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I. INTRODUCTION

The intensity response of a fluorescence microscope expresses the output digital signal to an input photon flux. It depends on many aspects of the microscope, either in the illumination path or the detection path.

In any fluorescence microscope, the overall intensity response may evolve over time. For example, variations in the illumination power, detector sensitivity, optical alignment, etc. can lead to intensity response fluctuations. The knowledge of the intensity response is therefore important when the intensity quantification in images of a biological sample, acquired at different times, is aimed to be performed.

Intensity response

The “intensity response” analysis provides the intensity response of the imaging system to 16 intensity levels following a linear evolution, as well as quantitative parameters such as ***intensity values*** and pattern ***dynamic range***. Monitoring these parameters allows to observe how the intensity response evolves over time, with respect to reference values.

II. IMAGE ACQUISITION PROCEDURE

The ***"intensity response"*** analysis is associated with the ***"4×4 intensity gradation"*** (Pattern C) and ***"2×16 intensity gradation"*** (Pattern D) patterns (see Figure 1).

CAUTION
INTENSITY-SENSITIVE PATTERN
TO BE IMAGED WITH CARE
See below the chapter on acquisition recommendations

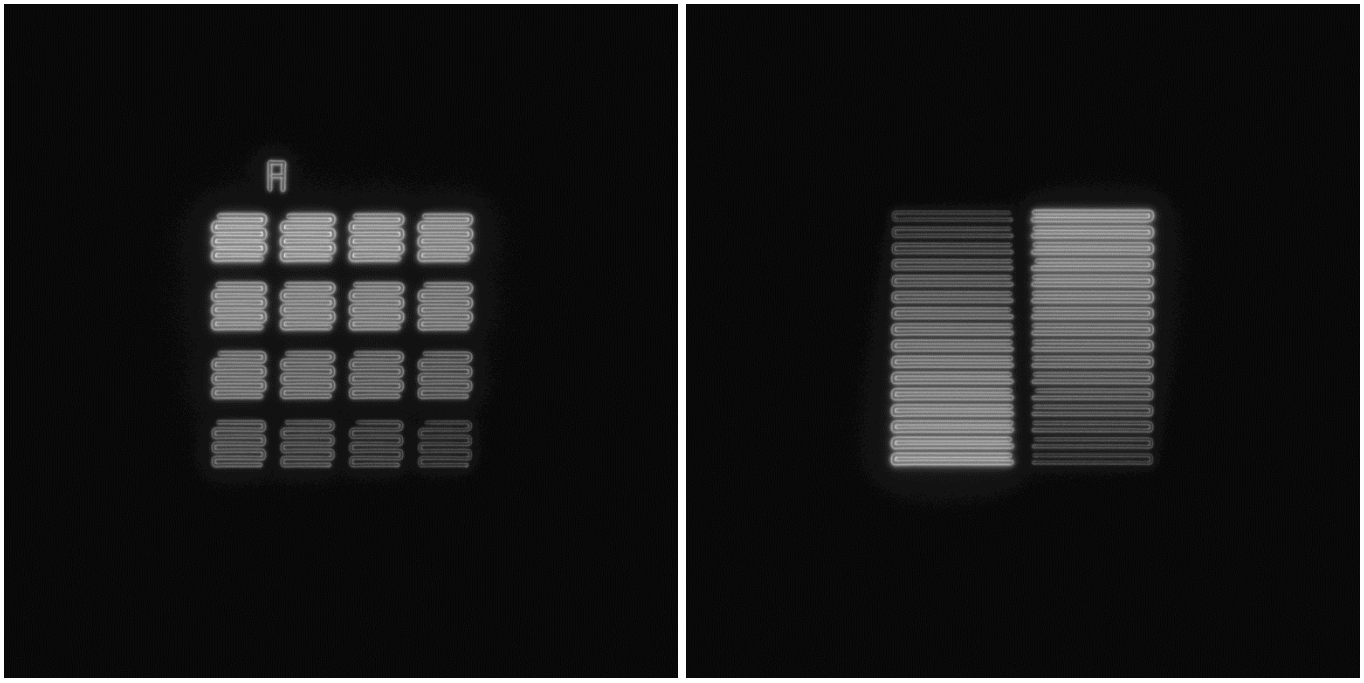


Figure 1: Image examples of intensity gradation patterns, fulfilling the acquisition recommendations. Left : "4×4 intensity gradation" pattern.Right : "2×16 intensity gradation" pattern.

1. ACQUISITION RECOMMENDATIONS

- **Recommended image type**

Z stack	Yes (if your microscope allows to do it)
Multi-channel	Recommended but not mandatory
Tiles	No

When a multi-channel Z-stack is acquired, the reader in Daybook separates each channel so that one Z-stack per channel can be analyzed.

Do not zoom in, this could damage the pattern.
The area of the scanned zone should not be smaller than the area of the pattern.

2. HOW TO IMAGE THE PATTERN?

1- Find the patterns

a) Start with a low mag objective (such as 10× or 20×). Set the DAPI (405 nm) or GFP (488 nm) channel.
b) Make coincide the center of the slide with respect to the objective.
c) Adjust focus through the eyepieces.
d) Switch to the objective you would like to use. Move the slide to the pattern.

2- Adjust your setup

a) Match the center of the pattern with the center of the field of view.
b) Adjust the focus.

The best focus usually corresponds to the Z-plane for which the pattern looks the clearest (qualitative approach) and/or for which the intensity histogram is the broadest (quantitative approach).

