FLUORESCENCE QUANTIFICATION WITH FIJI

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Madrid 2016

FLUORESCENCE QUANTIFICATION WITH FIJI 1. Image analysis – Digital image

CAO

- - Digital image
- Sample preparation 2.
- Image acquisition 3.
- Corrections 4.

FRUICIC

- 5. Fluorescence intensity quantification
 - Set Measurements
 - nit to Threshold
 - mages 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

ERVICIC

DIGITAL IMAGE (pixels). ayscale.

Dot mosaic (pixels).
– Color or grayscale.





confocal (SMOC). **DIGITAL IMAGE**

 \cap

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







Image analysis Digital image

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SAMPLE PREPARATION SMOCH same optimized of the second s Prepare samples the same day using exactly the same

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

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CONFOCAL SYSTEM

Same conditions and same day

Set conditions according to the brightest sample

🗢 🦸 Channel Channel Ch1-T1 Ch2-T2 Ch1-T3 Ch2-T4 -Pinhole 40.5 0.87 Airy Units = 0.7 µm sectio 1 AU Max Master Gain Digital Offset Digital Gain Track1 \checkmark 405 458 514 561 633 750 488 nm 1.8 🛟 561 nm 0.2 🛟 Acquisition Mode Plan-Apochromat 63x/1.40 Oil DIC M27 Objective Scan Mode Fram Frame Size X 1024 X*Y Y 1024 Line Step Ontimal Speed 6 0 Max Pixel Dwell 3 15 user Scan Time 1 min 1 ser Averaging Number Bit Depth Line Mode Direction Method Moor 100 Scan Area Image Size: 134.8 µm x 134.8 µm 700m

+ (SMOC)

CONFOCAL SYSTEM

- Same conditions and same day Set conditions according to the brightest sample
- Allow lasers to stabilize (switch on 1h before)

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

FRUICH

Range Indicator palette Red = saturation



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

CONFOCAL SYSTEM

- Avoid photobleaching
- Field centered
- 12 Bits

) 💳 Acquisition	Mode			• pro	1
Objective	Plan-Apochromat 63x/1.40 Oil DIC M27				
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Scan Area					
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MFOCAL (SMOC), **IMAGE ACQUISITION** WIDE-FIELD SYSTEM CAC Same conditions and same day Set conditions according to the brightest sample SERVICIO DE MICRI

IMAGE AC	QUISITION (SMOC)
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Acquire Image: 📑 Untitled	and the second s
Save Image Save to: D:\Wandosel\rojo_3.tif Set Save	CO.
Save w/Sequence Display Acquire Correct Annotate Special	
Exposure Time: 250	
Temp: n/a Setting [Modified]; Display Saturation Markers	Reset Display
Close Less << Setting: Load Save Save As	

M Acquire	
Acquire	Image: Mutitled
Save Image	Save to: D:\Wandosel\rojo_3.tif Set Save
Save w/Sequence	Display Acquire Correct Annotate Special
Exposure Time: 250 ms Auto Expose Binning: 1 Camera Area: -> Full Chip Center Quad. Use Active Region Show Live Live Bin: 1 Temp: n/a	Background Subtraction: None Constant Region Image Shading Correction None Image
Setting [Modified]:)	Do correction when live is running
Close Less <<	Setting: Load Save Save As

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

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



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

OPTI

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

	1AGE ACQUISITION	Me
Acquire		
Acquire	Image: 📑 Untitled	
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Save w/Sequence	Display Acquire Correct Annotate Special I	
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Live Bin: 1		
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Setting [Modified]:		
GFF Less «	Setting: Load Save Save As	



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CORRECTIONS

6P

.AC

- Background correction
- Shading correction

CORRECTIONS

Background correction

💱 Acquire	×
Acquire	Image: 📑 Acquired
Save Image	Save to: C:\MM\\Acquired001.tif Set Save
🗖 Save w/Sequence	Display Acquire Correct Annotate Special
Exposure Time: 5 AutoExpose Binning: 2 Camera Area: > Full Chip	Background Subtraction: ○ None ○ Constant ○ Region ○ Image □ isplay Background Image Load Background Image □ Offset Value: □ 🚔
Center Quad. Use Active Region Show Live Live Bin: 2	Shading Correction: None Acquire Shading Reference Display Shading Image Load Shading Image
Close Less (3)	Setting: Load Save Save As

METAMORPH

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

OCAL (SMOC

Images will be corrected for <u>camera</u> <u>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".



