FLUORESCENCE QUANTIFICATION WITH FIJI

Alfonsa Díaz Torres

Servicio Microscopía Óptica y Confocal

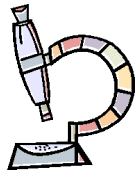
Centro Biología Molecular Severo Ochoa

Madrid 2016

FLUORESCENCE QUANTIFICATION WITH FIJI

1. Image analysis
   - Digital image
2. Sample preparation
3. Image acquisition
4. Corrections
5. Fluorescence intensity quantification
   - *Set Measurements*
   - *Limit to Threshold*
   - Images with multiple objects
   - Images with multiple planes

IMAGE ANALYSIS



Techniques for getting information from images.

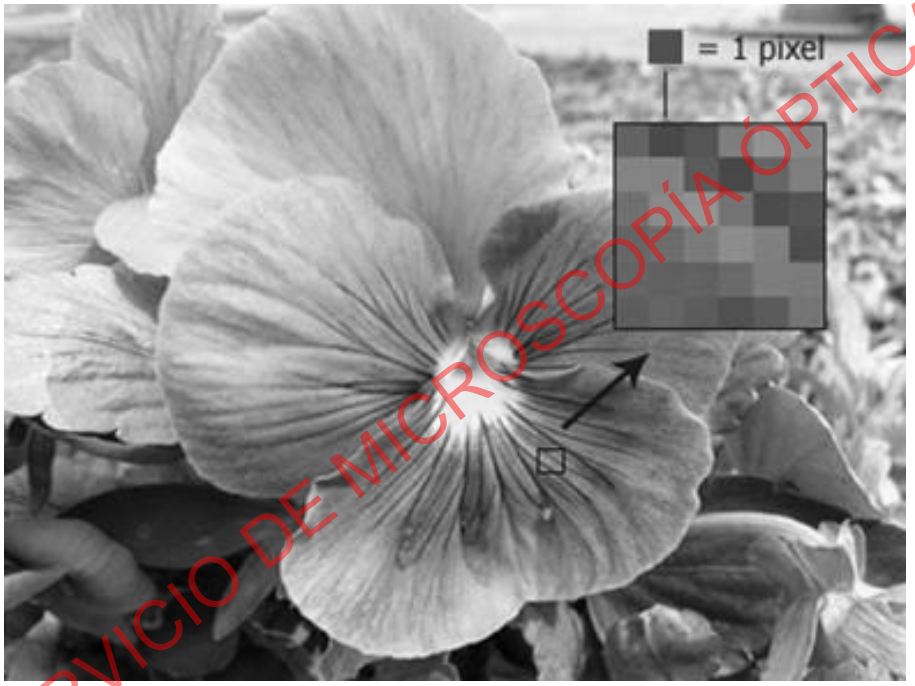
- Obtain quantitative data in numerical form
- Image capture and analysis software

DIGITAL IMAGE

- Dot mosaic (pixels).
  - Color or grayscale.

DIGITAL IMAGE

- Dot mosaic (pixels).
  - Color or grayscale.



8 bits = 2^8 = 256

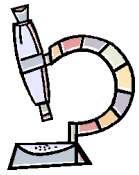
pixel Color	Decimal number
	0
	255

12 bits = 2^{12} = 4096

16 bits = 2^{16} = 65536

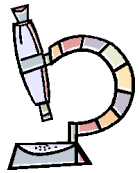
FLUORESCENCE QUANTIFICATION WITH FIJI

1. Image analysis
   - Digital image
2. Sample preparation
3. Image acquisition
4. Corrections
5. Fluorescence intensity quantification
   - *Set Measurements*
   - *Limit to Threshold*
   - Images with multiple objects
   - Images with multiple planes



SAMPLE PREPARATION

Prepare samples the same day using exactly the same protocol

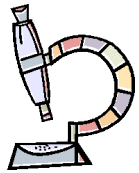


SAMPLE PREPARATION

Controls

AUTOFLUORESCENCE: Identical protocol without primary or secondary antibodies.

SECONDARY ANTIBODIES: Incubate the sample only with the secondary antibodies.



SAMPLE PREPARATION

Controls

CROSSTALK OR CHANNEL INTERFERENCE
Stain samples with each primary/secondary antibody combination separately and acquire images for all the channels with the same acquisition parameters as those used in double or triple-stained preparations

FLUORESCENCE QUANTIFICATION WITH FIJI

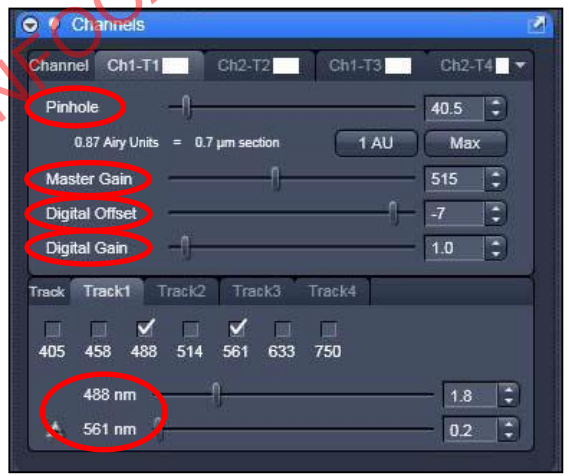
1. Image analysis
   - Digital image
2. Sample preparation
3. Image acquisition
4. Corrections
5. Fluorescence intensity quantification
   - *Set Measurements*
   - *Limit to Threshold*
   - Images with multiple objects
   - Images with multiple planes

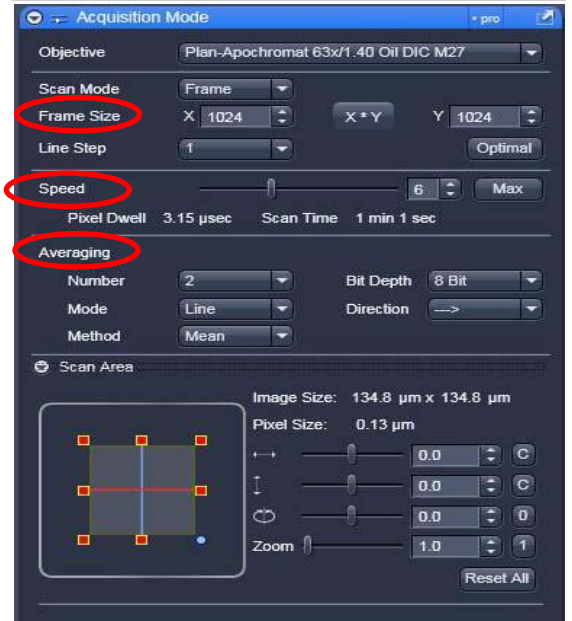
IMAGE ACQUISITION

CONFOCAL SYSTEM

- Same conditions and same day

Set conditions according to the brightest sample





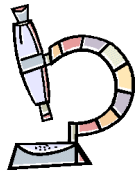


IMAGE ACQUISITION

CONFOCAL SYSTEM

- Same conditions and same day

Set conditions according to the brightest sample

- Allow lasers to stabilize (switch on 1h before)

IMAGE ACQUISITION

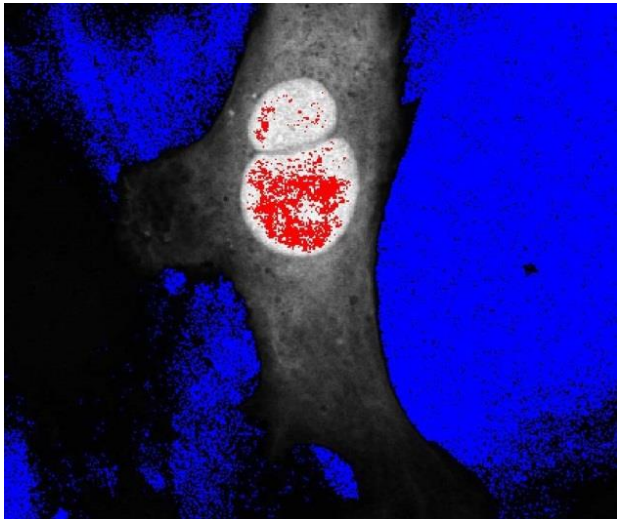
CONFOCAL SYSTEM

- Same conditions and same day

Set conditions according to the brightest sample

- Allow lasers to stabilize (switch on 1h before)

- No saturated pixels



Range Indicator palette

Red = saturation

IMAGE ACQUISITION

CONFOCAL SYSTEM

- Same conditions and same day

Set conditions according to the brightest sample

- Allow lasers to stabilize (switch on 1h before)
- No saturated pixels
- Check controls

IMAGE ACQUISITION

CONFOCAL SYSTEM

- Avoid photobleaching
- Field centered
- 12 Bits

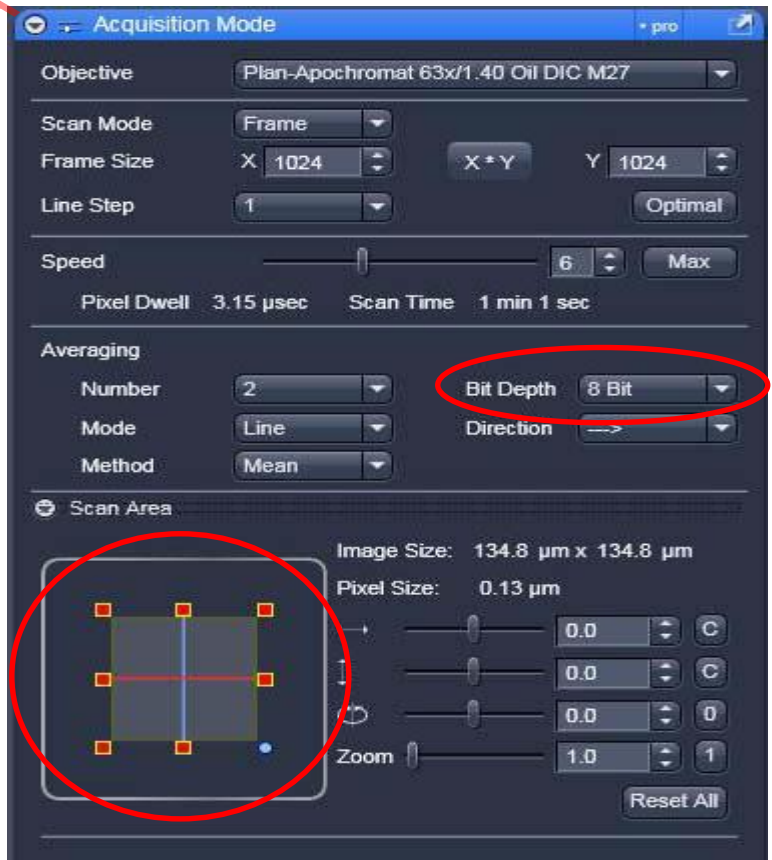


IMAGE ACQUISITION

WIDE-FIELD SYSTEM

- Same conditions and same day

Set conditions according to the brightest sample

IMAGE ACQUISITION

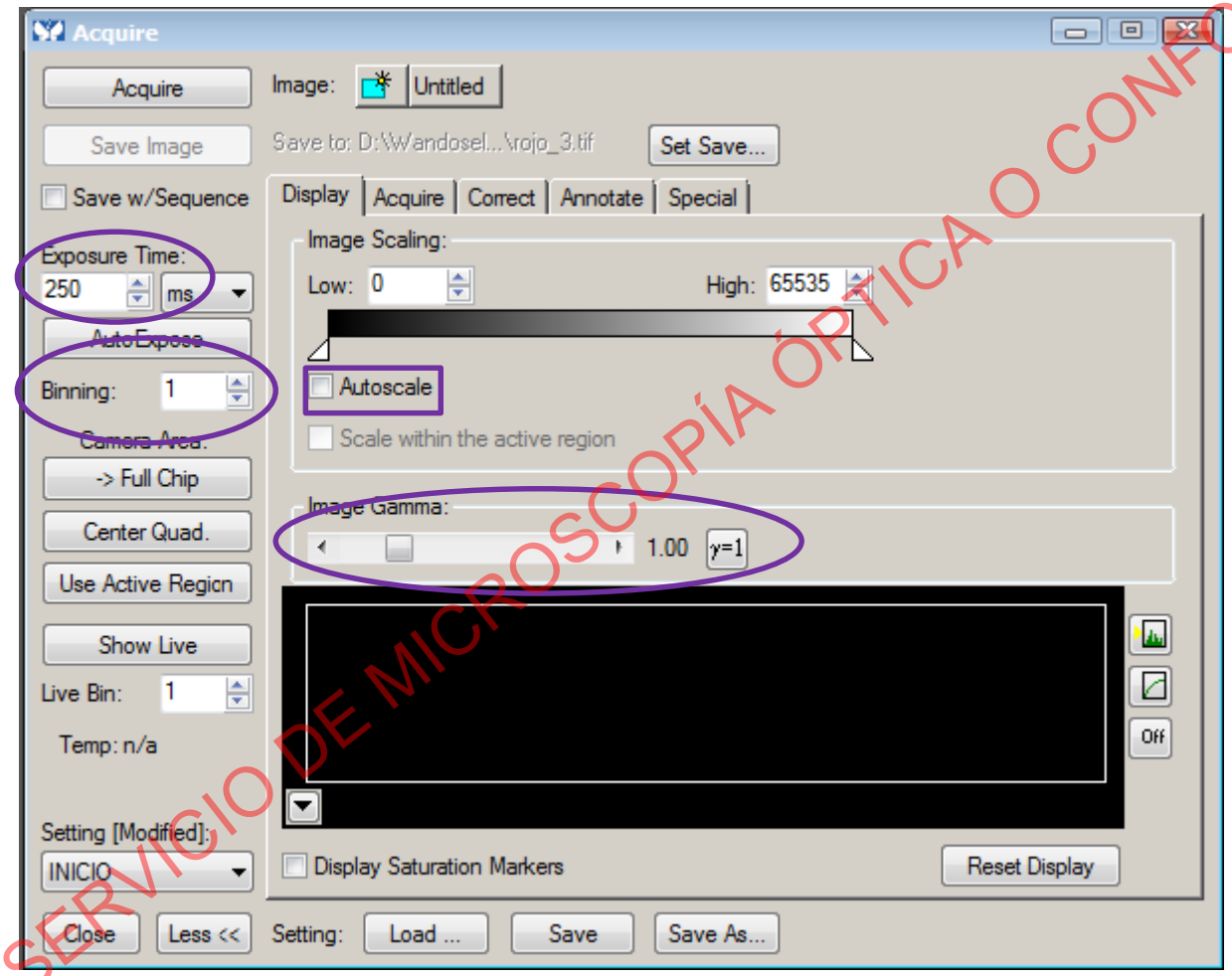
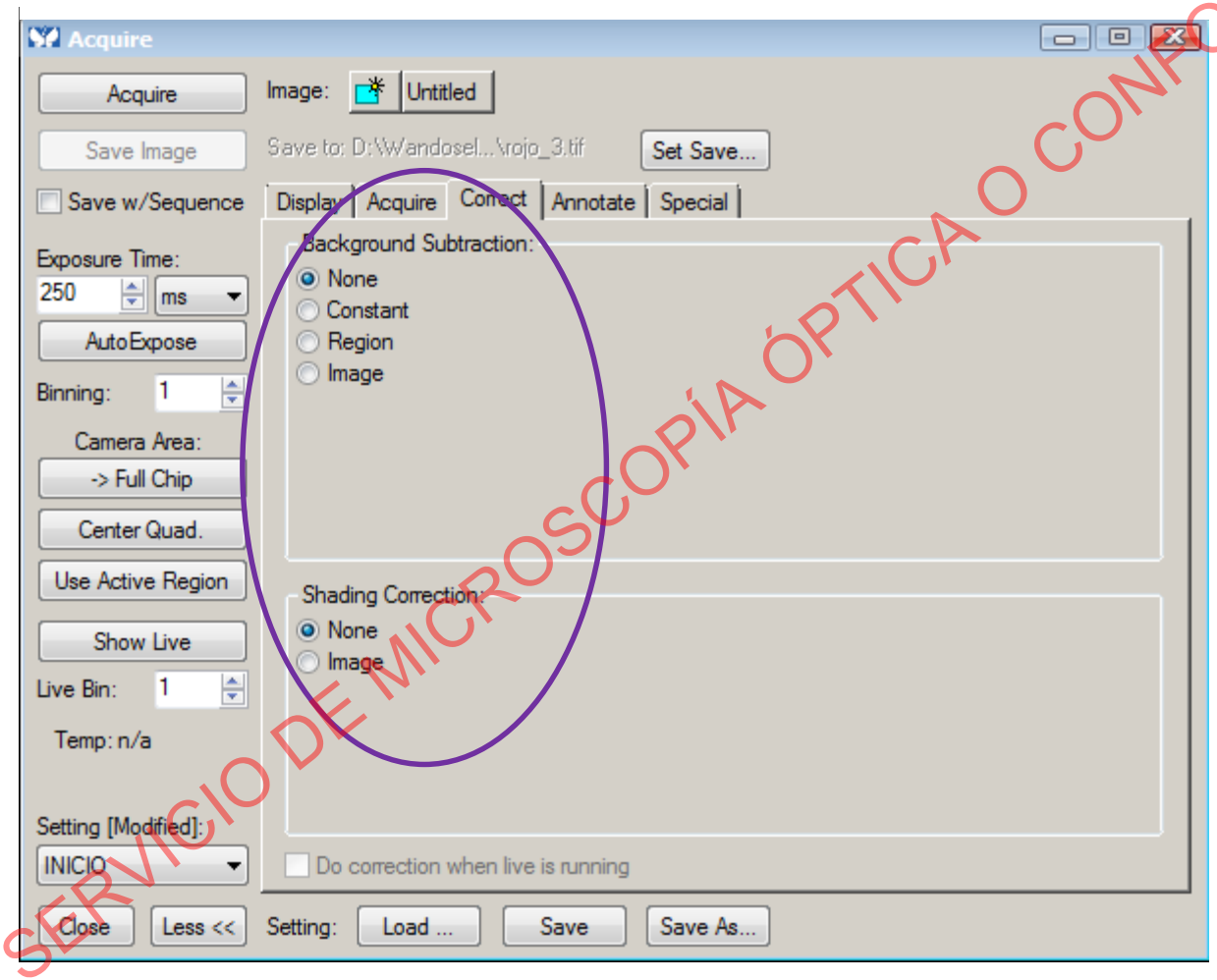


IMAGE ACQUISITION



If the camera has variable sensitivity, this value must also be the same between samples

IMAGE ACQUISITION

WIDE-FIELD SYSTEM

- Same conditions and same day
- Do not autoscale
- Allow lamp to stablize (Switch on 1h before use)
- No saturated pixels

IMAGE ACQUISITION

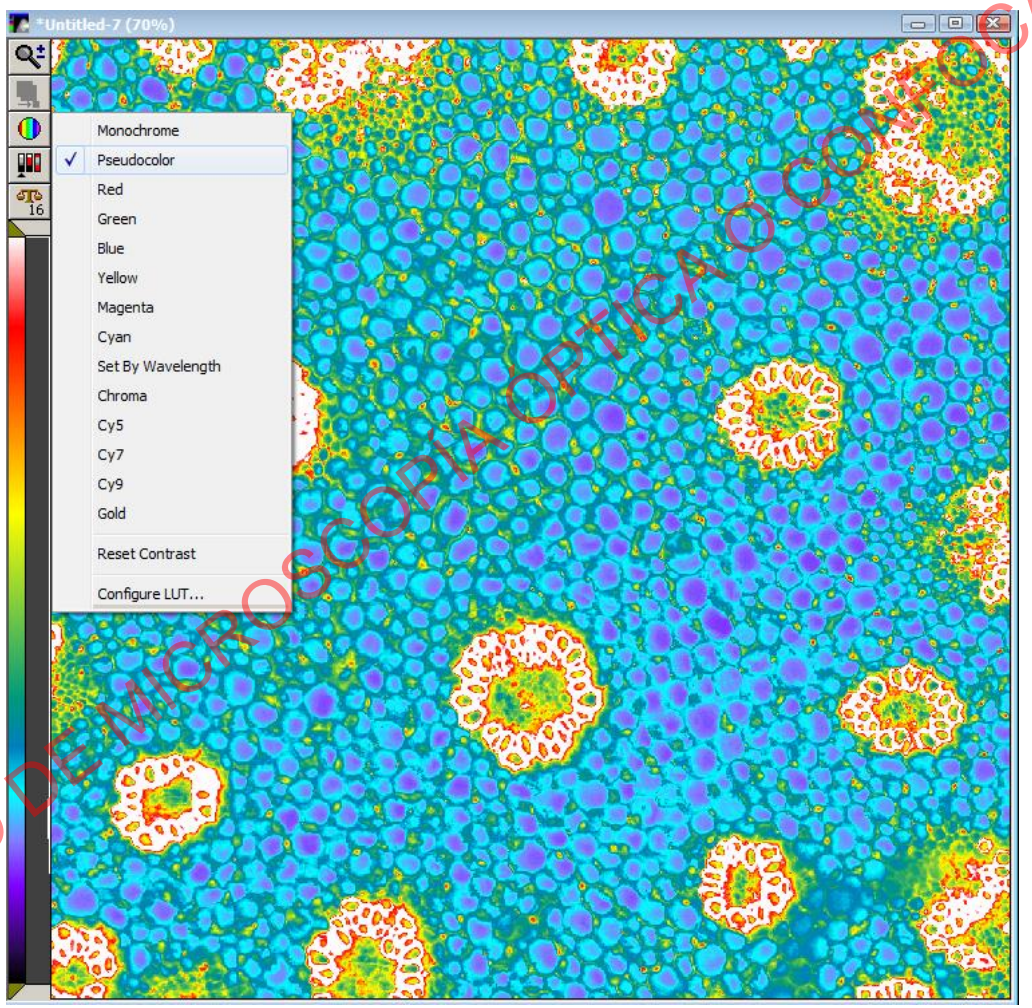


IMAGE ACQUISITION

WIDE-FIELD SYSTEM

- Same conditions and same day
- Do not autoscale
- Allow lamp to stablize (Switch on 1h before use)
- No saturated pixels
- Avoid photobleaching

IMAGE ACQUISITION

WIDE-FIELD SYSTEM

- Check controls
- Use the highest bit depth allowed by the system
- Select the center quadrant