3- Image the pattern and save the image

a) Image the pattern by following the acquisition recommendations.
b) Save the image into the acquisition software proprietary format or into a lossless compressed format. If saved into a lossless compressed format, the image file should have a dynamic range of 8 or 16 bits. Also, the metadata should be contained within the image file.

III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

a) Select “Intensity response” in the “Select analysis” list.

b) Upload your image(s) using the “Upload file” button.
Select the image to be analyzed.

c) Set the required and optional settings (see section III.2 “Analysis Settings”).

d) To proceed, click on “Start the analysis”.

e) If needed, select a region of interest (ROI) and click on “Crop” to crop the image (*cf.* Figure 2).

For “2×16 gradation” or “4×4 gradation (auto)”:

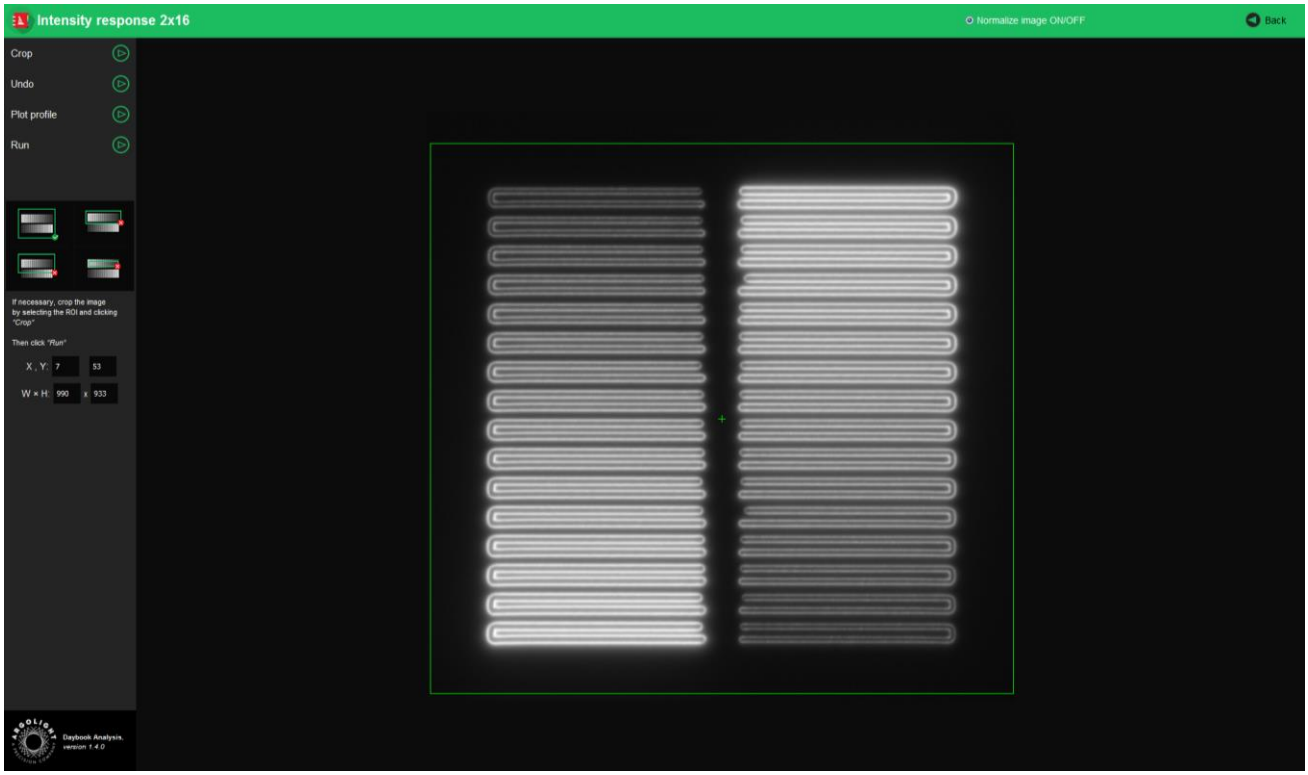


Figure 2: Crop window.

For “4×4 gradation (manual)”:
If the “4×4 intensity gradation (manual)” option is selected, crop the image to select the pattern without the A (*cf.* Figure 3, on the left). Then left-click between the 15th and the 16th most intense squares to place the round landmark (*cf.* Figure 3, on the right).