Shading





JCAL (SMOC)

CORRECTIONS

METAMORPH

Shading correction

💱 Acquire	
Acquire	Image: 📑 Acquired
Save Image	Save to: C:\MM\\Acquired001.tif Set Save
🔲 Save w/Sequence	Display Acquire Correct Annotate Special
Exposure Time:	Background Subtraction:
5 🕂 ms 💌	C None Acquire Background
AutoExpose	C Region V Keep Shutter Closed
Binning: 2 🕂	Image Display Background Image
Camera Area:	Load Background Image
-> Full Chip	Offset Value: 0 🕂
Center Quad.	
Use Active Region	- Shading Correction:
Show Live	None Acquire Shading Reference
Live Bin: 2 -	
T 1	Display Shading Image
Rkad Shd	Load Shading Image
Catting [Medified]:	
in vivo-Maria	Do correction when live is running
Close Less	Setting: Load Save Save As

Corrects defects in field illumination.

To acquire a shading image:

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

CORRECTIONS

METAMORPH

Shading correction

💱 Acquire		4	
Acquire	Image: 📑 Acquired		
Save Image	Save to: C:\MM\\Acquired001.tif Set Save	I.	
🔽 Save w/Sequence	Display Acquire Correct Annotate Special		
Exposure Time:	Background Subtraction:		
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AutoExpose	C Region V Keep Shutter Closed		
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Camera Area:	Load Background Image		
-> Full Chip	Offset Value: 0 🖶		
Center Quad.			
Use Active Region	Shading Correction:		
Show Live	None Acquire Shading Reference		
Temp: n/a	Display shading image		
Bka	Load Shading Image		
Setting [Modified]:			
in vivo-Maria 💌			
Close Less (3	Setting: Load Save Save As		

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

SCAL (SMOC) Background correction Fiji: Substract Background

🕎 (Fiji Is Just) ImageJ		
File Edit Image	Process Analyze Plugins Window He	
	Smooth Ctrl+Mayús+S	T 🖉 🔏 👌 💈
Straight, segmented	Sharpen	
	Find Edges	
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	Enhance Contrast	
	Noise	
	Shadows •	
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\sim	Math •	
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M	Batch	
	Image Calculator	
	Subtract Background	
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an'	Morphology •	
	Image Expression Parser	
GV	Multiple Image Processor	
	Enhance Local Contrast (CLAHE)	














Background correction

Analyze > Set measurements

We have to calculate:

- The average background value (usually using a ROI)
- Its standard deviation

	🗊 Set Measurements
	Area
	Standard deviation Modal gray value
1	Min & max gray value 🗖 Centroid
	Center of mass Perimeter
	🗖 Bounding rectangle 🛛 🗖 Fit ellipse
	🗆 Shape descriptors 🛛 🗖 Feret's diameter
	🗖 Integrated density 🔲 Median
	🗆 Skewness 👘 Kurtosis
	Area fraction Stack position
	🗆 Limit to threshold 🛛 🗖 Display label
	Invert Y coordinates Scientific notation
	Add to overlay
	Redirect to: None
	Decimal places (0-9): 3
	OK Cancel Help

SMOC



Background correction

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

Process
 Math
 Subtract

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		,	

(U) R	lesults		
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1	52.230	49.152	
.			

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.

Mean + (StdDev x 3)



SCAL (SMOC). Shading correction Fiji: Image calculator

😰 (Fiji Is Just) ImageJ							x
File Edit Image	Process Analyze	Plugins	Window	Help			
	Smooth	Ctrl+	•Mayús+S	UŢ 🥻	9 🔏	&	>>
Angle tool	Sharpen	~					
	Find Edges						
	Find Maxima	O,					
	Enhance Contrast	t					
	Noise			•			
	Shadows						
C	Binary			•			
\sim	Math			•			
	FFT			•			
NC.	Filters			•			
NI	Batch						
<u> </u>	Image Calculator						
\sim	Subtract Backgrou	und					
	Repeat Command	d Ctrl+	Mayús+R				
	Calculator Plus						
12	Morphology			•			
CK'	Image Expression	Parser					
3	Multiple Image Pro	cessor					
	Enhance Local Co	ontrast (CL	AHE)				

Shading correction

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

Process <-> image calculator



Image Calculator

Uncorrected image

Shading-corrected image

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).