IMAGE ACQUISITION

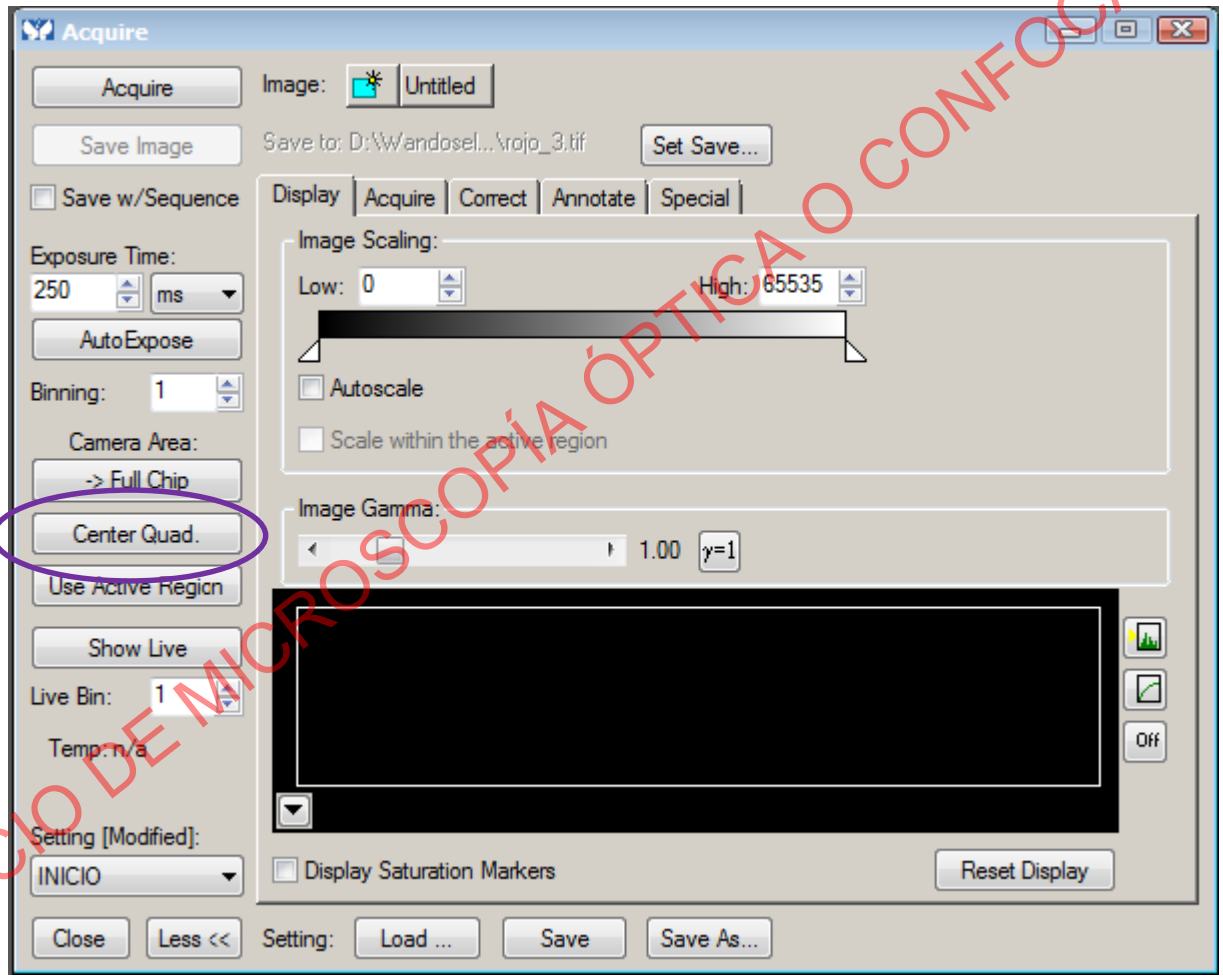
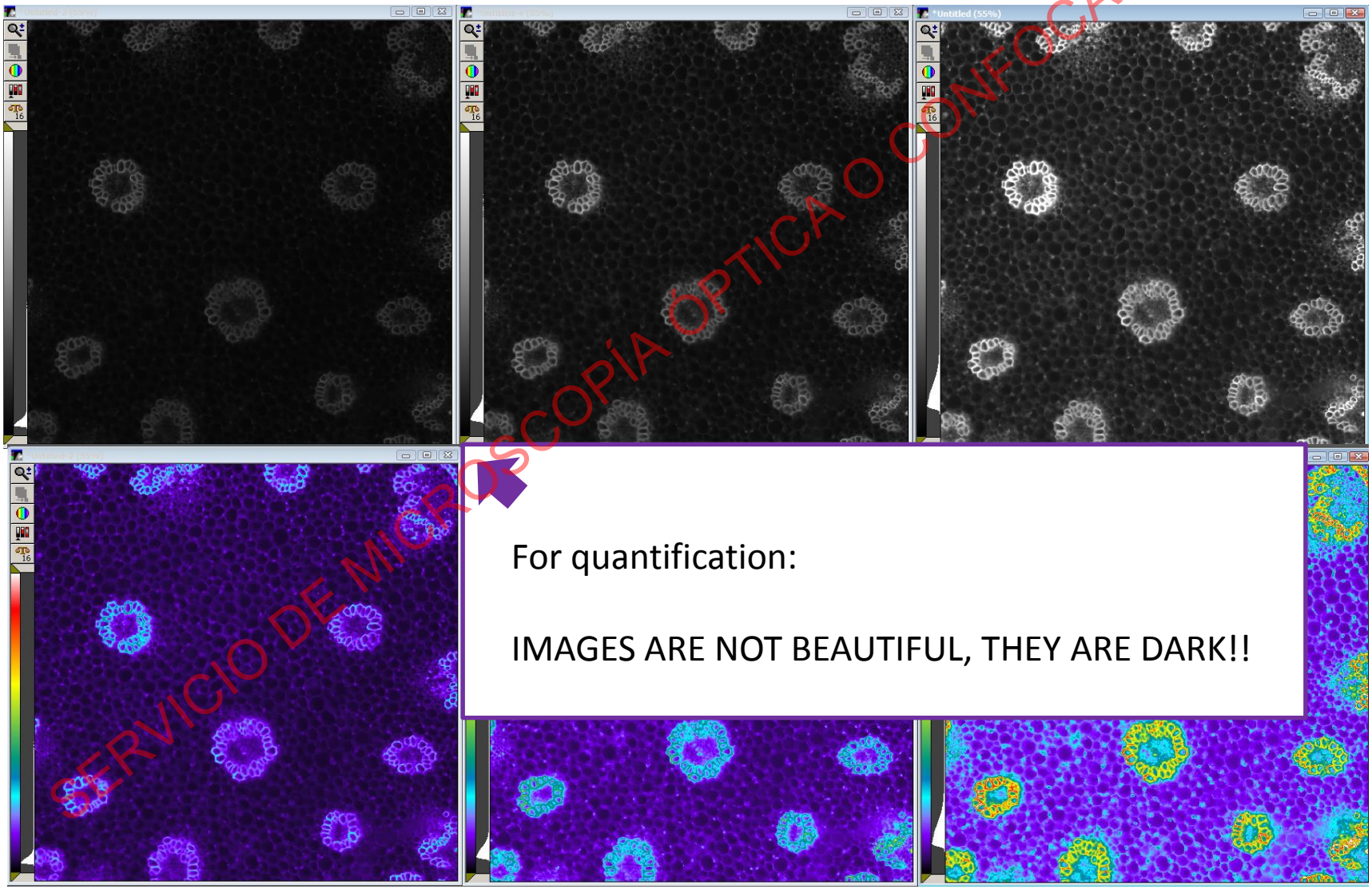


IMAGE ACQUISITION



For quantification:

IMAGES ARE NOT BEAUTIFUL, THEY ARE DARK!!

FLUORESCENCE QUANTIFICATION WITH FIJI

1. Image analysis
   - Digital image
2. Sample preparation
3. Image acquisition
4. Corrections
5. Fluorescence intensity quantification
   - *Set Measurements*
   - *Limit to Threshold*
   - Images with multiple objects
   - Images with multiple planes

CORRECTIONS

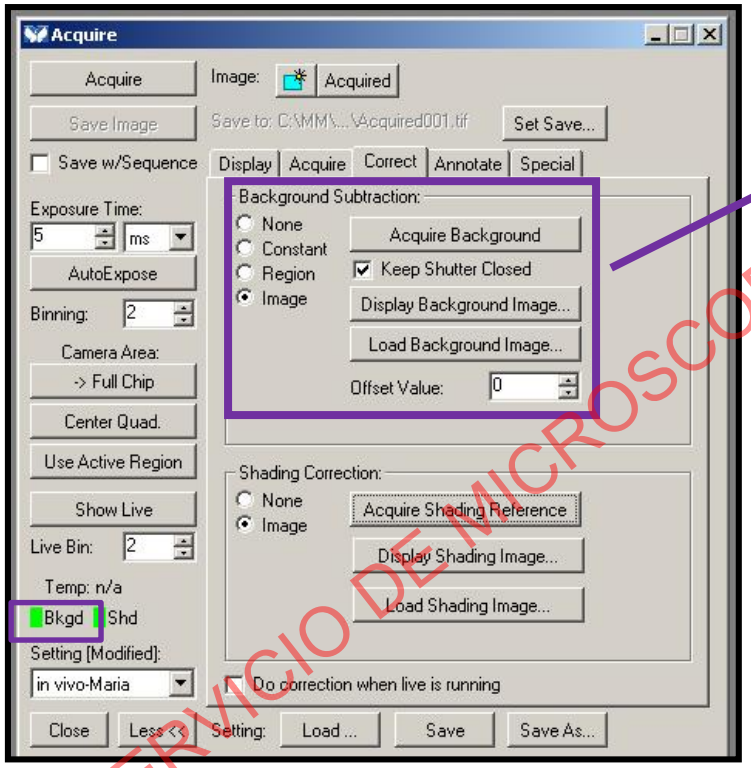
WIDE-FIELD SYSTEM

- Background correction
- Shading correction

CORRECTIONS

Background correction

METAMORPH



Select "*Image*"/"*Keep Shutter Closed*" and acquire image in "*Acquire Background*".

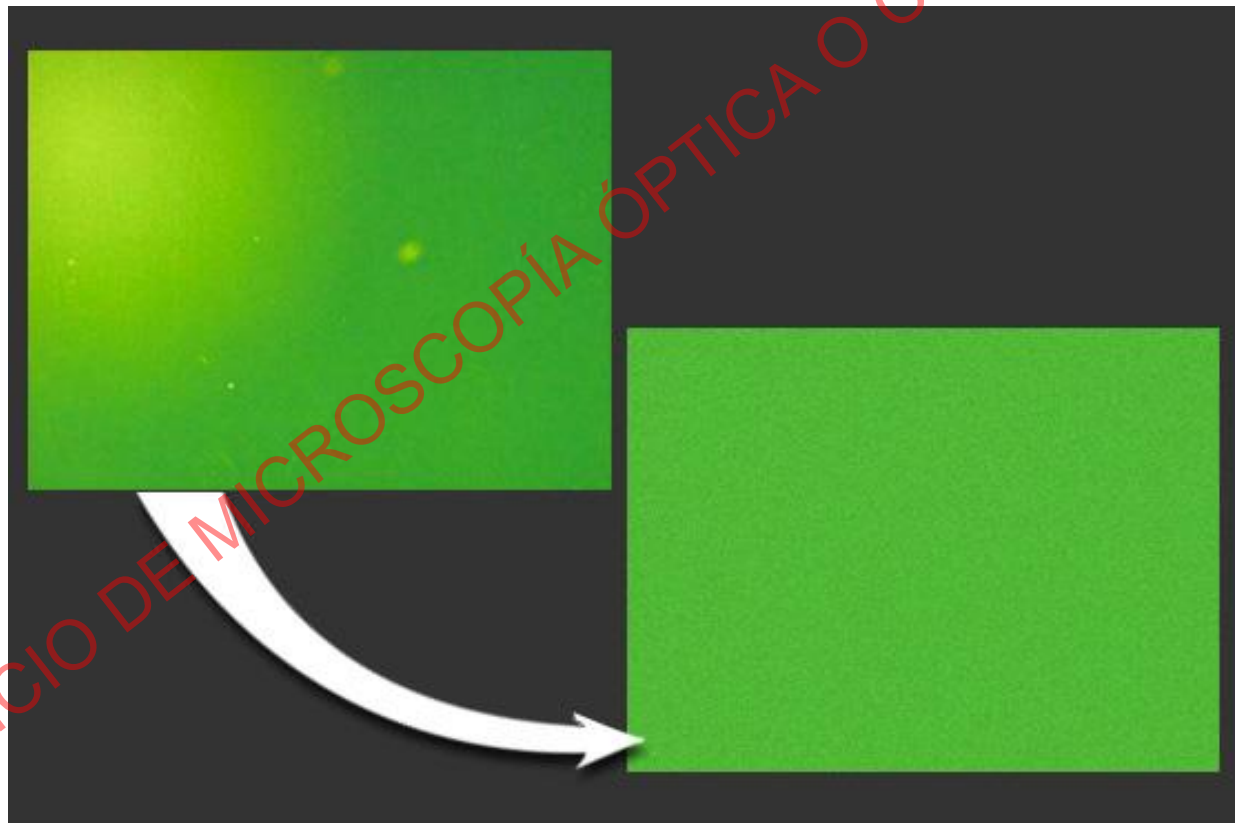
Images will be corrected for <u>camera background in the absence of light.</u>

To save the background image: "*Display Background Image*" and save that image.

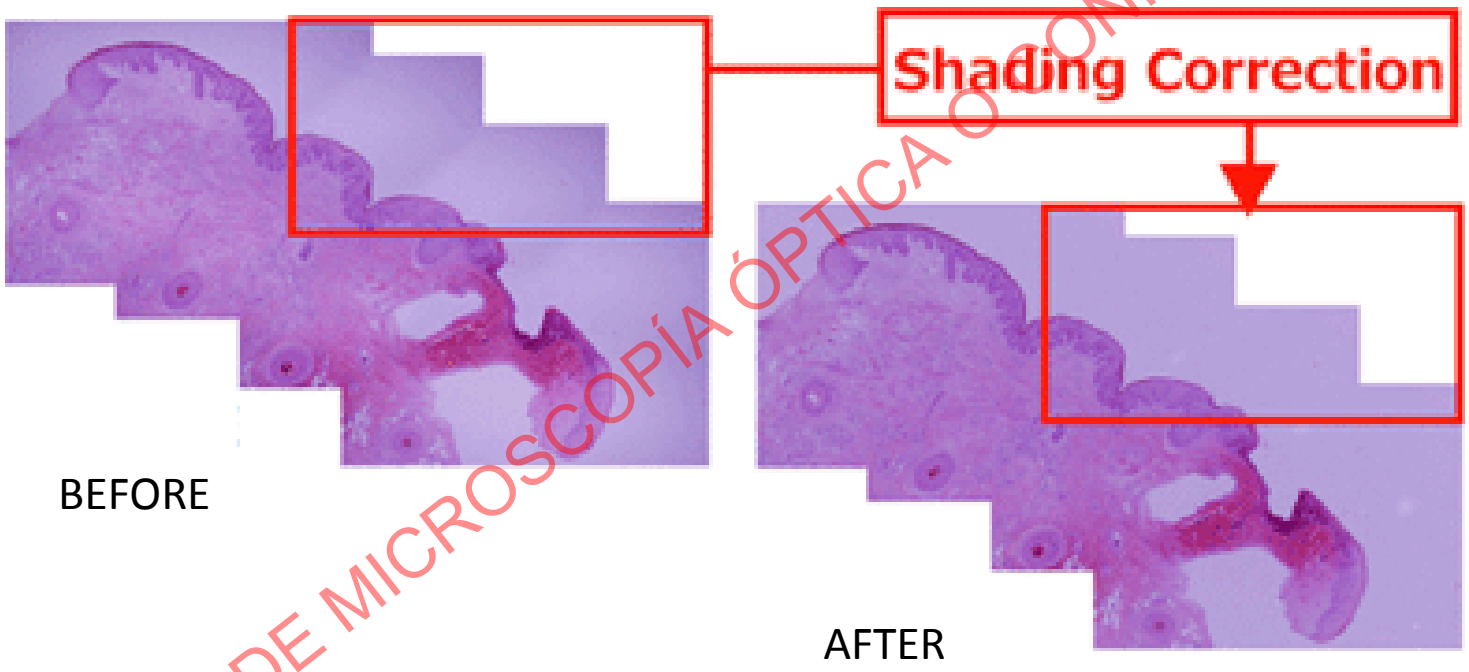
Acquisition conditions must be identical for the background image and the final one. Check that a green icon appears next to "*Bkgd*".

CORRECTIONS

Shading



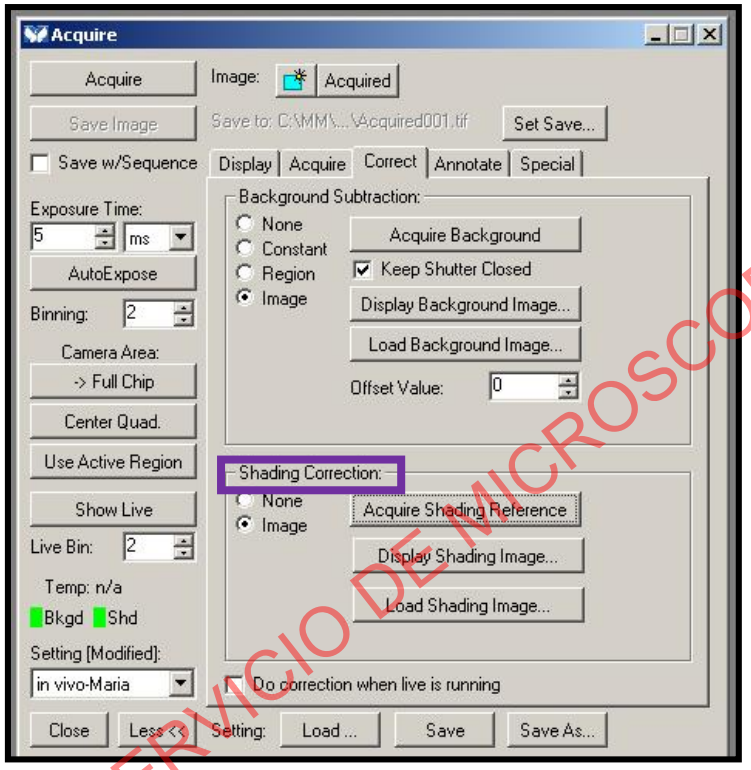
CORRECTIONS



CORRECTIONS

Shading correction

METAMORPH



Corrects defects in field illumination.

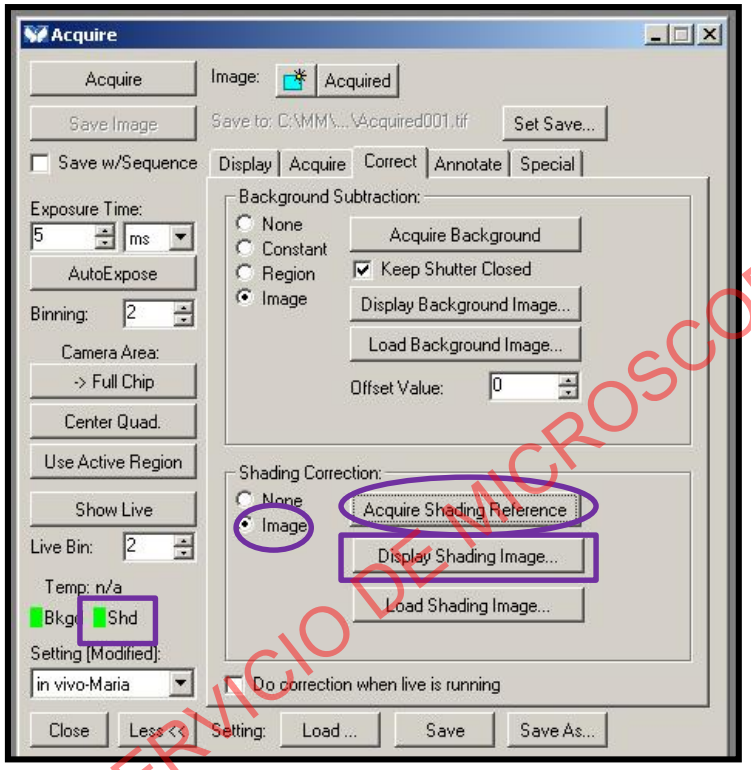
To acquire a shading image:

Defocus the preparation enough to see a uniformly illuminated background field.

CORRECTIONS

Shading correction

METAMORPH



Select "*Image*" and capture an image in "*Acquire Shading Reference*".

To save the reference image select "*Display Shading Image*" and save that image.

Acquisition conditions must be identical for the shading image and the final one. Check that a green icon appears next to "*Shd*".