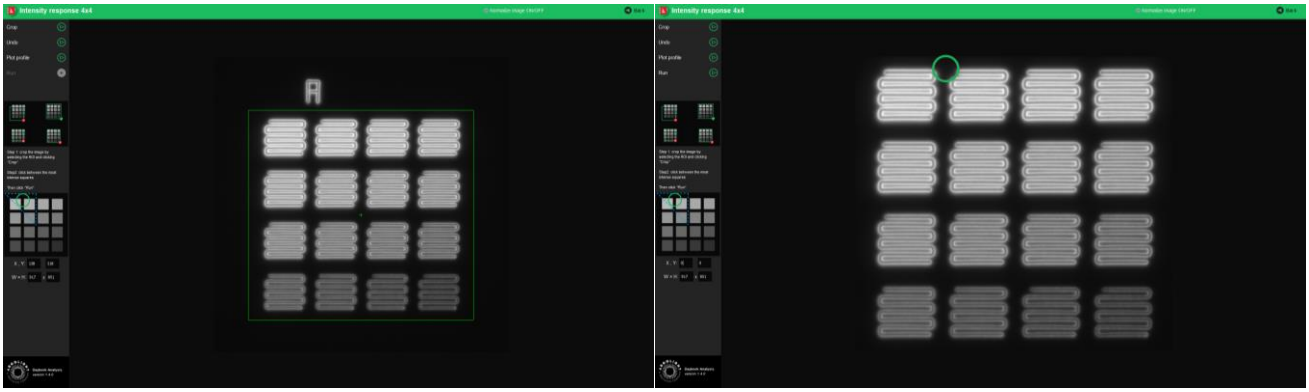


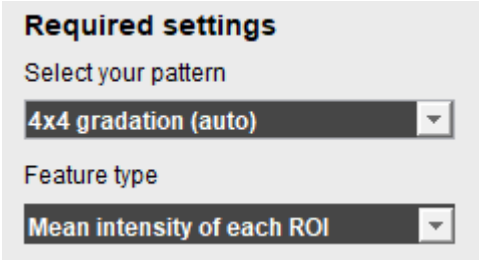
Figure 3: Procedure to carry out the analysis when the option “4×4 gradation (manual)” is selected.

f) Click on “Run” to run the analysis.
Results are displayed and can be saved as CSV, PDF, or transferred into Daybook Data Manager (if available in your package).

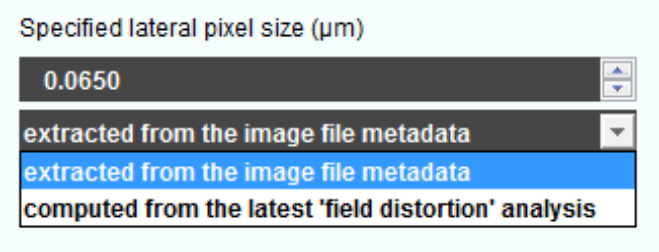
2. ANALYSIS SETTINGS

1- Required settings

- **Pattern selection**
Choose the pattern to be analyzed:
2×16 gradation (auto) or 4×4 gradation (auto).
4×4 gradation (manual) is advised for images with poor signal-to-background ration (SBR) or signal-to-noise ratio (SNR), or if you encounter any issue with the automated analysis.



- **Specified lateral pixel size**
There are two ways to get the lateral pixel size of the image to be analyzed:
- Either from the proprietary file:
Select *“extracted from the image file metadata”*.
- Or from a previous *“field distortion”* analysis:
Select *“computed from the latest ‘field distortion’ analysis”*.



2- Optional settings

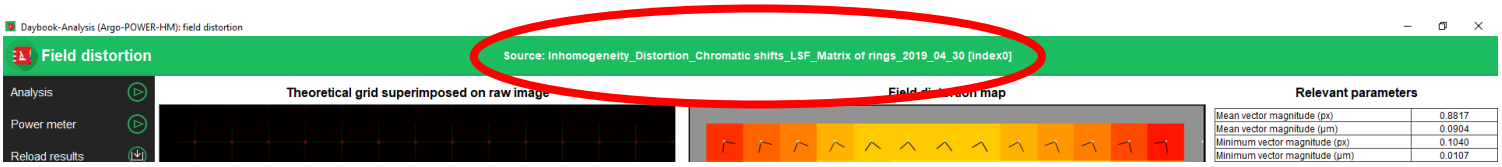
- **Background subtraction**

Subtracts the background in images where the signal-to-background ratio (SBR) is too low to be analyzed by Daybook Analysis.
It requires to acquire an image of an area where there is no fluorescent pattern (*i.e.* a dark image) with the same settings (channel, illumination power, exposure time, etc.) as the image of the pattern to be analyzed.
For multi-channel tests, a background image for each channel is required.

- **Hot pixels removal**
  Removes the very intense (*i.e.* hot) pixels that may cause analysis issues.
  Use this option only if you have such hot pixels in the image.

- **Specified axial pixel size**
  On Z-stacks analysis, the axial pixel size is determined from the proprietary file.

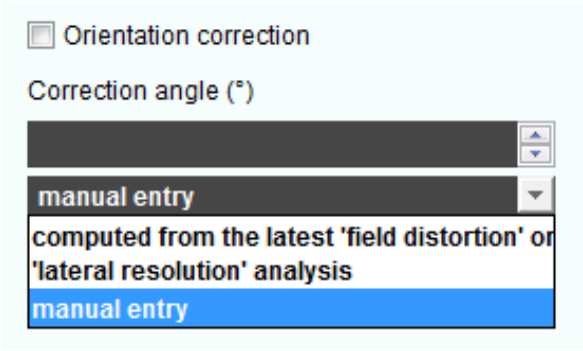
- **Best focus selection**
  Works only for mono- or multi-channel Z-stacks.
  It automatically selects from a Z-stack the image having the best contrast, corresponding to the best focus for the fluorescent pattern.
  The index of the selected image is displayed in the middle top of the results page (see figure below). Information about the selected image can also be found in the metrics and reports.



- **Intensity projection**
  Works only for mono- or multi-channel Z-stacks. It projects onto a 2D image the maximum or the mean intensity pixels of the images from the Z-stack. It is advised to use this option to prevent from any tilt that could be introduced if the lateral stage and/or the sample holder are not perfectly horizontal.

- **Orientation correction**
  There might sometimes be a tilt on the acquired images. Tick “Orientation correction” to button enable the correction angle option.

- **Correction angle**
  The correction angle is computed from other analyses (*field distortion* or *lateral resolution*). It can also be set manually.



IV. RESULTS PAGE DESCRIPTION

1. INTERFACE

The picture below shows the results page for this analysis (*cf.* Figure 4).
Results are displayed in the form of images, maps, graphs, and tables.

