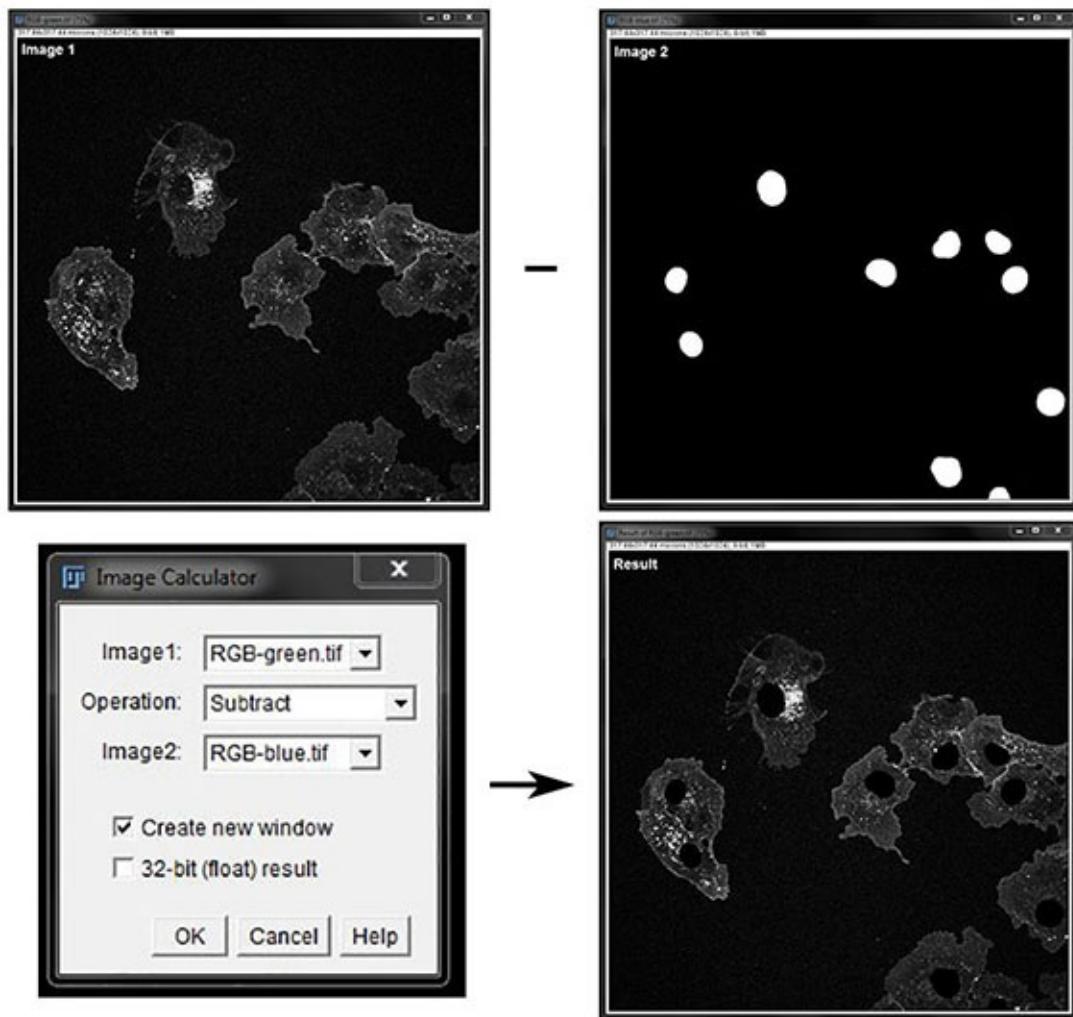
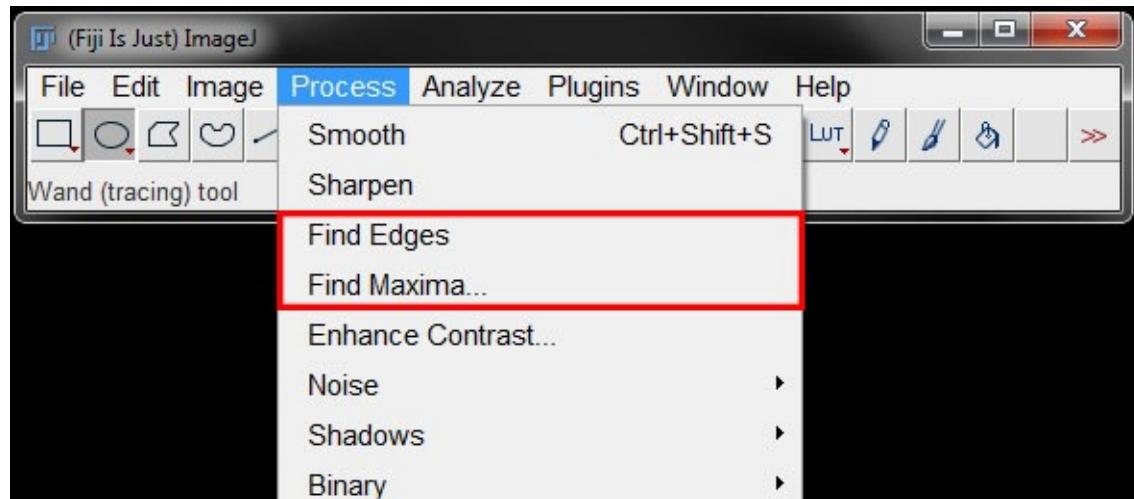


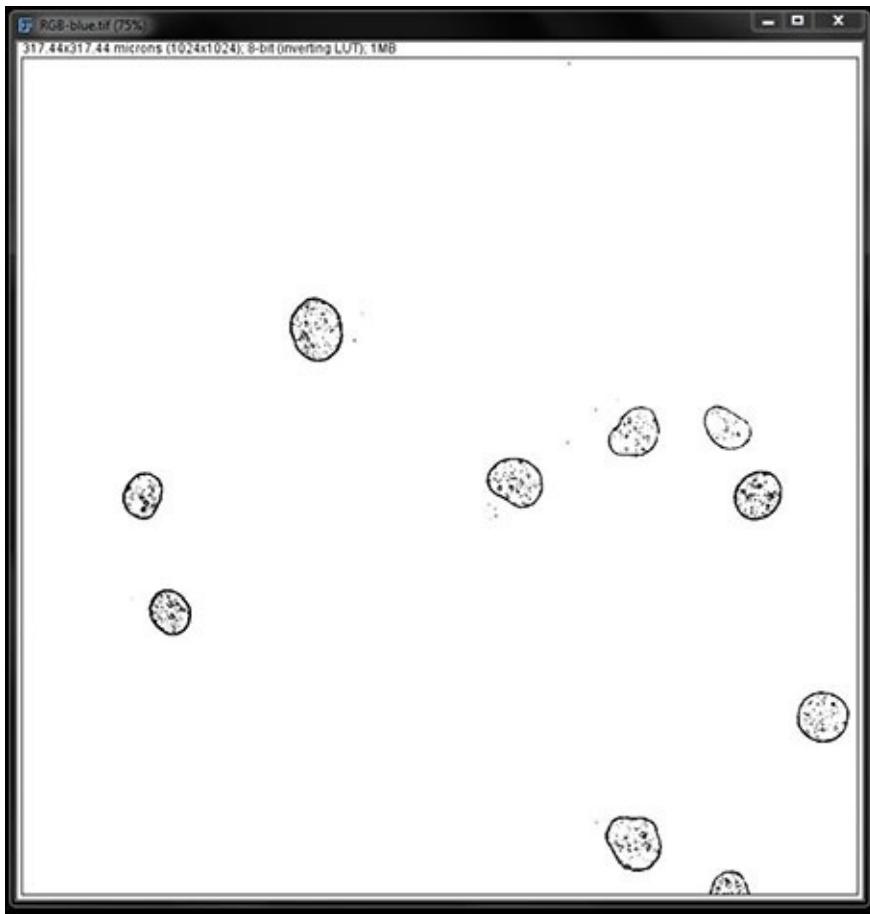
Image calculators can be useful in a number of different analysis techniques. Although not all examples can be shown here, they are worth taking some time to explore as a part of your image analysis.

## Find Edges and Find Maxima

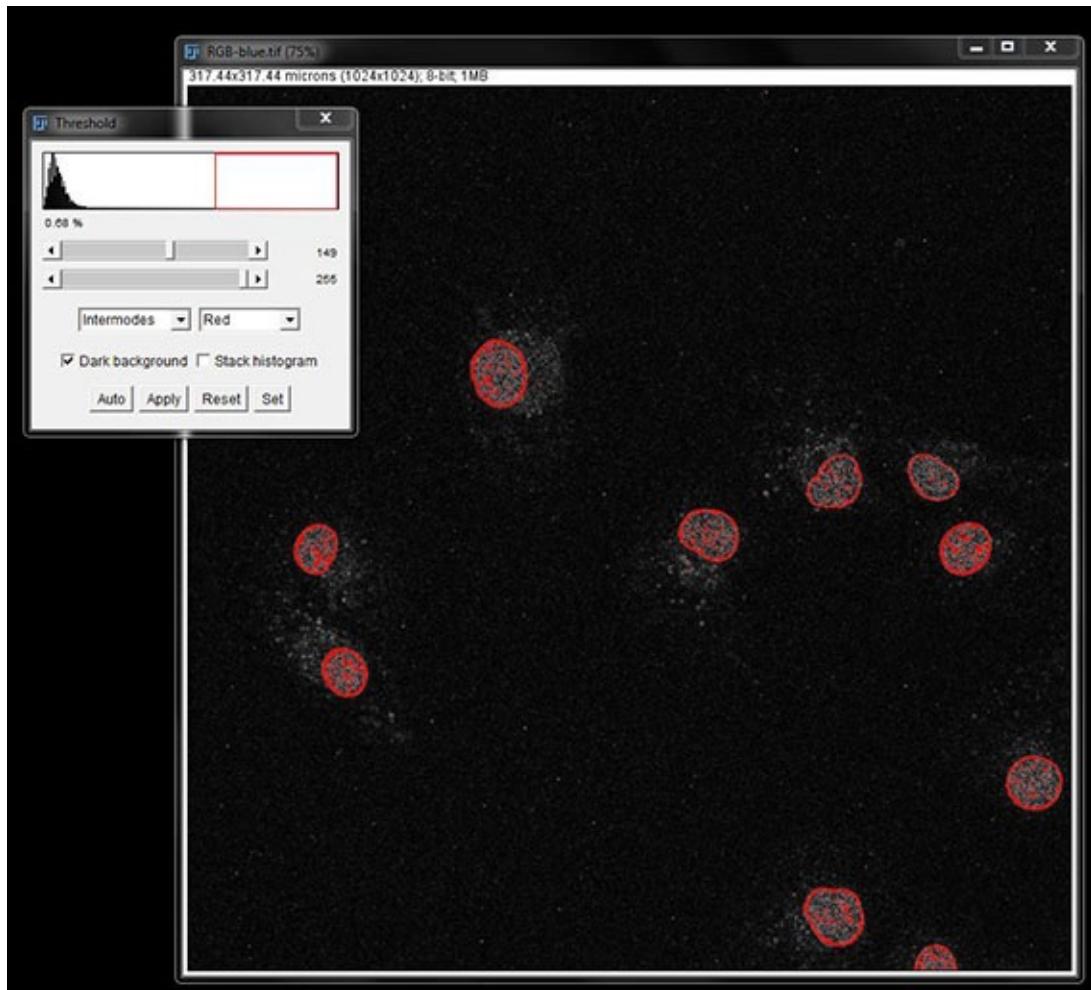
The **Find Edges** and **Find Maxima** tools can help you segment your images. Both of these tools can be found under the **Process** menu.



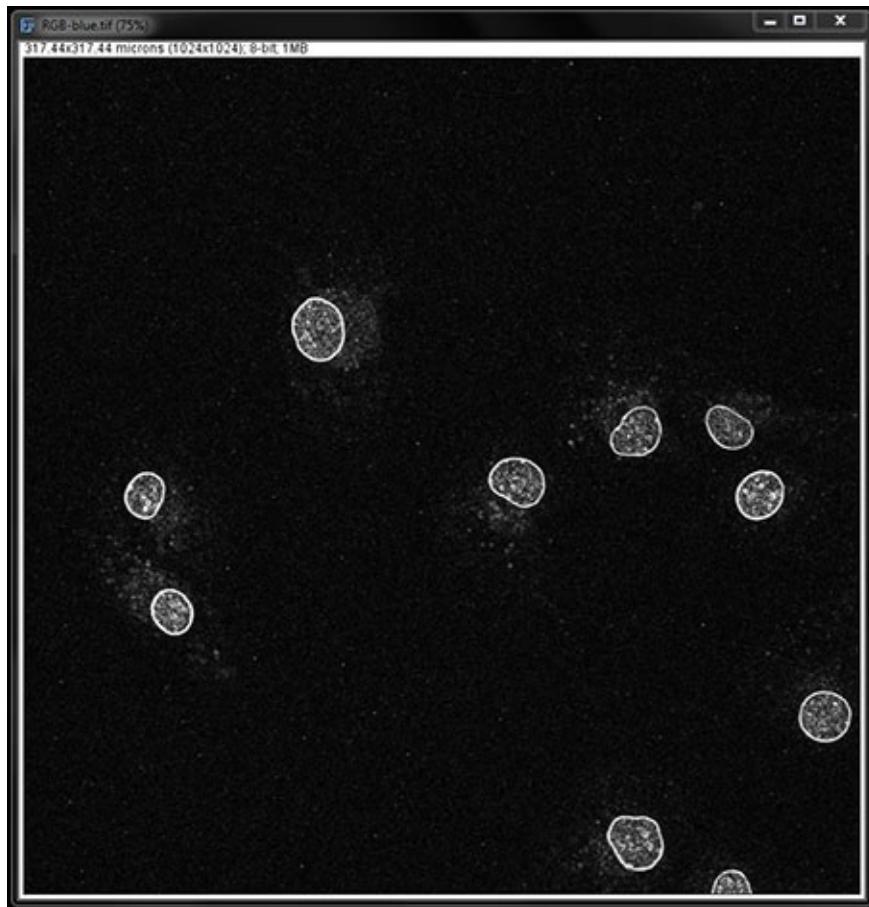
To segment the edges of your image, select the image and then select **Find Edges**. FIJI will ‘outline’ the edges of the signal in your image.



You can then threshold this image to select edges of the signal only.



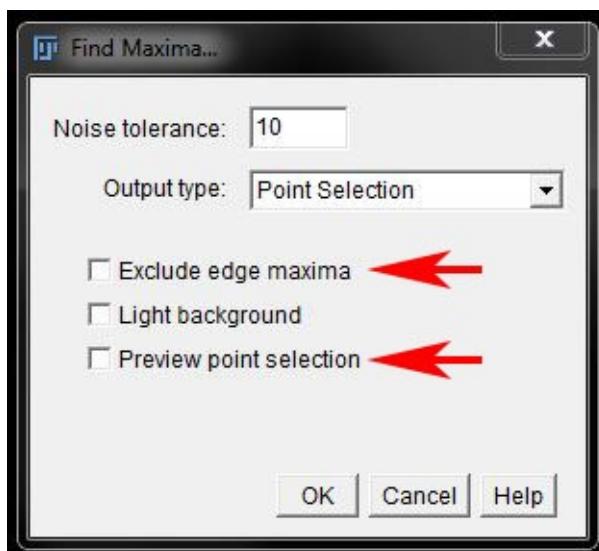
Once you are happy with the threshold, click **Apply** or create a mask or binary as previously shown.



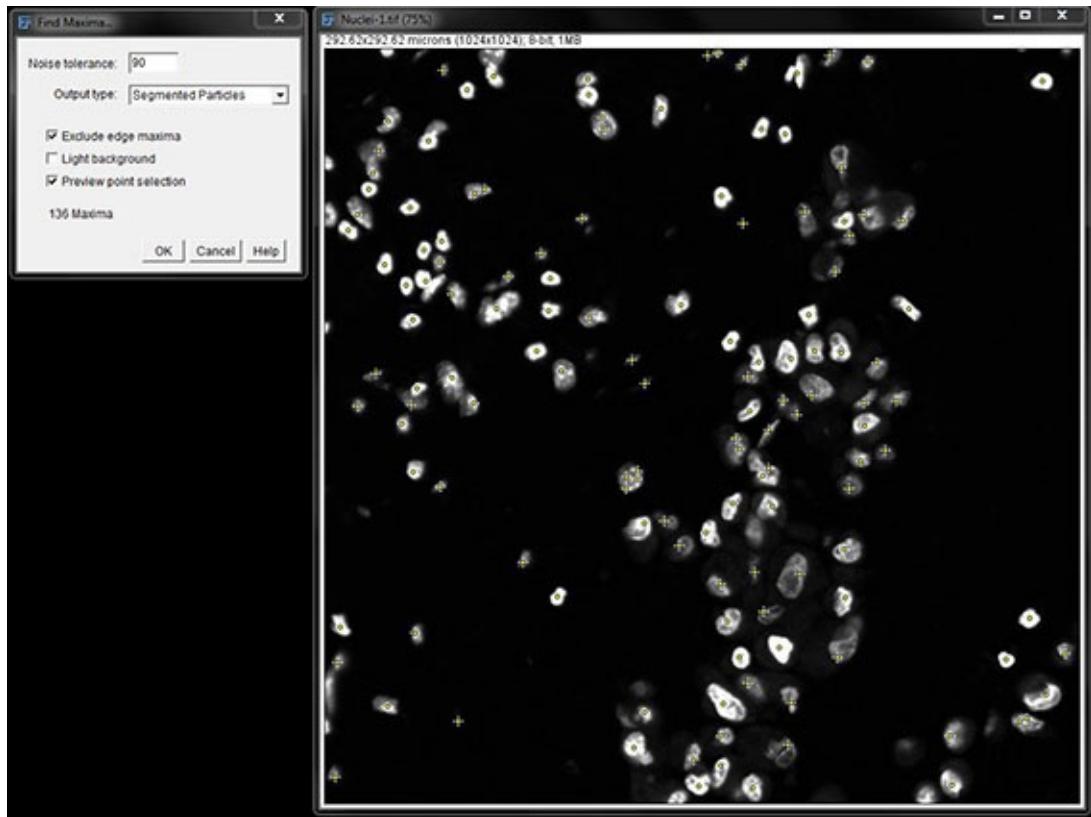
**Find Maxima** is particularly helpful for segmenting images of dense cell populations or objects.

Select your image and then go to **Find Maxima**.

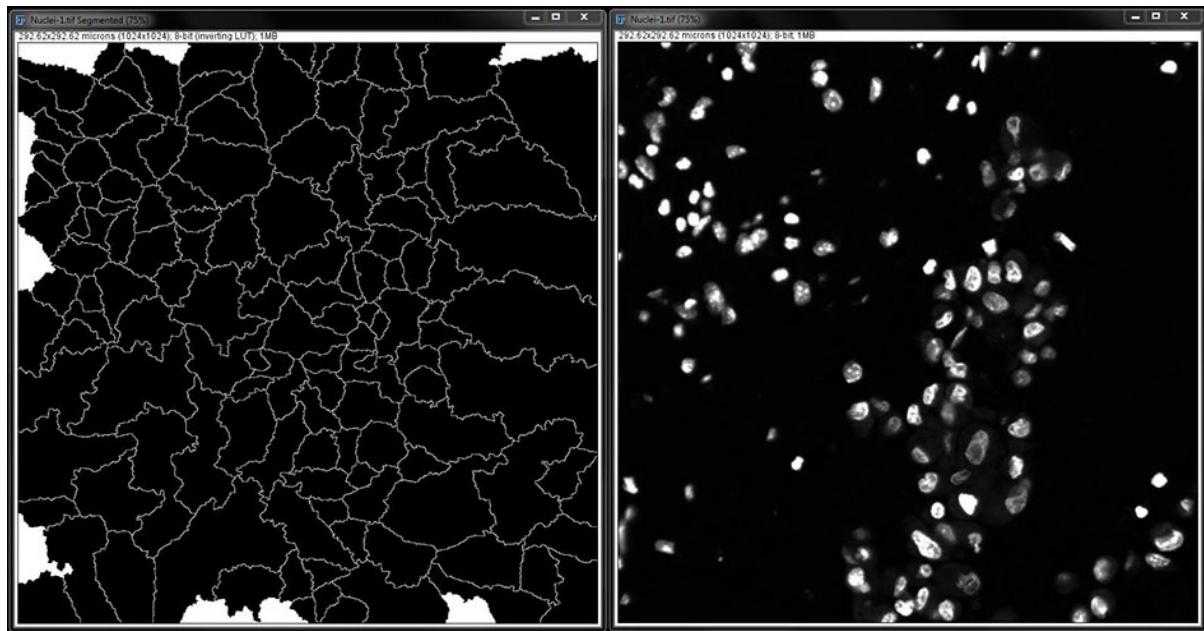
In the options window, select the box next to **Preview point selection** to see the default settings. These default settings will create a dense selection. Ensure **Exclude edge maxima** is selected.



Increase the **Noise tolerance** to reduce the point selection range on the image. For segmentation, 1 point per cell is ideal. Ensure that **Preview point selection** is still ticked on, then adjust the Noise tolerance to best fit your data. For segmentation, select **Segmented Particles** from the drop down list under **Output type**.



When you are happy with your selection click **OK** to generate a segmented image mask.



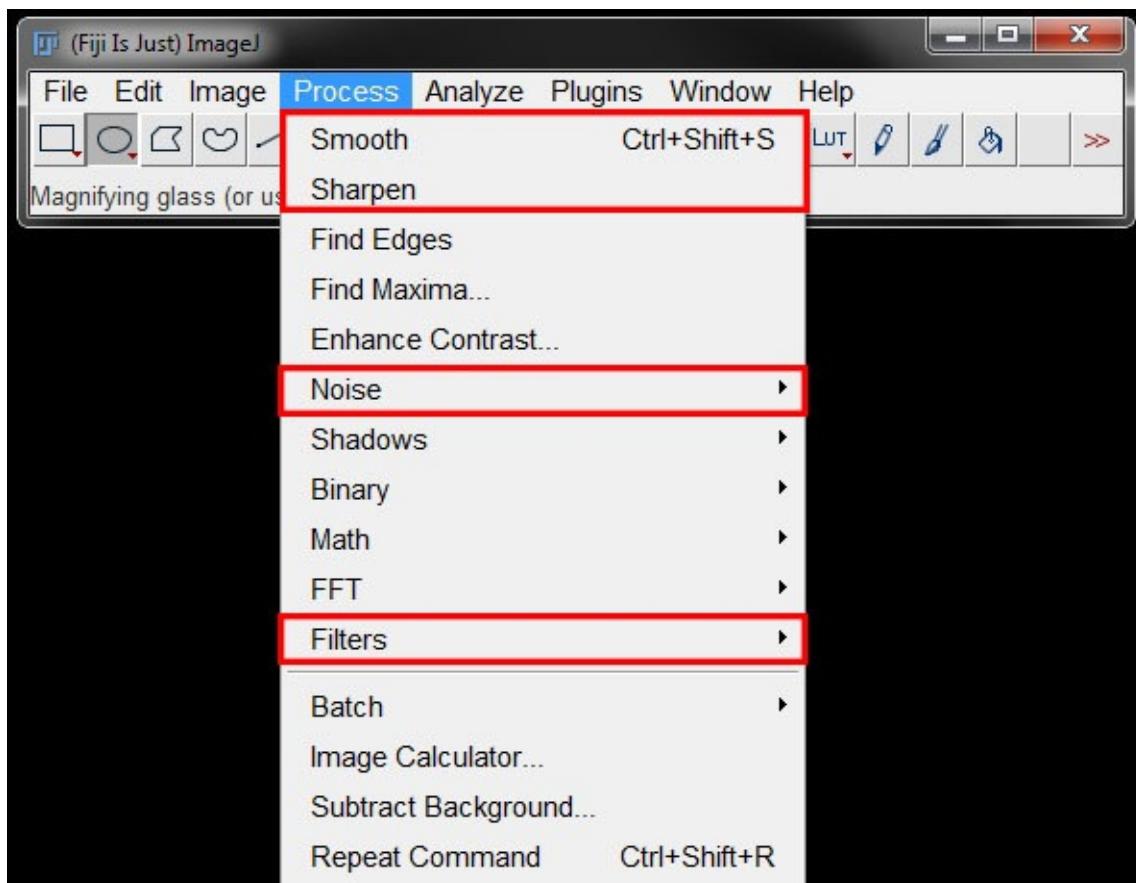
This mask can be used in combination with cell stains to segment and measure individual cells.

## Image Filters

Filters can be used to “smooth” your images before analysis. These are another helpful tool for removing blemishes or ensuring that your mask fits the data before analysis.

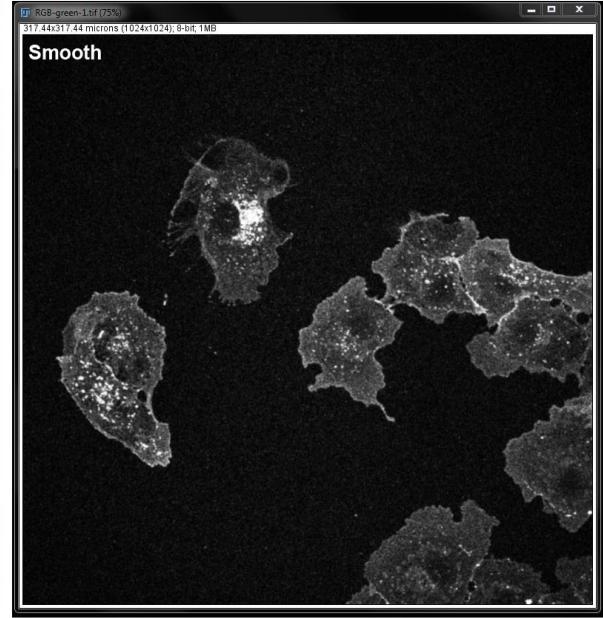
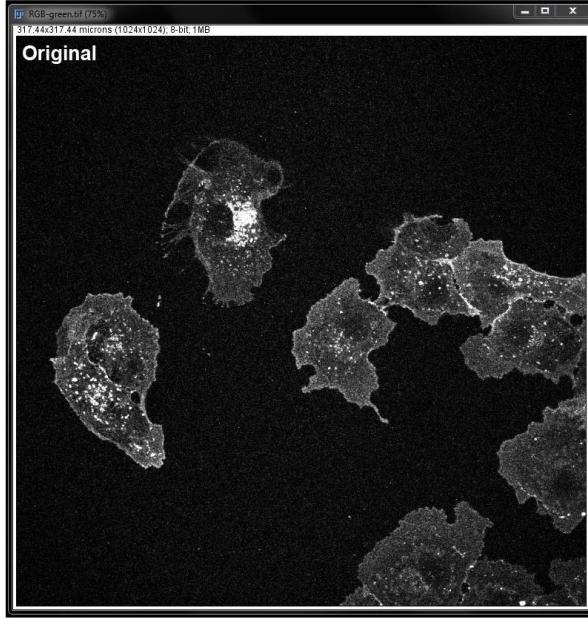
As with a lot of functions used to aid analysis, filters should be used with care and caution so as to not manipulate the outcome of the data. Filters should only be applied to allow the best fit to the image and not to force the image to fit a preconceived idea. Filters should never be used to alter or enhance images for publication purposes and should be applied prior to analysis ONLY to ensure accurate selection of data and NEVER to remove or enhance any feature that will alter the analysis result.

You can find some filters directly under the **Process** menu. Others are found under **Process -> Noise** or **Process -> Filters**.

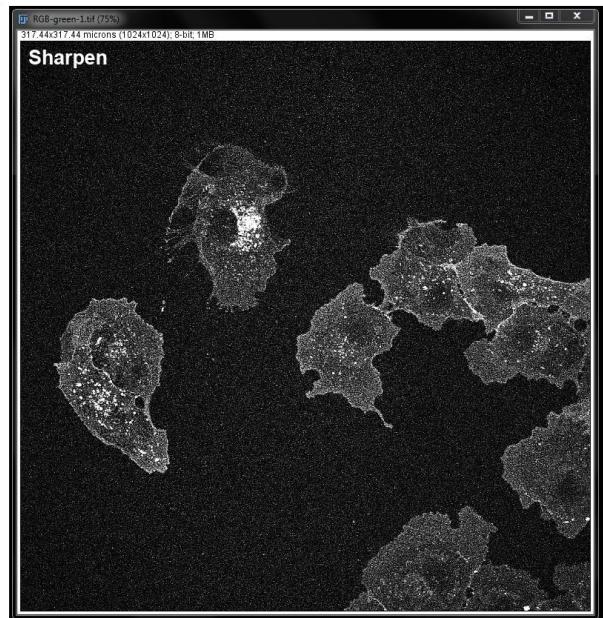
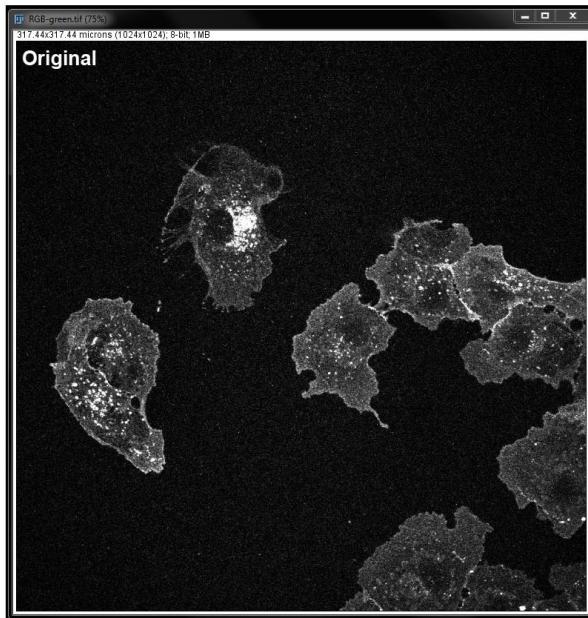


The different filters are demonstrated below with before and after images

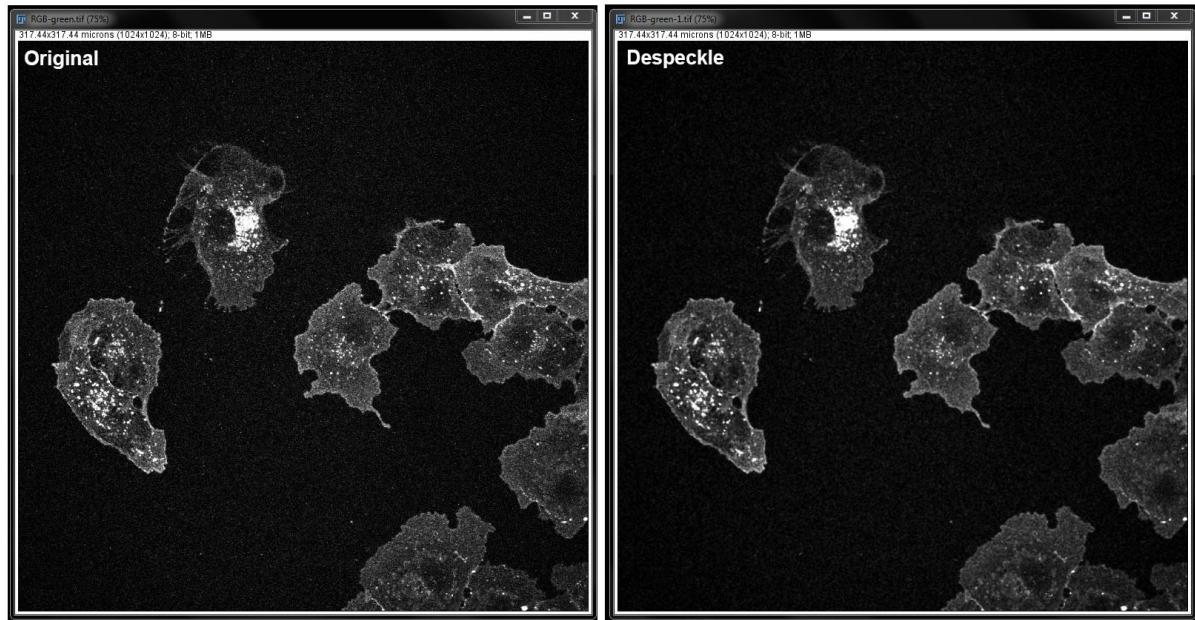
### **Smooth:**



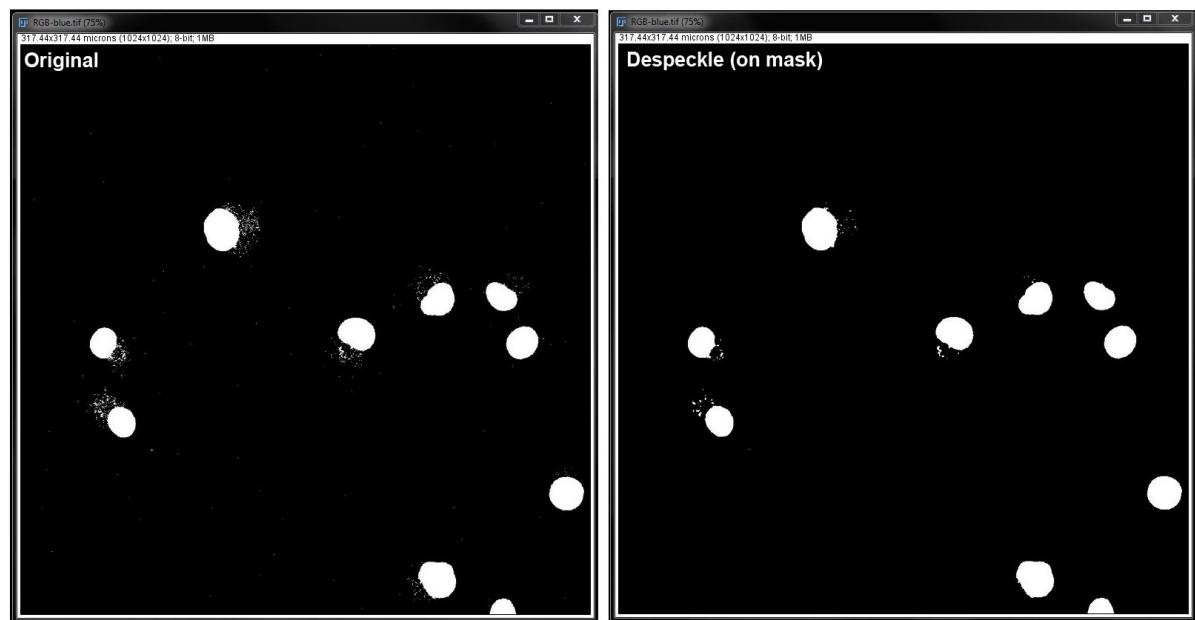
### Sharpen:



### Despeckle:

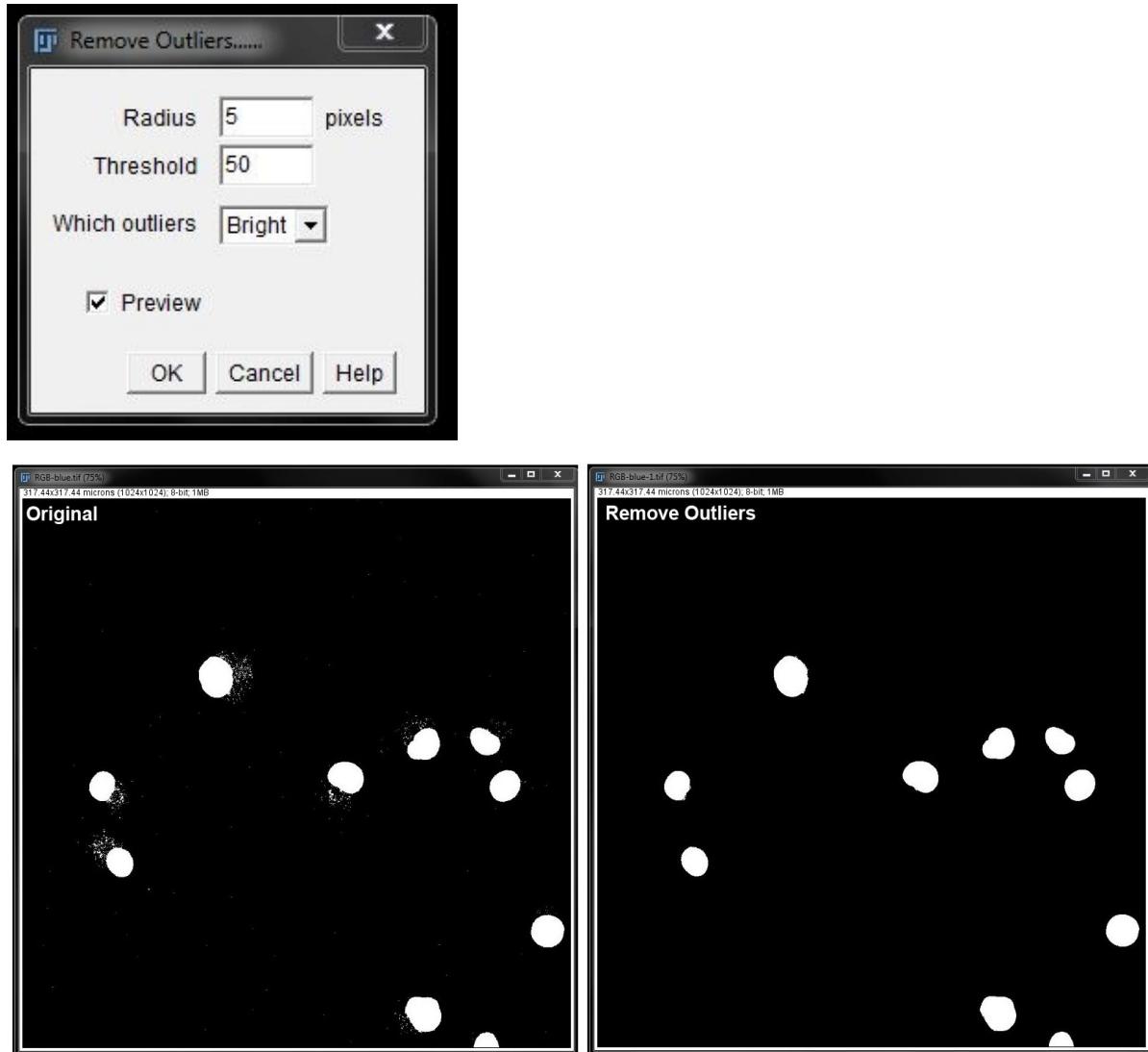


### Despeckle (mask):



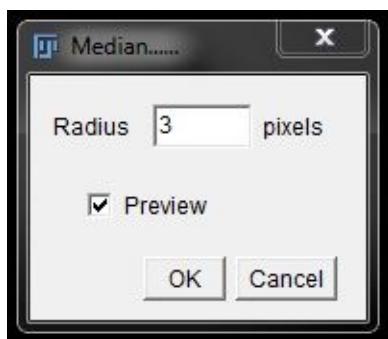
### Remove Outliers:

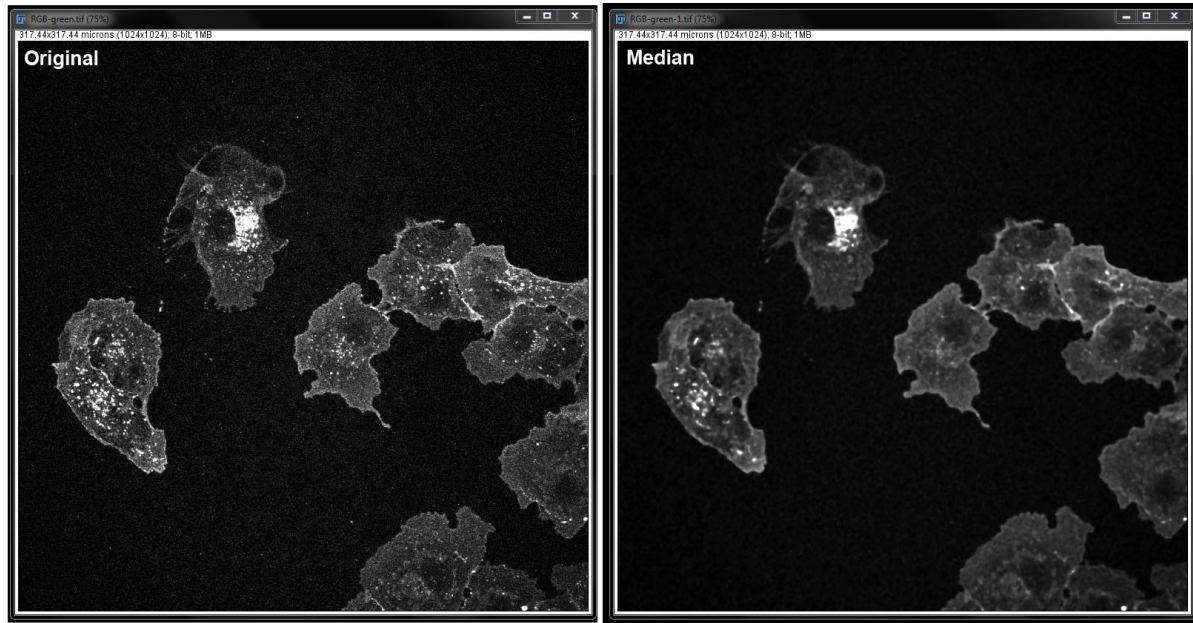
In this filter, you need to specify the outlier radius in pixels, and threshold. Select the checkbox next to **Preview** to see the changes before you apply them to the image. Click **OK** when you are happy with your changes.



### Median:

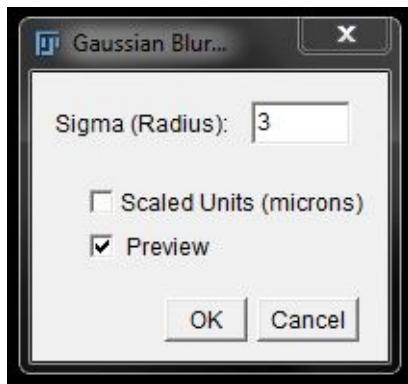
Again, in this filter, you need to specify the radius in pixels. Select the checkbox next to **Preview** to see the changes before you apply them to the image. Click **OK** when you are happy with your changes.

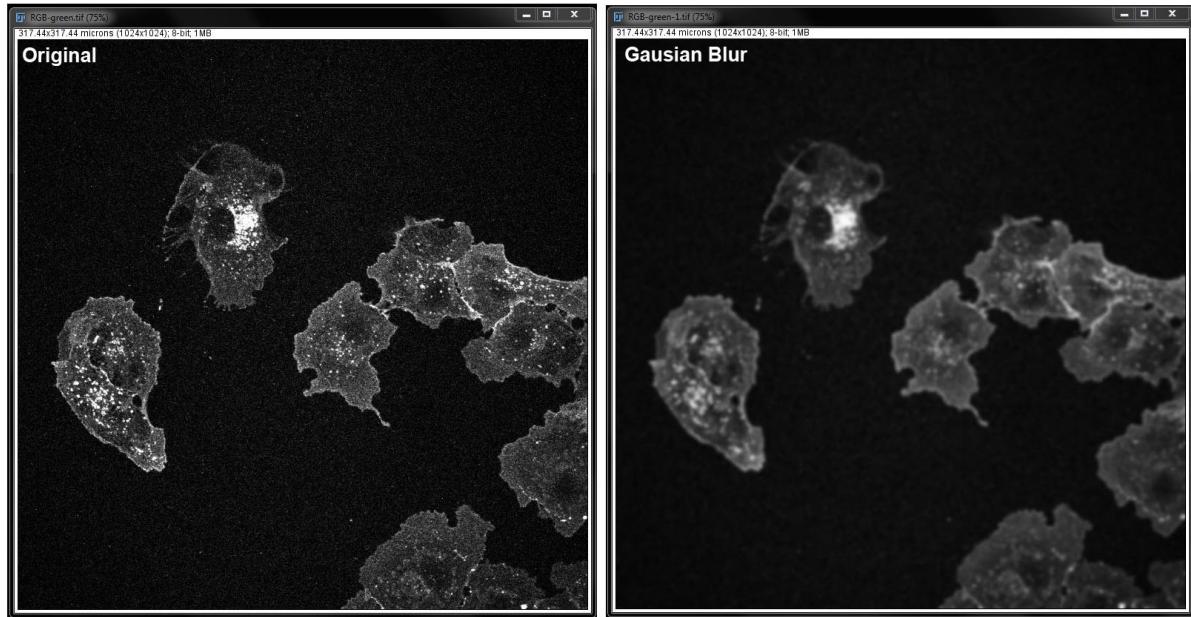




### Gaussian Blur:

This filter uses convolution to produce a smoothing effect. Here you again, need to specify the radius. Select the checkbox next to **Preview** to see the changes before you apply them and click **OK** when you want to apply changes to the image.





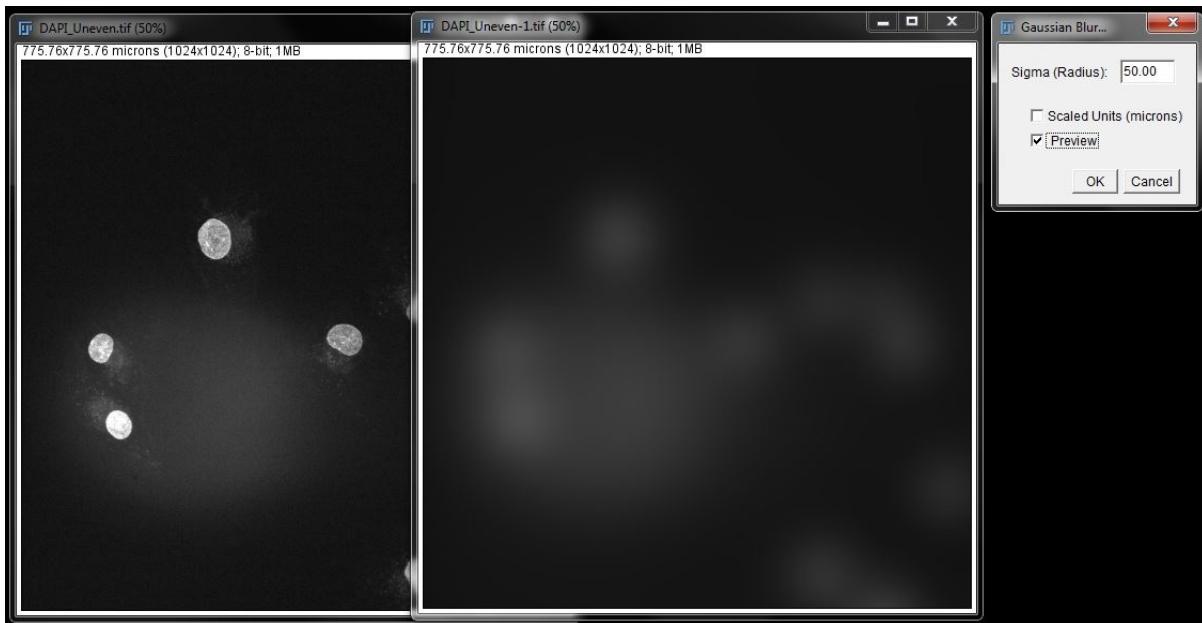
## PART 4: IMAGE CORRECTION

There are a number of tools in FIJI that can be useful for advanced correction of your images and image series. In this section we will go through the different tools available for correcting imaging artefacts and flaws. The demonstration images *DAPI\_Uneven.tif*, *Bleached.tif*, *Spheroid\_Shift.tif*, *Deconvolution\_Stack.tif* and *PSF.tif*.

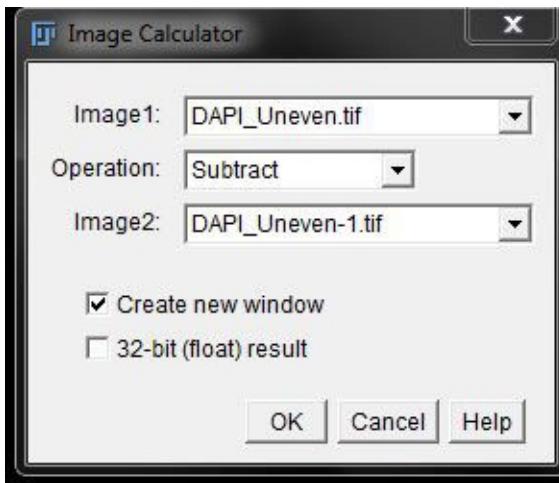
### Flatten

Sometimes, an uneven background in your image hinders image analysis because you can't threshold your features of interest without also thresholding some of the background. Flattening the image can help with that.

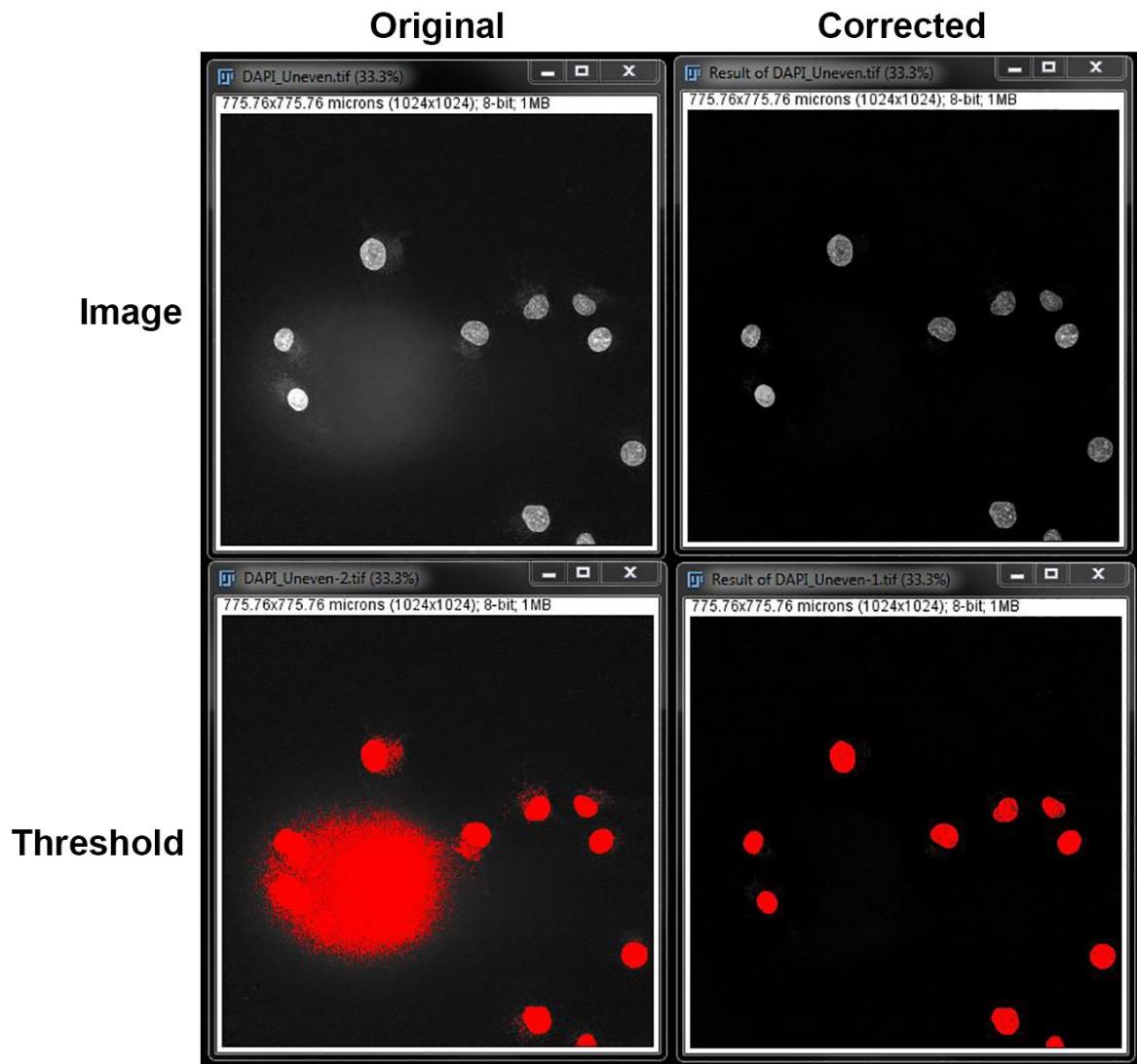
Open the image *DAPI\_Uneven.tif*. As shown in previous workshops/sections, duplicate the image and then apply a large Gaussian blur (e.g. radius 50) to the duplicated image.



