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I. INTRODUCTION

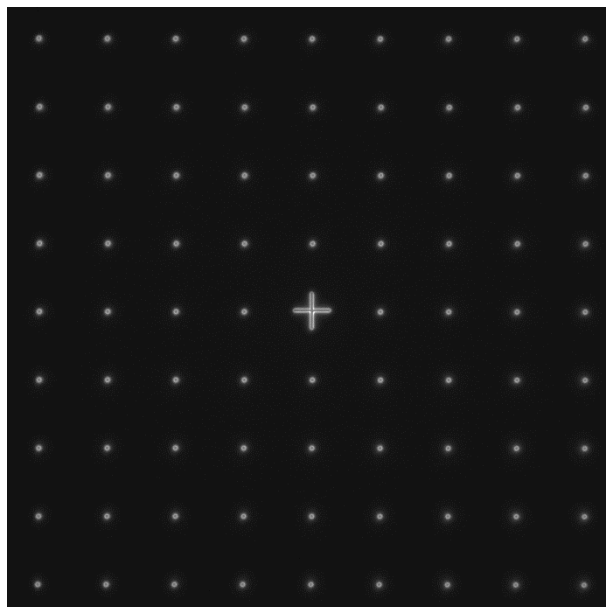
The ring spread function (RSF) gives access to the spreading behavior of the light by a ring object. Fitting the RSF with a mathematical function (double Gaussian, double Lorentzian, double Sech²) allows to extract the full width at half maximum (FWHM), which is a parameter related to the lateral resolution of the imaging system.

The “ring spread function” analysis provides information, in the XY plane, on how light spreads from a ring, as well as quantitative parameters such as the **FWHM** of the RSF and the **signal-to-noise ratio (SNR)** value in the image.



II. IMAGE ACQUISITION PROCEDURE

The “**ring spread function**” analysis is associated with the “**field of rings**” pattern (Pattern B - see Figure 1), or any **single ring** within any pattern.



CAUTION

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

Figure 1: Image example of the “field of rings” pattern, with a cross at the center, fulfilling the acquisition recommendations.

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.

- Alignment prior image acquisition**

Align precisely the detector orientation and/or the scanning with respect to the XY translation stage. The analysis, however, can correct a low XY orientation misalignment (a few degrees).

- Order of acquisition for different objectives**

If you would like to image the pattern with different objectives, we recommend starting to acquire images with the objective that has the highest magnification (e.g. 100×) then with the smallest magnification objective (e.g. 20×).





- **Signal-to-background ratio (SBR)**

Acquire images with enough contrast between the pattern and the background, e.g. a signal-to-background ratio higher than 2:1.

- **Signal-to-noise ratio (SNR)**

Acquire images with enough contrast between the pattern and the noise, e.g. a signal-to-noise ratio higher than 10:1.

- **Image intensity**

Acquire images within the linear response range of the detector, that is above the detection limit and below the saturation limit. If available in the acquisition software, use the color-coded pixels to adjust properly the image intensity. Note that Daybook Analysis cannot analyze images containing negative values.

- **Image dynamic range**

When possible, acquire images with a detector that captures raw data with a bit depth of 8 or 16 bits, the allowed image dynamic range for computers (1-byte and 2-byte chunks, respectively). If the detector captures raw data with a bit depth different from 8 or 16 bits, convert the images into 8- or 16-bit-dynamic range without losing any information. Note that if the image file weight is too big for the computational capacity of your computer, the analysis may not succeed.

- **Image sampling rate**

The sampling rate of the image should fulfill the Nyquist criterion, *i.e.* the image pixel size should be at least the half of the theoretical resolution limit. However, if possible, we recommend adjusting the image pixel size to one third of the theoretical resolution limit.

2. HOW TO IMAGE THE PATTERN?

1- Find the patterns

- a) Start with a low mag objective (such as 10x or 20x). 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 central cross 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 central cross looks the clearest (qualitative approach) and/or for which the intensity histogram is the broadest (quantitative approach).