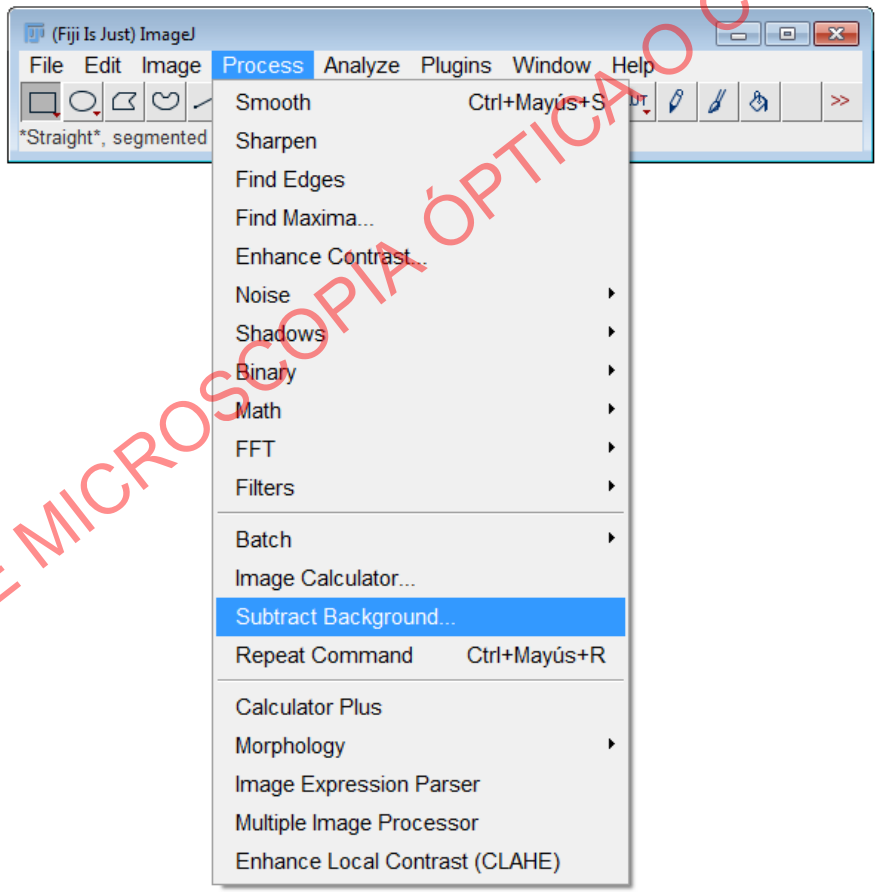
CORRECTIONS

FIJI

- Background correction
- Shading correction

CORRECTIONS

Background correction **Fiji: *Substract Background***

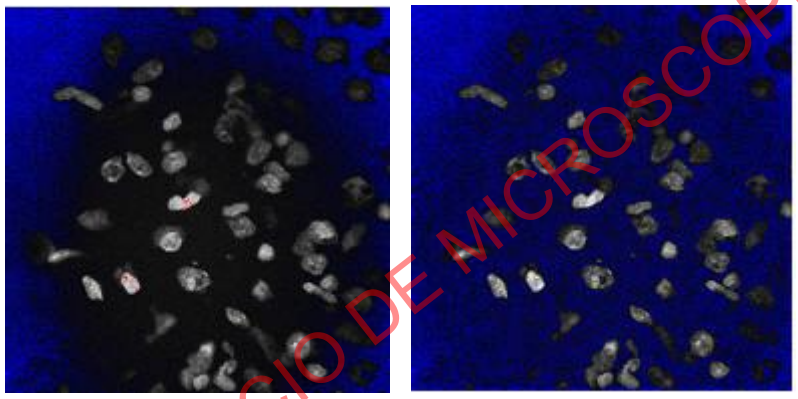


CORRECTIONS

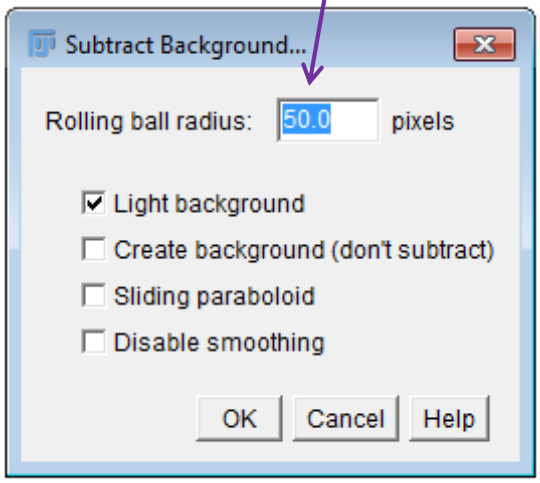
Background correction

- *Process* ▸ *Subtract background*

Size of the largest object that is not part of the background



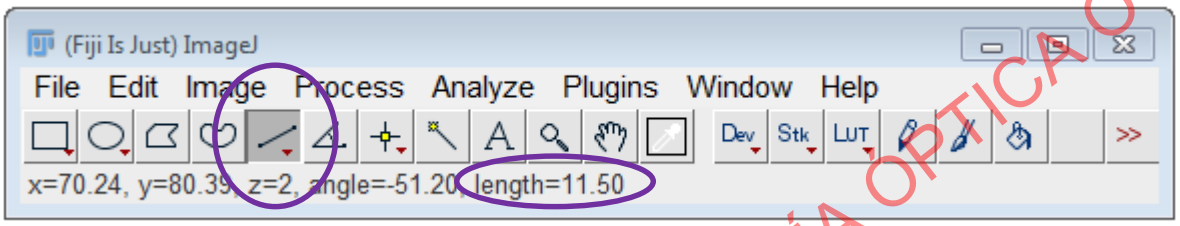
NON-UNIFORM BACKGROUND



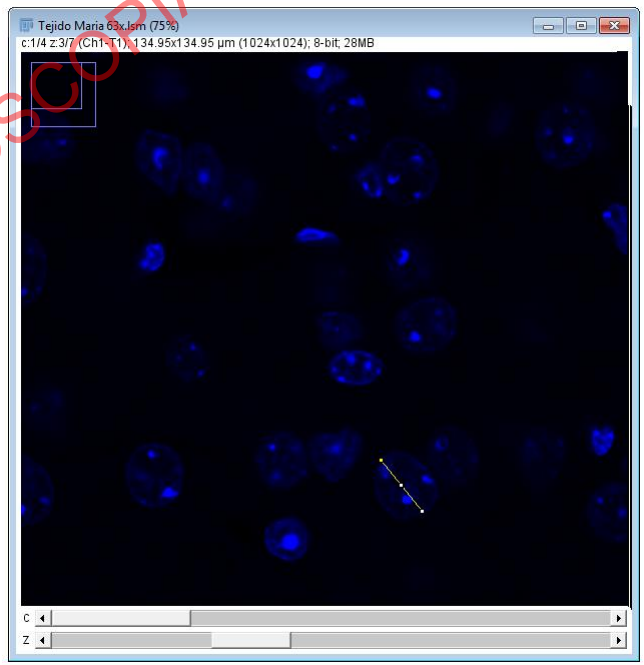
Rolling-ball algorithm

CORRECTIONS

Background correction

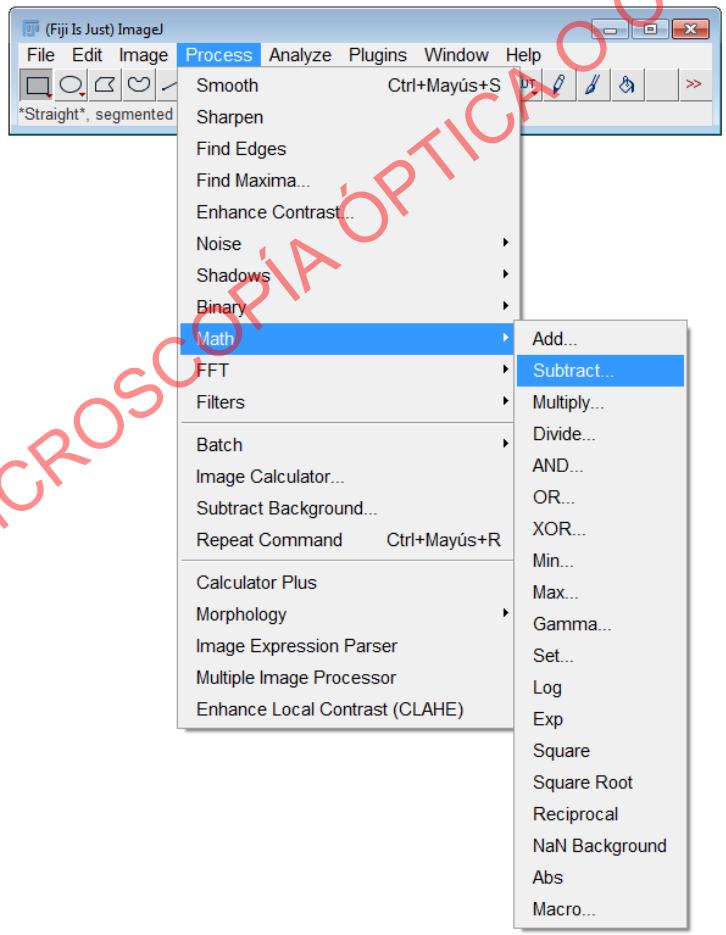


Size of the largest object that is not part of the background



CORRECTIONS

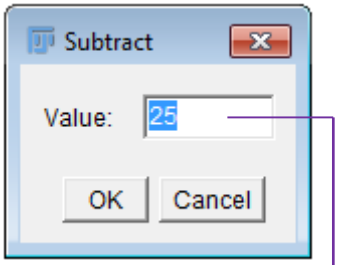
Background correction **Fiji : *Math***



CORRECTIONS

Background correction

- *Process* ▸ *Math* ▸ *Subtract*

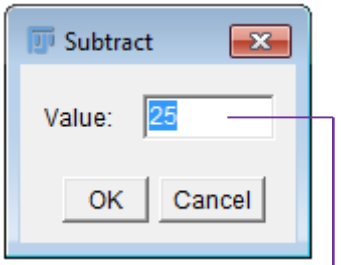


This will subtract the mean of the ROI from the image plus an additional value equal to the standard deviation of the ROI multiplied by the scaling factor you enter. The default value is 3.

CORRECTIONS

Background correction

- *Process* ▸ *Math* ▸ *Subtract*



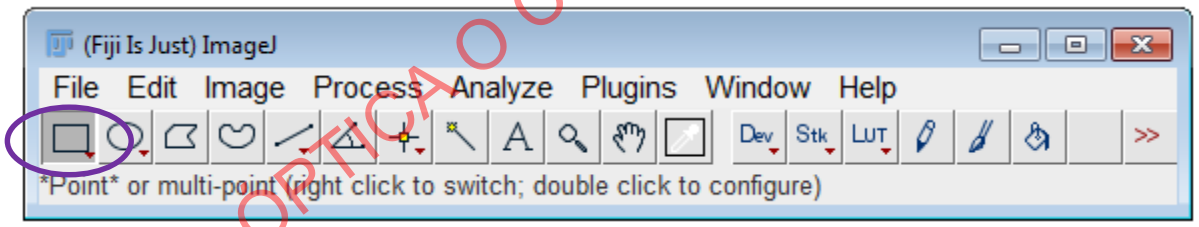
We have to calculate:
- The average background value (usually using a ROI)
- Its standard deviation

This will subtract the mean of the ROI from the image plus an additional value equal to the standard deviation of the ROI multiplied by the scaling factor you enter. The default value is 3.

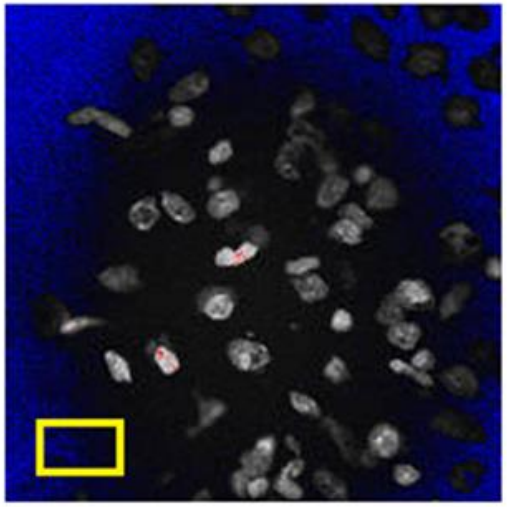
CORRECTIONS

Background correction

- *Drawing a ROI*



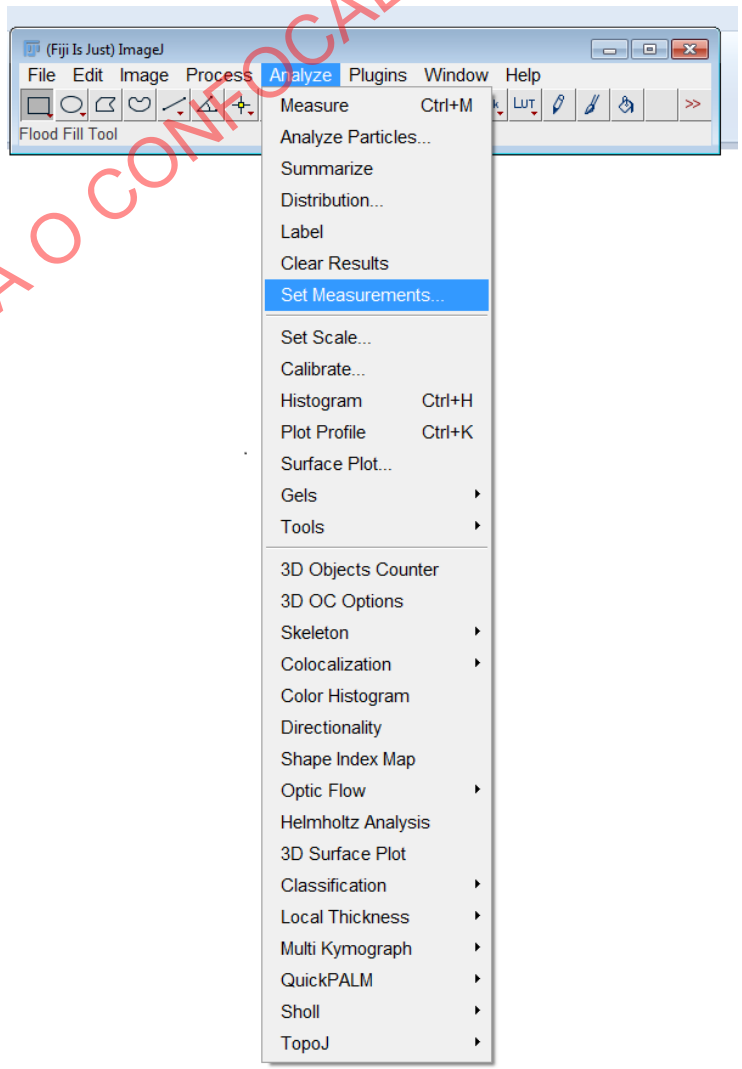
We have to calculate:
- The average background value (usually using a ROI)
- Its standard deviation



CORRECTIONS

Background correction

- *Analyze* ▸ *Set measurements*

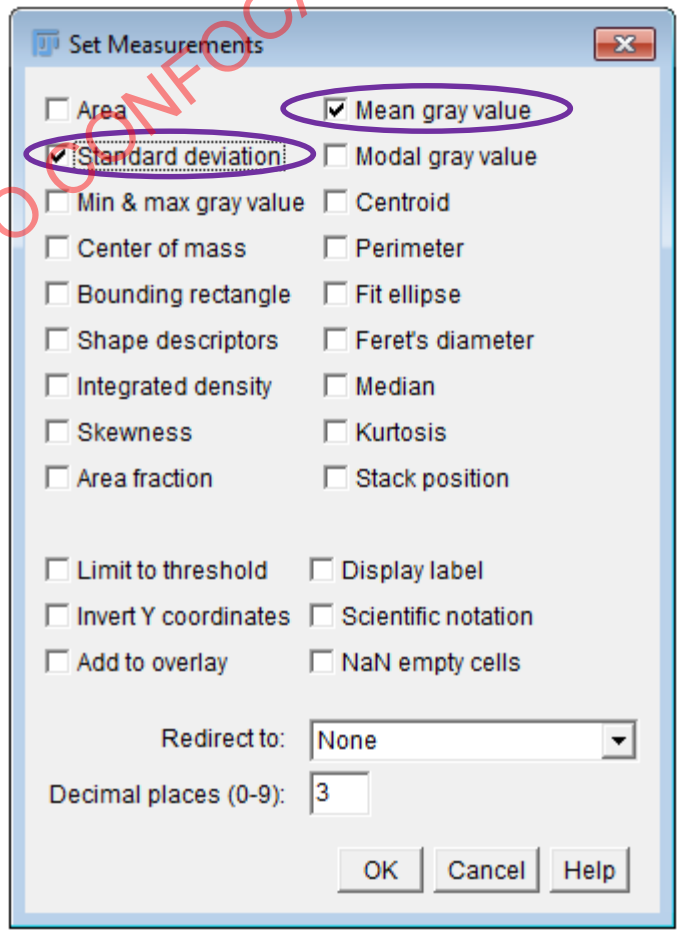


We have to calculate:
- The average background value (usually using a ROI)
- Its standard deviation

CORRECTIONS

Background correction

- *Analyze* ▸ *Set measurements*

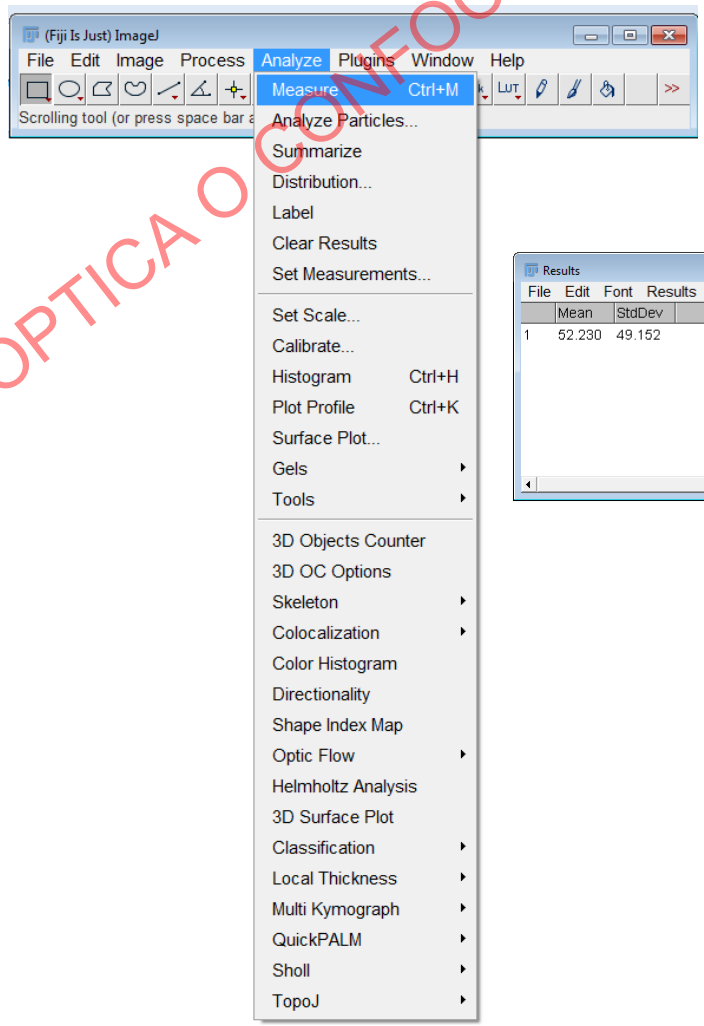


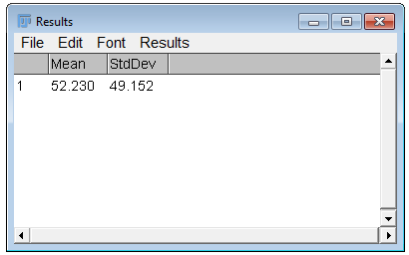
We have to calculate:
- The average background value (usually using a ROI)
- Its standard deviation

CORRECTIONS

Background correction

- *Analyze ▸ measure*





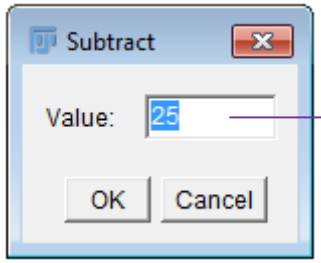
We have to calculate:
- The average background value (usually using a ROI)
- Its standard deviation

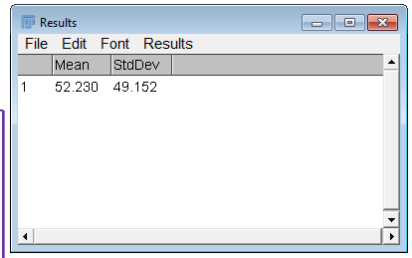
CORRECTIONS

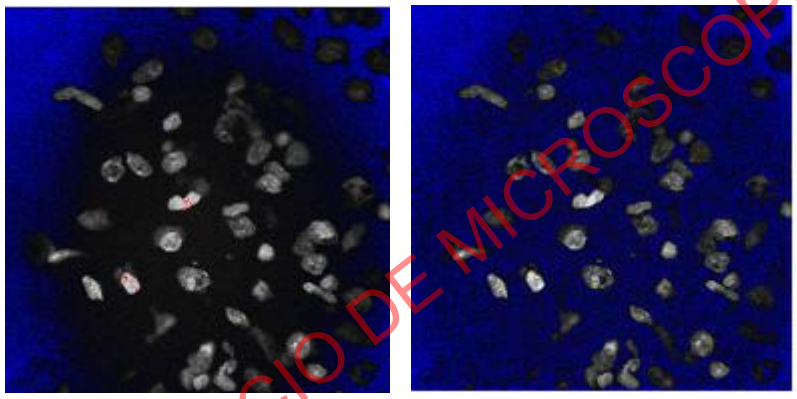
Background correction

Select a ROI in the background and calculate its mean value and standard deviation

- *Process* ▸ *Math* ▸ *Subtract*







This will subtract the mean of the ROI from the image plus an additional value equal to the standard deviation of the ROI multiplied by the scaling factor you enter. The default value is 3.

UNIFORM BACKGROUND

Mean +(StdDev x 3)

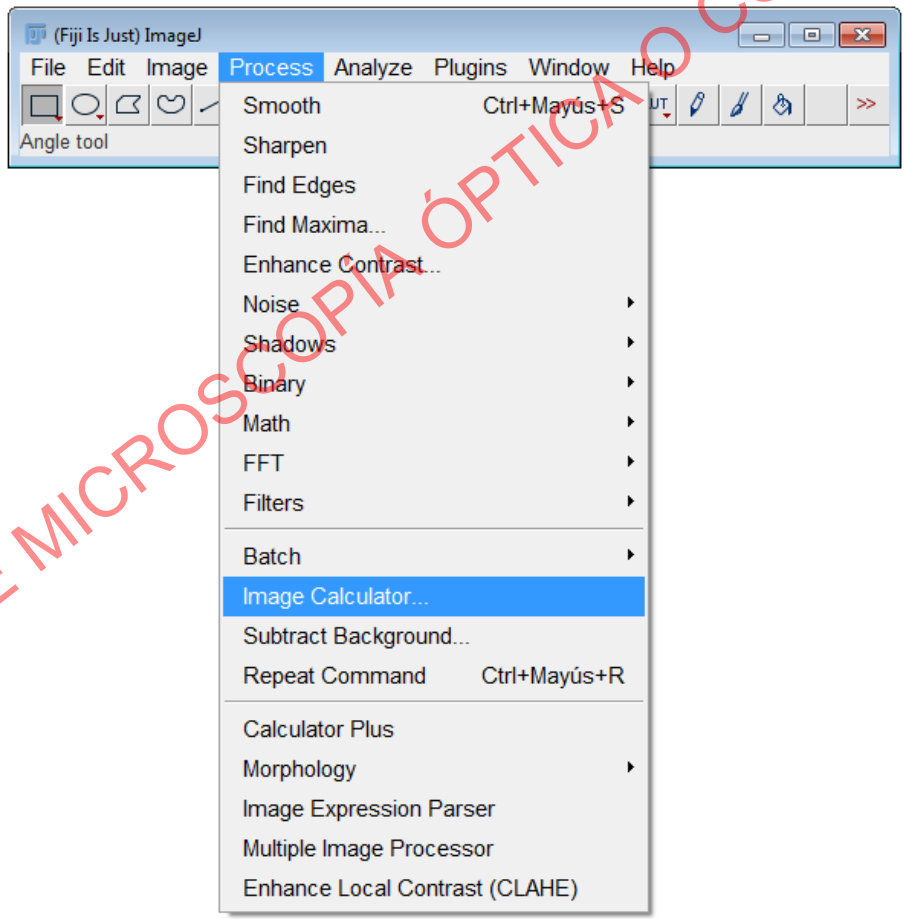
CORRECTIONS

FIJI

- Shading

CORRECTIONS

Shading correction **Fiji: *Image calculator***

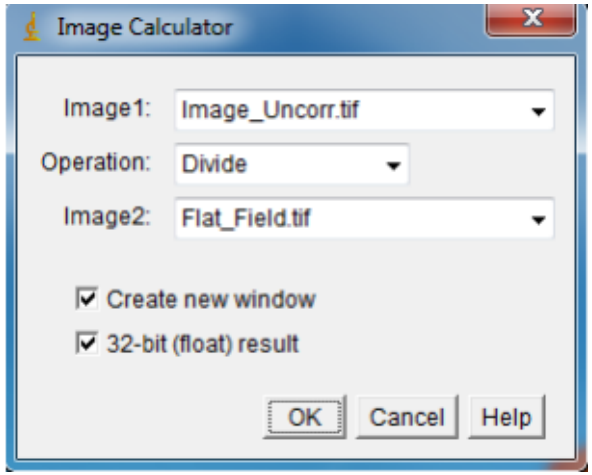


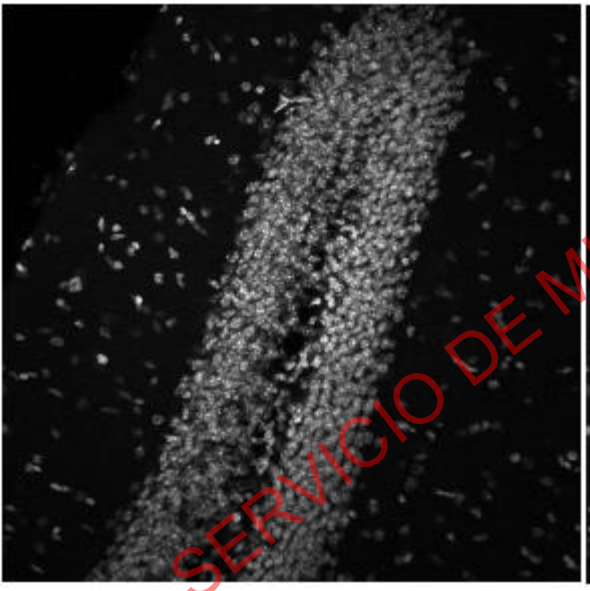
CORRECTIONS

Shading correction

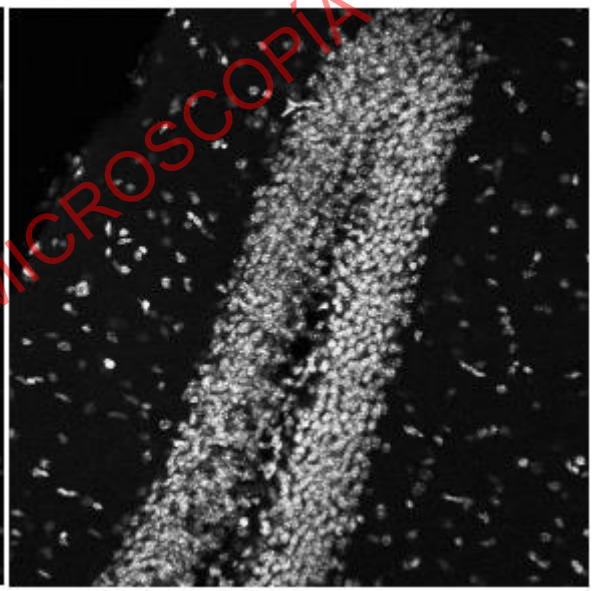
Open the uncorrected image and the flat-field image (shading image).