Using the image calculator described in Part 3 of this manual (**Process -> Image Calculator**), subtract the duplicate (Gaussian blurred) image from the original image.



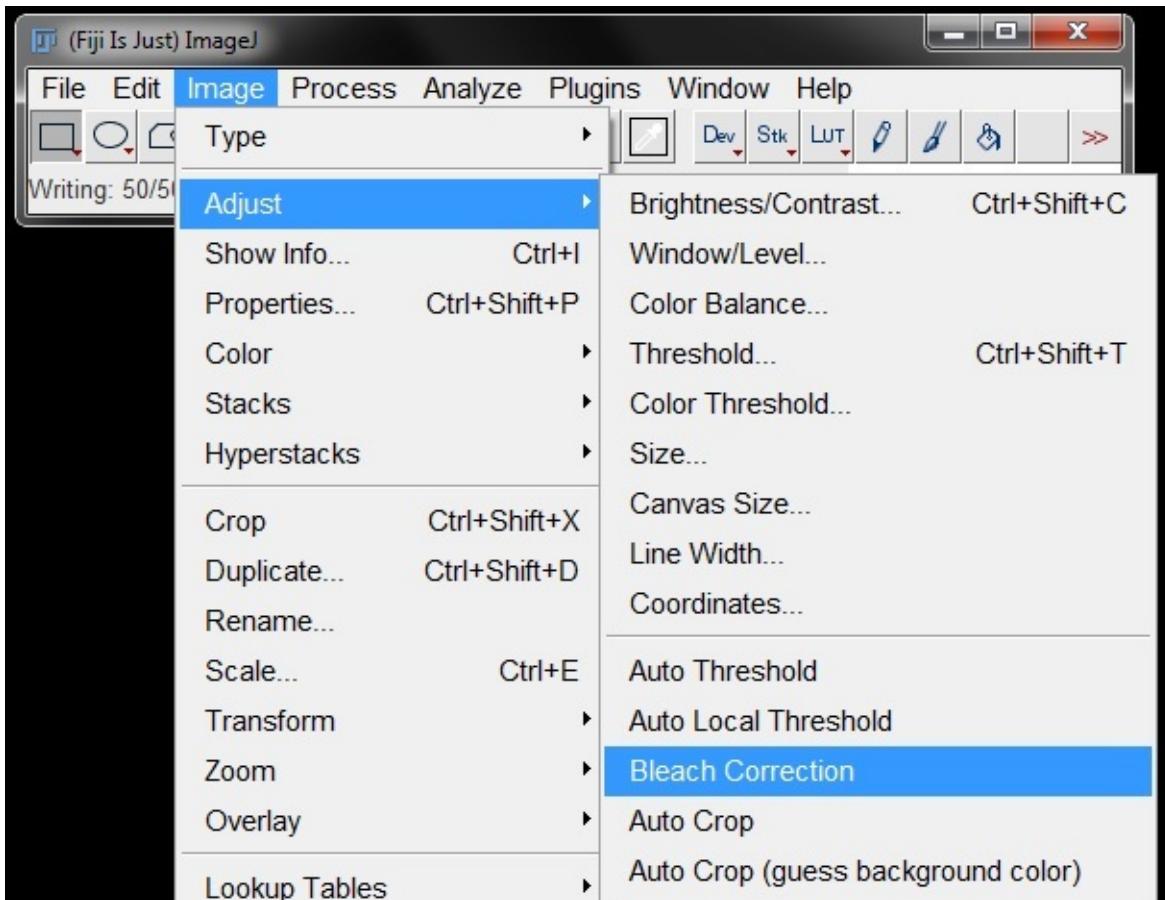
This results in a flattened image which will be easier to threshold accurately.



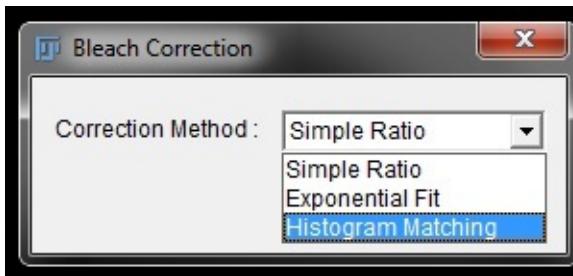
**Note:** There is also a background subtraction option in FIJI (**Process -> Subtract Background**). However, while this will remove even background it won't help with uneven background and consequent thresholding issues.

## Bleach Correction

Open the demonstration image *Bleached.tif* and go to **Image -> Adjust -> Bleach Correction**.

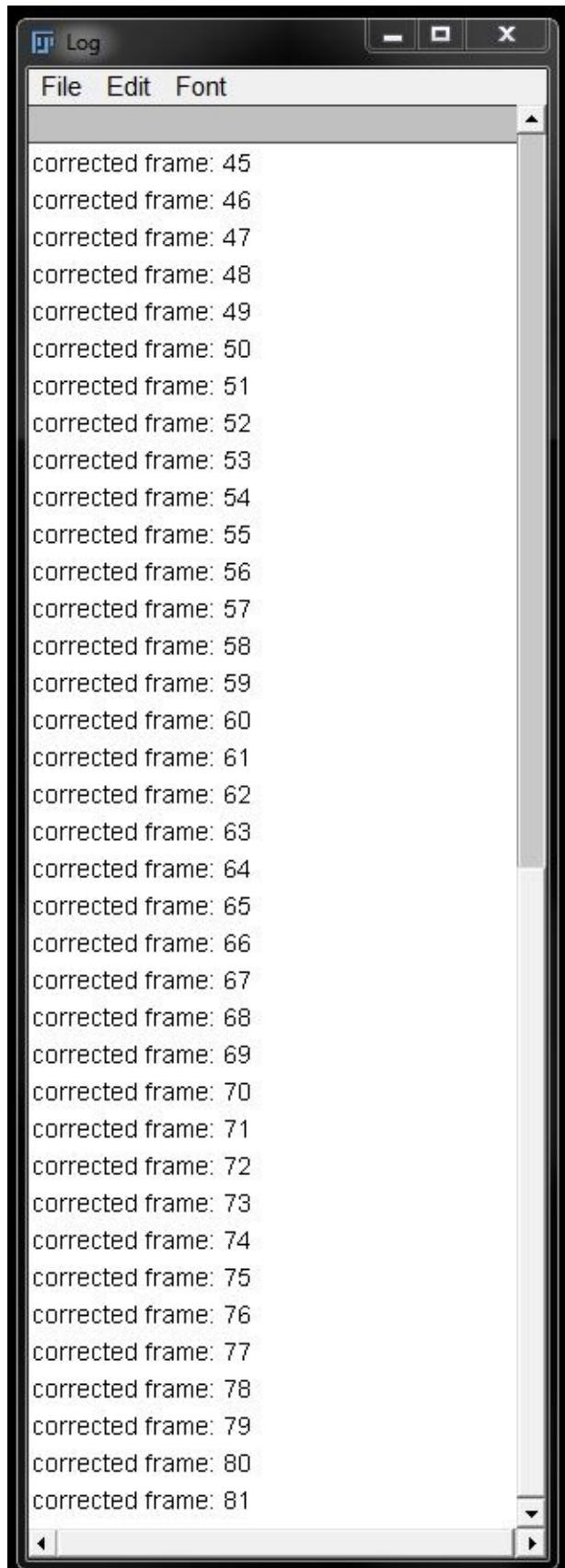


Choose **Histogram Matching** from the drop down menu in the pop-up window and then click **OK**.

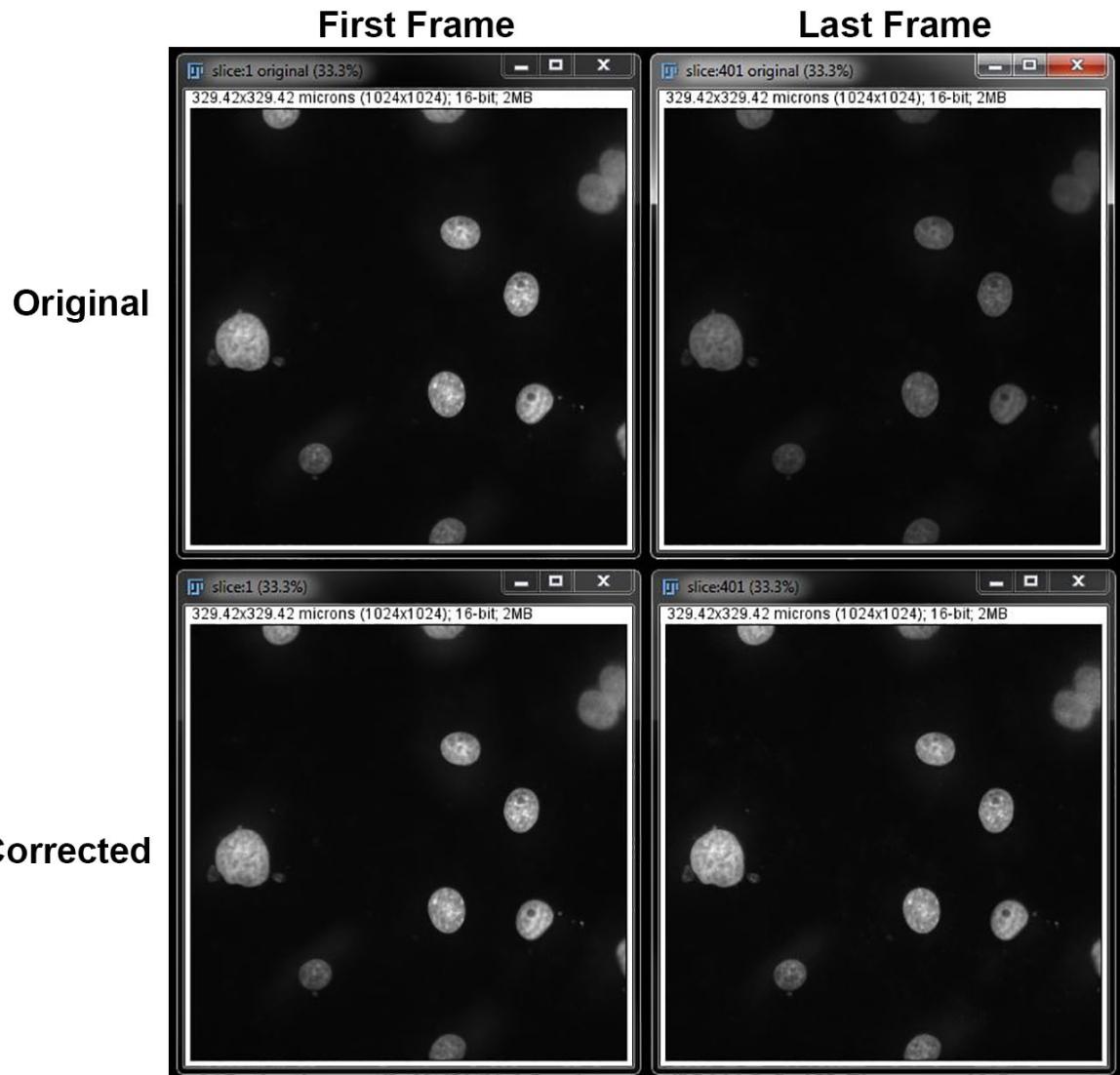


There are other options that you can try (Simple Ratio and Exponential Fit) which are quicker but in our experience don't work as well. For your own data you may need to try all options to see which fits best.

FIJI will open a log displaying the progress as each frame is corrected.



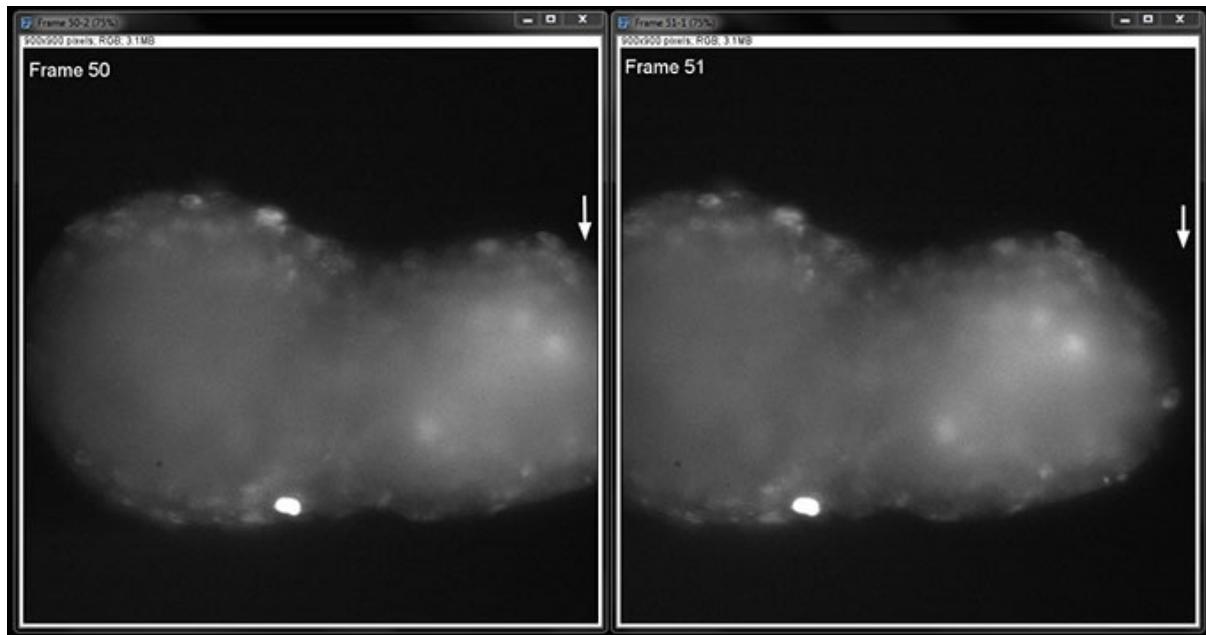
A new window will open with your result when the correction is completed. Here I have duplicated the final frame and show them side by side to demonstrate the difference between the original and corrected image.



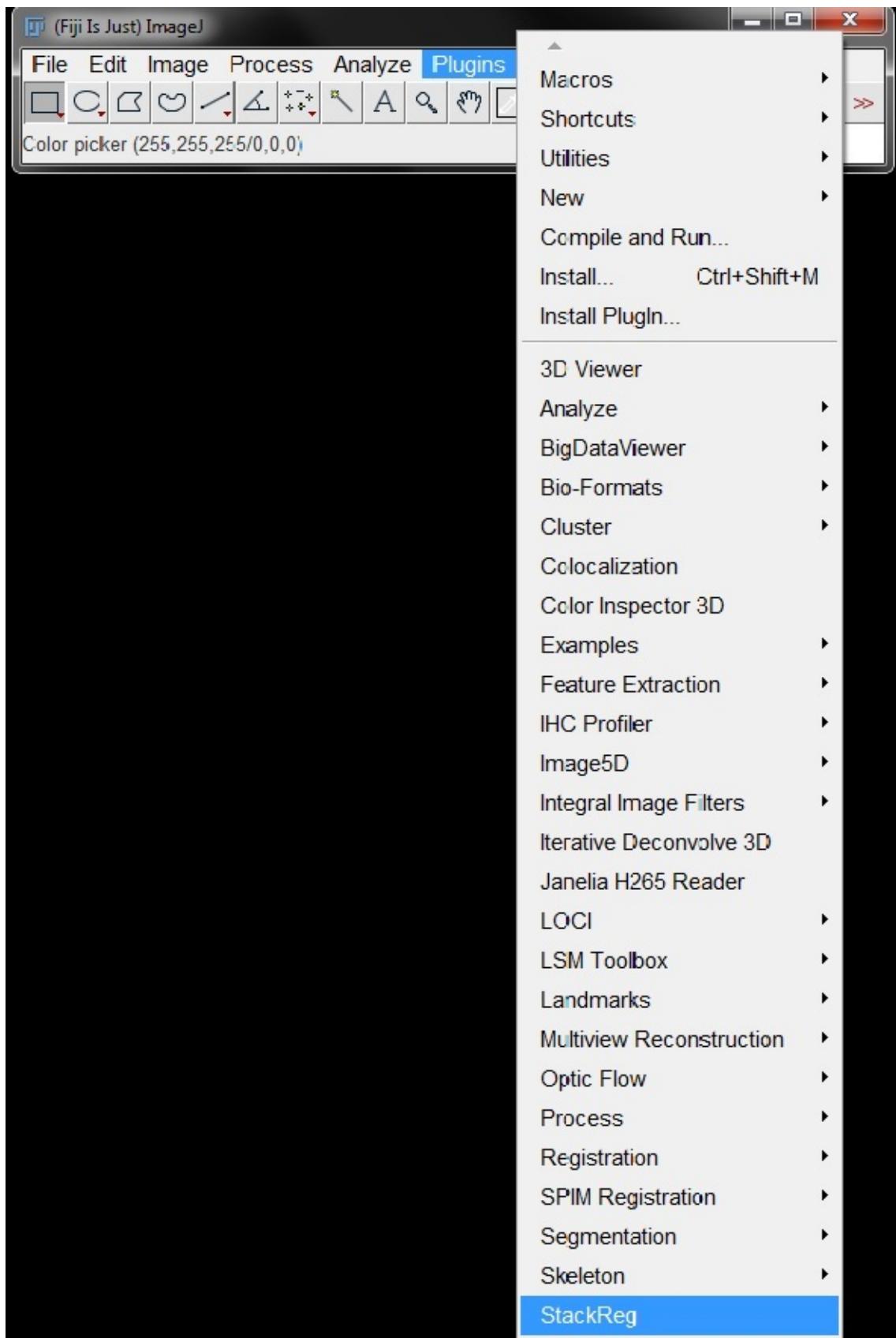
## Image Registration / Drift Correction

Correction of image shifts or drift during imaging requires installation of two stack registration plugins. If you have not already done this, do so now by Googling ‘StackReg FIJI’ and ‘TurboReg FIJI’ and download both plugins. They will appear as .zip files. Unzip both files and then move the .jar file from each into the plugins folder of your FIJI software and restart FIJI.

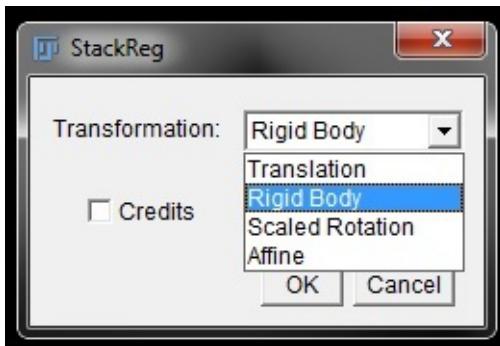
Once you have the plugins installed, open *Spheroid\_Shift.tif* and scroll through the stack. You will see that the image shifts (the spheroid makes a sudden jump to the left) at frame 51.



Go to **Plugins -> StackReg**.



Select **RigidBody** from the drop down menu next to **Transformation** in the pop up box in the pop-up window, then click **OK**.



**Note:** There are four selections for the type of transformation. You may need to test several on your data to determine the best option.

**Translation:** Will move planes in X and Y

**Rigid Body:** Will move planes in X and Y as well as rotate (most often provides the best correction without image distortion)

**Scaled Rotation:** Same as rigid body but will scale/zoom planes as well

**Affine:** Same as scaled rotation but can also deform images into trapezoid shapes

FIJI will move through the stack as it corrects for the unwanted movements. Results will display in the original image window. For this reason it can be useful to work with a duplicate in case the first correction is not acceptable.

This plugin can be used in the same way for slower drift over time.

## Image Deconvolution

Deconvolution is a mathematical process that takes into account the shape of the laser focus (called point spread function or PSF) and uses that to remove out-of-focus light from the image therefore improving resolution and signal-to-noise ratio.

To deconvolve an image you will need the plugin "Iterative Deconvolve 3D" and a PSF image from the microscope you captured your images on.

If you have not already done so, download *Iterative\_Deconvolve\_3D.class* from [http://imagej.net/Iterative\\_Deconvolve\\_3D](http://imagej.net/Iterative_Deconvolve_3D). Copy *Iterative\_Deconvolve\_3D.class* into your FIJI Plugins folder, then restart FIJI if it was already open.

The PSF can easily be recorded on a microscope by imaging 100nm fluorescent beads. For this example we have a PSF and matching image ready for you.

Open *Deconvolution\_Stack.tif* and *PSF.tif*, both found in the "Deconvolution" subfolder in the Demo images. Scroll through the PSF file to see what it looks like. It's a z-stack of a fluorescent bead.

Go to **Plugins -> Iterative Deconvolve 3D**.

Select *Deconvolution\_Stack.tif* under Image and *PSF.tif* under Point Spread Function from the drop down menus, then select number of iterations (try 10 and 100 and compare the resulting images). For this image you will also need to change **Wiener filter gamma** to 0, and **Terminate iteration if mean delta <x% to 0.01**. Click **OK** and wait for the deconvolution process to run.

A log of iterations will be displayed, which will tell you when deconvolution is completed. A new window with the deconvolved image will open.

Compare your deconvolved image to the original and repeat if necessary with increased or decreased iterations.

Save your final image when you are happy with the result.

## PART 5: IMAGE ALIGNMENT

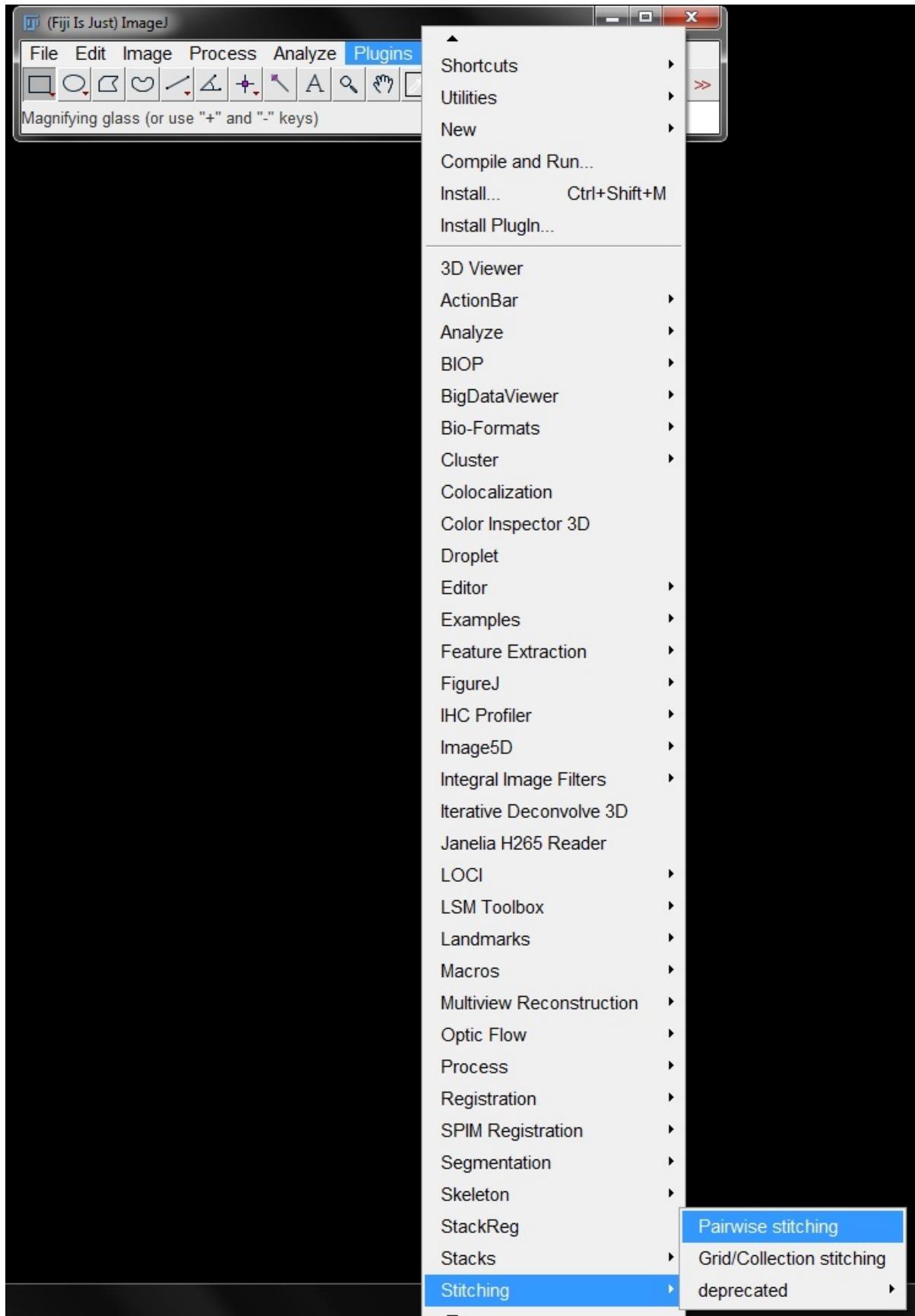
Sometimes we have multiple images that belong together in one larger image. Often times these can be captured and processed to create this larger image during acquisition, but in some circumstances we need to combine the images manually after acquisition. In those cases, we can use tools in FIJI to combine or align these images into a seamless single image.

For stitching we use the demo images *Tile\_01.tif* through to *Tile\_06.tif* found in the demo images sub-folder titled 'Manual Stitching'. For TrackEM2 alignment we use the image set found in the demo images sub-folder titled 'TrackEM2'.

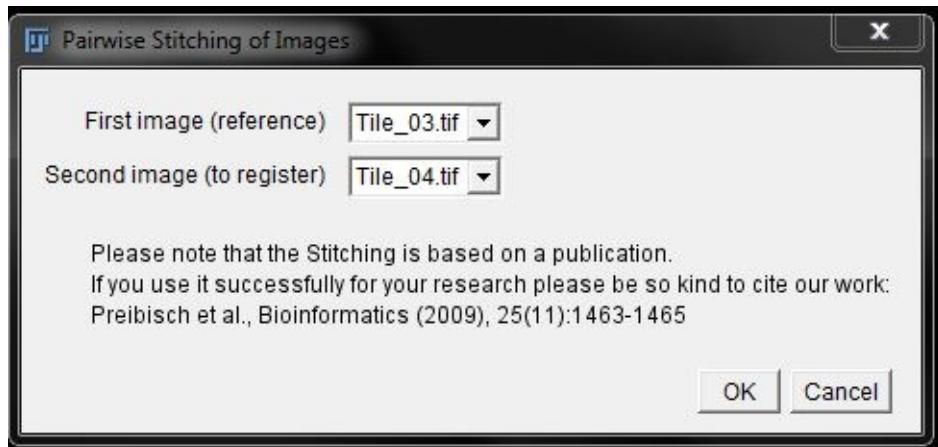
### Pairwise Stitching

Stitching combines multiple tiled image into a single large format image. One option to do this in FIJI is to use the **pairwise Stitching** plugin, found under the **Plugins -> Stitching** menu. This option allows you to combine images 2 at a time and is useful for small numbers of images, where there is no specific order.

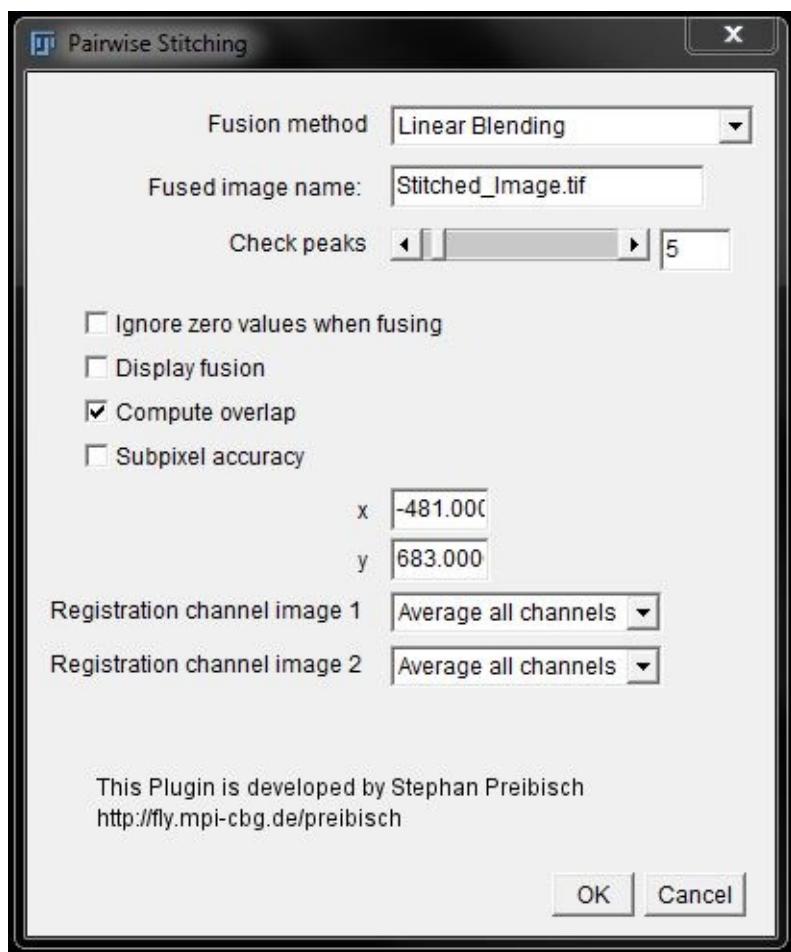
First you need to open at least 2 images that you want to stitch. Here we will use the two demonstration images *Tile\_03.tif* and *Tile\_04.tif*. Select Pairwise Stitching from the menu by going to **Plugins -> Stitching -> Pairwise Stitching**.



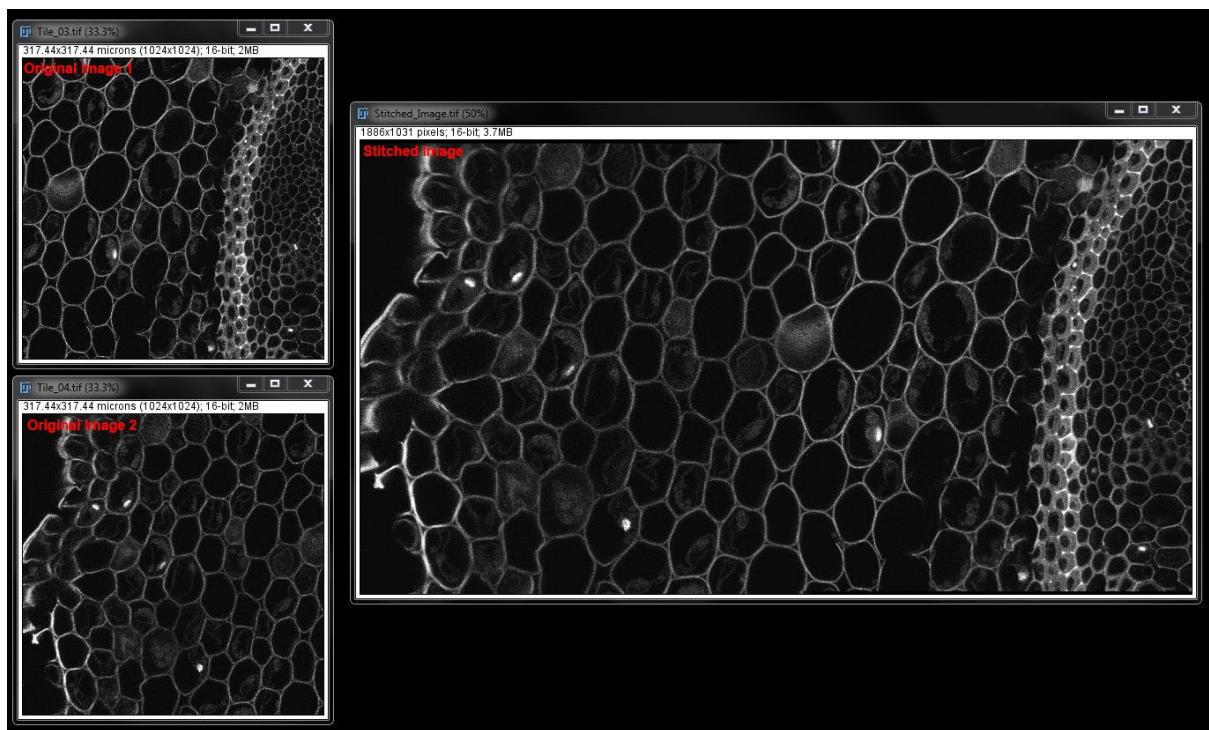
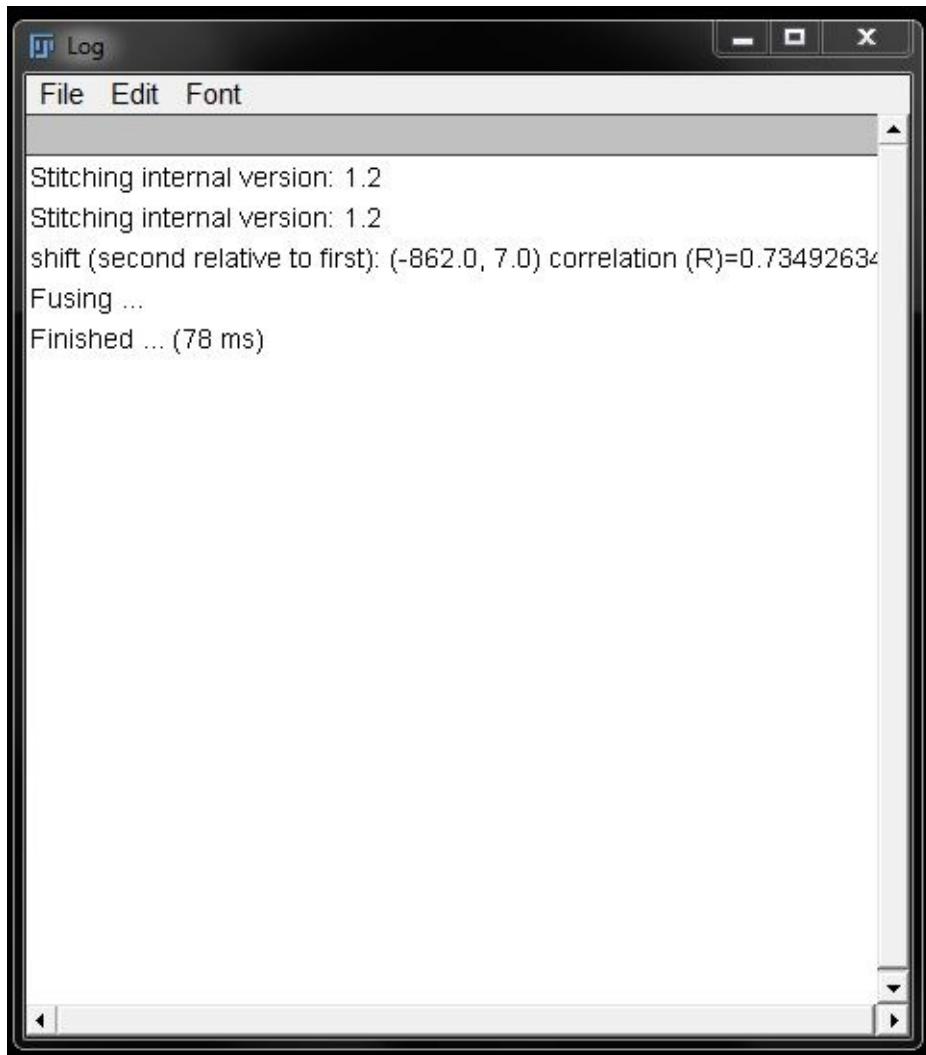
In the Pairwise stitching window, select Tile\_03 and Tile\_04 as your images from the drop down menus (the order doesn't matter) then click **OK**.



In the second options window, you can give your final image a new name, but no other settings should need adjusting. Click **OK** to begin the stitching process.



A log window will show progress and your stitched image will open in a new window upon completion.



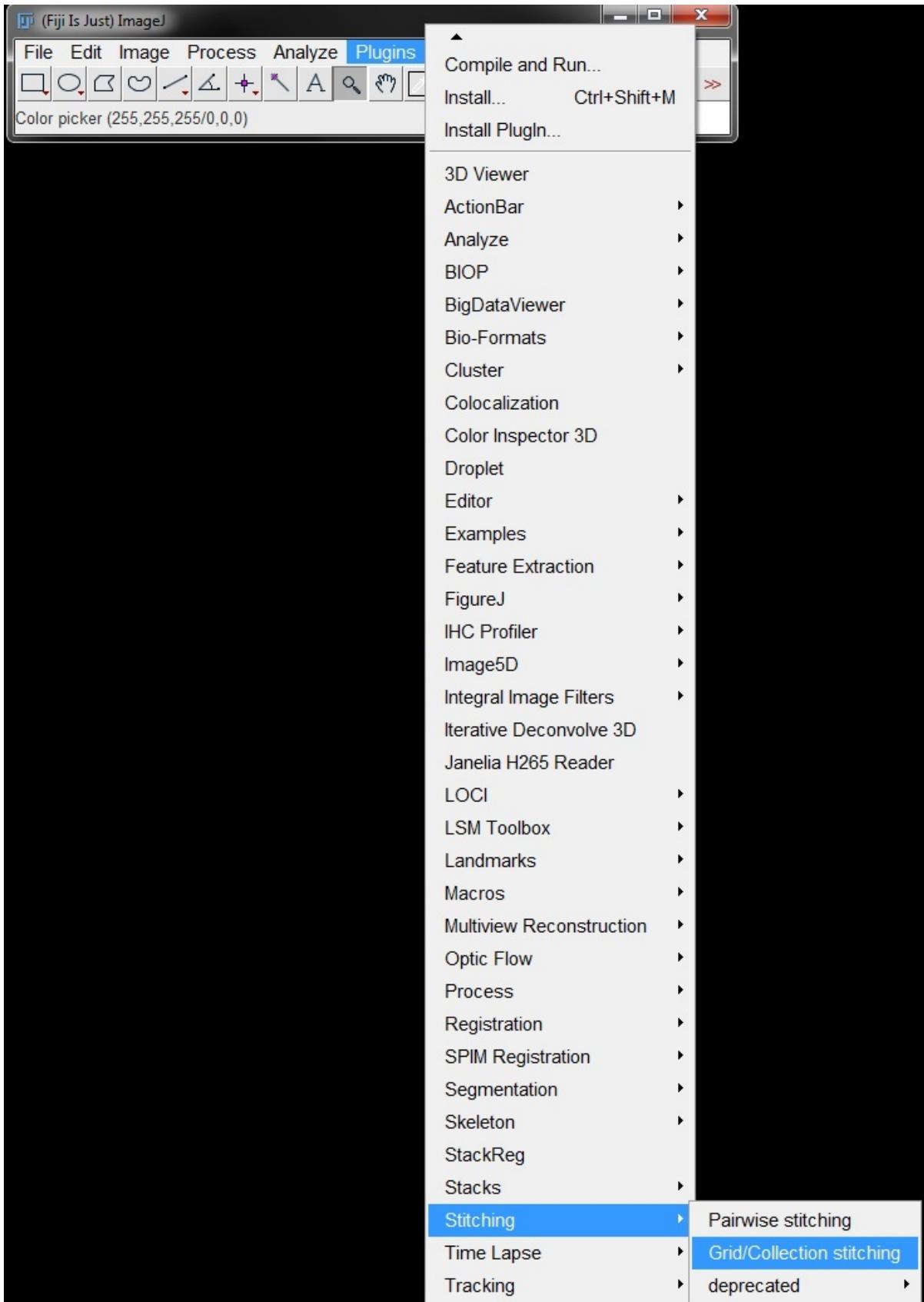
You can then repeat the steps to add other panels if you have more than two images, by opening the next image and using that plus your newly stitched image as your 2 images to combine.

## Grid Stitching

There also is a grid stitching option that you can use for larger numbers of images that were recorded with a motorized stage in a defined pattern. This is also found under the **Plugins -> Stitching** menu.

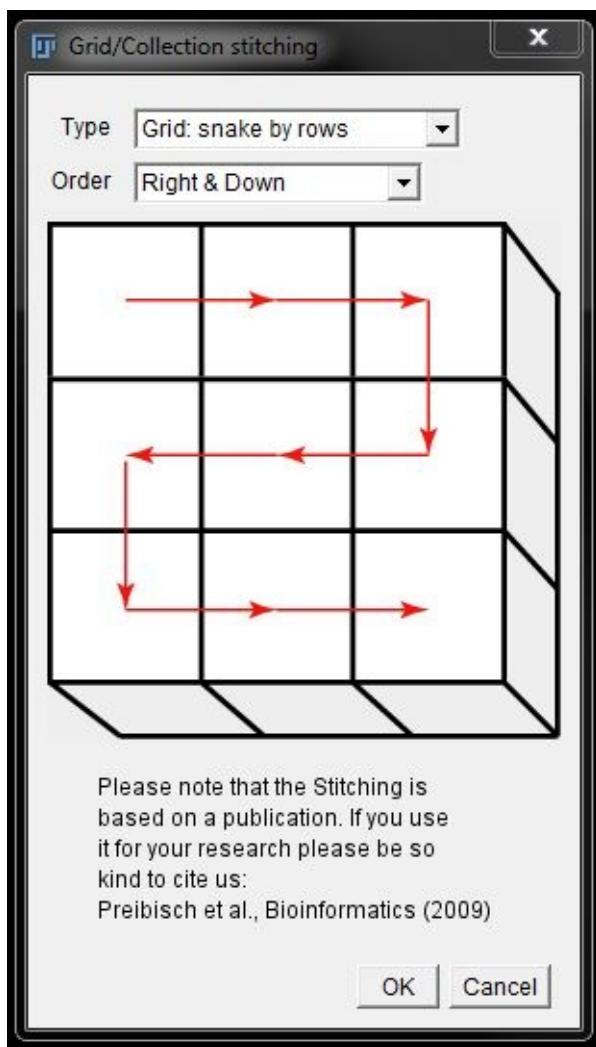
You do not need to have your images open in FIJI, but they must be named in specific manner and all must be named in the same format (ie: here they are all named Tile\_xx.tif).

Begin the process by going to **Plugins -> Stitching -> Grid/Collection stitching**.



In the first window, select the pattern of image acquisition from the drop down menus for **Type** and **Direction**. The image below these menus will illustrate the selected pattern for clarity. Here the images were captured as "Snake by Rows" and "Right & Down".

Click **OK** to proceed.

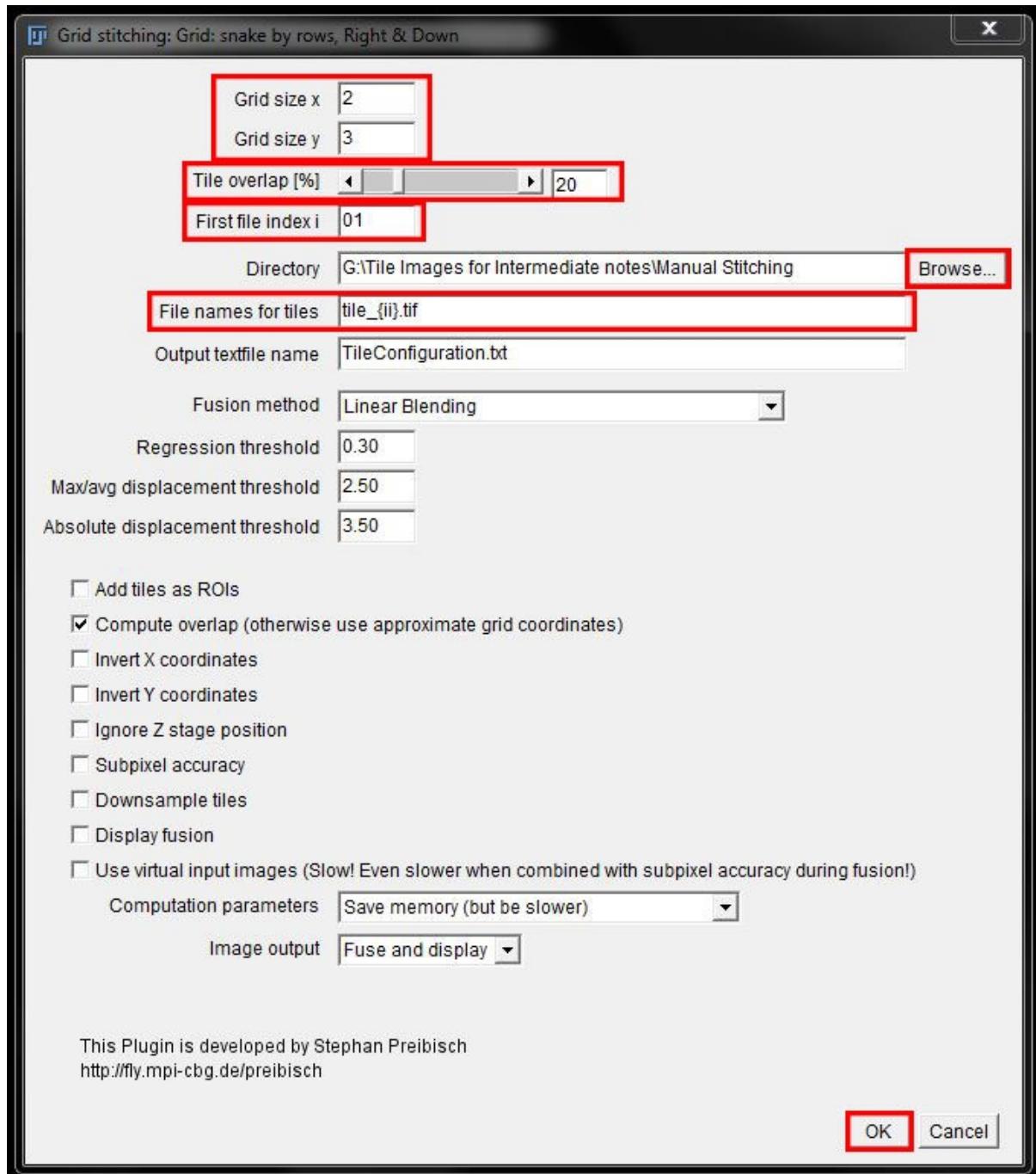


In the next options window, check the grid size is correct and adjust to the correct number of rows (x) and Columns (y) as needed. Here we had 3 rows x 2 columns.

Adjust the overlap to that used during automatic acquisition or an estimate (over-estimating is better than under) is captured manually. Here we use 20% overlap.

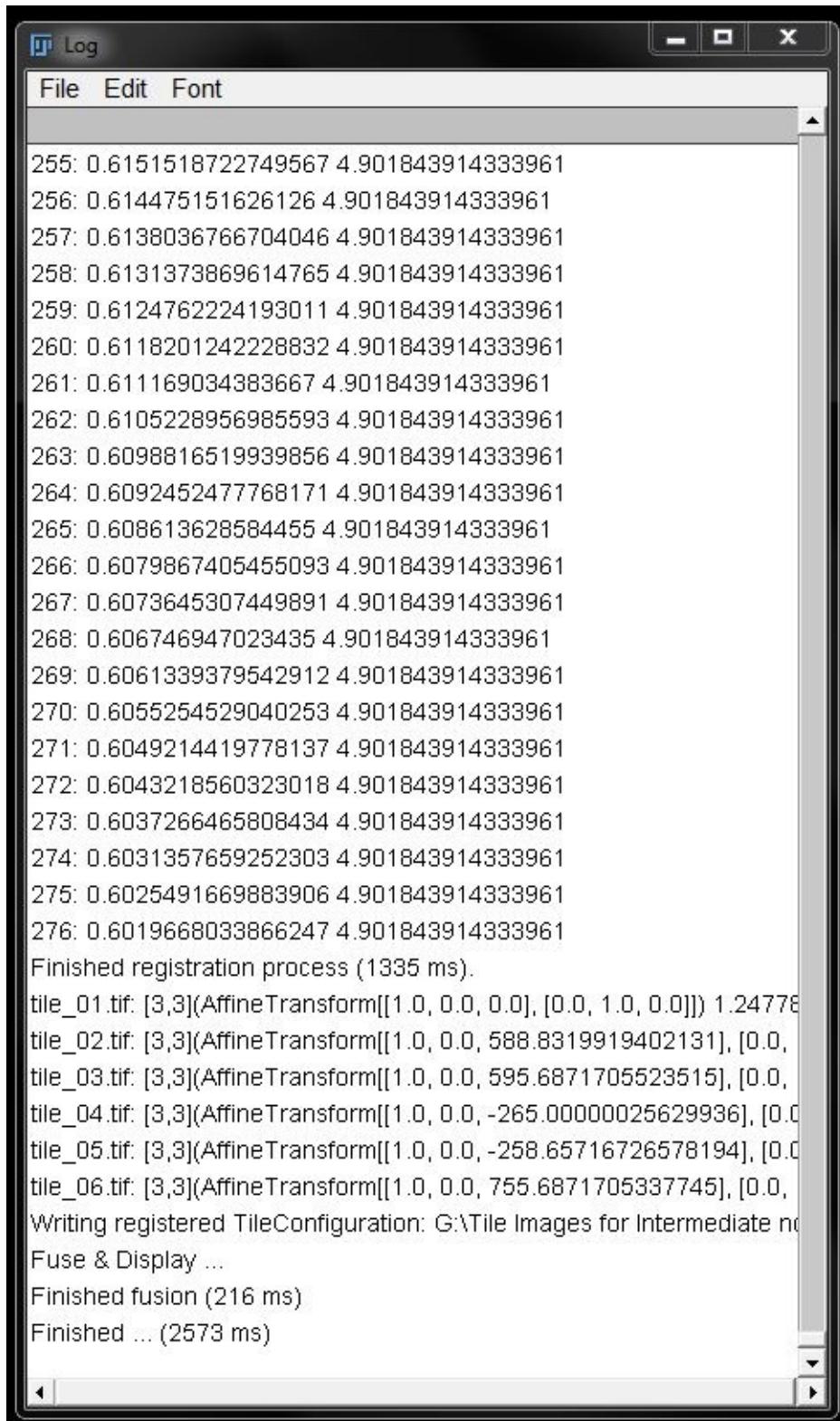
Enter the number of the first image in the sequence, or leave as 1 if all images are being included.

Select the folder where your images are saved by browsing under **Directory**. Then finally ensure the file name format is entered as used for your files.