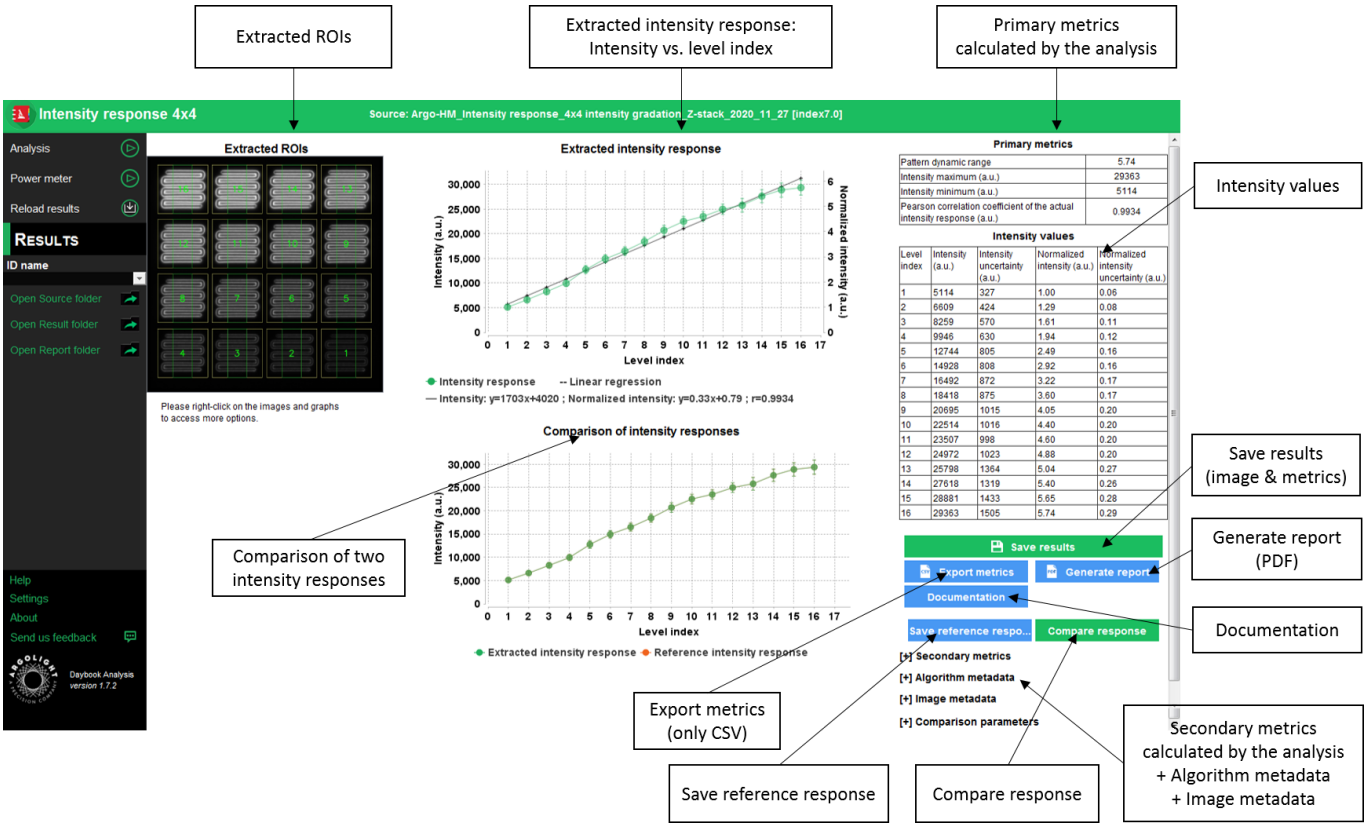


Figure 4: Results page.

2. OPTIONS

- **Saving options:**

When Daybook Data Manager is disabled, the results can be saved into a CSV file thanks to the "Save results" or "Export metrics" buttons.

Reports (in a PDF format) containing the results (maps, graphs, metrics) can be generated and saved by clicking on the "Generate report" button (*cf.* Figure 4).

By default, the results will be saved in the "Daybook Analysis\Daybook results" folder, located within the Daybook directory. To modify the default folder, go to the "Settings" menu at the bottom left corner.

When a valid Daybook Data Manager license key is registered, the “Save results” button becomes “Save into Data Manager”. Results are therefore transferred into Daybook Data Manager when clicking the “Save in Data Manager” button. To do that, in the saving window interface, select the system, the acquisition profile and the associated channel whose results you wish to save.

By default, the results are saved at the acquisition date of the image. If the acquisition date is not in the metadata of the image, it is possible to save the results at the upload date (date of the image upload), at the present date (date of the image analysis) or at a custom date (*cf.* Figure 5).

