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I. INTRODUCTION

This test concerns only the microscopes equipped with a motorized Z stage.

The point spread function (PSF) gives access to the spreading behavior of the light by a point-like object. Fitting the PSF with a mathematical function (Gaussian, Lorentzian, Sech²) allows to extract the full width at half maximum (FWHM) along X, Y and Z, which is a parameter that commonly describes the resolution of the imaging system.

The “point spread function” analysis provides information, in 3D, on how light spreads from a point-like object, as well as quantitative parameters such as the lateral and axial ***FHWM*** of the PSF and the ***signal-to-noise ratio (SNR)*** in the image.

II. IMAGE ACQUISITION PROCEDURE

The ***"point spread function"*** analysis is associated with any ***"point-like object"*** (see Figure 1).

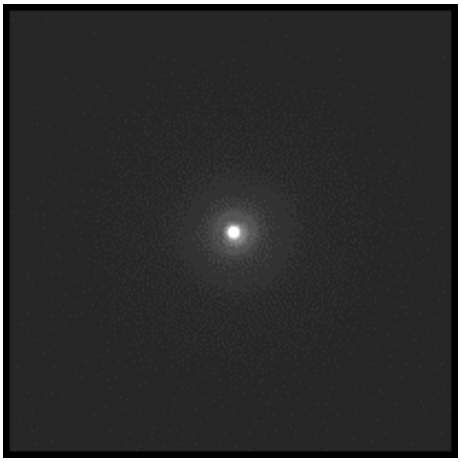


Figure 1: Example of an image of a "point-like object", fulfilling the acquisition recommendations.

1. ACQUISITION RECOMMENDATIONS

- **Recommended image type**

Z stack	Yes (mandatory)
Multi-channel	Recommended but not mandatory
Tiles	No

The Z-stack images should be acquired over a Z-range covering a distance larger than the size of the point-like object.
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.

- **Lateral pixel size**
The lateral pixel size of the Z-stack should be lower or equal to the half of the theoretical lateral resolution limit (Nyquist criterion). However, if possible, we recommend that you adjust the image lateral pixel size to one-third of the theoretical lateral resolution limit.

- **Axial pixel size (interval between each slice)**
The axial pixel size of the Z-stack should be equal to the half of the theoretical axial resolution limit (Nyquist criterion). However, if possible, we recommend adjusting the image axial pixel size to one-third of the theoretical axial resolution limit.

2. HOW TO IMAGE THE OBJECT OF INTEREST?

1- <u>Find the object of interest</u>

a) Start with a low mag objective (such as 10× or 20×).
b) Align the center of the slide with the objective.
c) Adjust focus through the eyepieces.
d) Switch to the objective you would like to use. Move the slide to the object of interest.

2- <u>Adjust your setup</u>

a) Match a single point-like object with the center of the field of view.
b) Adjust the focus.

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

3- <u>Image the object of interest and save the image</u>

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

III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

a) Select “Point spread function” 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) Click on “Start the analysis”.

e) Select within a region of interest (ROI) a single point-like object and click on “Crop” to crop the image (*cf.* Figure 2).

