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A taste of 

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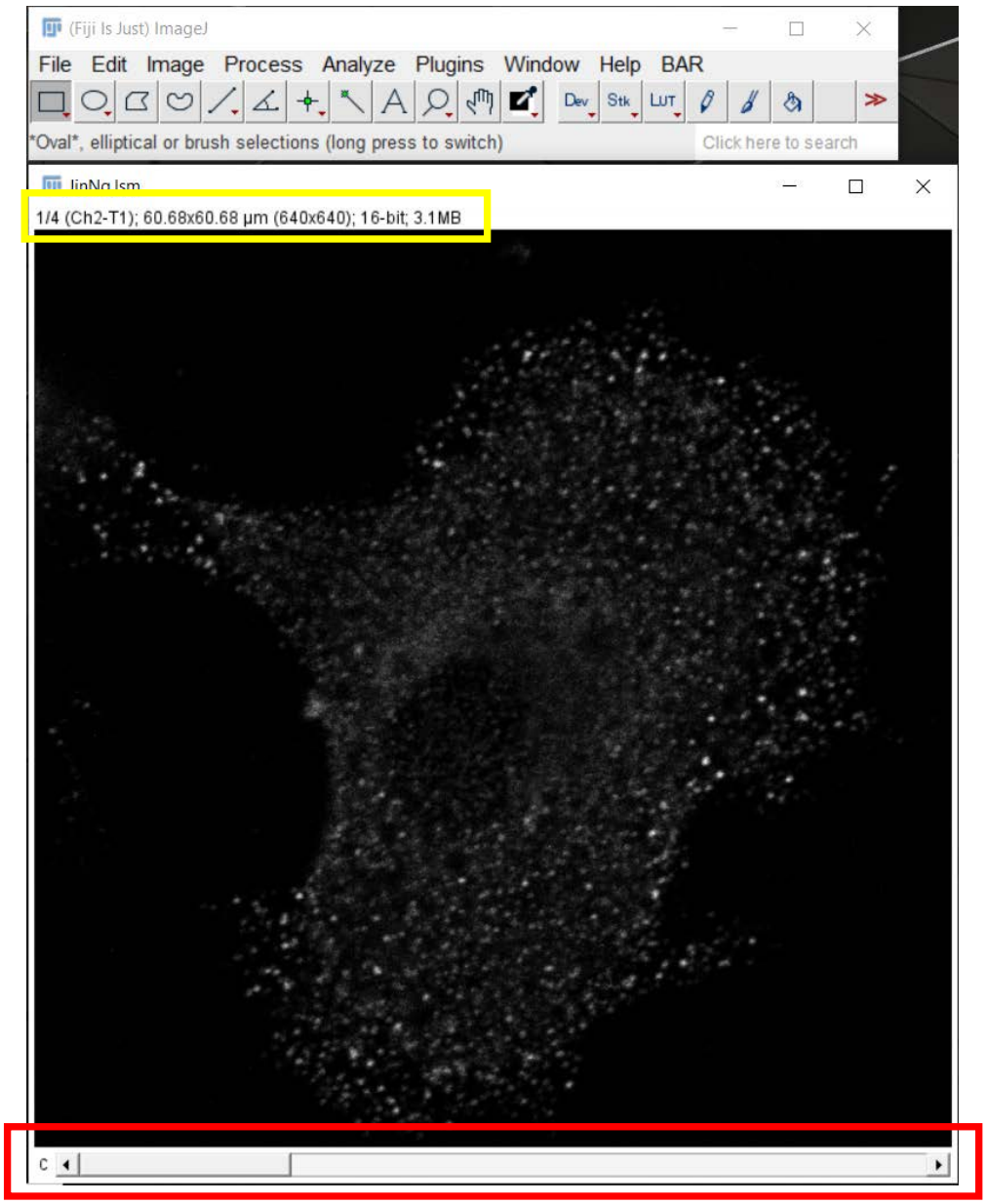
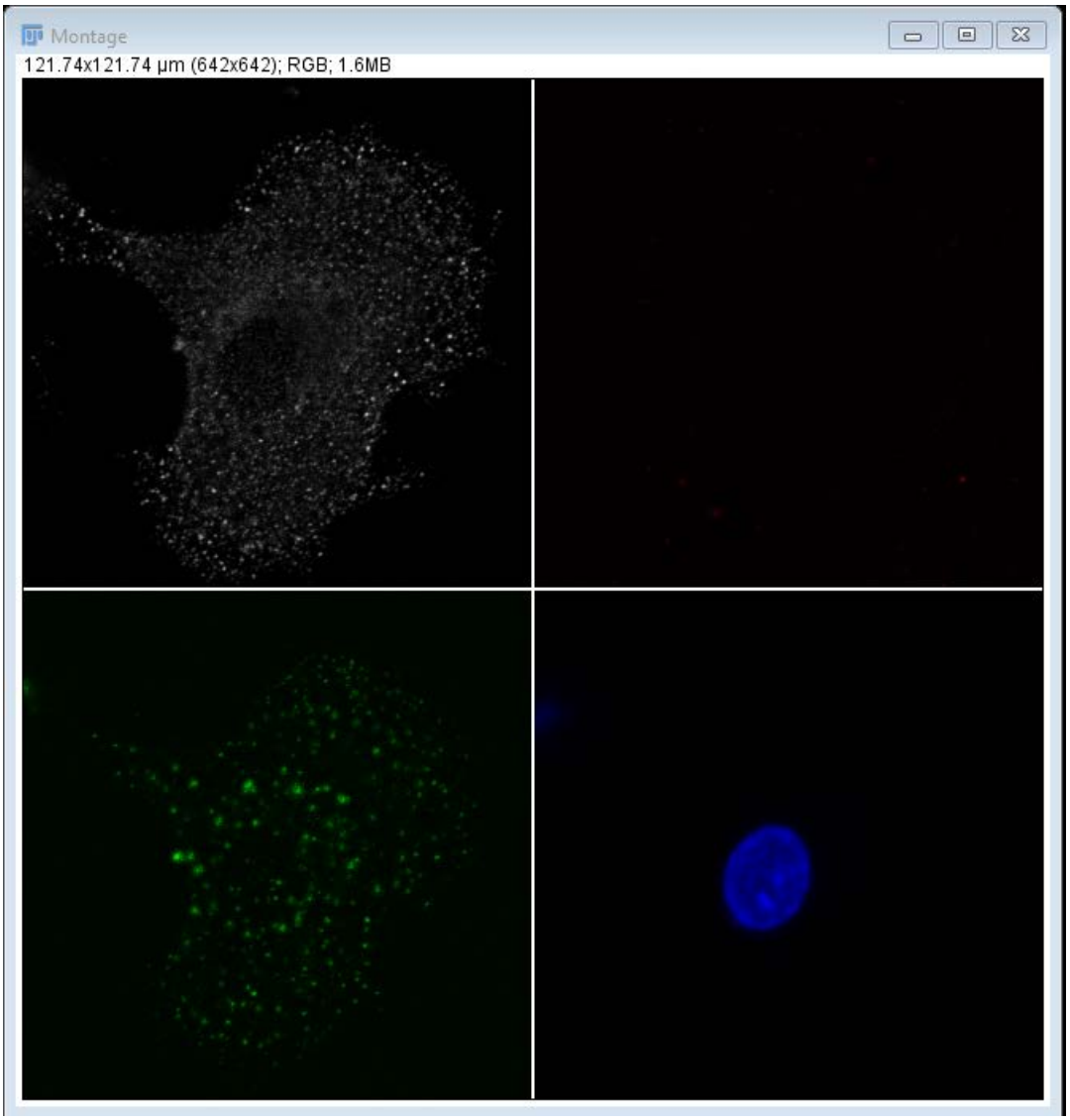
Getting started

This document is complementary to the webinar and provides written instructions for the example demonstrated during the webinar.

1. Open the file. Note that this image is already calibrated. If it wasn't, you would need to do this by going to **Analyze – Set Scale**. You would need an image of a micrometer slide or a scale bar on the image to do this.

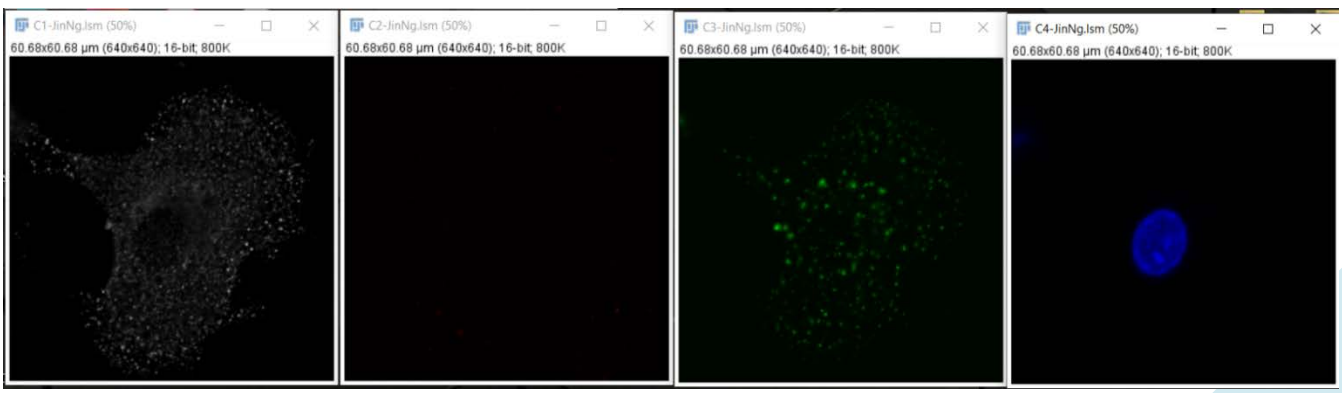


3. The data set has 4 channels.



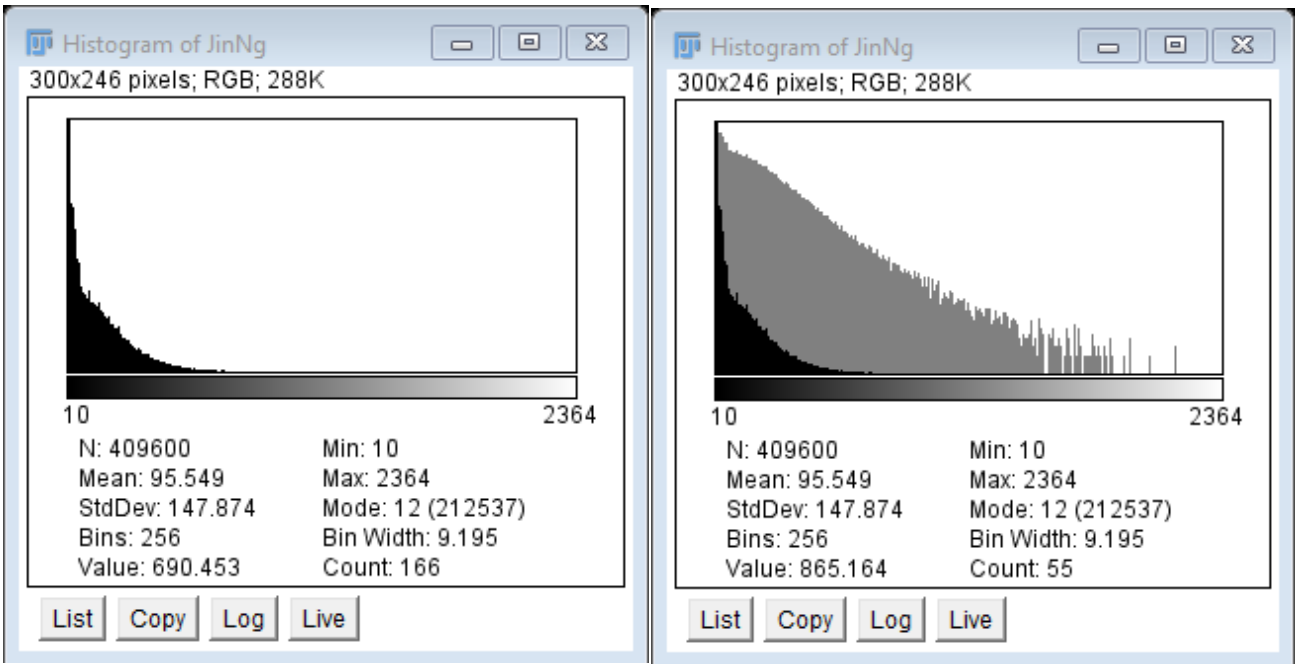
- Channel 1 – whole cell labelling but is punctate - white
- Channel 2 – punctate labelling - red
- Channel 3 – punctate labelling - green
- Channel 4 – nucleus – blue

4. Split the data set into the 3 components by going to **Image – Color – Split Channels**;



Histogram

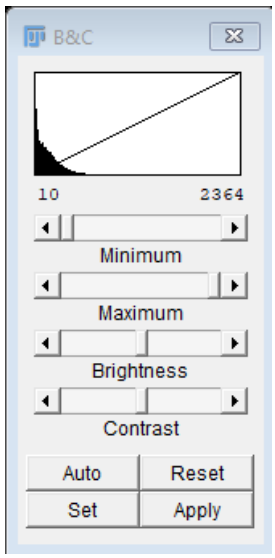
6. Select the C1 image (whole cell labelling).
7. You can check the histogram (frequency of pixel values) by going to **Analyze – histogram**. This provides important information about the image. You can also view this on a **Log scale**.



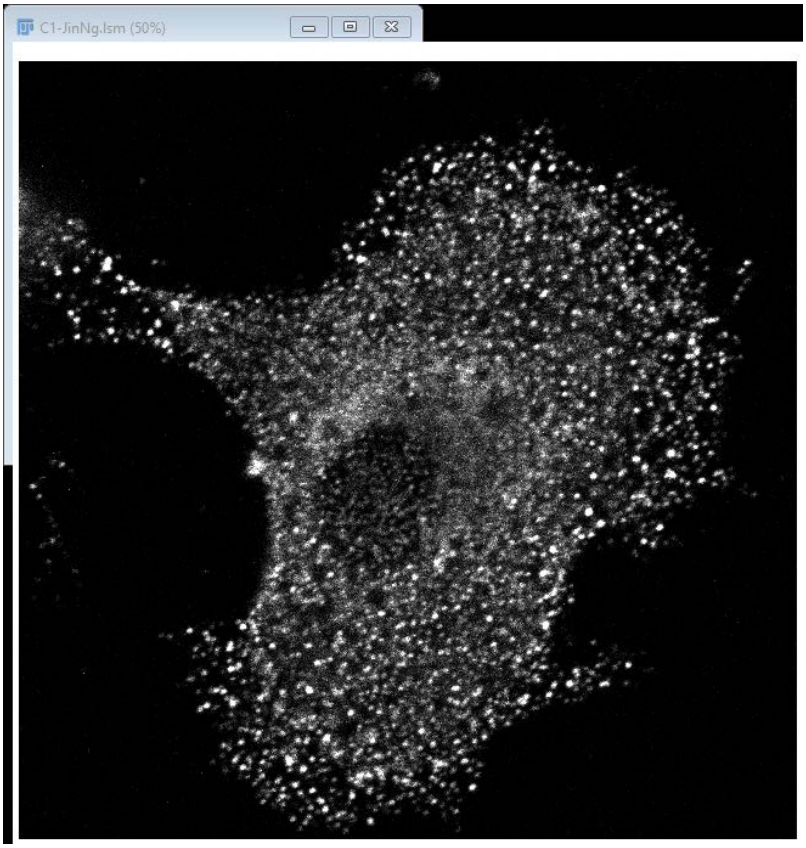
8. Use the **Magnifying tool** to make the image larger so that you are viewing it at 100%.
9. Go to **Image – Duplicate** to make a copy of the image. This is very useful so that you can compare the effects of any image processing.