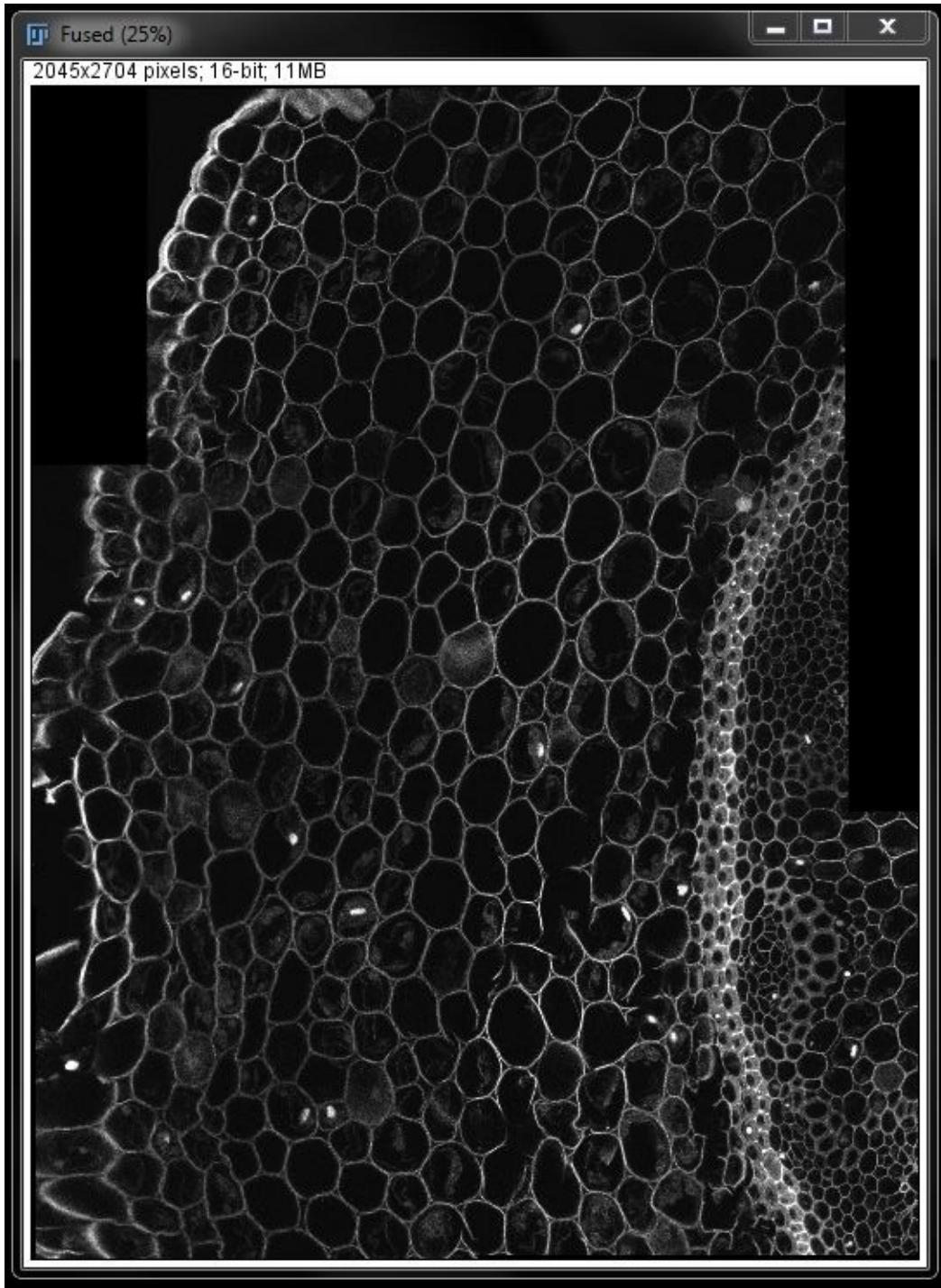


No other stitching/blending options should need to be altered from the default.

Click **OK** to proceed with stitching.



Again a log window will display your progress and the final image will open in a new image window upon completion.

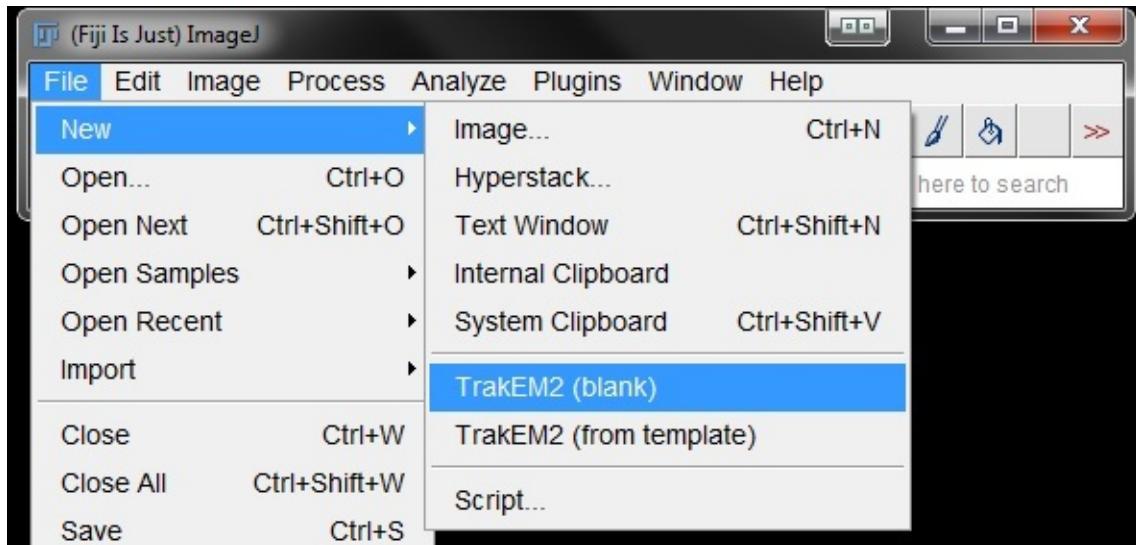


Often times, especially when images were acquired manually (as done here), there will be black space at the edges where shifts were required to line up images accurately. The final image may be cropped to ensure smooth edges without these blank sections.

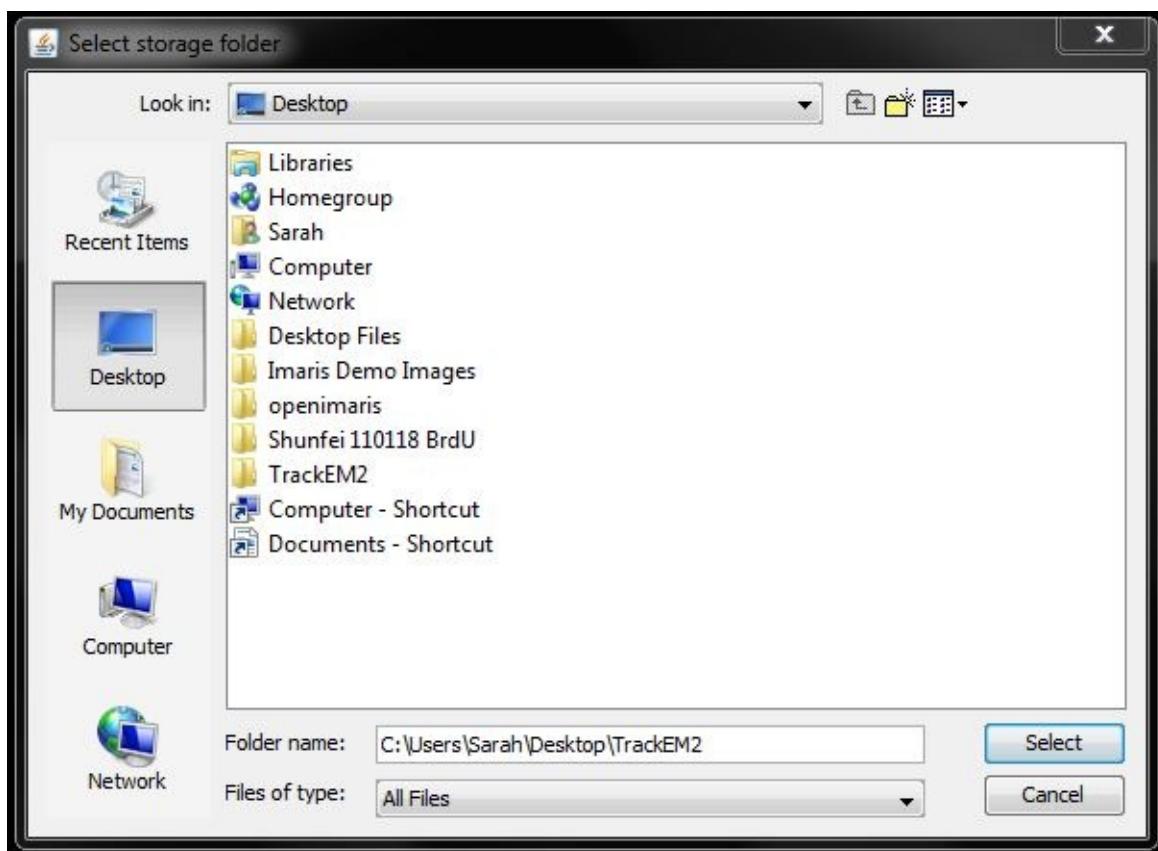
## TrakEM2 Advanced Image Alignment

To align z-stack images or histological serial sections that have been imaged in different orientations you can use the **TrackEM2** tool.

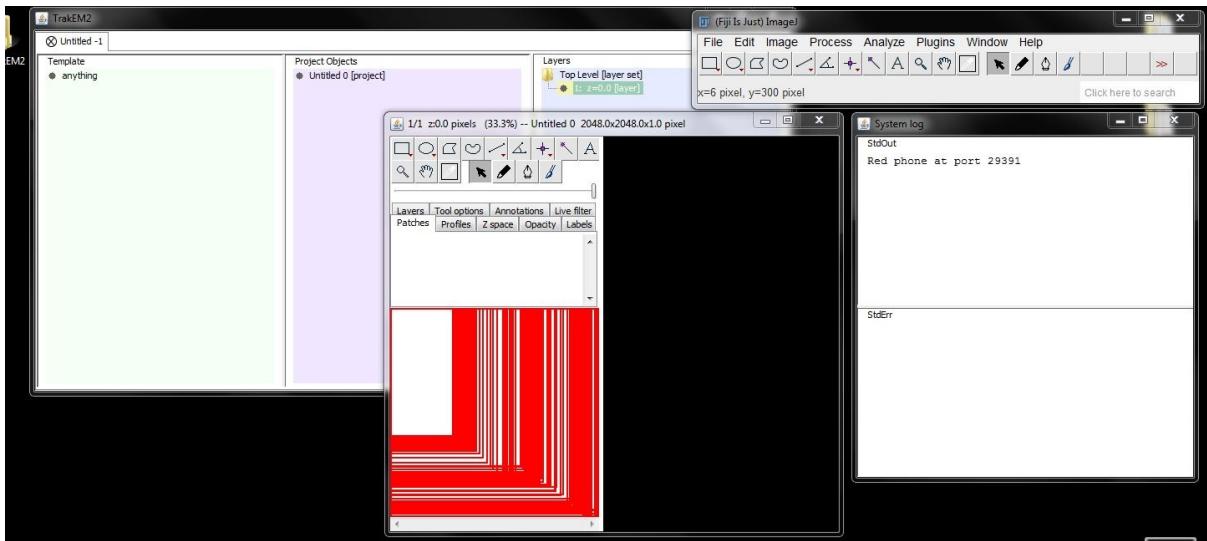
Go to begin go to **File -> New -> TrackEM2 (blank)**.



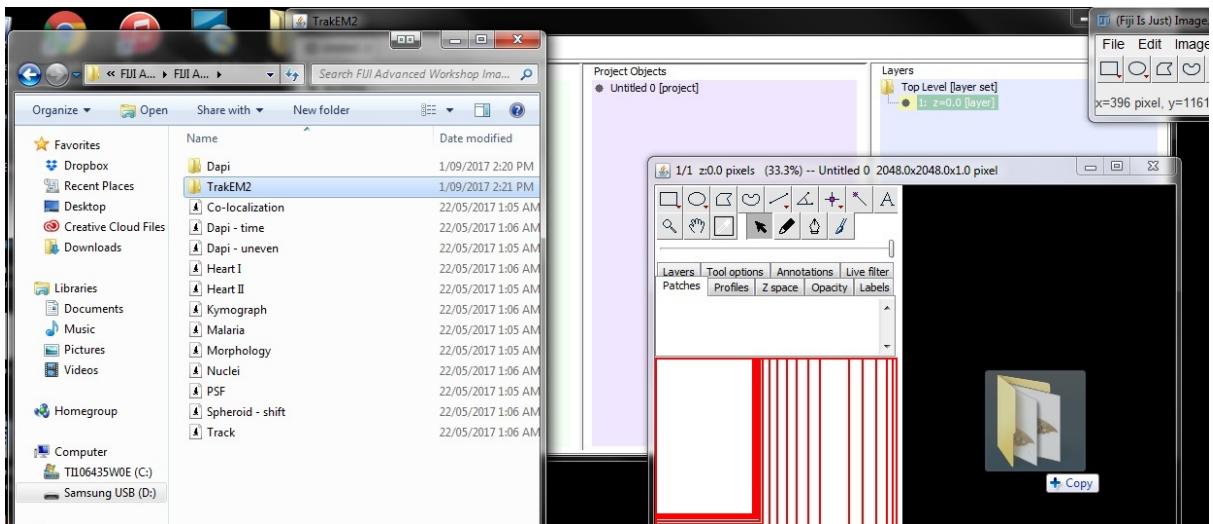
Create a storage folder in your desired directory as prompted by the software.



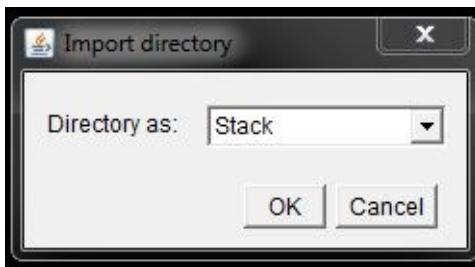
This will open a working area with several different windows.

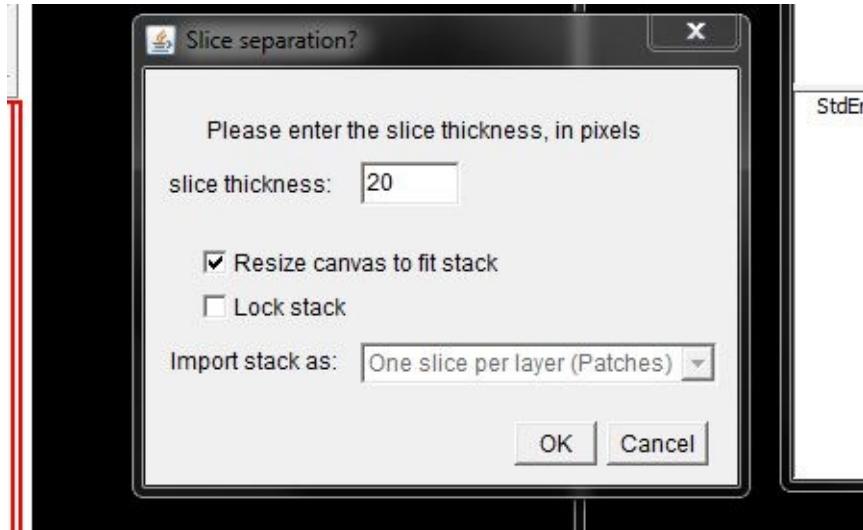


Drag and drop your folder containing all images (and only those images) into the main window (large black area). In the demo images we have a folder called *TrackEM2* with images for use here.

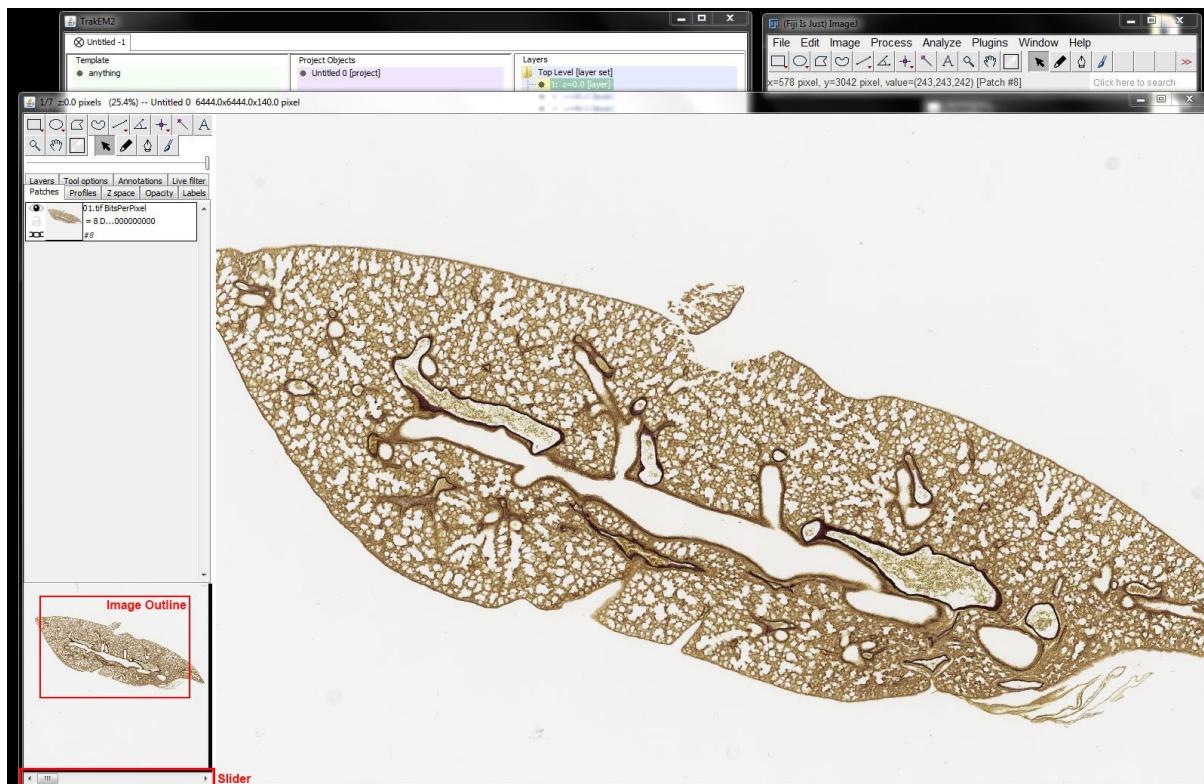


Select **Open as stack** when prompted. Then enter the distance between images (for z-stacks) or the thickness of your sections (for histological serial sections). For the workshop data, please enter 20um.

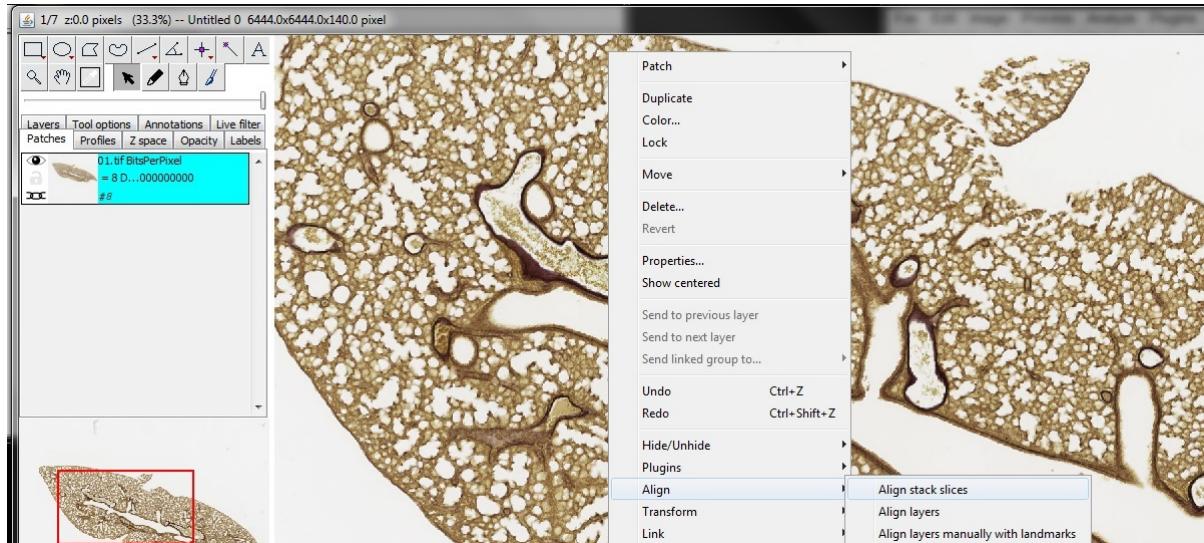




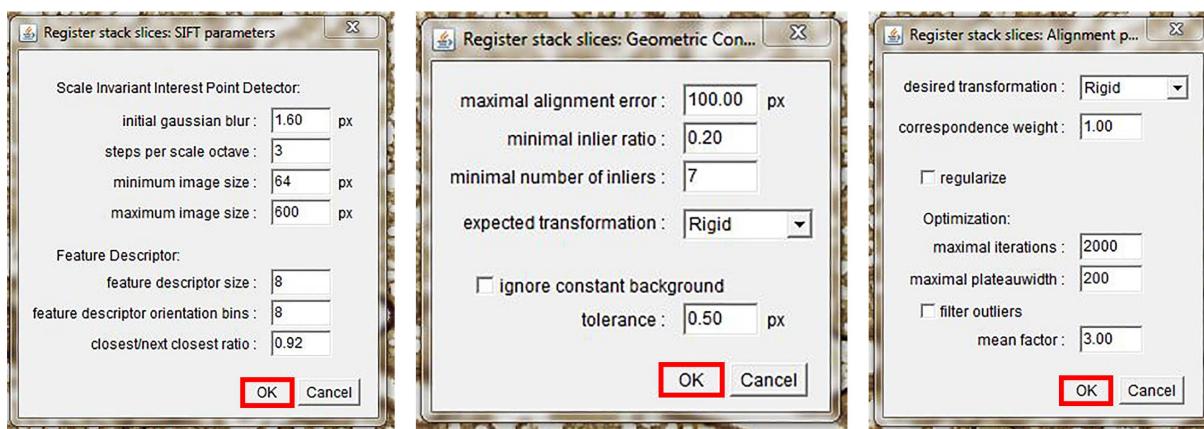
TrakEM2 will now load and display the images. You can zoom in and out by holding down Ctrl and turning the mouse wheel. You can play through the slices with the slider in the lower left corner of the main window, below the thumbnails. The red outline in the thumbnails shows the image area (which will enable you to easily see the area of each section within the image and how they do or don't line up). You can pan around by clicking with the mouse on the image and drag it around.



Left-click with the mouse on the image to activate the window, then right-click and choose **Align -> Align stack slices** from the right-click menu.

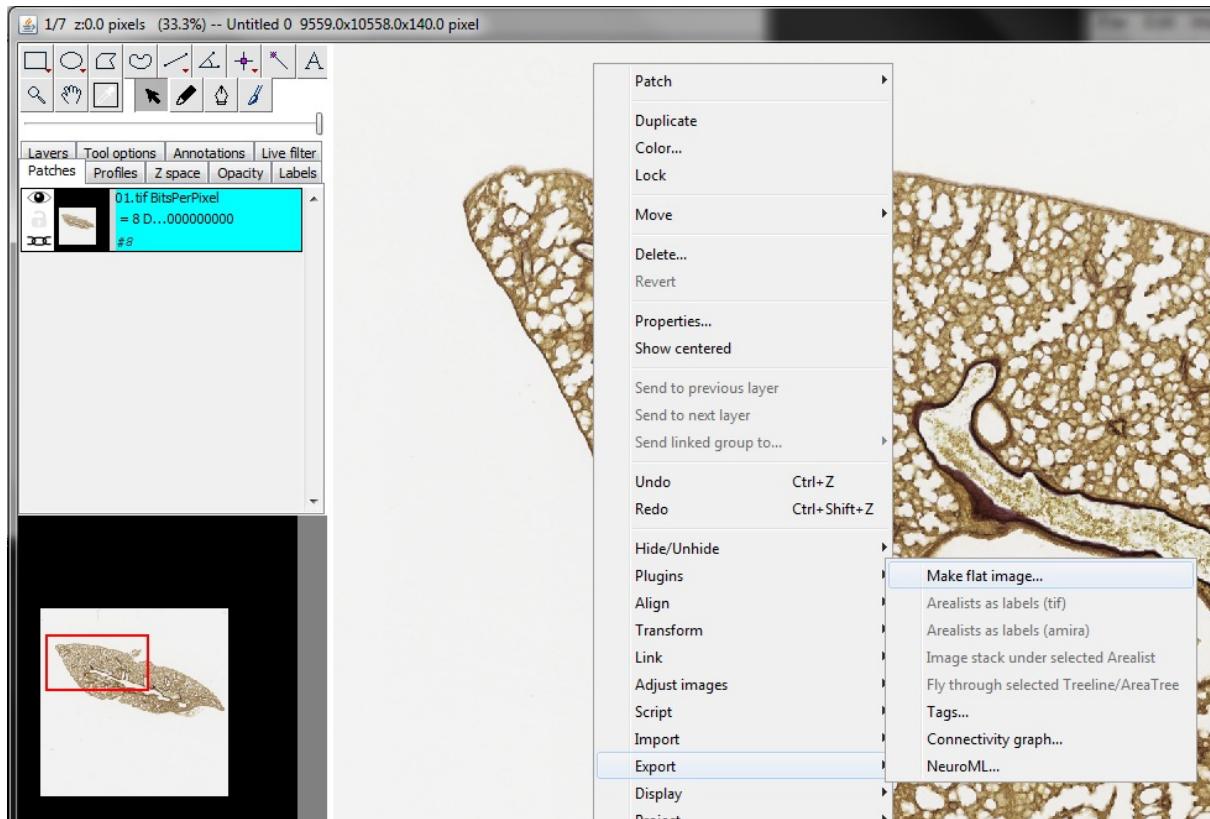


Accept all defaults by clicking **OK** three times.

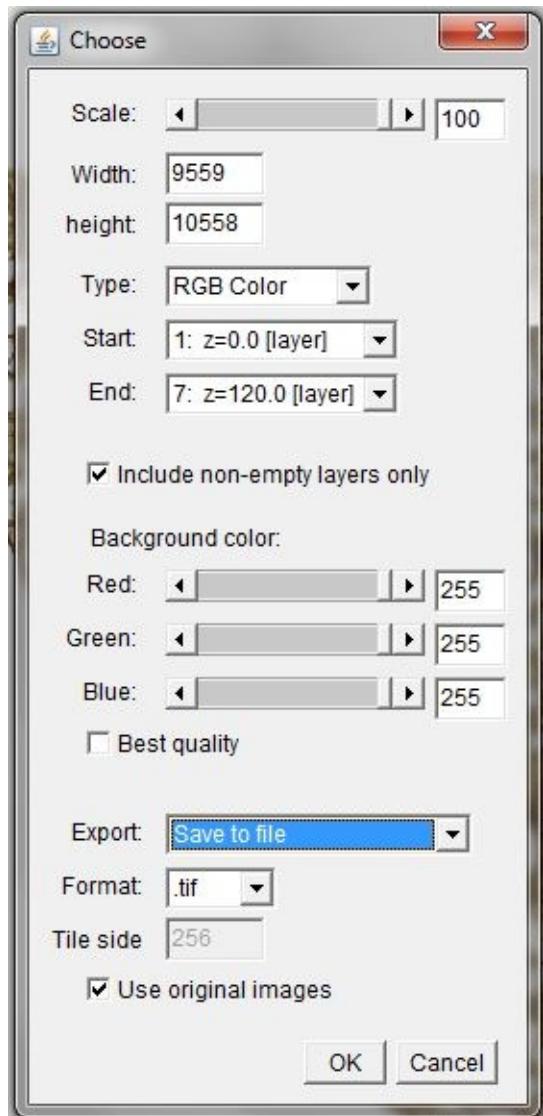


If you scroll through the slices now, you see that the sections are all aligned to each other.

To save your aligned image stack, right-click again, select **Export -> Make flat image...**



Make sure that **Scale** is set to 100% (you can reduce your image size here if you like but not recommended as you lose data) and select **Type:** RGB Color (for histology images) or 8-bit greyscale (for single channel fluorescence images). Set your first and last images as the **Start** and **End** layers. For **Background Color** set all three base colours to 255 to give a white background (for histology images) or set all three base colours to 0 to give a black background (fluorescent images). Tick '**Best Quality**' and ensure the final image is saving in .tif format, then click **OK** to save.



## PART 6: SIMPLE IMAGE MEASUREMENTS

Many of the simple FIJI functions that you have previously learnt about can be combined with other tools for more complex processes to analyse and measure your data. Here, our instructions become more specified as we go through several examples of common measurements using the different tools in FIJI.

In this section we use the images *RGB-blue.tif*, *RGB-green.tif*, *NeuralTubeRed.tif* and *Nuclei-1.tif* for demonstration.

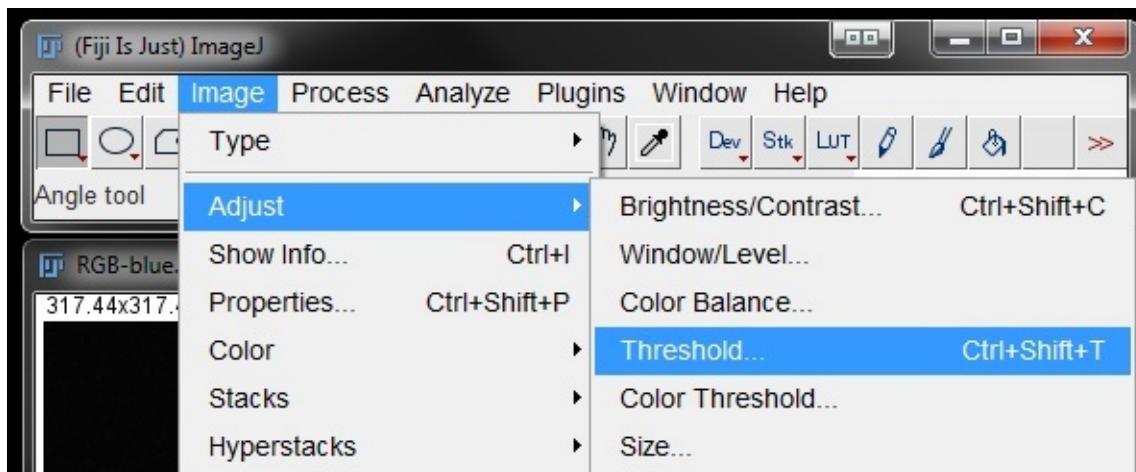
### Area Measurements

To perform any measurements on our image we must ensure the scale is calibrated. Checking and setting the scale calibration was covered in FIJI Basics.

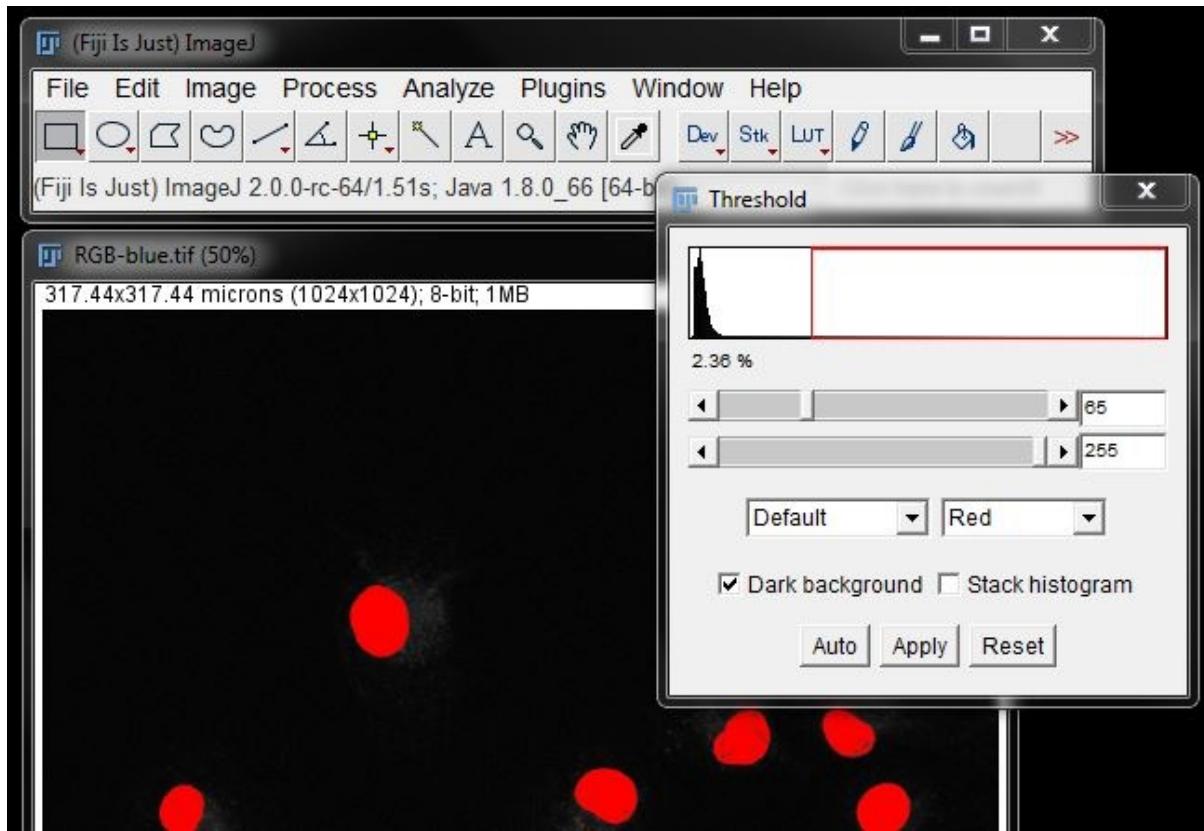
We also need to select an area within the image that we want to measure. Without a selection, the program would give an area measurement for the entire image, rather than just the area of interest. In this example we will use a threshold, but you can also select a specific area to measure by using an ROI.

Open the image *RGB-blue.tif*. Check that the scale is calibrated before carrying out any further steps.

For colour images I like to apply a grey LUT to allow better contrast between the stain and background during thresholding. You may also like to **Duplicate** the original image and work on a copy. This image is already grey, but apply a grey LUT if you are working with a colour image and duplicate the image if you would like. Then go to **Image -> Adjust -> Threshold** (or use short cut **Ctrl + Shift + T**).



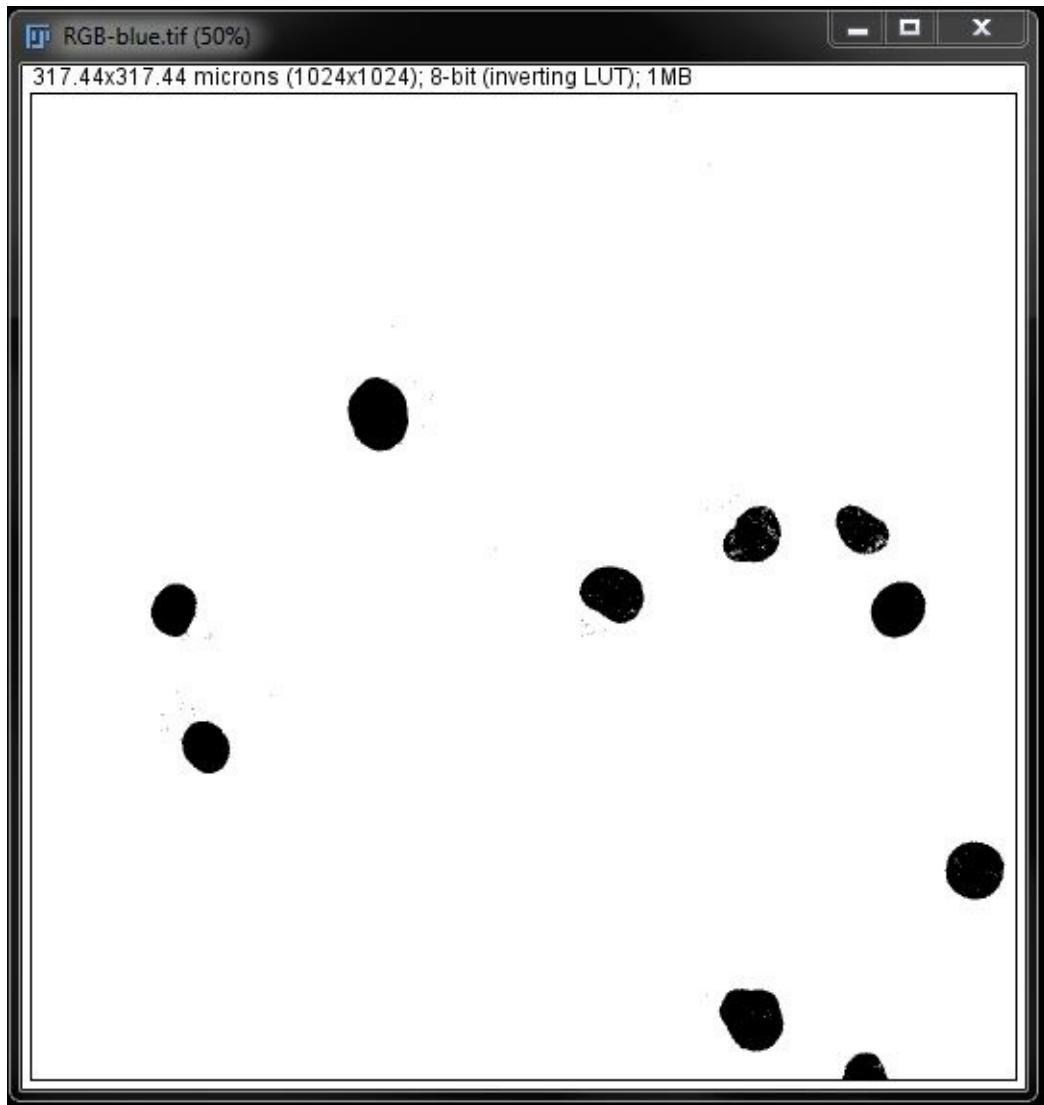
Fit your threshold to the data as best as possible. Here I have used the *Default* algorithm set at 65 and 255. For your own data you will need to find a threshold that works for you.



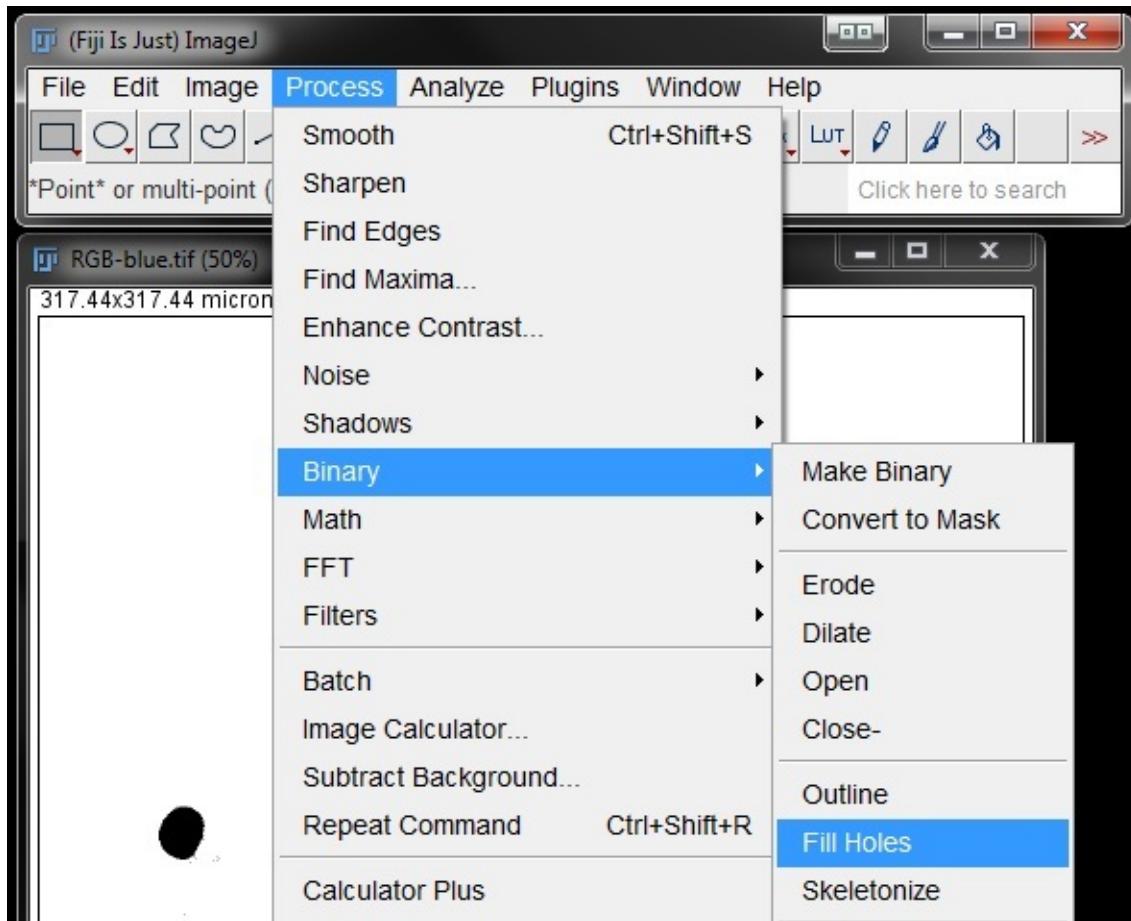
Close the options box to keep the selection as a threshold, or click **Apply** to generate mask as previously described.

If you choose to work with the threshold you can skip straight to measurements from here, however the threshold is not always a perfect selection of the data. Which is why we often create a mask instead.

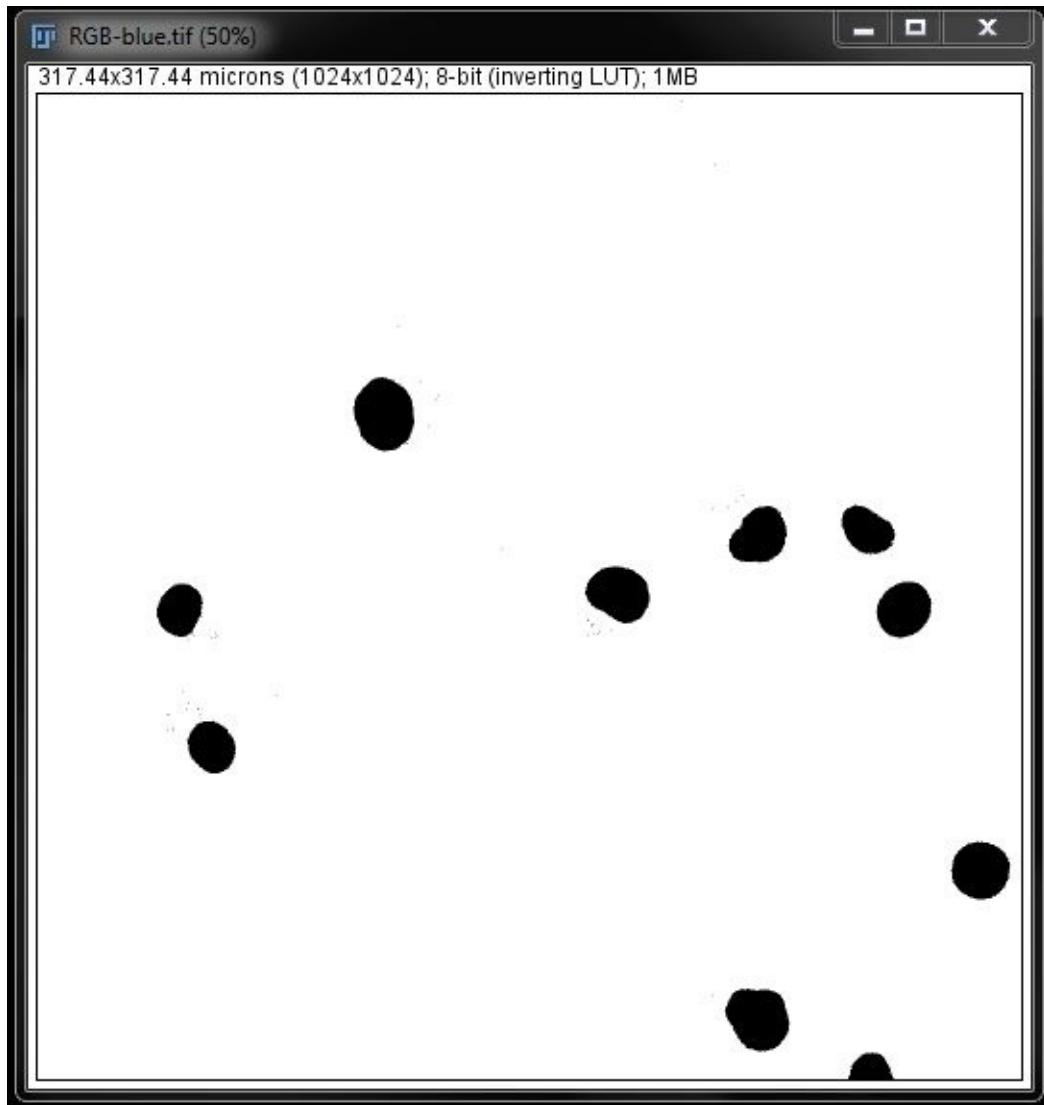
If you are working with a mask you can perfect your selection using filters. In this image mask there are a few blemishes that may affect the measurement. You can see below that we have some small speckled areas detected outside the nuclei, as well as some spots within the nuclei that are not masked completely.



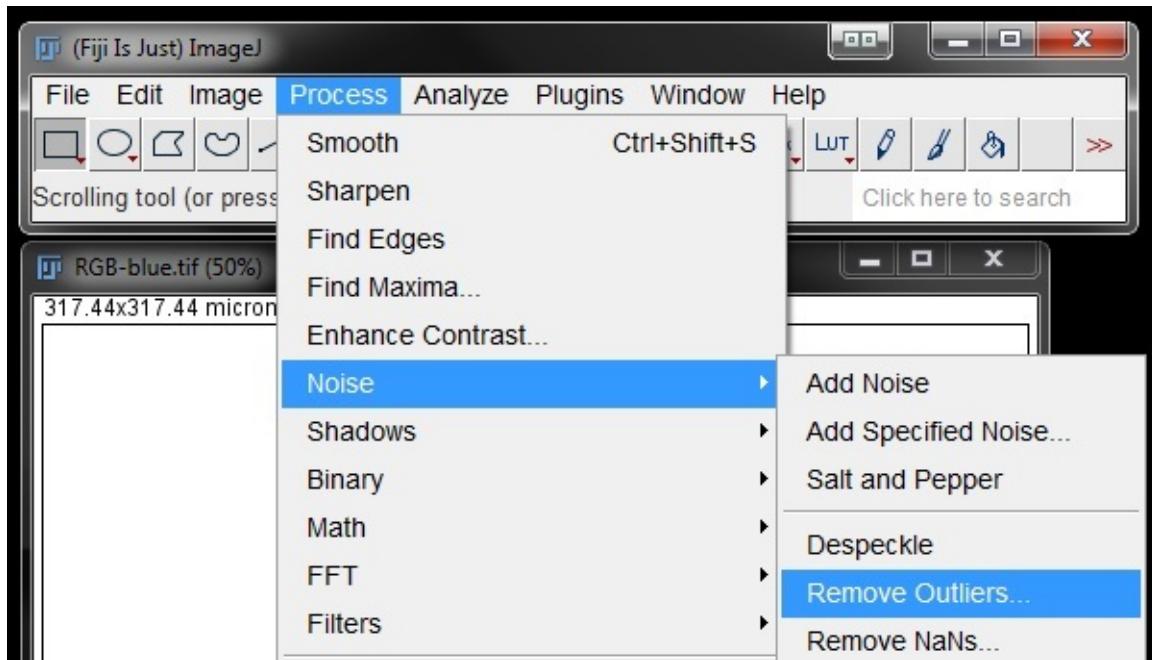
To take care of these 'gaps' in the nuclei mask we are going to use the **Fill Holes** option found under **Process -> Binary -> Fill Holes**.



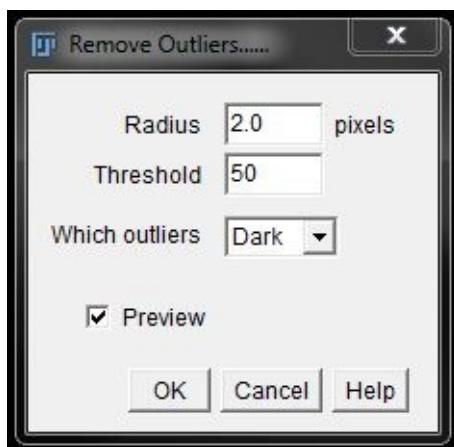
This will complete the mask over areas that contained gaps previously.



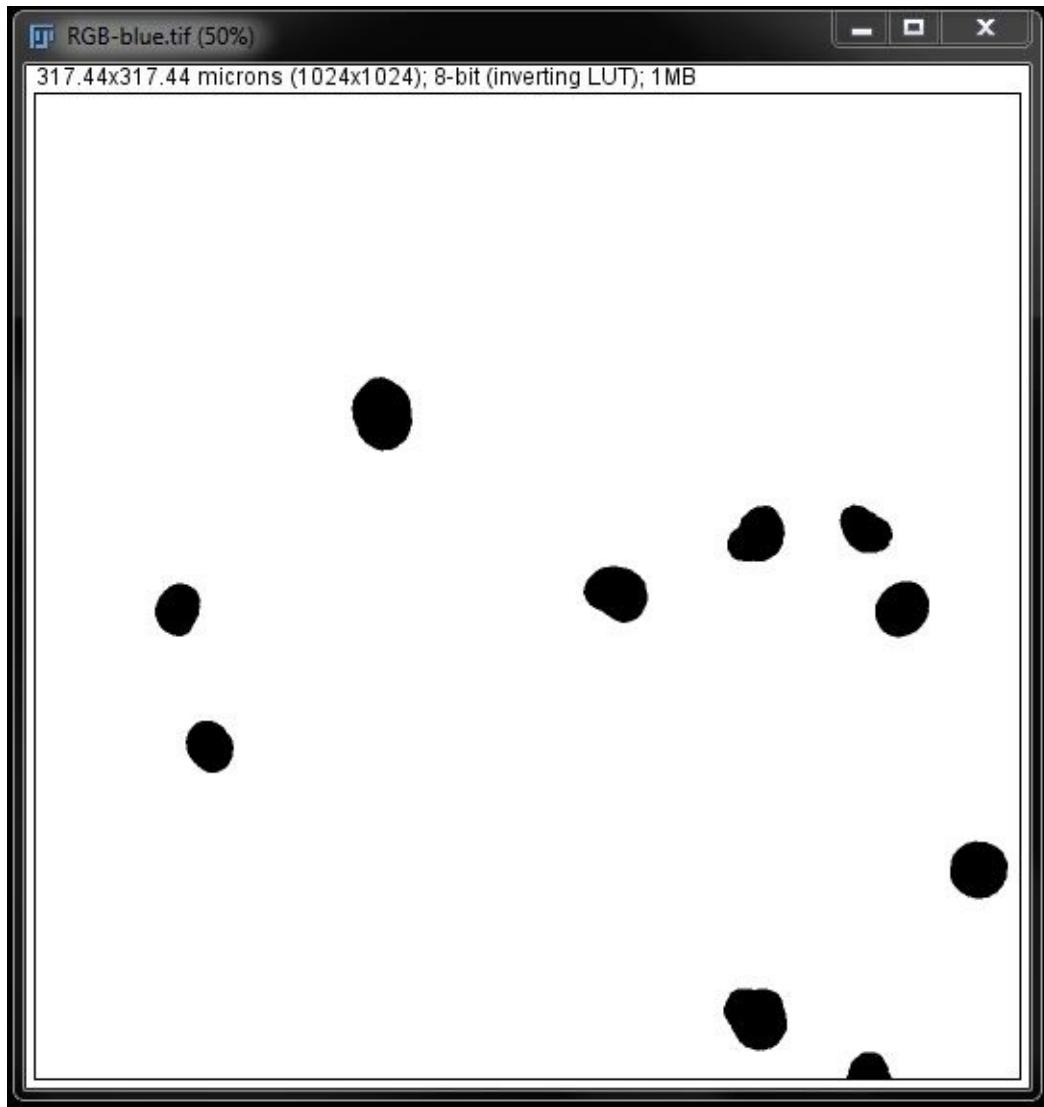
Next we will remove the small spots detected outside the nucleus using the **Remove Outliers** filter. Go to **Process -> Noise -> Remove Outliers**.