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

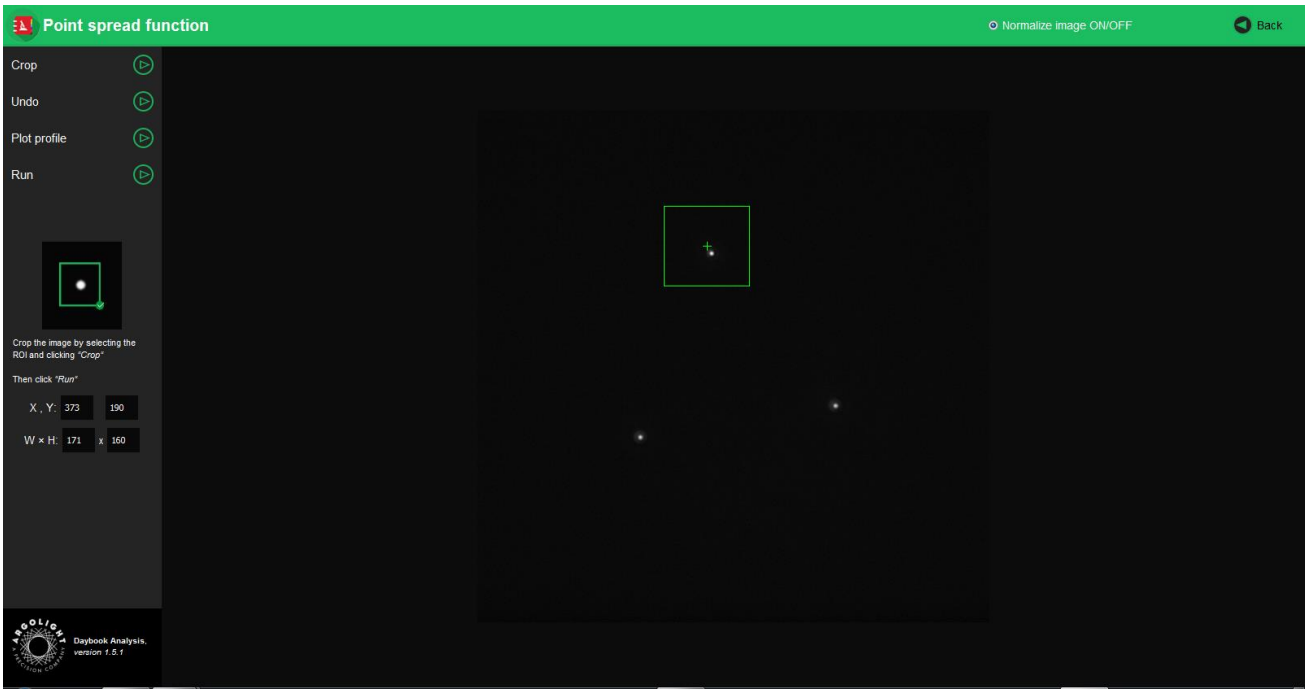


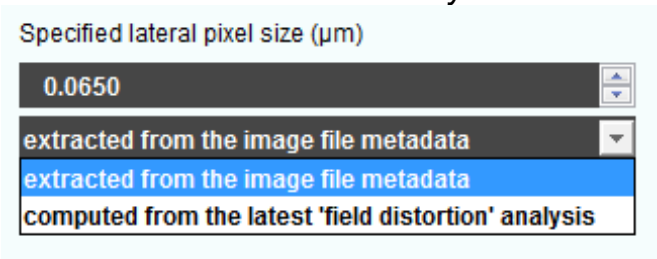
Figure 2: Crop window to select a point-like object.

2. ANALYSIS SETTINGS

1- <u>Required settings</u>

- **Specified lateral pixel size**
  There are two ways to get the lateral pixel size of the image to be analyzed:

- Either from the image file metadata:
Select *"extracted from the image file metadata"*.
- Or from a previous *"field distortion"* analysis:
Select *"computed from the latest 'field distortion' analysis"*.



- **Fitting model type**
Select the type of mathematical model used in the fitting of the raw intensity line profiles:
  - Single Gaussian → One Gaussian function.
  - Single Lorentzian → One Lorentzian function.
  - Single Sech² → One hyperbolic secant squared function.

- **PSF processed ROI width**
The PSF processed ROI width is the width of the Region Of Interest (ROI).

2- <u>Optional settings</u>

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

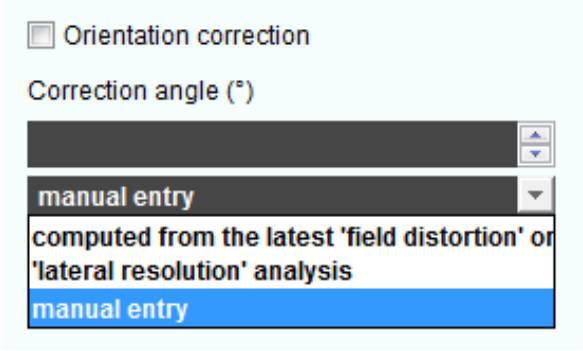
- **Hot pixels removal**
Remove the very intense (*i.e.* hot) pixels that may cause analysis issues.
Use this option only if you have such hot pixels in the image.

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

- **Best focus selection**
This 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 object of interest.
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.

- **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.