Image enhancement

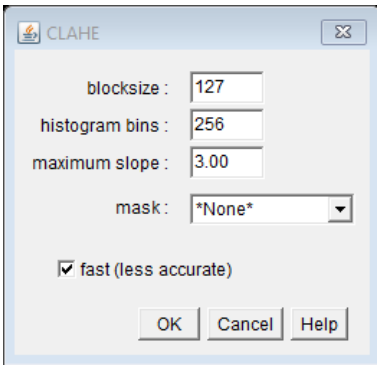
10. Using the duplicate image, go to **Image – Adjust – Brightness/Contrast**;



11. Click **Auto** or adjust **Minimum/Maximum** manually. Then click **Apply**. Note that this does change the pixel values so you wouldn't be doing this if you were going to measure intensity on this image. You can view the histogram if you want to see the changes.

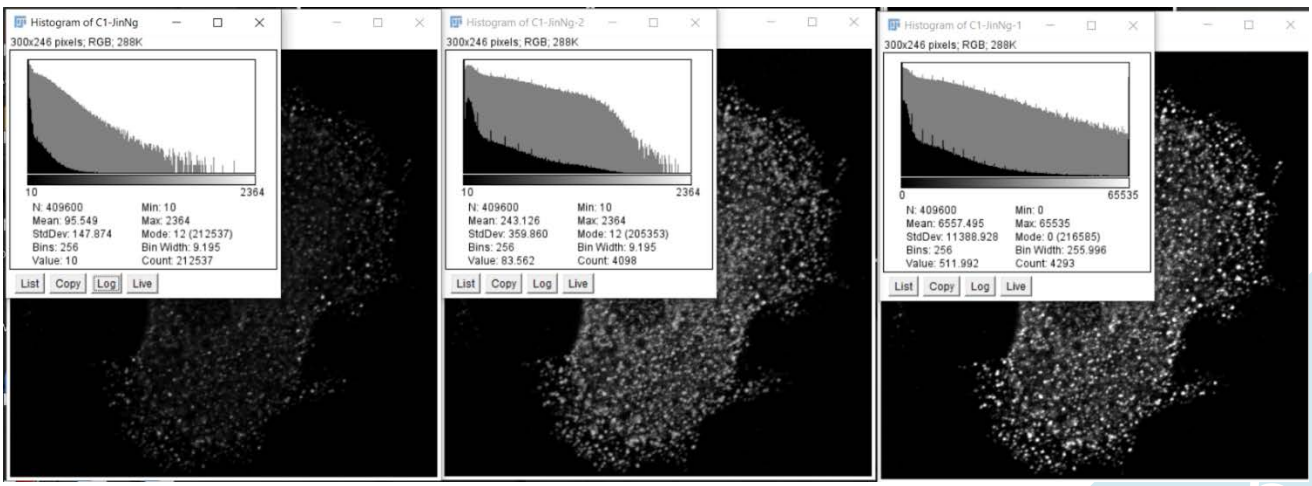


12. Another option to quickly enhance the image is to use local contrast enhancement.
13. Make another copy of the image (from the original) and go to **Process – Enhance Local Contrast** (CLAHE). **Contrast Limited Adaptive Histogram Equalization**



14. If the image is large, you might leave the option of **Fast** ticked. Otherwise, turn it off for better results.

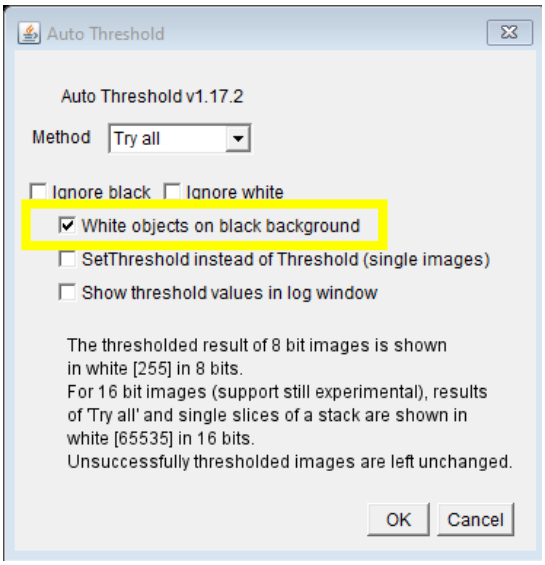
Original **CLAHE** **B/C**



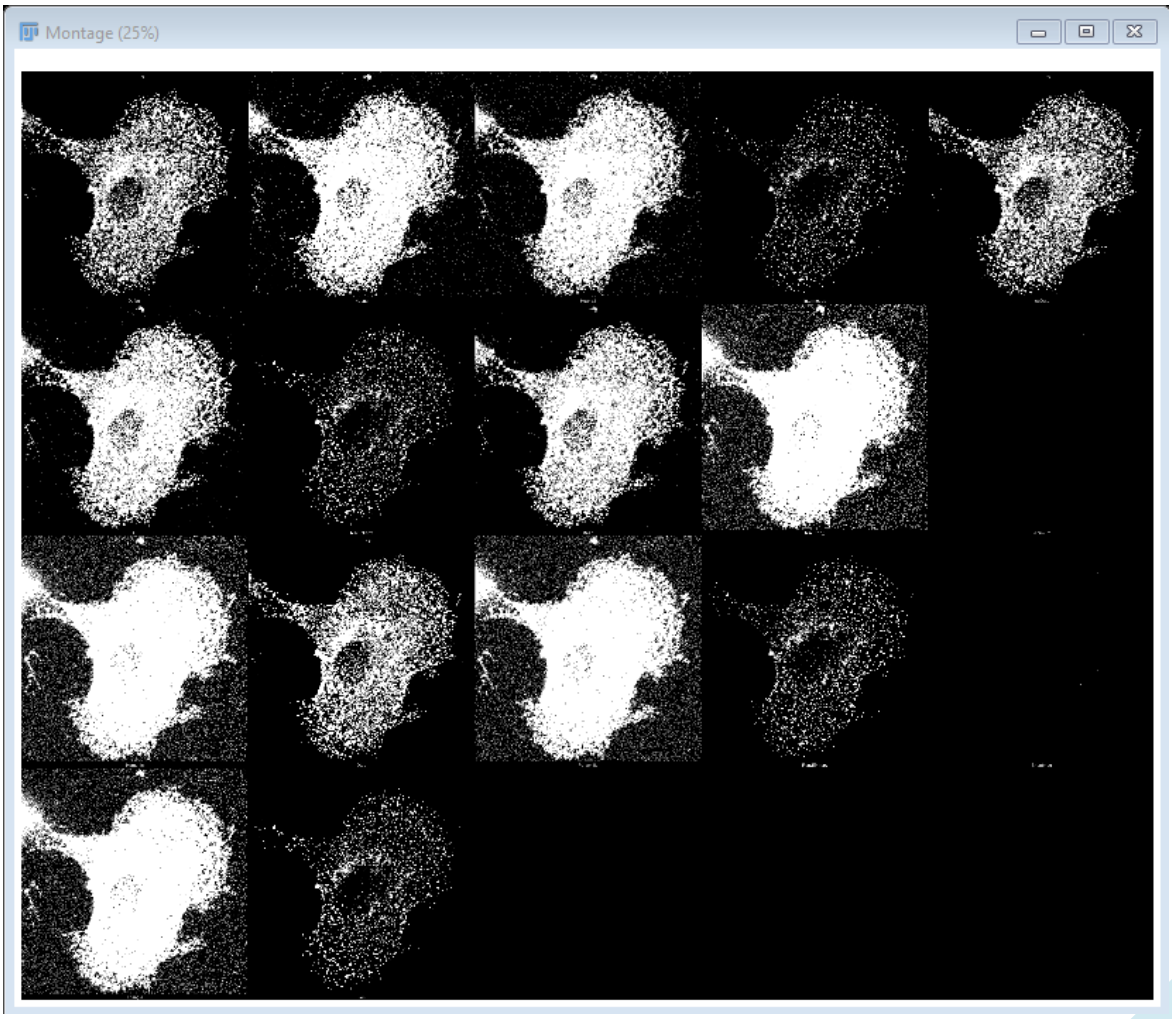
15. The next step is thresholding as we're going to use this image to define the cell area.

Thresholding

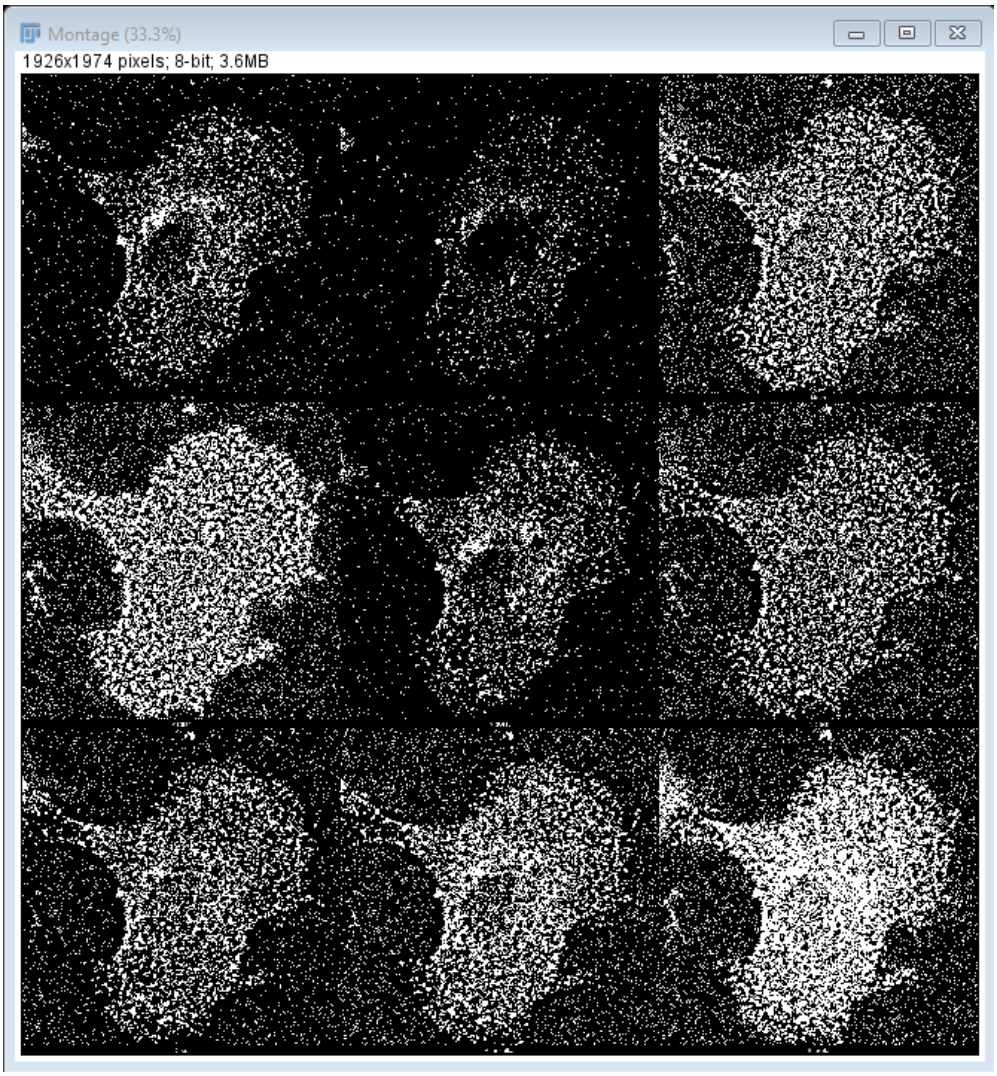
16. Using the CLAHE adjusted image, go to **Image – Adjust – Auto Threshold**. This will test out all of the available global thresholding methods. Select ***White objects on black background*** **as shown below**. For brightfield, you would turn this off.



17. The montage below shows the results for each method;

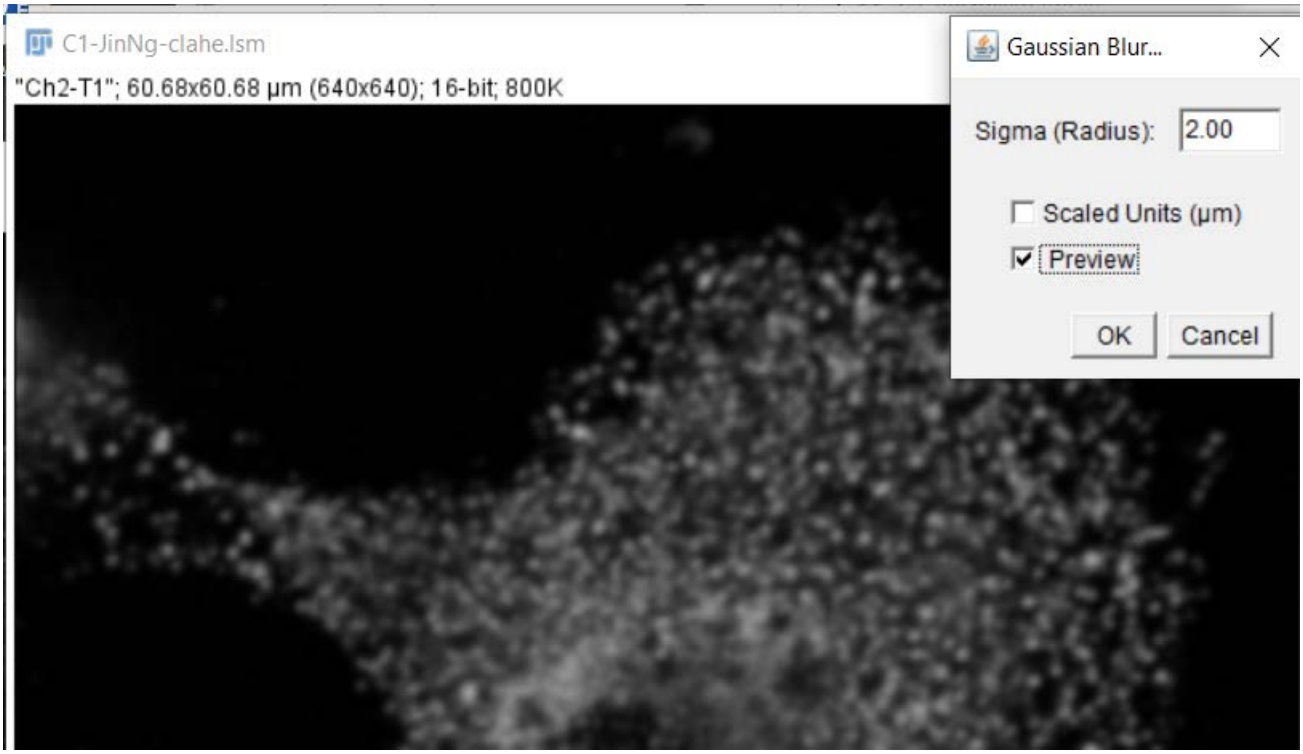


18. Another option is to use local thresholding. This only works on 8-bit. Make a duplicate of the CLAHE image (**Image – Duplicate**) and then go to **Image – Type-8-bit**. Then go to **Auto Local Threshold**. These methods are very sensitive to noise but the radius value is also important.
19. Note that any contrast enhancement technique can also enhance noise.