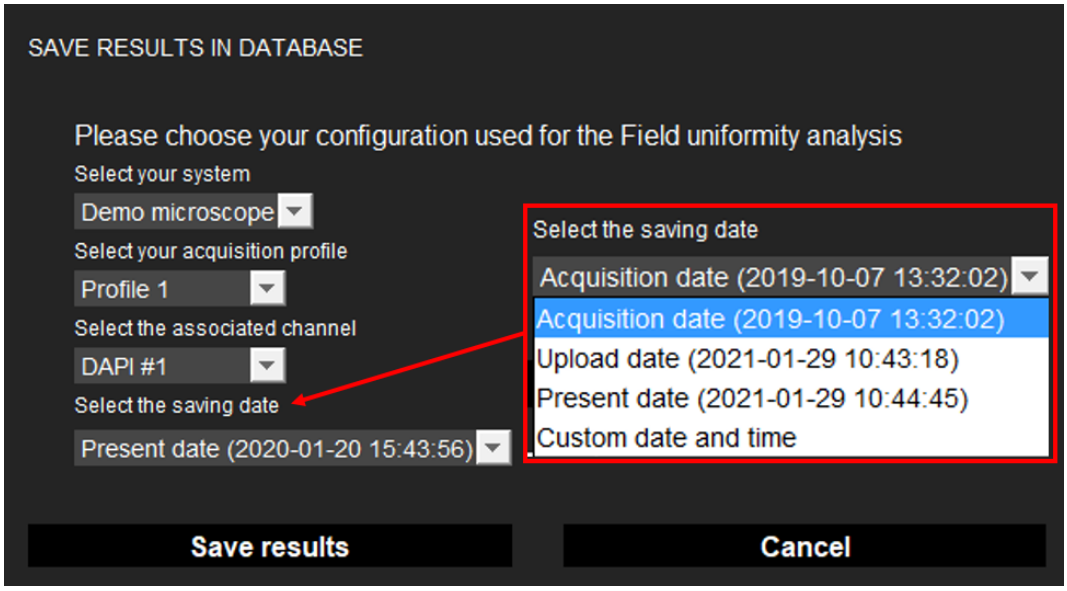


Figure 5: Interface window for saving the results in the database.

- **Image options:**
  - Zoom in and out. The images can be zoomed in and out by using the mouse roller.

- **Graph options:**
  - Zoom in and out: Hold the left or right button of the mouse and move it towards the bottom right to create a selection rectangle. To go back to the initial size, hold the left or right button of the mouse and move it towards any direction.
  - Optional features. Right click on the graph to have access to:
    - “Properties”: Edit the chart properties.
    - “Save as”: Save an image into a PNG or JPEG file, or the graph values into a TXT file.
    - “AutoRange”: Adjust automatically the ranges of the axes.

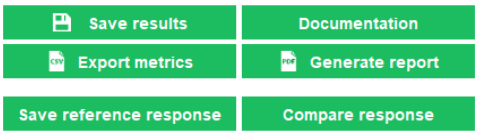
3. COMPARE TWO INTENSITY RESPONSE CURVES

It is possible to compare the extracted intensity response to a reference one, that you would have set as reference from a previous test.

1- <u>Use the results of a test as “reference intensity response”</u>

You can either save your current result as reference or upload a previous test result as reference.

- To save a current result as reference, after your analysis is finished, click on “Save reference response”.



- To save a previous result as reference, upload your previous result CSV file and lick on the “Save reference response” button.

2- Compare two responses

Use the “Compare response” button to compare your current result with the last reference intensity response saved. It is advised to store the results from the intensity response tests, so that they can be recalled as a reference.

Note:

- Only images with the same *image dynamic range* can be compared.
- As it is indicated in the name of the pattern, the 2x16 gradation pattern is composed of two gradations. The extracted intensity response is the mean of both gradations. Intensity values for each gradation are available within the CSV file.
- The intensity response of the 2×16 and 4×4 gradation patterns may have a different *pattern dynamic range* because:
    - the intensity measured in each ROI depends on the pattern shape,
    - the influence of the field uniformity on these two patterns is different, resulting in different intensity evolutions.

V. ANALYSIS ALGORITHM DESCRIPTION

1. DIAGRAM

The diagrams below describe the algorithm that allows the extraction of the intensity response from one image of a “4×4 intensity gradation” (*cf.* Figure 6) and one image of the “2x16 intensity gradation”, respectively (*cf.* Figure 7).

