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I. INTRODUCTION

Optical sectioning is the ability of an imaging system to distinguish in-focus signal from out-of-focus background, so that it can acquire clear slices of a thick object.

The metric used to quantify this is the optical sectioning strength, also called depth discrimination strength, optical slice thickness or depth of field (in wide-field microscopy). It does not have to be mixed up with the axial resolution, that is the minimal distance between two objects on top of each other that an imaging system can clearly measure, although these two metrics are related but not equivalent.

There are different ways to measure the optical sectioning strength. The method used in this analysis relies on the optical sectioning function, *i.e.* the measured contrast versus the specified distance between lines on the same plane and lines going deeper and deeper in the glass.

The “optical sectioning strength” analysis provides the ***optical sectioning strength*** between lines at different depths, for a given ***contrast*** value.

II. IMAGE ACQUISITION PROCEDURE

The ***"optical sectioning strength"*** analysis is associated with the ***"matrix of crosses"*** pattern (Pattern family F - see Figure 1).

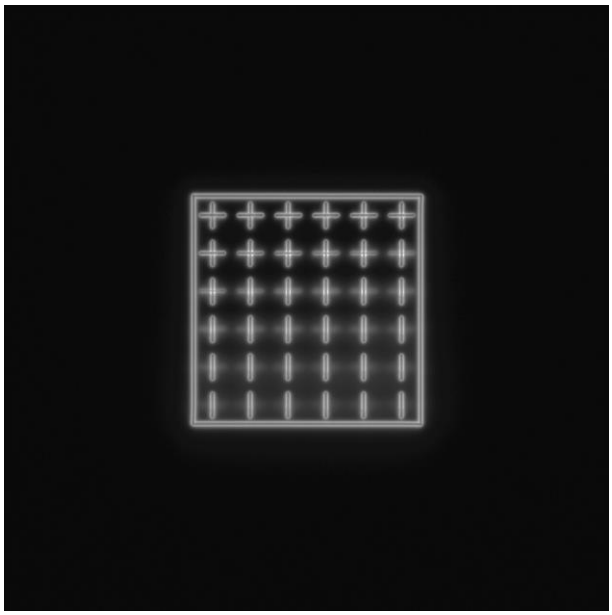


Figure 1: Example of an image of the "matrix of crosses" pattern, fulfilling the acquisition recommendations.

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.

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.

- **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 that you adjust the image axial pixel size to one-third of the theoretical axial resolution limit.

2. HOW TO IMAGE THE PATTERN?

1- <u>Find the patterns</u>

a) Start with a low mag objective (such as 10× or 20×). Set the DAPI (405 nm) or GFP (488 nm) channel.
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 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 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.

Important:
This pattern must be imaged entirely. Do not zoom into the pattern.
If the pattern is not imaged entirely, the analysis will not work.

III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

a) Select “Optical sectioning strength” 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) If needed, select a region of interest (ROI) 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).