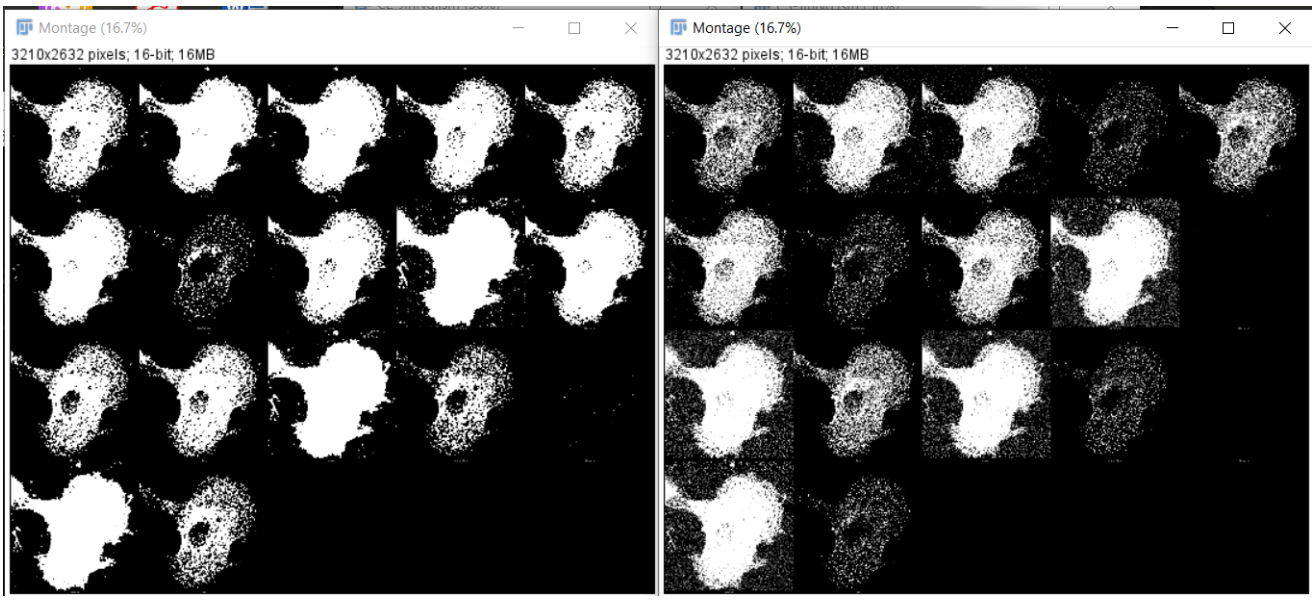


Filters

20. To improve the results, try using a smoothing filter first, e.g. **Process – Filters - Median** or **Gaussian**. Turn on **Preview** so that you can see the effect of changing the radius before applying it.

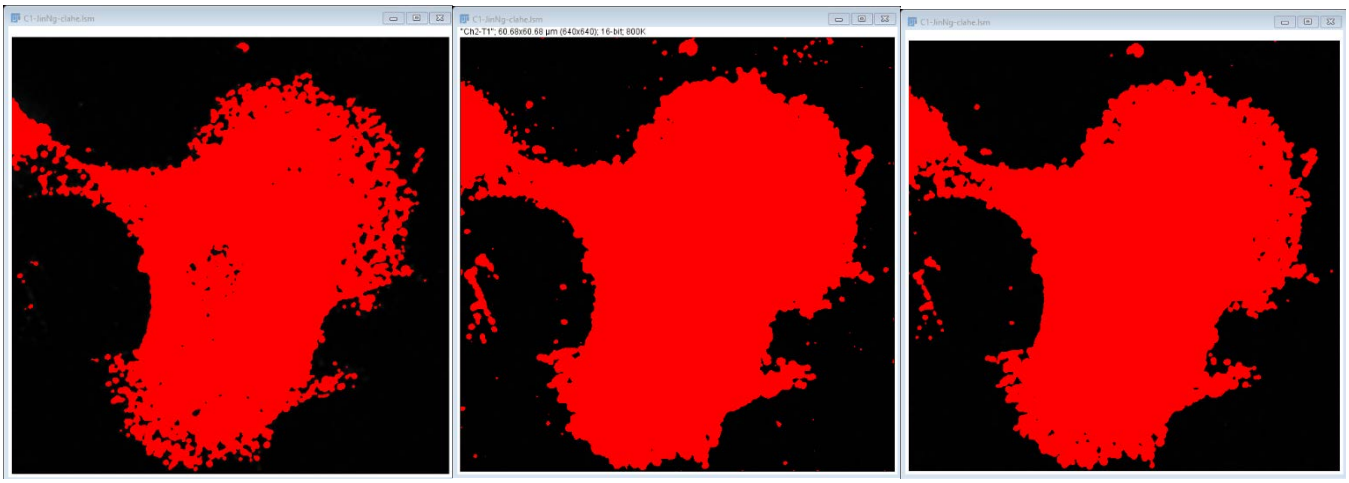


21. Then try the global thresholding again. Note the difference between the filtered and unfiltered reslts.
22. Remember, we just want to get an outline for the cell here.

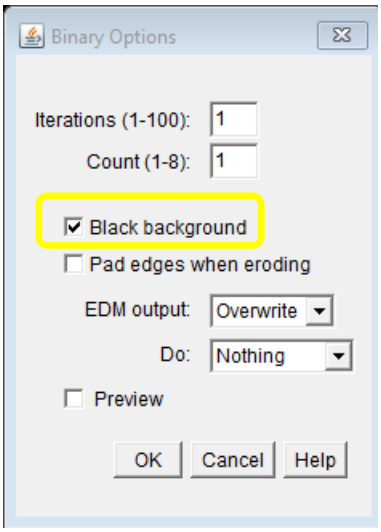


23. The best options look like **Minimum error**, **Percentile** and **Triangle**.
24. Go to **Image – Adjust Threshold** and select each of these methods from the drop-down menu.

Minimum Error **Percentile** **Triangle**



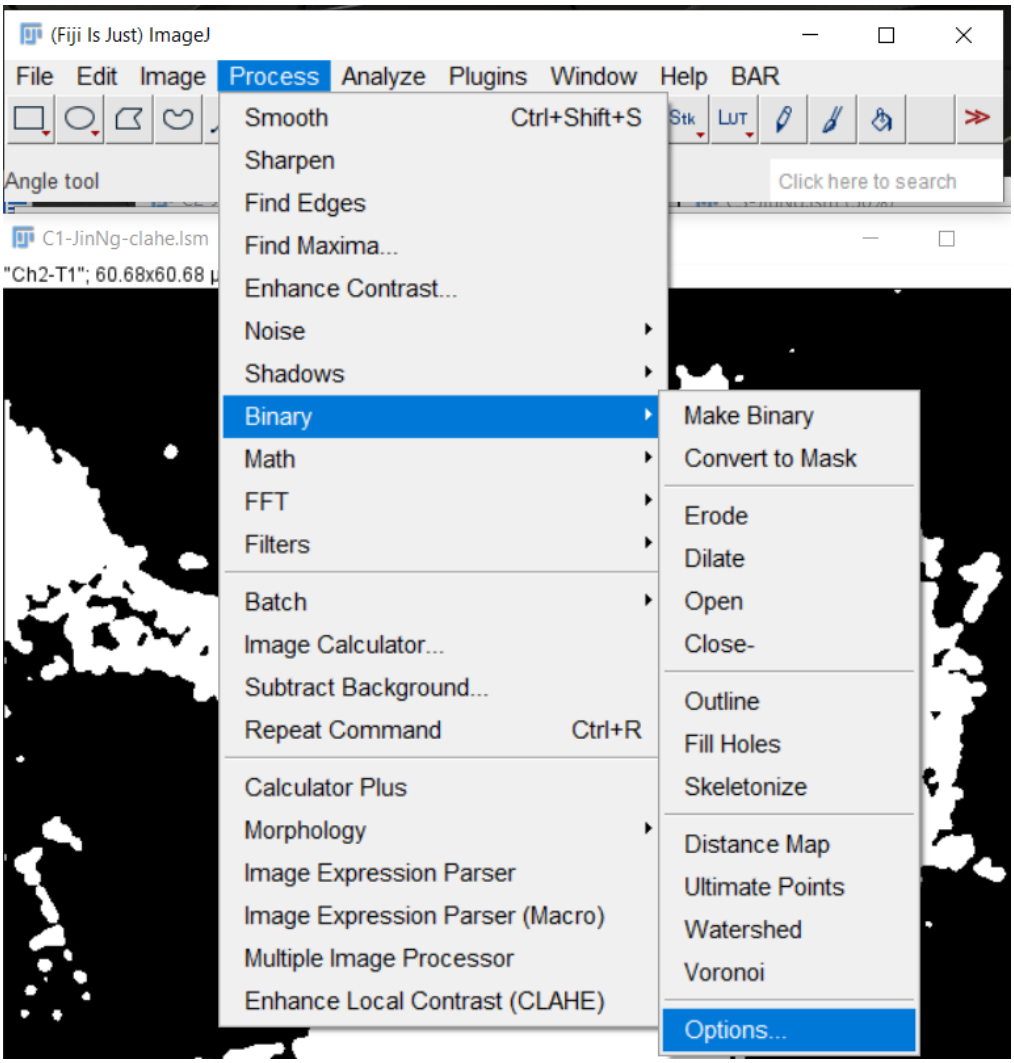
25. **Triangle** looks like the best option. The outline is pretty good but no attachments. **Percentile** has a bit attached and **Minimum** is too ragged, too many gaps to fill. Ideally you want to use an automatic method rather than having to Set it to particular values. However, in some circumstances you do need to do that.
26. You can choose whether your objects are white and background black (my preference) or the other way around. It doesn't matter as long as you are consistent and it's important to include this command in any macro that you write. Otherwise, if you give it to someone else to use and they have the reverse options, it won't work as expected! To change it go to **Process – Binary – Options**..;



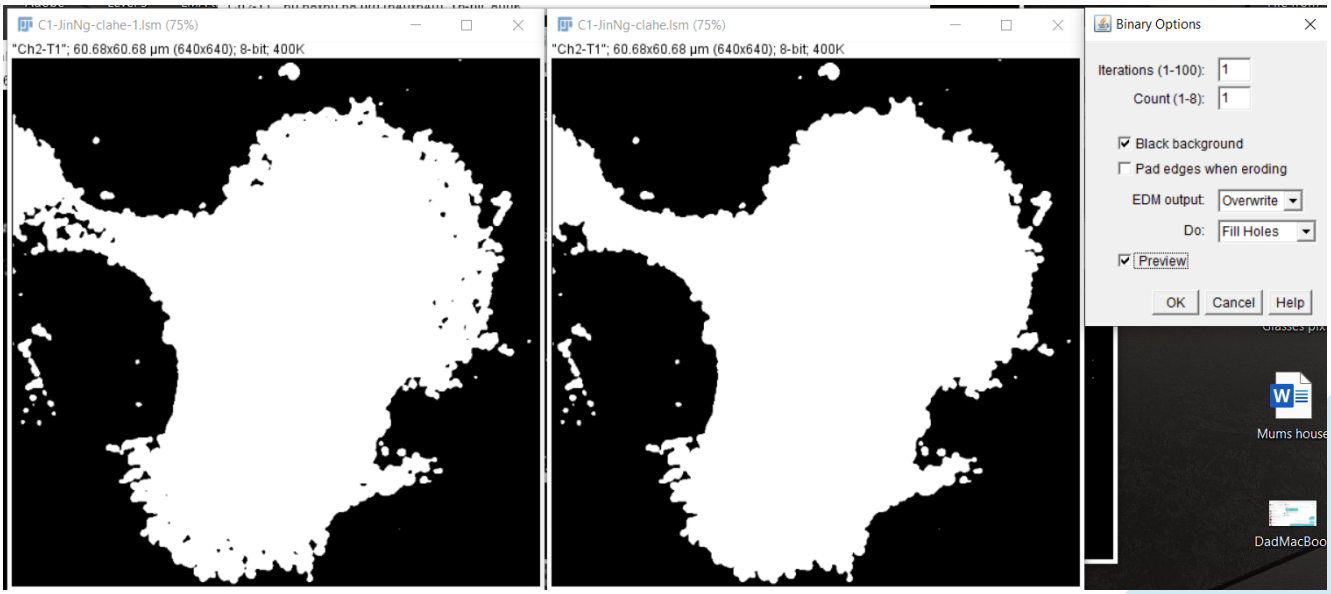
27. Close the **Threshold** window.

Morphological operations

28. The next step is to fill in any holes and smooth the outline. Under **Process – Binary** you can see a number of different morphological operations. However to try these out and to learn how they work, it's best to select **Options;**



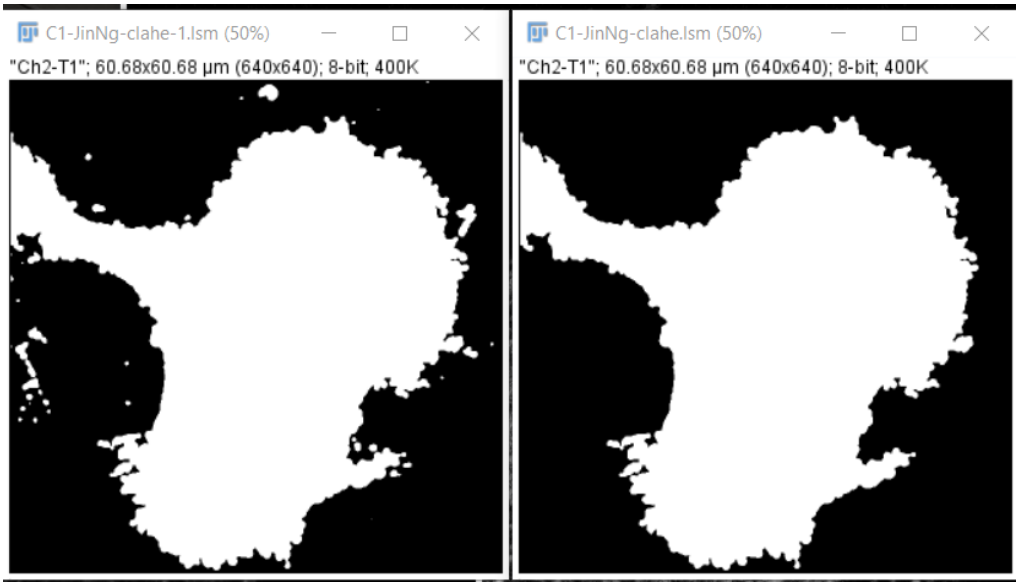
29. Make a duplicate of the image so it's easier to keep up with what the processing is doing. Or just turn **Preview** on and off.



