

Jacqueline Ross,

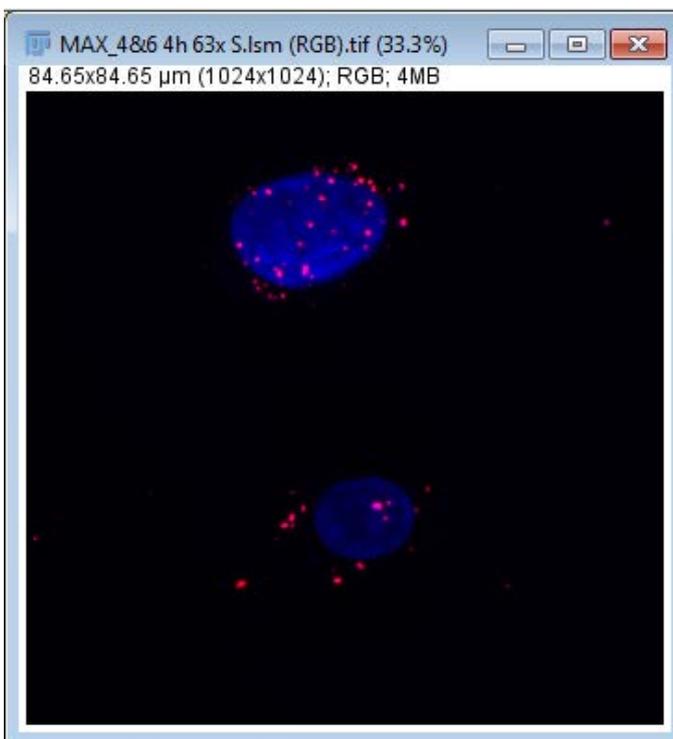
jacqui.ross@auckland.ac.nz

MICROSCOPY NEW ZEALAND INC. CONFERENCE WORKSHOP 2017

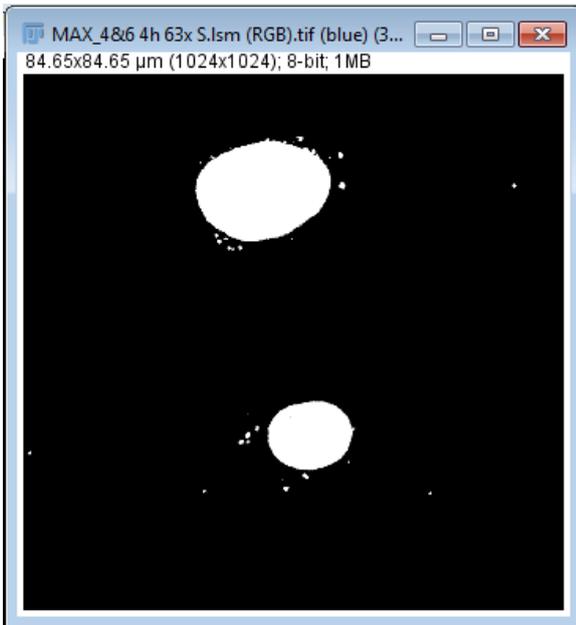
Measuring objects within objects with Fiji

31 January 2017

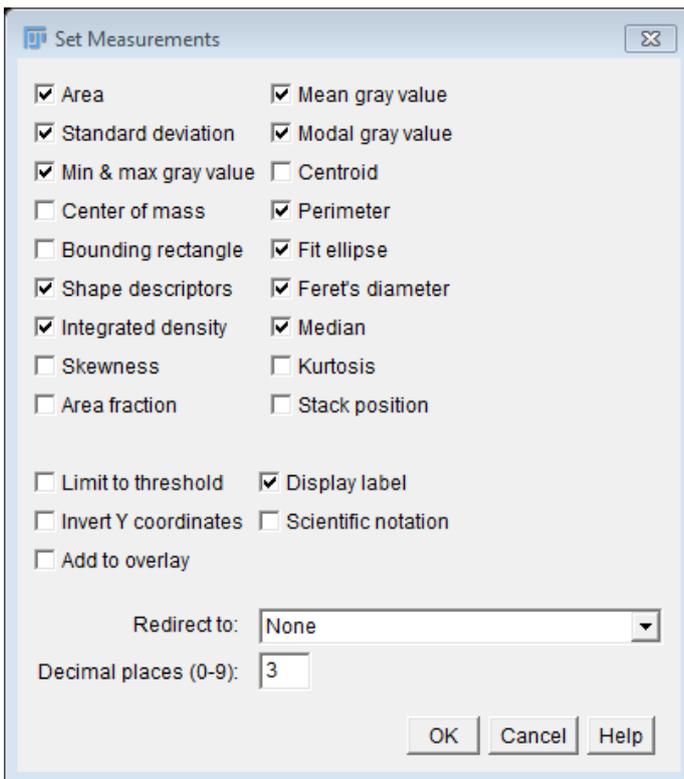
1. Open the file called **MAX_4&6 4H 63x S.Ism (RGB).tif**. Note that the image is already calibrated because it is a Zeiss .Ism file which is a format supported by Fiji.