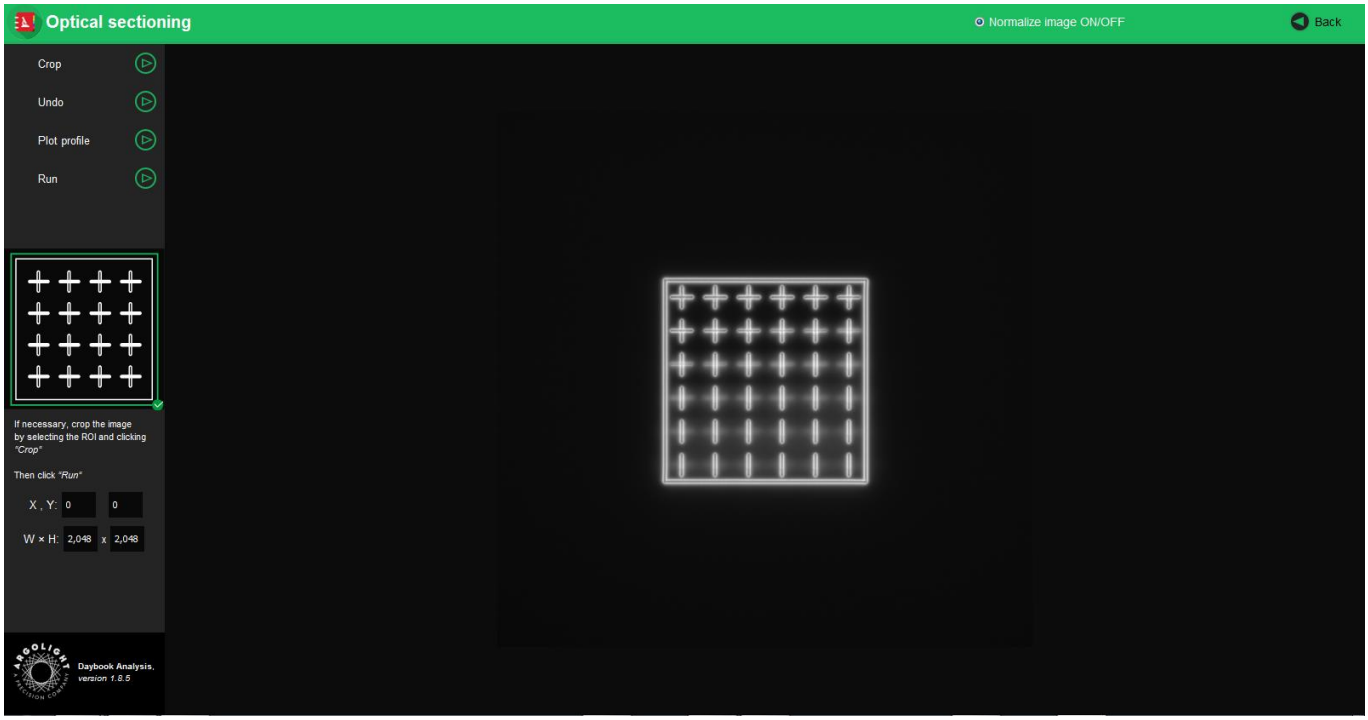


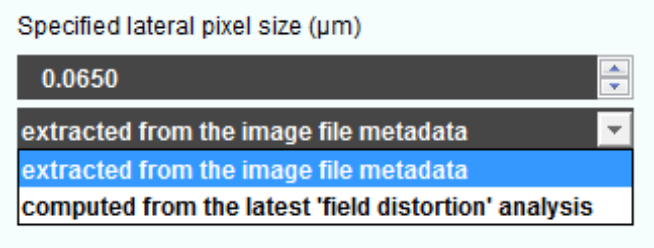
Figure 2: Crop window.

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"*.



- **Objective immersion medium**
Select the objective immersion medium used during the acquisition of the images. The theoretical refractive index (RI) of the immersion medium is used to calculate the expected dimensions along the Z axis.

- **Contrast criterion**
The user can define here the contrast value that he would like to apply, from 0 to 100 %. There are many criteria (Rayleigh → 26.5 %, Schuster → 99.9 %, Sparrow → 0.1 %, etc.) that can be applied to determine the optical sectioning strength.

2- Optional settings

- **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 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**
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 containing all the vertical lines of the crosses in the same plane.
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.

Optical sectioning strength

IV. RESULTS PAGE DESCRIPTION

1. INTERFACE

The picture below shows the results page for this analysis (*cf.* Figure 3).
Results are displayed in the form of images, maps, graphs and tables.