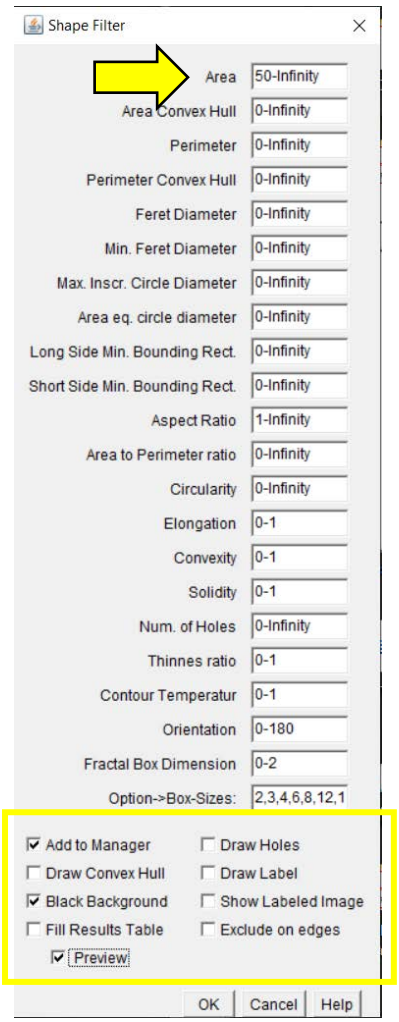
30. You could use more morphological processing to remove the small debris or use a lower limit with the **Particle Analyzer** to exclude these from being measured.

Shape Filter

31. A useful choice is the **Shape filter**, which can be used to remove objects based on size, shape, perimeter, etc. Check the link for more information.
32. After you have this installed, it will appear in your **Plugins** menu. Go to **Plugins – Shape filter**.



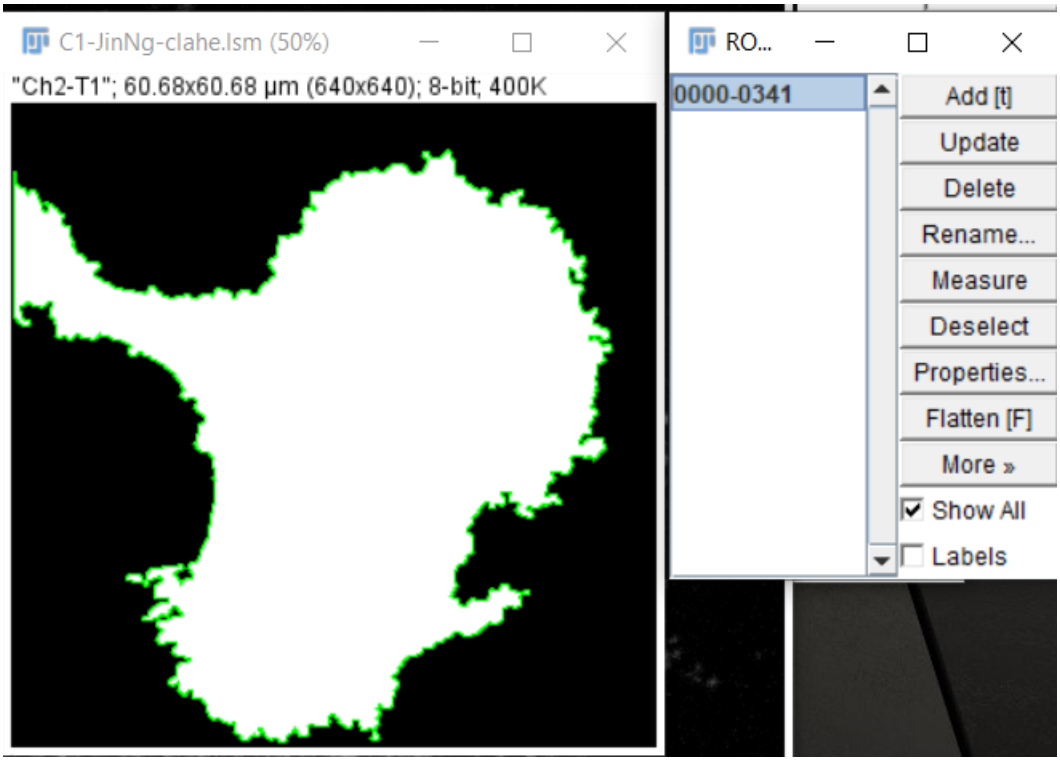
A simple **Area** limit removes the debris – see right hand image above.



33. Selecting **Add to Manager**, adds the object selection to the ROI Manager.

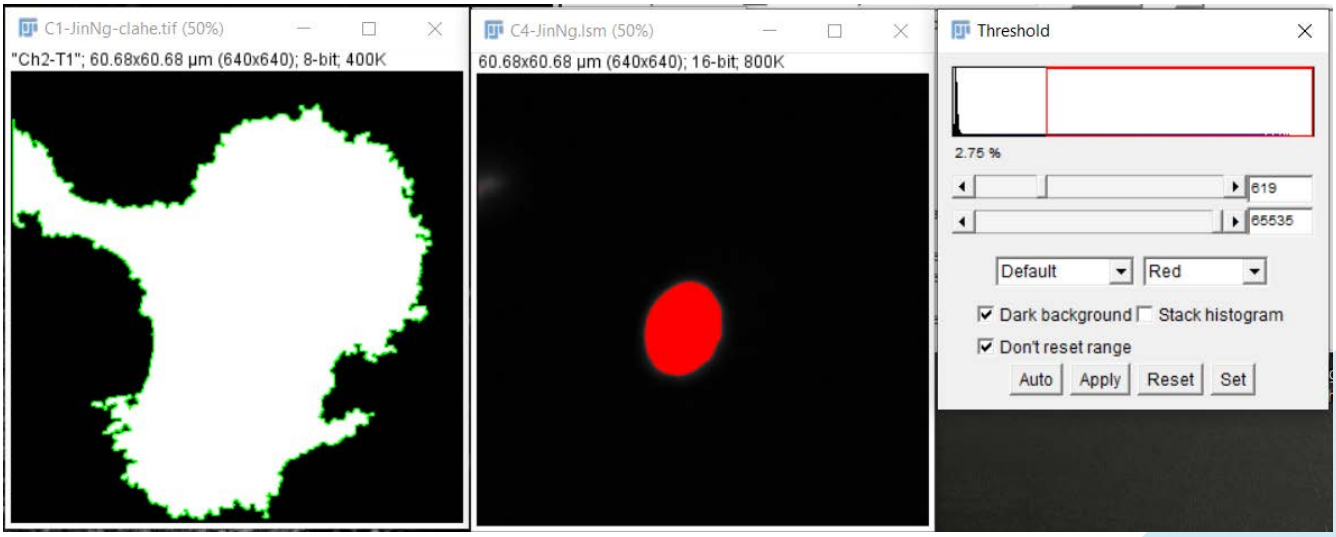
ROI Manager

34. The ROI Manager is how we store and control regions of interest.

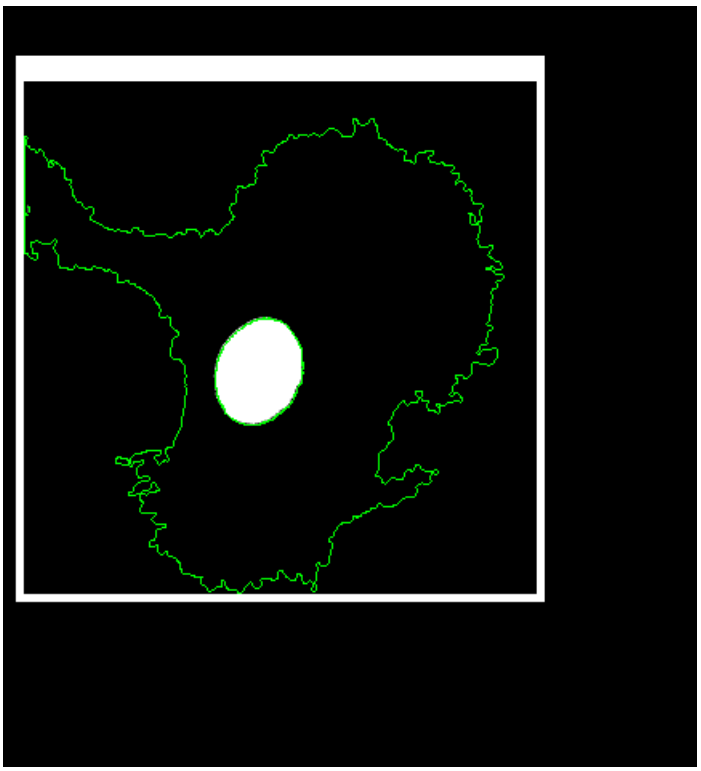


Boolean operations

35. What if we want to know how much signal is in the nucleus compared to the cytoplasm?
    We can make another ROI from the nucleus by using the **ROI Manager** and then using Boolean logic to combine these.
36. First, create a binary image from the image of the nucleus.
37. Go to **Image – Adjust – Threshold** and choose a method from the drop-down box.
38. The image is really clear so most methods will work. Click **Apply** to make the binary image.



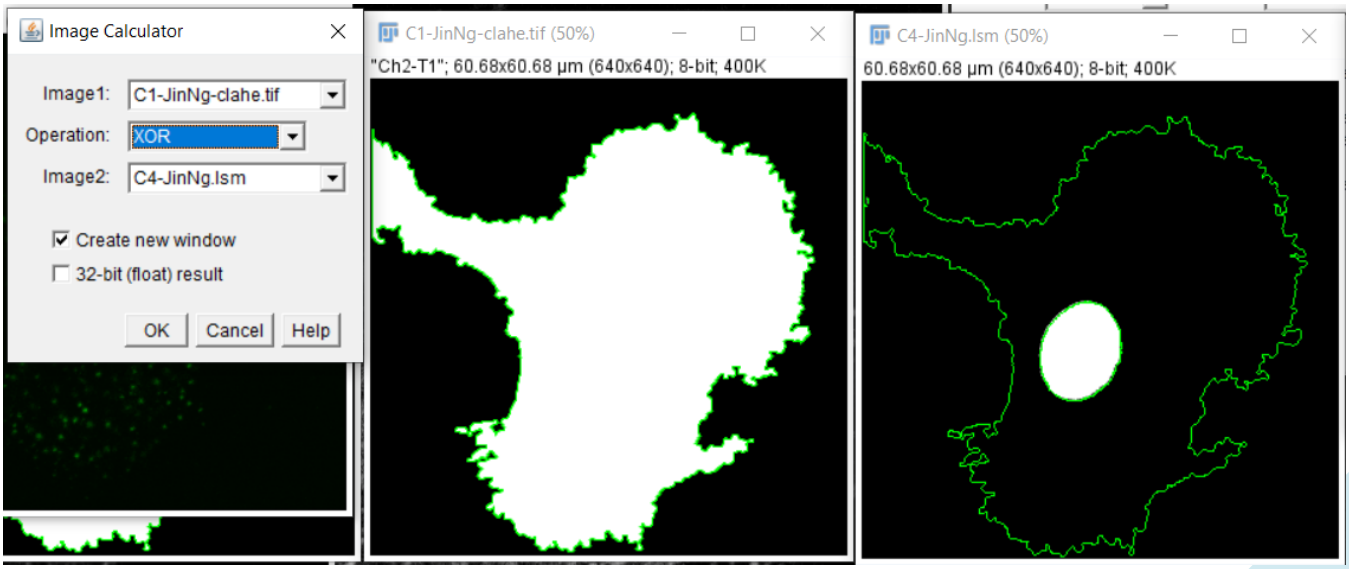
39. Go to **Edit – Selection – Create Selection**.
40. Then either go to **Edit – Selection – Add to Manager** or if the ROI Manager is already open, just click **Add** to add it.
41. Make sure none of the ROIs are selected or that both are selected (necessary if you have more than 2 there). Go to **More – XOR** to create a new selection which comprises the Cell ROI minus the Nucleus, i.e. the cytoplasm only.

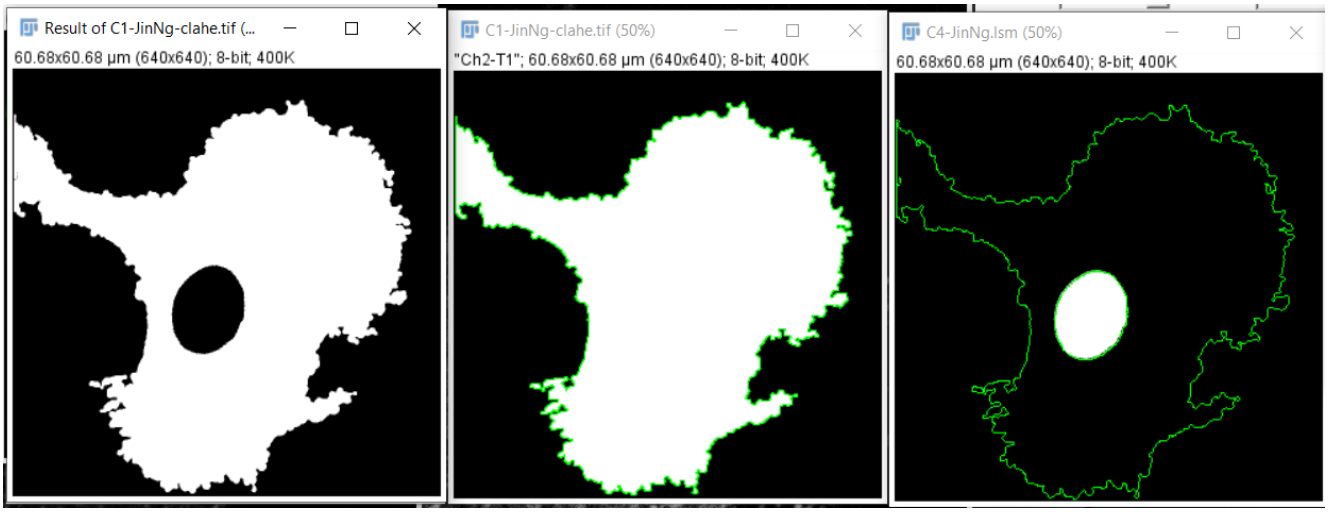


42. Then click **Add** to add this as a new ROI.

Image calculator

43. Another way to approach it is to use the **Image Calculator** and subtract the nucleus from the cell image.
44. Go to **Process – Image Calculator**
45. Select the options shown below;. Note you can use **XOR** or **Subtract**.