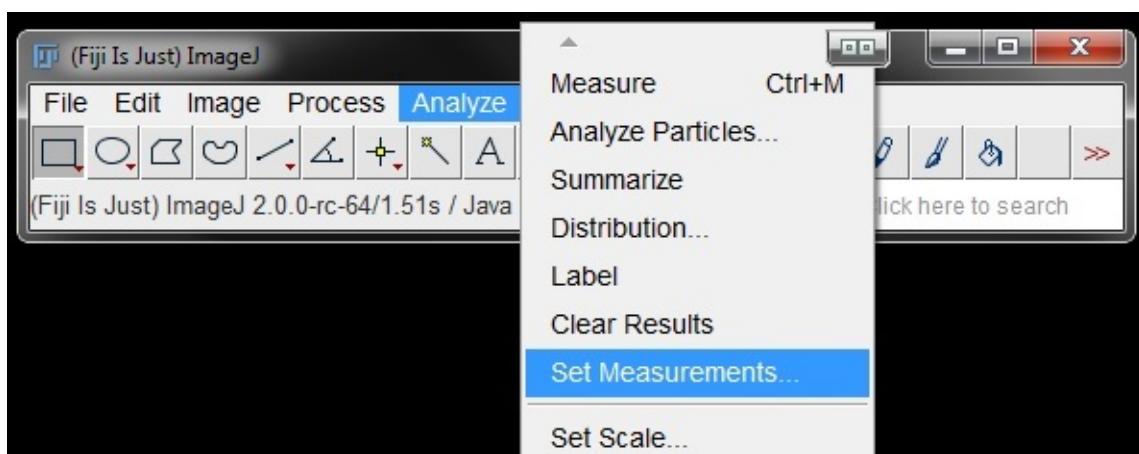
Turn on the preview, ensure it is set to act on the Bright or Dark part of the mask under the *Which outliers* drop down menu (this will be dependant on your mask colours - for me it needs to be set to Dark). Set a pixel radius that captures all spots outside the nuclei, in my example the default setting of 2 works nicely. Find an option that works for your image. Click **OK** to apply the filter.



You should now have a mask that nicely represents the nuclei in the original image.



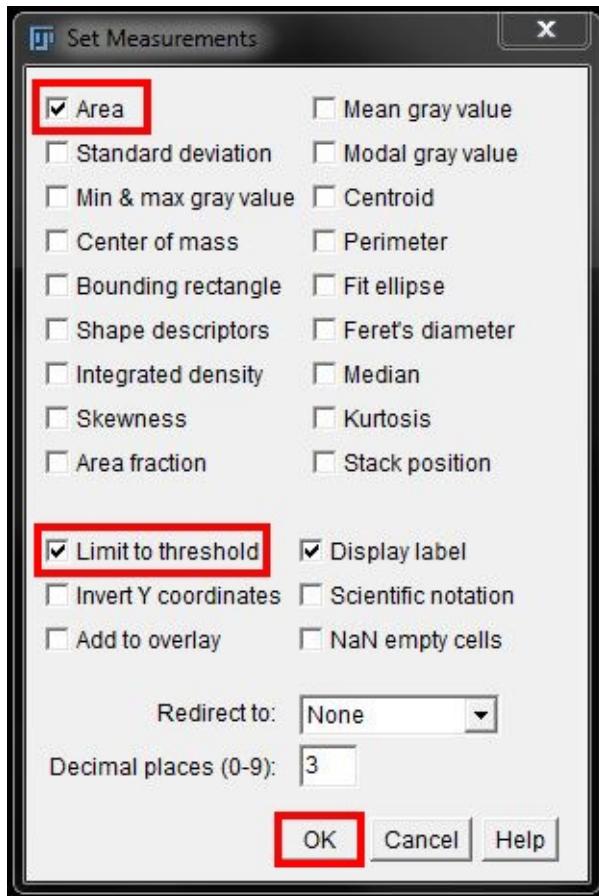
To measure the area in either your thresholded image or your mask, you first need to set your output parameters. Go to **Analyse -> Set Measurements**.



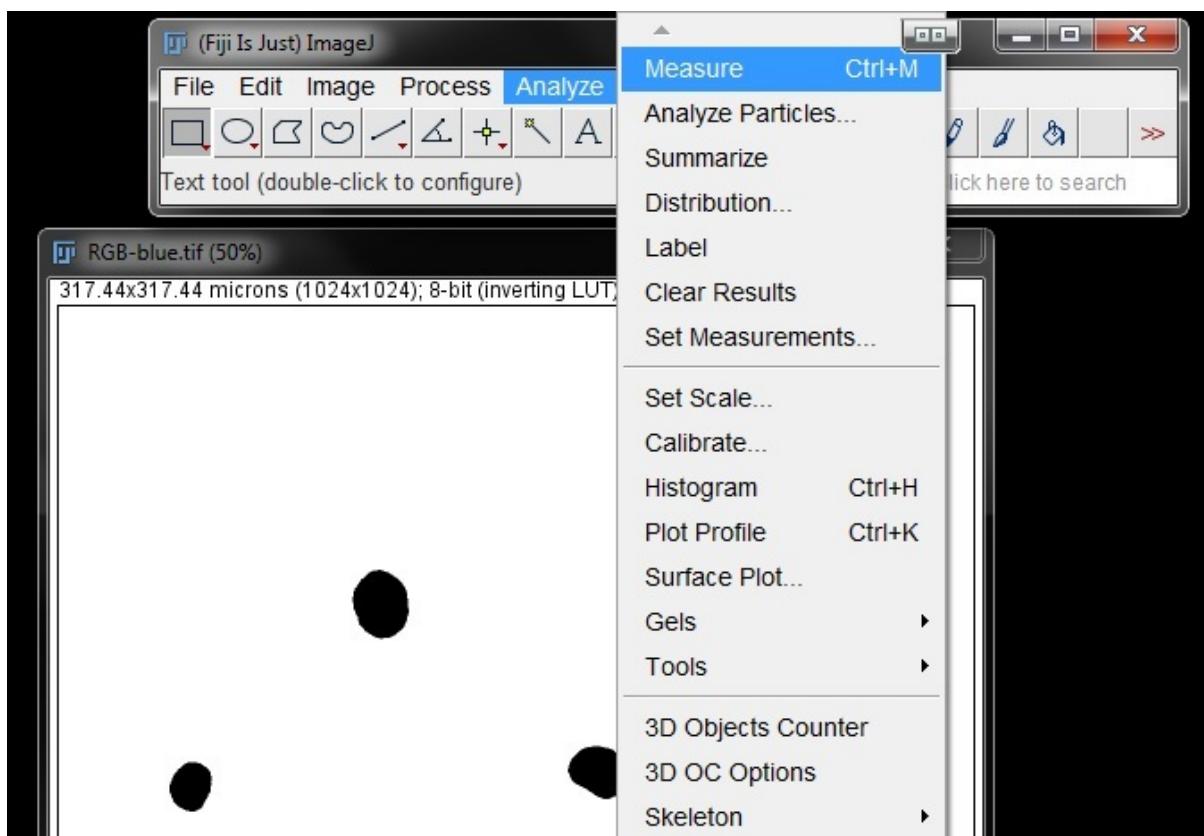
In the **Set Measurements** window you can choose what you want to measure by clicking on or off the checkboxes beside different parameters. Here we will turn on **Area** only.

Ensure you also have **Limit to threshold** selected in the options so that FIJI does not measure the entire image.

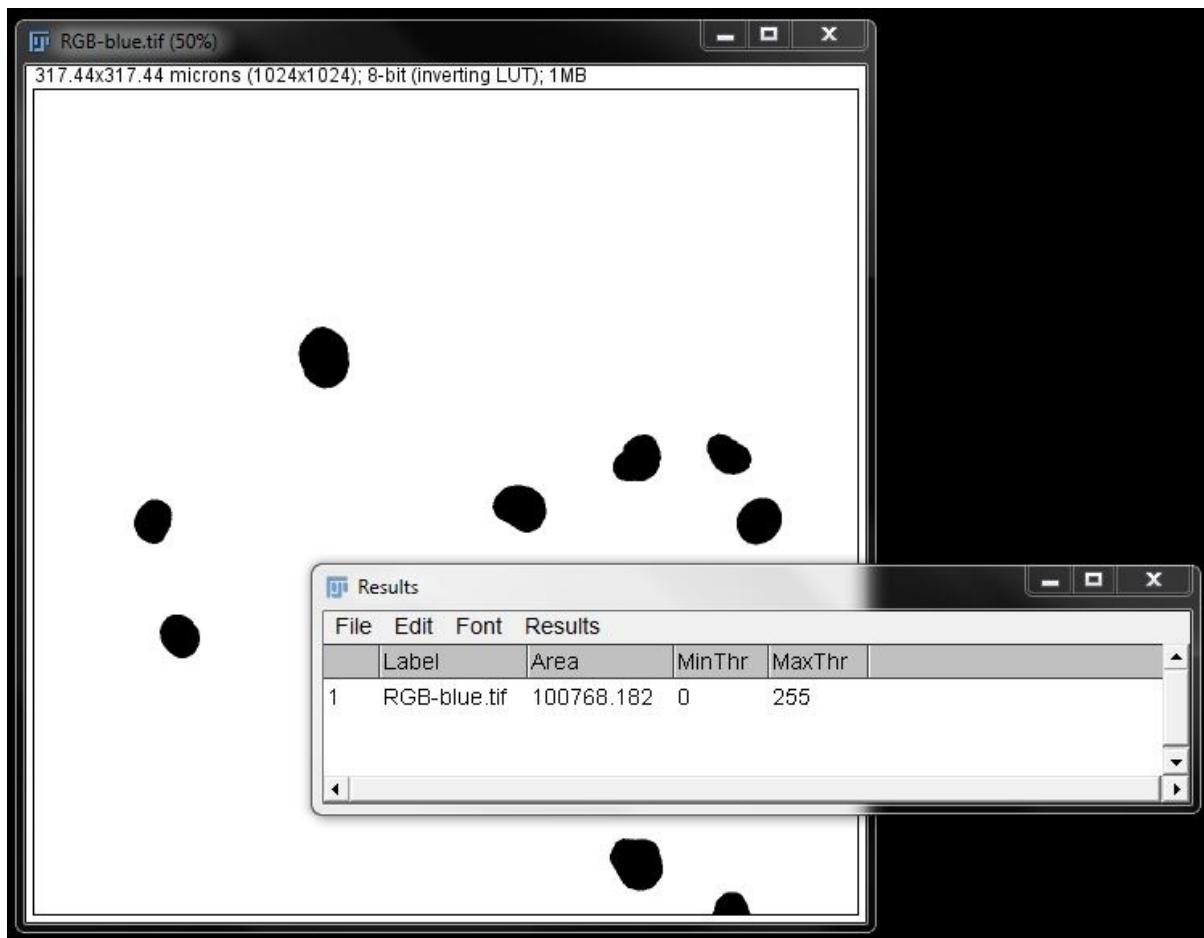
Click **OK** to save the output parameters.



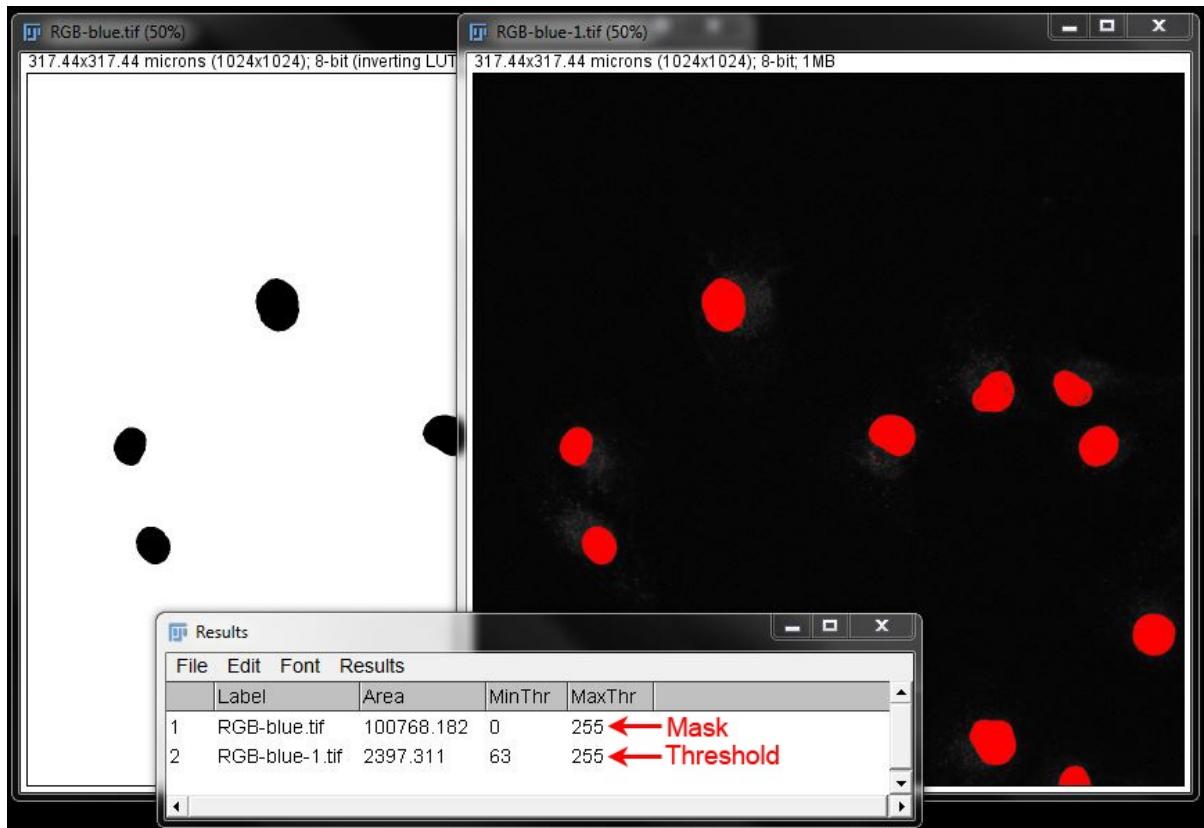
Now that the parameters are set, to measure the area of the nuclei using the parameters you have just set go to **Analyze > Measure** (or shortcut **Ctrl+M**).



This will give you a results table with the area of the nuclei. You will notice there is only one measurement. This is because this method measures the mask or threshold as a single entity.

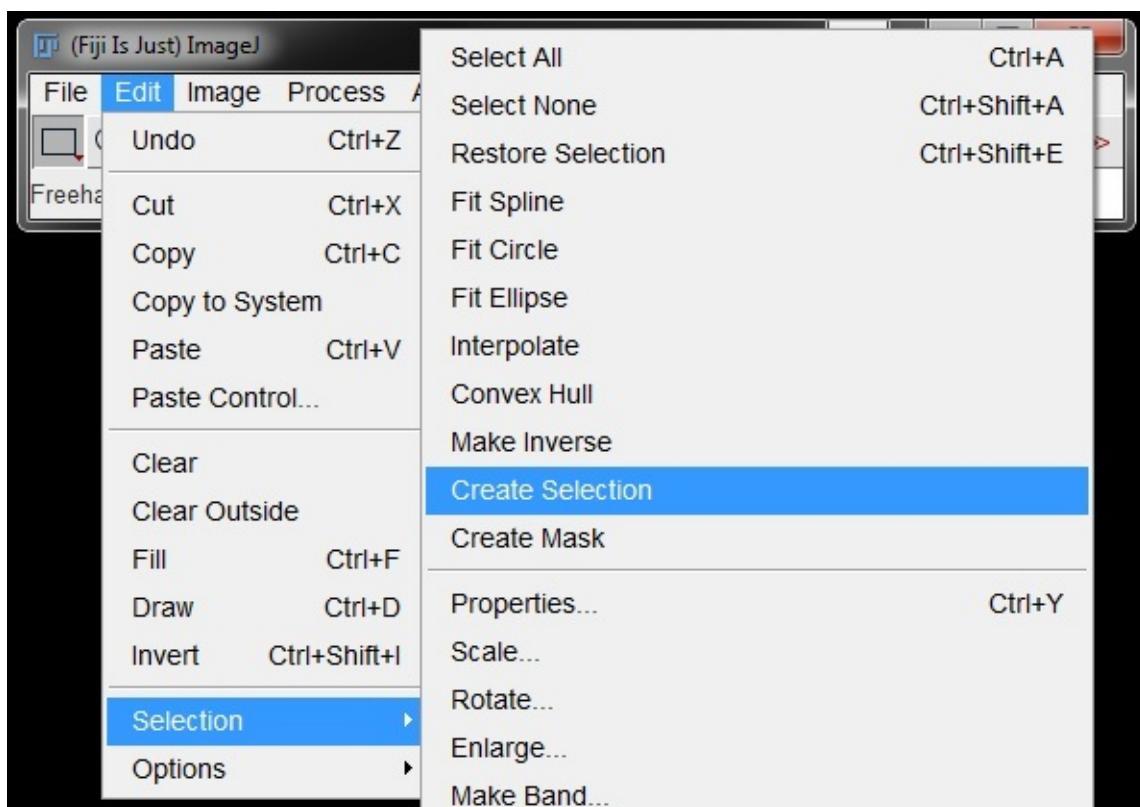


If you have used a mask for your measurement, you may notice a rather large number. Compare here the measurements from a Mask and a threshold on the same image.

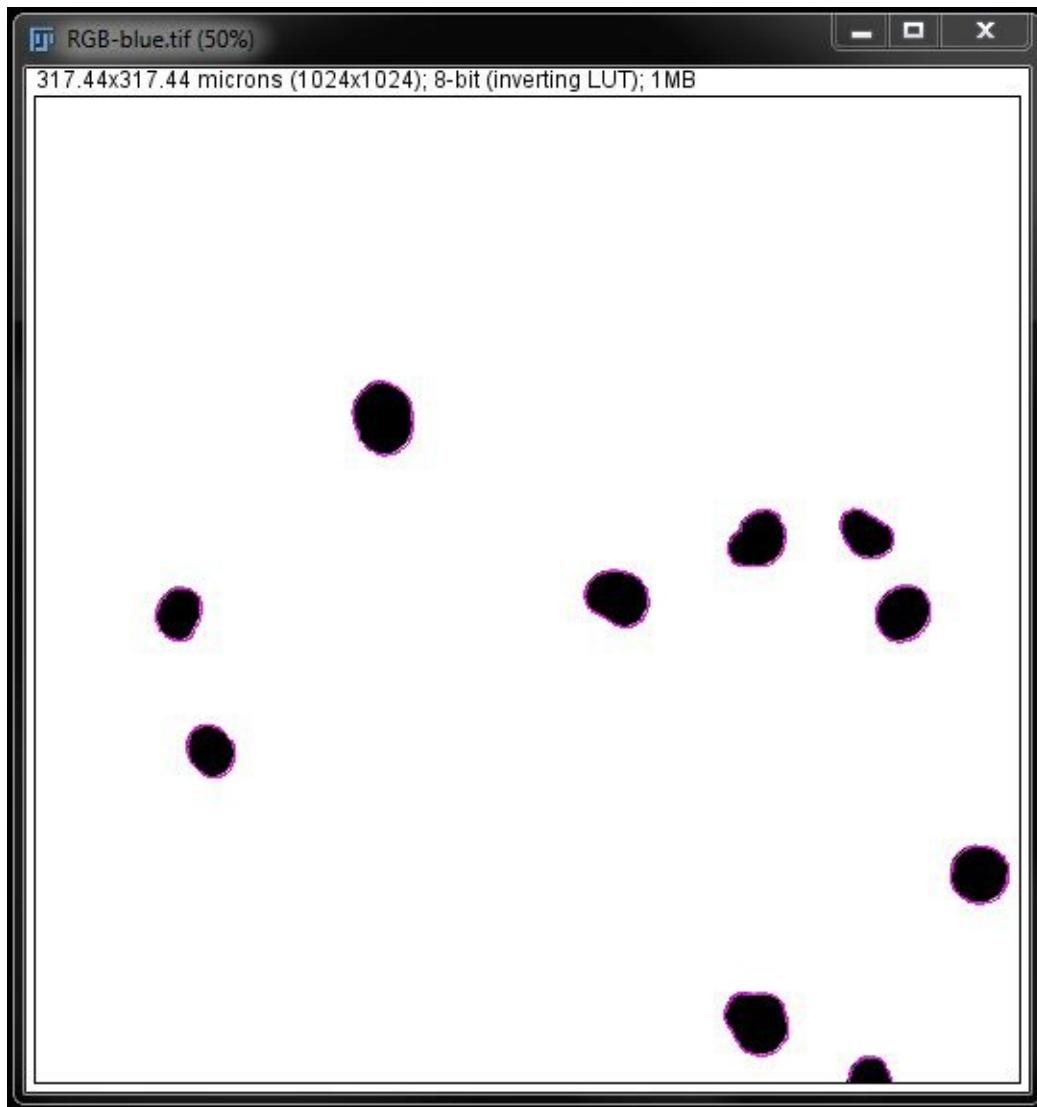


Sometimes FIJI will measure a mask as expected perfectly fine. But often It will still measure the entire image, disregarding the selected/masked area. You can fix this with a simple additional step.

Go to **Edit > Selection > Create selection**. (Masked area must be black on a white background here as the ROI selection will be for the black component of the image)



ROIs will appear around all of the masked nuclei.



Repeat the measurement (**Analyze -> Measure**). You should have the correct value now. Compare this result to the previous 2 values.

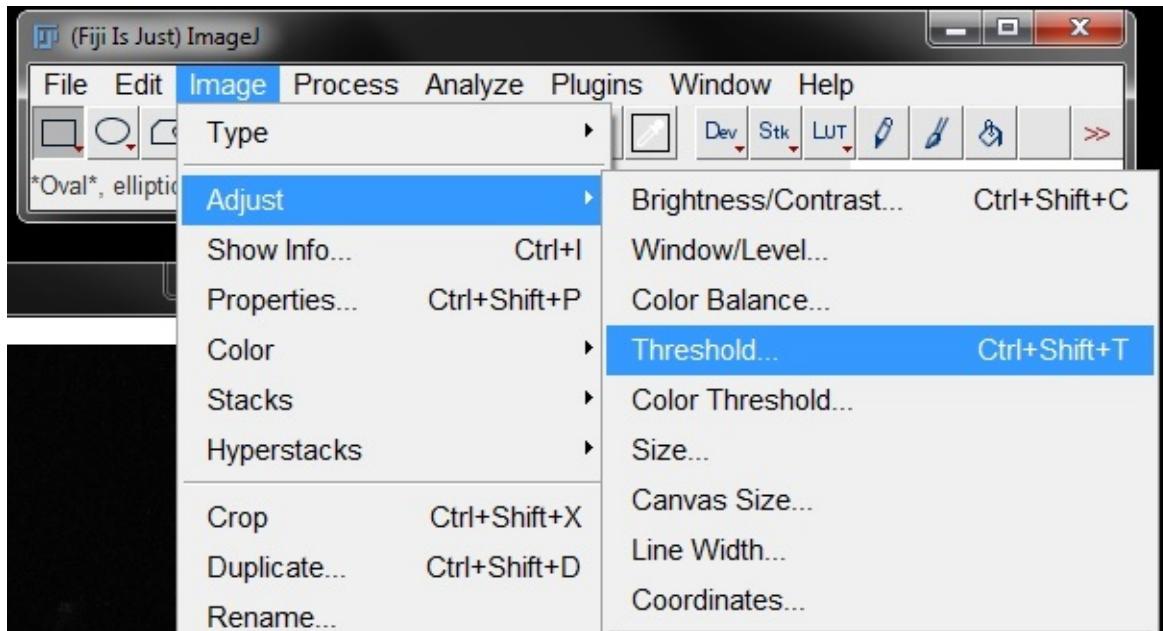
Results					
	Label	Area	MinThr	MaxThr	
1	RGB-blue.tif	100768.182	0	255	← Mask
2	RGB-blue-1.tif	2397.311	63	255	← Threshold
3	RGB-blue.tif	2395.197	255	255	← Mask Selection

## Mean Intensity Measurements

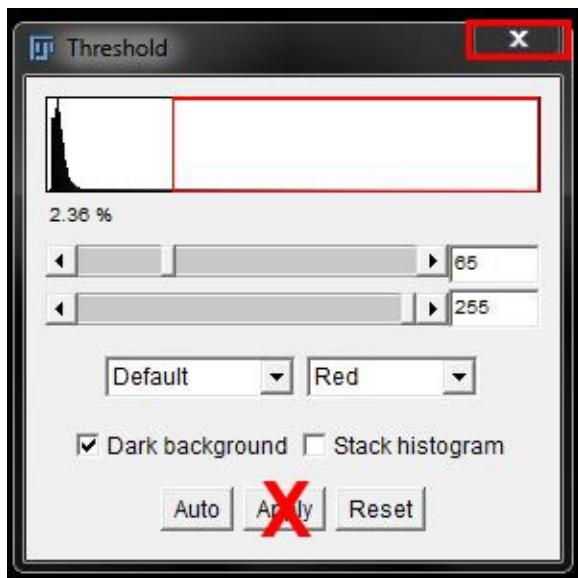
To generate a basic intensity measurement we apply a similar method as the basic area measurement. However, for intensity measurements, even with the output parameters set to **Limit to threshold**, or using ROIs, if we try to measure a mask we will only get intensity readings on the values within the mask itself (0 and 255 as it is a black and

white image). Therefore, when measuring intensity the threshold MUST be maintained, never converted to a mask or binary.

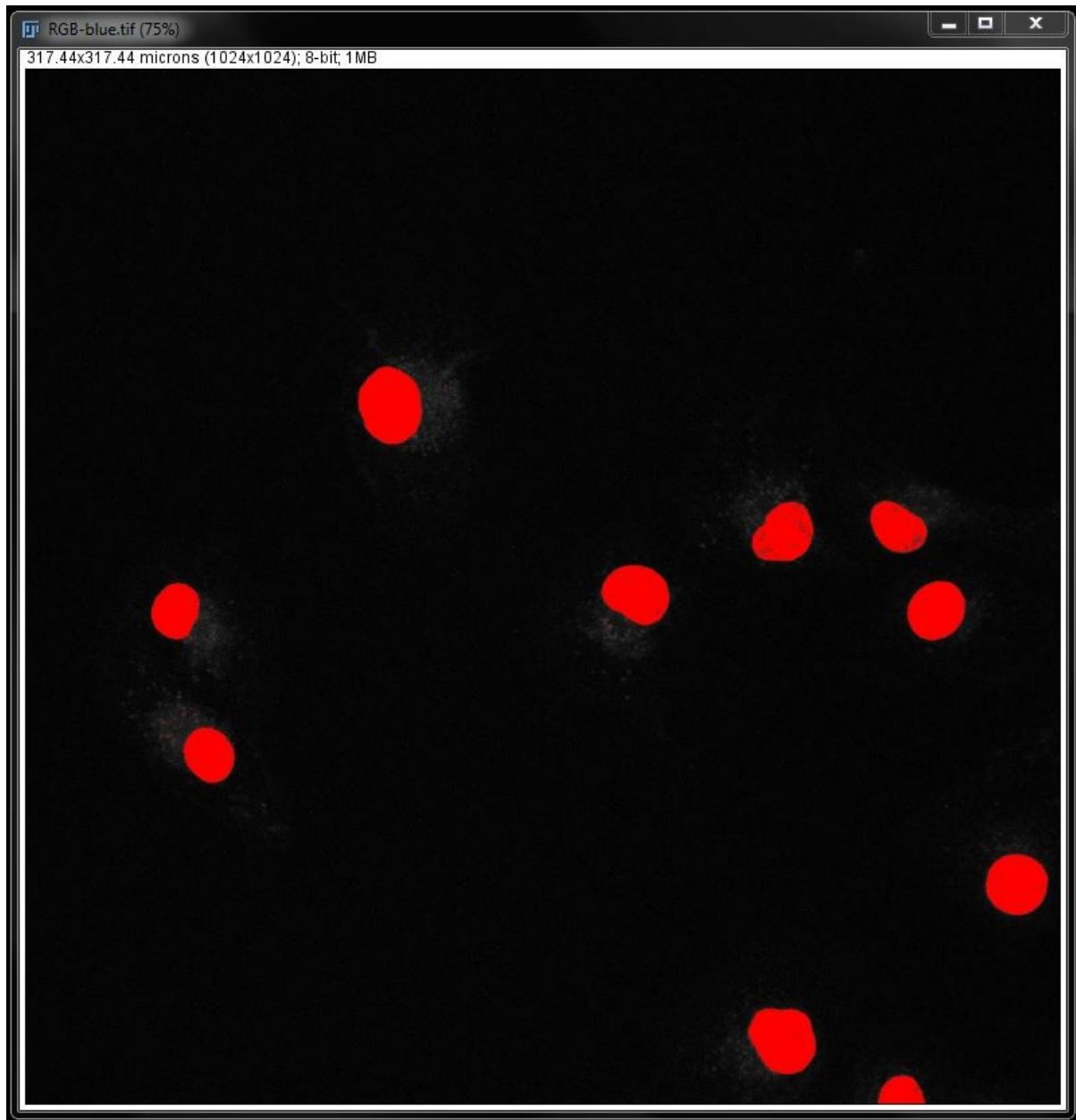
To measure intensity, open the image *RGB\_Blue.tif* and repeat your thresholding. Go to **Image > Adjust Threshold**.



Find the threshold to best fit the data (I have again used Default at 65 and 255). But DO NOT press apply! Close the threshold options box using the cross at the top right.

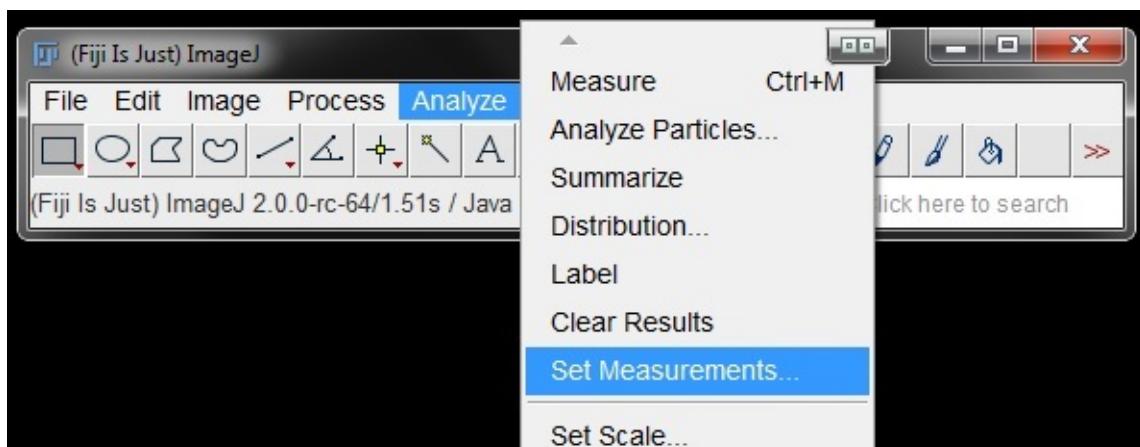


This will give you an image with the stain selected by threshold, but not converted to a mask.



With a threshold we can no longer make modifications in order to perfect our selection. What we see here is what we will measure.

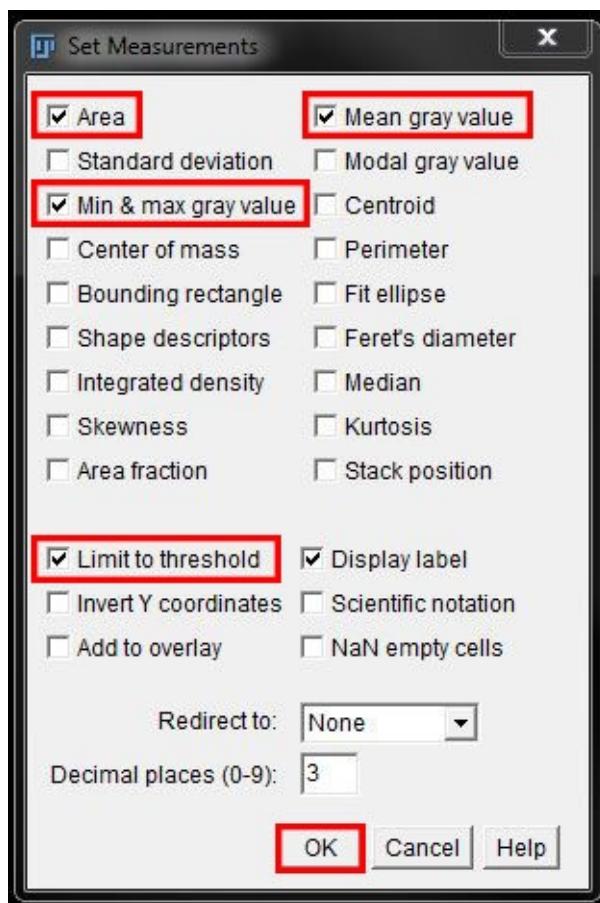
So we can now go straight to setting our measurement parameters. Again go to **Analyse -> Set Measurements**.



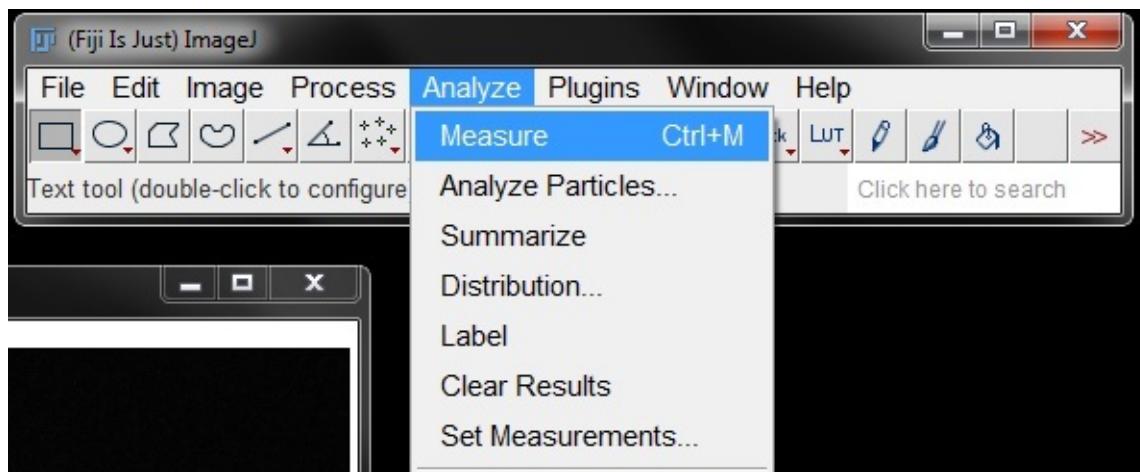
This time we want to set **Area**, **Min & max gray value** and **Mean gray value**.

Again ensure the checkbox next to **Limit to threshold** is selected.

Select **OK** to save the changes.



Repeat the measurement process as before by going to **Analyze -> Measure** (or shortcut **Ctrl+M**).



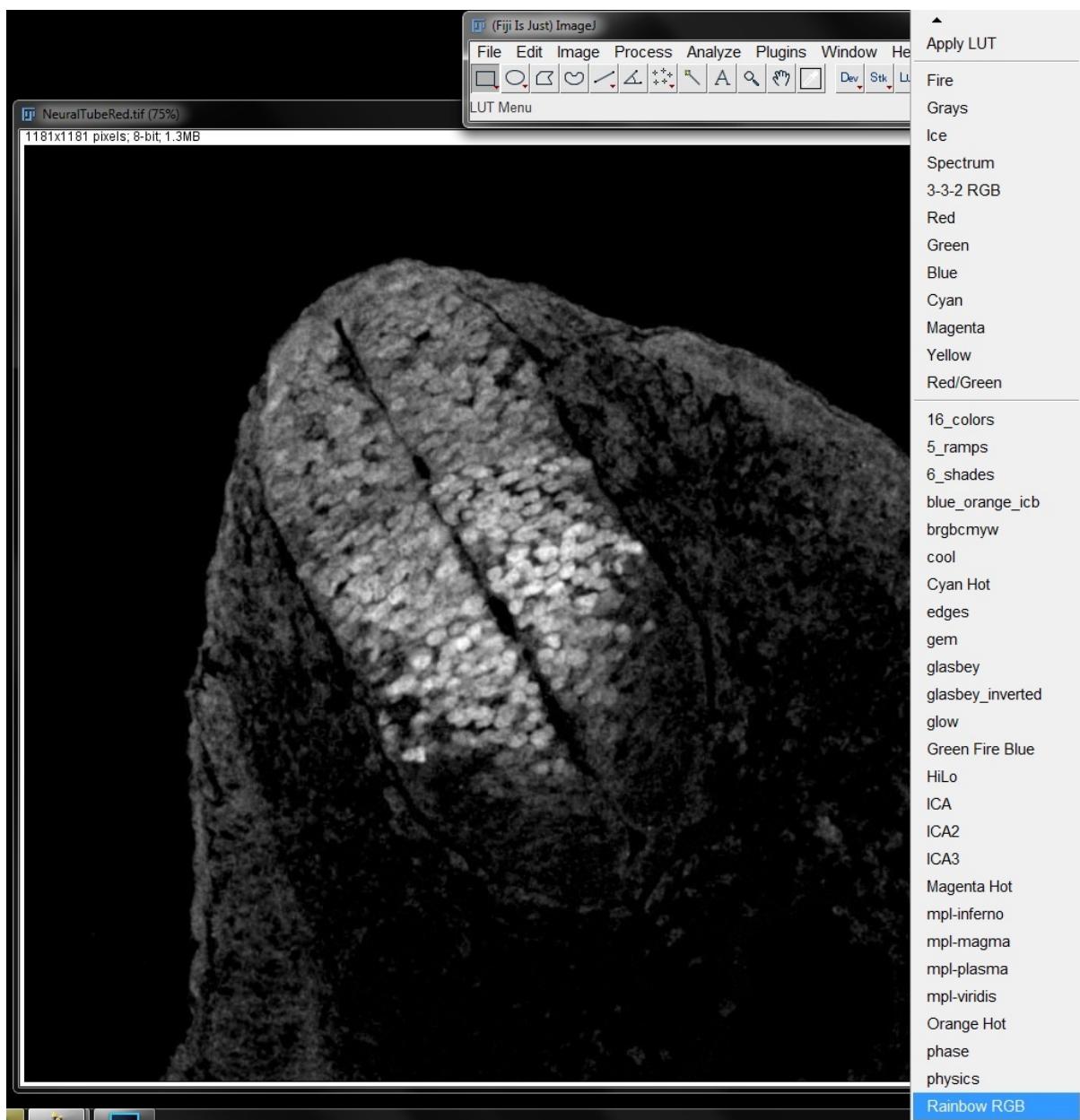
This will give you a measurement for the intensity in the original image, limited to the area under the threshold you have set.

Results								
	Label	Area	Mean	Min	Max	MinThr	MaxThr	
1	RGB-blue.tif	2382.608	118.357	65	255	65	255	

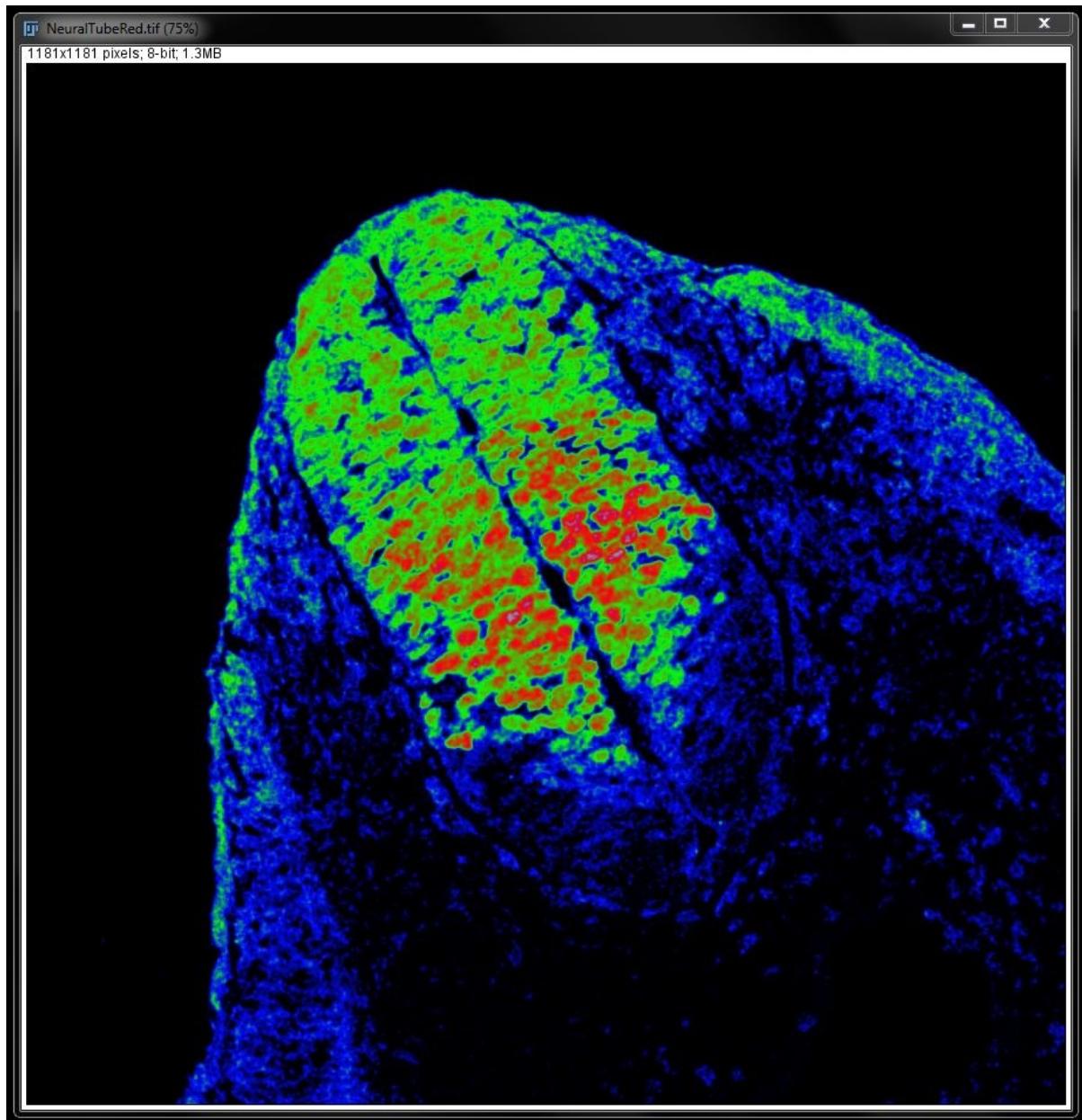
## Intensity Map (Rainbow RGB LUT)

For a visual representation of the intensity, you can map the intensities across the image very simply using a LUT.

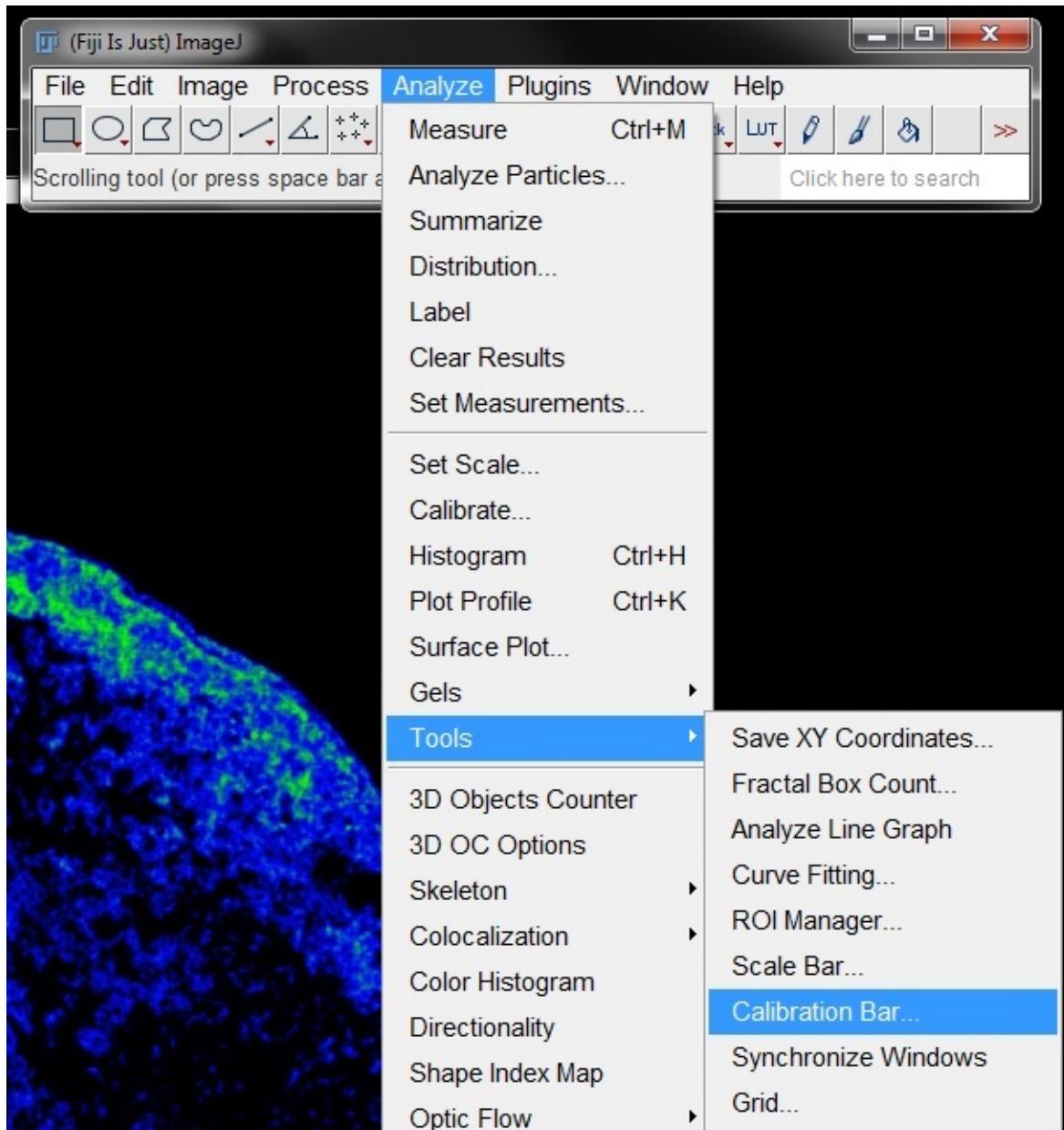
Open the image *NeuralTubeRed.tif* and go to **Image -> Look up Tables** and select the LUT **Rainbow RGB** from the bottom of the list (or select the Rainbow LUT from the LUT menu in the tool bar).



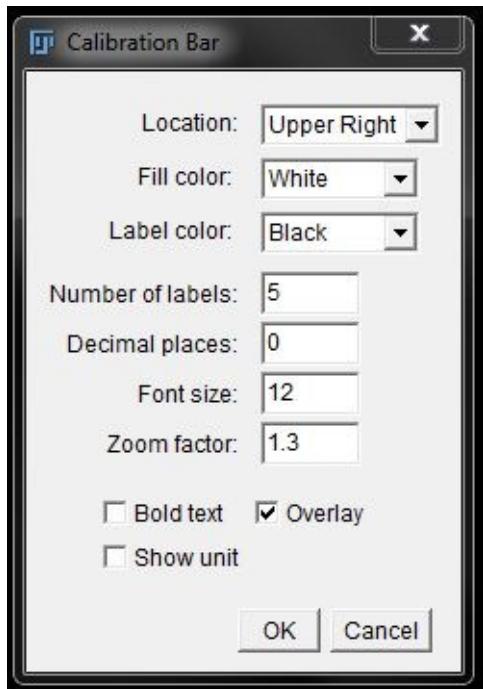
This will apply a multi-coloured LUT to your image, in shades of red, blue or green. The highest 33% of intensities are reds, the middle 33% are greens and the bottom 33% are blues. This gives the viewer the ability to easily see the range of intensities present in the image.