3- <u>“Redo the fit” window</u>

To optimize or to check the influence of the fitting parameters (interpolation factor, smoothing factor, low threshold, X and Y centroid location, ROI length and width) on the results, you can click on the “Redo” button in the results page and re-run the analysis (*cf.* Figure 3).

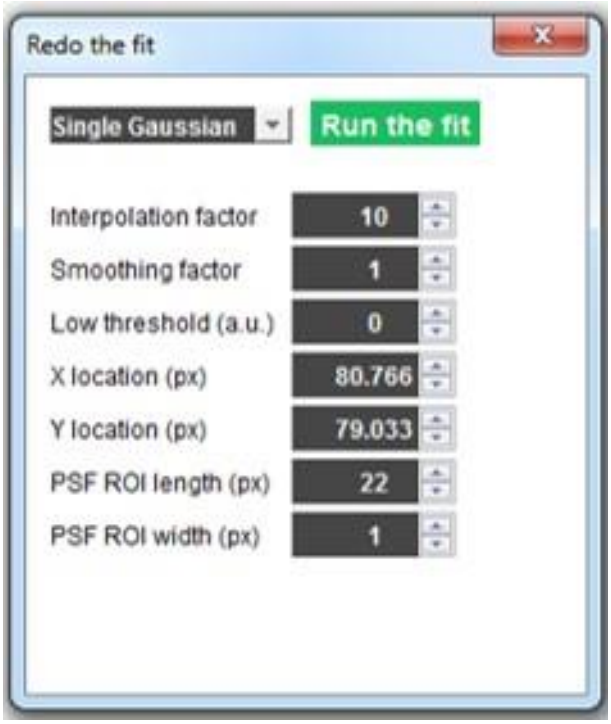


Figure 3: “Redo the fit” window.

- **Interpolation factor**

The interpolation factor, also known as resampling factor, allows the increase of the number of points of the intensity line profile, by applying a bicubic interpolation.
It is automatically calculated when launching the analysis, but it can also be set manually in the “Redo the fit” window.
An interpolation factor of 1 does not change the data.

- **Smoothing factor**
  The smoothing factor allows to improve the peak detection before the fitting, in the presence of noise.
  It is set at 1 by default.
  A smoothing factor of 1 does not change the data.

- **Low threshold**
  The low threshold is the intensity value above which the experimental data are fitted. If it is set at zero, all the data are fitted.
  It is set at 0 by default.

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.