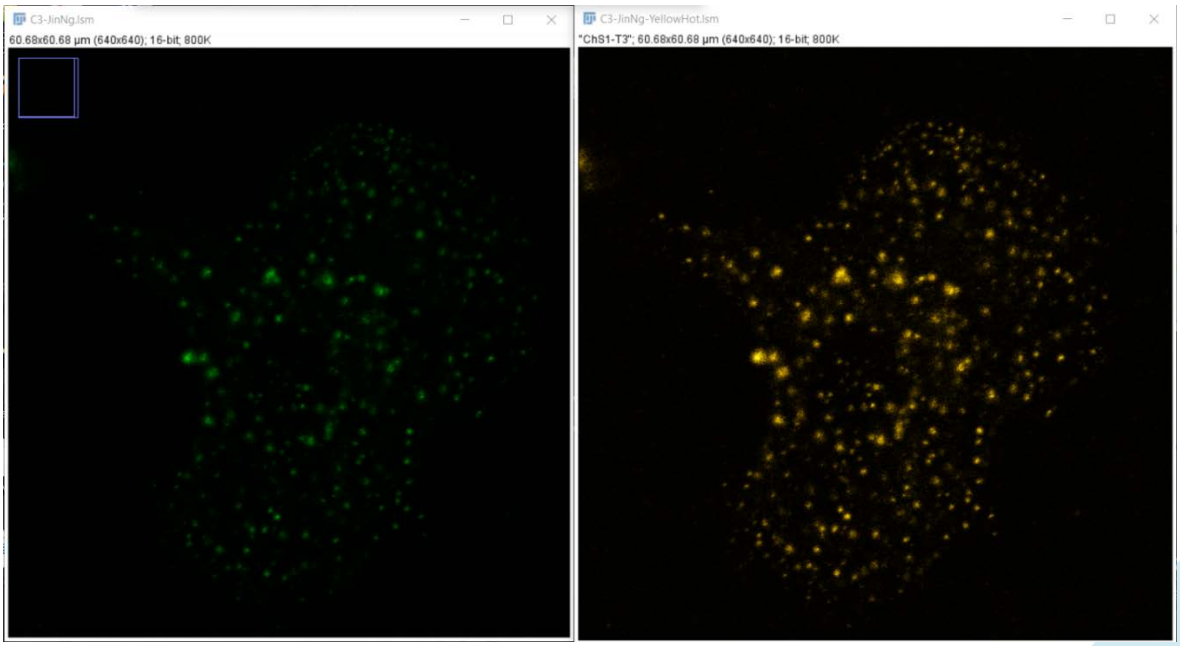
46. The next step is to look at the other markers.

Measurements

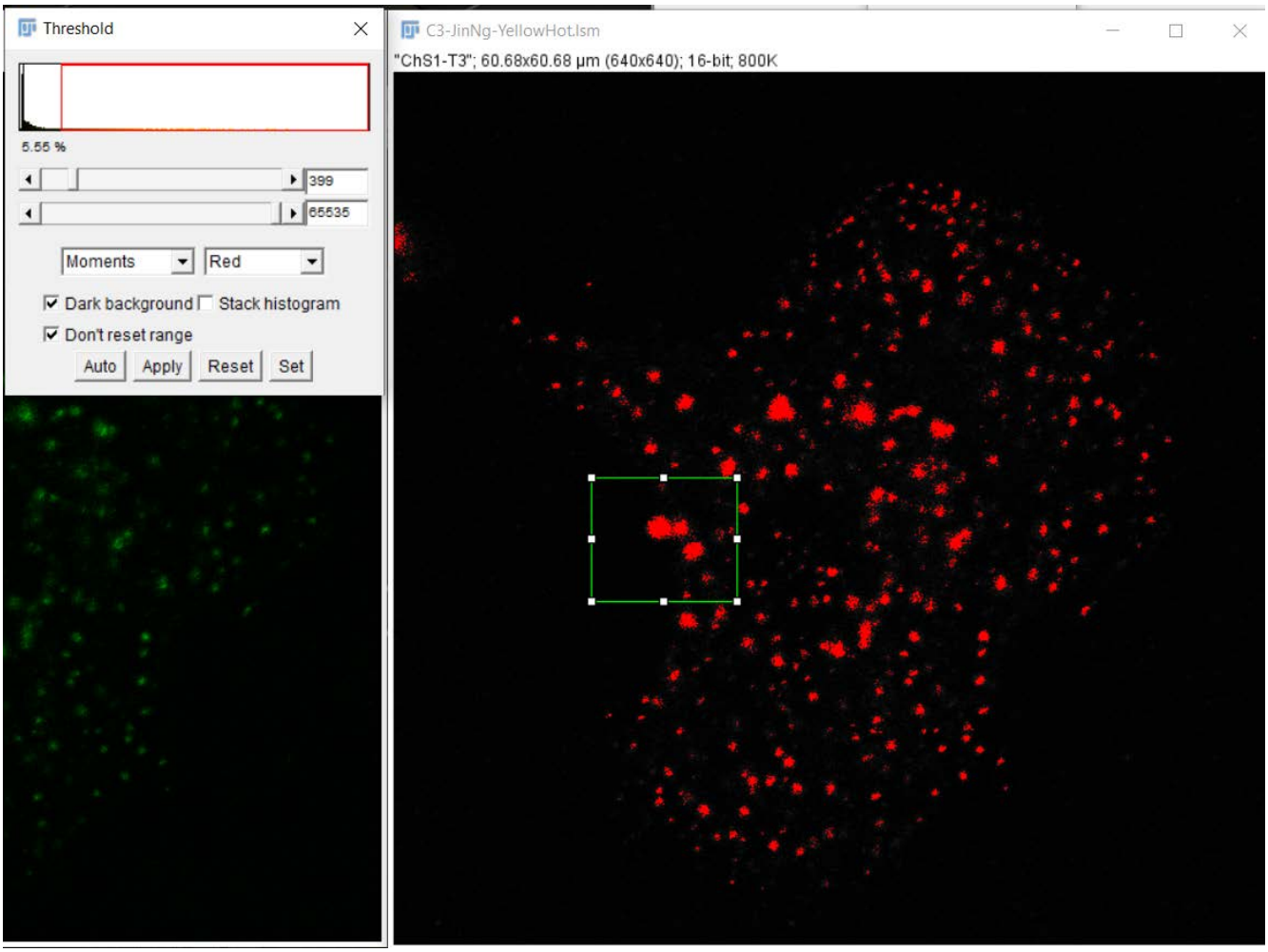
47. For C2 and C3, the process is basically the same. What's important is whether you want intensity information or just number of spots.
48. If we assume that you want to know how many spots, what the intensity is and what the size is, then it's important not to enhance the image as this will change the pixel values and potentially the size. So, we just need to use the threshold option to identify the foci.
49. Select the C3 image.

Look Up Tables

50. When you have tiny objects, using a different **Look Up Table (LUT)** can really help when you are working things out. Go to **Image – Lookup Tables** and select one that works well for your vision. I like the ones that have "Hot" in the name.
51. So, I have selected **Yellow Hot.**
52. This also demonstrates the issue of your own vision and why it's so important not to select the threshold based on your own eyes. It's far too subjective. Using an "**Auto**" option or determining the best method based on a trial set of images and then (based on background values) using the **Set** option with the same values for all images, is the most reliable.
53. It also shows why you might change the LUT for images for presentation or publication so that features are more visible. The two images below have the same pixel values at the same coordinates. The only difference is that the pixel values are mapped to different colours.

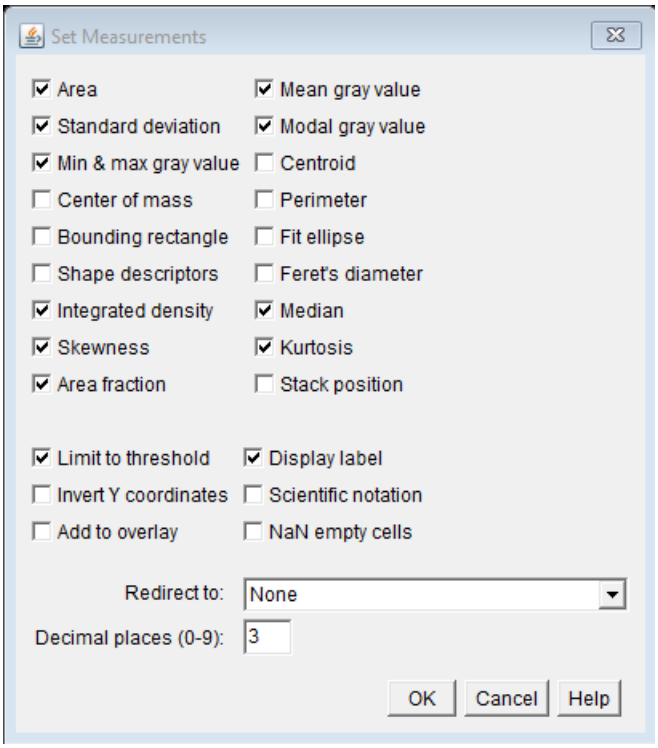


54. You can test out all of the threshold options as shown earlier but with small structures like this, it's often better to just check them one by one using the drop-down box. **Moments** looks like a good option as it picks up all the smaller spots but also doesn't blow out the larger ones. Look at the objects in the ROI to see if it's working well enough.

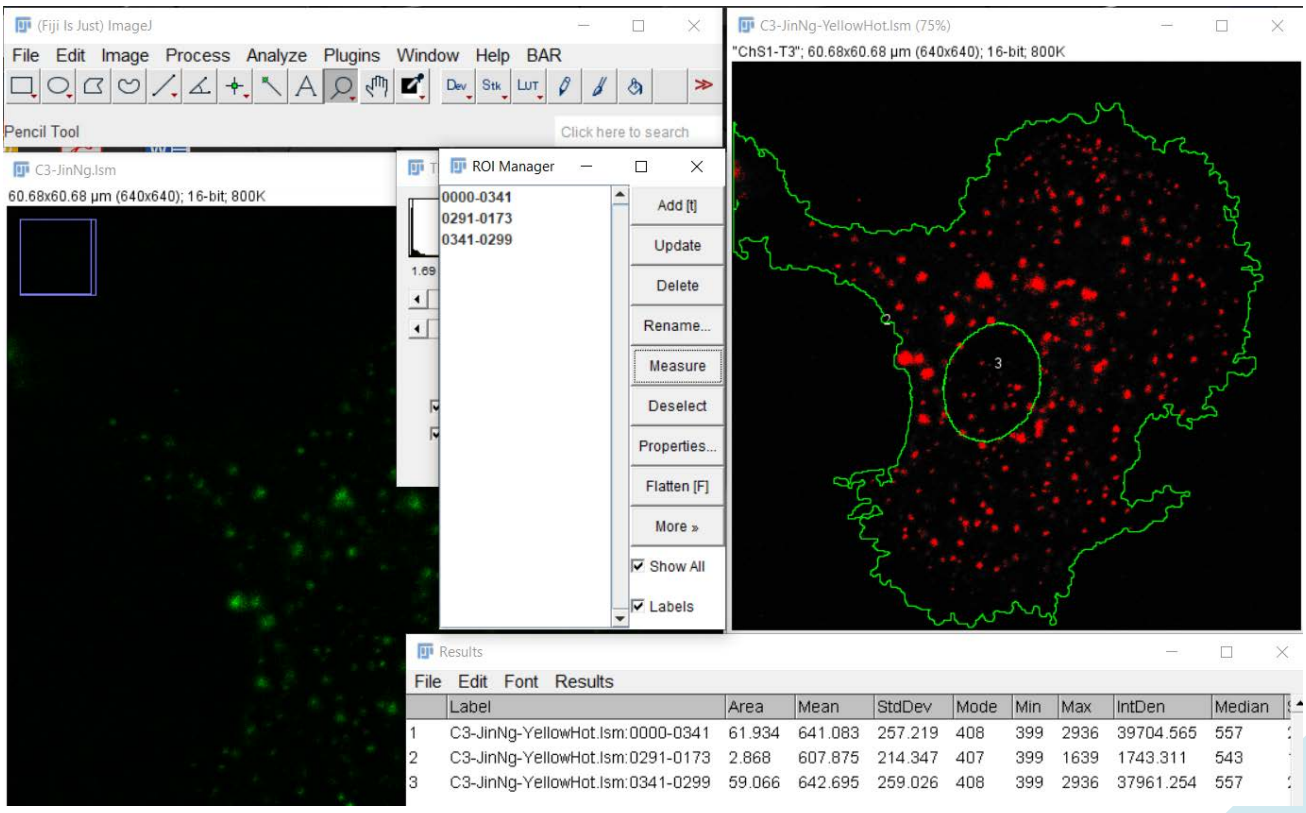


Setting measurment parameters

56. Go to **Analyze – Set Measurements**. Select all of the gray value parameters (intensity) and **Standard deviation** (of the mean). Choose **Limit to threshold** so that only the pixels under the threshold overlay will be measured. Otherwise, the entire image will be measured.

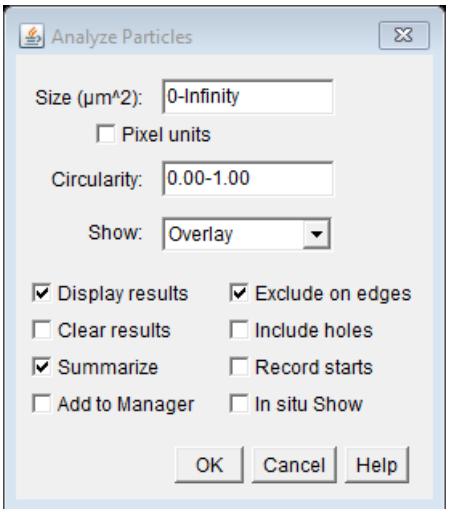


57. Go to **Analyze – Measure** if you want values for the whole image.
58. Otherwise, click **Measure** in the **ROI Manager**. You will get measurements for each ROI.