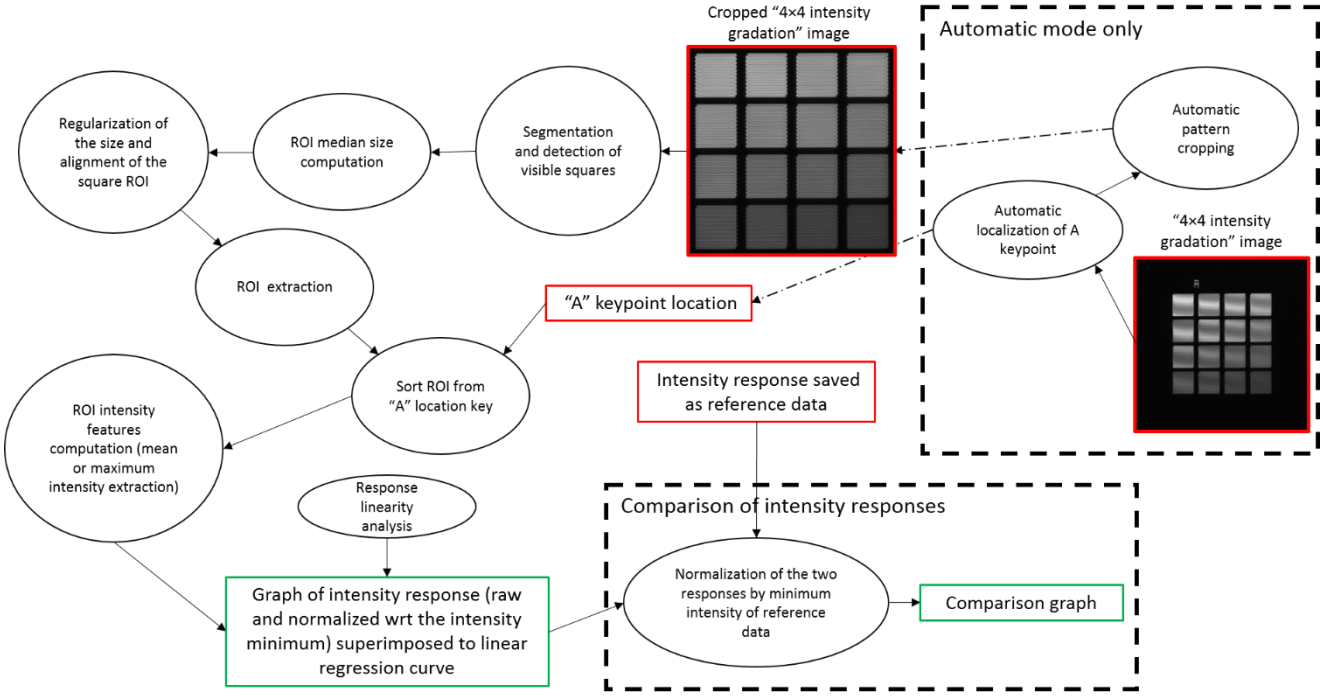


Figure 6: Schematic description of the different steps of the analysis algorithm.

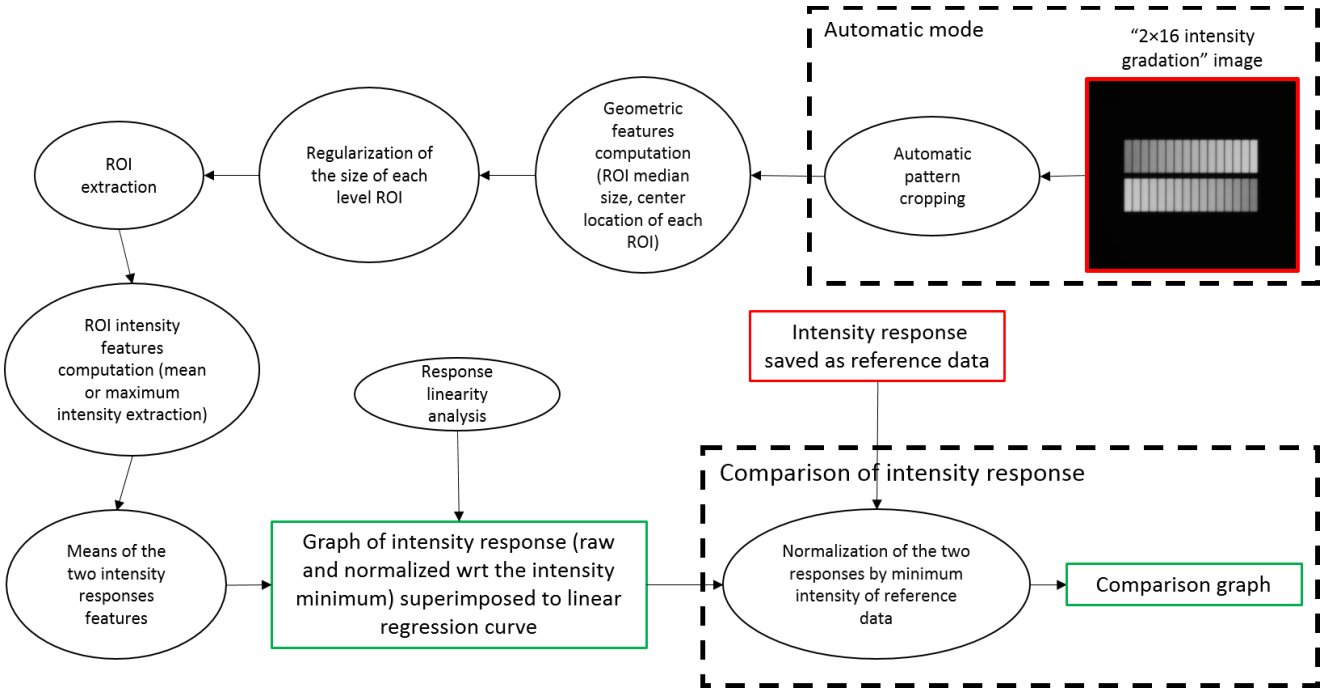


Figure 7: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It detects and segments the squares (for the 4×4 gradation) or the rectangles (for the 2×16 gradation) in the image. This is marked by the yellow ROIs surrounding the squares and both the green and yellow ROIs surrounding the rectangles.
- It measures the average and standard deviation of the peak (maximum) intensities from the intensity profile perpendicular to the lines, in the ROI of each level of each square (green ROI) or rectangle (green and yellow ROIs).
- It displays the average of the maximum intensities versus the square/rectangle index level into the "Extracted intensity response" graph, with error bars corresponding to three times the standard deviation.

VI. OUTPUT METRIC DESCRIPTION

1. PRIMARY METRICS

- *Pattern dynamic range* is the ratio between the maximum intensity level and the minimum intensity level of the "intensity gradation" pattern. It is unitless, and is given by the following equation:

$$Pattern\ dynamic\ range = \frac{I_{max}}{I_{min}}$$

Where I_{max} and I_{min} are the maximum and minimum intensities, respectively, of the "intensity gradation" pattern.

- *Intensity maximum* is the maximum intensity of one of the intensity levels in the image. It is expressed in arbitrary unit.

- *Intensity minimum* is the minimum intensity of one of the intensity levels in the image. It is expressed in arbitrary unit.