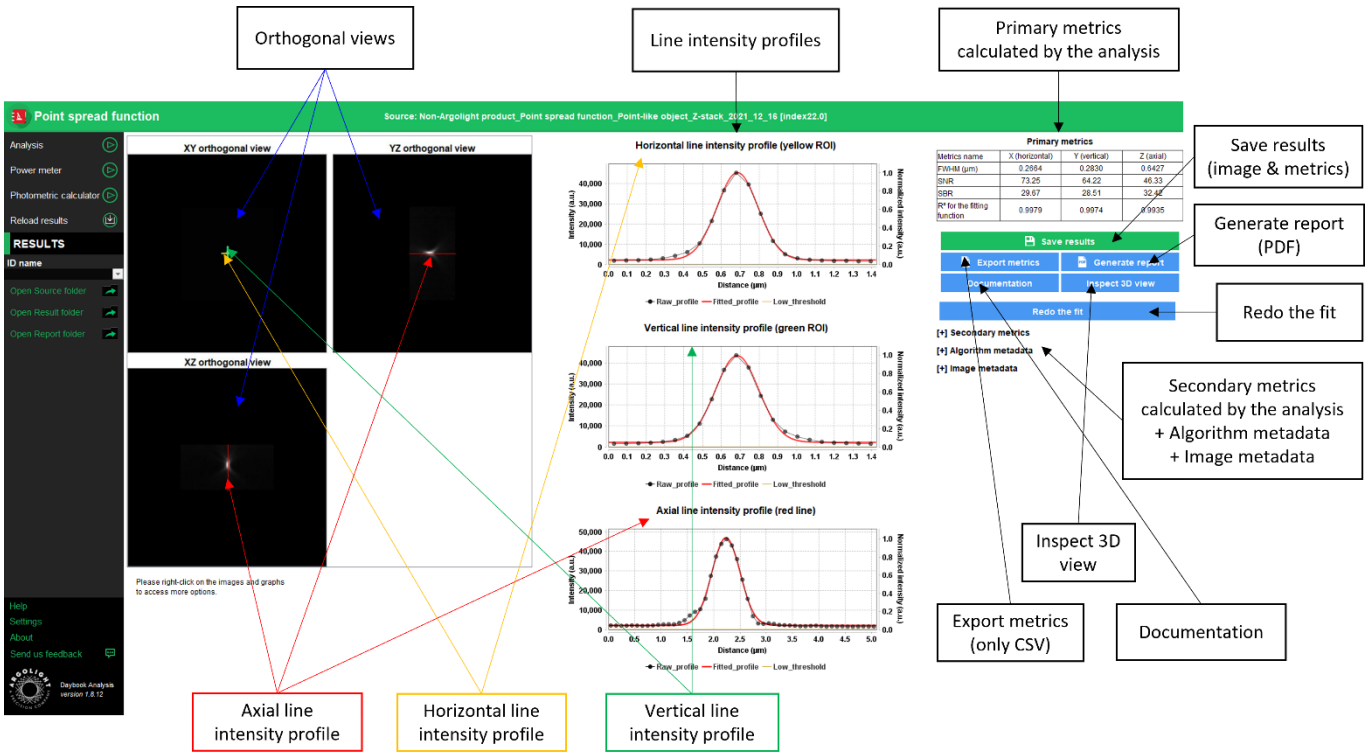


Figure 4: Results page.

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).

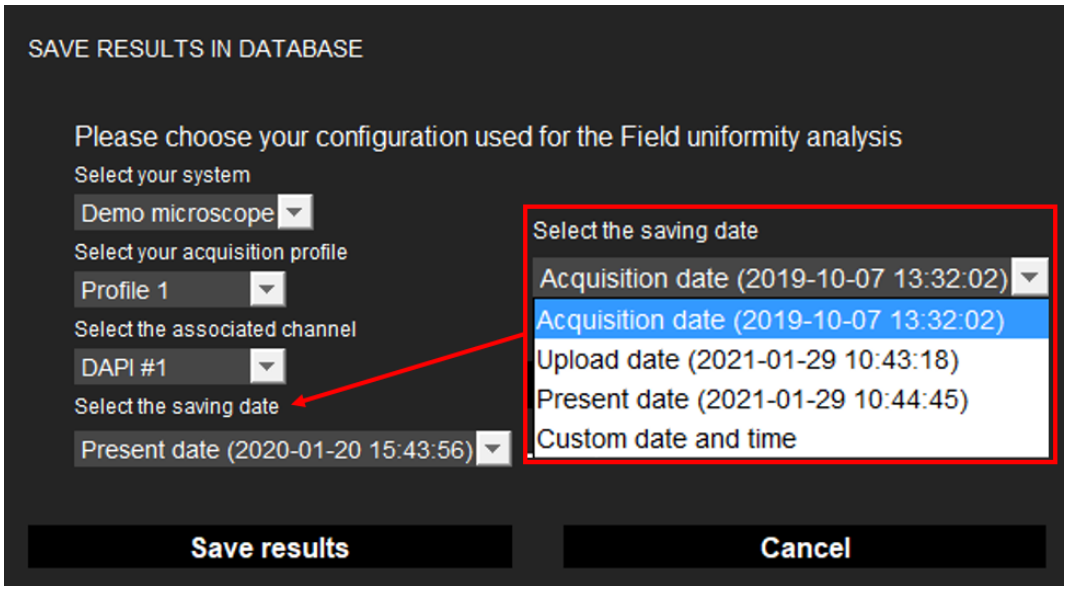


Figure 5: Interface window for saving the results in the database.

- **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.

- **Redo the fit:**

It is possible to redo the fit with other parameters than the default ones. These parameters are:

- The ***fitting model***:
  - Single Gaussian → One Gaussian function.
  - Single Lorentzian → One Lorentzian function.
  - Single Sech² → One hyperbolic secant squared function.
- The ***interpolation factor***: change (increase or decrease) the number of values of the fit, and therefore make it appropriately sampled. The interpolation is bicubic.
- The ***smoothing factor***: in the presence of noise, improve the peak detection before the fitting.
- The ***low threshold***: change the intensity value above which the experimental data are fitted.