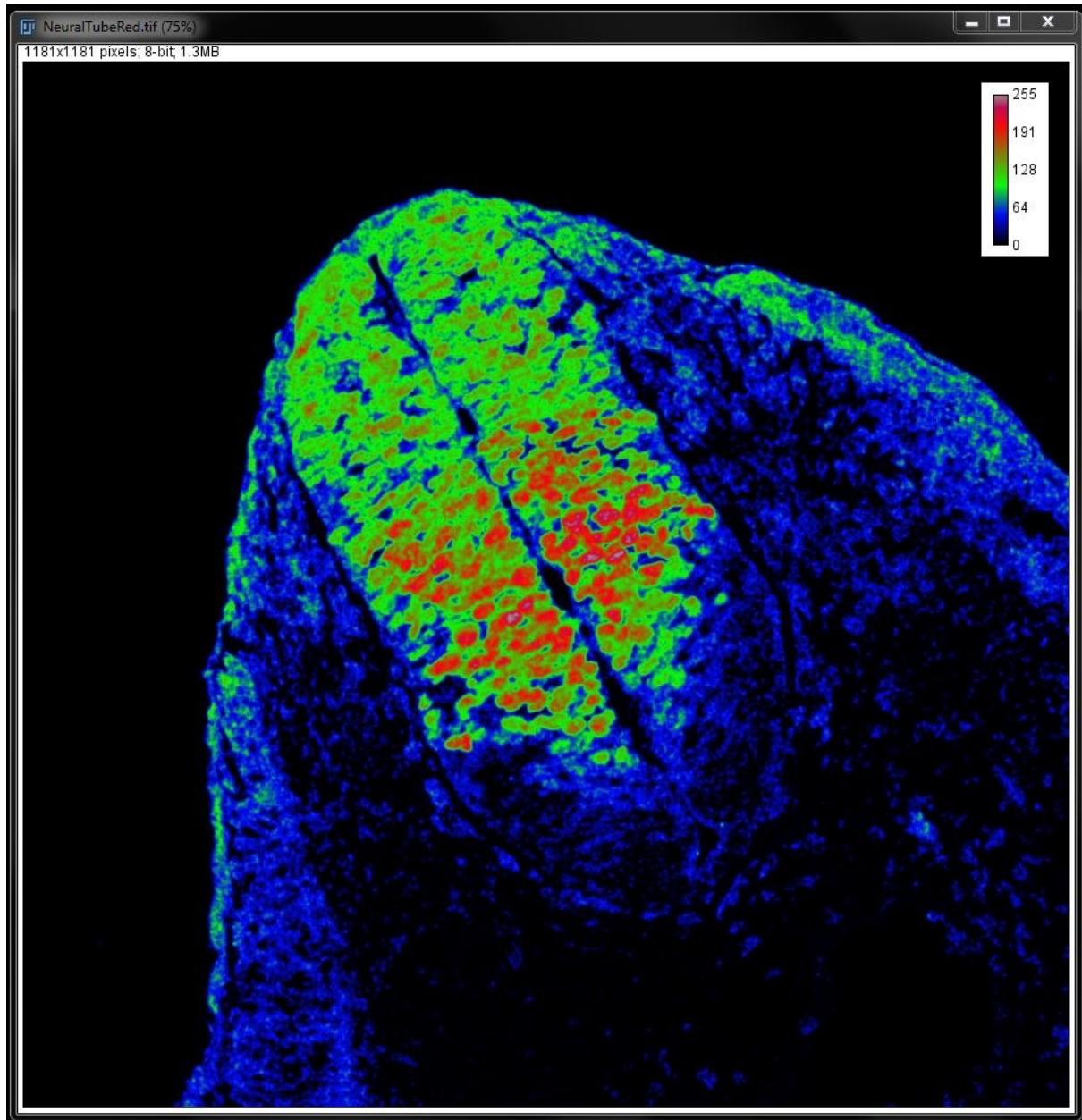
You can add a calibration bar for the intensities by going to **Analyze -> Tools -> Calibration Bar**.



In the **Calibration Bar** window you can specify the position and configuration of the calibration bar. The **Overlay** tick box allows you to create the bar as an overlay of the original image, instead of burning it into the image permanently.



Click **OK** to add the calibration bar to your image.

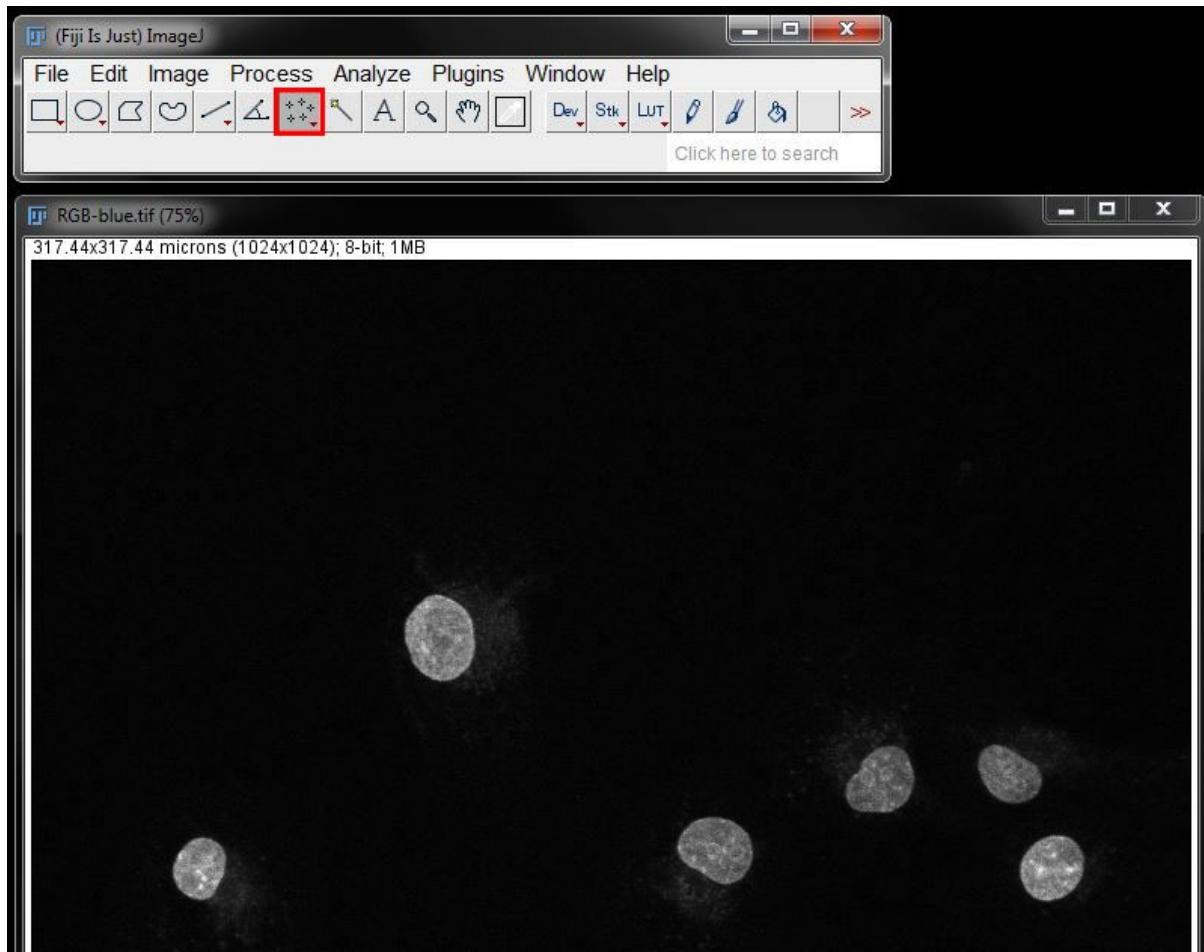


## Simple Counting

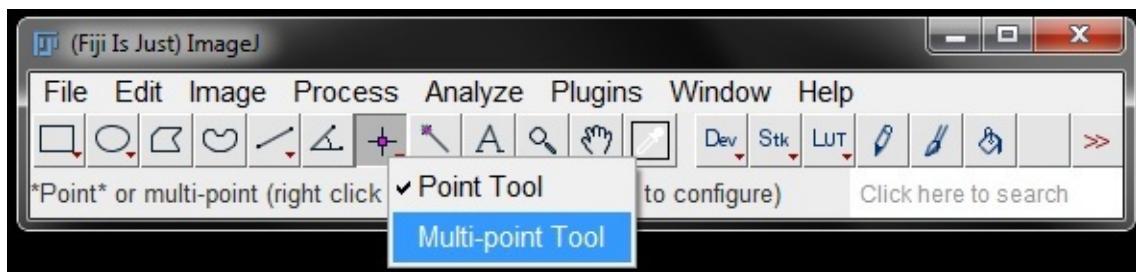
Often we want to know how many cells or objects we have in an image. For a low number of objects you can easily count manually using the multi-point tool.

Open *RGB-Blue.tif* and select the multi-point tool from the FIJI tool bar (as demonstrated in FIJI Basics).

If multiple points are showing in the tool icon (as below), you can simply click it on.



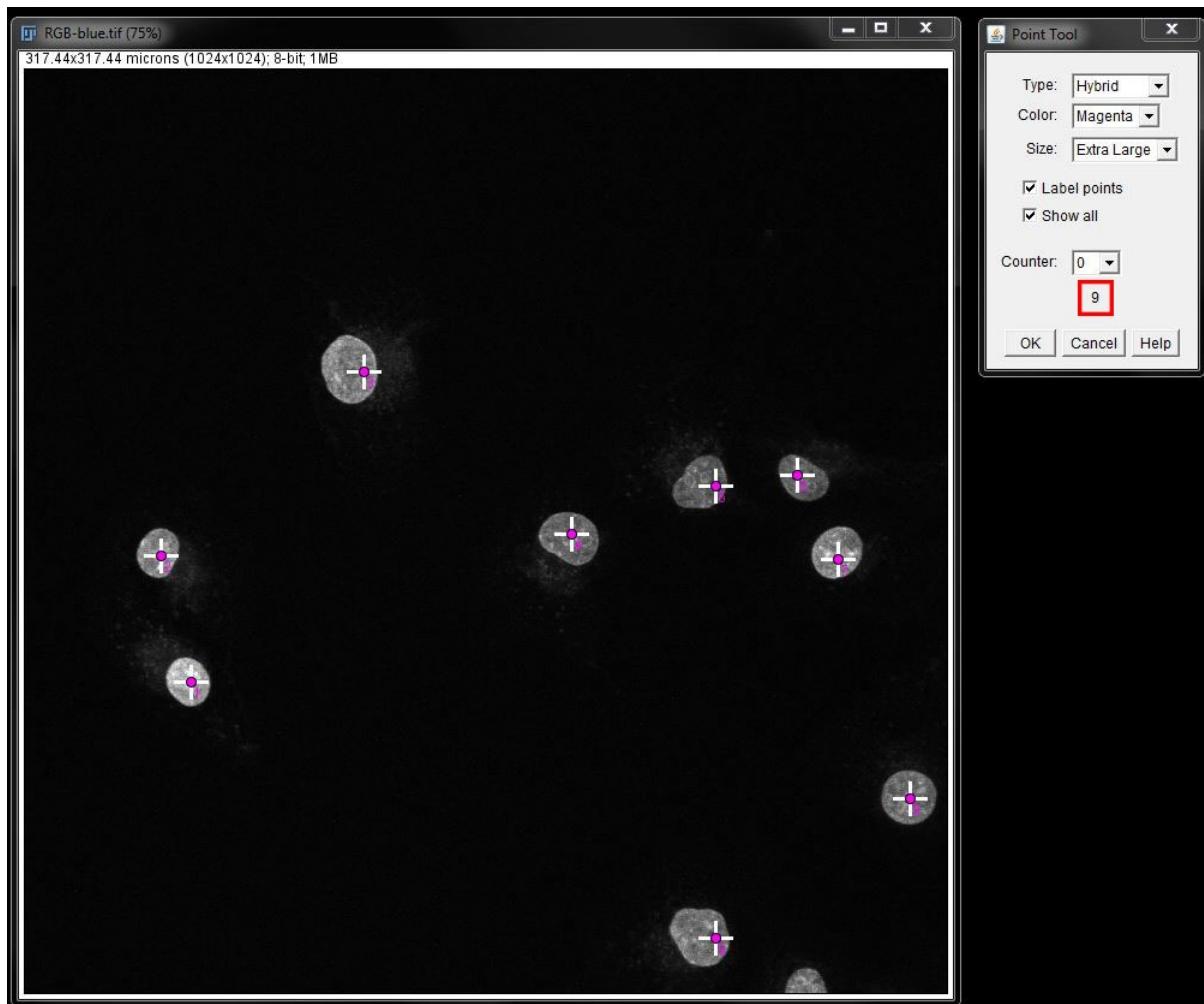
If only a single point is visible on the icon, switch it to multi-point by right clicking and selecting **Multi-point Tool** from the options.



Once the tool is active (indented), double click on the icon again to open the **Point Tool** options window. Set up your points as you want them to appear on your image by selecting colour and size from the drop down menus. Ensure **Label Points** and **Show All** are selected. Leave the window open.



Return to your image and click once on every object you want to count. A point will appear on the image for each click. As you click you will see a counter increasing at the bottom of the **Points Tool** window. Once you have clicked on all of your objects, the final number will be your object count. Each object will also be marked in the image as a check of whether you correctly counted the objects.



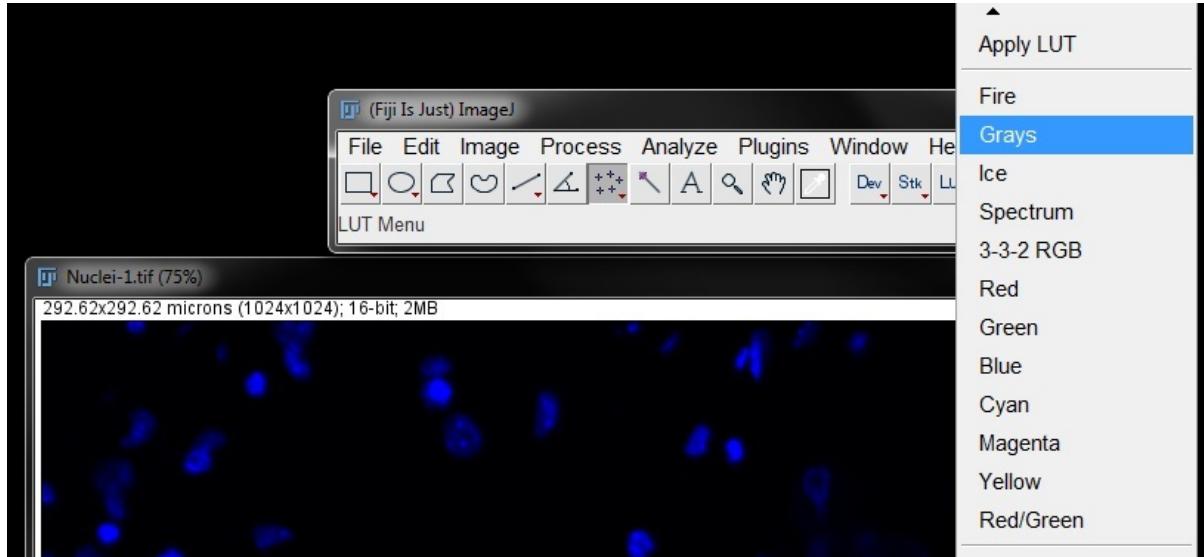
The window shows that we have 9 nuclei in this image.

**Note:** We always exclude partial nuclei/cells/objects at the edges of an image from analysis such as counts.

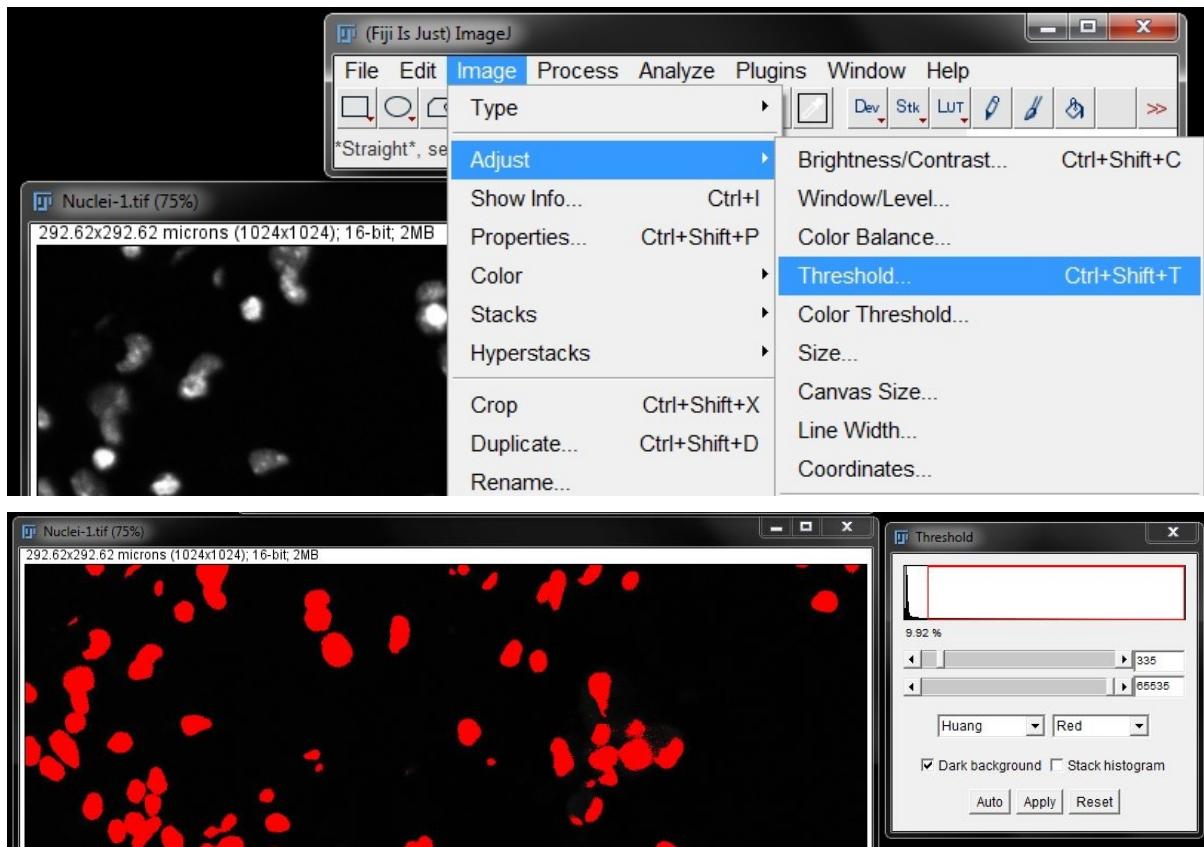
## Analyse Particles

For an image with a large number of objects manually clicking to count can be a laborious task. For these images you can use the **Analyze Particles** tool. This tool also enables you to measure other aspects of the objects (such as area) at the same time as counting.

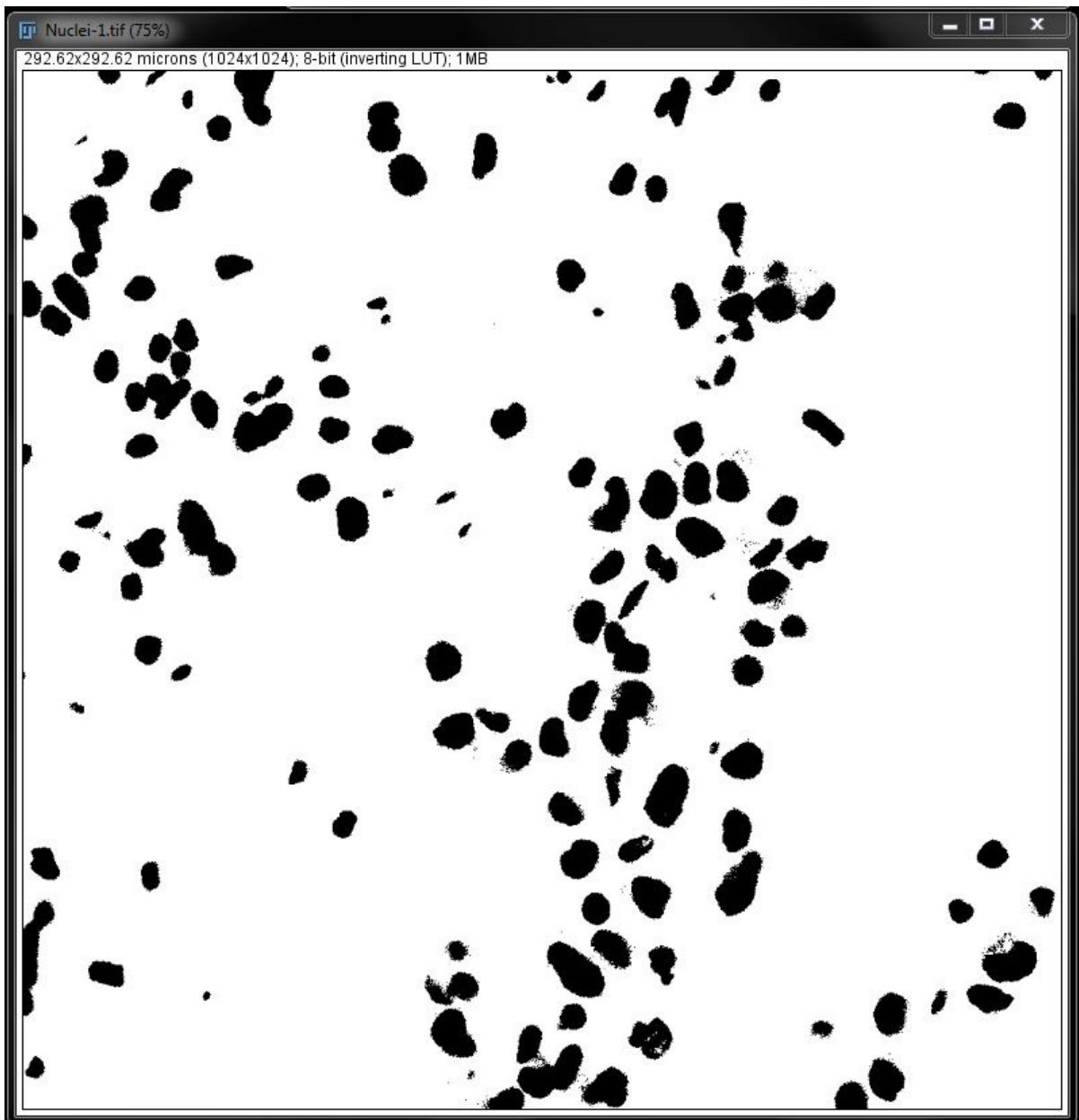
To use this tool, you first need to threshold your image as previously shown. Open the image *Nuclei-1.tif*. Apply a grey LUT if you desire. I find this makes thresholding easier.



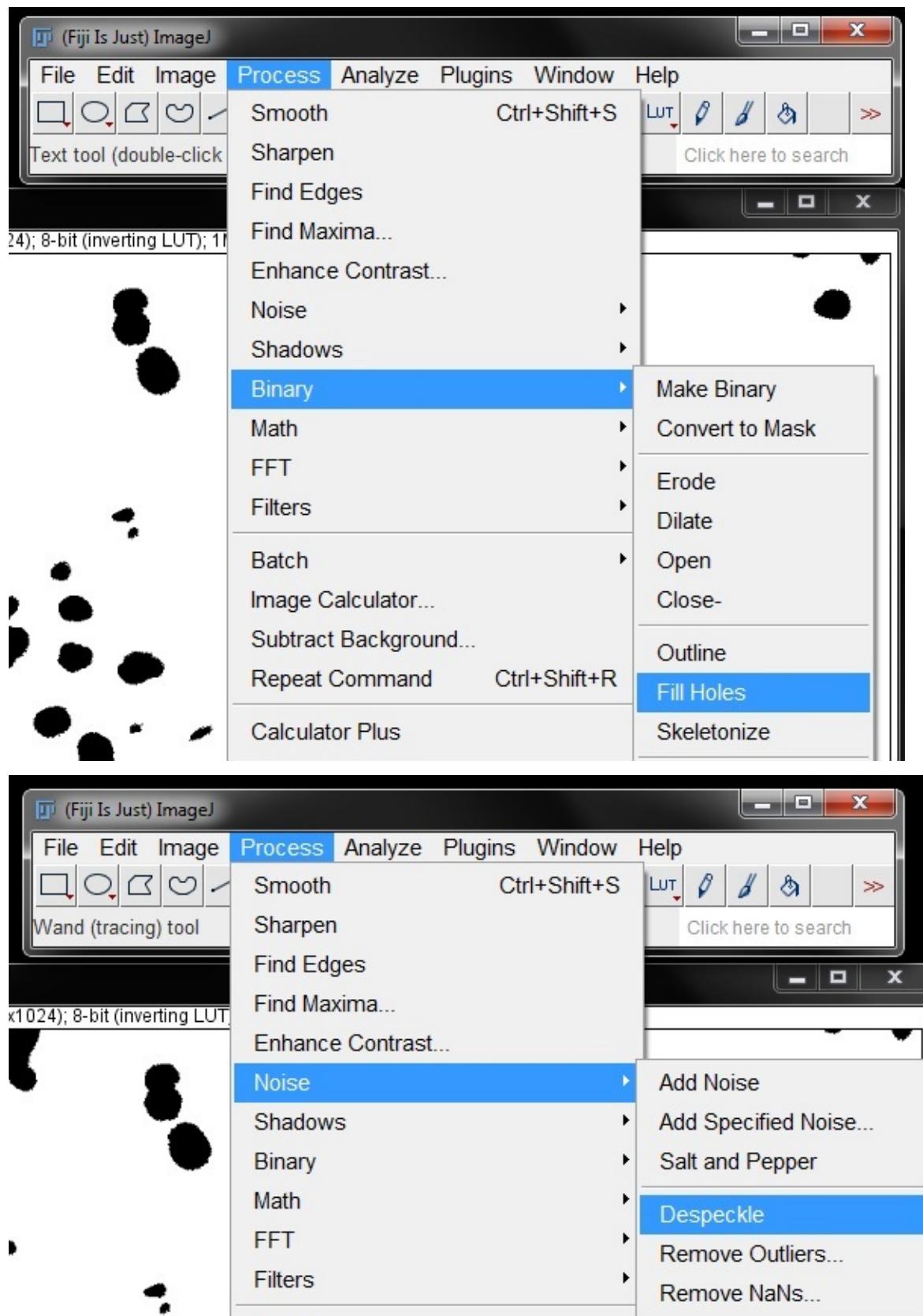
Select a threshold that fits your data then click **Apply** to create a mask (here, I used the Huang algorithm).



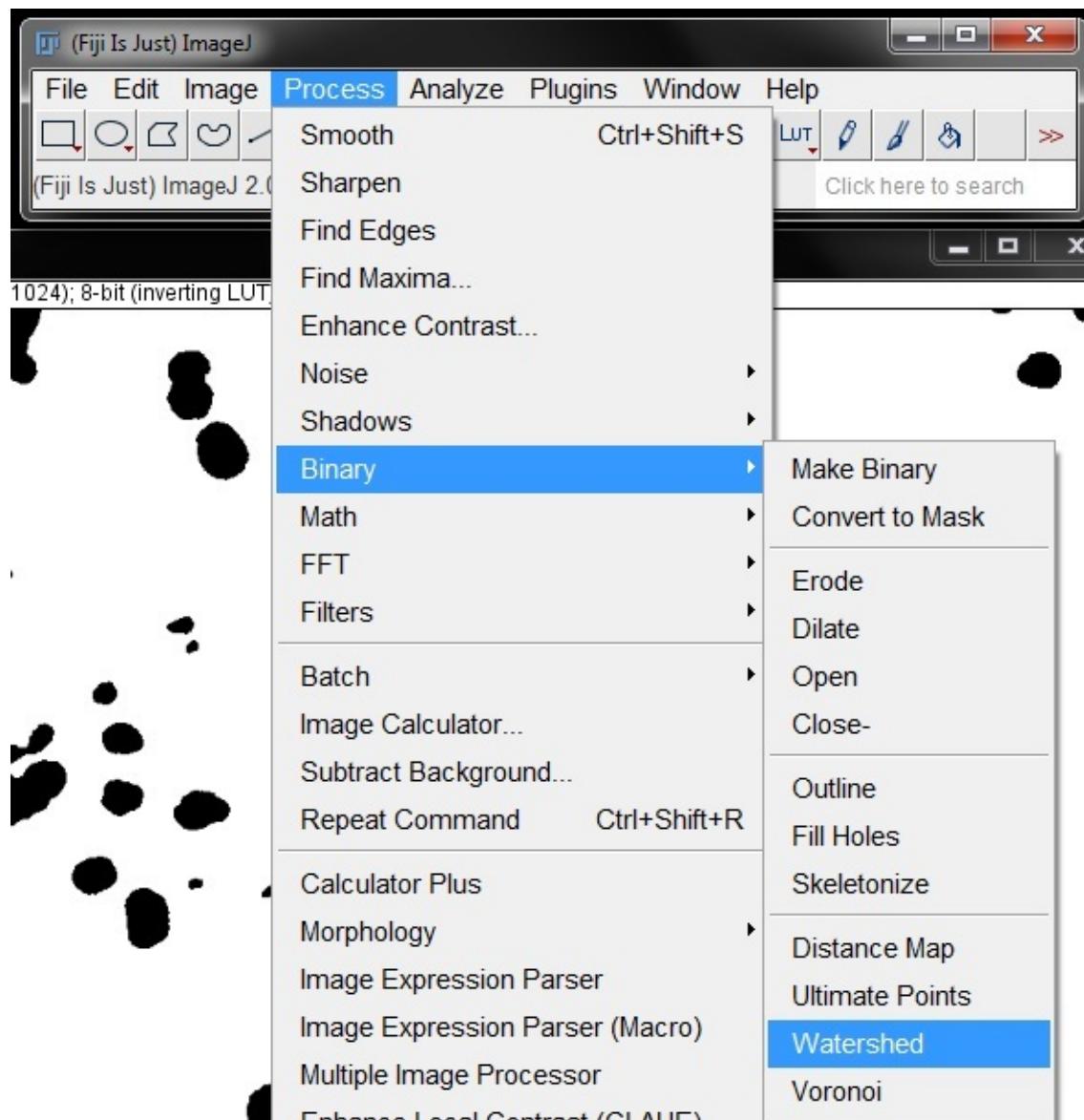
You will see several issues with this mask; gaps within the nuclei and small particles detected outside the nuclei.



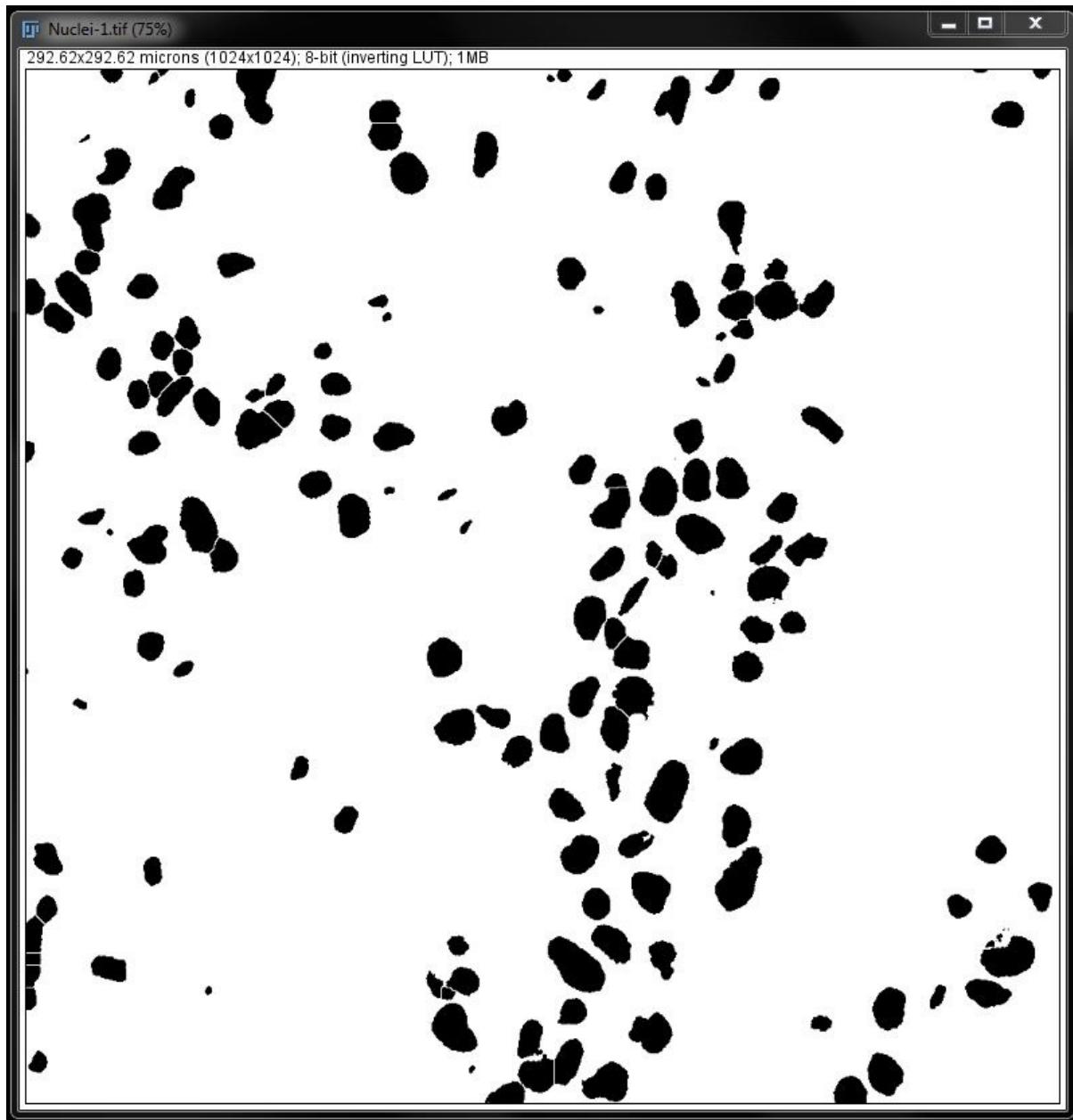
For counting these small particles can be problematic as they will be counted as an object, when they are in fact a false positive. To clean up the mask a little, we will apply the **Fill Holes** and **Despeckle** tools to the mask. Find these under the **Process** menu.



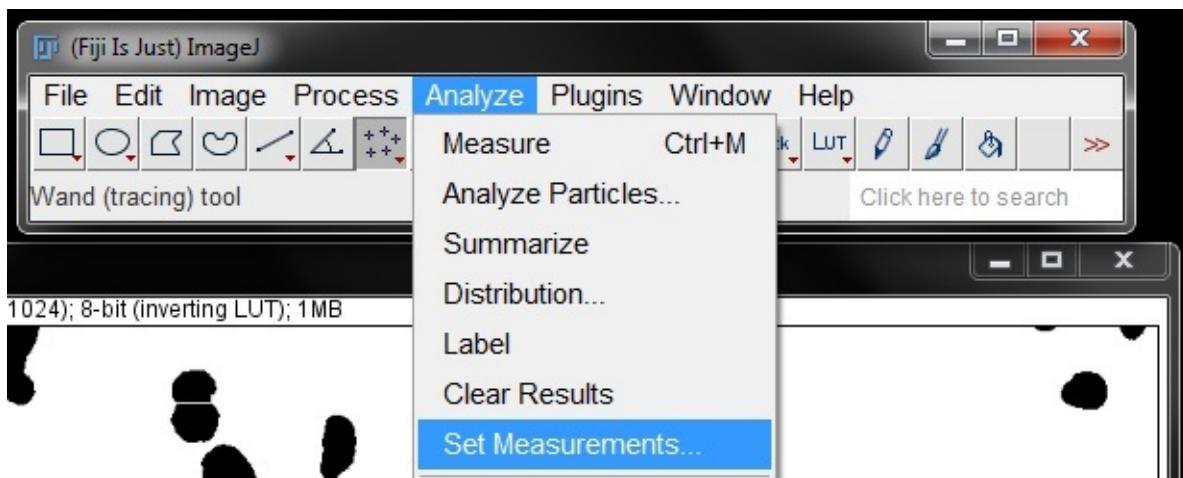
Another problem that will result in a false count with this mask is several areas where nuclei were touching, resulting in them being masked as a single object. To separate these we need to apply a **Watershed** binary filter to the mask. Select this from the **Process -> Binary** menu as previously described to separate touching objects.

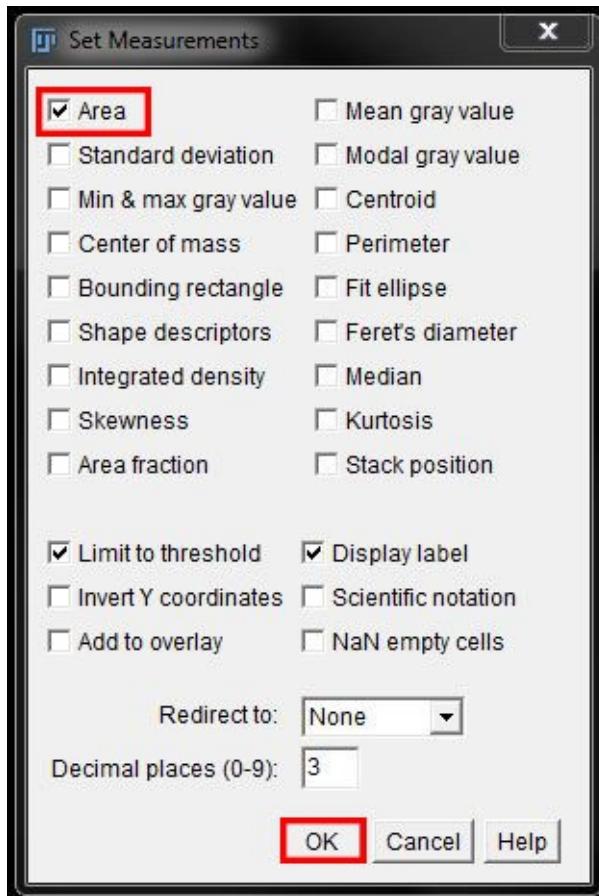


This gives us a fairly neat mask for analysis. There are still a number of small objects that will be counted as false positives but we can exclude them during the analysis process.

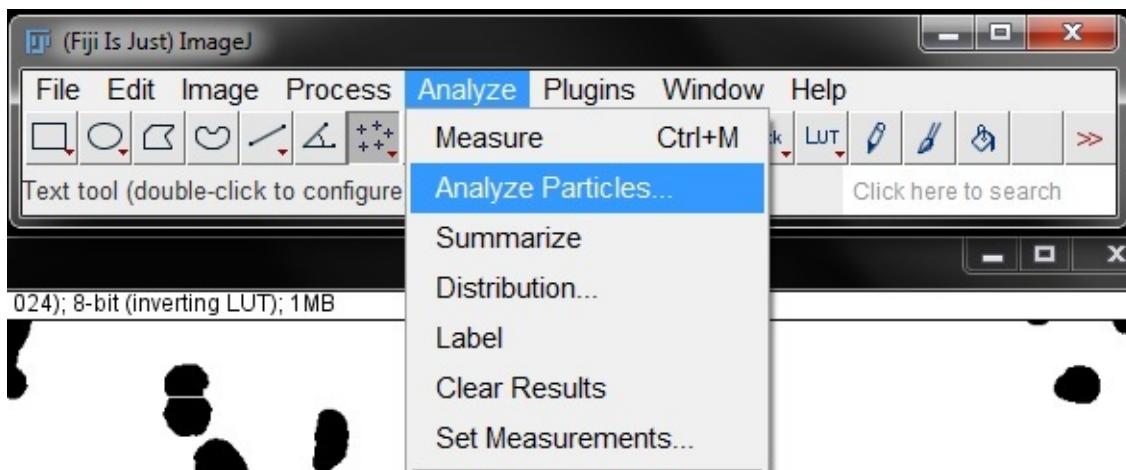


Before we do our analysis, check the measurements you need are set by going to **Analyze -> Set Measurements**. Here we will only check **Area**.

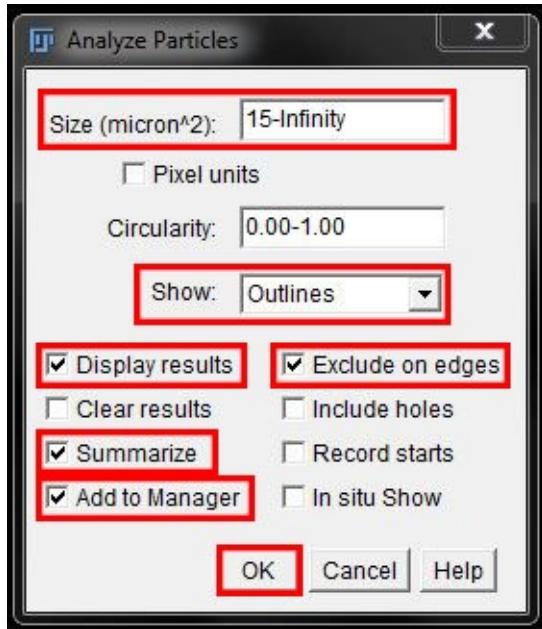




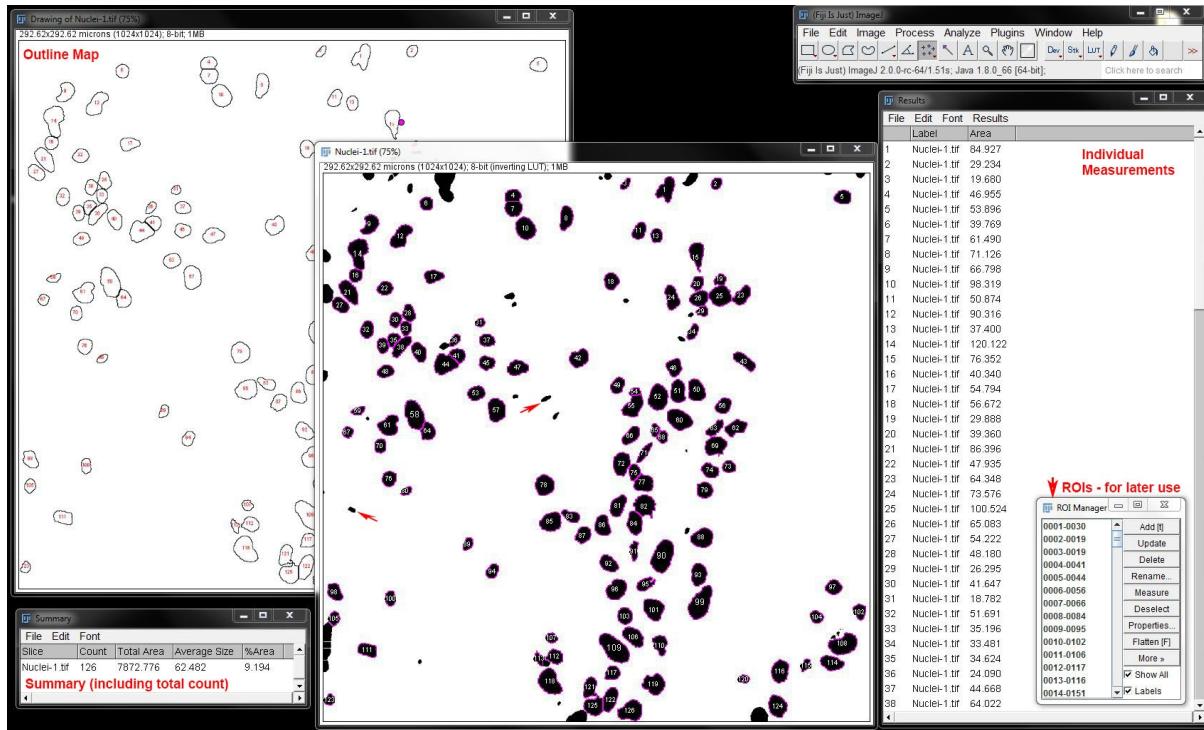
We can now count our objects using **Analyze Particles** tool. Find this under the **Analyze** menu.



In the options window, we can adjust the size to exclude smaller objects. This eliminates those false positive specks that remained in the mask. I have set the lower limit to 15 for this example. Leave the upper limit unchanged (infinity). Leave **Circularity** at the default values. Set **Show:** to 'Outlines' and tick the boxes to **Display Results**, **Summarize**, **Add to Manager** and **Exclude on Edges** as shown below. Press **OK** to measure the objects.



This set up will result in a number of new "results" windows. you will now have a results table displaying the selected measurements for each individual object detected (in this example, Area only). The Summary box will display the total count for you. An image of the outlines, numbered to match the results will be created, which you can save for future reference. We also have all objects added to the ROI manager which can be overlayed other images for measurements (ie intesity in another channel), or saved for future use.



You can also see in the original image that the small outliers that we didn't want to count have been excluded from the analysis (no ROI outline) by adjusting the size. We would use the Outline map and the original image here to ensure we have measured only the objects we want, and adjust our optins and re-measure if needed.

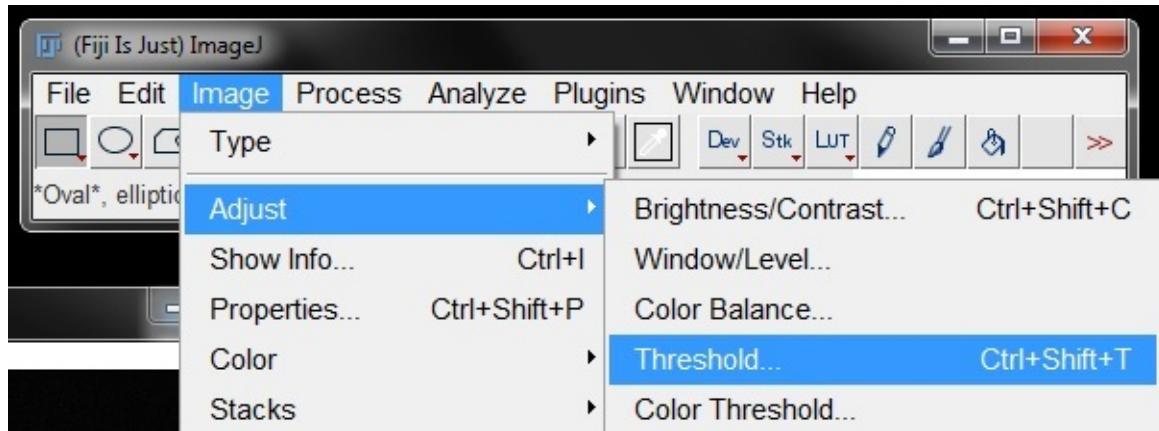
## Redirect Measurements

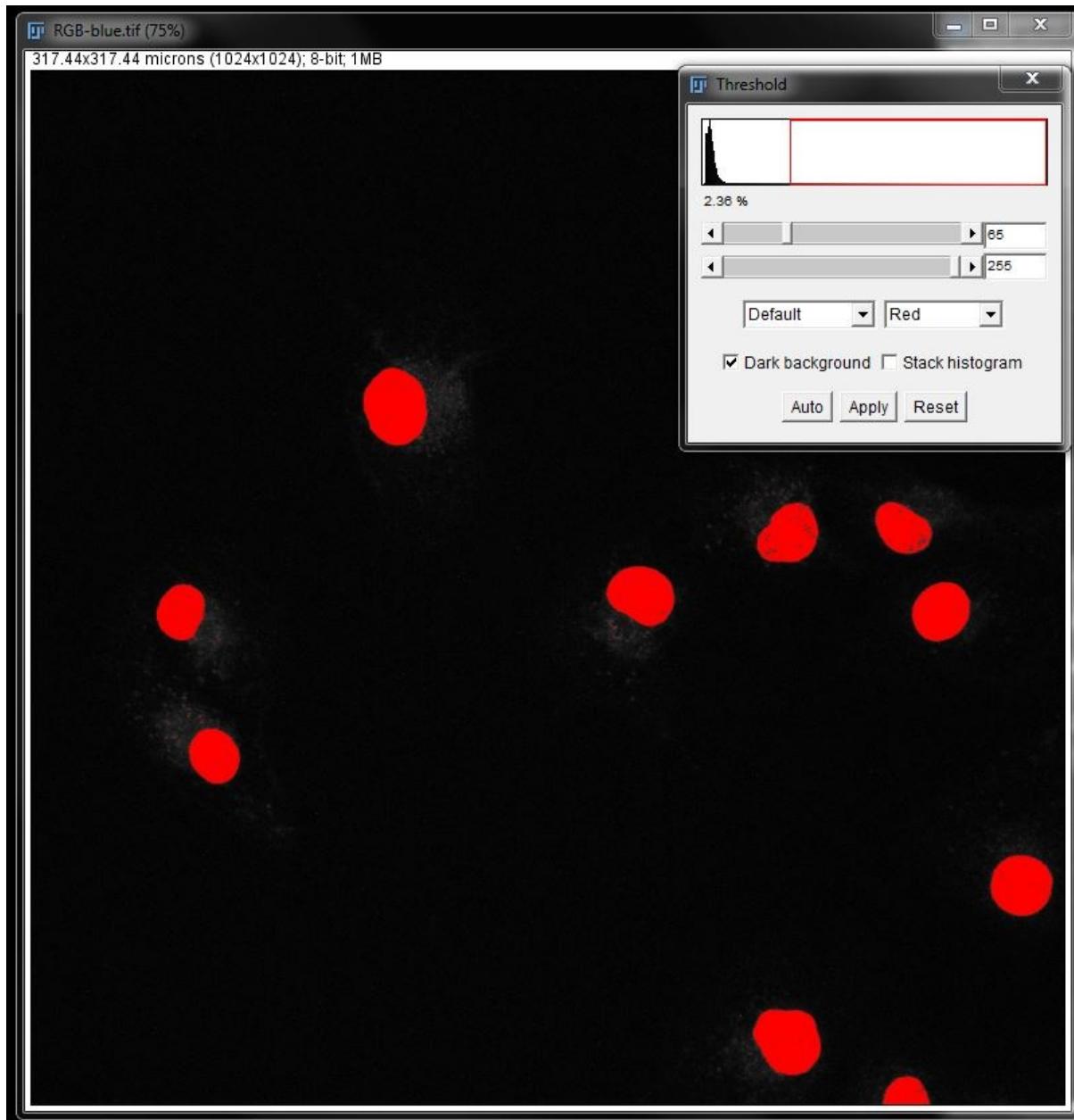
Masks, thresholds or ROIs generated on one image can also be used to make measurements on another image. This allows you to measure the same area in different channels or images.

One method for applying your selection to a different image during measurement is to use 'redirect'.

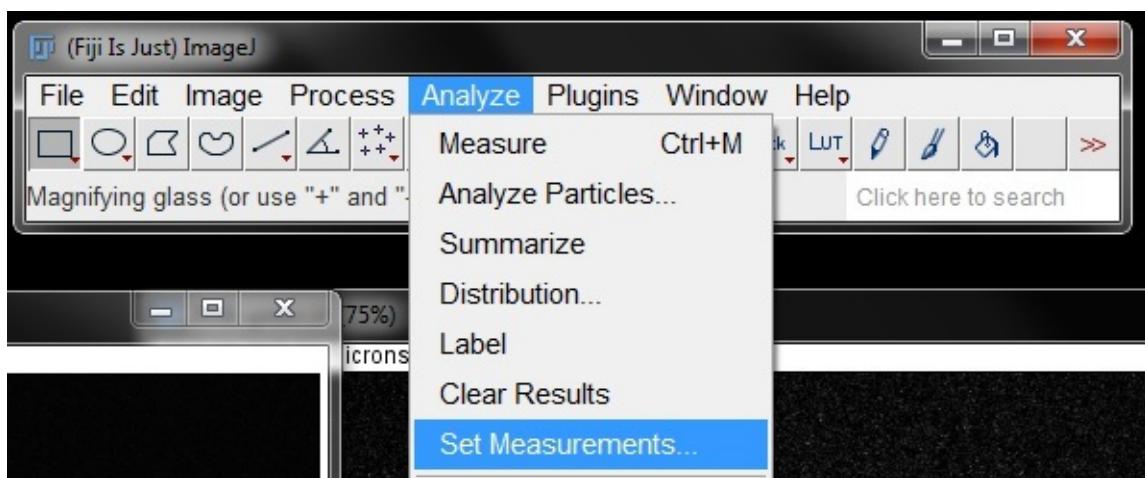
To demonstrate this we will use the images *RGB-blue.tif* and *RGB-green.tif*. We will generate a threshold of the nuclei (blue) image and use that to measure the intensity of the stain in the "nucleus" only on the green image. We will also measure the area under the threshold in both the original image and the green image to confirm it is measuring the same area in both.

Open both images and generate a threshold for your nuclei using the methods previously described. In this instance leave it as a threshold only, don't generate a mask.