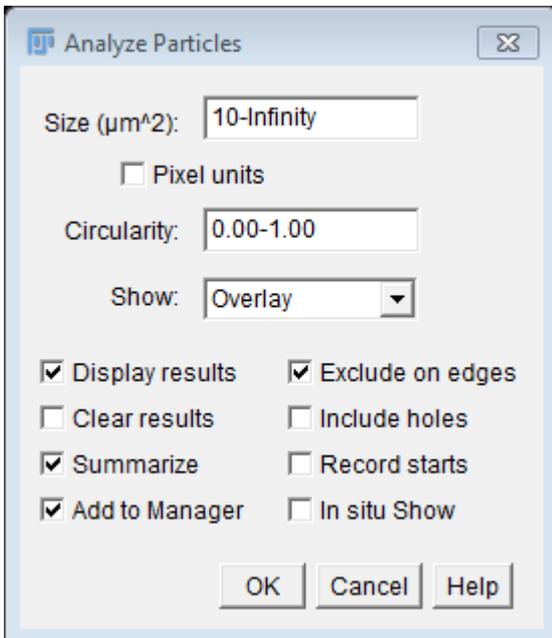
2. Split the channels (*Image – Color – Split Channels*) and discard the **Green** image.
3. The **Blue** nuclei image will be used to create the **Regions of Interest** because we are interested in the spots that are located inside the nucleus.
4. Make sure that you have **Black background** selected in your **Binary Options** (*Process – Binary – Options*).
5. Threshold the image so that both nuclei are filled completely, e.g. *Huang* and apply the threshold to create a binary image (or mask);



6. Go to **Analyze – Set Measurements** and select the parameters you want to measure. You don't need to select **Limit to Threshold** but you need **Display Label**. Since the objects within the objects (**Red** channel) will also go into this spreadsheet, it's sometimes easier to select everything that might be relevant to both images, i.e. if in doubt, select it anyway! Then click **OK**.



7. Now go to **Analyze – Analyze Particles**.
8. Enter a minimum value to exclude small fragments and select the other options you want. Show **Overlay Outlines** will draw the ROIs onto the image. This can be saved with the binary image as a record of what has been measured.



9. The two nuclei will be measured and **Regions of Interest** will be created and stored in the **ROI Manager**;

Results

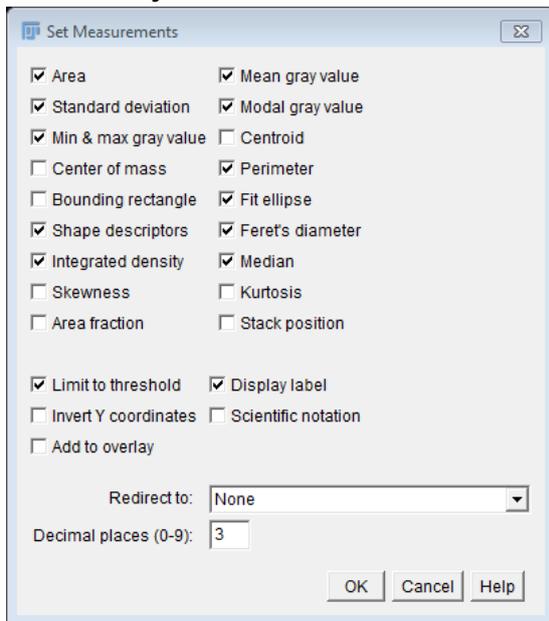
Label	Area	Mean	StdDev	Mode	Min	Max
MAX_4&6 4h 63x S.Ism (RGB).tif (blue)	259.175	255	0	255	255	255
MAX_4&6 4h 63x S.Ism (RGB).tif (blue)	115.029	255	0	255	255	255