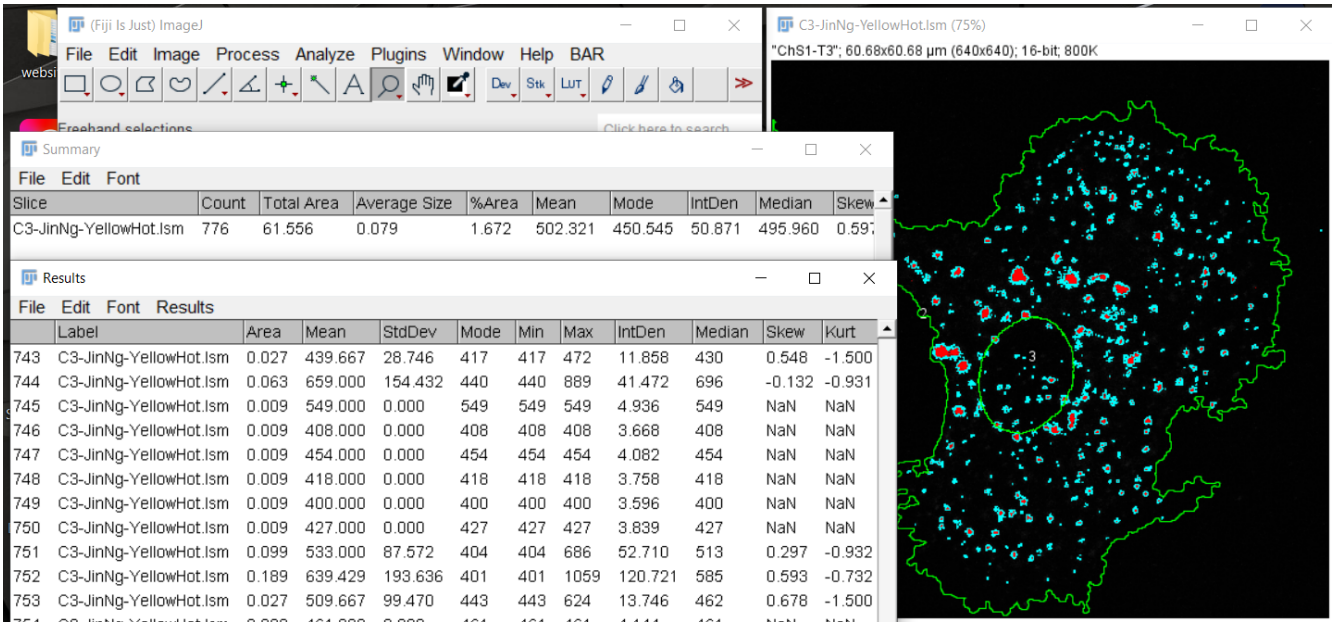


Particle analysis

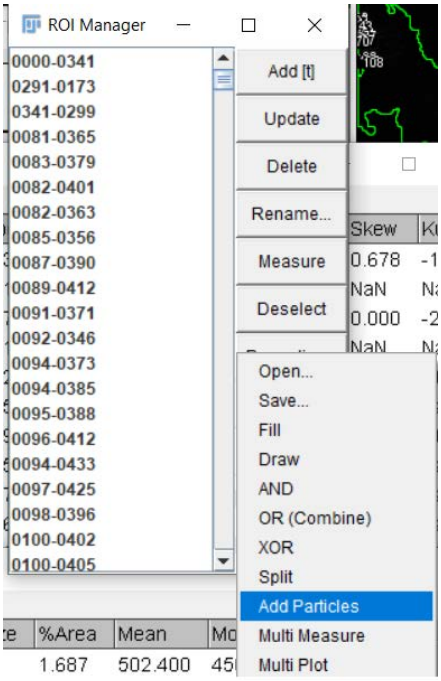
59. To get measurements of the individual spots, go to **Analyze – Analyze Particles**. You can exclude very tiny objects or very large ones using the Size limits. You can also select Pixel units. Circularity can also be used to exclude objects based on shape. There is a plugin called the **BioVoxxel Toolbox,** which has an advanced **Particle Analyzer** option which has more options such as perimeter, aspect ratio, etc. So this can be used instead of using the **Shape filter** or to measure different shaped objects in the same image.
The entire image will be analysed because a region of interest has not been selected. So, this will measure all of the objects in the whole cell.



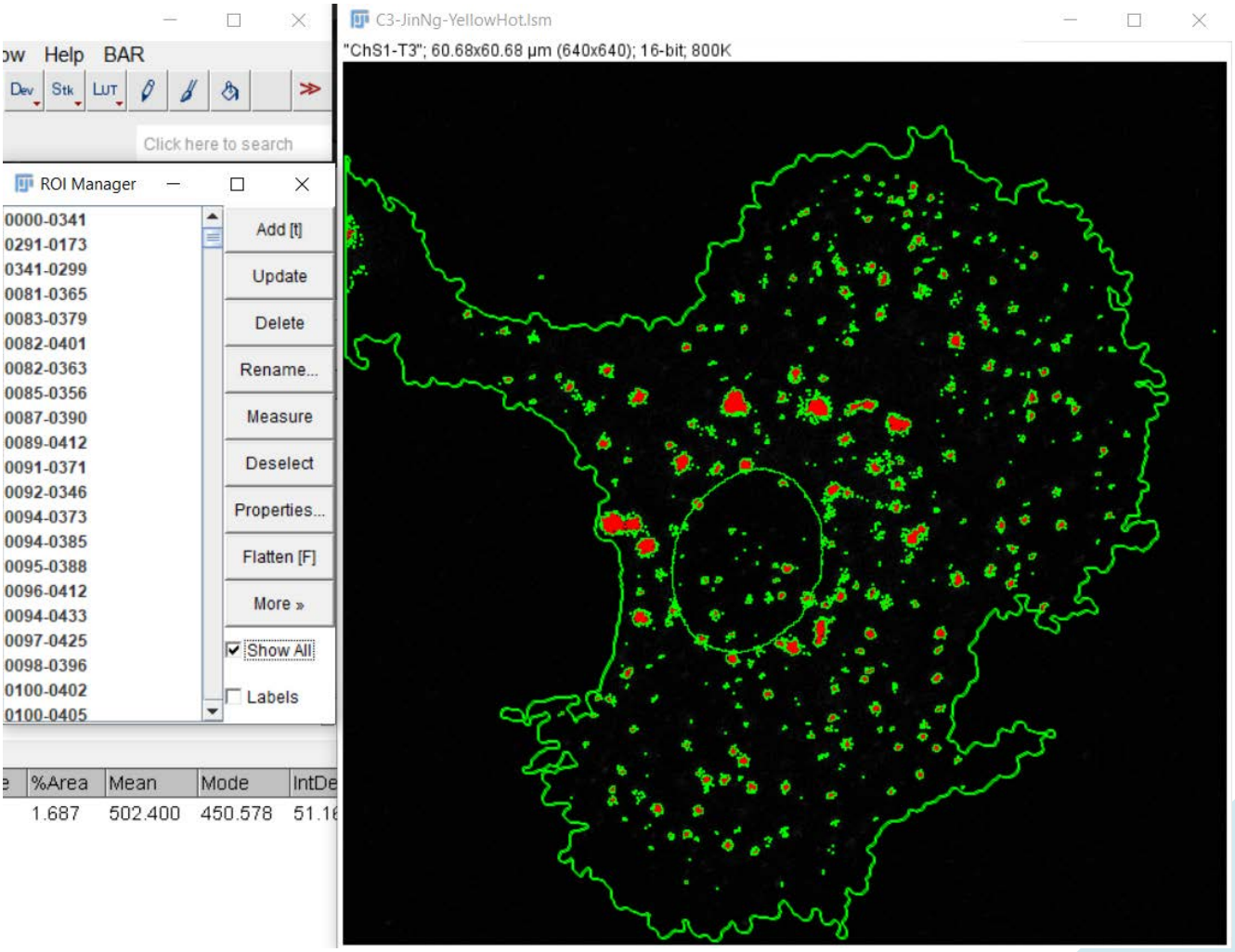
Summary and Results tables and image with overlay and ROI showing



61. If you haven't selected **Add to Manager,** you can add the selections as ROIs by going to **Overlay – To Manager.** Or if you select **Record Starts**, you can **Add Particles**.

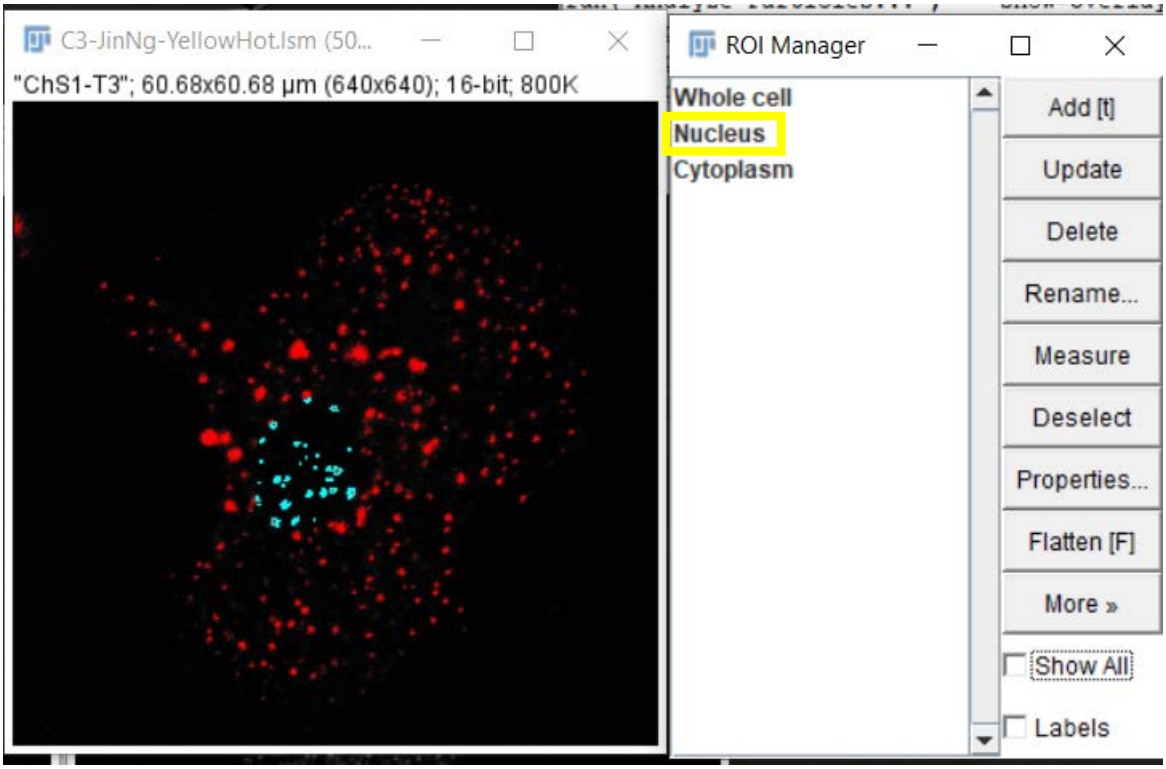


62. If you save the image as TIFF with the overlay shown, it will still be there if you open the image again so this is a useful record of what you have measured.

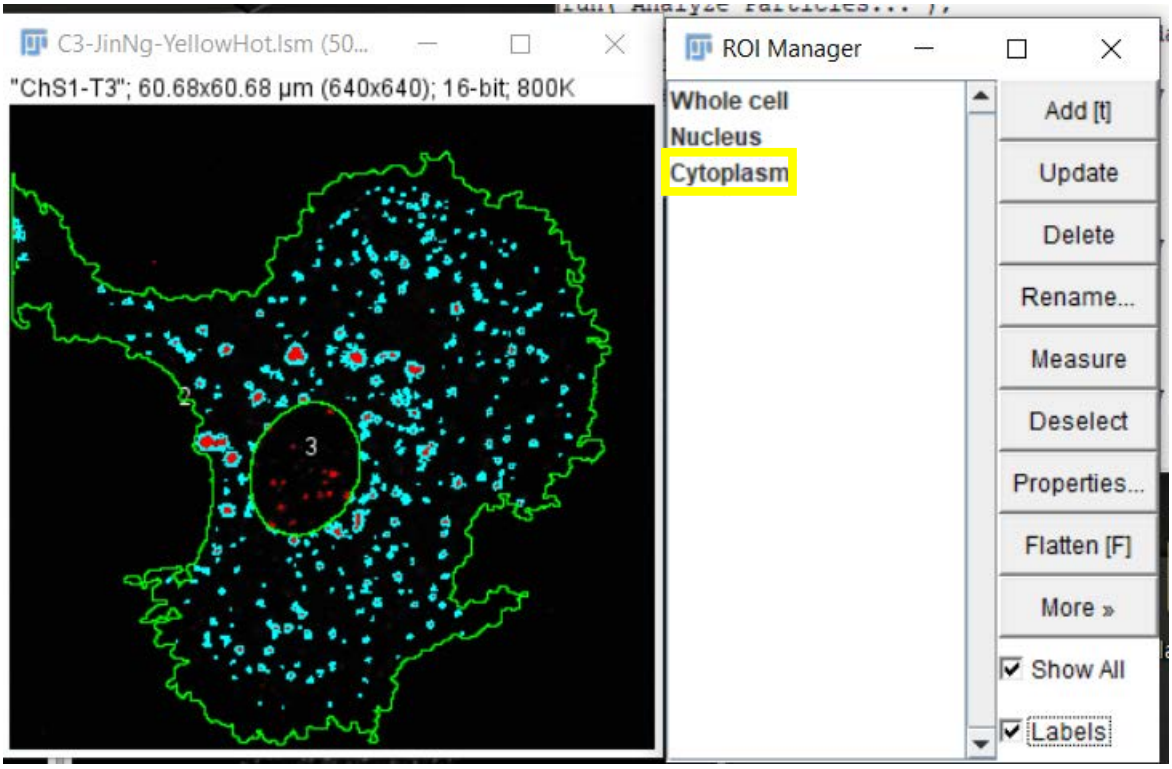


63. To restrict measurements to a particular region of interest in the **ROI Manager**, highlight the ROI and then go to **Analyze – Analyze particles**. Only the objects inside the ROI will be measured.