- *Process ▸ image calculator*





Uncorrected image



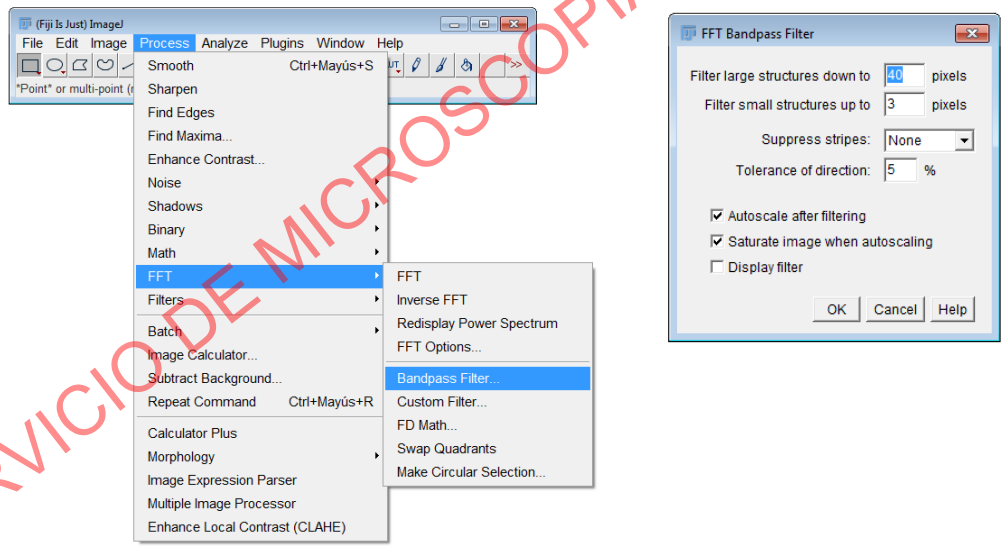
Shading-corrected image

CORRECTIONS

Shading correction

If you do not have a reference *shading image,* you can use the FFT Bandpass function as an alternative method of shading correction. It is less ideal but still produces acceptable results in most cases.

- *Process ▸ FFT ▸ Bandpass Filter*

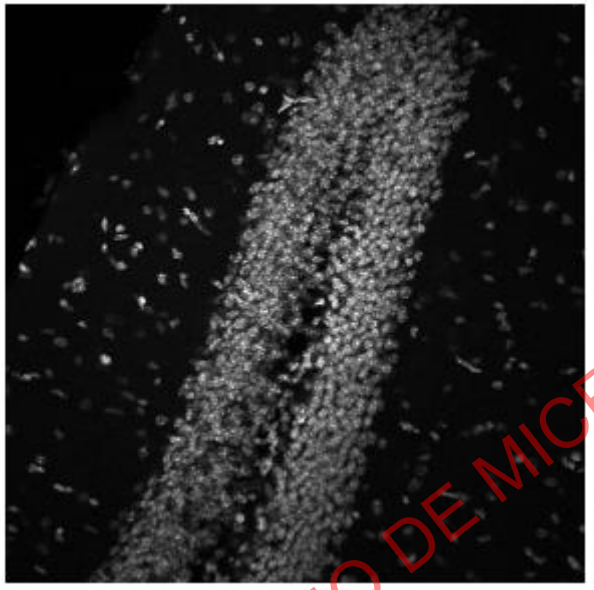


This tool removes high spatial frequencies (blurring the image) and low spatial frequencies (similar to subtracting a blurred image).

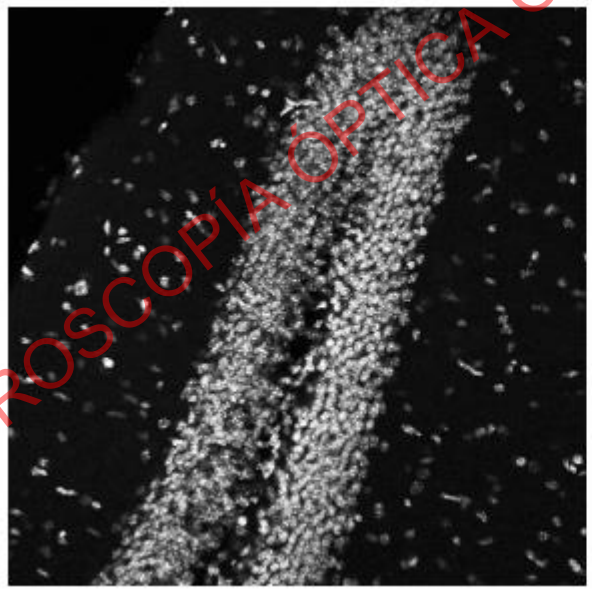
It can also suppress horizontal or vertical stripes that were created by scanning an image line by line.

CORRECTIONS

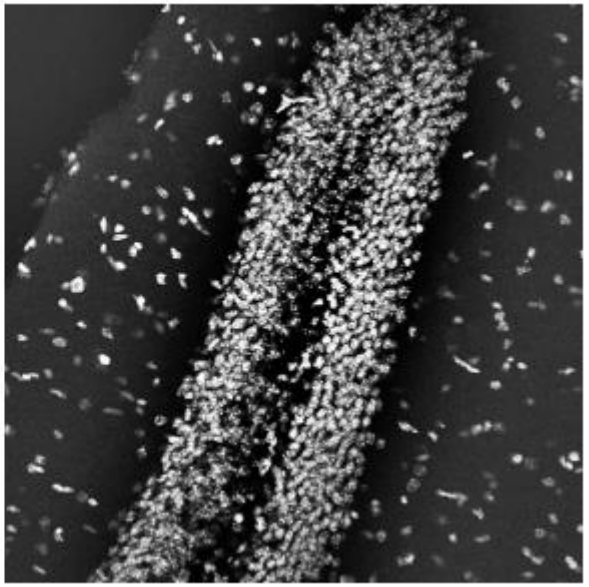
Shading correction



Uncorrected image



Shading corrected in ImageJ

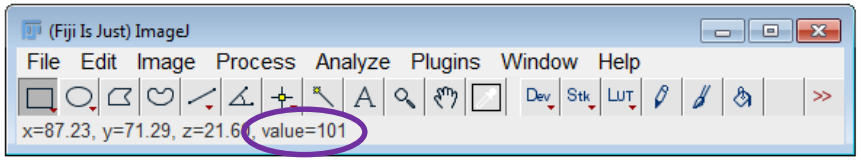


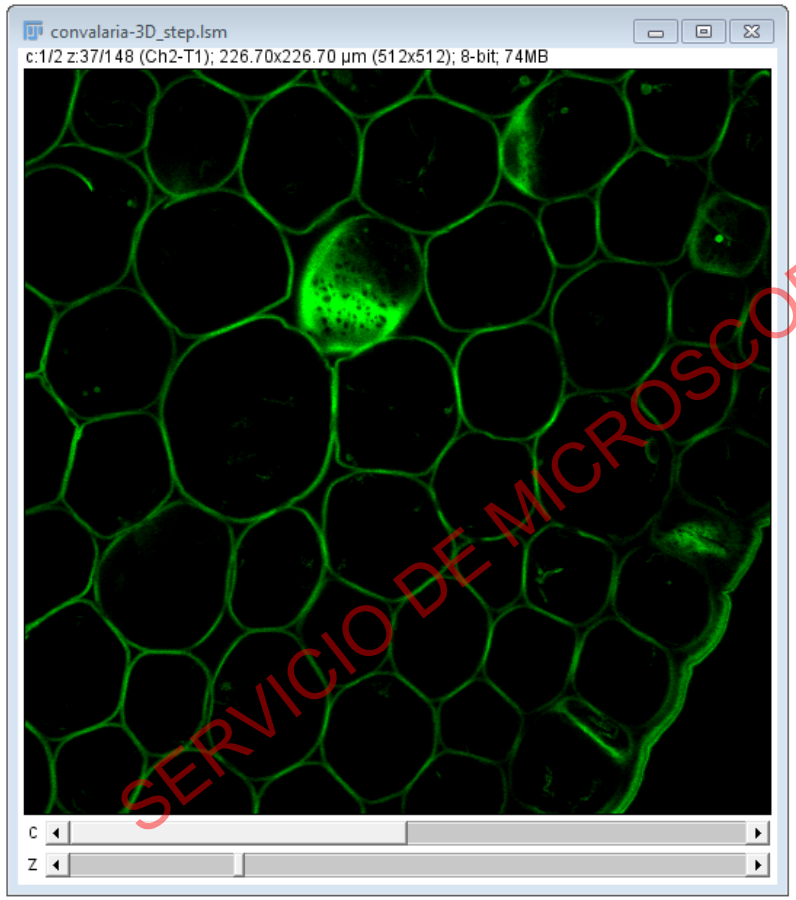
FFT Method

FLUORESCENCE INTENSITY QUANTIFICATION WITH IMAGEJ-FIJI

1. Image analysis
   - Digital image
2. Sample preparation
3. Image acquisition
4. Corrections
5. Fluorescence intensity quantification
   - *Set Measurements*
   - *Limit to Threshold*
   - Images with multiple objects
   - Images with multiple planes

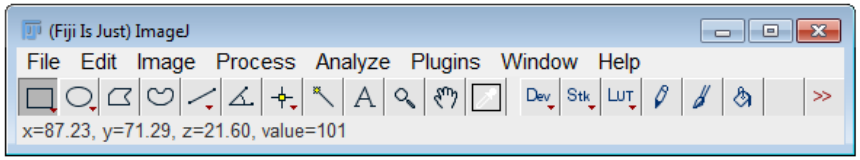
FLUORESCENCE INTENSITY QUANTIFICATION

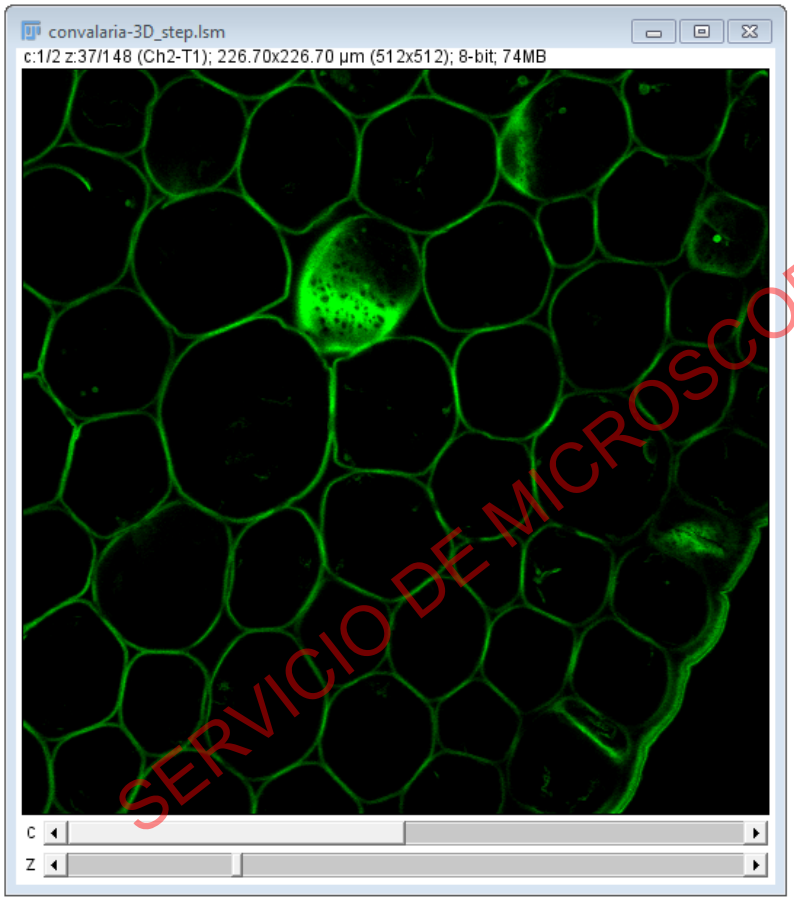




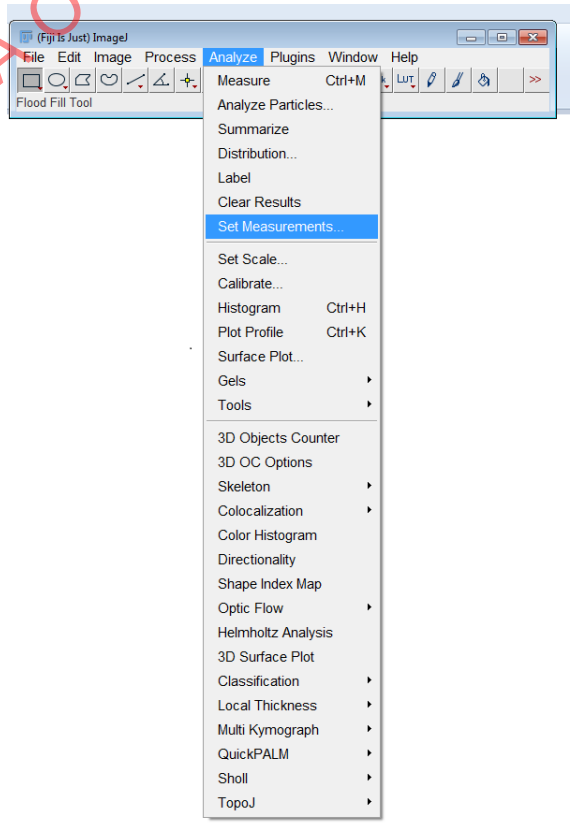
- 1) You can simply hover the cursor over a given area in the image and read out the pixel intensity at that pixel on the toolbar.
  - For RGB images, there will be three numbers: red, green and blue.

FLUORESCENCE INTENSITY QUANTIFICATION

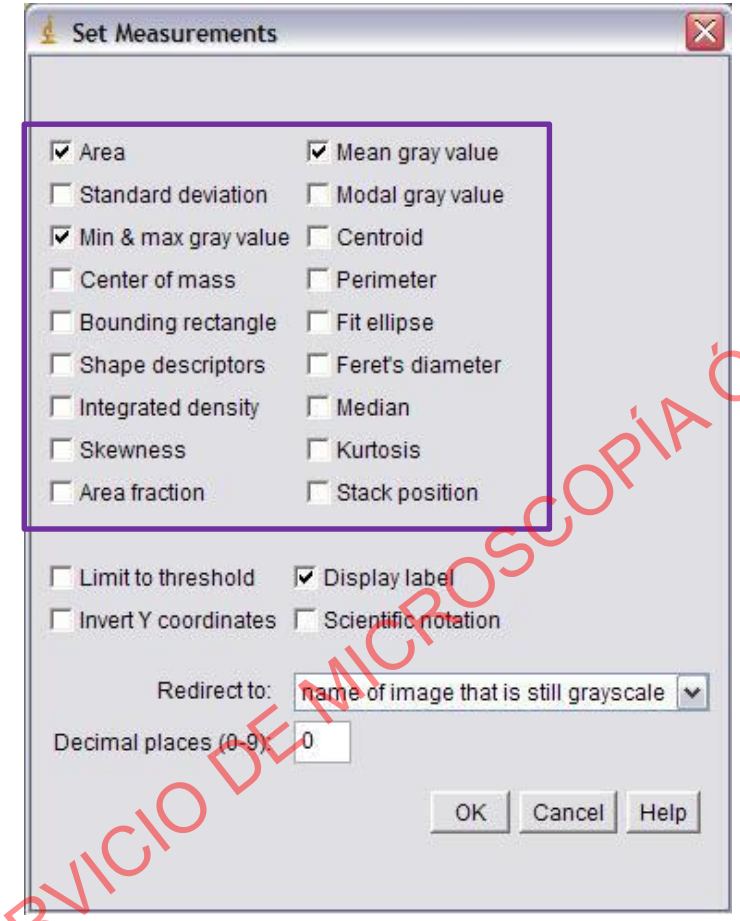




- 2) *Analyze option*
  - Go to *Analyze/Set Measurements.*



FLUORESCENCE INTENSITY QUANTIFICATION



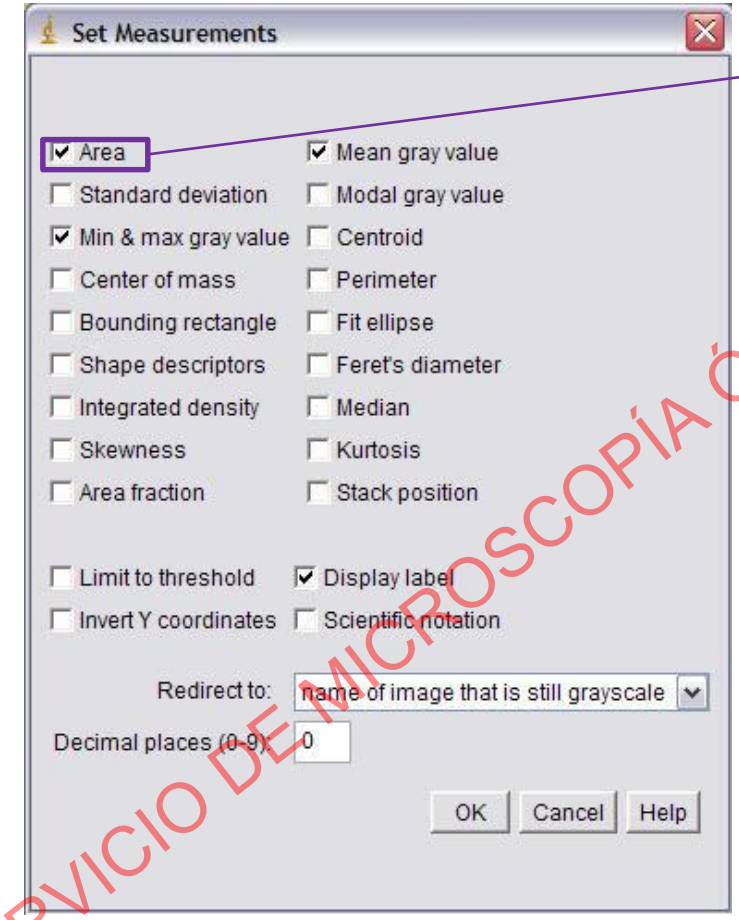
2) *Analyze option*

– Go to *Analyze/Set Measurements.*

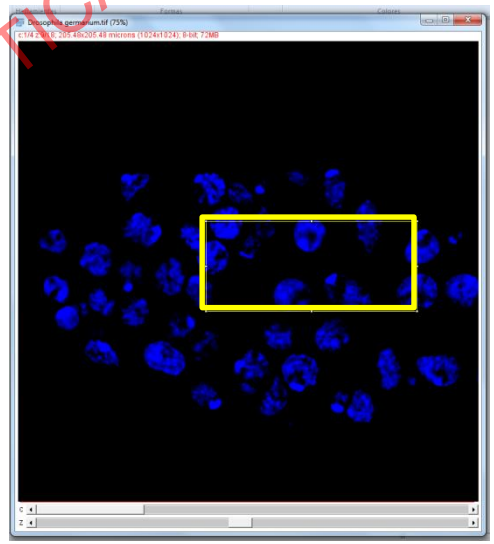
Check the boxes for the information you want.

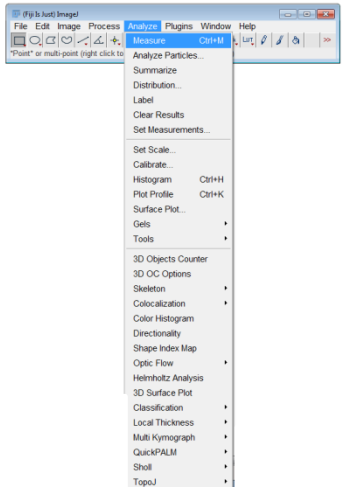
You can get information on area, diameter, perimeter and other factors as well as intensity information.

SET MEASUREMENTS OPTIONS

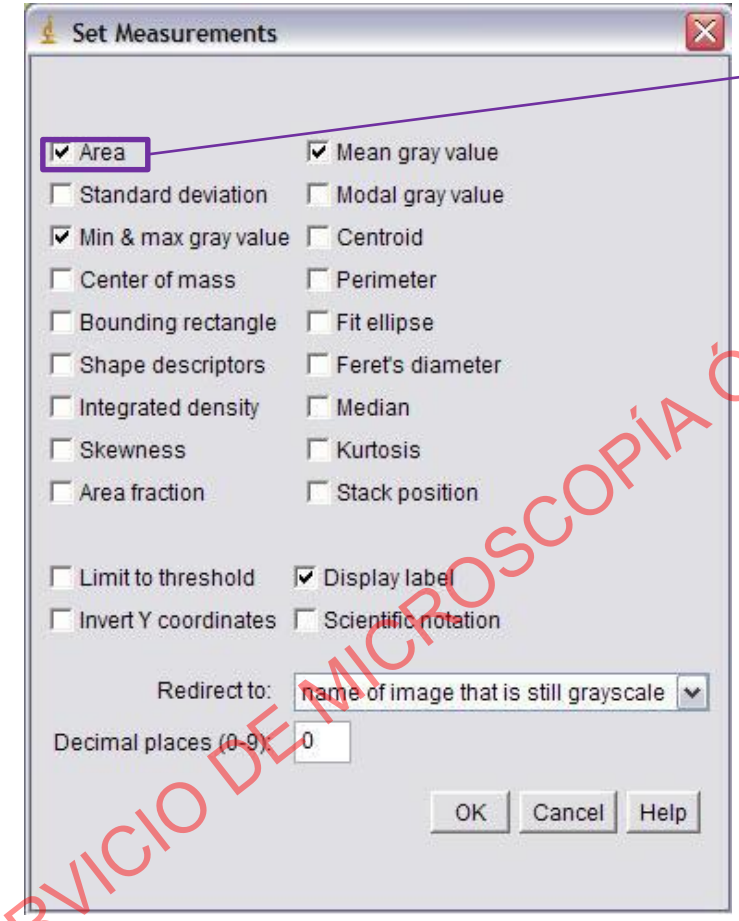


Area in pixels squared or in measurement units of the selected image or area.

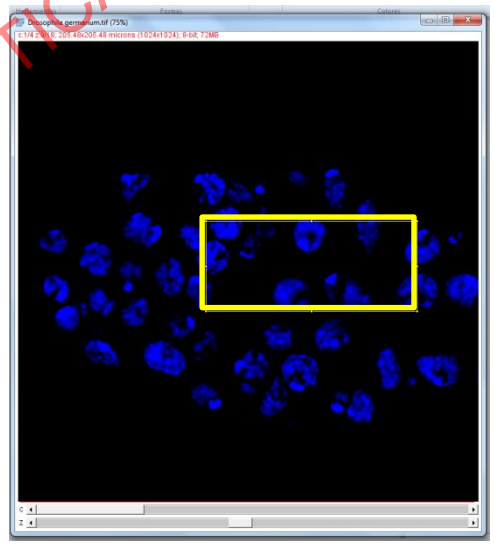


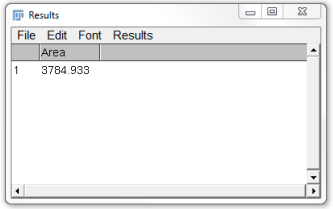


SET MEASUREMENTS OPTIONS

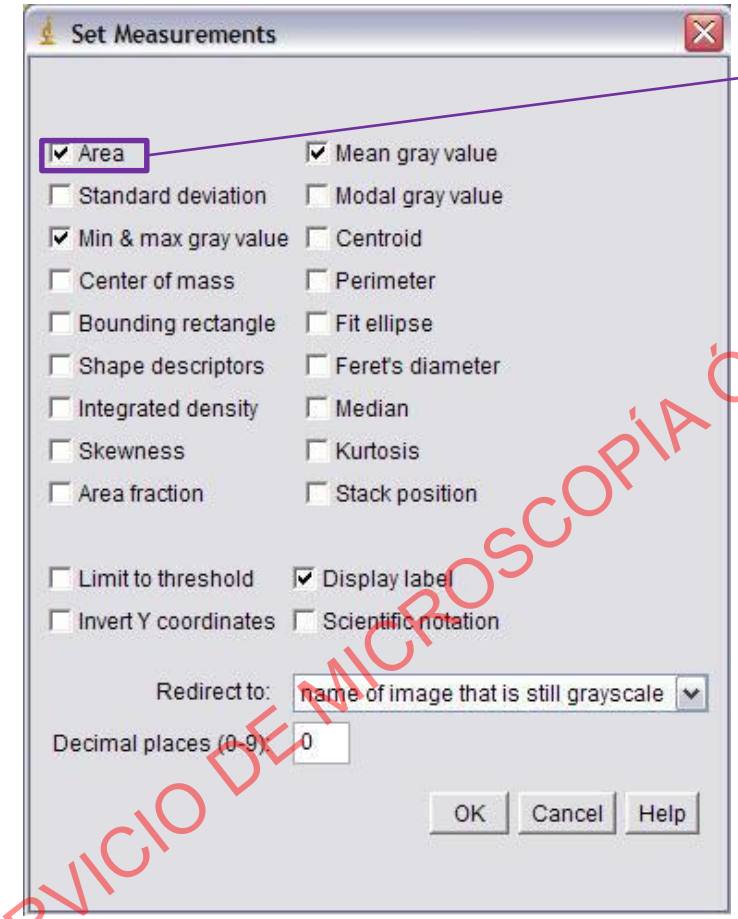


Area in pixels squared or in measurement units of the selected image or area.