3- Image your pattern

- a) Image the pattern by following the acquisition recommendations.
- b) Save images into a raw, non-compressed format (the best is the acquisition software proprietary format) or into a lossless compression format (e.g. TIFFs can be lossless, depending on the technique chosen for storing the pixel data). The image file must have a dynamic range of 8 or 16 bits.





III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

- a) Select “Ring spread function” in the “Select analysis” list.

Ring spread function Start the analysis ☒ Normalized image On/Off

- b) Upload your image(s) using the “Upload file” button.
Select the image to be analyzed.
- c) Set the required and optional settings (see chapter 2 “Analysis Settings”).
- d) Click on “Start the analysis”.
- e) Select within a region of interest (ROI) a single ring 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).

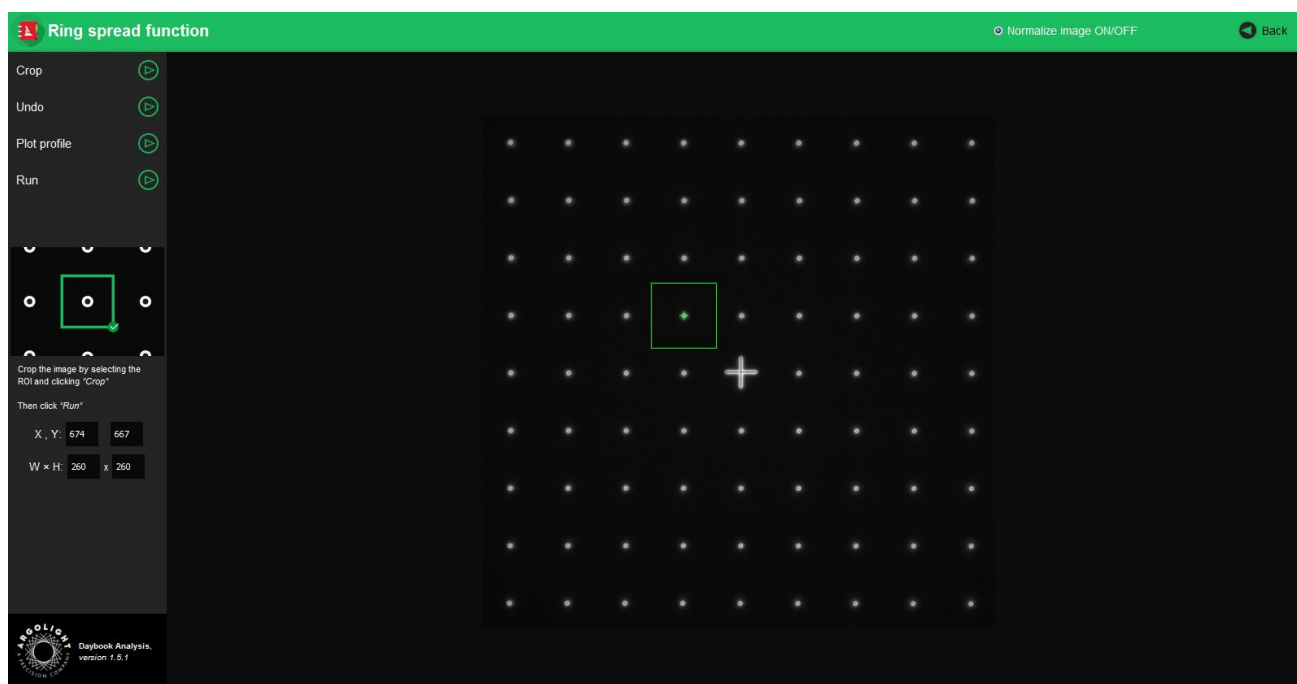


Figure 2: Crop window to select a single ring.