.OCAL (SMOC)

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

Shading correction





Shading corrected in ImageJ

FFT Method



FLUORESCENCE INTENSITY QUANTIFICATION WITH IMAGE JFOCA

CAC

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- 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.



 \mathbf{X}

✓ Area	🔽 Mean gray value
Standard deviation	Modal gray value
Min & max gray value	☐ Centroid
Center of mass	☐ Perimeter
Bounding rectangle	☐ Fit ellipse
Shape descriptors	Feret's diameter
Integrated density	T Median
Skewness	T Kurtosis
Area fraction	T Stack position
Invert Y coordinates	Scientific notation
Redirect to:	name of image that is still grayscal
Decimal places (0-9)	0
VICION	OK Cancel H

Set Measurements

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			Area in	pixels sq	uared	or ir
			measurem	ent units o	of the s	elected
I✔ Area	I▼ Mean gray value		image or a	rea.		
Standard deviation	🖵 Modal gray value		\sim			
Min & max gray value	Centroid					
Center of mass	F Perimeter		Portias unstif (73%)	Colores		
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Shape descriptors	🖵 Feret's diameter				"Point" or multi-point (right click to	Analyze Particles Summarize Distribution
Integrated density	F Median	$\mathbf{\nabla}$				Label Clear Results Set Measurements
Skewness	☐ Kurtosis			* *		Set Scale Calibrate
Area fraction	Stack position		10 10 10	60 S		Plot Profile Ctri+K Surface Plot
	c C		S 41 42 9 4			Tools · · · · · · · · · · · · · · · · · · ·
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Invert Y coordinates	Scientific notation					Color Histogram Directionality Shape Index Map
r	<u></u>			2.00		Optic Flow Helmholtz Analysis O Surface Plot
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Decimal places (0-9)	0					Multi Kymograph QuickPALM



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IT Mi	n & max grav value	e Centroid						
L Ce	enter of mass	F Perimeter		Ternes	Colores			
ГВо	ounding rectangle	☐ Fit ellipse	C:1742 (1) (5:205	gernarium.bf (73%) 5.46x205.48 microns (1024x1024); 8-bit; 1	7388	File Edit	t Font Results	
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ust equal 70 micron 0.200662 0.2006620 1.5106198

OK Cancel

SET MEASUREMENTS OPTIONS

✓ Area	🔽 Mean gray value
Standard deviation	🖵 Modal gray value
Min & max gray value	☐ Centroid
Center of mass	☐ Perimeter
Bounding rectangle	☐ Fit ellipse
Shape descriptors	Feret's diameter
Integrated density	T Median
Skewness	T Kurtosis
Area fraction	Stack position
Limit to threshold	🔽 Display labe
Invert Y coordinates	Scientific notation
Redirect to:	name of image that is still grayscale
Decimal places (0-9)	0
	OK Cancel Help
110	

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

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

Image/properties

(Fiji Is Just)	ImageJ					<u>×</u>]	🗊 Drosophila ger	mariu
ile Edit	Image Process	Analyze Plugins	Window He	lelp				
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xt tool (dot	Adjust	•		_			Slices (z):	18
	Show Info	Ctrl+I					Frames (t):	1
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	Crop	Ctrl+Mavús+X					Pixel width:	10.200
	Duplicate	Ctrl+Mayús+D					Pixel height:	0.2006
	Rename						Voxel depth:	1.510
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	Drawing	•						
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	Axes	•						
	Convert							
	Convolve							
	Threshold	•						

SET MEASUREME	ENTS OPTIONS
Set Measurements	Average gray values of the selection
 ✓ Area ✓ Mean gray value ✓ Standard deviation ✓ Modal gray value ✓ Min & max gray value ✓ Centroid ✓ Center of mass ✓ Perimeter ✓ Bounding rectangle ✓ Fit ellipse ✓ Shape descriptors ✓ Feret's diameter 	Sum of pixel gray levels from the selected zone divided by the number of pixels.
 ☐ Integrated density ☐ Median ☐ Skewness ☐ Kurtosis ☐ Area fraction ☐ Stack position ☐ Limit to threshold ☐ Display label ☐ Invert Y coordinates ☐ Scieptific notation 	Σ pixel values pixel number
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SET MEASUREMENTS OPTIONS

sophila germarium.tif (75%)

Area	Mean gray value
Standard deviation	Modal gray value
Min & max gray value	Centroid
Center of mass	F Perimeter
Bounding rectangle	☐ Fit ellipse
Shape descriptors	Feret's diameter
Integrated density	T Median
Skewness	T Kurtosis
Area fraction	□ Stack position
Limit to threshold	Display labe
Invert Y coordinates	Scientific notation
Redirect to:	name of image that is still grayscale 💌
Decimal places (0-9)	•
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Average gray values of the selection.