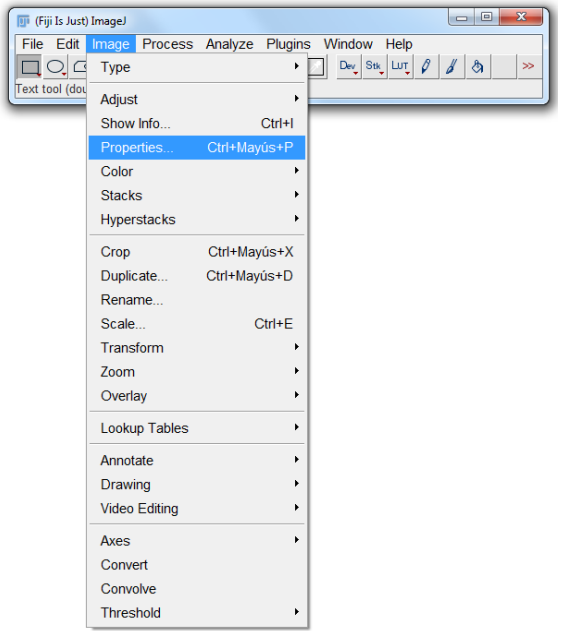
SET MEASUREMENTS OPTIONS

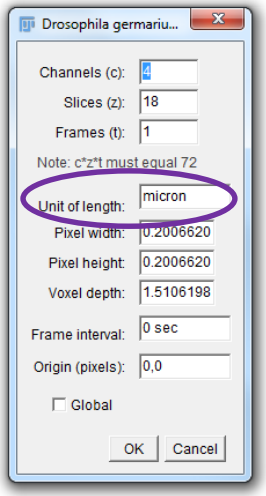


Area in pixels squared or in measurement units of the selected image or area.

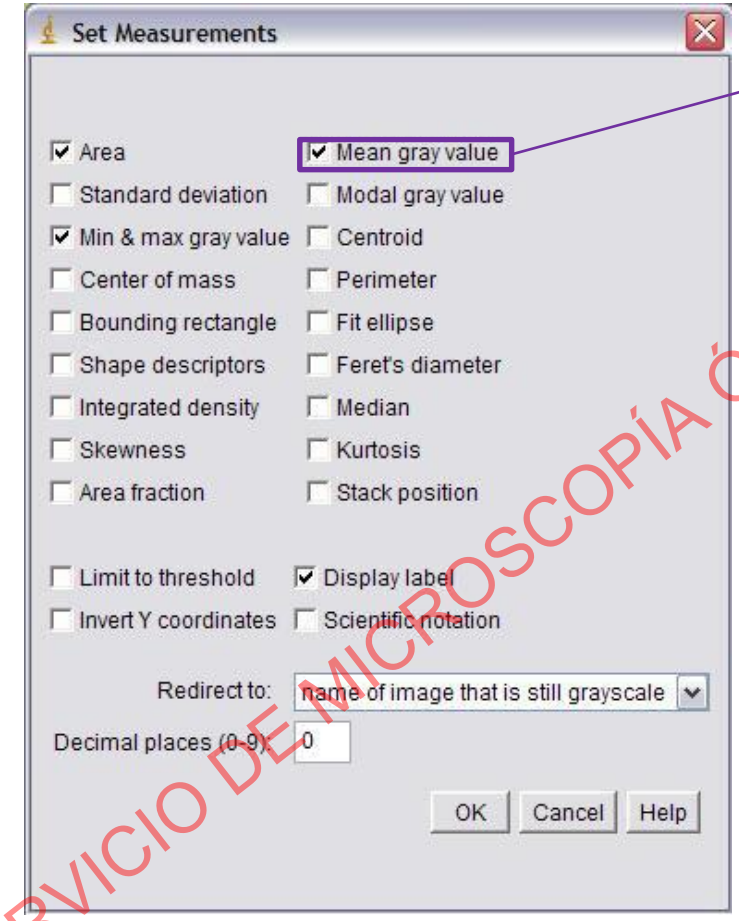
To see if the image is calibrated and the measurement units :

Image/properties





SET MEASUREMENTS OPTIONS



Average gray values of the selection.

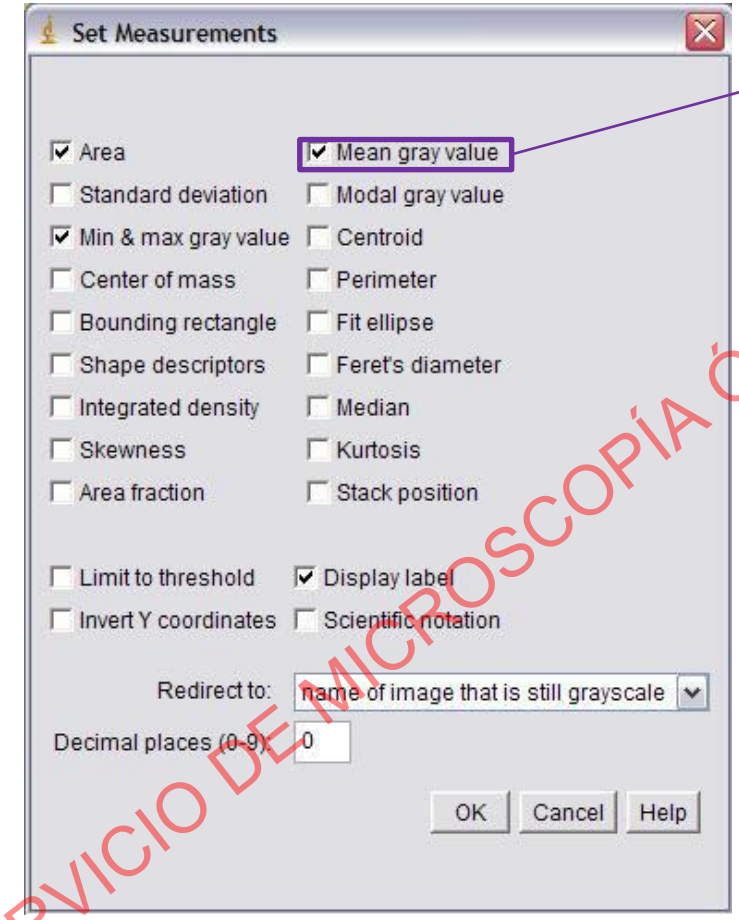
Sum of pixel gray levels from the selected zone divided by the number of pixels.

$$\frac{\sum \text{pixel values}}{\text{pixel number}}$$

5	10
15	0

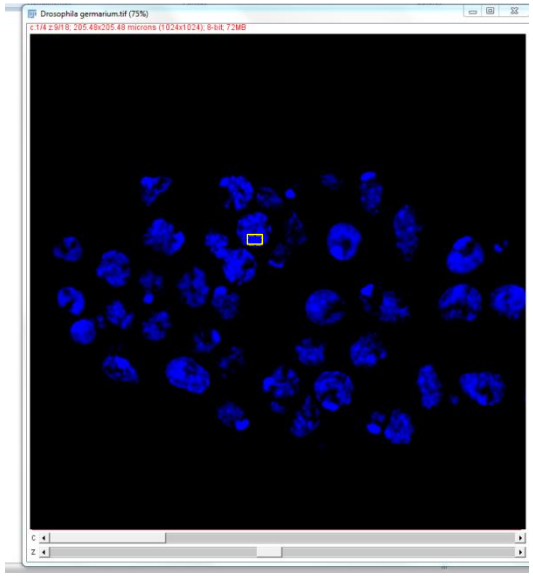
$$= \frac{5+10+15+0}{4} = 7{,}5$$

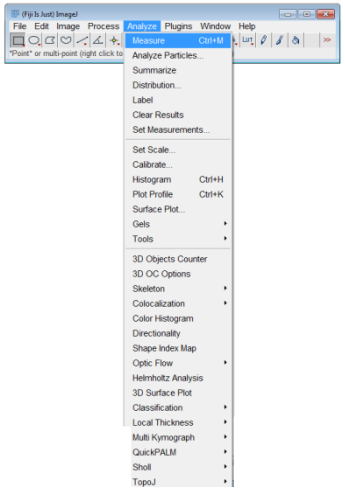
SET MEASUREMENTS OPTIONS



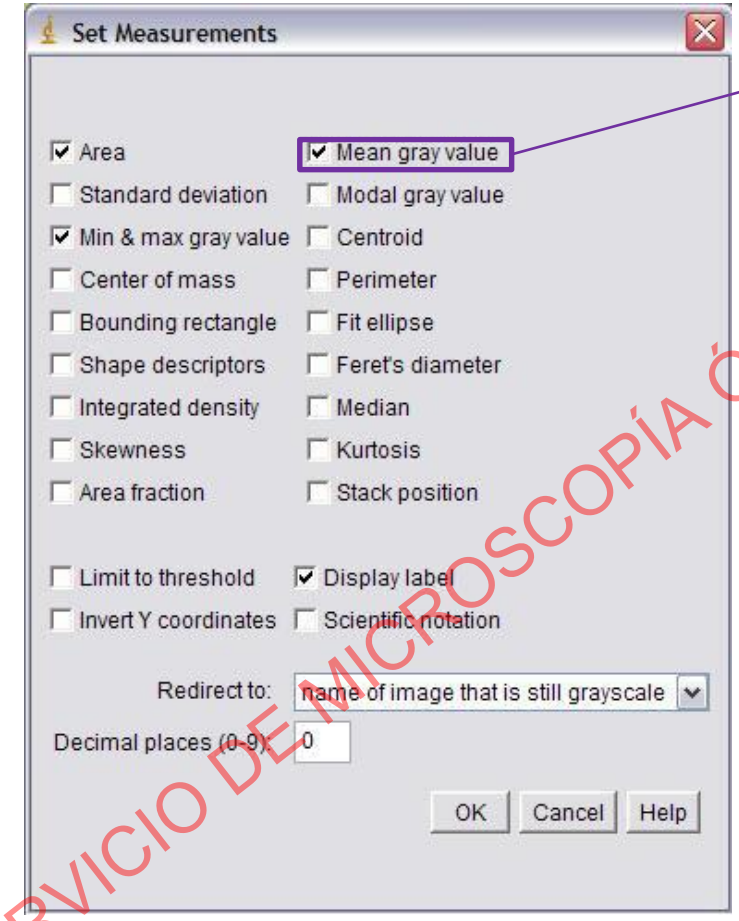
Average gray values of the selection.

Sum of pixel gray levels from the selected zone divided by the number of pixels.



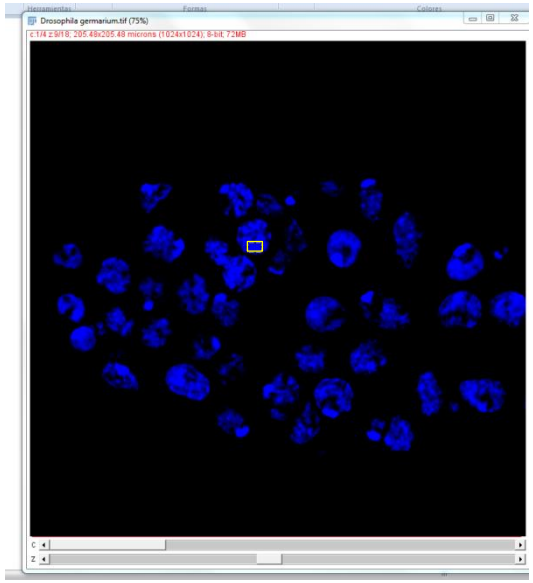


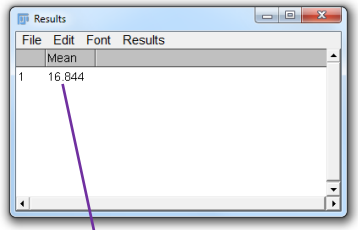
SET MEASUREMENTS OPTIONS



Average gray values of the selection.

Sum of pixel gray levels from the selected zone divided by the number of pixels.

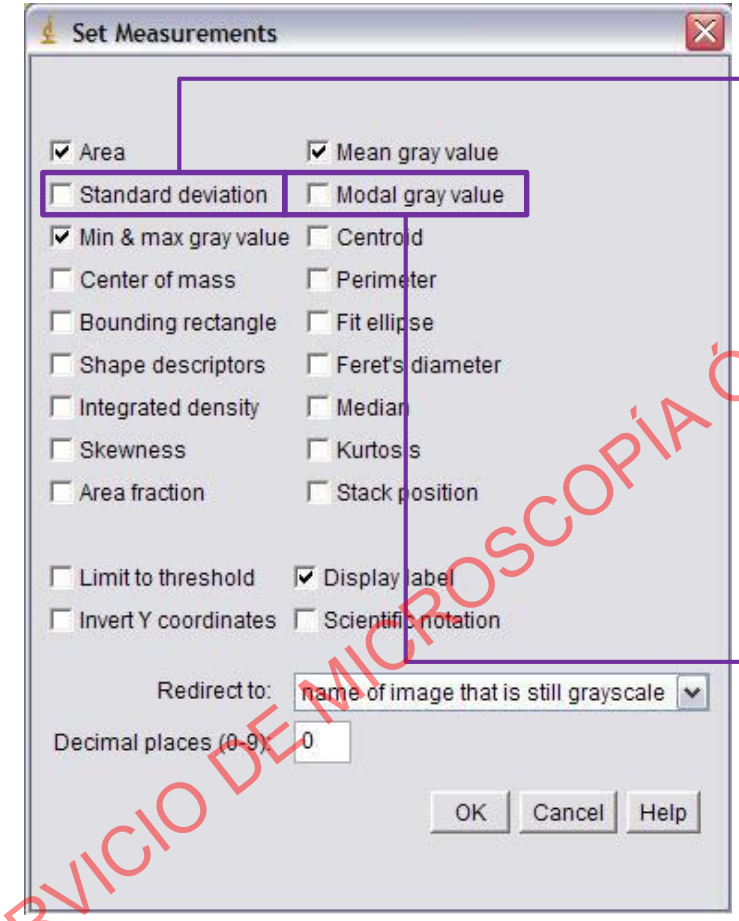




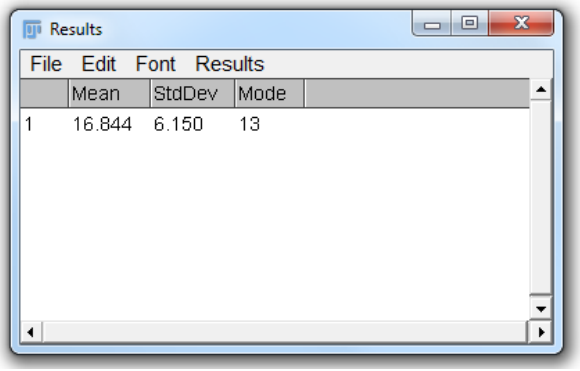
$$\frac{\sum \text{pixel values}}{\text{pixel number}}$$

Different ROI sizes can be compared

SET MEASUREMENTS OPTIONS

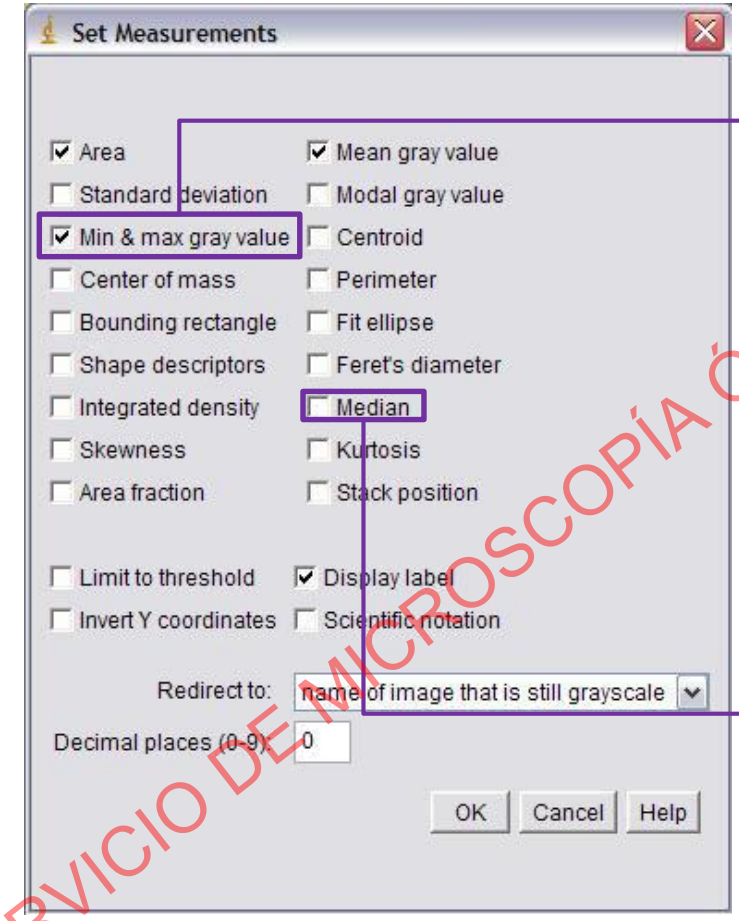


Standard deviation of the values used to generate the gray value mean.

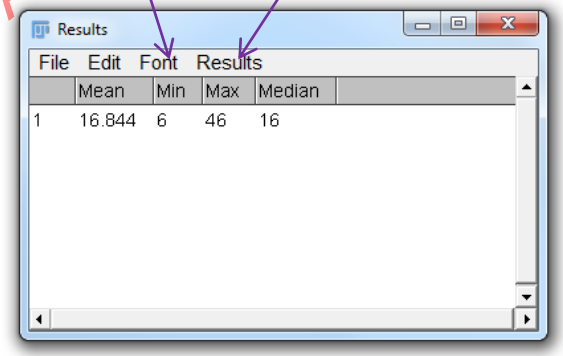


Most frequent gray value in the selected area.

SET MEASUREMENTS OPTIONS

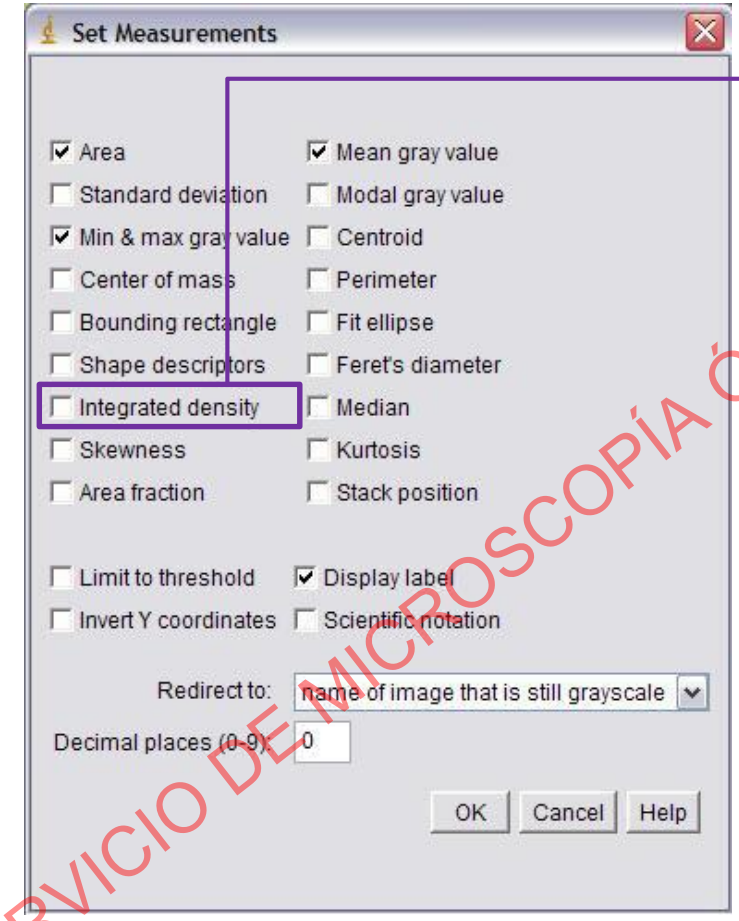


Minimum and maximum gray values in the selected area.



Median value.

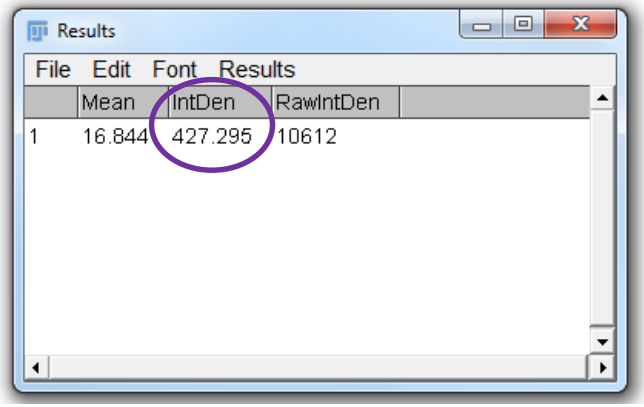
SET MEASUREMENTS OPTIONS



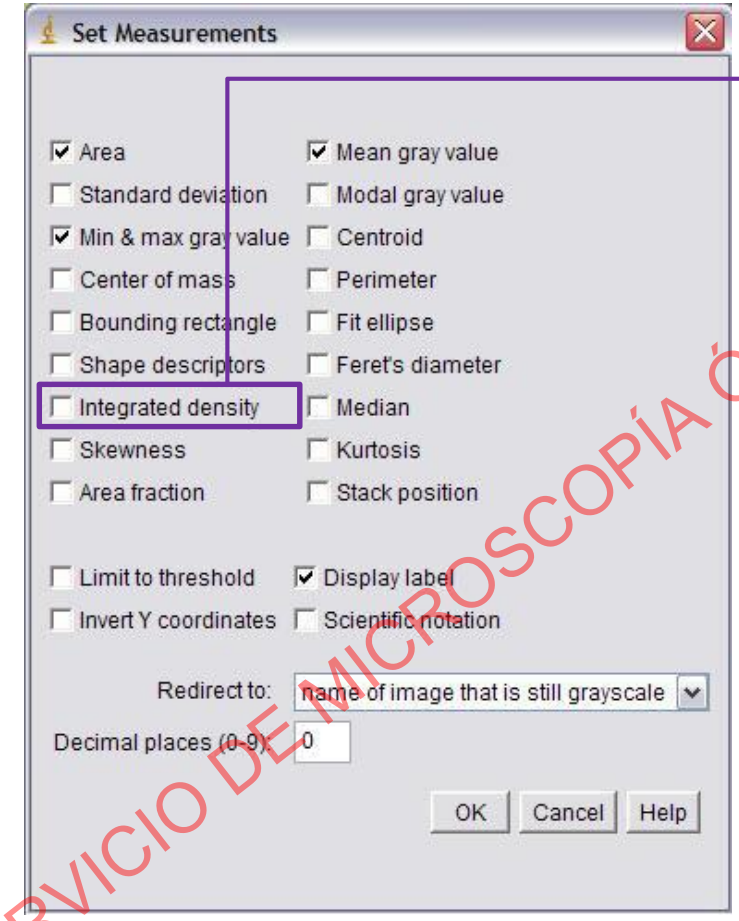
Provides two values:

IntDen

This is equivalent to the product of **Area** and **Mean Gray Value**.



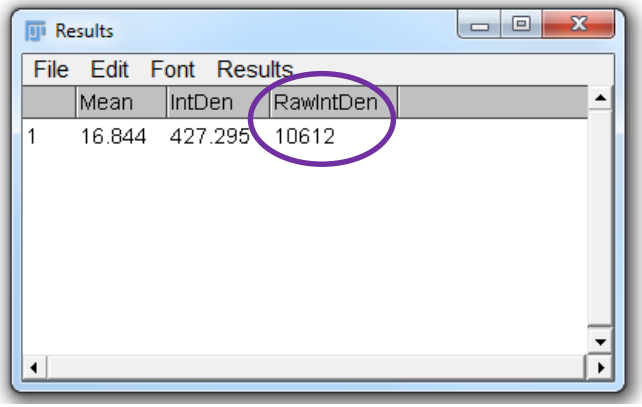
SET MEASUREMENTS OPTIONS



Provides two values:

IntDen

This is equivalent to the product of **Area** and **Mean Gray Value**.