2. ANALYSIS SETTINGS

1- Required settings





- **Specified lateral pixel size**

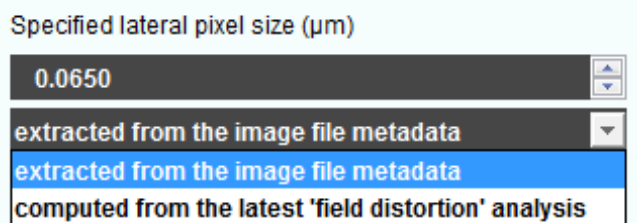
There are two ways to get the lateral pixel size of the image to be analyzed:

- 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*”.



- **Fitting model type**

Select the type of mathematical model used in the fitting of the raw intensity line profiles:

- Double Gaussian (*) → Sum of two Gaussian functions, which the magnitude of one of them is set to the maximum of the intensity profile.
- Double Lorentzian (*) → Sum of two Lorentzian functions, which the magnitude of one of them is set to the maximum of the intensity profile.
- Double Sech² (*) → Sum of two hyperbolic secant squared functions, which the magnitude of one of them is set to the maximum of the intensity profile.
- Double Gaussian → Sum of two Gaussian functions.
- Double Lorentzian → Sum of two Lorentzian functions.
- Double Sech² → Sum of two hyperbolic secant squared functions.

The (*) symbol means that the magnitude of one of the two mathematical functions is set to the maximum value of the intensity profile.

- **RSF processed ROI width**

The RSF processed ROI width is the width of the Region Of Interest (ROI).

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 background 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**





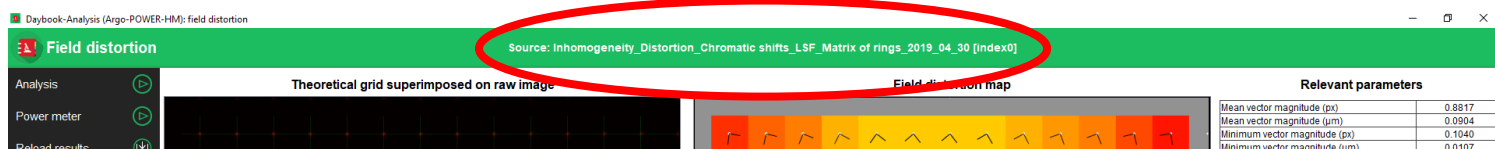
On Z-stacks analysis, the axial pixel size is determined from the proprietary file.

- **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.

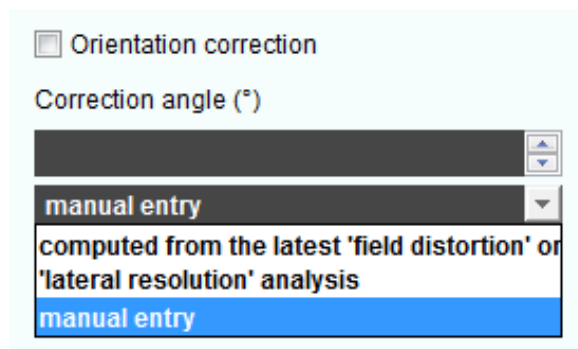


- **Orientation correction**

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- “Redo the fit” window

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).