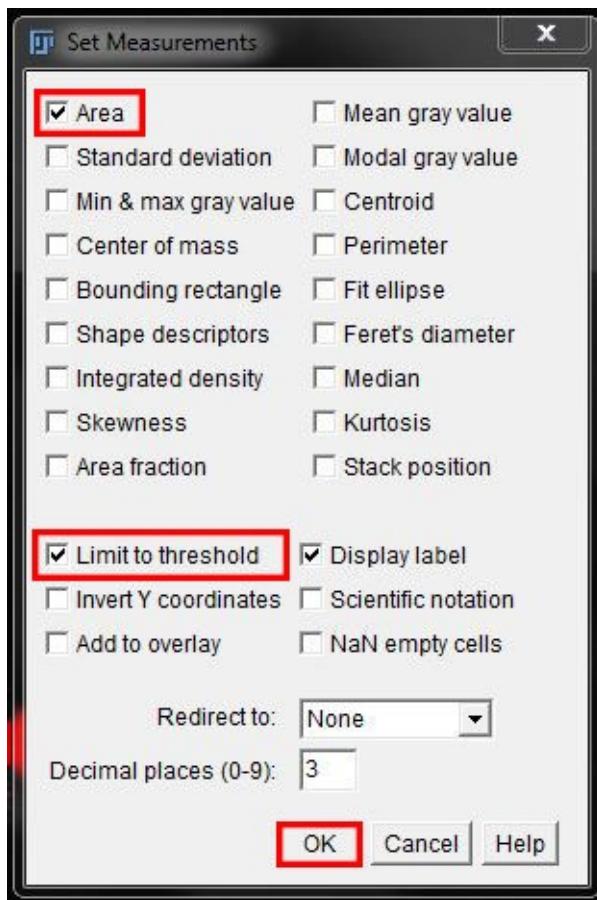




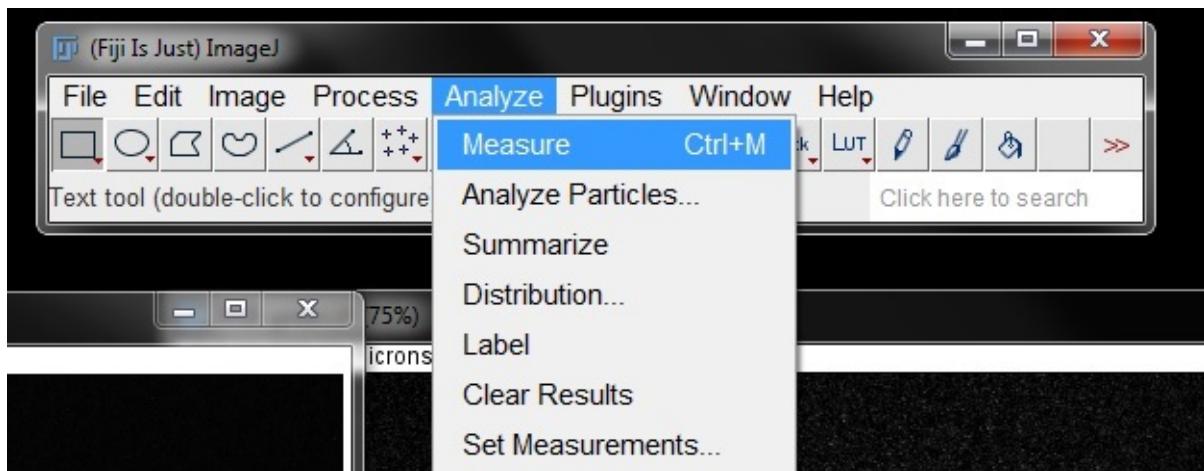
Ensure the thresholded nuclei image is active (click on the image) then go to **Analyze > Set Measurements**.



In the **Set Measurements** window, select **Area** as the measurement criteria. Make sure you have **Limit to Threshold** ticked, then click on **OK** to set the parameters.



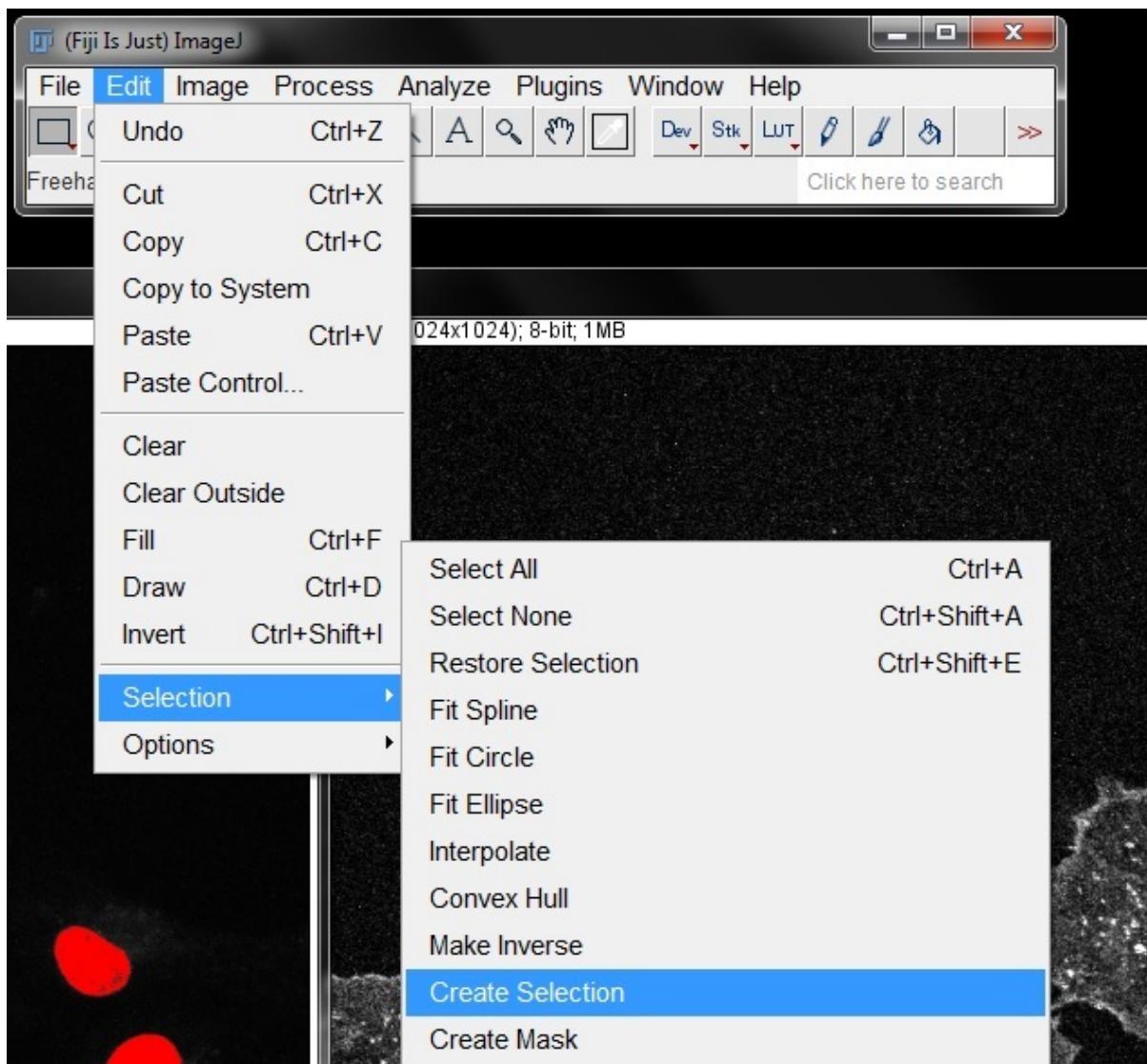
Once your parameters are set, go to **Analyze > Measure** to generate the measurement.



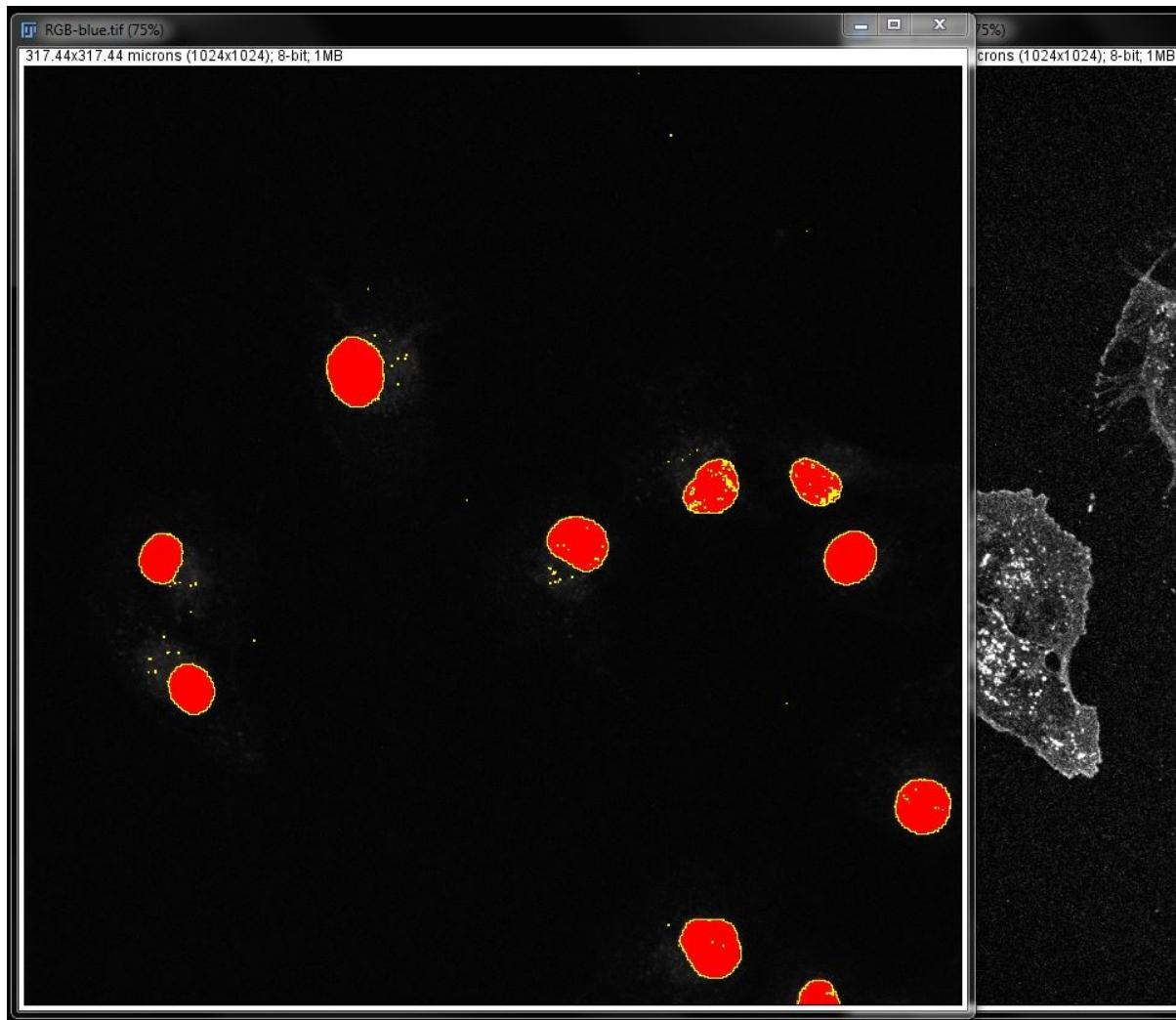
We now have the area of the "threshold" as a control for ensuring we are measuring the same part on the green image.

	Label	Area	MinThr	MaxThr
1	RGB-blue.tif	2397.311	63	255

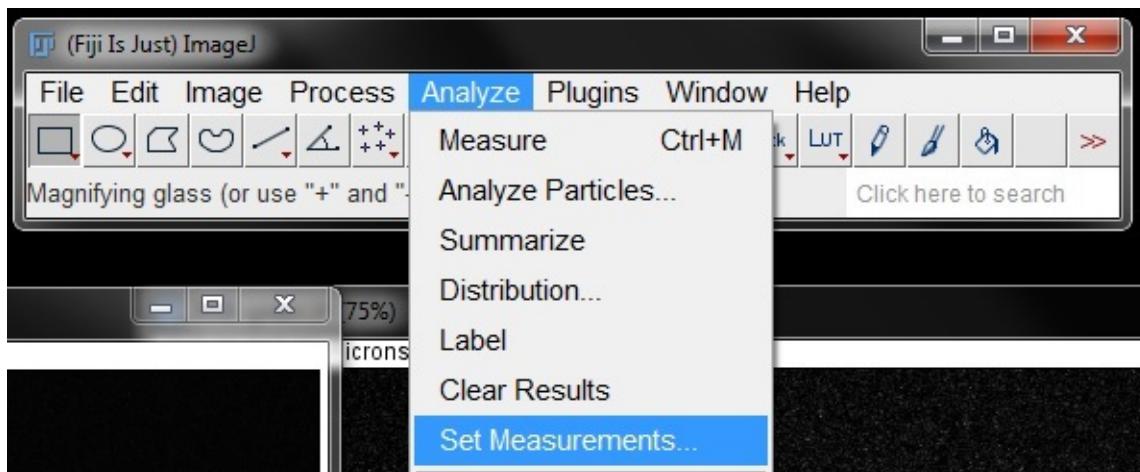
To make sure this is the case, we want to generate a selection fo the thresholded area. To do this, go to **Edit -> Selection -> Create Selection**.



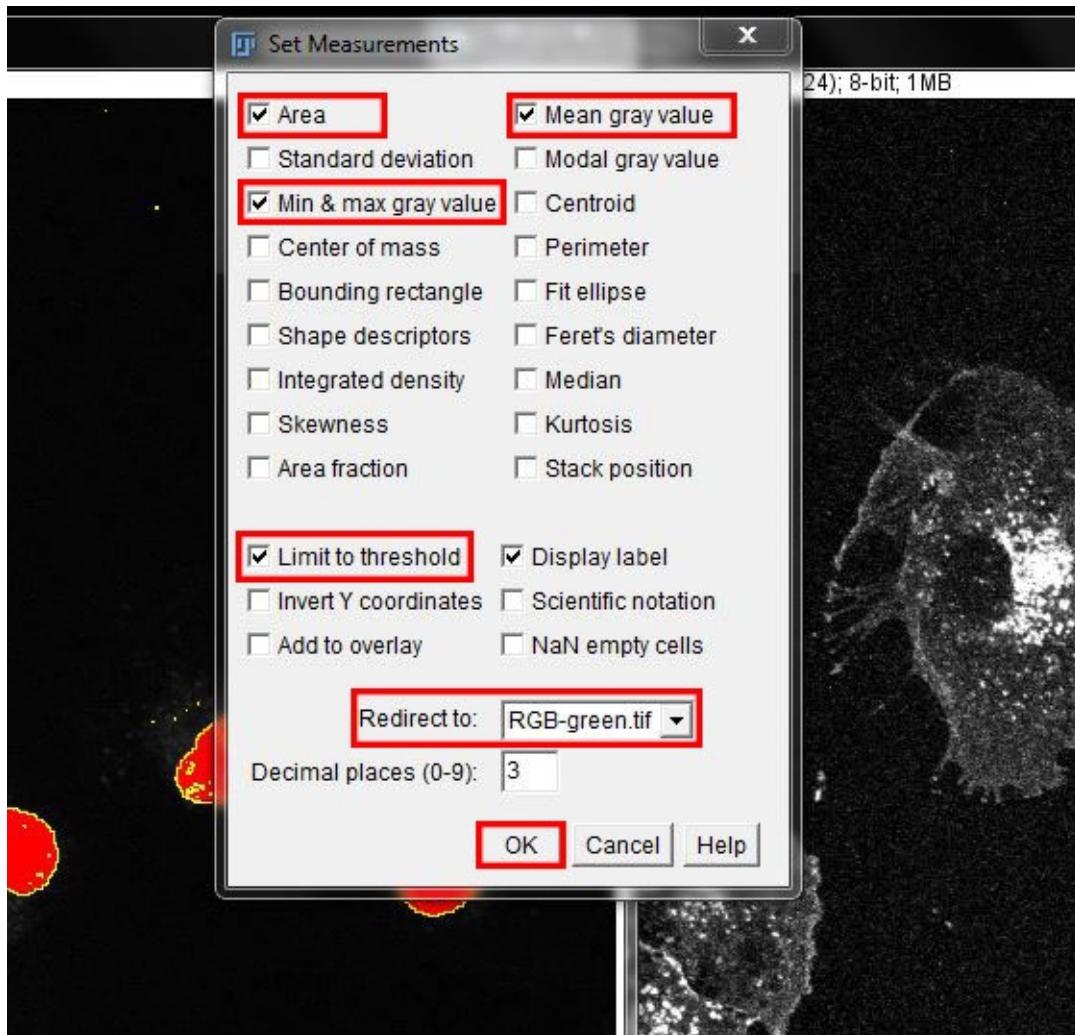
This will 'outline' the thresholded area as an ROI in the nuclei image.



We can now tell FIJI to measure any parameters we want in the green image, using this selection. To do this we open the **Set Measurements** settings again.



In **Set Measurements**, add **Mean Grey Value** to the measurement settings, then select **RGB-Green.tif** from the drop down menu under **Redirect to:**.



Click **OK** to perform the measurements on the second image. You will now get measurement for the second image in the areas under the original mask.

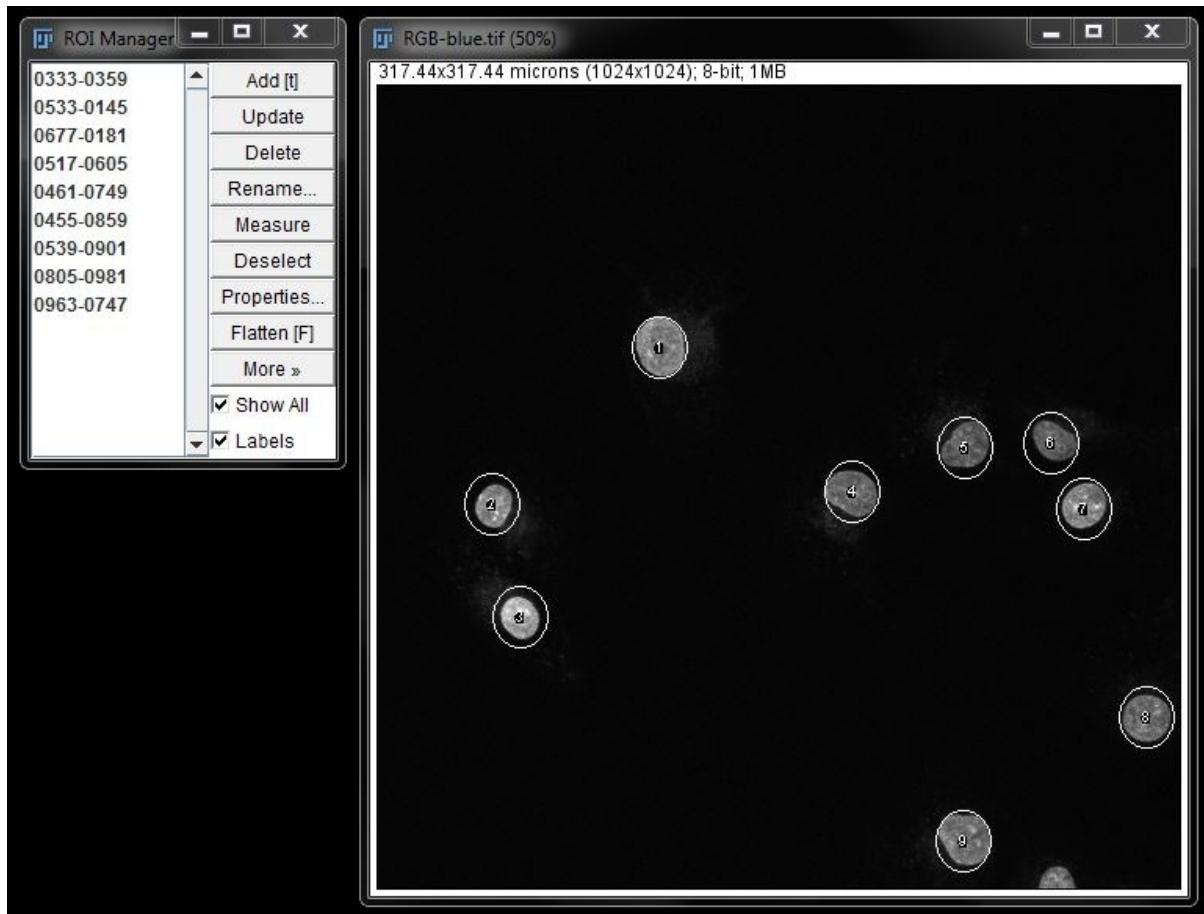
	Label	Area	Mean	Min	Max	MinThr	MaxThr
1	RGB-blue.tif	2397.311	0	0	0	63	255
2	RGB-green.tif	2397.311	45.461	0	255	0	255

You can see from the comparison with the blue image measurement that we have measured the exact same area in the green image. And we now have the mean intensity for the green image pixels (ie: your green stain) within that area.

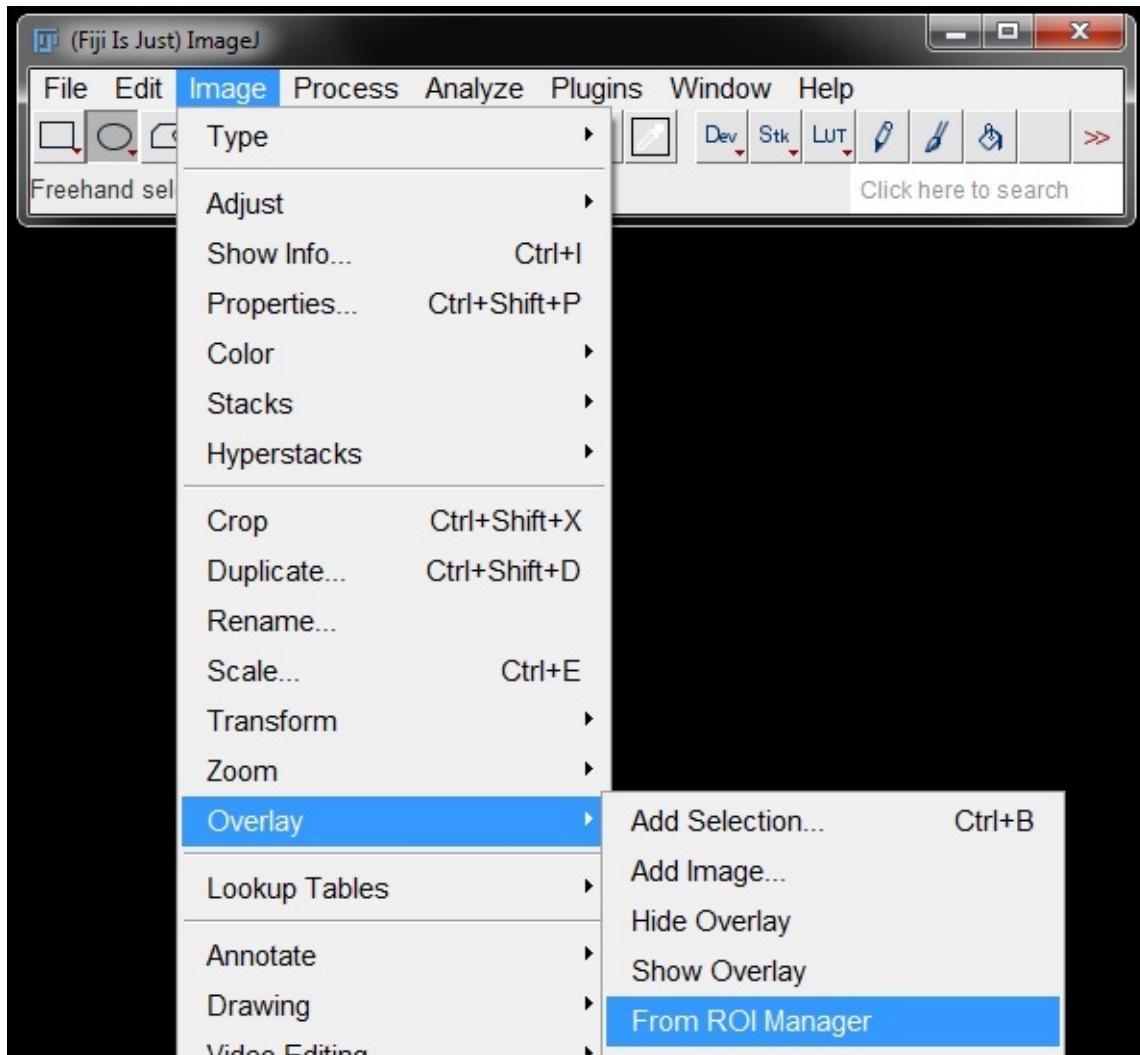
## Overlay ROIs

You can also overlay ROIs from the original image onto the second image using the ROI manager.

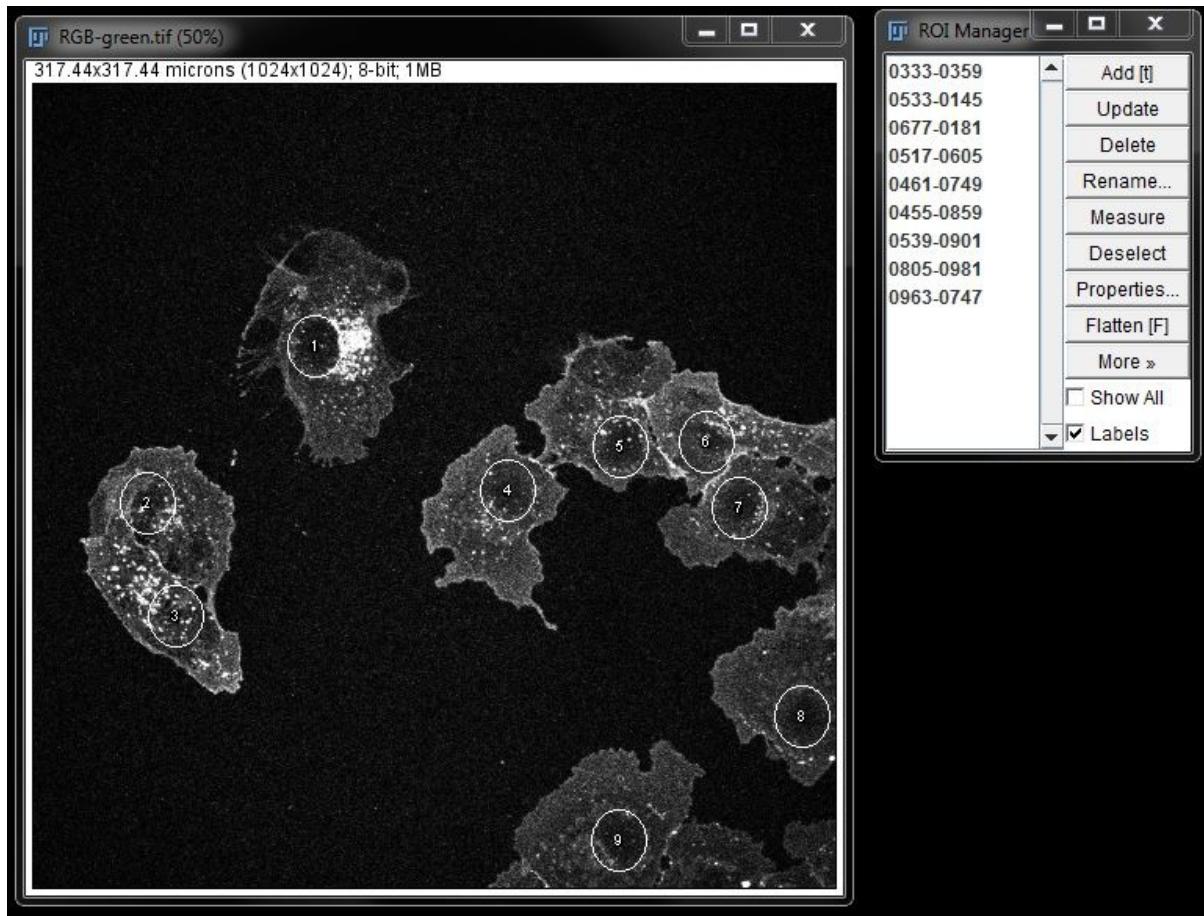
Open your two images (here we use RGB-Blue and RGB-Green again) and draw the required ROIs on image 1 (RGB-Blue), adding each to the ROI Manager as you go (as shown in FIJI Basics). Measure these in image 1 if needed.



To apply the ROIs to another image, select your second image (RGB-Green) and go to **Image -> Overlay -> From ROI Manager**.



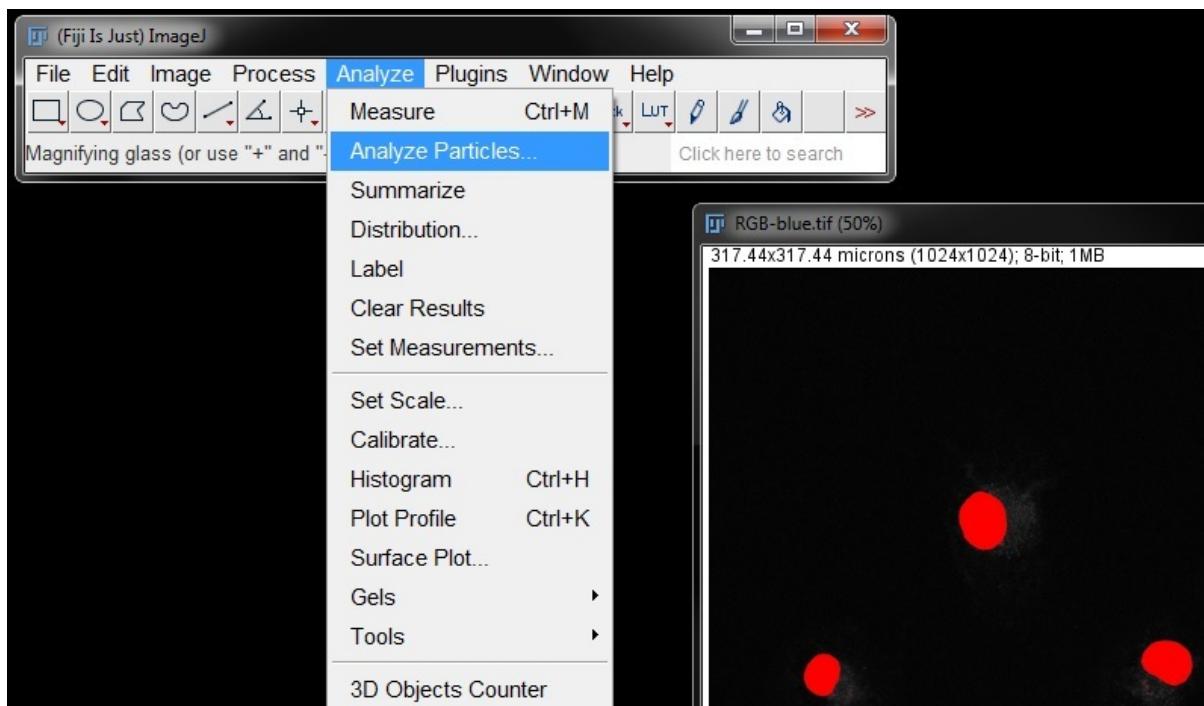
The ROIs generated from the mask will be applied to the second image and you can now set your measurements for the second image and measure the same area.



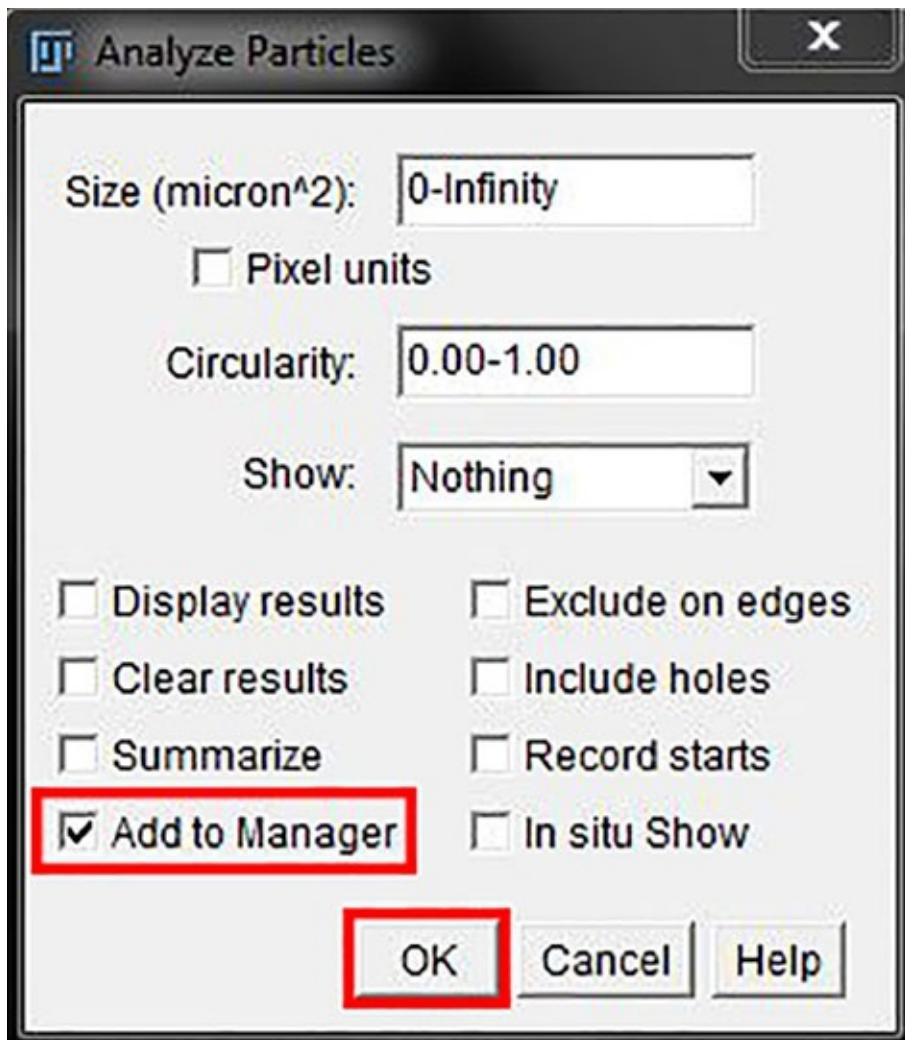
**Note:** you can also activate teh second image by clicking on it, then select an individual ROI from manager to display and measure it individually in the second image.

## ROIs from Particles

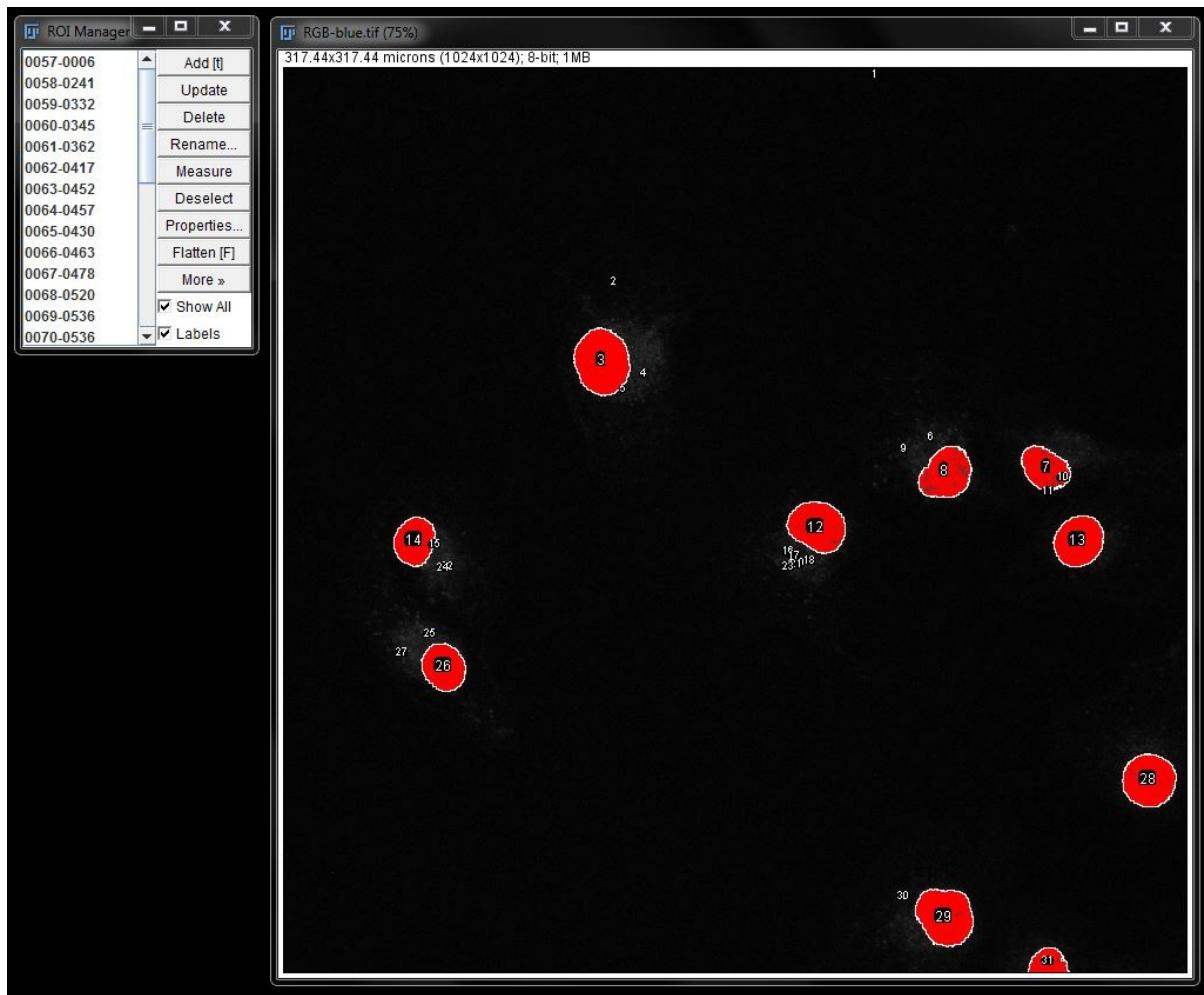
You can also generate ROIS to overlay to a second image using the **Analyze Particles** tool.



This time in the **Analyze particles** window, tick off every options box except **Add to Manager**. Select **OK**.

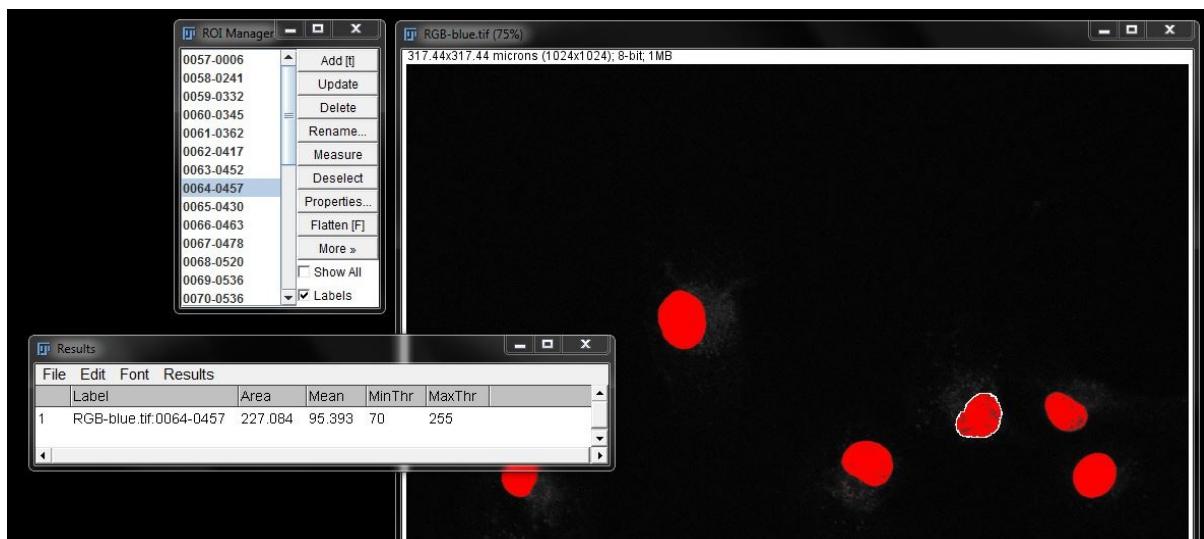


The mask or threshold will be converted to ROIs in the ROI manager.



When using a mask, you can use filters to remove any unwanted particles before creating ROIs. When maintaining a threshold, you can clean up any unwanted ROIs by changing the particle size in Analyze Particles options as shown before or selecting them in the ROI manager and deleting.

You can now make individual measurements using the ROI manager as normal.



**Note:** Remember that intensity measurements can't be made from a mask. To perform intensity measurements using this method, you should maintain a threshold before creating ROIs (as shown here) or you can overlay or redirect the ROIs created with a mask to a copy of the original image.

## PART 7: MEASURING MOVEMENT IN LIVE CELLS

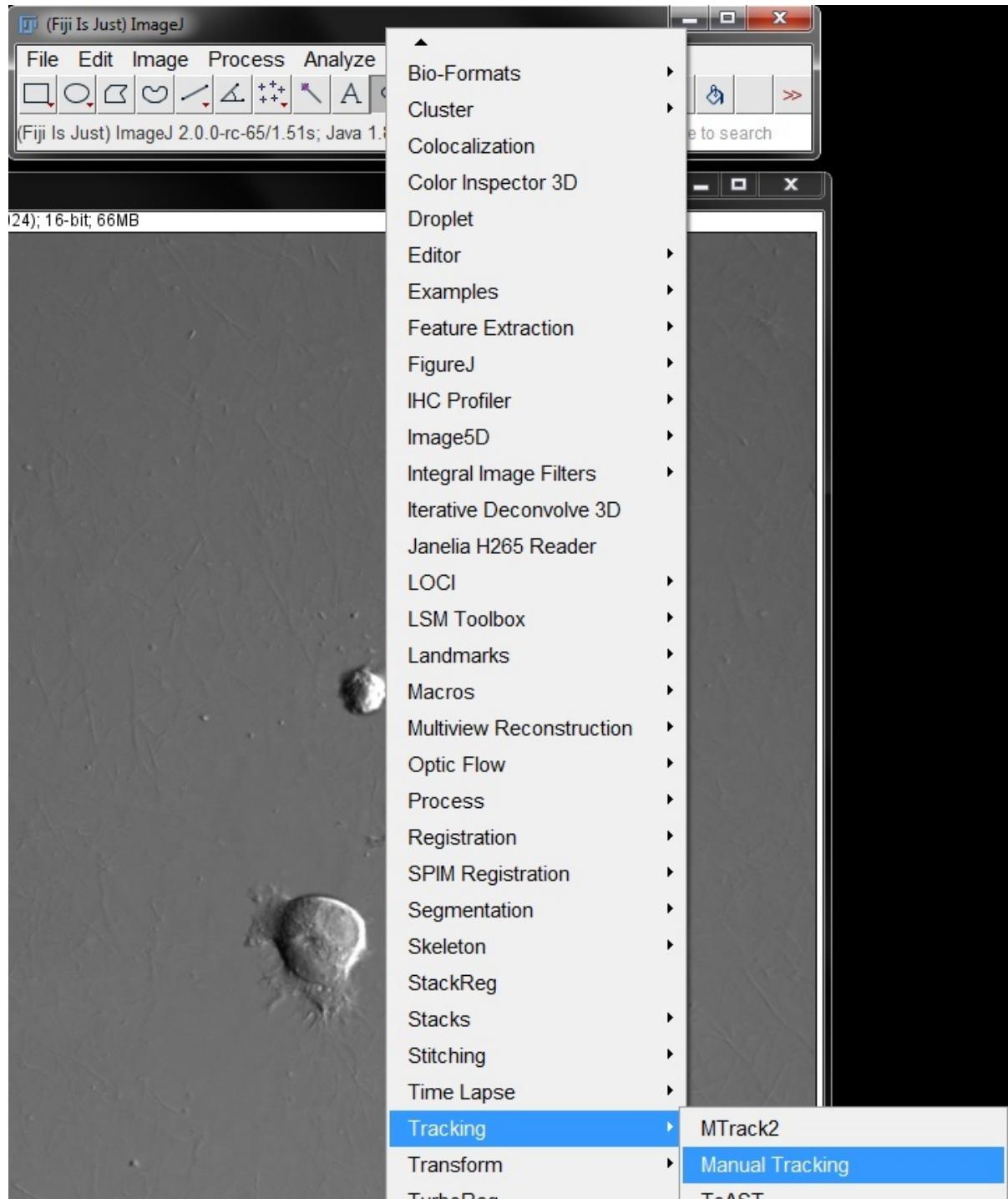
When we capture cells over time, also known as times series or live cell imaging, we are presented with more analysis options. along with the other types of analysis presented above, we can also get several pieces of information from time series by tracking objects over time or examining the dynamics of the cells or their organelles.

In this section we will show you two different methods for tracking objects in time series, as well as how to present the dynamics of a cellular component in a single image (known as a kymograph). We will use the images *MovieStack.tif*, *AutoTrack.tif* and *Kymograph.tif* for demonstration.

### Manual Tracking

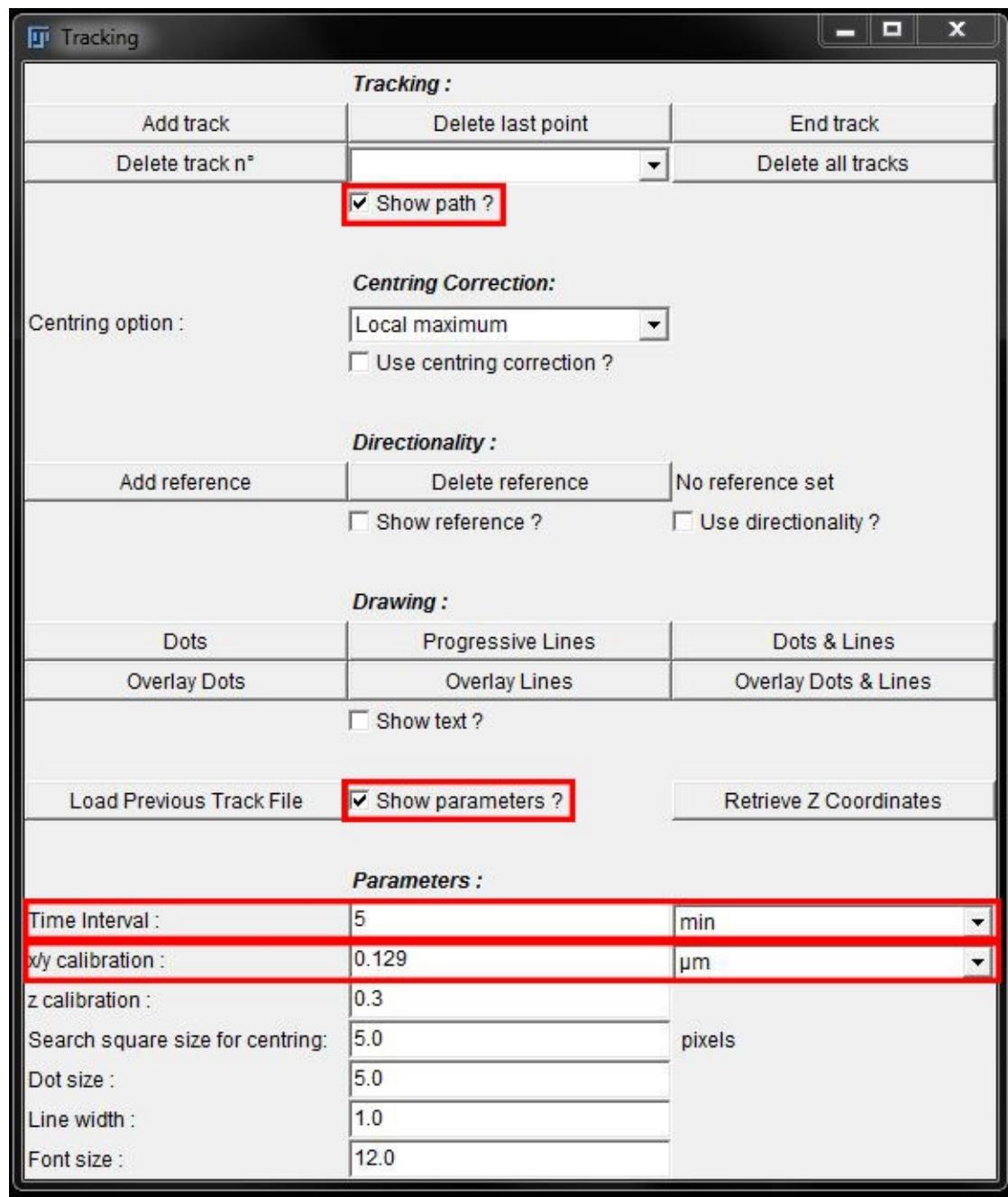
The simple method for tracking objects over time is to manually track them using the **Manual Tracking** tool.

Open the time series *MovieStack.tif* and then open the **Manual Tracking** tool from the menu **Plugins -> Tracking**.

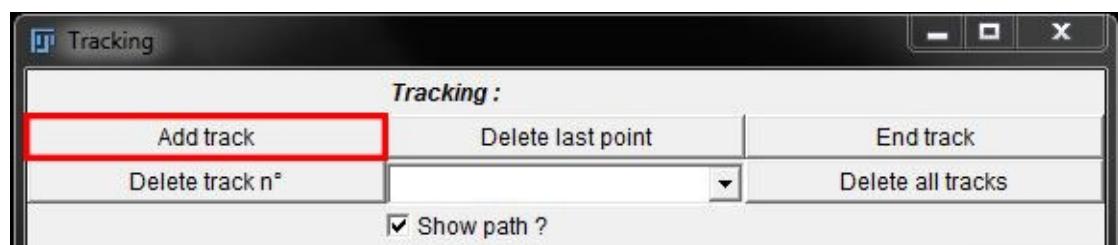


In the **Tracking** window, tick on **Show Parameters?** to see and set all calibration settings. You can then set your parameters, including **Time Interval** (5mins for this example). If the xy calibration (scale) is not set automatically, enter the calibration value too (in this example it is automatically set correctly).

Select the option to show the tracks on the image if required by checking the box next to **Show path?**



When you have set your parameters, click **Add Track** to start your first track.