Summary

Slice	Count	Total Area	Average Size	%Area	Mean
MAX_4&6 4h 63x S.Ism (RGB).tif (blue)	2	374.204	187.102	5.223	255

10. Select the **Red** image. Choose a threshold method but don't apply it to make the image binary this time. Leave it in grayscale as we might be interested in intensity information.

11. Go to **Analyze – Set Measurements** and select **Limit to Threshold**. Click **OK**.



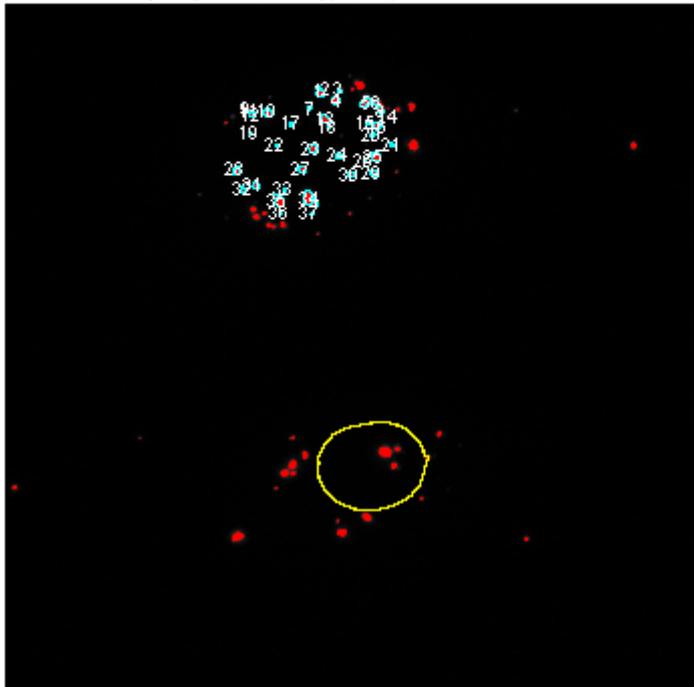
12. Highlight the first ROI in the ROI Manager list, then go to **Analyze – Analyze Particles**;

	Mean	StdDev	Mode	Min	Max	Perim
MAX_4&6 4h 63x S.Ism (RGB).tif (blue)	259.175	255	0	255	255	77.99:
MAX_4&6 4h 63x S.Ism (RGB).tif (blue)	115.029	255	0	255	255	44.71:

Slice	Count	Total Area	Average Size	%Area	Mean	Mode
MAX_4&6 4h 63x S.Ism (RGB).tif (blue)	2	374.204	187.102	5.223	255	255

13. Then highlight the second ROI in the list and repeat **Analyze – Analyze Particles**;

MAX_4&6 4h 63x S.lsm (RGB).tif (red) (33....
84.65x84.65 µm (1024x1024); 8-bit; 1 MB



ROI Manager

- 0001-0219
- 0002-0691

Add [t]
Update
Delete
Rename...
Measure
Deselect
Properties...
Flatten [F]
More »
 Show All
 Labels

	Area	Mean	StdDev	M
7 MAX_4&6 4h 63x S.lsm (RGB).tif (red):0001-0219	1.421	166.534	74.888	25
8 MAX_4&6 4h 63x S.lsm (RGB).tif (red):0001-0219	0.123	73.833	17.757	60

Summary

File Edit Font

Slice	Count	Total Area	Average Size	%Area	M
MAX_4&6 4h 63x S.lsm (RGB).tif (blue)	2	374.204	187.102	5.223	25
MAX_4&6 4h 63x S.lsm (RGB).tif (red)	37	15.914	0.430	6.138	10

MAX_4&6 4h 63x S.lsm (RGB).tif (red) (33...
84.65x84.65 µm (1024x1024); 8-bit; 1MB

ROI Manager

- 0001-0219
- 0002-0691

Buttons: Add [t], Update, Delete, Rename..., Measure, Deselect, Properties..., Flatten [F], More »