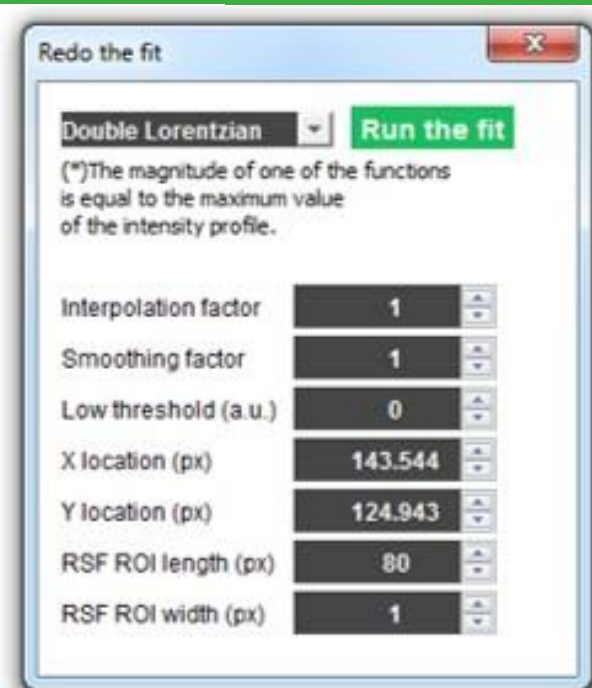


Figure 3: “Redo the fit” window.

- **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 linear 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.

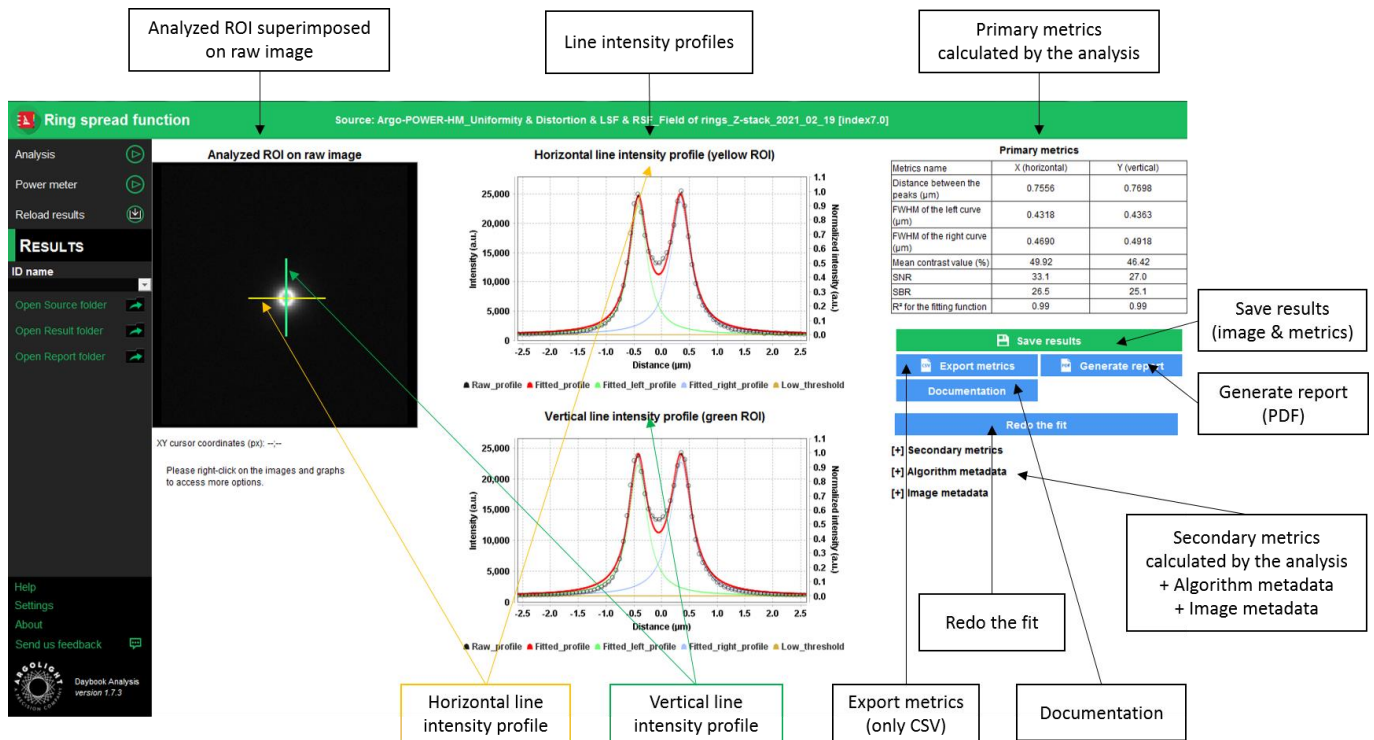


Figure 4: Results page.