- *Pearson correlation coefficient (r) of the actual intensity response* is the correlation coefficient of the extracted intensity response. It measures the linearity of the intensity response evolution. It can have values between +1 and −1, where +1 corresponds to a total positive correlation (perfect linear evolution), 0 to no correlation at all (totally nonlinear evolution), and −1 to a total negative correlation. It is unitless, and is given by the following formula:

$$r = \frac{mean(Level\ index \times Level\ intensity) - mean(Level\ index) \times mean(Level\ intensity)}{\sigma(Level\ index) \times \sigma(Level\ intensity)}$$

Where *mean* denotes the mean (average) value and *σ* the standard deviation.

2. INTENSITY VALUES

The measured intensity values, both raw and normalized, plotted in the "Extracted intensity response" graph, as well as their associated uncertainties, are displayed in the "Intensity values" table. The measured intensity values and their associated uncertainties correspond respectively to the average and to three times the standard deviation of the peak (maximum) intensities from the intensity profile perpendicular to the lines.

3. SECONDARY METRICS

- *Pattern dynamic range wrt detector bit depth* is the difference between the maximum and

minimum intensities, normalized with respect to the detector bit depth. It is expressed in %, according to the following formula:

$$Pattern\ dynamic\ range\ wrt\ detector\ bit\ depth = 100 \times \frac{I_{max} - I_{min}}{2^{Detector\ bit\ depth}}$$

- *a* is the slope of the linear regression curve. It is expressed in arbitrary unit.
- *b* is the y-intercept of the linear regression curve. It is expressed in arbitrary unit.

4. ALGORITHM METADATA

- *Analysis date* is the date at which the analysis has been performed.
- *Software version* is the version of the software.
- *Product type* is the type of Argolight product selected in the panel settings.
- *Angle value used for the orientation correction* is the angle value applied to the analyzed image to correct a small rotation/tilt of the pattern, usually due to camera or laser scanning misalignment in microscopes. This angle value can either be automatically calculated by some of the algorithms and/or previously set in the analysis settings. It is expressed in degree.
- *Background subtraction* indicates if the "Background subtraction" option has been activated or not.
- *Hot pixels removal* indicates if the "Hot pixels removal" option has been activated or not.
- *Best focus selection* indicates if the "Best focus selection" option has been activated or not.
- *Index of the selected image in the stack* indicates the index of the image in the stack that has been selected when activating the "Best focus selection" option.
- *Intensity projection* indicates if the "Intensity projection" option has been activated or not.
- *Intensity projection type* indicates the type (mean or maximum) of intensity projection when activating the "Intensity projection" option.
- *X coordinate of the ROI* is the coordinate along X (starting from the top left corner) of the cropped area in the image. A null value corresponds to an uncropped image. It is expressed in pixel.

- *Y coordinate of the ROI* is the coordinate along Y (starting from the top left corner) of the cropped area in the image. A null value corresponds to an uncropped image. It is expressed in pixel.

- *ROI width* is the width of the cropped area in the image. A value equal to the image width corresponds to an uncropped image. It is expressed in pixel.

- *ROI height* is the height of the cropped area in the image. A value equal to the image height corresponds to an uncropped image. It is expressed in pixel.

5. IMAGE METADATA

- *Acquisition date* is the date at which the acquisition of the image has been performed. If this information is not contained in the metadata of the image, then the note “unknown” is displayed.

- *Specified lateral pixel size* is the size of one pixel, provided by the metadata associated to the raw image. It is expressed in µm.

- *Specified axial pixel size* is the interval between each slice of the stack, provided by the metadata associated to the raw image. It is expressed in µm.

- *Image dynamic range* is the dynamic range of the image, provided by the metadata associated to the raw image. It is expressed in bits (8 or 16 bits).

- *Detector bit depth* is the data capturing range of the detector, provided by the metadata associated to the raw image. It is expressed in bits. For example, a 16-bit detector can capture 2^{16} = 65536 intensity levels.

- *Image width* is the width of the image, provided by the metadata associated to the raw image. It is expressed in pixel.

- *Image height* is the height of the image, provided by the metadata associated to the raw image. It is expressed in pixel.

6. COMPARISON PARAMETERS

- *Comparison grade* is one among many other parameters providing information about how close the extracted intensity response is from a reference one, previously saved as the reference response. It is expressed in %, according to the following formula:

$$Comparison\ grade\ = 100 \times \left[\left(1 - \frac{1}{16\sqrt{I_{norm}}}\right) \times \sum_{i=1}^{16} \sqrt{\left|I_{ref}(\mathrm{i}) - I_{extr}(i)\right|} \right]$$

Where $I_{ref}(i)$ and $I_{extr}(i)$ are the reference and extracted intensities, respectively, for the level index i, and I_{norm} is given by the following expression:

$$I_{norm} = \left[Max(I_{ref}) - Min(I_{ref})\right] + \left[Max(I_{extr}) - Min(I_{extr})\right]$$

- *Partial correlation coefficient (r')* is another parameter providing information about how close the extracted extracted intensity response is from the one previously saved as the reference response. It is unitless, and is given by the following formula:

$$r' = \frac{mean(Intensities_{ref} \times Intensities_{extr}) - mean(Intensities_{ref}) \times mean(Intensities_{extr})}{\sigma\left(Intensities_{ref}\right) \times \sigma(Intensities_{extr})}$$

Where *mean* denotes the mean (average) value and *σ* the standard deviation.

- *Pearson correlation coefficient (r) of the reference response* is the Pearson correlation coefficient of the reference intensity response, calculated the same way as the *Pearson coefficient (r) of the actual intensity response*.