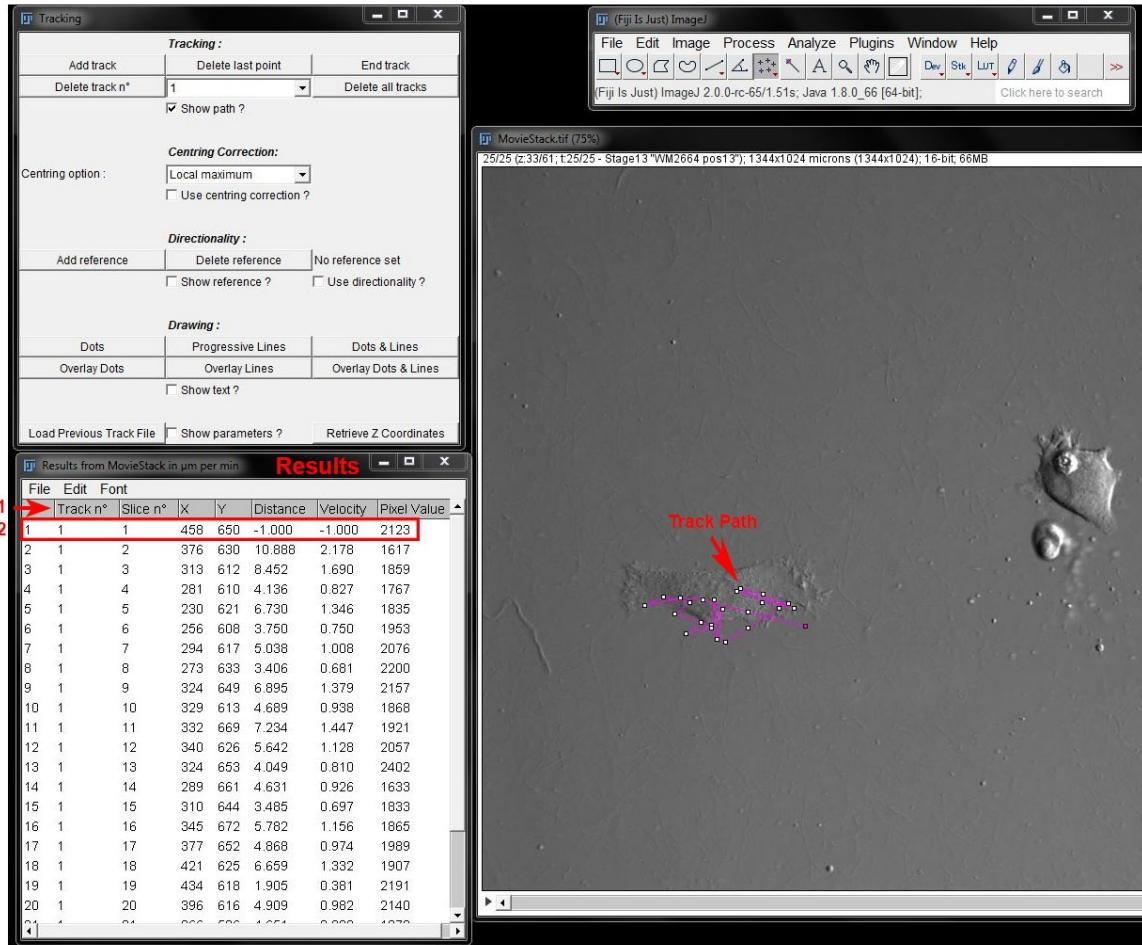
Then to begin tracking, click on the image in the centre of the object you want to track. The series will automatically move to the next frame. Click the centre of the object again.

Continue until you have reached the end of the series. If you have selected an option to show the track it will be visible overlaid in the image as a segmented line. Measurements for the object will be displayed in a results table.

Measurements will be displayed separately for each time point, these can be exported to excel and averaged.

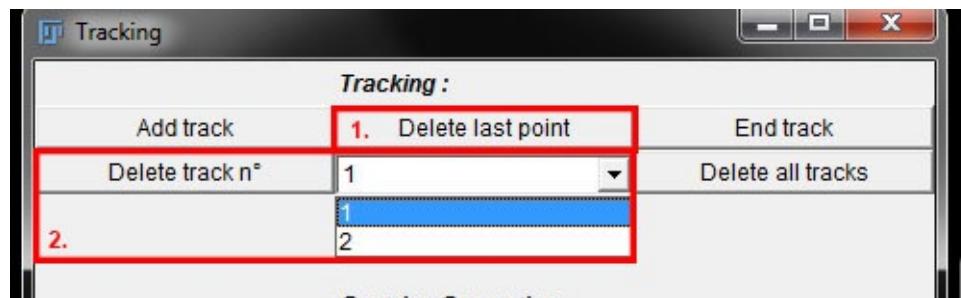
**Note 1:** Manual tracking results are cumulative. You can check the "track number" column in the results to see which results belong to which track, or save and then clear results between each cell or each image to ensure you know which results belong to which cell.

**Note 2:** The first measurements will not be accurate as the object had not moved yet (ie: first measurement for distance is -1) and these should be removed from the data before averaging, and is not considered manipulation of data as it is not an actual measurement.



Repeat the process above (from **Add track**) for each cell you want to track in the image. Generally we would avoid tracking cells that divide, leave or enter the field of view. If cells cross paths, make sure you are able to determine which cell is which once they separate again, otherwise exclude these from tracking as well.

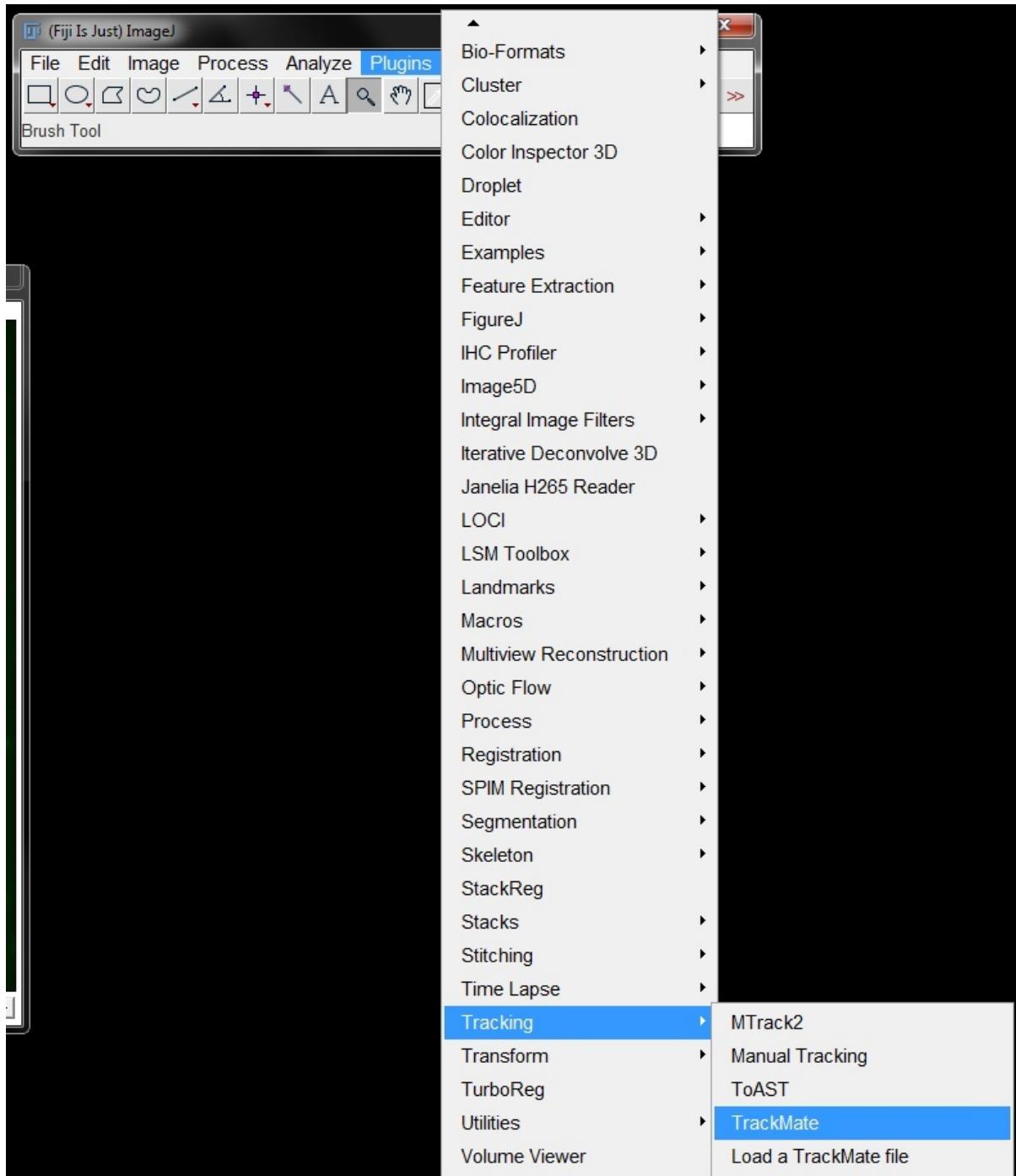
If you make a mistake during tracking you can **Delete the last point** (1 below) or select the track number from the drop down list and click on **Delete track** (2 below) to remove it entirely and start again.



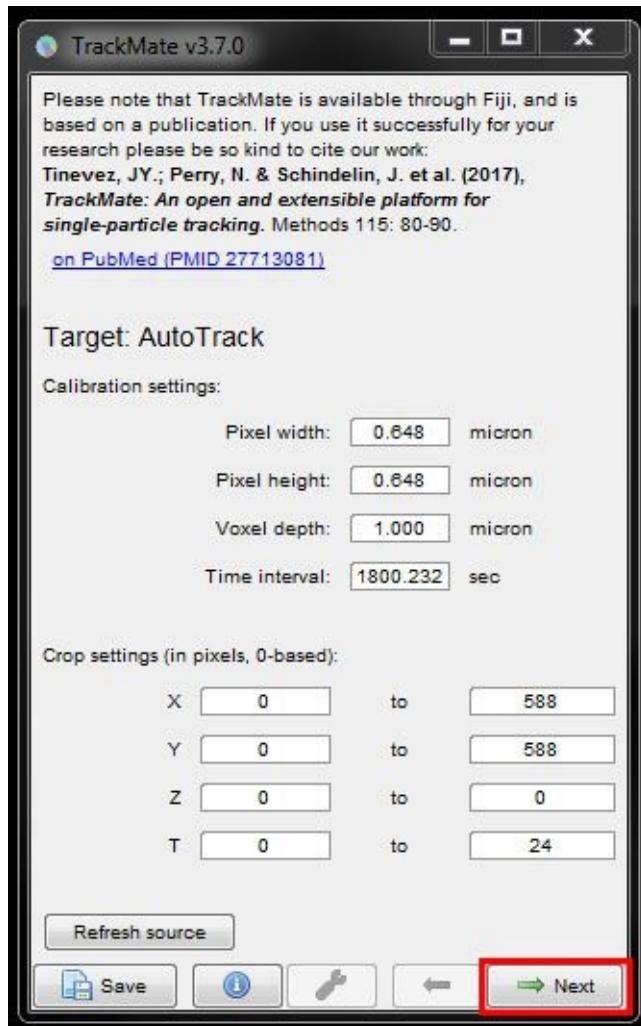
## TrackMate: Automated Live Cell Tracking

For large numbers of cells, very fast movement or long imaging times it can be difficult to track cells manually. For these circumstances you can do automated tracking.

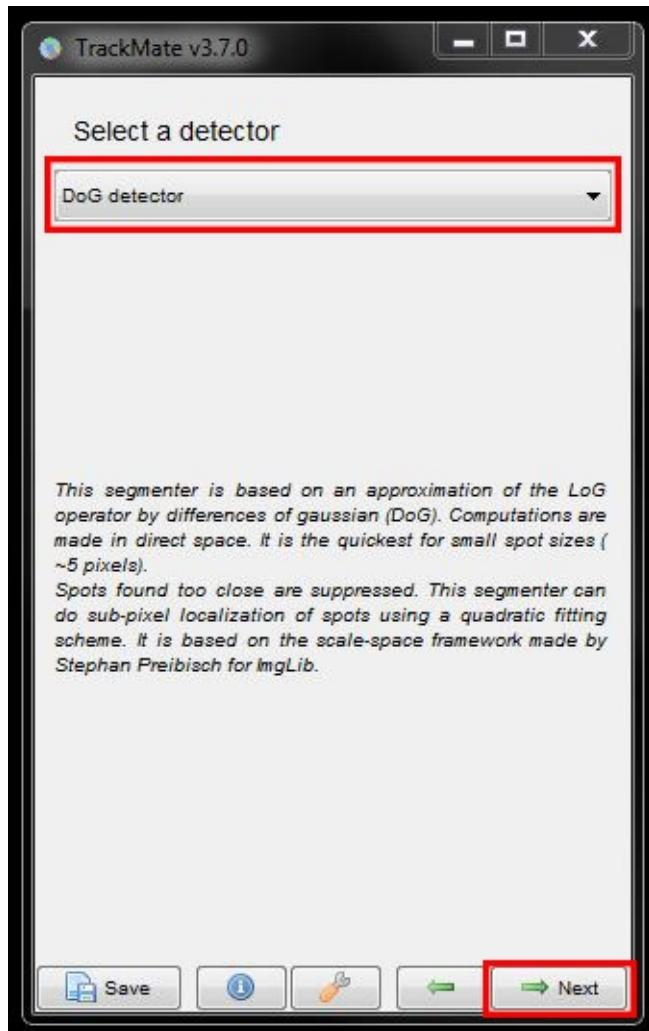
First open `_AutoTrack.tif` in Fiji, then go to **Plugins -> Tracking -> TrackMate**.



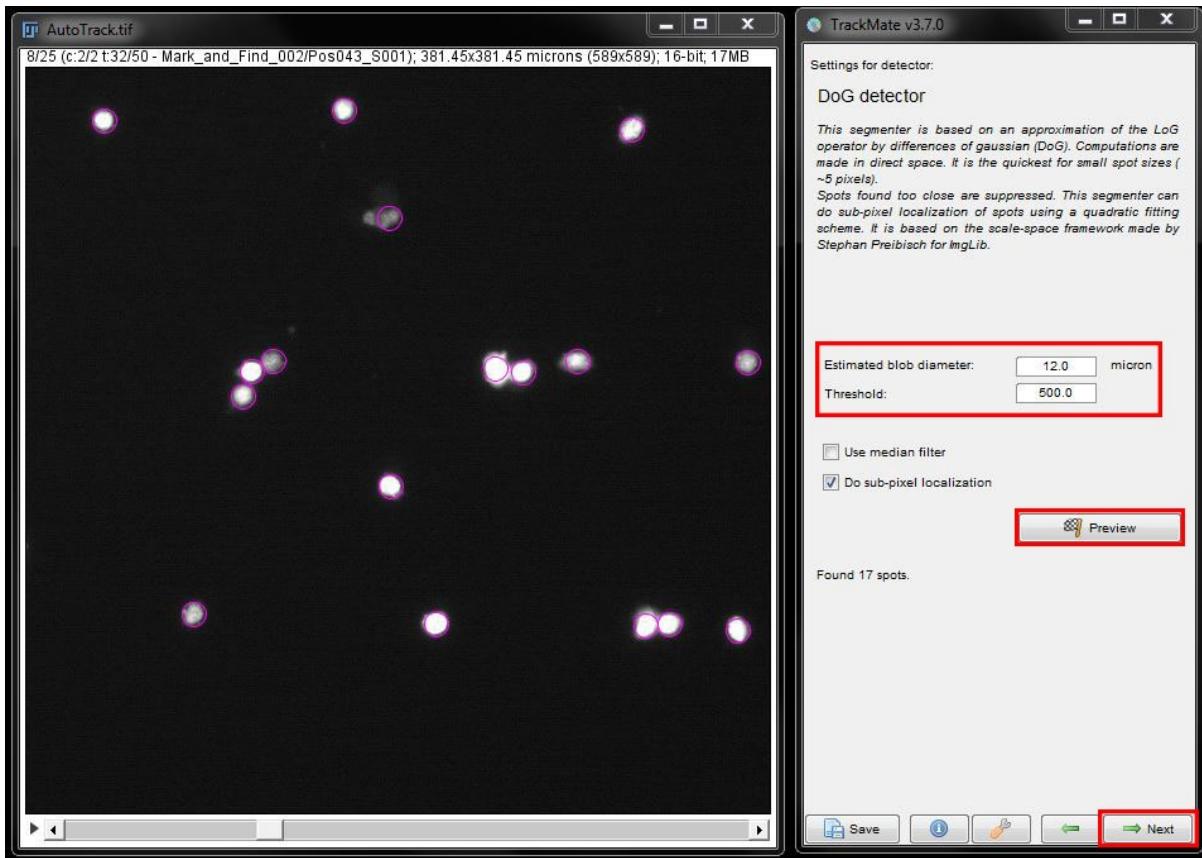
In the first window, you can set the scale of your image (should be pre-filled if your image is scaled). You can also add a region of interest here if you only want to analyse part of your image. As our image is scaled correctly and we want to analyse the whole image we don't need to make any changes here. Click **Next** to continue.



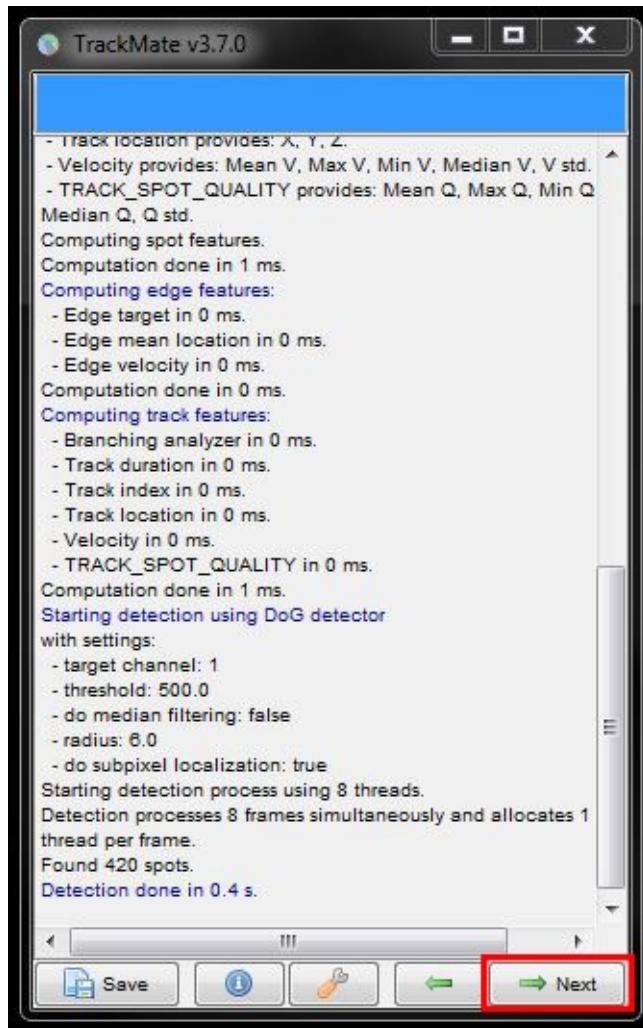
There's different methods of detecting cells, the most common ones are LoG (Laplacian of Gaussian) and DoG (Difference of Gaussian). For this image choose **DoG detector** from the drop down menu the click **Next**.



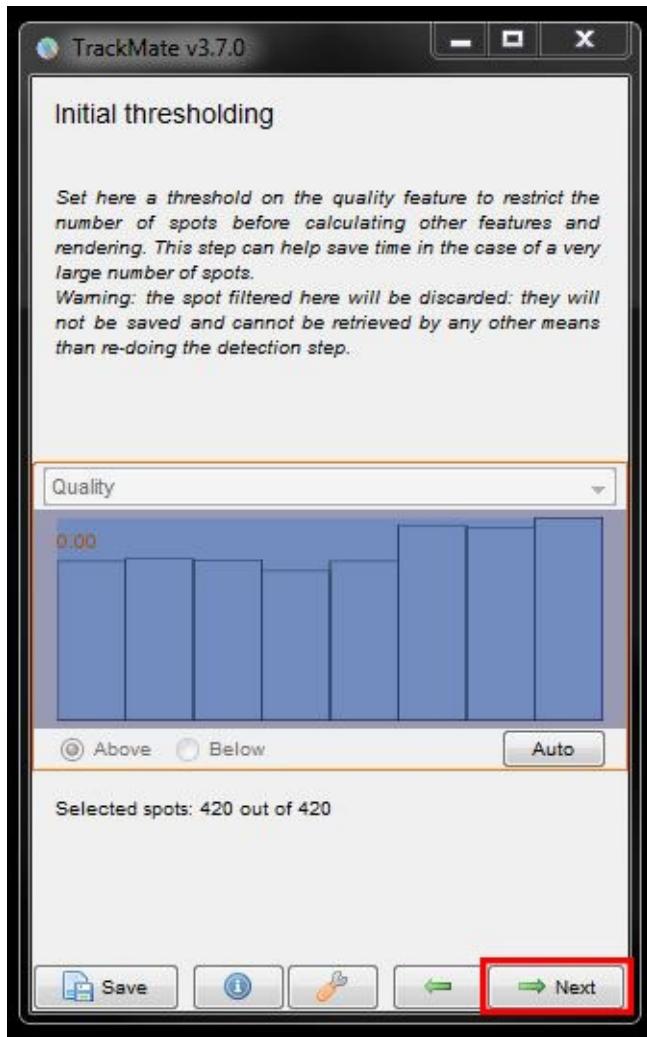
In the next window, enter the expected size of your cells and a threshold. Here, please enter a diameter of 12 microns for cell size and threshold of 500. Click on **Preview** to see what it detects. Repeat this at several different time points and adjust the diameter and threshold if needed to better detect the cells and eliminate any background signal that gets detected. When you are happy with your selection click **Next** to continue.



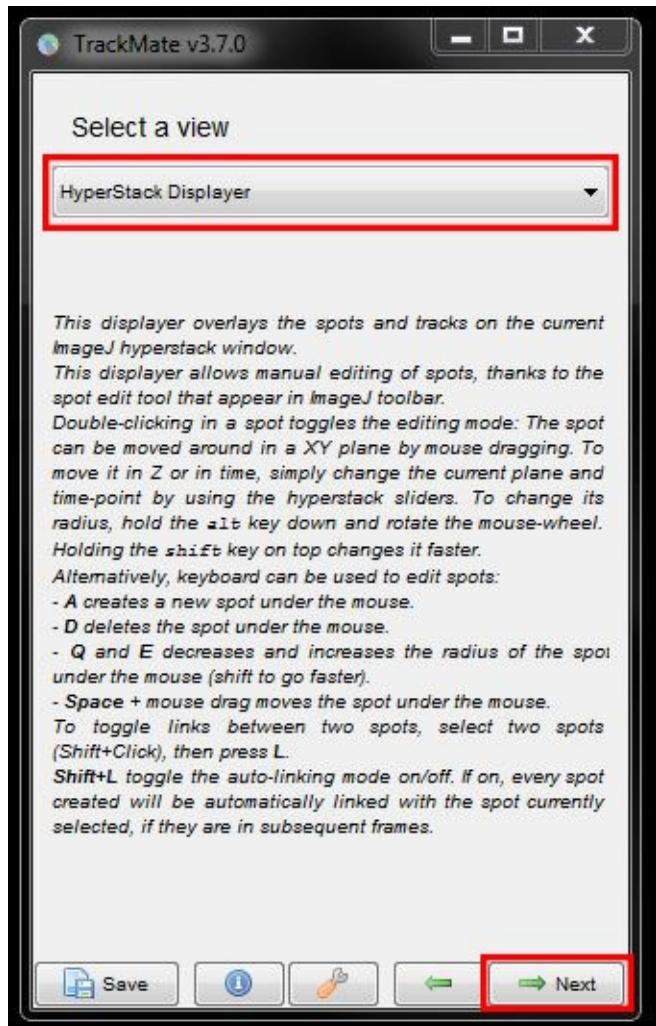
The TrackMate plugin will analyse each image in the time series and detect all cells with the given parameters. Once the detection is finished, press **Next** again.



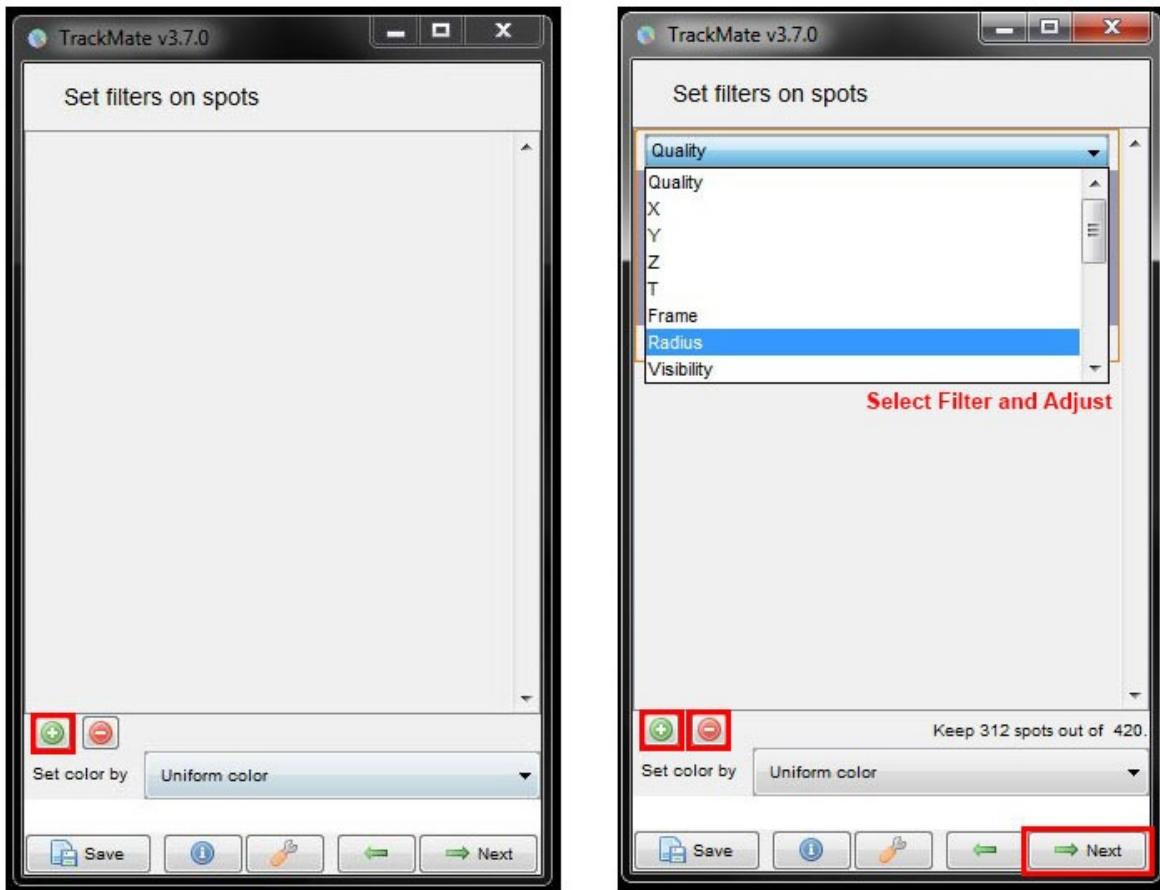
Now there's options to filter out cells according to their quality. You can adjust the filter threshold using the sliders or try the **Auto** threshold settings by clicking on the **Auto** button. For this data set though, we'll ignore this filter and press **Next**.



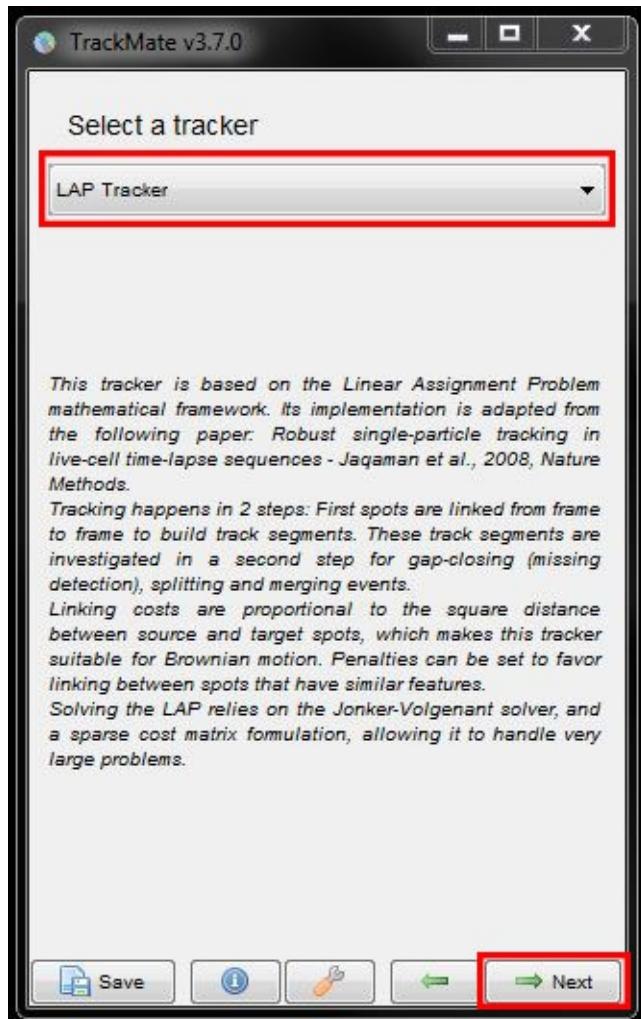
The next window will ask you to chose the view mode from a drop down menu. Here we will leave it as Hyperstack and again click **Next** to continue.



Another set of filter options will come up. To add a filter, click on the green plus button then select the filter from the drop down menu and adjust the corresponding threshold. You can add several filters by repeating this process for each. For this data set, we're again going to ignore these filters. So remove any filters you have added, by pressing the red minus button, and then click **Next**.



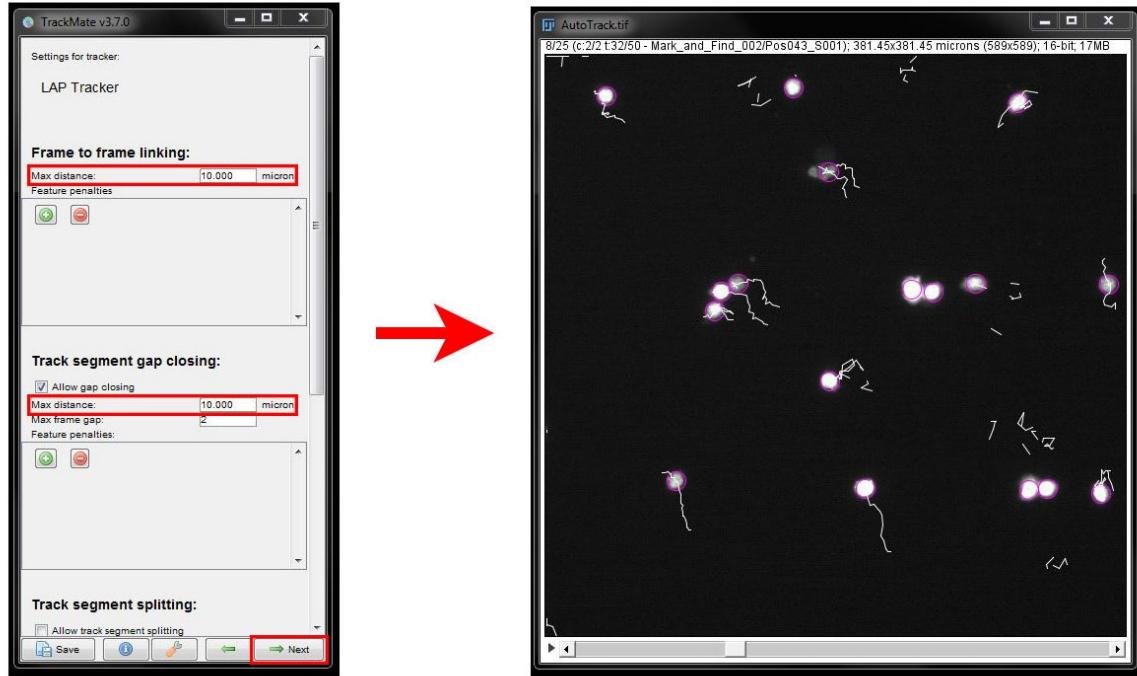
This brings us to the tracking options. All the options are different algorithms on how to connect the detected spots. Descriptions of the algorithm and how they work are given below the selected algorithm. **Simple LAP Tracker** will work well in most cases but here, we want to use specific parameters and penalties, so we need to choose the **LAP Tracker** (LAP = Linear Assignment Problem). Select this option from the drop down menu then click **Next** to continue.



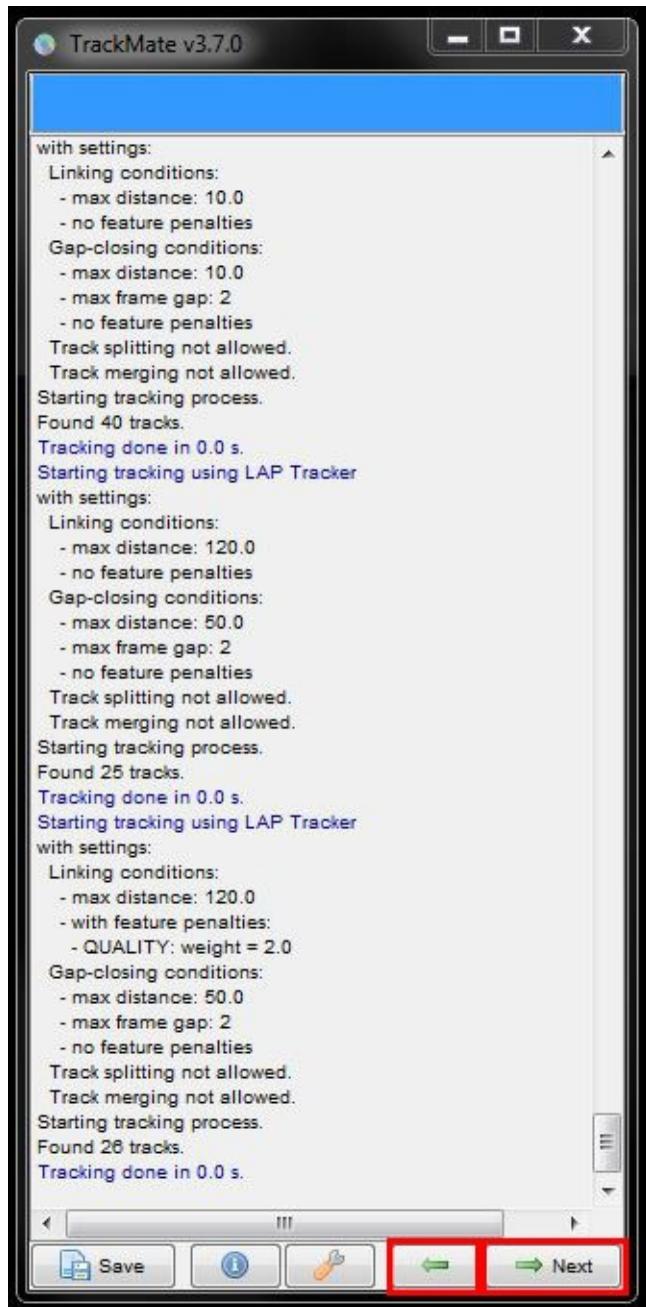
In the next window, you can set distances on how far you expect your cells to travel per frame. Set your distances for **Frame to frame linking** and **Gap closing** and click **Next** to assess the tracking accuracy.

We can try a few different settings here to see what best fits our data:

Under **Frame to frame linking** set the Max Distance as 10 microns and under **Gap closing** again set 10 microns.

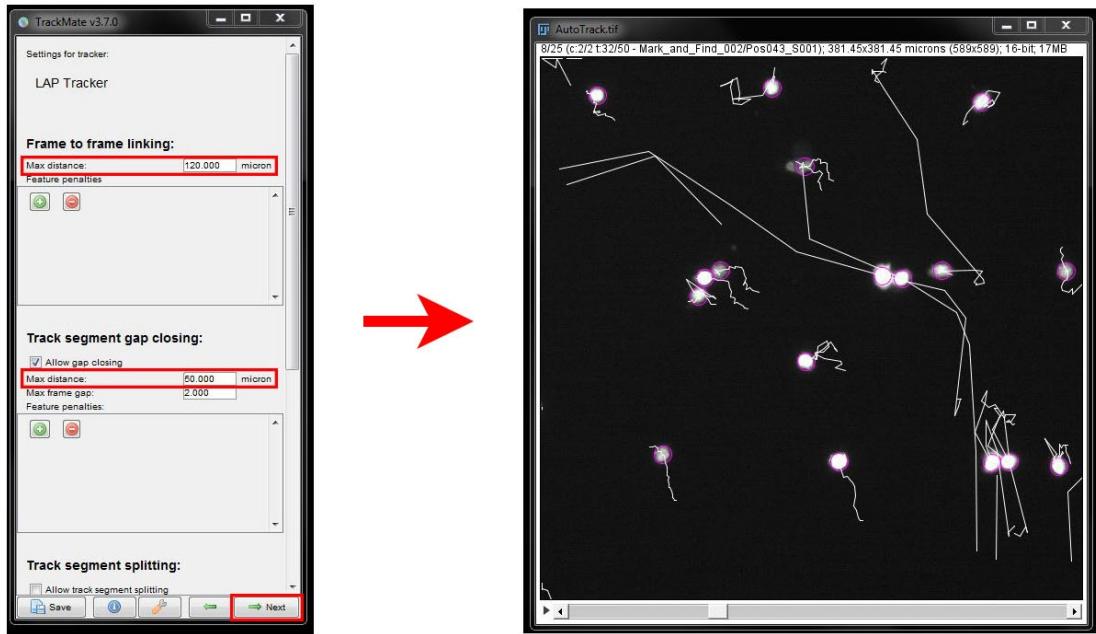


At any point here you can click the **Back** arrow after tracking (in the log window) to return and change the parameters or **Next** (in the log window) to keep the tracks and continue.



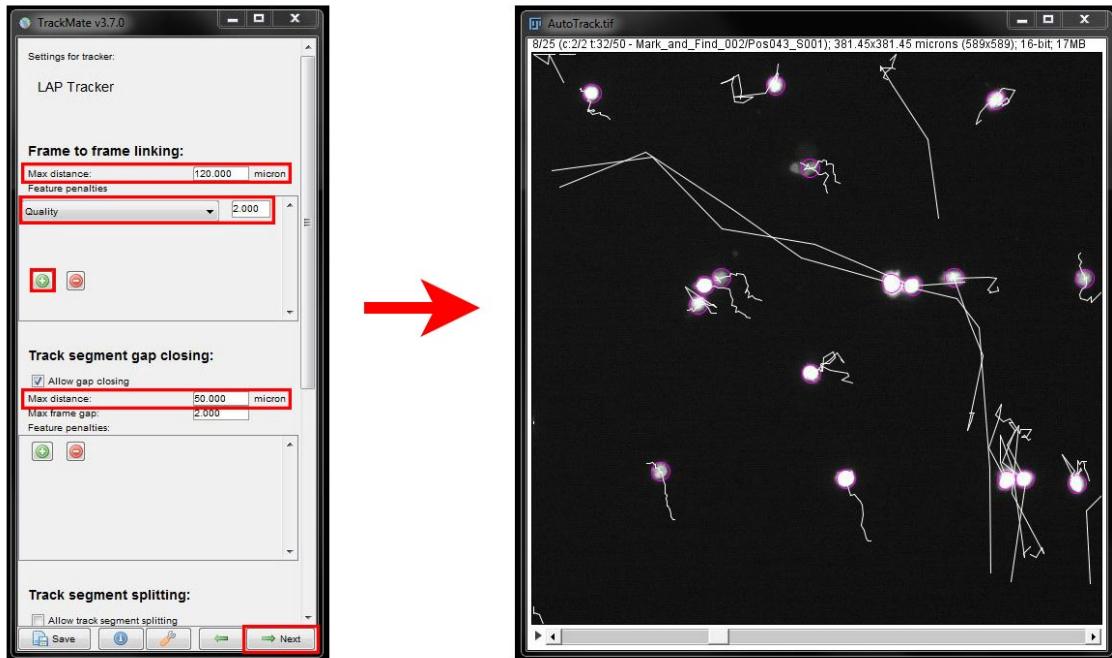
Our initial settings didn't track our cells very well. You can see that some tracks are broken up into several tracks as the cells moved more than 10um between frames and some cells are not tracked for the entire time series. So lets try some different settings:

Under **Frame to frame linking** set the Max Distance as 120 microns and under **Gap closing** again set 50 microns.



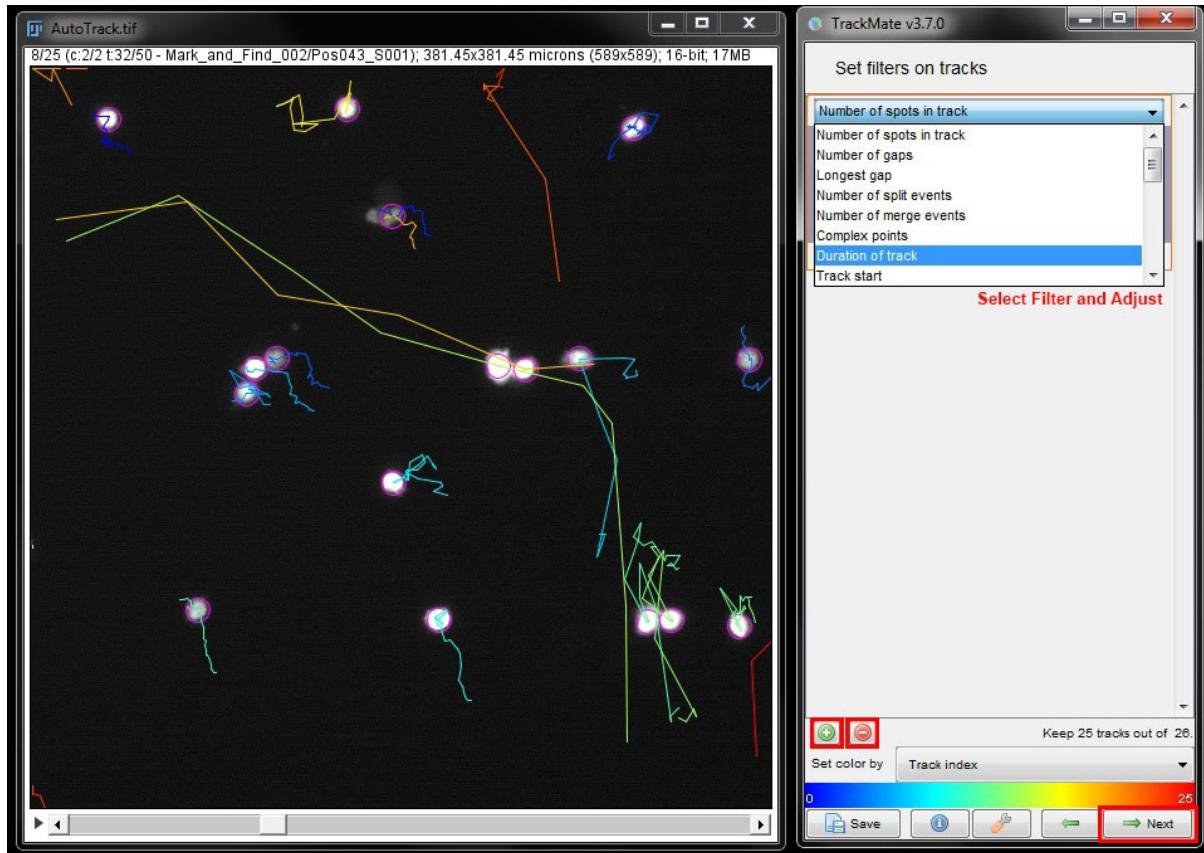
Here we see that the tracks are now better connected. Scroll through the time series a few times and look for any errors in the tracking.

We do have a couple of cells that are not being tracked accurately. To amend this we will use **Feature Penalties**. Go back to the tracker settings using the **Back** arrow button. Under **Frame to frame linking** click the green plus button. From the new drop down menu select the **Quality** filter and adjust the threshold for the filter to 2. Click **Next** to see the result.

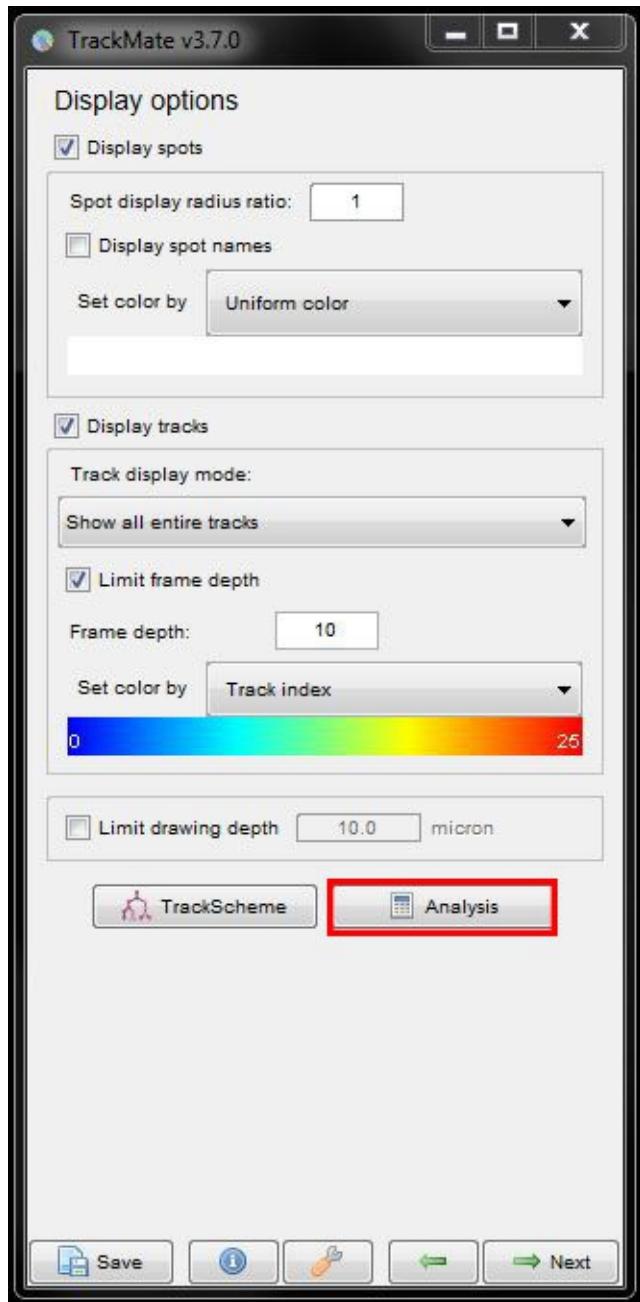


The two long tracks in the centre of the field of view are now a more accurate representation. As always, you will have to find the right settings for your data each time you use an analysis tool. When you are happy with your tracks, click **Next** in the log window to continue.

In the next step you will find **Filter tracks**, similar to the spot selection filters before. To add a track filter, follow the same protocol as spot filters; press on the green plus button, choose your filter from the drop down menu and adjust the threshold. Repeat for any additional filters you want to add. I am not going to use any additional track filters on this data, so use the red minus button to delete any you have added, then click on **Next**.



Now we can export the results of our analysis. In this window click on the **Analysis** button.



This will open tables with all the information on all your detected tracks and spots. You can save these as .csv and open in Excel or Prism for further analysis. The summary of the results is best found in the Track Statistics window. We have 26 tracks listed with results such as duration, displacement, and speed.

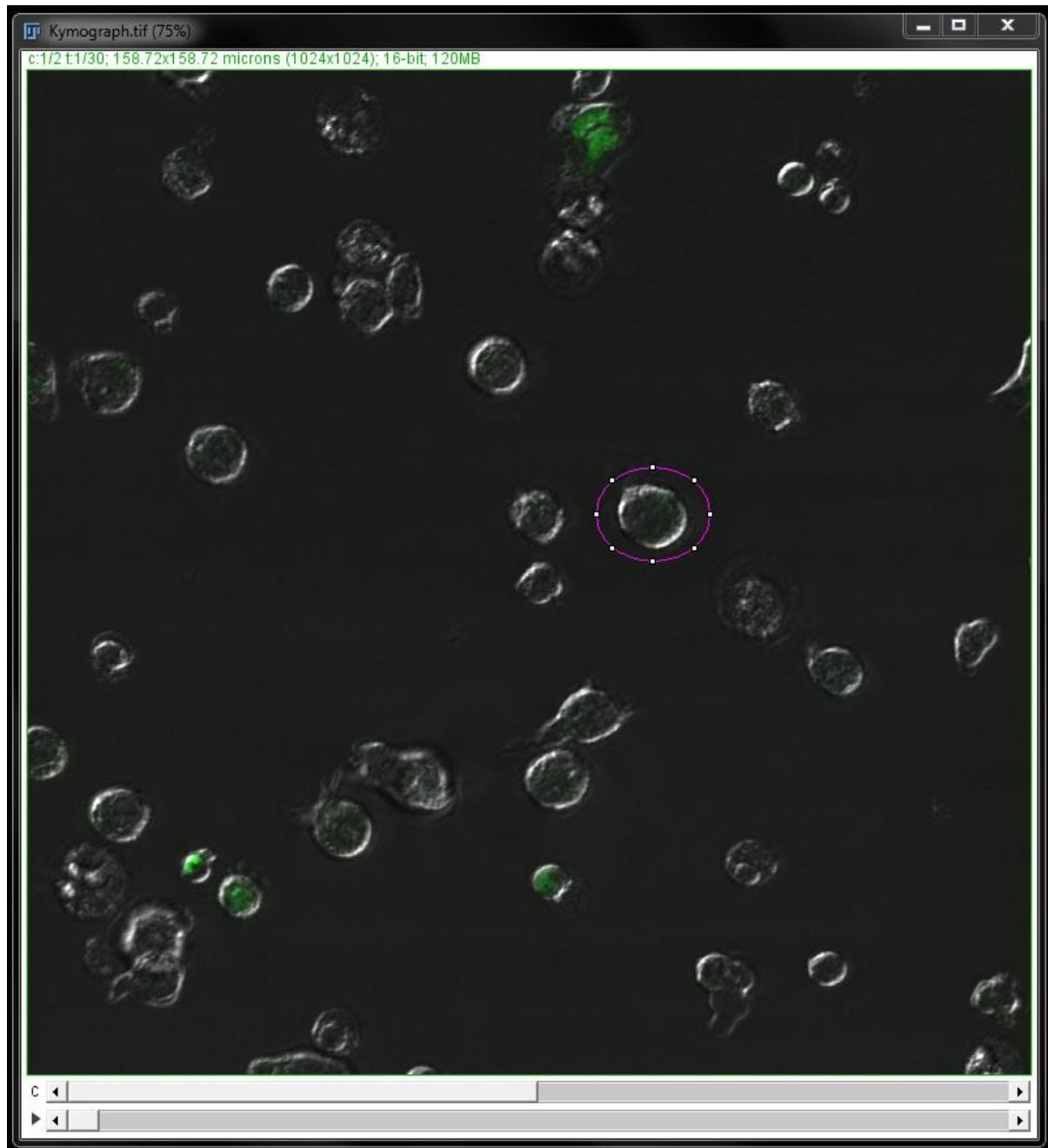
	Label	NUMBER_SPOTS	NUMBER_GAPS	LONGEST_GAP	NUMBER_SPLITS	NUMBER_MERGES	NUMBER_COMPLEX	TRACK_DURATION	TRACK_START	TRACK_STOP	TRACK_DISPLACEMENT	TRA
1	Track_852	25	0	0	0	0	0	43205.568	0.000	43205.568	28.117	0
2	Track_853	25	0	0	0	0	0	43205.568	0.000	43205.568	30.004	1
3	Track_854	25	0	0	0	0	0	43205.568	0.000	43205.568	16.467	2
4	Track_855	25	0	0	0	0	0	43205.568	0.000	43205.568	33.223	3
5	Track_856	20	0	0	0	0	0	34204.408	0.000	34204.408	29.406	4
6	Track_857	25	0	0	0	0	0	43205.568	0.000	43205.568	19.362	5
7	Track_858	25	0	0	0	0	0	43205.568	0.000	43205.568	42.650	6
8	Track_859	15	0	0	0	0	0	25203.248	0.000	25203.248	85.337	7
9	Track_860	25	0	0	0	0	0	43205.568	0.000	43205.568	17.392	8
10	Track_861	25	0	0	0	0	0	43205.568	0.000	43205.568	41.815	9
11	Track_862	25	0	0	0	0	0	43205.568	0.000	43205.568	41.893	10
12	Track_863	25	0	0	0	0	0	43205.568	0.000	43205.568	71.650	11
13	Track_864	25	0	0	0	0	0	43205.568	0.000	43205.568	8.589	12
14	Track_865	14	0	0	0	0	0	23403.016	0.000	23403.016	88.973	13
15	Track_866	9	0	0	0	0	0	14401.856	5400.696	19802.552	387.644	14
16	Track_867	2	0	0	0	0	0	1800.232	5400.696	7200.928	66.818	15
17	Track_868	3	0	0	0	0	0	3600.464	14401.856	18002.320	0.648	16
18	Track_869	19	0	0	0	0	0	32404.176	10801.392	43205.568	34.498	17
19	Track_870	6	0	0	0	0	0	9001.160	10801.392	19802.552	286.621	18
20	Track_871	16	0	0	0	0	0	27003.480	16202.088	43205.568	22.241	19
21	Track_872	9	0	0	0	0	0	14401.856	23403.016	37804.872	19.790	20
22	Track_873	4	1	1	0	0	0	7200.928	23403.016	30603.944	9.714	21
23	Track_874	10	0	0	0	0	0	16202.088	27003.480	43205.568	98.640	22
24	Track_875	7	0	0	0	0	0	10801.392	32404.176	43205.568	14.380	23
25	Track_876	5	0	0	0	0	0	7200.928	36004.640	43205.568	7.403	24
26	Track_877	3	0	0	0	0	0	3600.464	37804.872	41405.336	59.841	25

## Kymographs

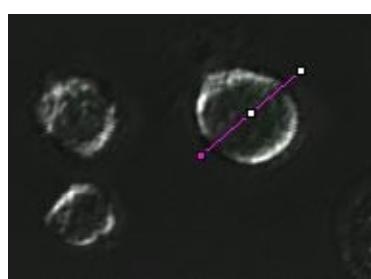
Kymographs are a way to represent a dynamic process as a single image. They are particularly useful to monitor and characterize the movement of a cell, membrane or organelle or a change in fluorescence over time. They can be seen as an  $x-t$  scan, where the intensity along a given line is plotted for all images of a time series. Each time point gives an intensity line, plotted e.g. along the  $x$  axis of the kymograph. These lines are stacked along the  $y$  axis for all frames.