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 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, acquisition profile and associated channel for which you would like to save the results.

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).

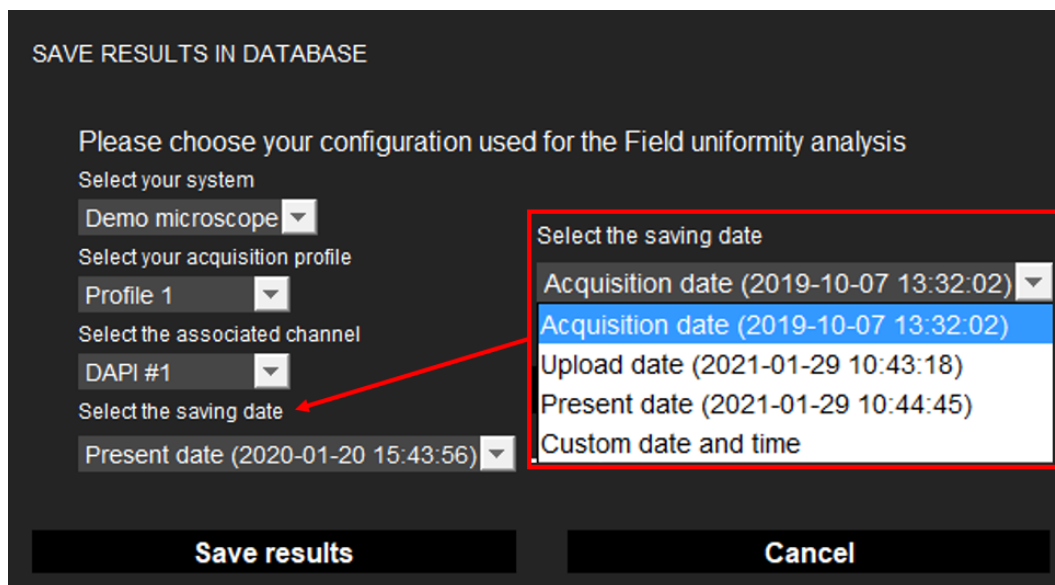


Figure 5: Interface window for saving the results in the database.

- **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**:
 - Double Gaussian (*) → Sum of two Gaussian functions, which the magnitude of one of them is set to the maximum of the intensity profile.
 - Double Lorentzian (*) → Sum of two Lorentzian functions, which the magnitude of one of them is set to the maximum of the intensity profile.





- Double Sech² (*) → Sum of two hyperbolic secant squared functions, which the magnitude of one of them is set to the maximum of the intensity profile.
- Double Gaussian → Sum of two Gaussian functions.
- Double Lorentzian → Sum of two Lorentzian functions.
- Double Sech² → Sum of two hyperbolic secant squared functions.

The (*) symbol means that the magnitude of one of the two mathematical functions is set to the maximum value of the intensity profile.

- The **interpolation factor**: change (increase or decrease) the number of values of the fit, and therefore make it appropriately sampled. The interpolation is linear.
- 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 **RSF 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 ring spread function from a ring image (*cf.* Figure 6).