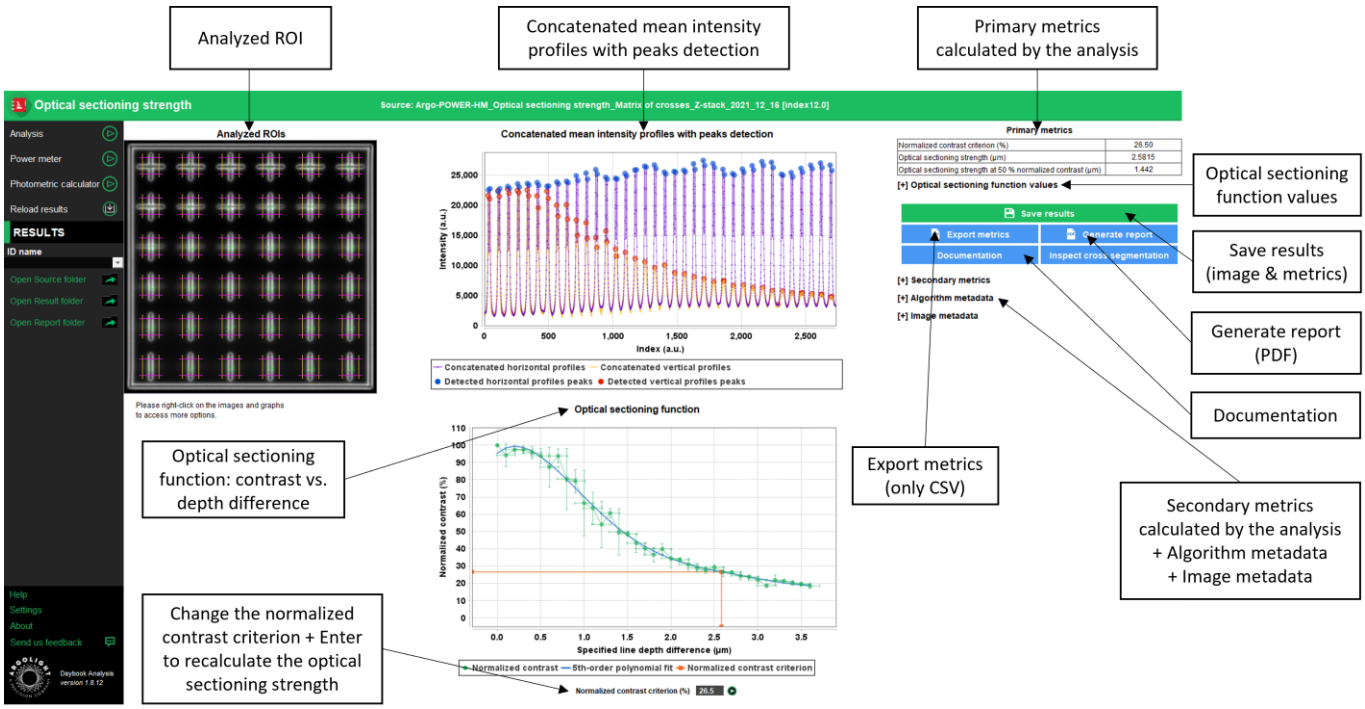


Figure 3: Results page.

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 3).

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 4).

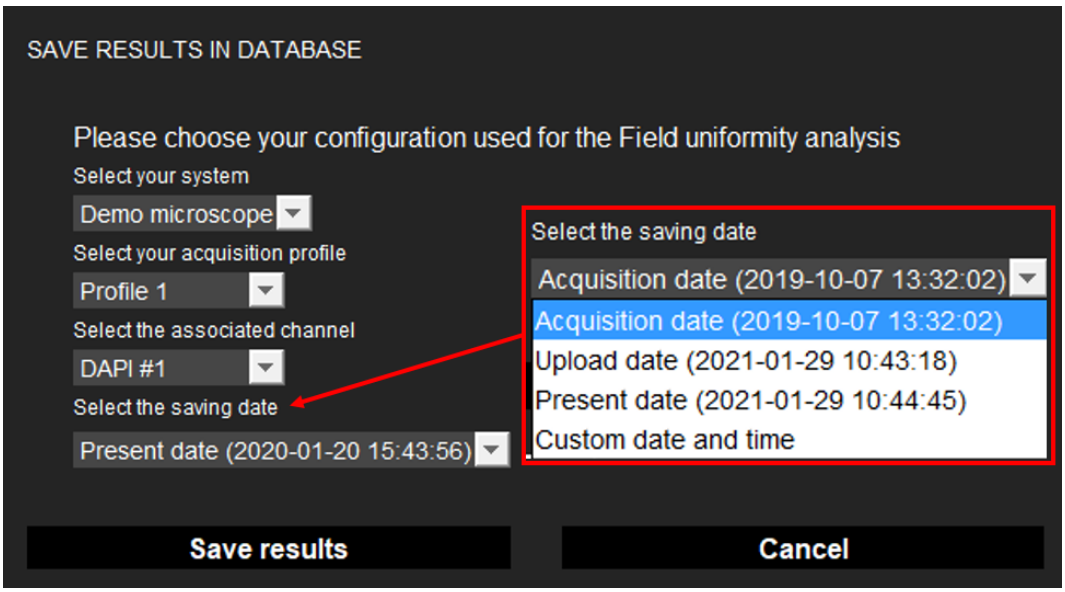


Figure 4: Interface window for saving the results in the database.