RawIntDen

The sum of all pixel values in the image or selection.

SET MEASUREMENTS OPTIONS

IntDen

This is equivalent to the product of **Area** and **Mean Gray Value**.

15	20
40	5

Area = 10
Mean = 20
Int Den = **200**

0	1	8	3
2	0	0	2

Area = 100
Mean = 2
Int Den = **200**

Different ROI sizes can be compared

RawIntDen

The sum of the values of the pixels in the image or selection.

15	20
40	5

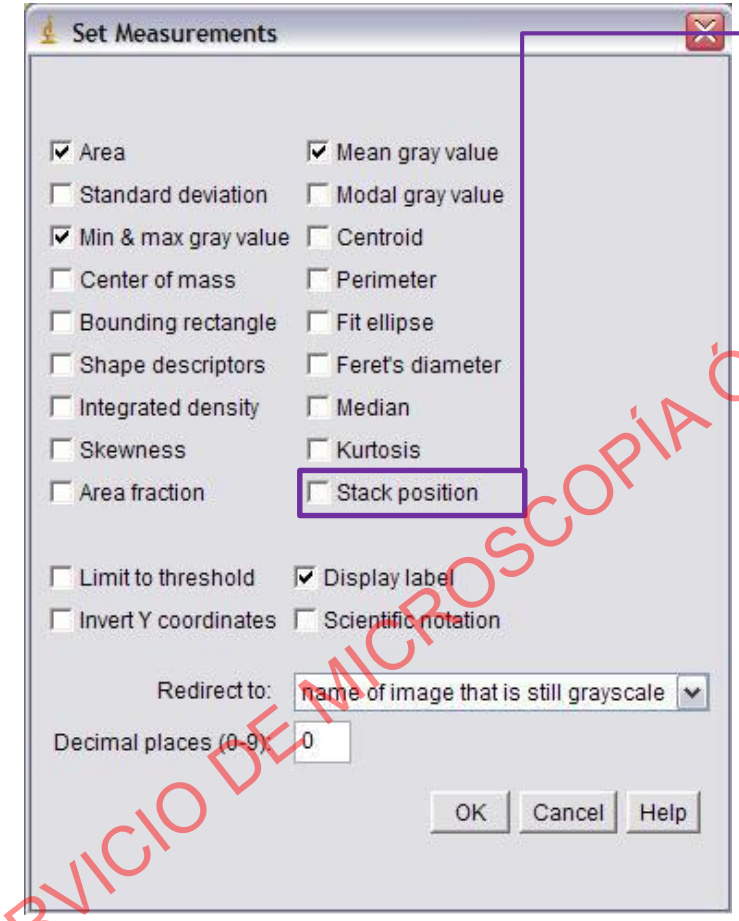
Area = 10
Mean = 20
Raw Int Den = **80**

0	1	8	3
2	0	0	2

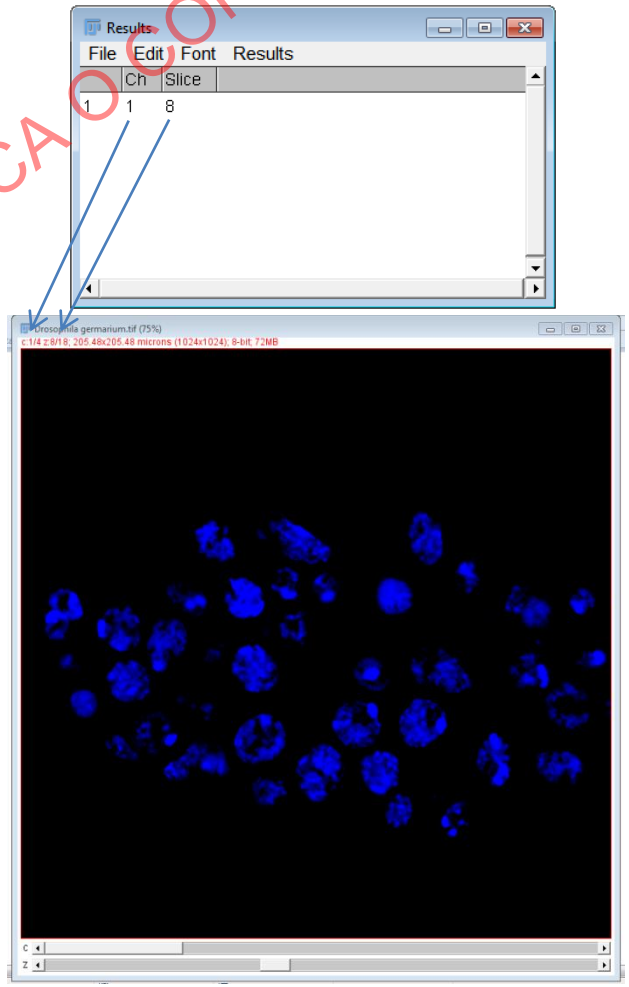
Area = 100
Mean = 2
Raw Int Den = **16**

Different ROI sizes can not be compared

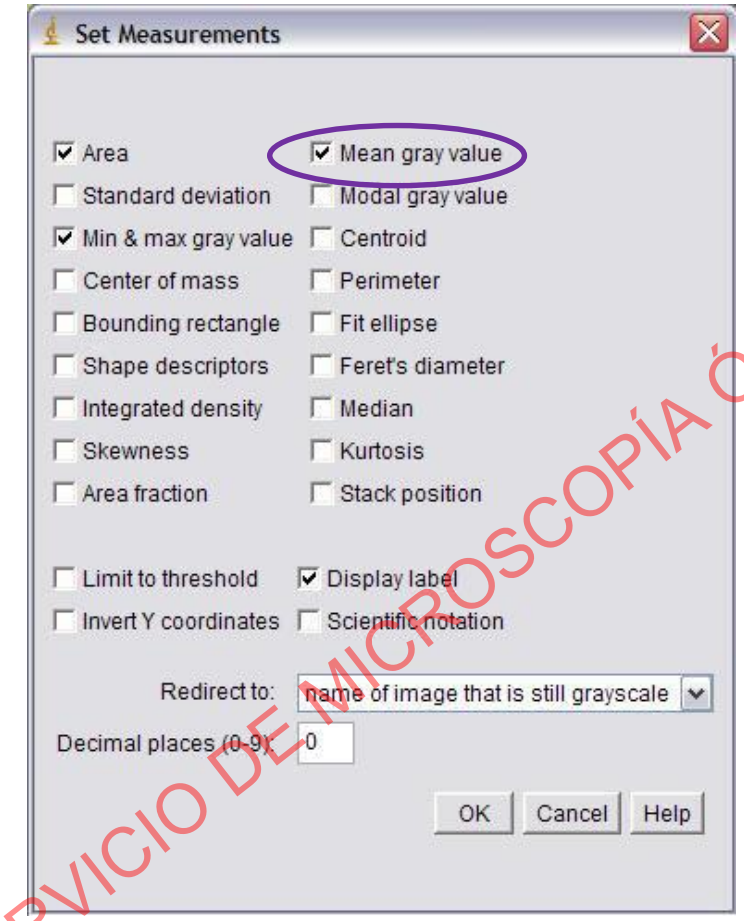
SET MEASUREMENTS OPTIONS



The position (slice, channel and frame) in the stack or hyperstack of the selection.



FLUORESCENCE INTENSITY QUANTIFICATION

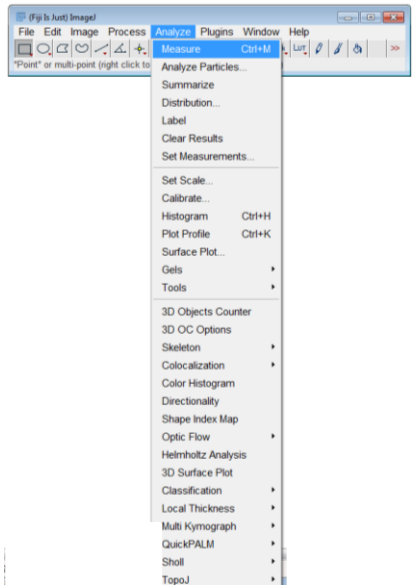


2) *Analyze* option

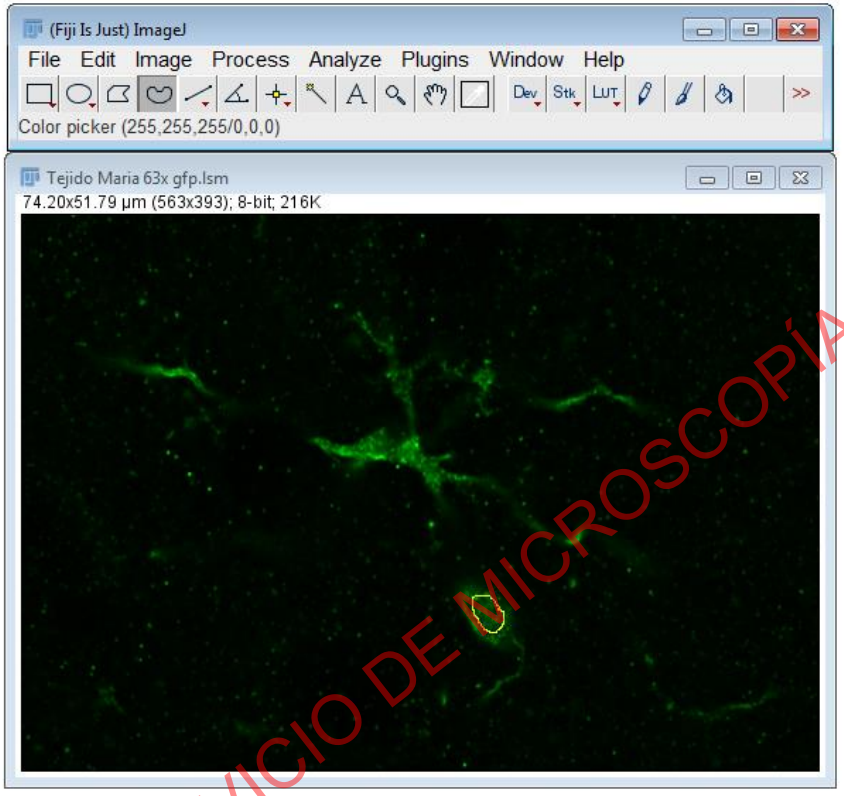
– Go to *Analyze/Set Measurements.*

Mean grey value

Then selecting *Analyze/Measure*, you will get information on the entire image.

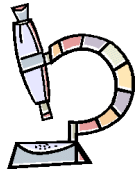


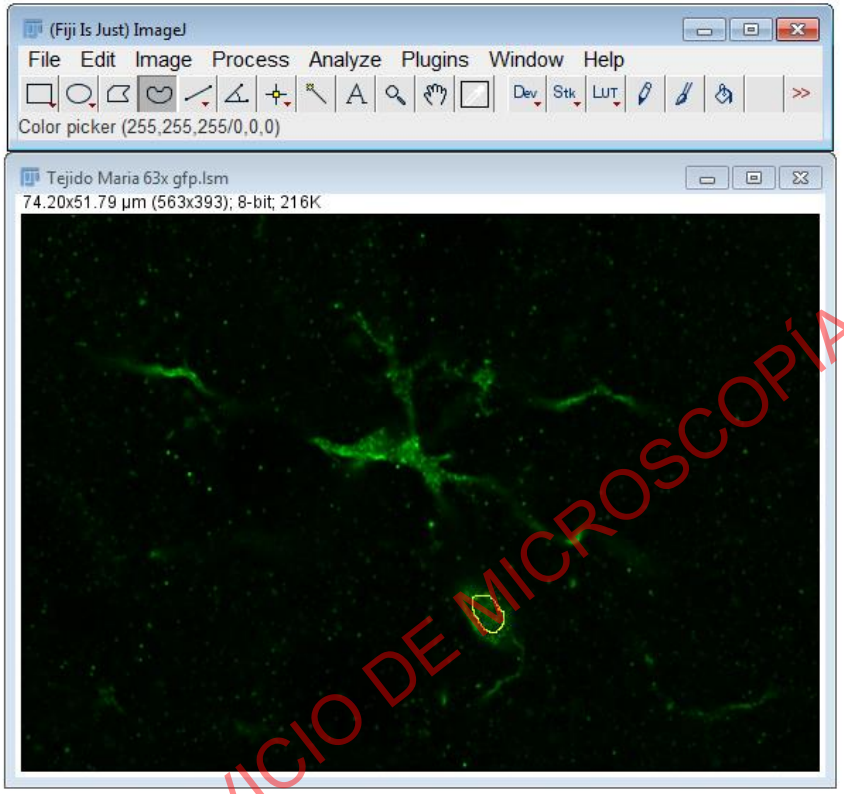
FLUORESCENCE INTENSITY QUANTIFICATION



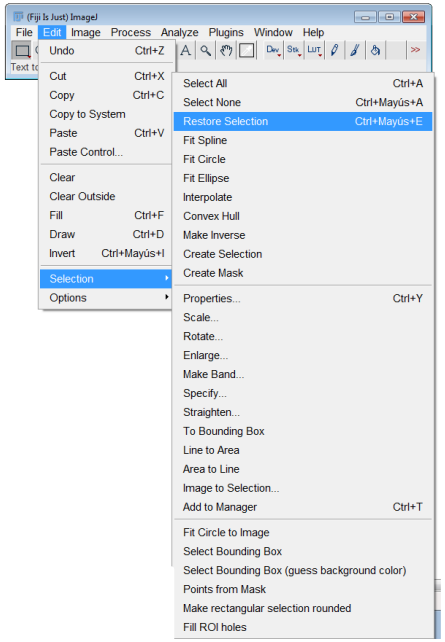
- 3) Limit your measured area
  - Draw a region of interest (ROI) around your object of interest with the drawing tools .
    - *Analyze/Measure*

FLUORESCENCE INTENSITY QUANTIFICATION





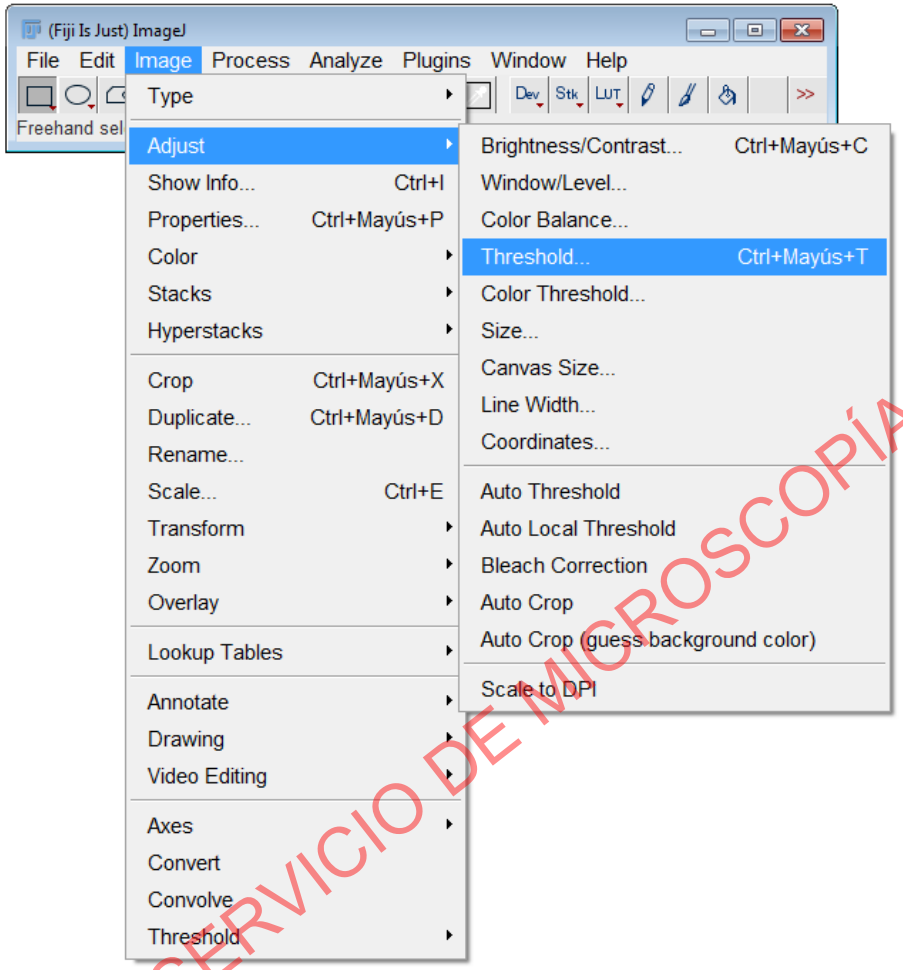
- 3) Limit your measured area
  - To copy/paste the shape or ROI to another image in order to compare equivalent regions in different images
    - *Edit/Selection/Restore Selection*



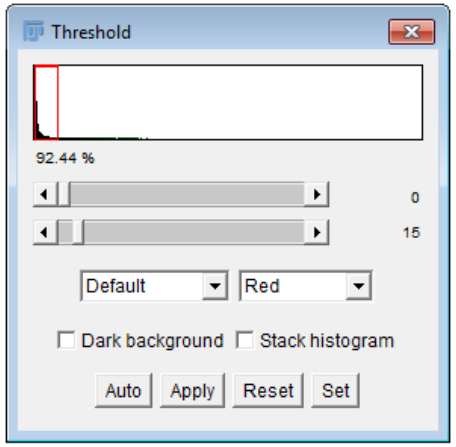
FLUORESCENCE INTENSITY QUANTIFICATION

- 3) Limit your measured area
  - *"Limit to Threshold"*
    - *Analyze/Set Measurements check Limit to Threshold*

FLUORESCENCE INTENSITY QUANTIFICATION



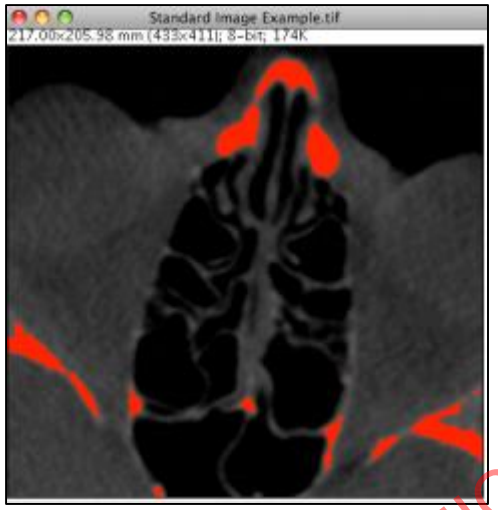
- 3) Limit your measured area
  - *"Limit to Threshold"*
    - *Image/Adjust/Threshold.* To highlight the area you want to analyze.

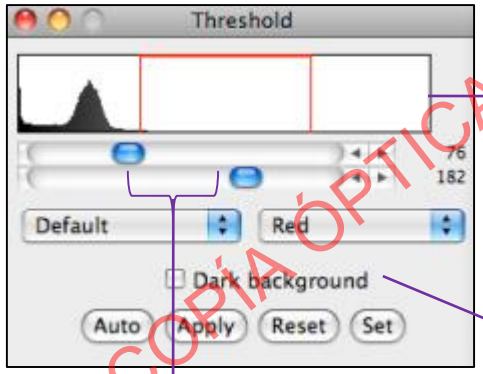


- *Analyze/Measure.* Will give you intensity measurements only in your thresholded area.

FLUORESCENCE INTENSITY QUANTIFICATION

- 3.1) Using *"Limit to Threshold"*





Histogram: represents the distribution of pixel intensities in the image.
0 = black
255 = white

Select *dark background* if the background is highlighted in red

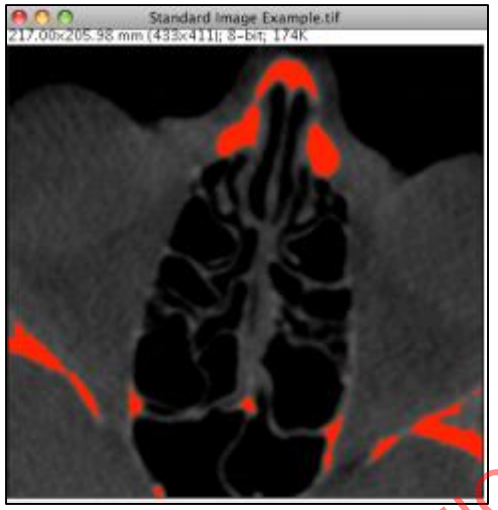
Dragging the sliders selects different regions within the greyscale.

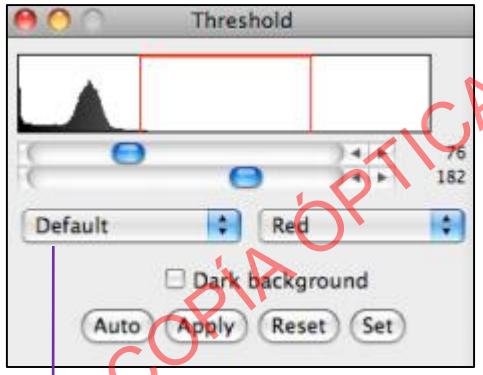
In this case, all the pixels between 76 (dark grey) and 182 (mid grey) are highlighted in red.

Maintains the same limits for all images

FLUORESCENCE INTENSITY QUANTIFICATION

- 3.1) Using *"Limit to Threshold"*

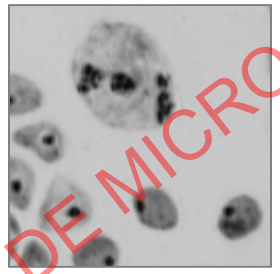




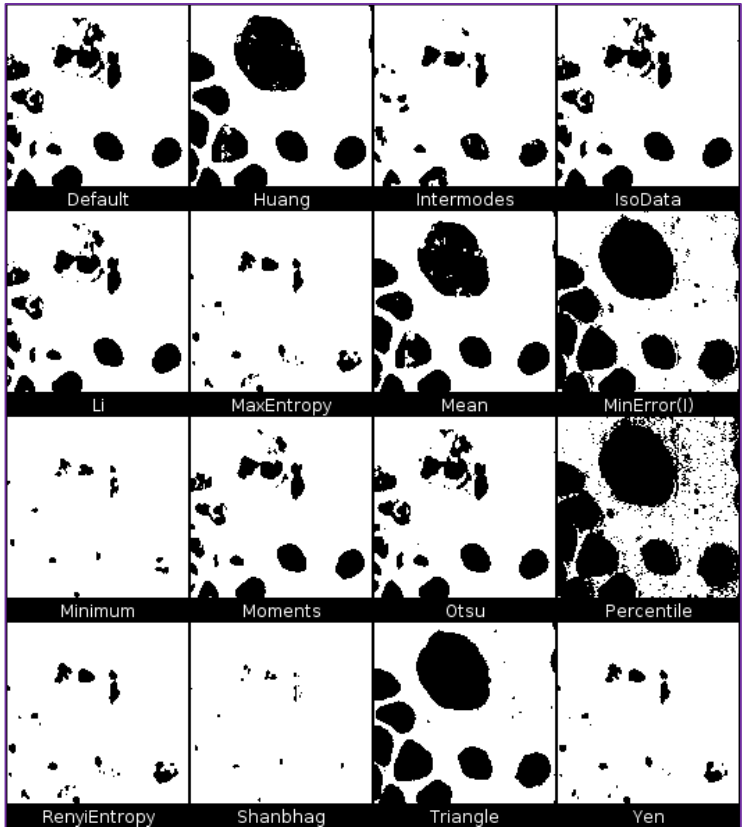
There are many algorithms you can use to calculate the threshold without introducing user-bias.