- The ***X and Y locations***: change the central locations of the profiles, in the X (horizontal) and Y (vertical) directions. Use the position indication cursor in the raw image to guess these values.
- The ***PSF ROI height and width***: change the size of the green and yellow regions of interest (ROI) in the raw image.

V. ANALYSIS ALGORITHM DESCRIPTION

1. DIAGRAM

The diagram below describes the algorithm that allows the extraction of the point spread function from a “point-like object” image (*cf.* Figure 6).

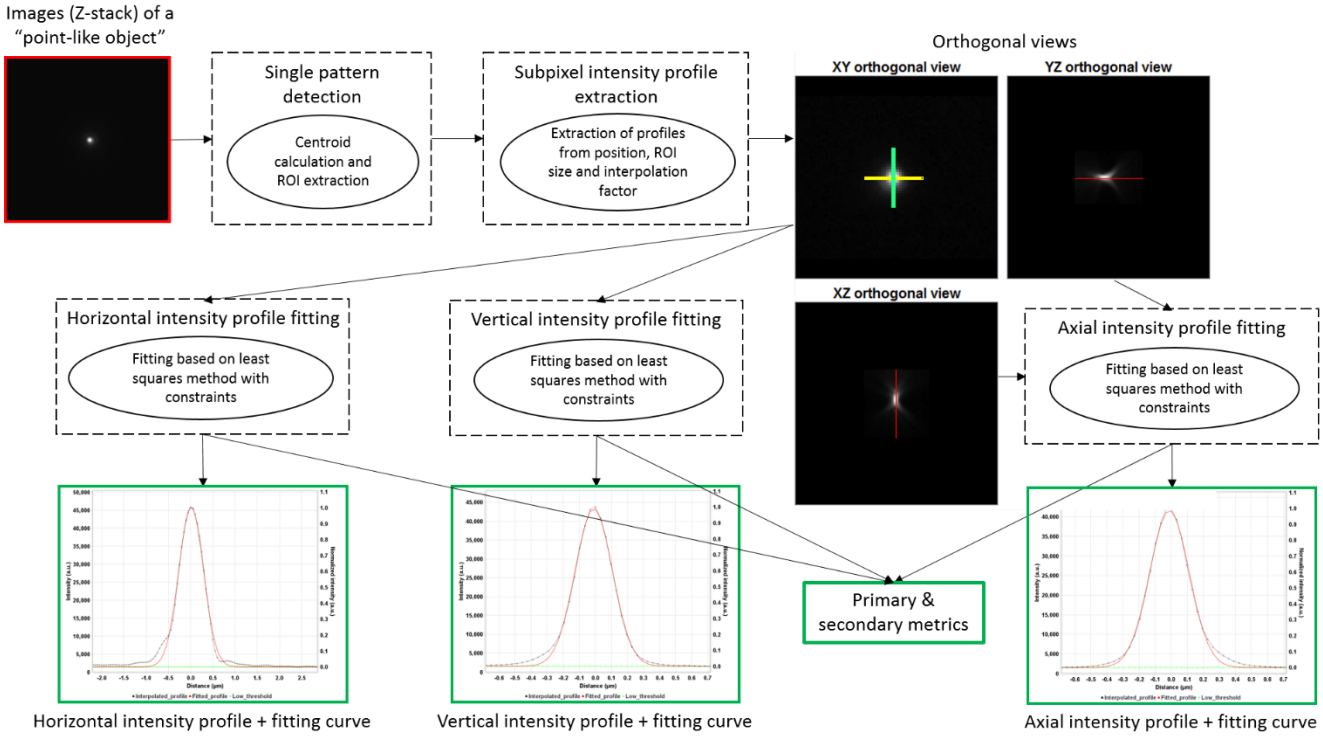


Figure 6: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It detects and segments the point-like object in the image.
- It plots three intensity line profiles, passing through the centroid of the point-like object, in the horizontal (green), the vertical (yellow) and the axial (red) directions.
- It fits the horizontal, vertical and axial intensity line profiles with the selected mathematical model:
    - “Single Gaussian”
    - “Single Lorentzian”
    - “Single Sech²”

    An automatic bicubic interpolation is applied, so that there are about 200 points per fitting function.
- It displays the raw intensity line profiles and the fitting functions into three graphs.