Options: Show All, Labels

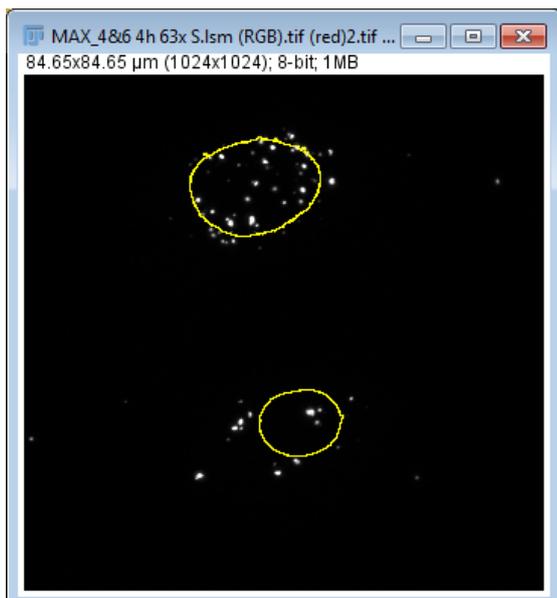
	Area	Mean	StdDev	Mc
1 MAX_4&6 4h 63x S.lsm (RGB).tif (red):0002-0691	0.369	129.444	57.958	65
2 MAX_4&6 4h 63x S.lsm (RGB).tif (red):0002-0691	0.109	113.062	36.346	55

Summary

File Edit Font

Slice	Count	Total Area	Average Size	%Area	Mc
MAX_4&6 4h 63x S.lsm (RGB).tif (blue)	2	374.204	187.102	5.223	25
MAX_4&6 4h 63x S.lsm (RGB).tif (red)	37	15.914	0.430	6.138	11

14. Save the image with the ROIs drawn on it.



15. This can all be carried out automatically by creating a macro.

Macro for measuring objects within objects

1. In order to get Fiji to measure the objects inside each ROI, you need to insert the following text before the **Analyze – Analyze Particles** step. This will sequentially select each ROI in the list and then run the Particle Analyzer.

```
for (i=0 ; i<roiManager("count"); i++) {
```

```
roiManager("select", i);
```

```
6 dir2 = getDirectory("Choose Destination Directory ");
7 list = getFileList(dir1);
8 setBatchMode(true);
9 for (i=0; i<list.length; i++) {
10 showProgress(i+1, list.length);
11 open(dir1+list[i]);
12 imgName=getTitle();
13 run("Options...", "iterations=1 count=1 black edm=Overwrite");
14 //Separates the RGB image into red, green and blue channel images.
15 run("Split Channels");
16 //Nuclei image is selected = blue channel
17 selectWindow(imgName + " (blue)");
18 //Blue channel image is thresholded, mask is created and particle analysis run to crea
19 setAutoThreshold("Huang dark");
20 setOption("BlackBackground", true);
21 run("Convert to Mask");
22 run("Set Measurements...", "area standard perimeter feret's display redirect=None deci
23 run("Analyze Particles...", "size=100-Infinity show=[Overlay Outlines] display exclude
24 //Binary image of nuclei is saved for future reference.
25 title = getTitle;
26 dotIndex = indexOf(title, ".");
27 title = substring(title, 0, dotIndex);
28 newtitle= title + "-Nuclei";
29 run("Rename...", "title=[" +newtitle+"]");
30 saveAs("TIFF", dir2 +newtitle);
31 close();
32 //Green channel image is closed.
33 selectWindow(imgName + " (green)");
34 close();
35 //Red channel image of foci is selected.
36 selectWindow(imgName + " (red)");
37 //Red channel image is thresholded but not converted to binary as intensity measuremen
38 setAutoThreshold("Huang dark");
39 run("Set Measurements...", "area mean standard modal min perimeter feret's median limi
40 //ROIs are selected sequentially in the ROI Manager - particles in each ROI are measur
41 for (i=0 ; i<roiManager("count"); i++) {
42     roiManager("select", i);
43     for (i=0 ; i<roiManager("count"); i++) {
44         roiManager("select", i);
45 run("Analyze Particles...", "size=0-Infinity show=[Overlay Outlines] display exclude s
46 //end of macro
```