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

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i Is Just) ImageJ					
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or multi-point (right click to	Analyze Particles	k			
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	Surface Plot				
	Gels	•			
	Tools	,			
	3D Objects Cour	nter			
	3D OC Options				
	Skeleton	•			
	Colocalization	•			
	Color Histogram				
	Directionality				
	Shape Index Map				
	Optic Flow	•			
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SET MEASUREMENTS OPTIONS

🗸 Area	l✔ Mean gray value
Standard deviation	Modal gray value
Min & max gray value	Centroid
Center of mass	F Perimeter
Bounding rectangle	Fit ellipse
Shape descriptors	Feret's diameter
Integrated density	T Median
Skewness	T Kurtosis
Area fraction	Stack position
Limit to threshold	 Display label Scientific notation
Redirect to:	name of image that is still grayscale 💌
Decimal places (0-9)	<i>•</i>
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Average gray values of the selection.

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



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Different ROI sizes can be compared



SET MEASUREN	IENTS OPTIONS
 ✓ Set Measurements ✓ Area ✓ Mean gray value ✓ Standard deviation ✓ Modal gray value ✓ Min & max gray value ✓ Centroid ✓ Center of mass ✓ Perimeter 	Standard deviation of the values used to generate the gray value mean.
Bounding rectangle Fit ellipse Shape descriptors Feret's diameter Integrated density Median Skewness Kurtos s Area fraction Stack position	File Edit Font Results Mean StdDev Mode
Climit to the should be propay label Invert Y coordinates Scientific notation Redirect to: Redirect to: Redirect to: OK Cancel Help	Most frequent gray value in the selected area.

Set Measurements OPTIONS



Set Measurements

🛓 Set Measurements 🛛 🔀	
	————————————————————————————————————
I ✓ Area I ✓ Mean gray value I ✓ Standard deviation I ✓ Modal gray value I ✓ Min & max gray value I ✓ Centroid I ✓ Center of mass I ✓ Perimeter	IntDen This is equivalent to the produ
□ Bounding rectangle □ Fit ellipse	of Area and Mean Gray Value.
□ Shape descriptors □ Feret's diameter □ Integrated density □ Median □ Skewness □ Kurtosis □ Area fraction □ Stack position □ Limit to threshold ☑ Display labe	Results File Edit Font Results Mean IntDen RawIntDen 1 16.844 427.295 10612
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SET MEASUREMENTS OPTIONS

Set Measurem	ents 🛛 🔛
🔽 Area	🔽 Mean gray value
Standard devia	tion 🔽 Modal gray value
🔽 Min & max gray	value 🔽 Centroid
Center of mas	s 🔽 Perimeter
Bounding recta	ngle 🦵 Fit ellipse
Shape descrip	tors 🛛 🗂 Feret's diameter
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Skewness	T Kurtosis
Area fraction	🗆 Stack position
Limit to thresh	old IV Display labe
Redire	ct to: 🕅 name of image that is still grayscale 💌
Decimal places (0-91 0
, <i>ClO</i>	OK Cancel Help

Provides two values:

IntDen

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

I Results	
File Edit Font Results	
Mean IntDen RawIntDen	<u>▲</u>
1 16.844 427.295 10612	
•	+ }

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.





Set Maximum



Set Measurements	X	2
🔽 Area 🕻	I Mean gray value	
Standard deviation	Modal gray value	
Min & max gray value	e 🔽 Centroid	
Center of mass	T Perimeter	
Bounding rectangle	☐ Fit ellipse	6
Shape descriptors	🗆 Feret's diameter	\mathbf{N}
Integrated density	T Median	Pη
Skewness	T Kurtosis	'
Area fraction	☐ Stack position	Y
Limit to threshold	I Display label	6
Invert Y coordinates	C Scientific notation	
Redirect to:	name of image that is still grayscale 💌	
Decimal places (0-9)	0	
	OK Cancel Help	
110		

2) Analyze option
Go to Analyze/Set Measurements.
Mean grey value
Then selecting Analyze/Measure, you will get information on the entire image.