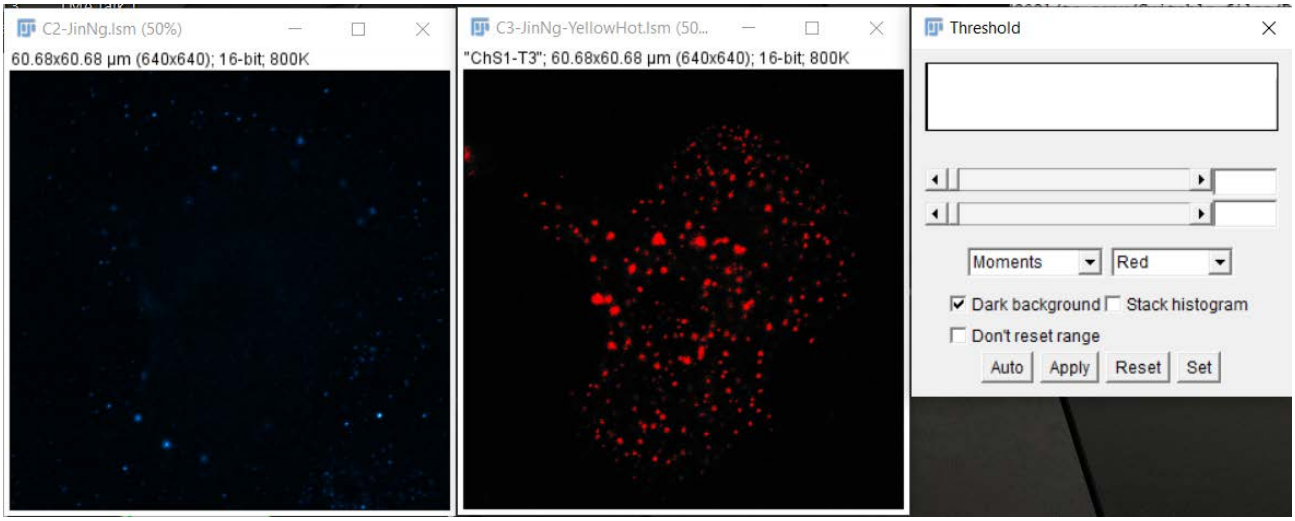
Measurements restricted to the nucleus



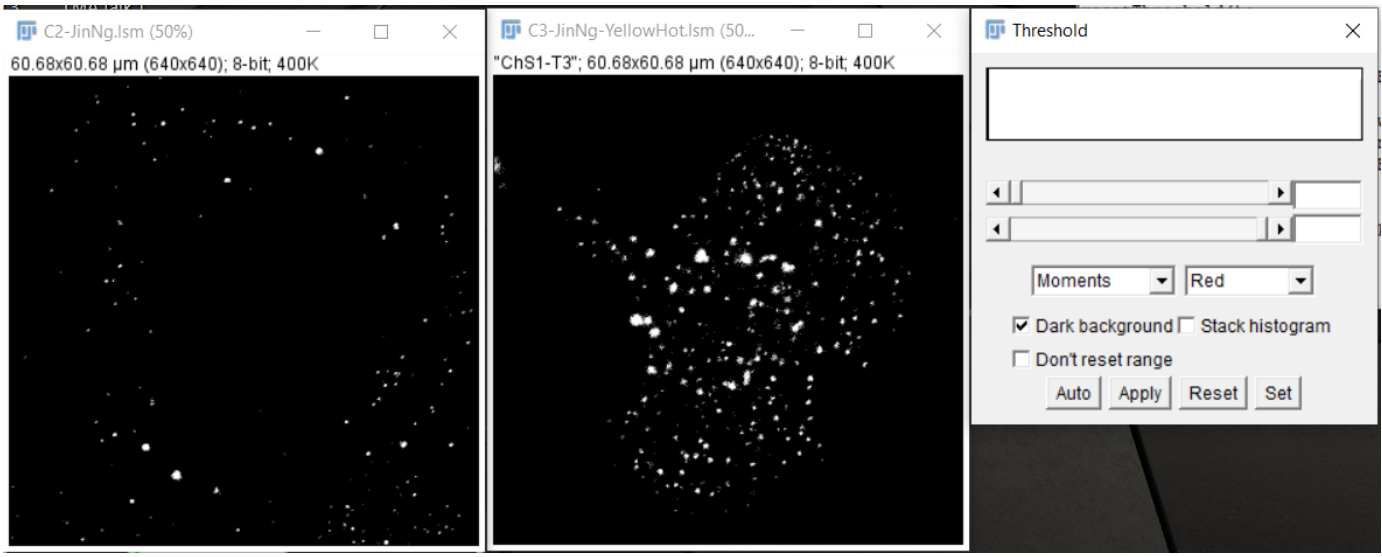
Measurements restricted to the cytoplasm



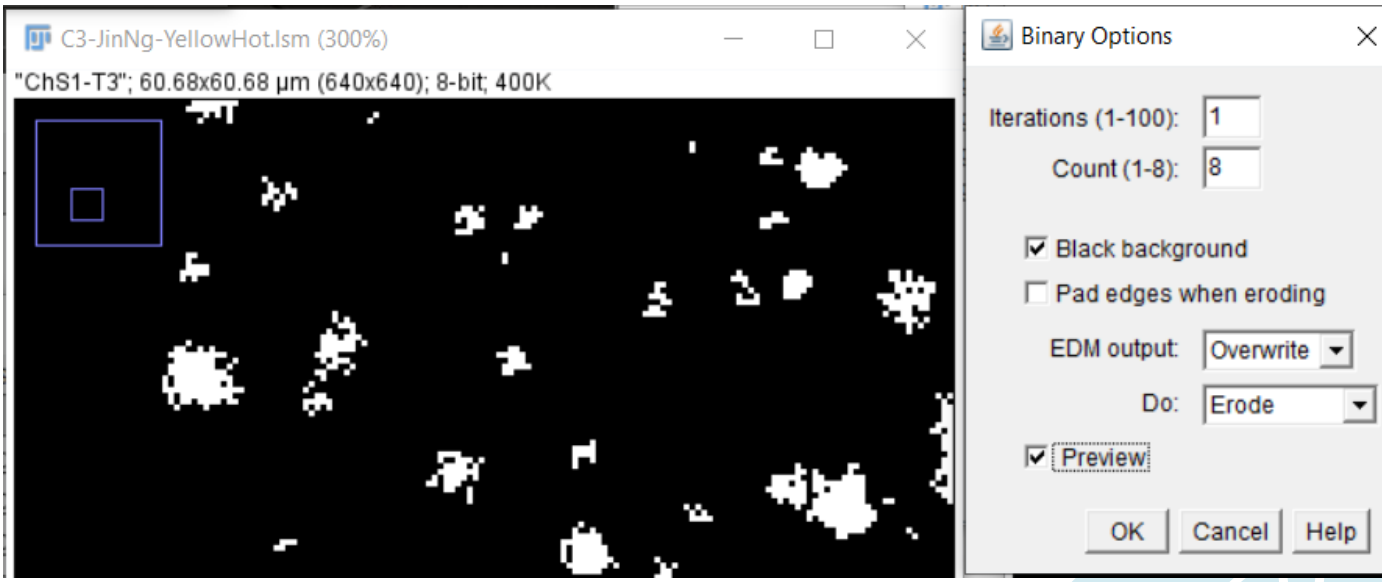
65. Next is to count the number of double-labelled spots (green and red). Not this is not the same as intensity-based colocalisation analysis where you are interested in correlation coefficients, e.g. Pearson's/Manders, etc.
66. Create binary images of both images by using thresholding as detailed earlier (**Image – Adjust – Threshold**).



67. **Moments** works well for both images. **Apply** the threshold to create 2 binary images.

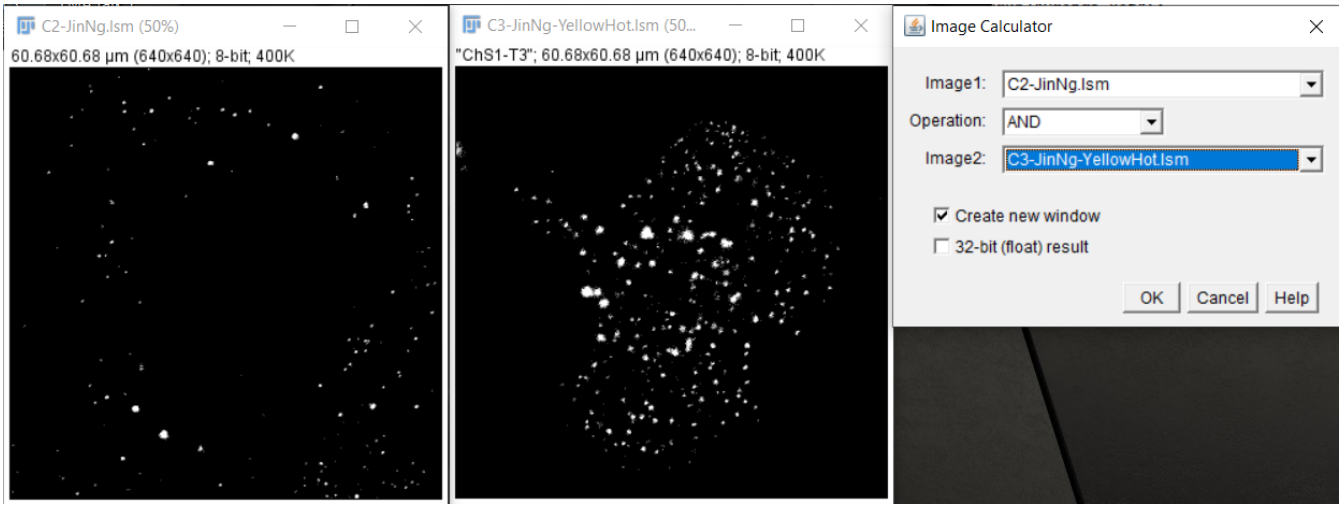


68. To remove single (isolated) pixels, go to **Process – Binary – Options**. Use **Erode** or **Open** – note the **Count** (8) restricts the operation to isolated pixels only (those "object pixels" that have no neighbours).

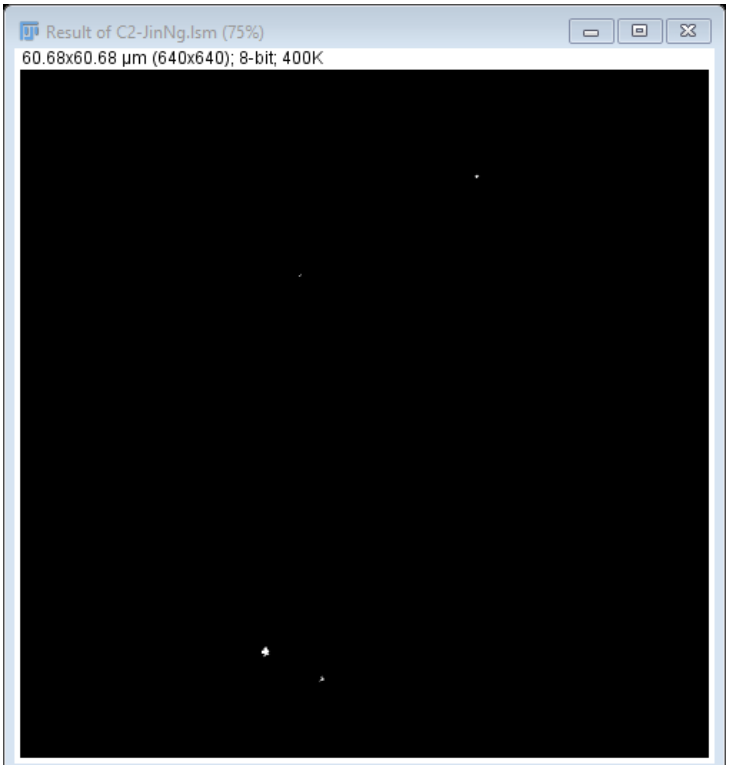


69. Use Boolean logic to create an image that only contains spots that are in both images.

Go to **Process – Image Calculator.** Use the **AND** operation to create an image where both images have white (object) pixels in the same locations.

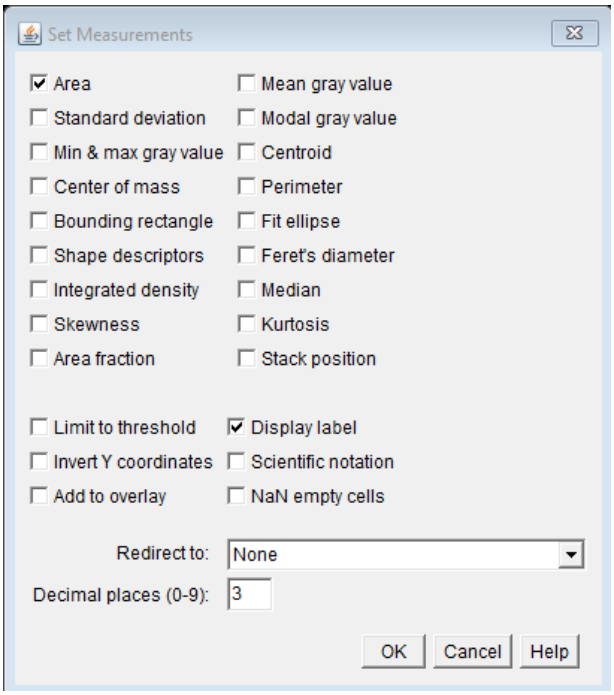


70. Here is the result image;

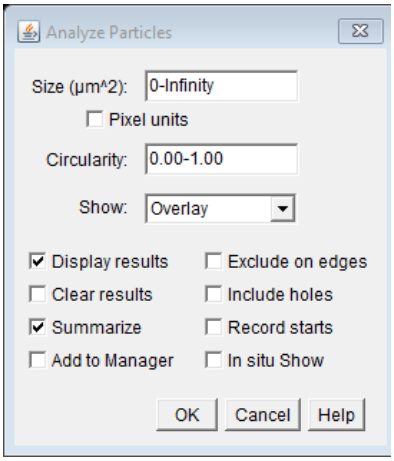


71. Then use the **Particle Analyzer** to measure the number of spots.
72. Since the image is binary, you don't need to use a threshold but you might decide to if you think it's confusing. Any threshold option will work with a binary image.