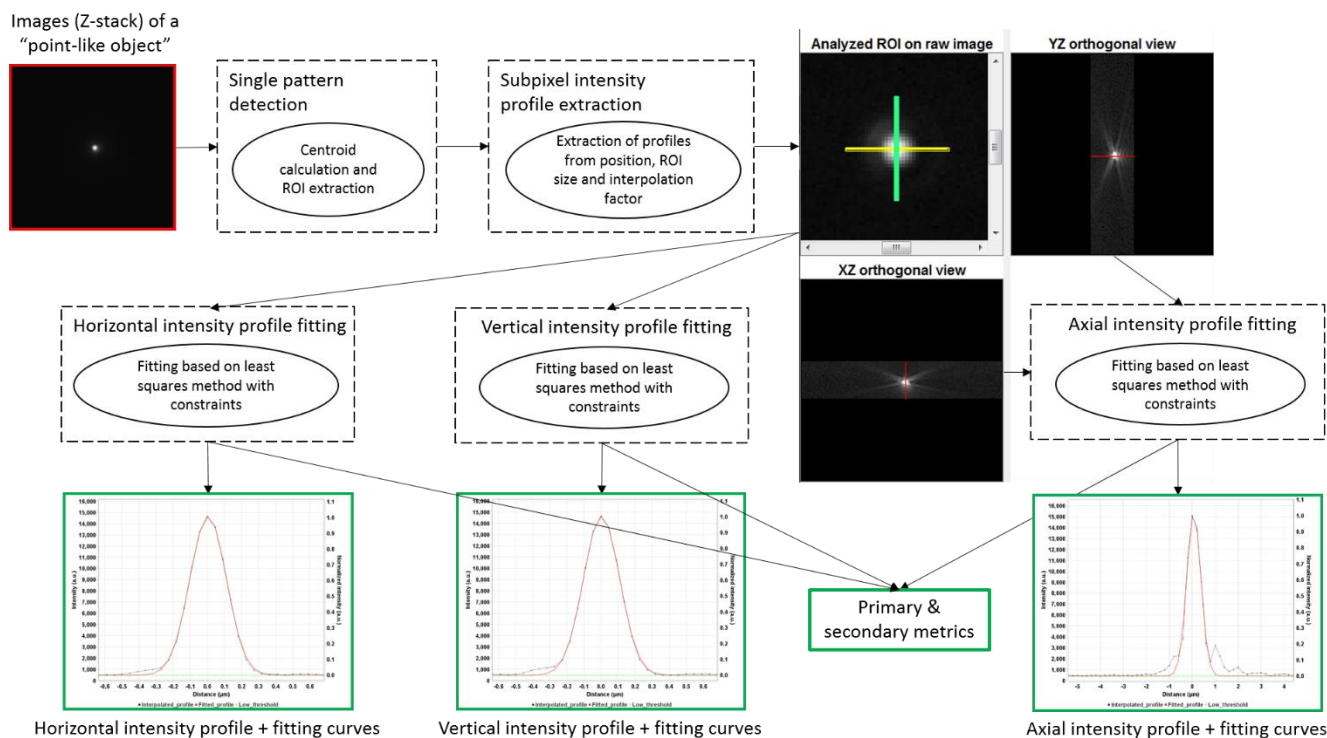


Figure 6: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It detects the ring.
- It plots one intensity line profile, both in the horizontal (green) and the vertical (yellow) directions.
- It fits both the horizontal and vertical intensity line profiles with the selected mathematical model:
 - “Double Gaussian (*)”
 - “Double Lorentzian (*)”
 - “Double Sech² (*)”
 - “Double Gaussian”
 - “Double Lorentzian”
 - “Double Sech²”

The (*) symbol means that the magnitude of one of the two mathematical functions is set to the maximum value of the intensity profile.





- It plots the raw intensity line profiles and the fitting functions into two 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.