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





- 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

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	Paste	Ctrl+V	Fit Spline	
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	Clear		Fit Ellipse	
	Clear Outside		Interpolate	
	Fill	Ctrl+F	Convex Hull	
	Draw	Ctrl+D	Make Inverse	
	Invert Ctrl+	Mayús+I	Create Selection	
	Selection	•	Create Mask	
	Options	•	Properties	Ctrl+Y
			Scale	
			Rotate	
			Enlarge	
			Make Band	
			Specify	
			Straighten	
			To Bounding Box	
			Line to Area	
			Area to Line	
			Image to Selection	
			Add to Manager	Ctrl+T
			Fit Circle to Image	
			Select Bounding Box	
			Select Bounding Box (guess backg	round color)
			Points from Mask	
			Make rectangular selection rounded	1
			Fill ROI holes	

CAL (SMOC) **FLUORESCENCE INTENSITY** QUANTIFICATION

🛓 Set Measurements		• 3) Lin	nit your me	easured area
		— "Li	mit to Thresho	old"
 Area Standard deviation Min & max gray value Center of mass Bounding rectangle Shape descriptors Integrated density Skewness Area fraction 	 Mean gray value Modal gray value Centroid Perimeter Fit ellipse Feret's diameter Median Kurtosis Stack position 	- "Li	mit to Thresho Analyze/Set check Limit to	old" Measurements Threshold
Redirect to: Decimal places (0=9)	name of image that is still grayscale OK Cancel Help			

area

🗾 (Fiji Is Just)	Imagel		
File Edit	Image Process Type	Analyze Plugin	s Window Help
reenand sel	Adjust	•	Brightness/Contrast Ctrl+Mayús+C
	Show Info	Ctrl+I	Window/Level
	Properties	Ctrl+Mayús+P	Color Balance
	Color	+	Threshold Ctrl+Mayús+T
	Stacks	+	Color Threshold
	Hyperstacks	+	Size
	Crop	Ctrl+Mavús+X	Canvas Size
	Duplicate	Ctrl+Mavús+D	Line Width
	Rename	,	Coordinates
	Scale	Ctrl+E	Auto Threshold
	Transform	•	Auto Local Threshold
	Zoom	+	Bleach Correction
	Overlay	•	Auto Crop
	Lookup Tables	•	Auto Crop (guess background color)
	Annotate	•	Scale to DPI
	Drawing	-	
	Video Editing		
	Avoc		
	Convert		
	Convolve		
	Threshold	*	
	moshou		1

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

🗊 Threshold	×
92.44 %	
•	0
▲ ▶	15
Default 💌 Red 💌	
🗖 Dark background 🗍 Stack histogram	
Auto Apply Reset Set	

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

• 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

confocal (SMOC). **FLUORESCENCE INTENSITY QUANTIFICATION**

3.1) Using "Limit to Threshold"





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

scoptA'



We must choose the most appropriate method or algorithm to segment our image



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


FLUORESCENCE INTENSITY OCAL (SMOC) **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.

• 3.2) Combining "Threshold" and multiple ROIs



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



 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



Topo, J







🗊 (Fiji Is Just) ImageJ	
File Edit Image Process Analyze Plugins Window Help	
	1 3 >>
(Fiji Is Just) ImageJ 2.0.0-rc-54/1.51g; Java 1.8.0_66 [64-bit];	

🎹 Tejido Maria 63x qfp.lsm

55.88x26.88 µm (424x204); 8-bit; 84K

ERVIC

- 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

(Fiji Is Just) Imagel		
le Edit	Image Process	Analyze Plugin	s Window Help
	Туре	•	Dev Stk LUT 0 8 81 >>
gle tool	Adjust	•	
	Show Info	Ctrl+I	
	Properties	Ctrl+Mayús+P	
	Color	•	
	Stacks	Þ	Add Slice
	Hyperstacks	•	Delete Slice
	Crop	Ctrl+Mavús+X	Next Slice [>]
	Duplicate	Ctrl+Mavús+D	Previous Slice [<]
	Rename	our majao o	Set Slice
	Scale	Ctrl+E	Images to Stack
	Transform	•	Stack to Images
	Zoom	•	Make Montage
	Overlay	•	Reslice [/]
	Lookup Tables	•	Orthogonal Views Ctrl+Mayús+H
	Annotate	,	3D Project
	Drawing	•	Plot 7-axis Profile
	Video Editing	•	Label
	Axes	•	Statistics
	Convert		Animation •
	Convolve		Tools
	Threshold	,	Radial Reslice
			Dynamic Reslice
			Series Labeler
			Kalman Stack Filter
			Time Stamper
			Reslice Z
			View5D