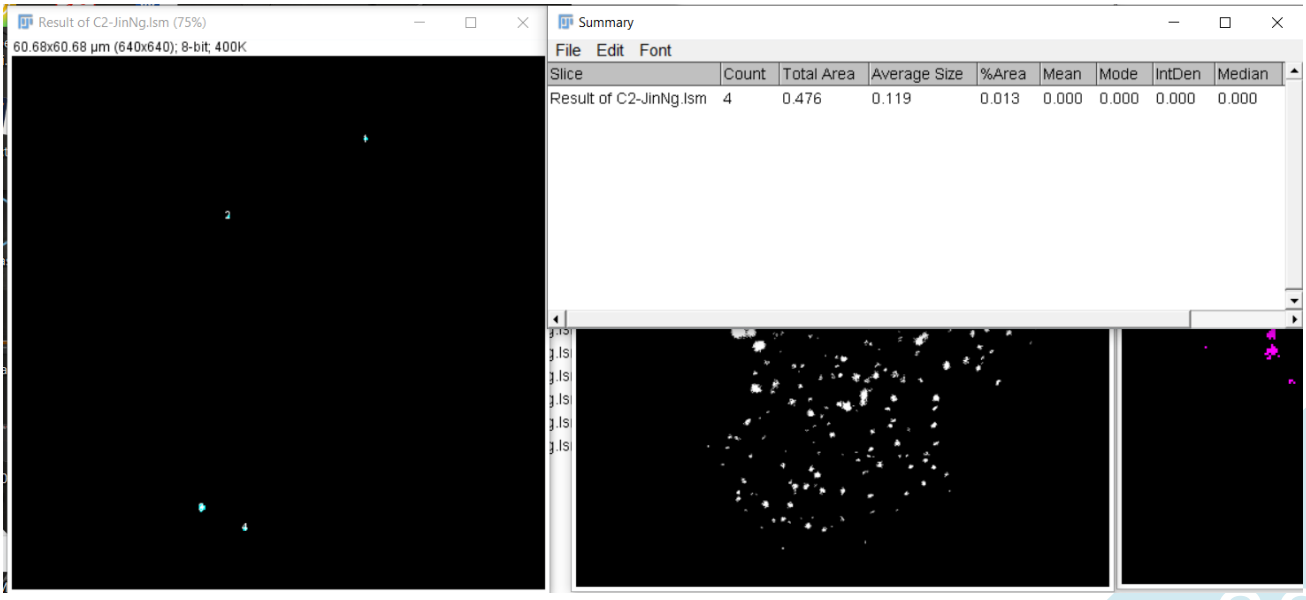
74. Go to **Analyze – Set Measurements;**



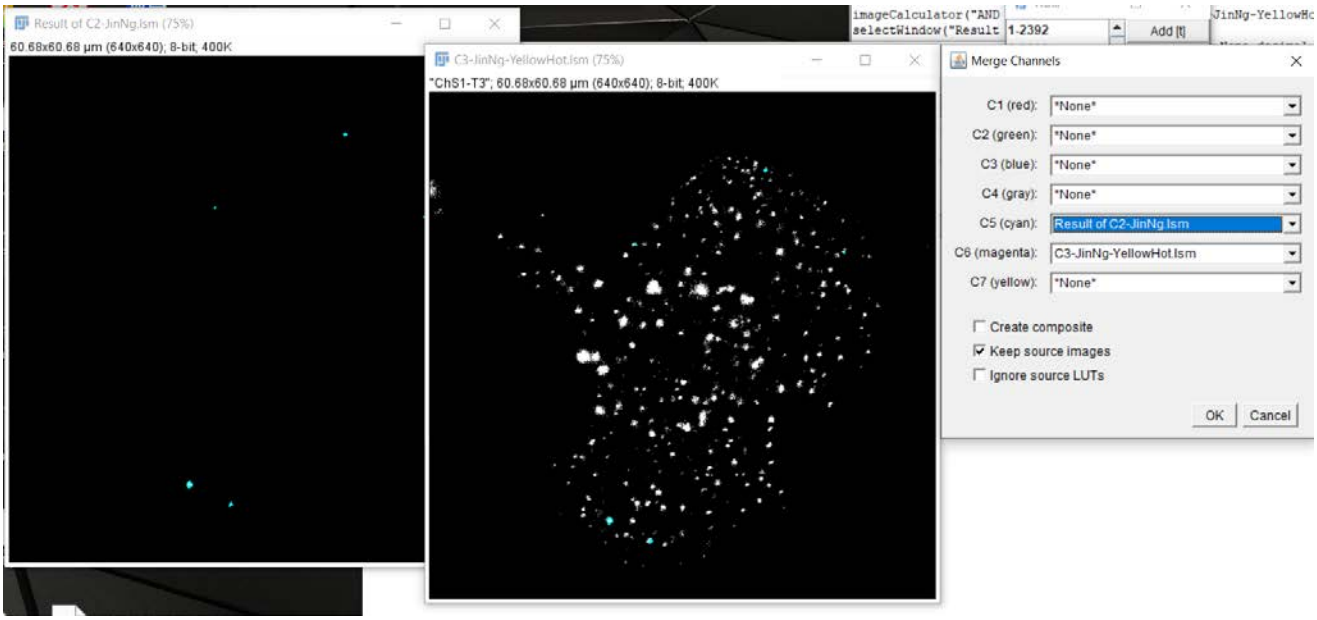
75. You can include an upper or lower limit if necessary to exclude very tiny objects or very large ones.



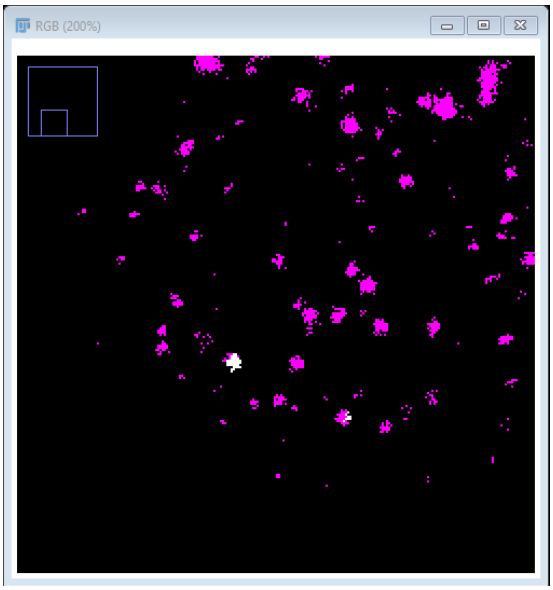
Results



76. If you didn't select **Add to Manager**, you can still move the overlay to the **ROI Manager**. Go to **Image – Overlay – To ROI Manager.**
77. If you want to visualise where the double-labelled objects are, you can use **Image – Color – Merge Channels.**

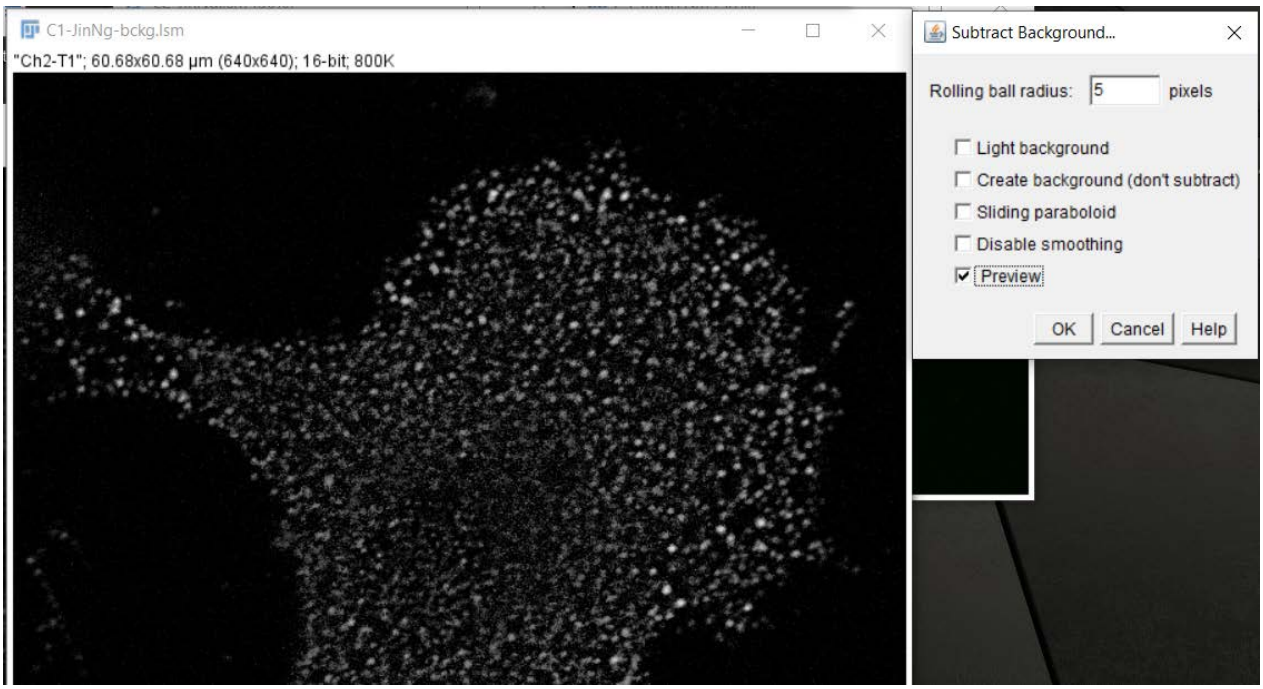


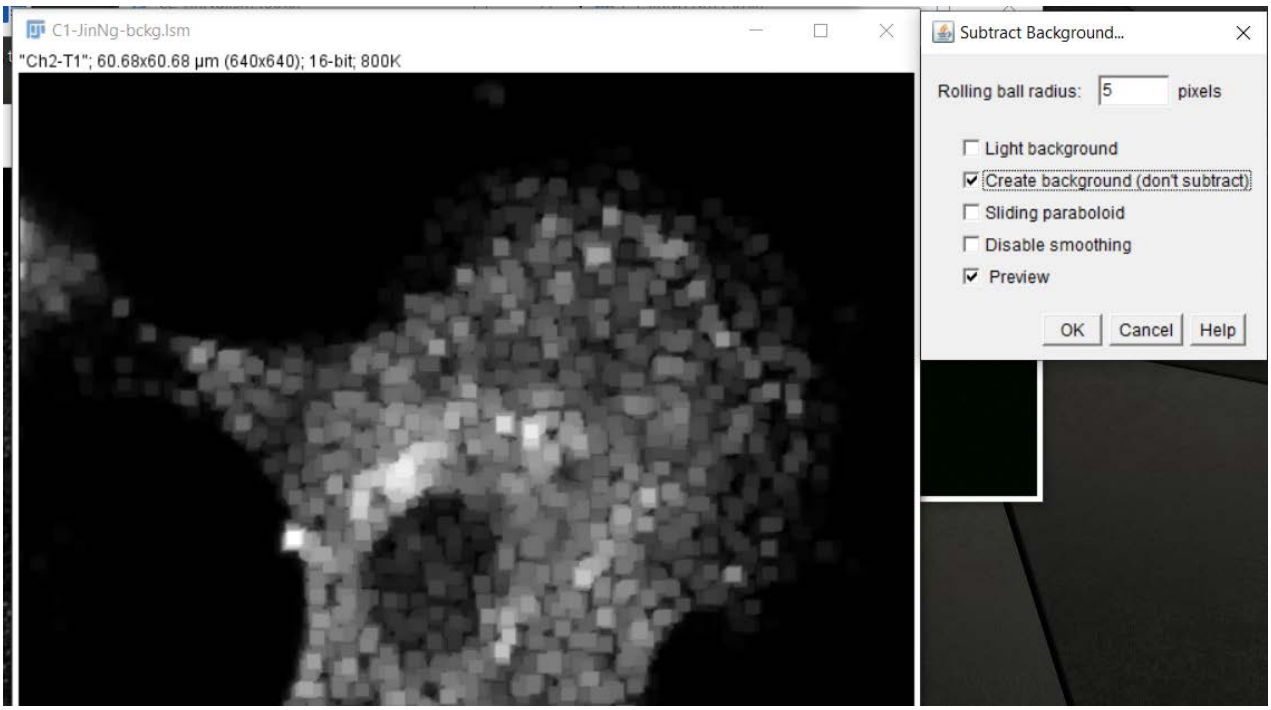
78. Note that if you want to know the intensity of each channel in these areas, you can measure back on the original image either by using the ROI Manager (create a selection) or by using the result image as a mask and selecting the **Redirect To** option in **Analyze – Set Measurements.**



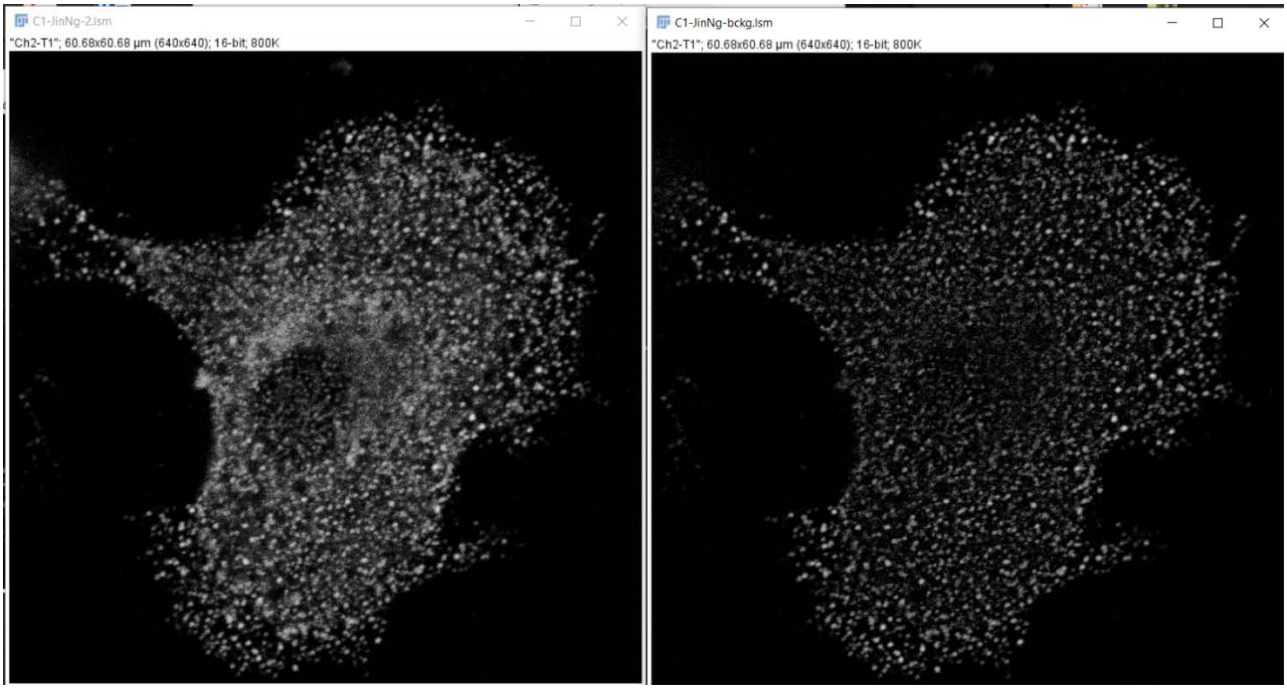
Background subtraction

80. Background subtraction can be used to better define the spots if you want to know if they are colocated with another marker.
81. Using the CLAHE image, go to **Process – Subtract background**. Turn on **Preview** and note the difference in the image. Turn on **Create background** to help you understand what the radius means. To remove unwanted blur, this works well. This is also used to remove shadows/background. If you have a brightfield image, choose **Light** background.





83. **Before** **After**



Macro recorder

84. Go to **Plugins – Macros – Record.**
85. All actions will be recorded in this window.
86. After you have finished, click **Create** to create a new macro which will appear in the **Script** window. The default language is ImageJ1 but you can use others.
87. This is also a good way of keeping track of your trial attempts.
88. Once you have worked out the best way to carry out the analysis, you can add in the necessary code to enable the script to run over many images, e.g. batch processing.
89. For example, the text below can be used to select all of the files;

GetDirectories_.ijm | GetImageName.ijm | SelectsAll_ROIs_.ijm

```
1 //Selection of directories and gets file list
2 dir1 = getDirectory("Choose Source Directory ");
3 dir2 = getDirectory("Choose Destination Directory ");
4 list = getFileList(dir1);
5 setBatchMode(true);
6 for (i=0; i<list.length; i++) {
7 showProgress(i+1, list.length);
8 open(dir1+list[i]);
```

90. The code below allows you to get the name of the image so that you can select images based on this;

GetDirectories_.ijm | GetImageName.ijm | S

```
1 imgName=getTitle();
2
```

91. As you can see, the colour-coding helps to highlight the different commands. Fiji also has a library of commands so you can type into the script window and different options will appear. You can easily select the process that you want.