- *Ratio between the slopes* is the ratio between the slopes of the linear regression curves from the extracted and reference responses. It is unitless.

- *Ratio between the intercepts* is the ratio between the y-intercepts of the linear regression curves from the extracted and reference responses. It is unitless.

VII. HOW TO CORRECT AN INTENSITY RESPONSE FLUCTUATION?

The intensity response fluctuation of a fluorescence microscope can have an impact on the intensity in images of a biological sample acquired at different times. To correct for it, one can use the comparison function within the “intensity response” analysis.

An intensity response fluctuation is relative, *i.e.* it is measured with respect to a reference intensity response. It is therefore necessary to acquire a reference image of an “intensity gradation” pattern at a judicious time t_0, in this case at the beginning of an imaging campaign of a biological sample. The imaging campaign, defined as the acquisition of several images of the same biological sample, can last one hour, one day, one week, one month, etc.

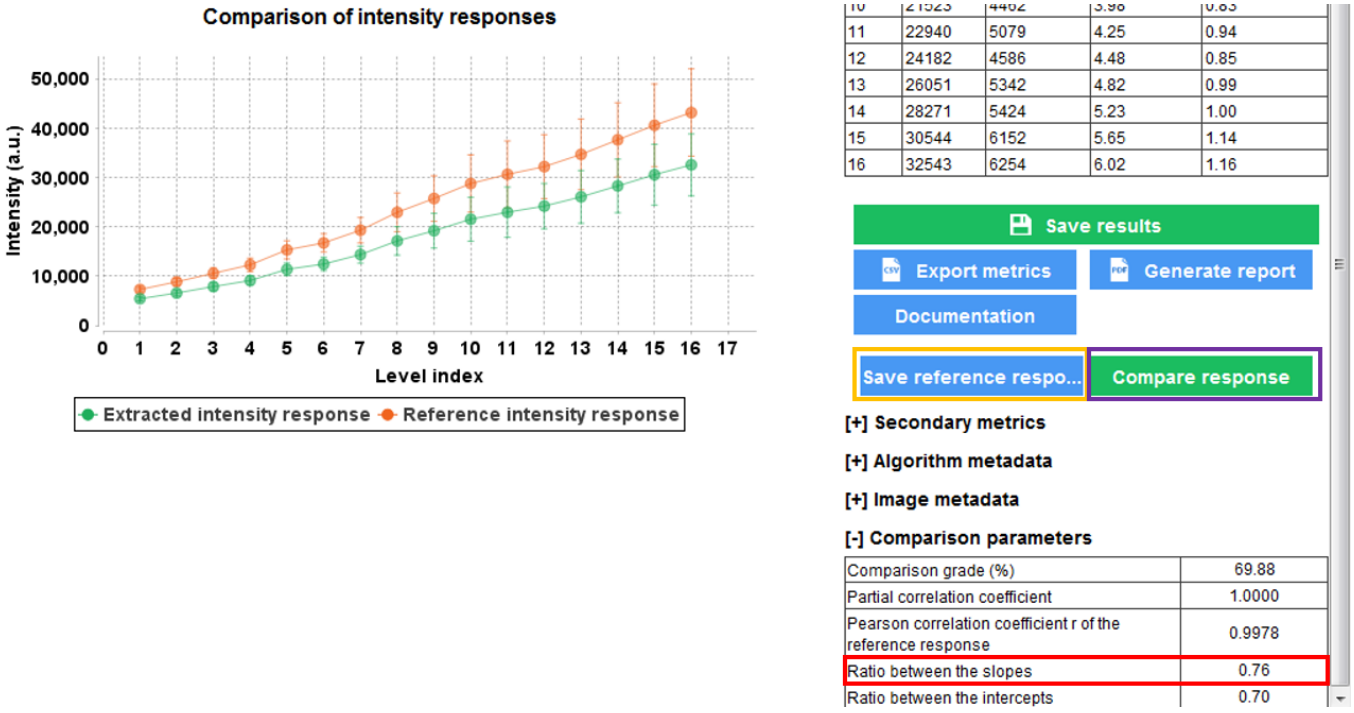


Figure 8: Relevant buttons and metric provided by the analysis to correct an intensity response fluctuation.

Here are the different steps to follow:

1. Acquire an image of an “intensity gradation” pattern at t_0. This image will be considered as the “reference image”.
2. From this “reference image”, perform an “intensity response” analysis. Save this response by clicking on the “Save reference response” button (orange frame in Figure 6). Note that the generated reference response will remain in the memory of Daybook Analysis until you save another reference response.
3. Acquire an image of the same “intensity gradation” pattern, using the same acquisition parameters (objective used, illumination power, exposure time, etc.) as for the reference image, just after acquiring the image of a biological sample to be corrected.
4. From this second image, perform an “intensity response” analysis. Compare this response to the reference one by clicking on the “Compare response” button (purple frame in Figure 6). Daybook Analysis displays the two responses (the reference one and the new extracted one) in the graph “Comparison of intensity responses”. The “Comparison parameters” table

displays the resulting metrics. Among them, the metric “ratio between the slopes” (red frame in Figure 6) can be used as a correction coefficient.

5. Multiply the image of a biological sample to correct by this coefficient, to compensate from a possible intensity response fluctuation of the microscope.

Note:

This method works better if the reference and extracted responses are generated from images which background has been subtracted (see the section III.2.2 “Background subtraction” for more details).

Encountered an issue or a question when using Daybook Analysis?
Please send a screenshot and your problem description to:
customer@argolight.com