See below a description of the different mathematical models used to fit the intensity line profiles. The same equations are used for the Y and Z directions, with x switched for y and z, respectively.

Single Gaussian:

$$I(x) = I_{offset} + I_0\, exp\left[-4\, ln(2)\left(\frac{x - x_0}{FWHM_x}\right)^2\right]$$

Single Lorentzian:

$$I(x) = I_{offset} + I_0 \frac{1}{1 + \left(\frac{x - x_0}{0.5\, FWHM_x}\right)^2}$$

Single Sech²:

$$I(x) = I_{offset} + I_0\, sech^2\left[\frac{2\, arccosh\left(\sqrt{2}\right)\,(x - x_0)}{FWHM_x}\right]$$

The following parameter is set at a fixed value:
x_0: position of the peak.

The following parameters are set as free:
$\boldsymbol{I_{offset}}$: offset value.
$\boldsymbol{I_0}$: magnitude of the peak.
$FWHM_x$: full width at half-maximum.

VI. OUTPUT METRIC DESCRIPTION

The primary and secondary metrics are given for the X, Y and Z axes.

1. PRIMARY METRICS

- *FWHM* is the full width at half-maximum of each fitting function. It is expressed in µm.

- *SNR* is the signal-to-noise ratio. There are different ways to define it. Here, it is defined as the ratio between the normalized intensity maximum (*i.e.* 1 a.u.) and the RMSE for the fitting function, defined hereinafter. It is unitless and is given by the following equation:

$$SNR = \frac{1}{RMSE}$$

- *SBR* is the signal-to-background ratio, *i.e.* the ratio between the intensity maximum and the offset value. It is unitless.

- *R squared for the fitting function* is the coefficient of determination between the fitting function and the experimental data in the vertical and horizontal intensity line profiles. It is another measurement of the goodness of the fit. It is unitless and is given by the following equation:

$$R^2 = 1 - \frac{\sum_{i=1}^{n}\left(I_{measured\_i} - I_{fit\_i}\right)^2}{\sum_{i=1}^{n}\left(I_{measured\_i} - I_{mean}\right)^2}$$

Where $I_{measured\_i}$ is the measured intensity value of the ith experimental point, I_{fit_i} the intensity value of the ith experimental point from the fitting function, and I_{mean} the mean (average) intensity value.

2. SECONDARY METRICS

- *Position of the curve's peak* is the X position of the fitting function. It is expressed in pixel.

- *Magnitude of the curve's peak* is the magnitude of the fitting function. It is expressed in arbitrary unit.

- *Offset value* is the offset value used for the fitting. It is expressed in arbitrary unit.

- *Intensity maximum* is the maximum intensity in the raw image. It is expressed in arbitrary unit.

- *Intensity minimum* is the minimum intensity in the raw image. It is expressed in arbitrary unit.

- *RMSE for the fitting function* is the root mean square error between the fitting function and the experimental data in the vertical and horizontal intensity line profiles. It is a measurement of the goodness of the fit. It is expressed in arbitrary unit and is given by the following equation:

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(I_{measured\_i} - I_{fit\_i}\right)^2}$$

Where *n* is the number of experimental points, $I_{measured\_i}$ the measured intensity value of the ith experimental point and I_{fit_i} the intensity value of the ith point from the fitting function.

3. 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 object of interest, 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.
- *Fitting model type* is the type of mathematical model used in the fitting of the raw intensity line profiles: “Single Gaussian”, “Single Lorentzian”, or “Single Sech²”.
- *Interpolation factor* is the factor used to interpolate the horizontal, vertical and axial intensity line profiles. It is unitless, and automatically calculated according to the following formula:

  $$Interpolation\ factor = \text{Round up}\left\{\frac{200\ (number\ of\ fitting\ points\ ;px)}{PSF\ ROI\ length\ (px)}\right\}$$

  It can also be set manually in the “Redo the fit” window.
- *Smoothing factor* is the factor set to smooth the horizontal and vertical intensity line profiles. It is unitless.
- *Low threshold* is the threshold below which the raw data are not fitted. It is expressed in arbitrary unit.

- *PSF ROI length* is the length set for the region of interest within the “point-like object”. It is expressed in pixel.

- *PSF ROI width* is the width set for the region of interest within the “point-like object”. It is expressed in pixel.

- *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.

4. 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.

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