OBJECTS



modified blobs sample



Threshold to highlight all the structures you want to measure

Dev Stik LUT 0 8 8

Ctrl+

Ctrl+Mayús+X

Video Editi

Image/Adjust/Threshold

Make a copy of your image

Image/Duplicate

- Manually
- Using algorithms

OBJECTS



 If you have particles that have merged together

Apply (This will create a binary version

of the image)

🗊 Threshold
13.72 %
▲ <u>255</u>
▲ ▶ 255
Default 💌 Red 💌
🔽 Dark background 🔲 Stack histogram
Auto Apply Reset Set

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

OBJECTS

File



 If you have particles that have merged together

Process/Binary/Watershed

ji Is Just) ImageJ					
Edit Image	Process Analyze Plugins Window H	lelp			
0,00/	Smooth Ctrl+Mayús+S	vī, 0 8 8 🚿 🚿			
tool	Sharpen				
	Find Edges				
	Find Maxima				
	Enhance Contrast				
	Noise				
	Shadows •				
		Make Binary			
	Math +	Convert to Mask			
	FFT •	Frode			
	Filters +	Dilate			
	Batch	Open			
	Image Calculator	Close-			
	Subtract Background				
	Repeat Command Ctrl+Mavús+R	Outline			
		Fill Holes			
	Calculator Plus	Skeletonize			
	Morphology	Distance Map			
	Image Expression Parser	Ultimate Points			
	Multiple image Processor	Watershed			
	Ennance Local Contrast (CLAHE)	Voronoi			
		Options			
		·			

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

🛓 Set Measurements		
🔽 Area	🔽 Mean gray value	
Standard deviation	Modal gray value	
✓ Min & max gray value	Centroid	
Center of mass	F Perimeter	
Bounding rectangle	Fit ellipse	
Shape descriptors	Feret's diameter	
Integrated density	🗂 Median	
Skewness	🗂 Kurtosis	S
Area fraction	Stack position	$\sim 0^{-1}$
		CK
Limit to threshold	 Display label 	
Invert Y coordinates	Scientific notation	
Redirect to:	name of image that is a	till gravecale
Desired allower (0.0)	name of mege that is a	un grayscale
Decimal places (0-9):		
	ок	Cancel Help
SV		

- 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.

OBJECTS

iji Is Just) ImageJ							
Edit Image Process	Analyze Plugins	Window	Help				
0,00/4+	Measure	Ctrl+M	k, Lut	0	1 3	>>	
tool (double-click to configure	Analyze Particles	;					
	Summarize						
	Distribution						
	Label						
	Clear Results						
	Set Measuremen	ts					
	Set Scale						
	Calibrate						
	Histogram	Ctrl+H					
	Plot Profile	Ctrl+K					
	Surface Plot						
	Gels	•					
	Tools	•		•			
	3D Objects Cour	nter					
	3D OC Options					(
	Skeleton	•				2	
	Colocalization	•			. (
	Color Histogram				$\sim \sim $		
	Directionality			1	\mathcal{V}		
	Shape Index Map						
	Optic Flow	- -	NV				
	Helmholtz Analys	is					
	3D Surface Plot	U					
	Classification	•					
	Local Thickness	•					
	Multi Kymograph	+					
	QuickPALM	+					
C	Sholl	+					
	ТороЈ	•					

File Text

 Click on the binary or thresholded image to select it, then go to:

Analyze/Analyze Particles



- 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 measurement²

• To check if your image is calibrated: Image/Properties

FLUORESCENCE INTENSITY QUANTIFICATION FOR EACH OBJECT IN IMAGES WITH MULTIPLE **OBJECTS**





Compressed TIFF



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



FLUORESCENCE INTENSITY QUANTIFICATION FOR Z STACK IMAGES

Z stack images

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.

1	TProjection	
	Start slice: 1 Stop slice: 4	
l	Projection type Average Intensity -	
_	Max Intensity Max Intensity Sum Slices	
	Standard Deviation Median	

Proceed for projection as for one plane images

Image/stack/Z-Project



NEVER SAVE YOUR IMAGES AS FOG





TIFF

JPG