FLUORESCENCE INTENSITY QUANTIFICATION

- 3.1) Using *"Limit to Threshold"*
  - We must choose the most appropriate method or algorithm to segment our image



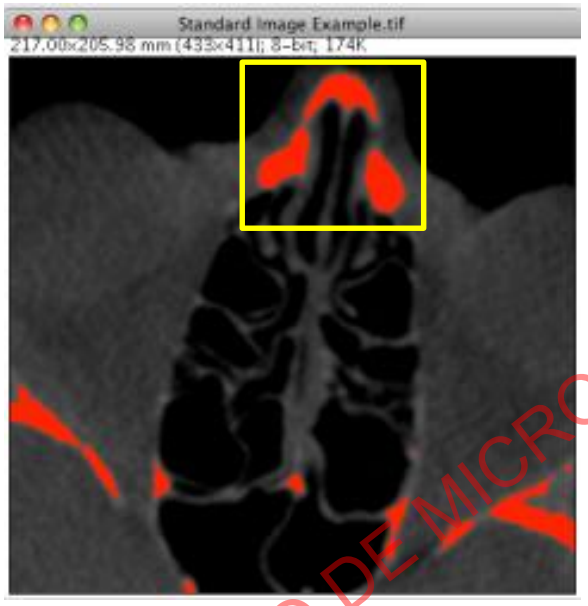
Original image



Test algorithms with several of our images to decide which is the best

FLUORESCENCE INTENSITY QUANTIFICATION

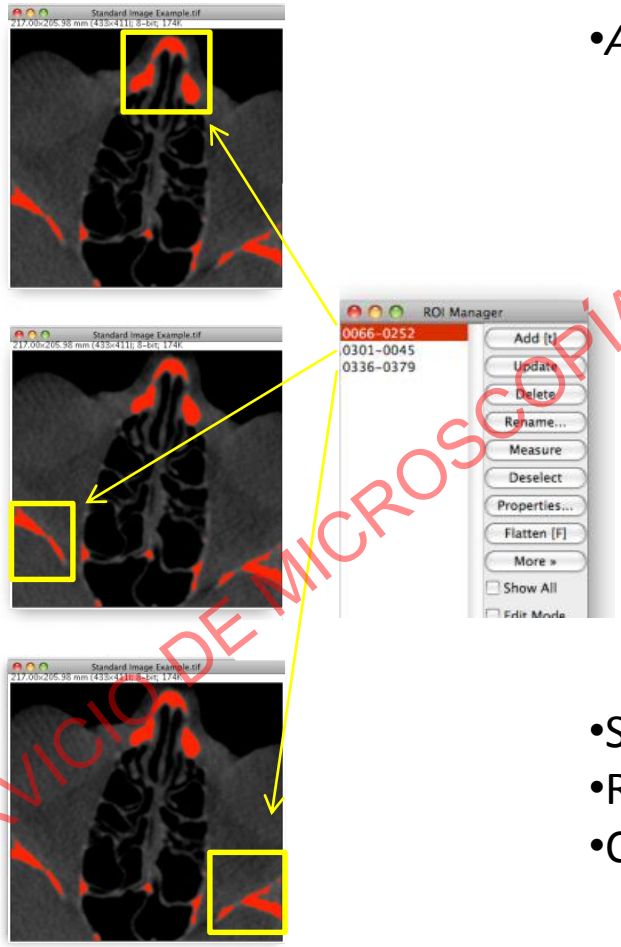
- 3.2) Combine *"Threshold"* and *ROI*



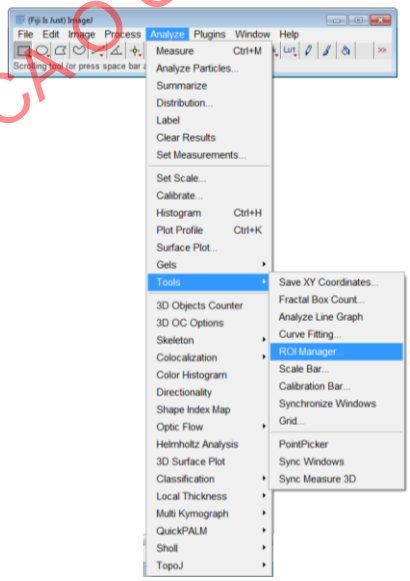
Use a selection tool to mark your ROI. Measurements will now be limited to pixels which fall within the selected area and are within the selected threshold intensity range.

FLUORESCENCE INTENSITY QUANTIFICATION

- 3.2) Combining *"Threshold" and multiple ROIs*



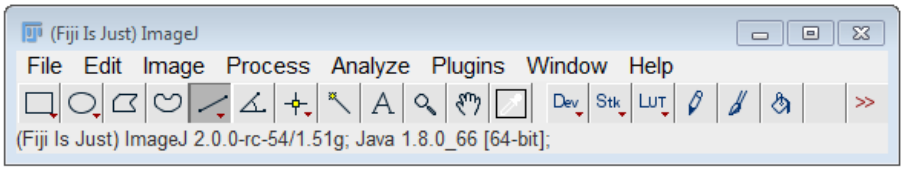
•*Analyse / Tools / ROI Manager*

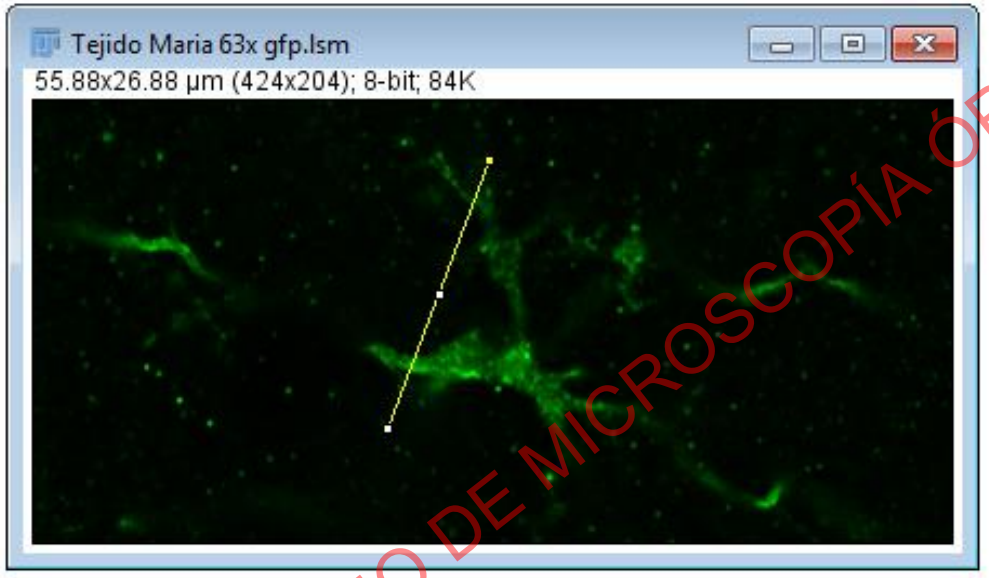


•Select a ROI and add it to *ROI Manager: "Add" Button*
•Repeat as required
•Click the measure button to see the measurements

It gives us the measurements we have selected in Set Measurements

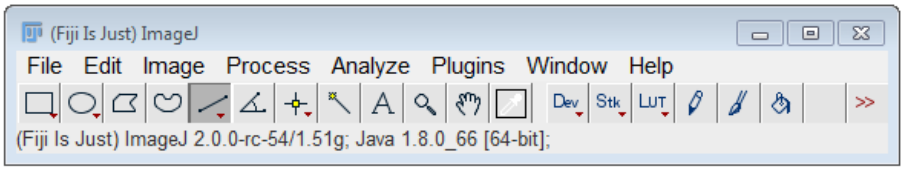
FLUORESCENCE INTENSITY QUANTIFICATION

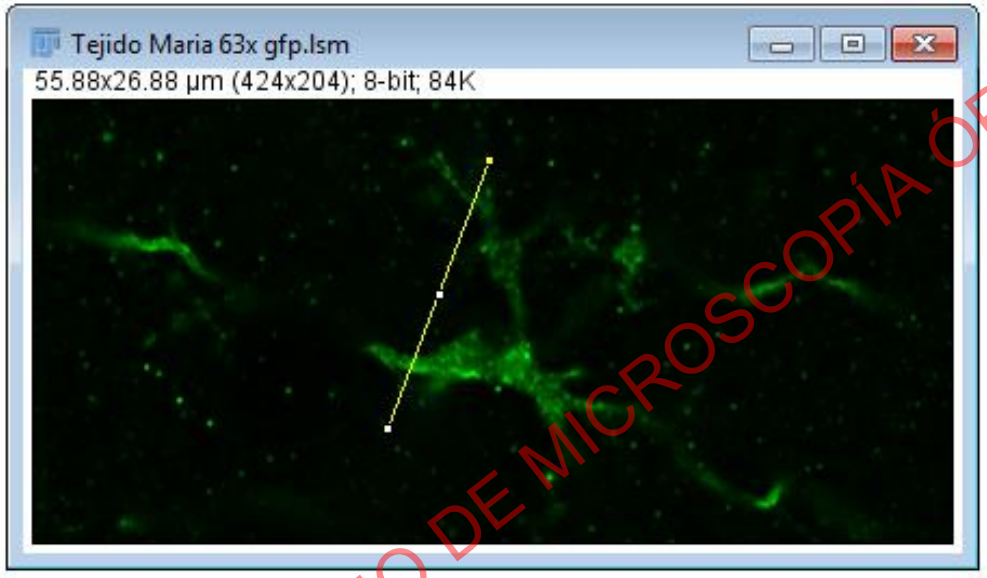




- 4) To create a plot of intensity values across features in your image.
  - The plot gives intensity values along the line drawn across the image.
    - *Analyze/Plot profile*
  - To obtain a similar plot for intensity values through a z or time stack, or within an ROI drawn on a stack.
    - *Image/Stacks/Plot z-axis profile*

FLUORESCENCE INTENSITY QUANTIFICATION

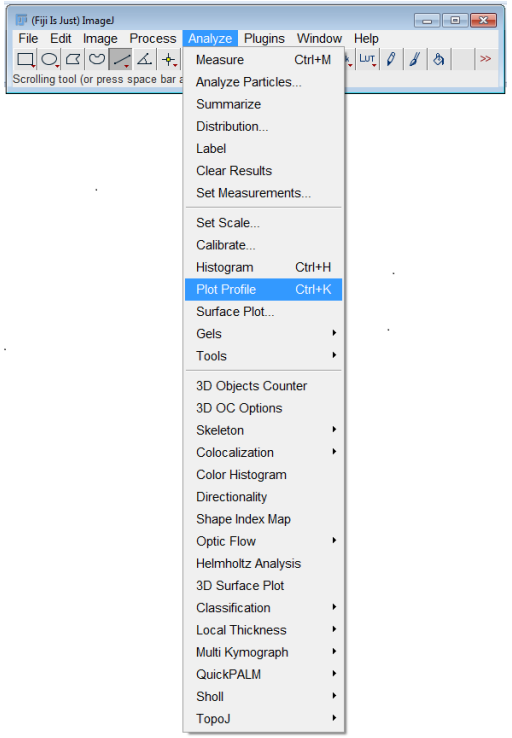




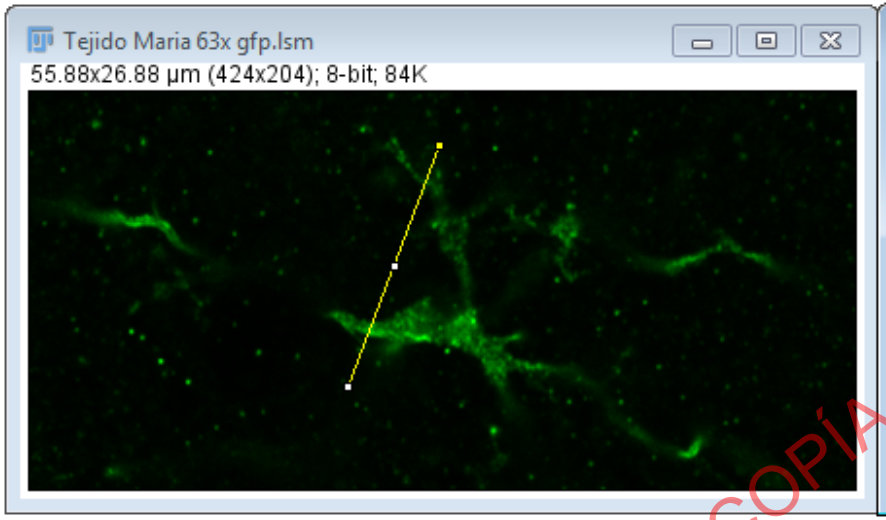
- 4) To create a plot of intensity values across features in your image.

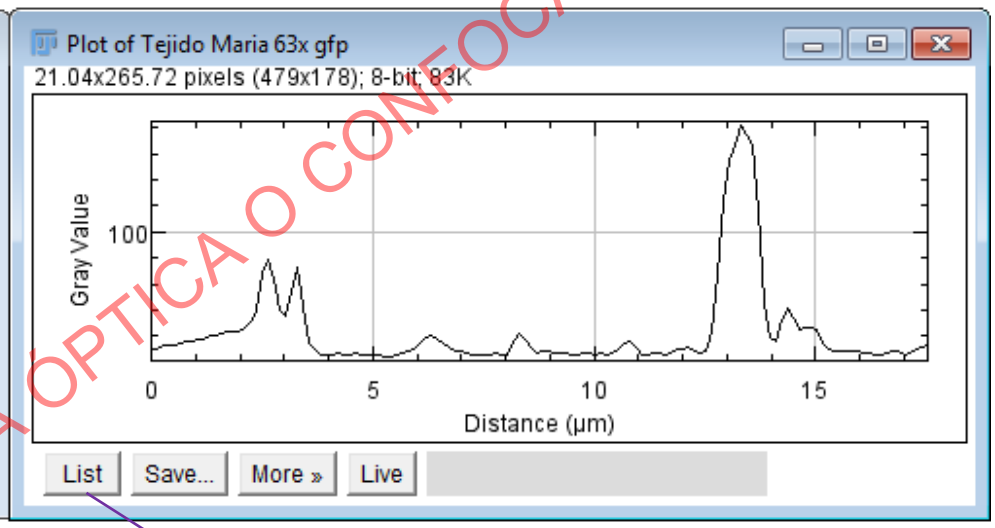
- Draw a line in the area to be analyzed with the drawing tools.

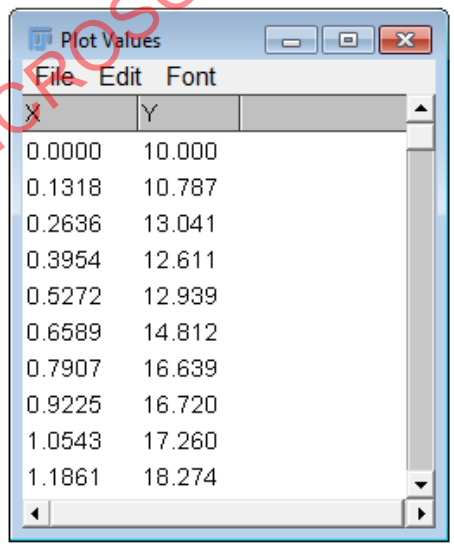
- *Analyze/Plot profile*



FLUORESCENCE INTENSITY QUANTIFICATION



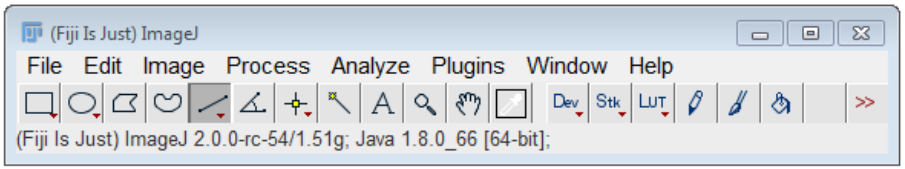


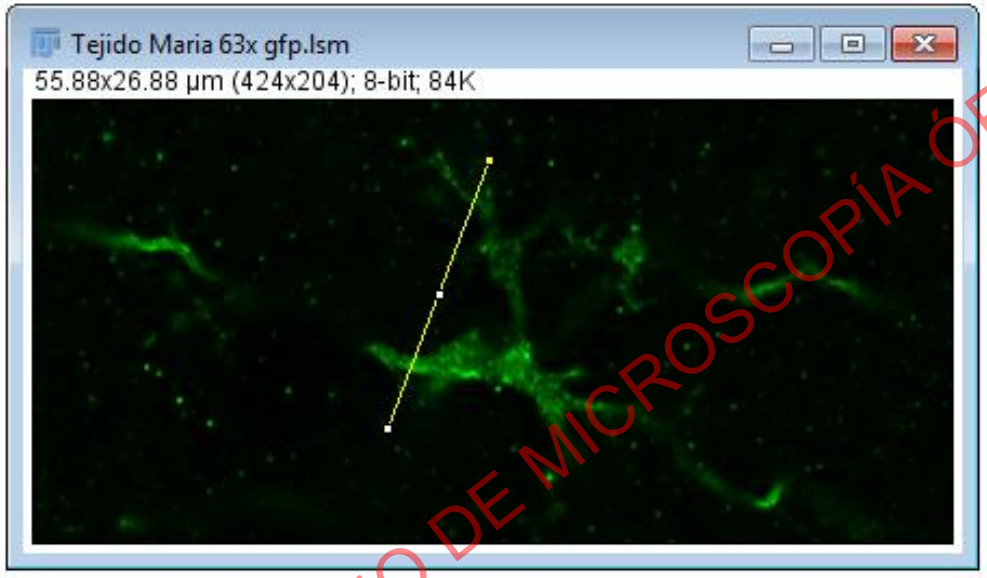


List button

Gives a list of the intensity values used to create the graph.

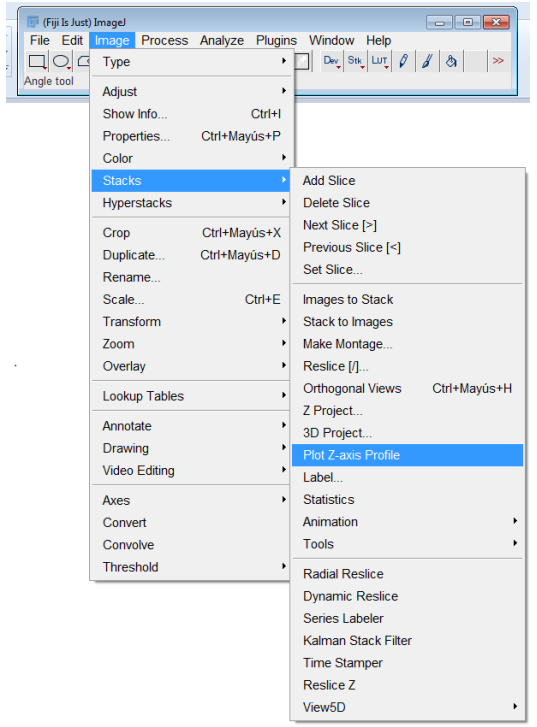
FLUORESCENCE INTENSITY QUANTIFICATION



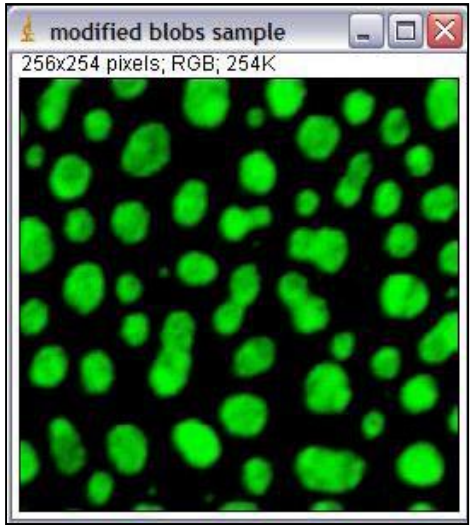


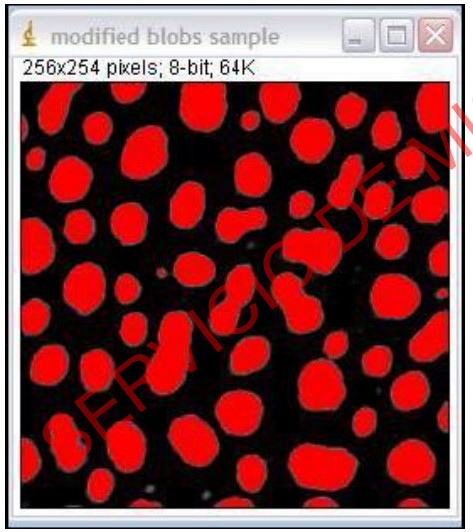
- 4) To create a plot of intensity values across features in your image.

- Draw a line in the area to be analyzed with the drawing tools.
- *Analyze/Plot profile*
- *Image/Stacks/ Plot z-axis profile*

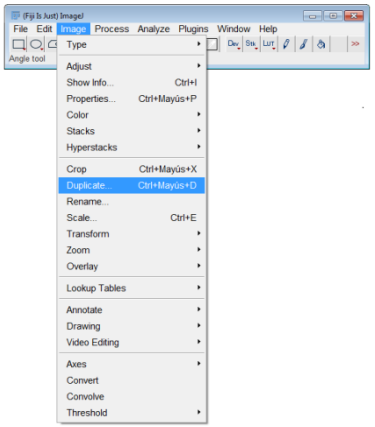


FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS





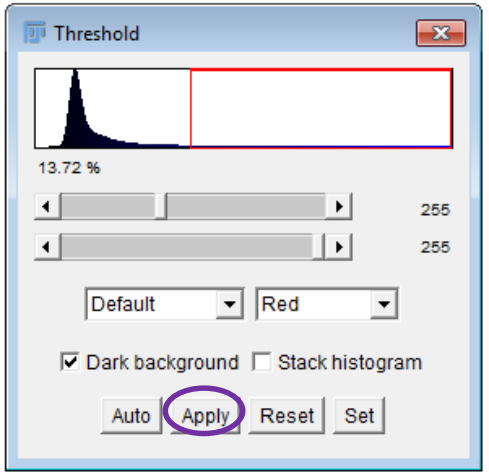
- Make a copy of your image
  - *Image/Duplicate*



- Threshold to highlight all the structures you want to measure
  - *Image/Adjust/Threshold*
    - Manually
    - Using algorithms

FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS

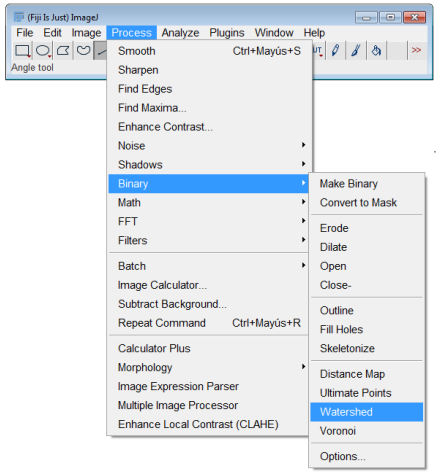
– If you have particles that have merged together
  - *Apply* (This will create a binary version of the image)



- *Two pixel intensities: black (=0) and white (=255).*

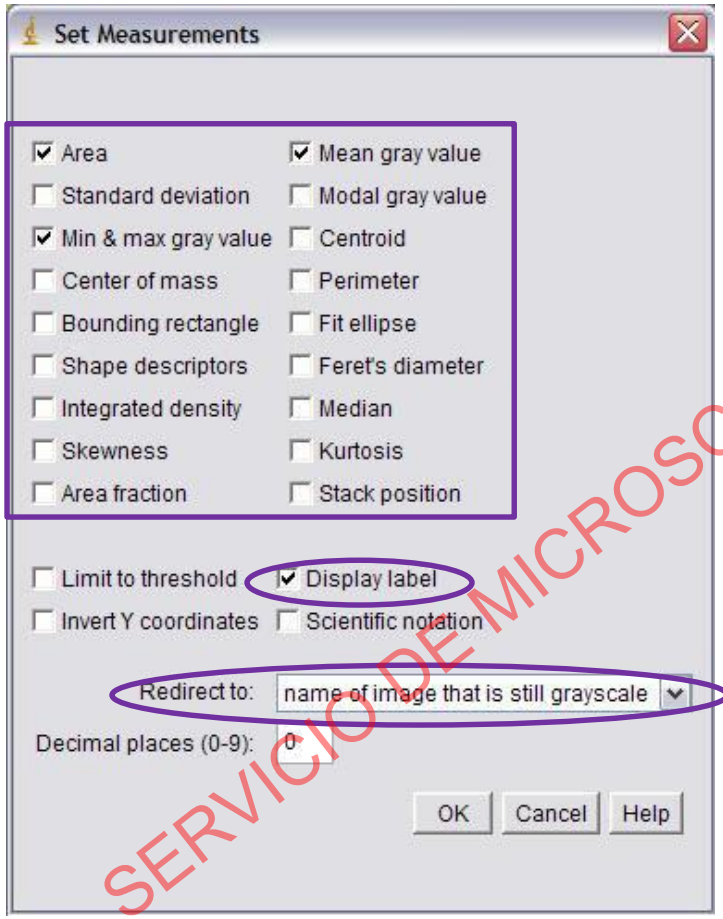
FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS

– If you have particles that have merged together
  - *Process/Binary/Watershed*



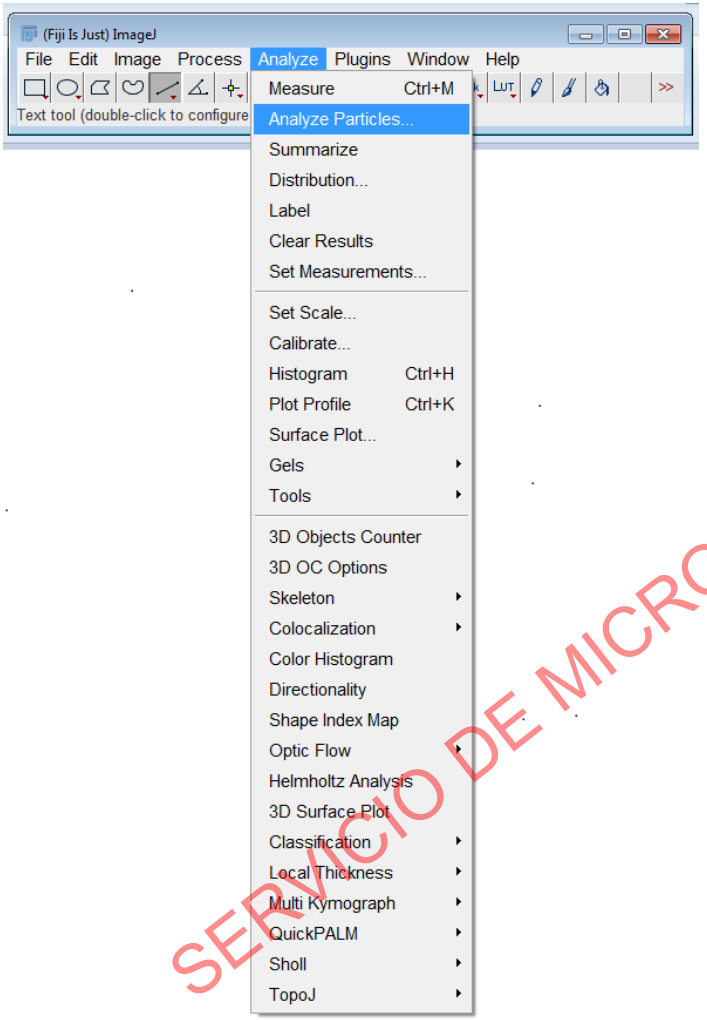
Watershed can often accurately separate particles by adding a 1 pixel thick line where it calculates the division should be.

FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS



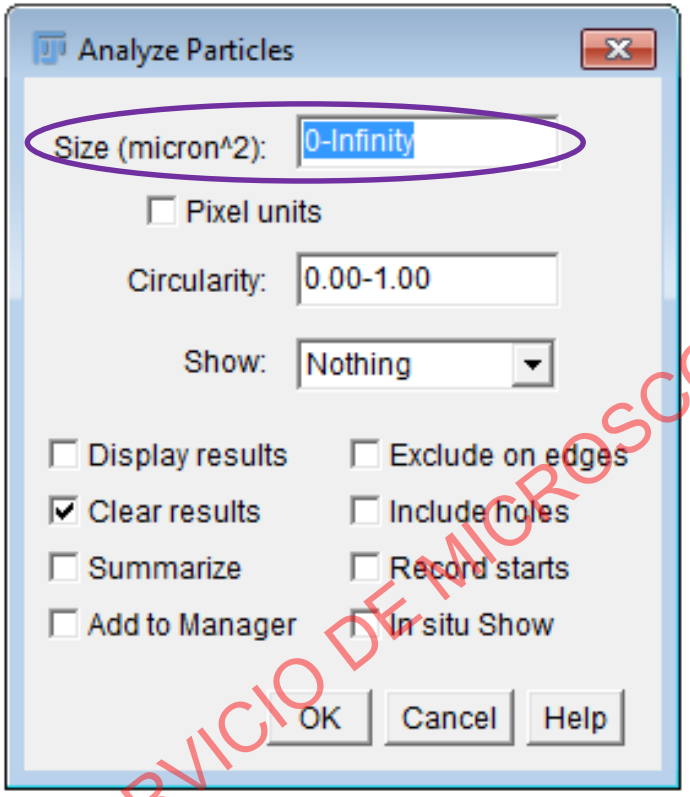
- *Analyze/Set measurements*
  - Set the "Redirect to" line to the name of the copy of the image that is still in grayscale.
    - If you don't do this, your intensity values will be read from the binary image, and they will all be 255!
  - Checking "display label" will label your data table with the image name and particle number.
  - Use the checkboxes to select which statistics you want from your image.

FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS



- Click on the binary or thresholded image to select it, then go to:
  - *Analyze/Analyze Particles*

FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS



- Click on the binary or thresholded image to select it, then go to:
  - *Analyze/Analyze Particles*

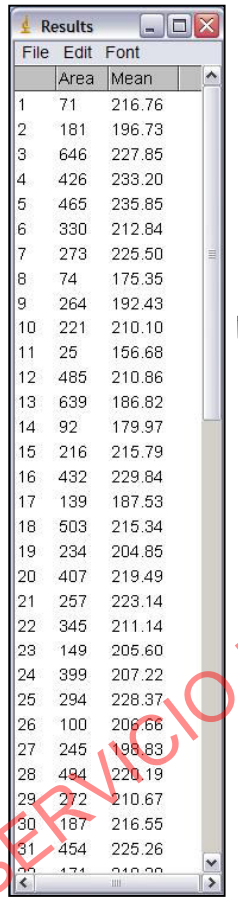
    -Size

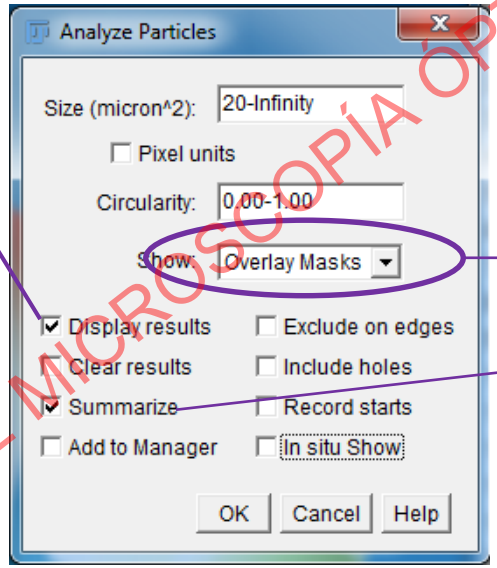
    Particles smaller than that value are ignored

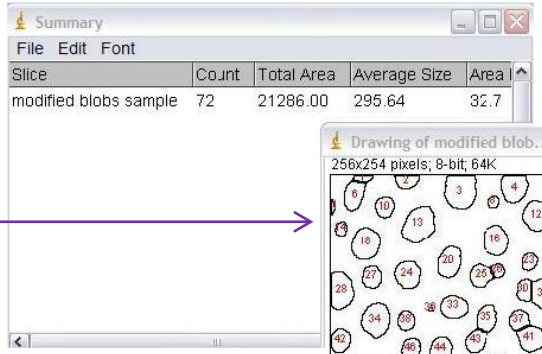
    It will either be in pixels, or, if your image is calibrated, in a unit of measurement^2

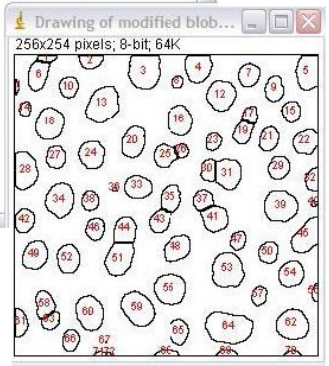
  - To check if your image is calibrated: *Image/Properties*

FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS





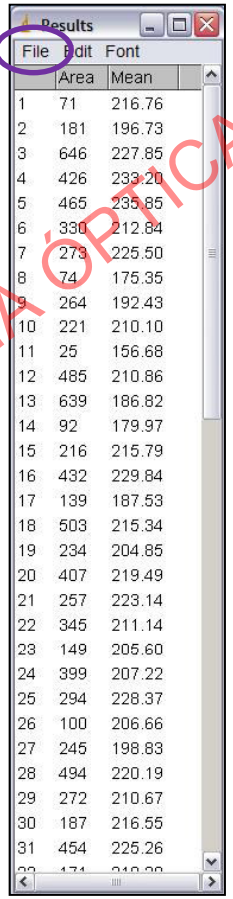


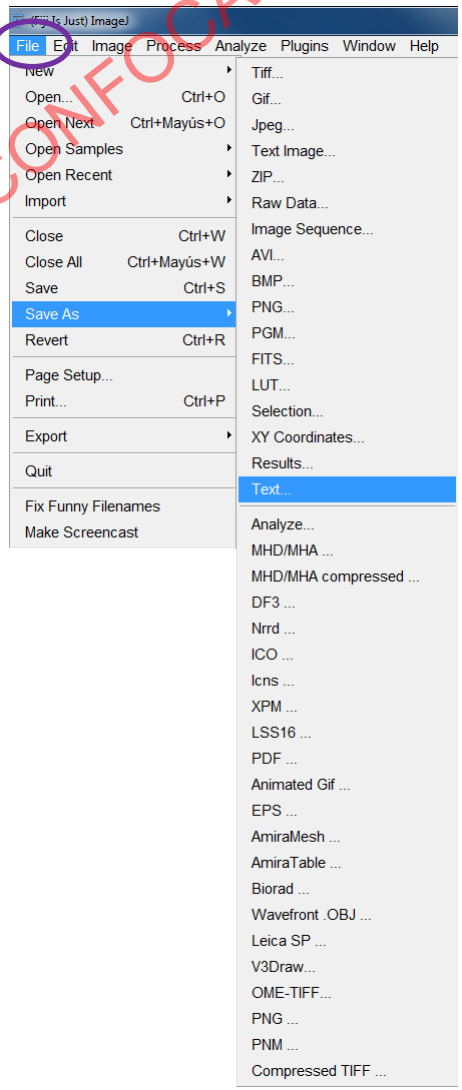


FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS

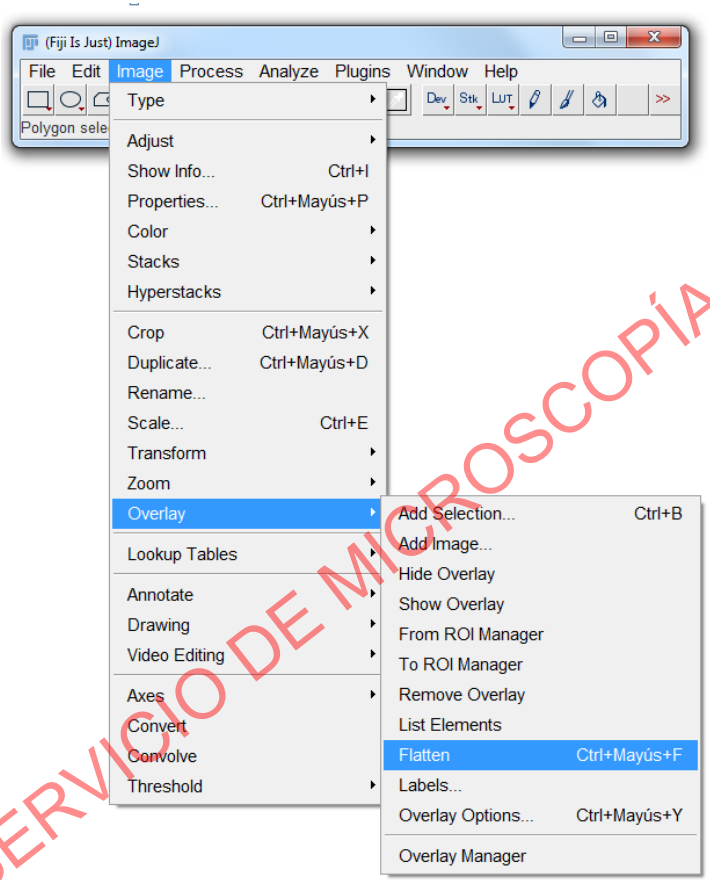
To save the results window

- *File/Save as text*



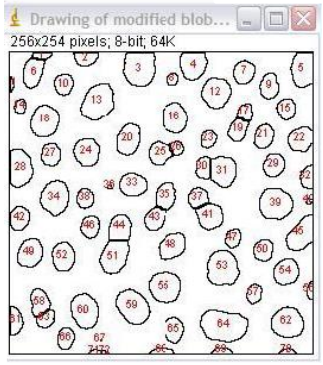


FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE OBJECTS

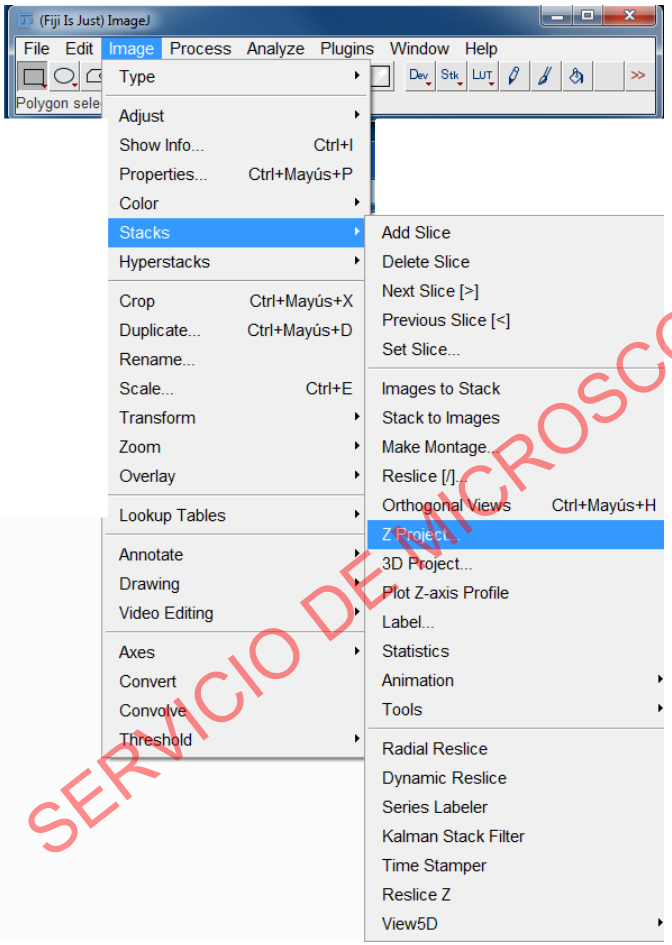


To save the image with the numbers

- *Image/Overlay/Flatten*
- *File/Save as/Tiff*



FLUORESCENCE INTENSITY QUANTIFICATION FOR Z STACK IMAGES

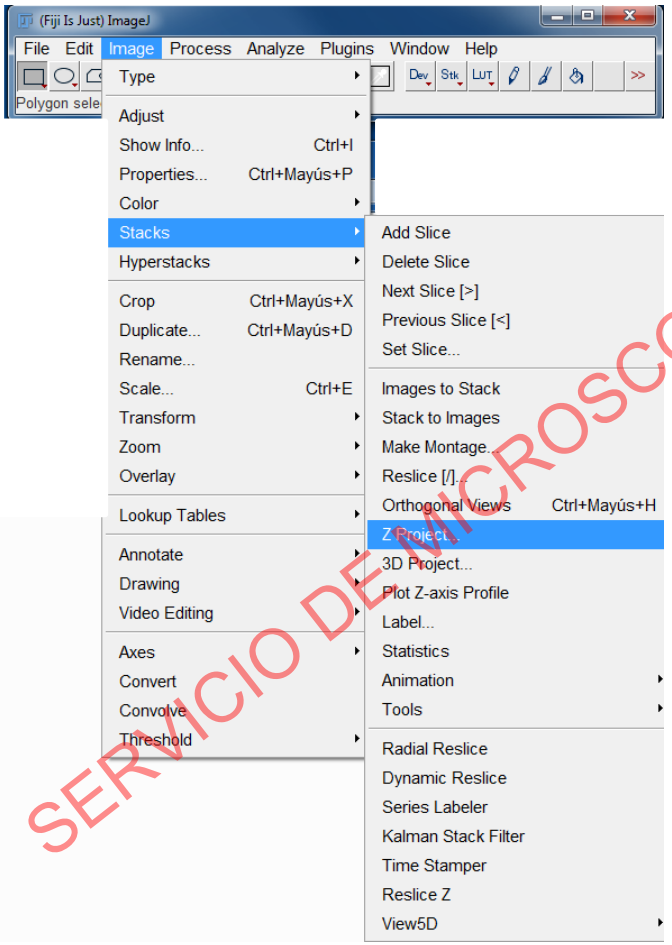


Z stack images

- *Image/stack/Z-Project*

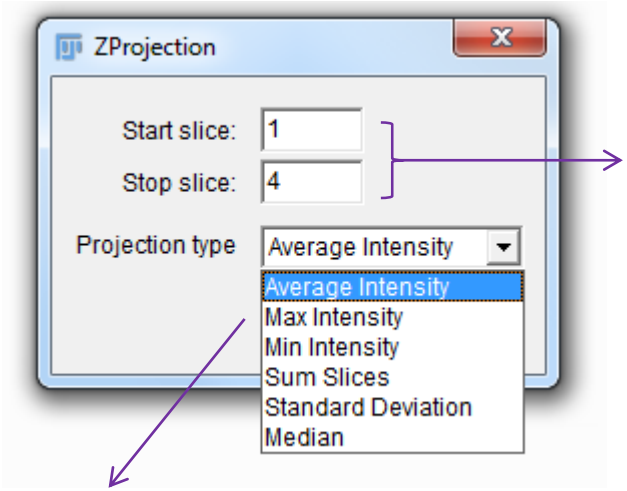
Z Project is a method of analyzing a stack by applying different projection methods to the pixels within the stack

FLUORESCENCE INTENSITY QUANTIFICATION FOR Z STACK IMAGES



Z stack images

- *Image/stack/Z-Project*



will determine the range of the stack that will be included in the z projection

There are six different projection types to choose from

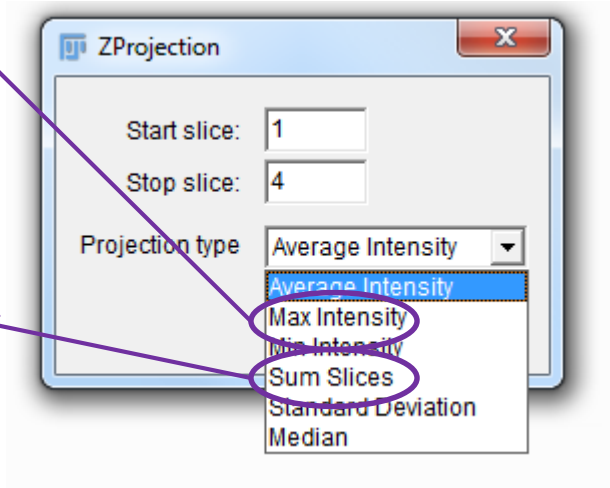
FLUORESCENCE INTENSITY QUANTIFICATION FOR Z STACK IMAGES

Z stack images

- *Image/stack/Z-Project*

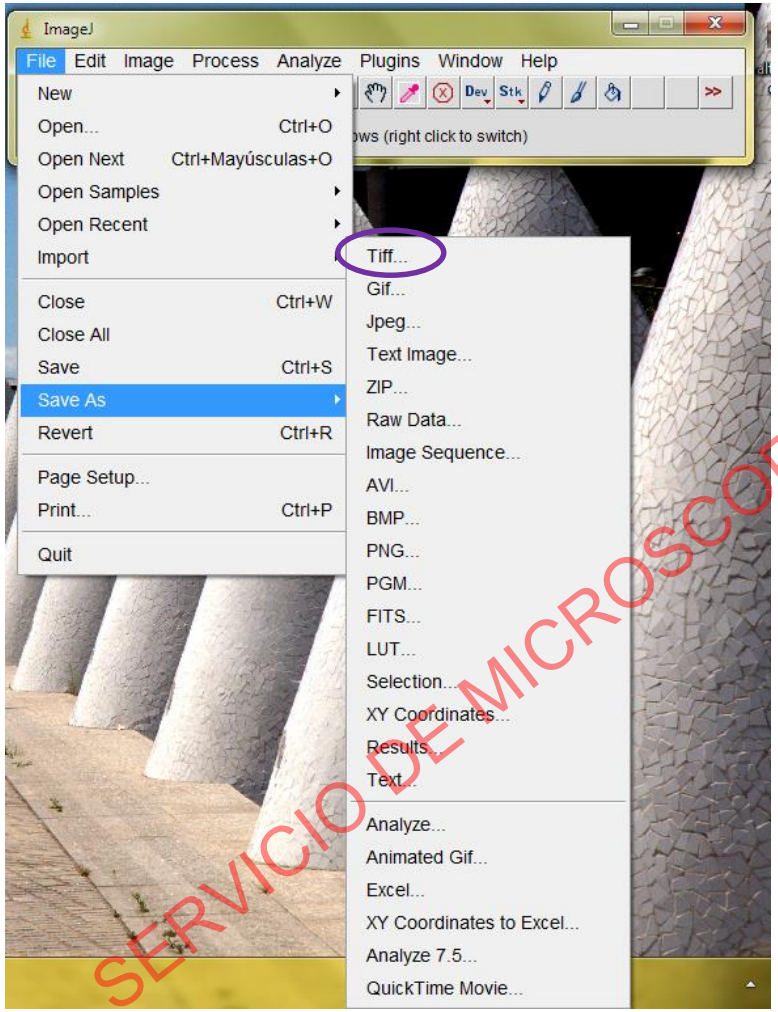
Maximum Intensity projection creates an output image whose pixels correspond to the maximum value of each pixel position (in xy) across all the stack images (z).

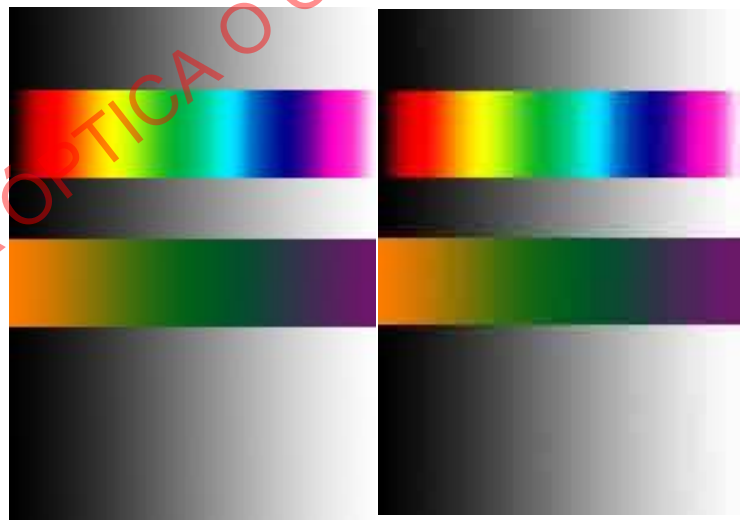
Sum Slices projection creates an image that is the sum of the selected slices in the stack.



Proceed for projection as for one plane images

NEVER SAVE YOUR IMAGES AS JPG





TIFF JPG

ACKNOWLEDGEMENTS

Javier Díez-Guerra

Ángeles Muñoz

Teresa Villalba

Carmen Sánchez

Alejandro Molina

Laboratorio 310: 91 196 4643
E-mail: confocal-cbm@listas.csic.es