FLUORESCENCE QUANTIFICATION WITH FIJI

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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 = $2^8 = 256$

<table>
<thead>
<tr>
<th>Pixel Color</th>
<th>Decimal number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>255</td>
<td>255</td>
</tr>
</tbody>
</table>

12 bits = $2^{12} = 4096$

16 bits = $2^{16} = 65536$
FLUORESCENCE QUANTIFICATION WITH FIJI

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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.
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   – 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
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 stabilize (Switch on 1h before use)

• No saturated pixels
IMAGE ACQUISITION

[Image of a microscope interface with pseudocolor options]

SERVICIO DE MICROSCOPÍA ÓPTICA O CONFOCAL (SMOC)
IMAGE ACQUISITION

WIDE-FIELD SYSTEM

• Same conditions and same day
• Do not autoscale
• Allow lamp to stabilize (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

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   – Digital image
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CORRECTIONS

WIDE-FIELD SYSTEM

• Background correction

• Shading correction
Background correction

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

Images will be corrected for camera background in the absence of light.

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

METAMORPH
CORRECTIONS

Shading
CORRECTIONS

BEFORE

AFTER

Shading Correction
Shading correction

Corrects defects in field illumination.

To acquire a shading image:

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

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: Subtract Background**
CORRECTIONS

Background correction
• *Process ▶ Subtract background*

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

Rolling-ball algorithm

NON-UNIFORM BACKGROUND
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
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*
Shading correction

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

- **Process ➤ image calculator**

**CORRECTIONS**

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

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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.
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.
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.

\[
\frac{\sum \text{pixel values}}{\text{pixel number}} = 7.5
\]

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.

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**.

---

![Image of measurement options dialog box]

- **IntDen**
- **Mean gray value**
- **Centroid**
- **Perimeter**
- **Fit ellipse**
- **Feret's diameter**
- **Median**
- **Stack position**

Results table:

<table>
<thead>
<tr>
<th>File</th>
<th>Edit</th>
<th>Font</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean IntDen</td>
<td>RawIntDen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.644</td>
<td>427.295</td>
<td>10612</td>
<td></td>
</tr>
</tbody>
</table>
**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:

\[
\text{Int Den} = \text{Area} \times \text{Mean}
\]

<table>
<thead>
<tr>
<th>Area</th>
<th>Mean</th>
<th>Int Den</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20</td>
<td>200</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>200</td>
</tr>
</tbody>
</table>

Different ROI sizes can be compared.

*RawIntDen*

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

\[
\text{Raw Int Den} = \sum \text{Values of Pixels}
\]

<table>
<thead>
<tr>
<th>Area</th>
<th>Mean</th>
<th>Raw Int Den</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

Different ROI sizes cannot be compared.
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.
3) Limit your measured area
   - Draw a region of interest (ROI) around your 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
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.
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.
• 3.1) Using “Limit to Threshold”

There are many algorithms you can use to calculate the threshold without introducing user-bias.
3.1) Using “Limit to Threshold”

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.
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

- 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*
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**

There are six different projection types to choose from.

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

- Image/stack/Z-Project

Proceed for projection as for one plane images
NEVER SAVE YOUR IMAGES AS JPG

TIFF

JPG
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