So we get an image where we move through space in the  $x$  direction and time in the  $y$  direction.

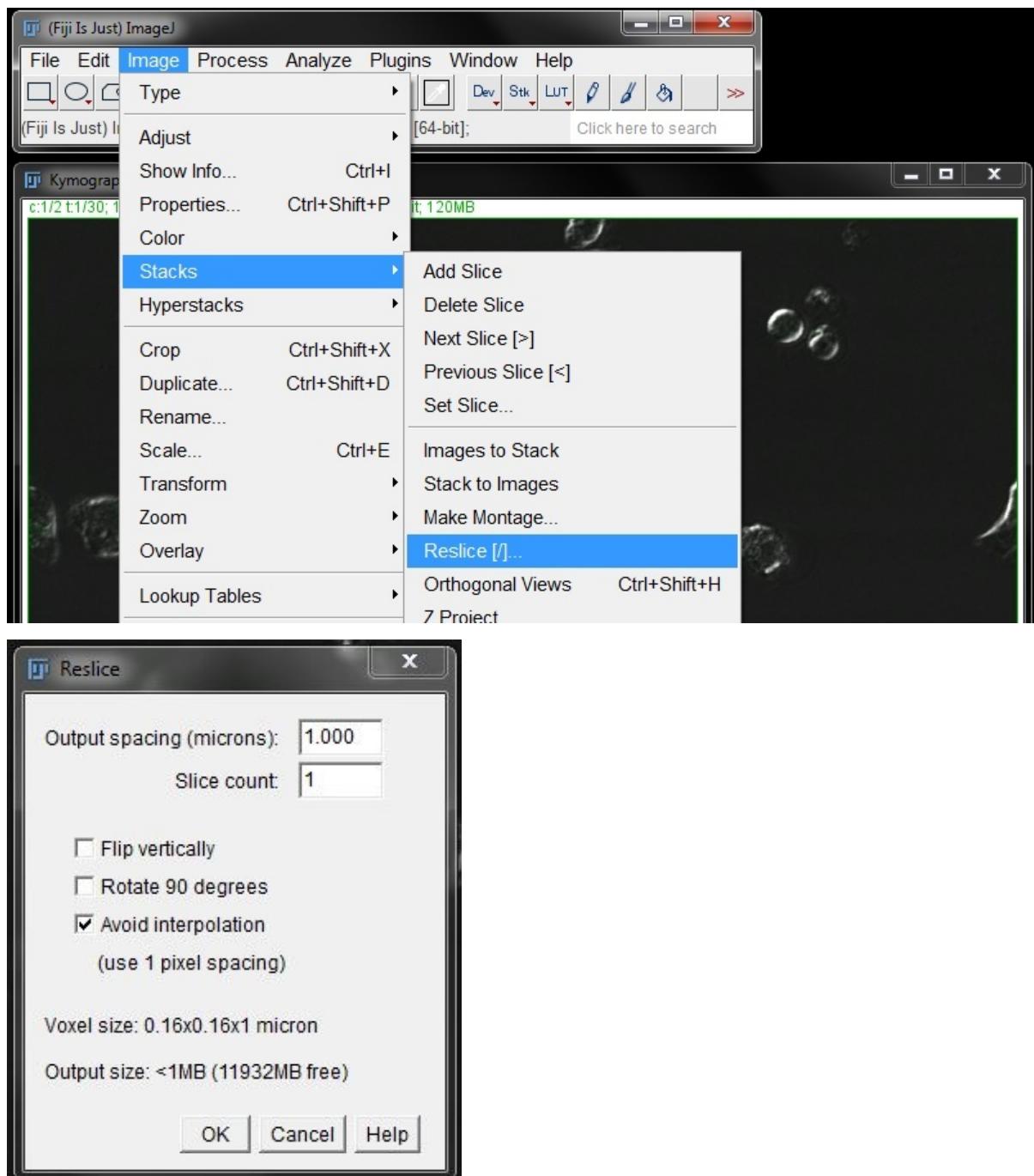
Open *Kymograph.tif* and scroll through time to find a cell that stays in the same area for the whole time series. An example of one of these cells is shown inside the ROI below.



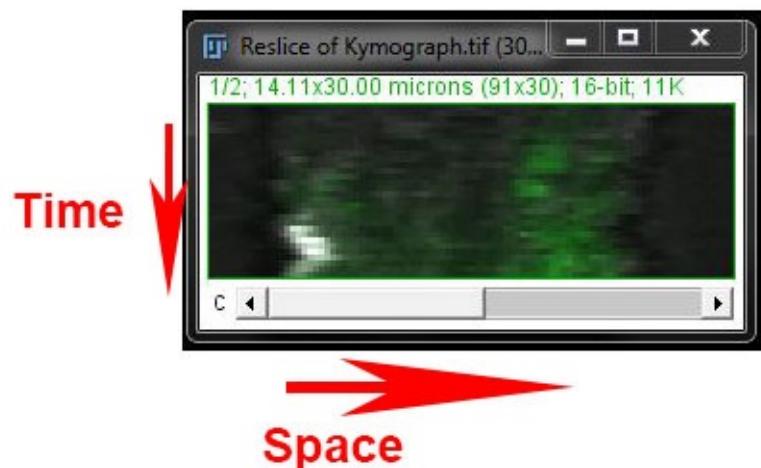
Switch to line ROI and draw a line through the cell you want to analyse.



Go to **Image -> Stacks -> Reslice** and press **OK** in the resulting window.



The resulting image displays space on the x-axis, an intensity profile across the cells in brightfield and fluorescence. Time is displayed on the y-axis (top: start, bottom: end), you can see the green fluorescence intensity increase over time and the cell move left and right in the brightfield image.



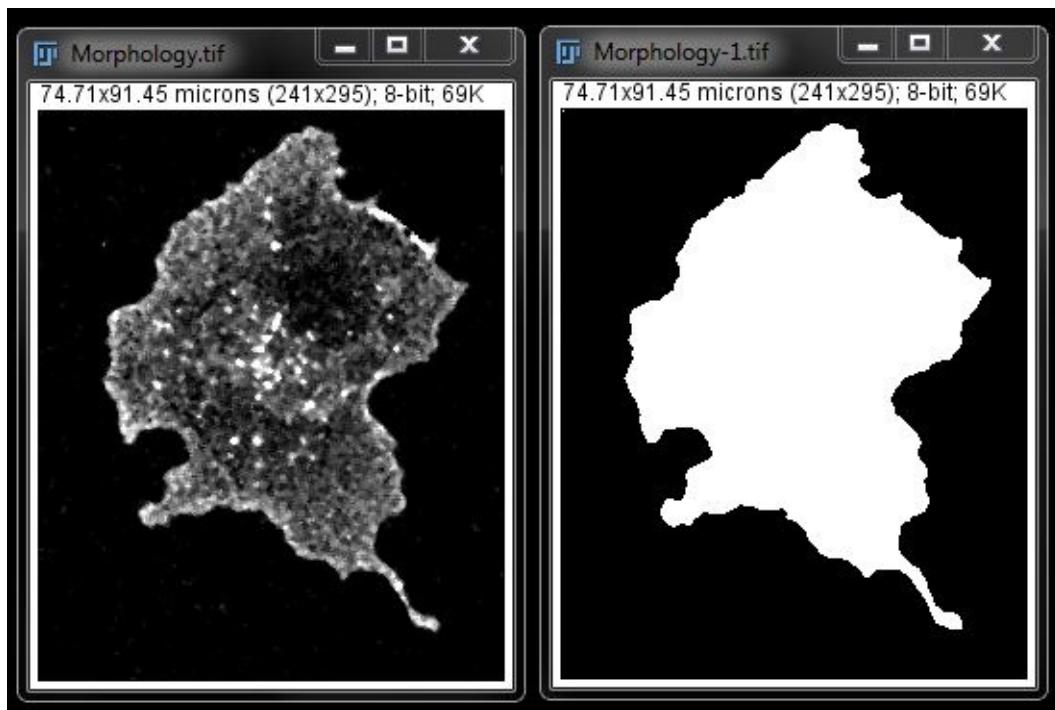
## PART 8: MORPHOLOGY MEASUREMENTS

Some of the basic analysis tools we have already used can also be adapted to give us more detailed information about the data we are analysing. For example, we previously used the **Analyse Particles** tool to count nuclei. You can use the same tool for analysing the morphology of your cells.

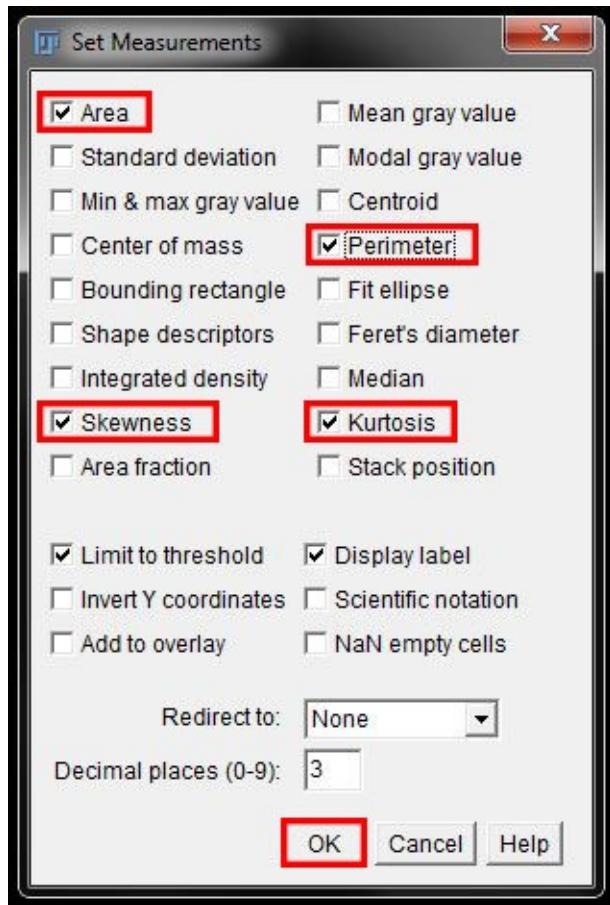
In this section we use the image *Morphology.tif* for demonstration.

### Morphology Changes

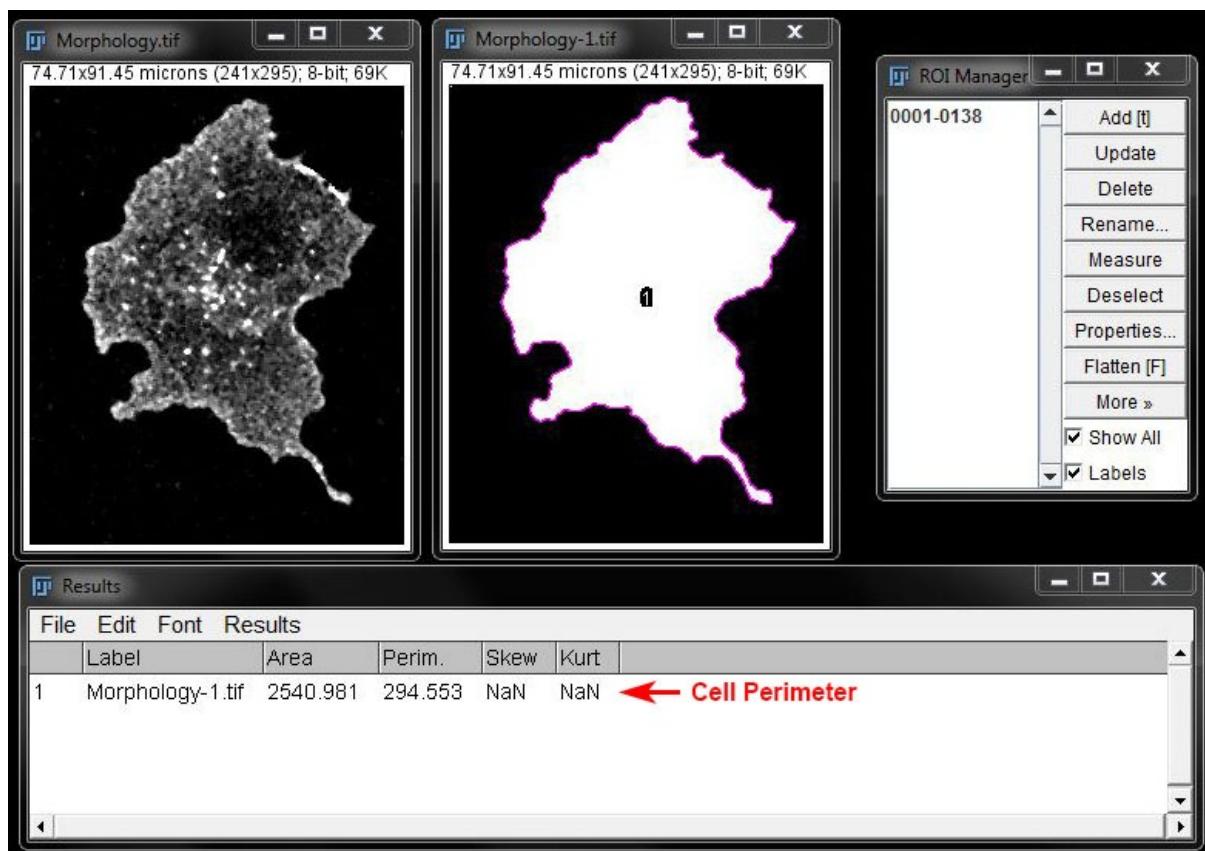
Open the demo image *Morphology.tif*. Duplicate it, then set a threshold and create a binary of the image. Here I have used the Huang algorithm set at 12 and 255. Following binary creation I have used the 'fill holes', 'despeckle' and 'remove outliers' filters to clean up the binary.



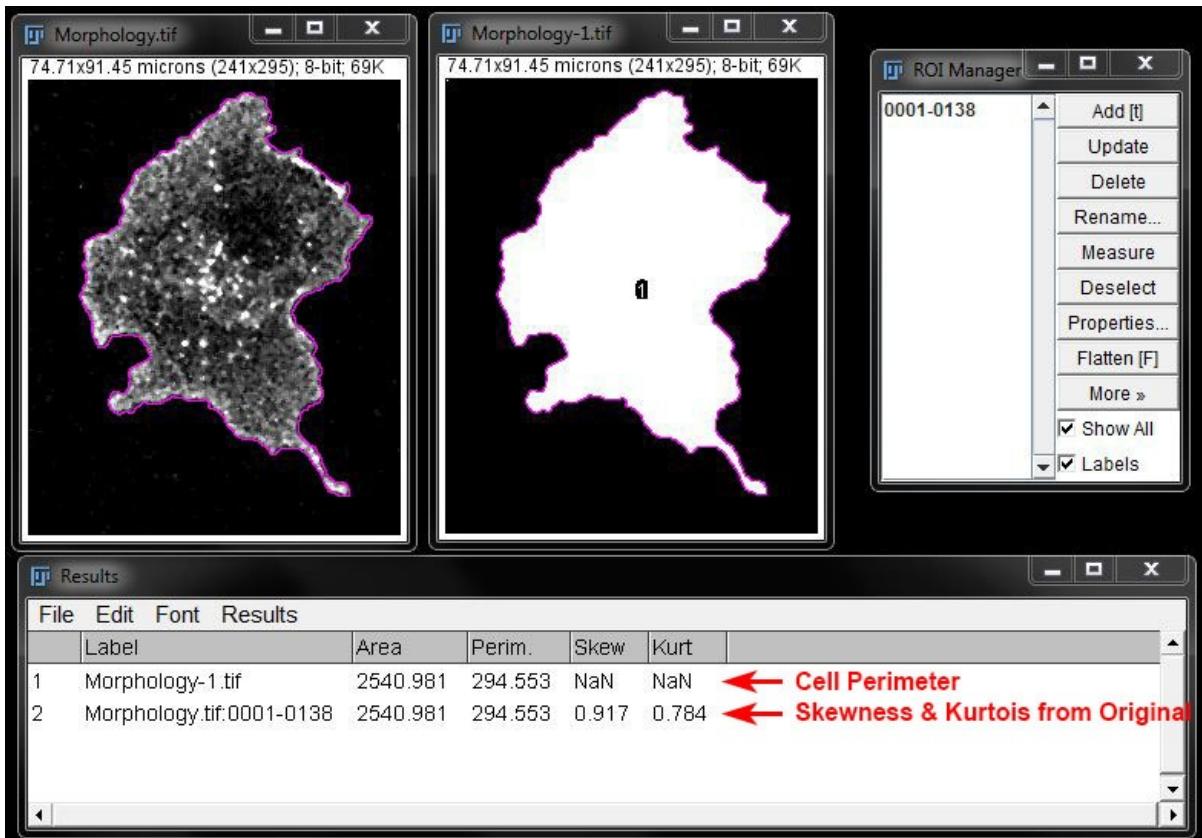
Under **Analyze -> Set Measurements** select Area, Perimeter, Skewness and Kurtois and click **OK**.



Open the ROI Manager and check it is empty, then run **Analyze Particles** on the binary image. You will get one ROI that outlines the cell, the results window shows you the perimeter of the cell.

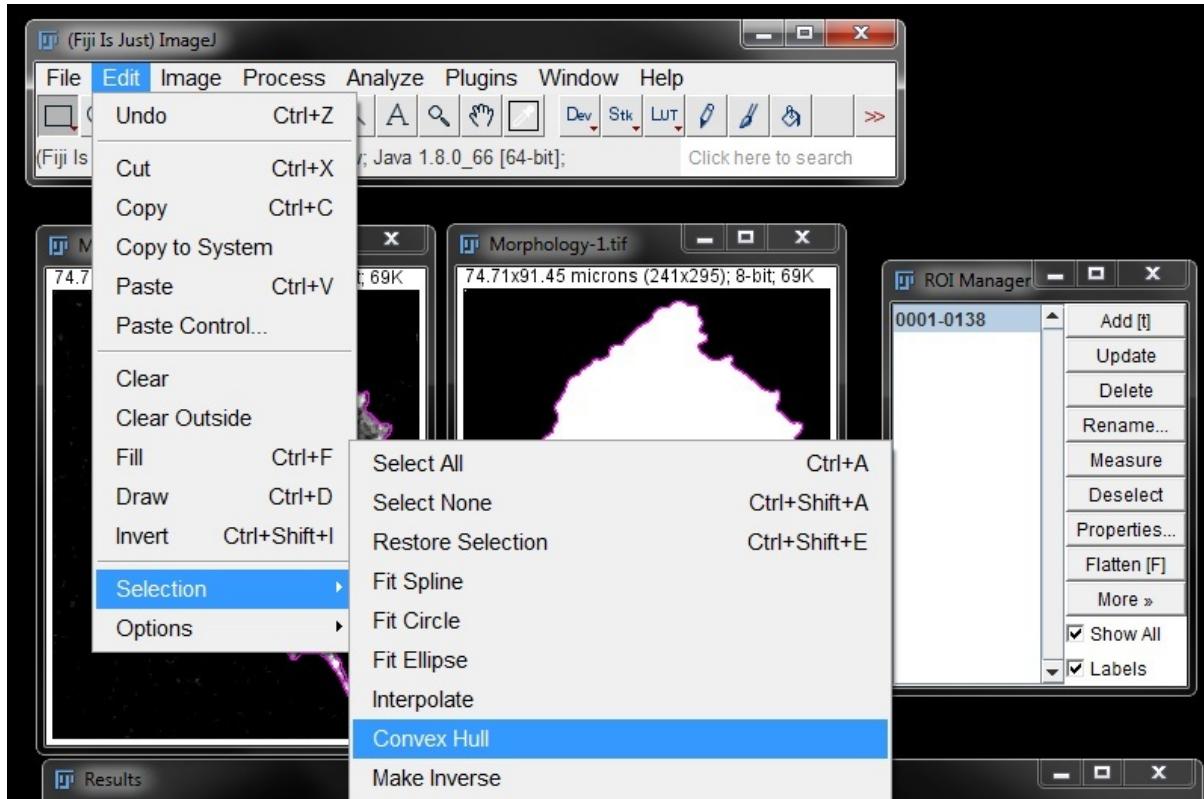


Click on the original image to activate it and press **Measure** in the ROI Manager. You will get a second results line that gives you Skewness and Kurtosis of the cell.

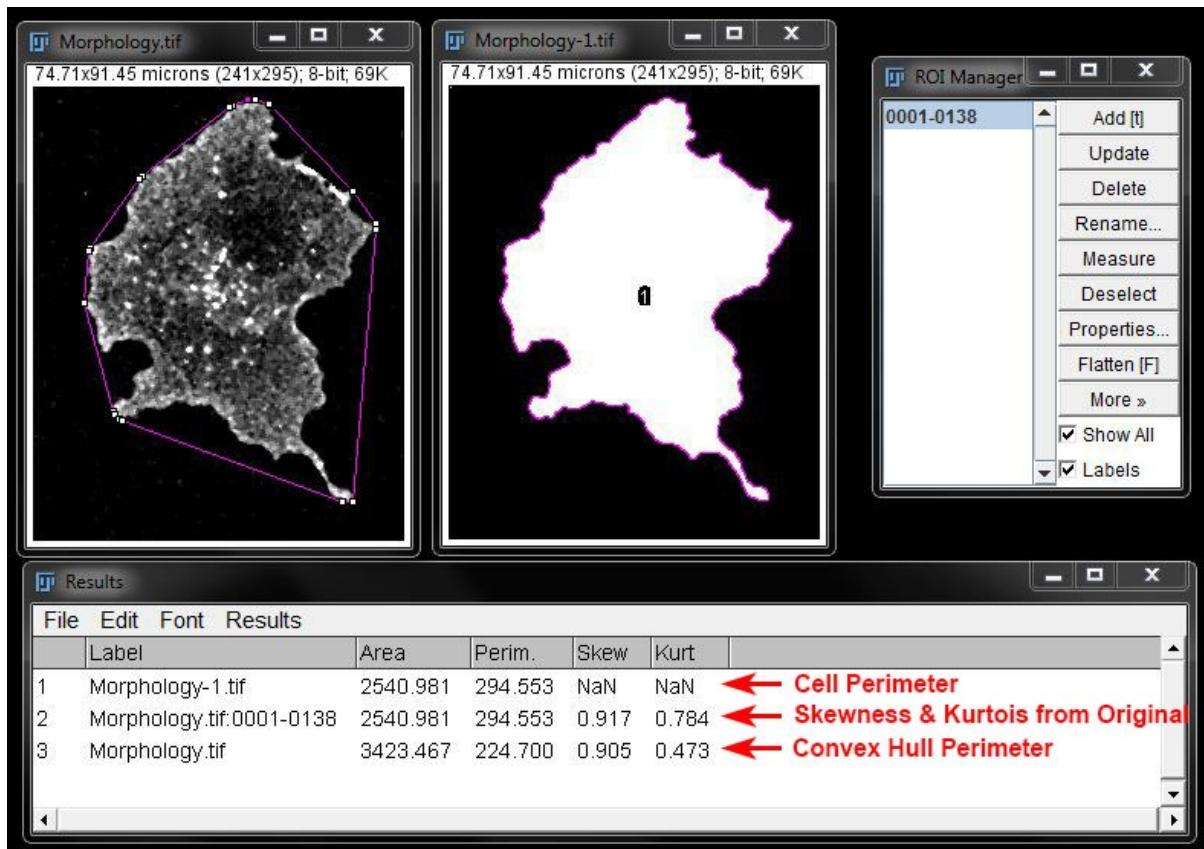


Skewness and Kurtosis provide information on the intensity distribution within the cell. **Skewness** is a measure of symmetry, or more precisely, the lack of symmetry. A distribution, or data set, is symmetric if it looks the same to the left and right of the centre point. **Kurtosis** is a measure of whether the data are heavy-tailed or light-tailed relative to a normal distribution.

Now select the ROI in the ROI Manager and go to **Edit -> Selection -> Convex Hull**. This will draw a closed circumference around the ROI.



Go to **Analyse -> Measure**, this will give you a third line in the results window with the perimeter of the convex hull.



The ratio between convex hull perimeter and cell ROI perimeter is a measure of the sphericity or 'spikiness' of the cell. If the ratio is close to 1 then the cell is almost spherical, the closer the ratio goes towards zero, the spikier the cell.

If you have a time series of images, you can also look at morphology changes over time with this method.



## PART 9: CO-LOCALISATION ANALYSIS

For fluorescent images we can examine whether two fluorophores (hence; two proteins of interest) are interacting by using co-localisation analysis.

In this section we use the image *Colocalisation.tif* for demonstration.

### Important Considerations

It is important to remember that co-localisation analysis is not an exact measurement, and numerous factors can alter the outcome of your analysis. Before you undertake co-localisation analysis there are several important factors that need to be considered.

*Resolution and the Limitations of the Microscope:* Resolution refers to the ability to distinguish two separate objects as separate. The size of proteins and fluorophores within your cells will be smaller than the resolution limitations on a light microscope (widefield or confocal) as such two tagged proteins that are not actually occupying the same space but are close can appear to be co-localised, simply because they could not be resolved. The higher the resolution of an image, the more accurate the result will be, with super-resolution images being the highest standard for determining true co-localisation.

*The Size of Antibodies and Tags:* Antibodies and fluorescent tags are bigger than you might expect. The addition of an antibody (or antibodies) or fluorescent tag to your protein of interest can add considerable size and it is important to remember that we are viewing that fluorescent marker with our imaging, not the protein of interest directly. As such, two fluorescent markers sitting side by side or even within the same space may not necessarily mean that your two proteins also overlap. There may still be considerable distance between proteins that is being occupied and diminished by the size of the antibodies or tags.

*Spectral Overlap:* Although fluorescent markers have a 'peak' emission the actual emission spectrum often has a long tail. This means that it can also be detected at a higher wavelength, often overlapping with a fluorophore in the next 'colour' range. Therefore it is important to select your fluorescent markers carefully when imaging for co-localisation to ensure minimal spectral overlap. It is also important to use image acquisition parameters that minimise the potential for any overlap (such as sequential scanning on a confocal microscope). Failure to do this may mean your "co-localisation" is simply the same dye being imaged in both channels.

*Acquisition Parameters and Differences in Fluorophores:* As co-localisation analysis is based on intensity it is important to ensure that all acquisition parameters remain equal between images. It is also important to keep in mind that different dyes and fluorophores have quite different parameters that can affect imaging and subsequent analysis. There is no way to avoid these differences, but you can try to minimise variations by following the same protocols and imaging parameters each time if possible.

### Co-localisation Analysis Algorithms

This workshop will concentrate on two different algorithms to calculate co-localization: Manders and Pearsons.

#### Manders...

...thresholds both images and calculates how many pixels above threshold in channel 1 are also above threshold in channel 2 and vice versa. You get two Manders coefficients, one for channel 1 and one for channel 2. Values will range between 1, for complete co-localization i.e. all channel 1 pixels above threshold are also above threshold in channel 2, and 0, for no co-localization i.e. no channel 1 pixels above threshold are above threshold in channel 2.

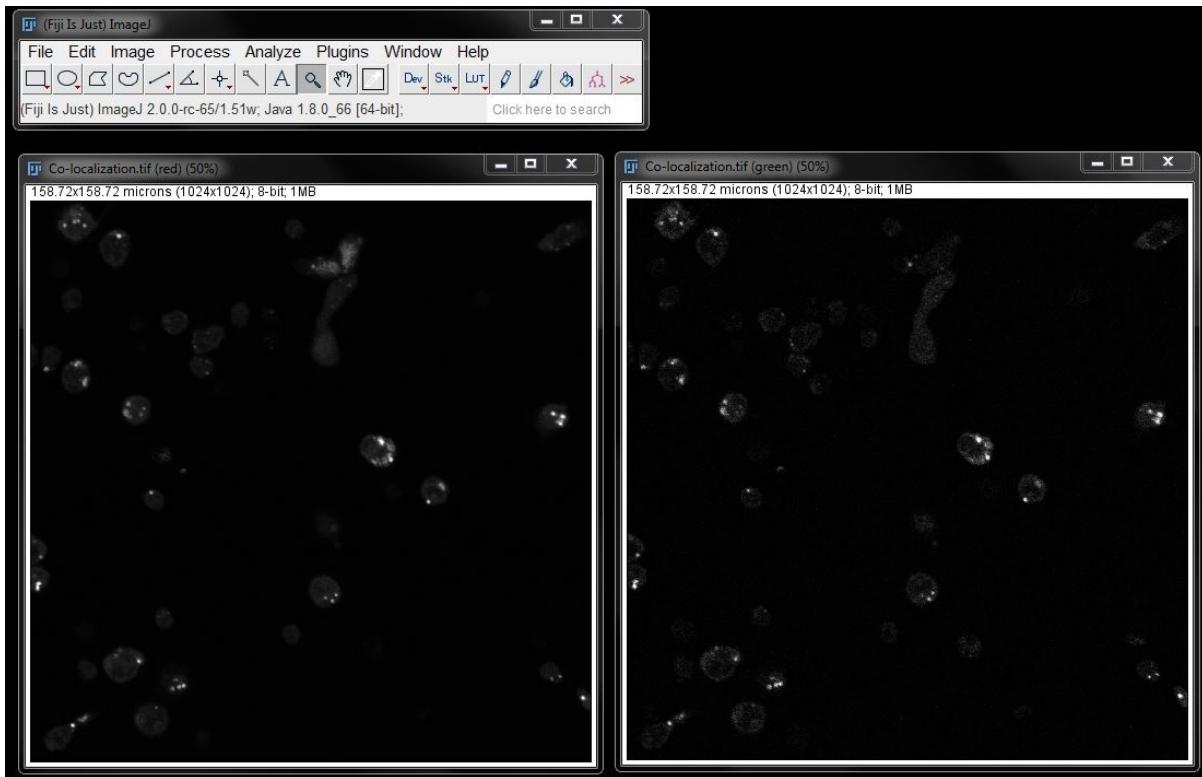
## Pearsons...

...takes into account the fluorescence intensity of each pixel. If all bright pixels in channel 1 are also bright in channel 2, and all dim pixels in channel 1 are also dim in channel 2 then the Pearson's coefficient is 1. If there's no co-localization, i.e. fluorescence intensities are randomly distributed in channel 1 and 2, then the Pearson's coefficient is 0. The two channels can also be anti-correlated, which means that all bright pixels in channel 1 are dim in channel 2, and vice versa. In other words, where there's green signal, there is no red signal. In this case, the Pearson's coefficient is -1. Pearson's co-localization does not require thresholding and is therefore more robust than Manders. It also provides you with more information as fluorescence intensity is being taken into account and you get information on anti-correlation.

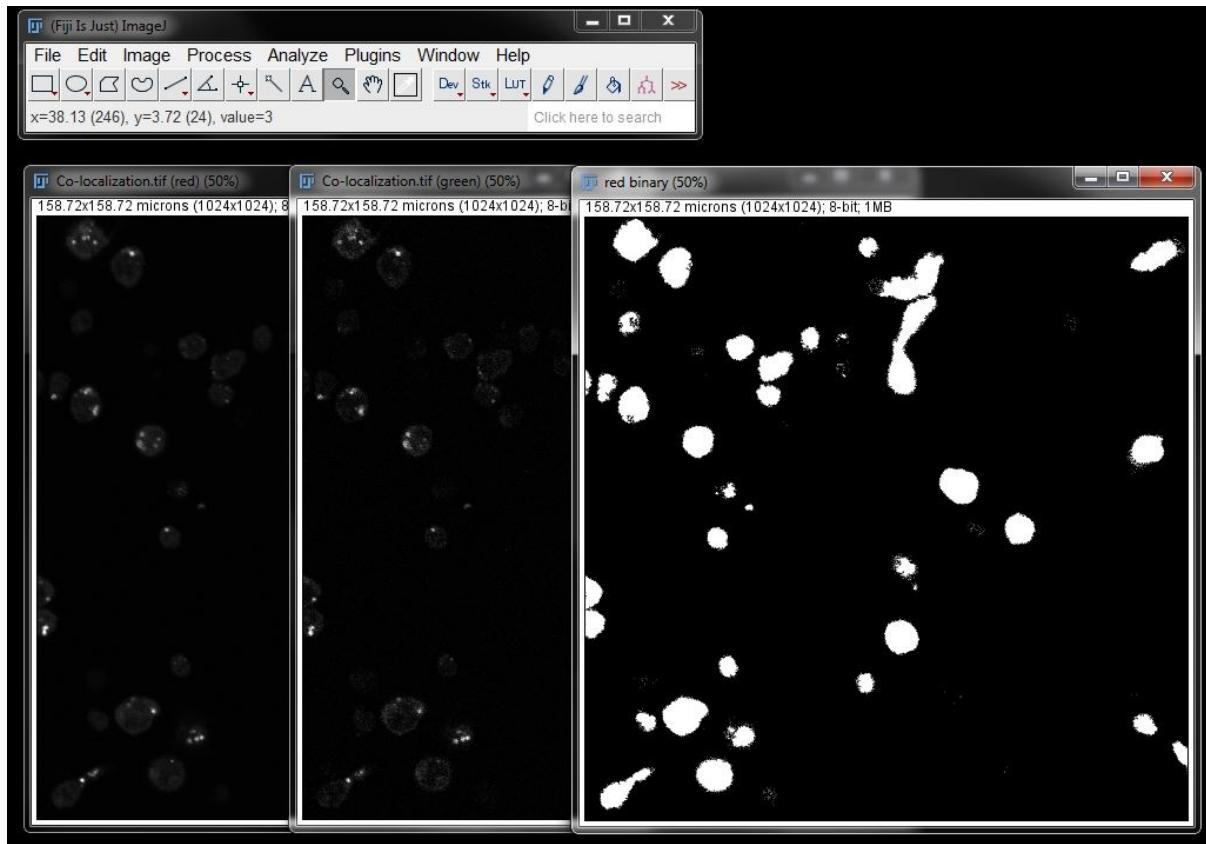
## Co-localisation Analysis

FIJI has a co-localisation plugin available that will allow you to carry out analysis in a few steps.

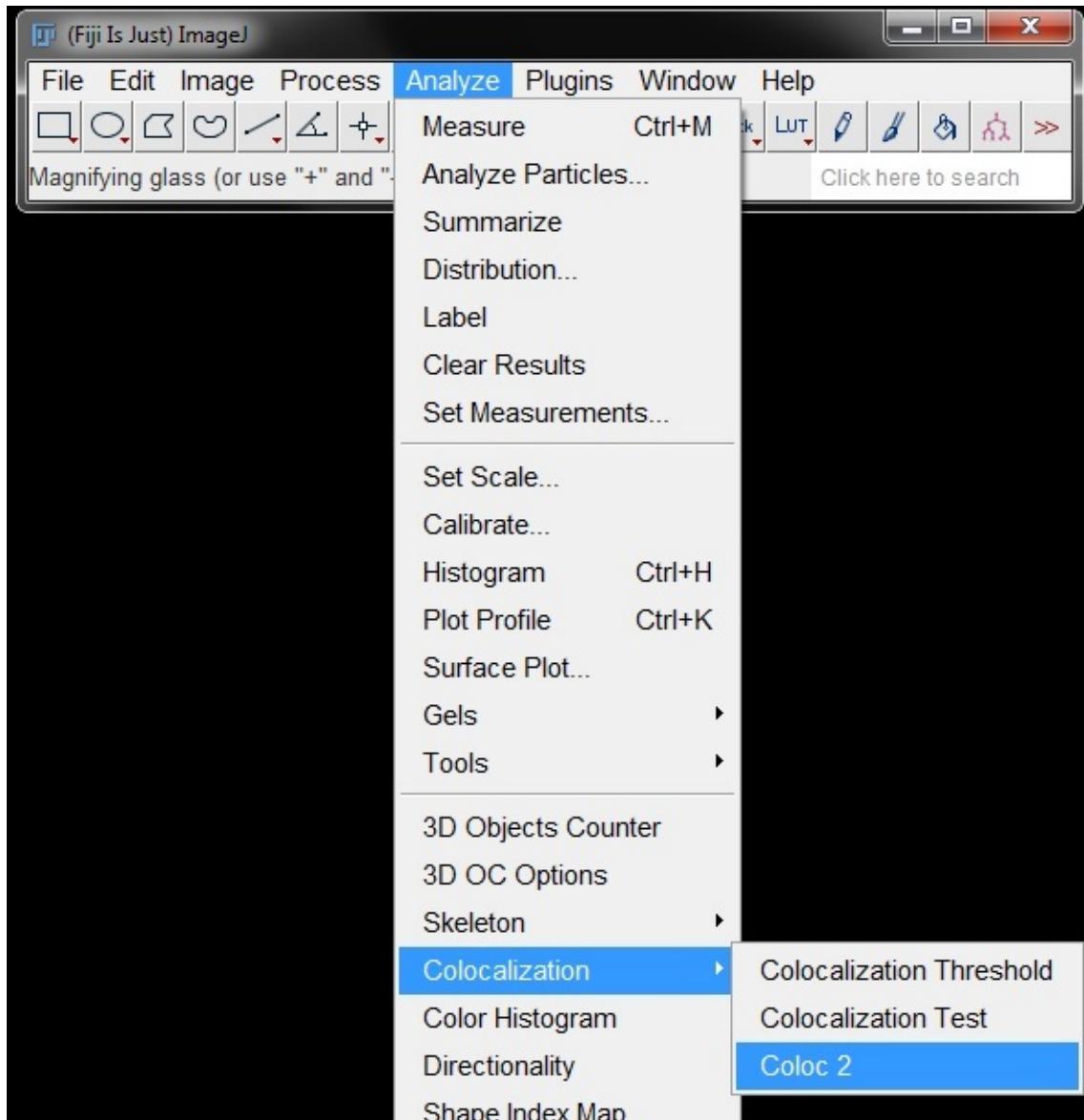
To begin analysis, open the demo image *Colocalization.tif* and split channels. We only need the red and green channels here, so close the image for the blue channel.



Our first step is to create a binary of the red channel to allow the plugin to separate the cells from the background. To do this, first duplicate the red channel, then threshold and create a mask/binary of the duplicate. Make any necessary adjustments to your binary to ensure accurate representation of the cells.

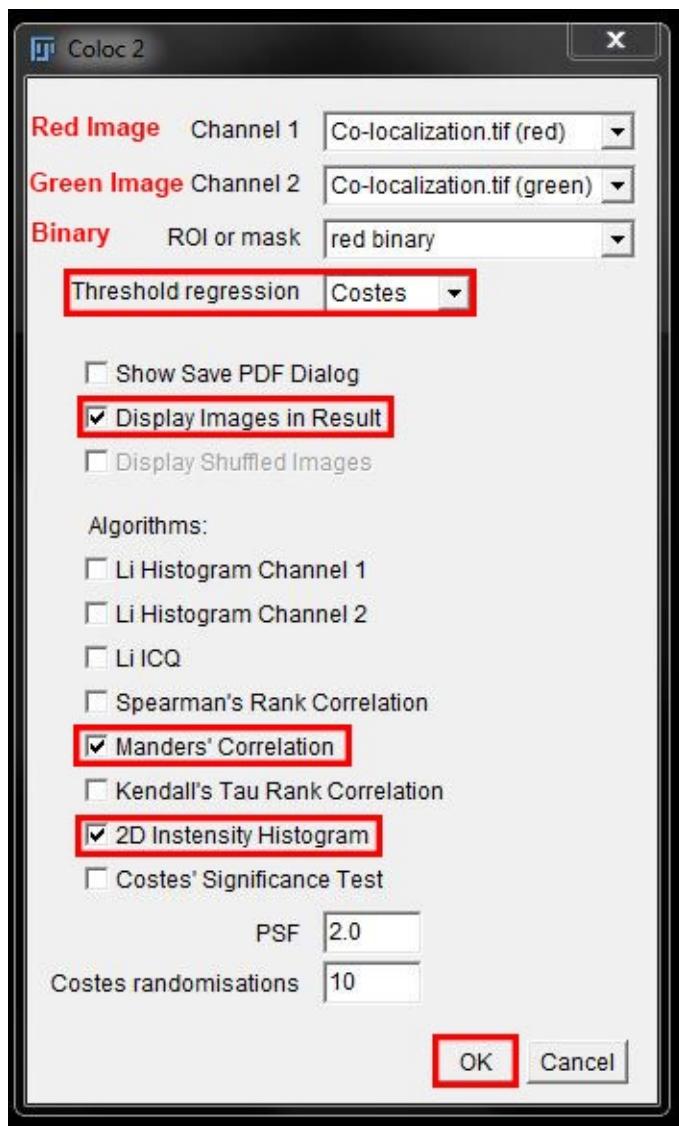


Go to **Analyze -> Colocalization -> Coloc2**.

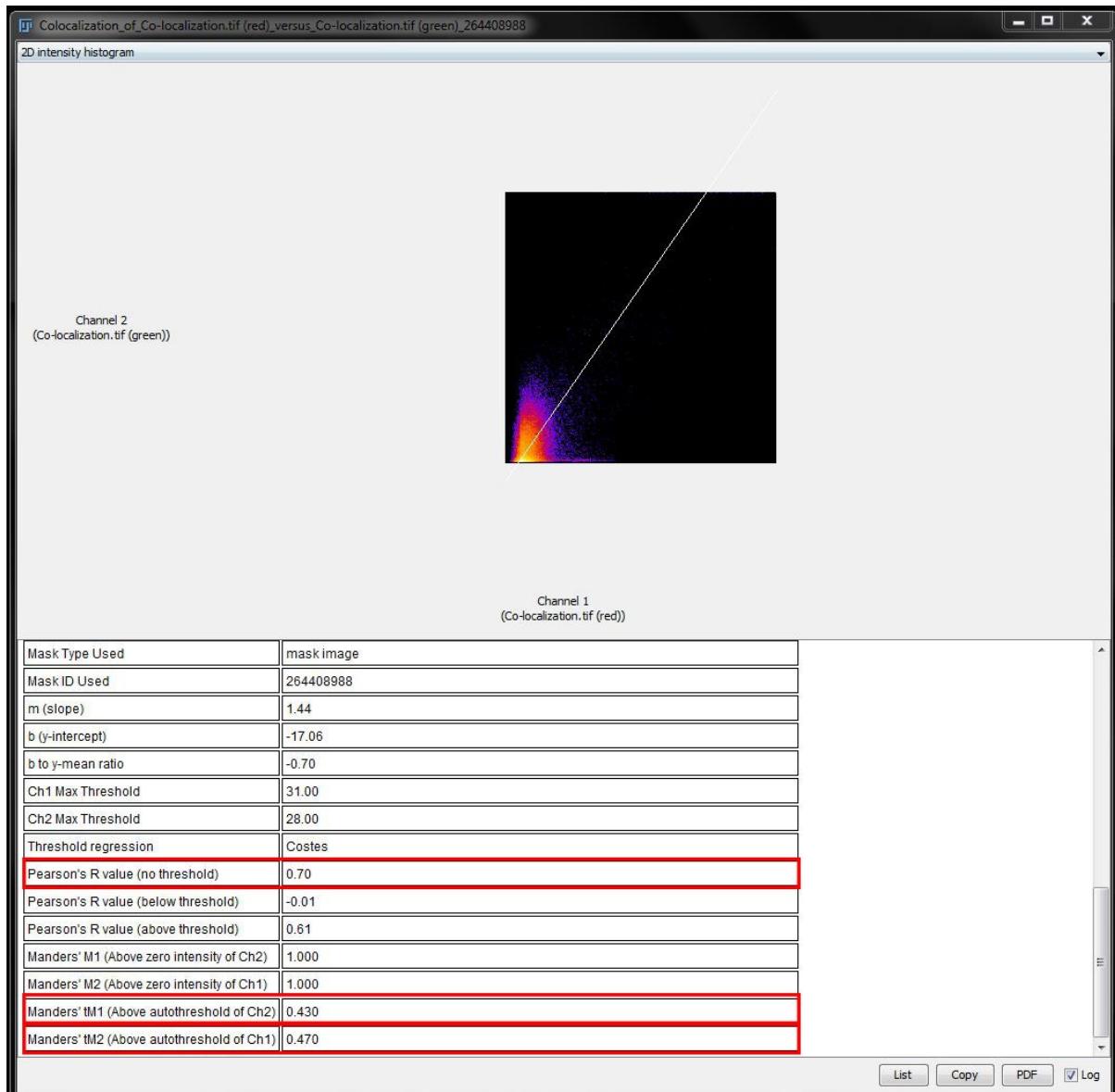


In the options window, choose the red image as channel 1, the green image as channel 2, and your mask/binary as mask from the drop down menus. Coloc2 will use autothresholding to calculate the Manders coefficients, leave this option as the default **Costes** method. Tick on the following options (if not already on by default): **Display Images in Result, Mander's Correlation, 2D Instensity Histogram**. Leave the **PSF** and **Costes randomisations** at 2 and 10 respectively.

Once your options are set, click on **OK** to begin the calculation.



After calculation, a results window will open, containing a graph and a table of values.



Let's try to understand these values. Manders coefficients without thresholds is 1. That will be the case for almost all images and just means that there is some kind of signal in all pixels. Not useful!

Manders with thresholds is better: we have a Manders coefficient of 0.43 for the red channel and 0.47 for the green channel. That means that inside the mask, 43% of the red pixels are also green, 47% of the green pixels are also red.

Pearson's coefficient without threshold is 0.70, which means the signal in both channels is correlated, the above and below values are not really crucial, but a nice control. Above threshold is also correlated, while below threshold is randomly distributed.

The scatter plot shows fluorescence intensity of one channel on the x-axis and fluorescence intensity of the other channel on the y-axis. Correlated or co-localized signal will show up along the white line.

## Acknowledgements

These workshop notes were prepared by Dr Sarah Creed, with contribution to content by Dr Kirstin Elgass, of Monash Micro-Imaging at the Monash Health Translation Precinct . Preparation of these notes was made possible by generous contributions from Cameron Nowell, Monash Institute of Pharmaceutical Sciences and the contribution of demonstration images by researchers.

## Use and Distribution of Workshop Notes

These notes may be used and distributed by workshop attendees to their co-workers and associates, provided they remain intact and are not altered in any way. Only the workshop notes may be used and distributed in this way.

Demonstration images CANNOT be distributed to others with the notes. Distribution and use of demonstration images is not permitted under any circumstances, but the notes can be easily applied to your own images.

# Demonstration Images

All of the images used for the FIJI Intermediate Image Analysis Workshop are made available to workshop attendees under the condition that they are used for demonstration purposes in the workshop only. Images cannot be redistributed or used for any other purpose, personal or professional, by workshop attendees at any time.

Demonstrations Images were generously provided by the following contributors:

<b>Spheroid.tif</b> <sup>1</sup>	Kirstin Elgass, <i>Monash Micro-Imaging at MHTP</i>
<b>HyperStack.tif</b> <sup>2</sup>	Sarah Creed, <i>Monash Micro-Imaging at MHTP</i>
<b>Nuclei-1.tif</b>	Kirstin Elgass, <i>Monash Micro-Imaging at MHTP</i>
<b>RGBStackProjection.tif</b> <sup>3</sup>	Sarah Creed, <i>Monash Micro-Imaging at MHTP</i>
<b>DAPI_Uneven</b> <sup>4</sup>	Kirstin Elgass and Sarah Creed, <i>Monash Micro Imaging at MHTP</i>
<b>Bleached.tif</b>	Kirstin Elgass, <i>Monash Micro Imaging at MHTP</i>
<b>NeuralTubeRed.tif</b>	Catherine Cochrane, CCR, <i>Hudson Institute of Medical Research</i>
<b>AutoTrack.tif</b>	Nils Nesheim, <i>Monash University</i>
<b>Kymograph.tif</b> <sup>5</sup>	Rebecca Lim, <i>The Ritchie Centre, Hudson Institute of Medical Research</i>
<b>Image Set: Deconvolution</b>	Kirstin Elgass, <i>Monash Micro-Imaging at MHTP</i>
<b>Image Set: Manual Stitching</b>	Sarah Creed, <i>Monash Micro Imaging at MHTP</i>
<b>Image Set: TrackEM2</b>	Anqi Li, <i>_T_he Ritchie Centre, Hudson Institute of Medical Research</i>

<sup>1</sup>. Captured at the UWA Centre for Microscopy, Characterisation and Analysis. Also used to generate Spheroid\_Shift.tif ↵

<sup>2</sup>. Captured at The Lineberger Comprehensive Cancer Centre, UNC, Chapel Hill, NC USA. Also used to generate MovieStack.tif ↵

<sup>3</sup>. Also used to generate the image set RGB-Blue.tif, RGB-Green.tif and RGB-Red.tif and Morphology.tif ↵

<sup>4</sup>. Pseudo image created by Sarah Creed as a demonstration image from a combination of Spheroid.tif background and RGB-Blue.tif ↵

<sup>5</sup>. Also used to generate Colocalisation.tif ↵