Double Gaussian:

$$I(x) = I_{offset} + I_1 \exp \left[-4 \ln(2) \left(\frac{x - x_1}{FWHM_{x_1}} \right)^2 \right] + I_2 \exp \left[-4 \ln(2) \left(\frac{x - x_2}{FWHM_{x_2}} \right)^2 \right]$$

Double Lorentzian:

$$I(x) = I_{offset} + I_1 \frac{1}{1 + \left(\frac{x - x_1}{0.5 FWHM_{x_1}} \right)^2} + I_2 \frac{1}{1 + \left(\frac{x - x_2}{0.5 FWHM_{x_2}} \right)^2}$$

Double Sech²:

$$I(x) = I_{offset} + I_1 \operatorname{sech}^2 \left[\frac{2 \operatorname{arccosh}(\sqrt{2}) (x - x_1)}{FWHM_{x_1}} \right] + I_2 \operatorname{sech}^2 \left[\frac{2 \operatorname{arccosh}(\sqrt{2}) (x - x_2)}{FWHM_{x_2}} \right]$$

The following parameters are set as free:

I_{offset} : offset value.

I_1 : magnitude of the left curve's peak.

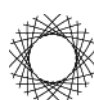
I_2 : magnitude of the right curve's peak.

x_1 : position of the left curve's peak.

x_2 : position of the right curve's peak.

$FWHM_{x_1}$: full width at half-maximum of the left curve.

$FWHM_{x_2}$: full width at half-maximum of the right curve.





VI. OUTPUT METRIC DESCRIPTION

The primary and secondary metrics are given for both vertical and horizontal directions.

1. PRIMARY METRICS

- *Distance between the peaks* is the distance between the two peaks of the fitting function. It is expressed in μm .
- *FWHM of the left curve* is the full width at half-maximum of the left fitting function.
- *FWHM of the right curve* is the full width at half-maximum of the right fitting function.
- *Mean contrast value* is a measure of the contrast between the mean (average) of the two peaks magnitude $\overline{I_{peak}}$ and the valley magnitude I_{valley} . It is expressed in % and is given by the following equation:

$$Contrast = 100 \times \frac{\overline{I_{peak}} - I_{valley}}{\overline{I_{peak}}}$$

- *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:

$$R^2 = 1 - \frac{\sum_{i=1}^n (I_{measured_i} - I_{fit_i})^2}{\sum_{i=1}^n (I_{measured_i} - I_{mean})^2}$$

Where $I_{measured_i}$ is the measured intensity value of the i^{th} experimental point, I_{fit_i} the intensity value of the i^{th} experimental point from the fitting function, and I_{mean} the mean (average) intensity value.

3. SECONDARY METRICS



- *Position of the left curve's peak* is the X position of the left double fitting function. It is expressed in pixel.
- *Position of the right curve's peak* is the X position of the right double fitting function. It is expressed in pixel.
- *Magnitude of the left curve's peak* is the magnitude of the left fitting function. It is expressed in arbitrary unit.
- *Magnitude of the right curve's peak* is the magnitude of the right 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:

$$RMSE = \sqrt{\frac{1}{n} \sum_{i=1}^n (I_{measured_i} - I_{fit_i})^2}$$

Where n is the number of experimental points, $I_{measured_i}$ the measured intensity value of the i^{th} experimental point and I_{fit_i} the intensity value of the i^{th} point from the fitting function.

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.
- *Fitting model type* is the type of mathematical model used in the fitting of the raw intensity line profiles: “Double Gaussian (*)”, “Double Lorentzian (*)”, “Double Sech² (*)”, “Double Gaussian”, “Double Lorentzian”, or “Double Sech²”. The (*) symbol means that the magnitude of one of the two mathematical functions is set to the maximum value of the intensity profile.
- *Interpolation factor* is the factor used to linearly interpolate the horizontal and vertical intensity line profiles. It is unitless, and automatically calculated according to the following formula:

$$\text{Interpolation factor} = \text{Round up} \left\{ \frac{800 (\text{number of fitting points ; px})}{\text{RSF ROI length (px)}} \right\}$$

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.
- *RSF ROI length* is the length set for the region of interest within the “ring” pattern. It is expressed in pixel.
- *RSF ROI width* is the width set for the region of interest within the “ring” pattern. 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.

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.





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