- **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.

V. ANALYSIS ALGORITHM DESCRIPTION

1. DIAGRAM

The diagram below describes the algorithm that allows the extraction of the optical sectioning strength from the "matrix of crosses" image (*cf.* Figure 5).

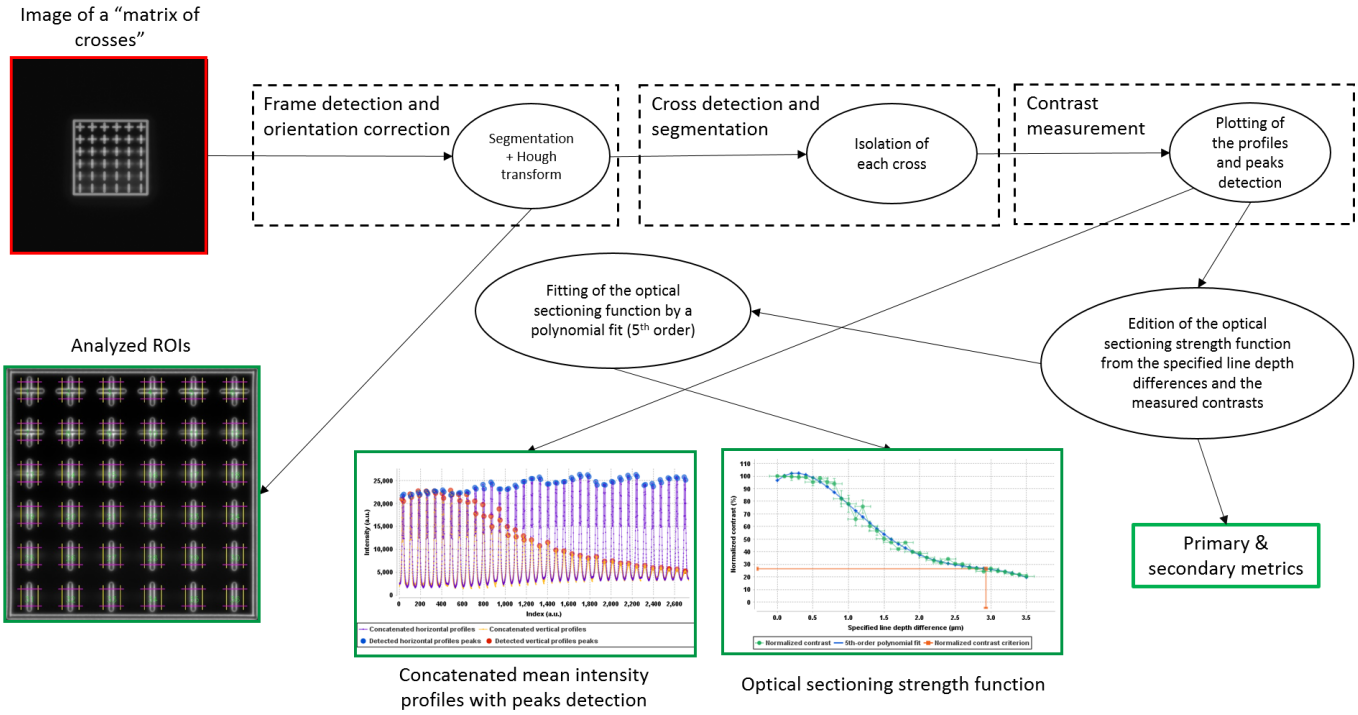


Figure 5: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It detects the frame around the "matrix of crosses" in the image.
- It determines the orientation of the frame via a Hough transform.
- It applies an automatic orientation correction, if necessary.
- It detects and segments the crosses in the image.
- For each cross, it draws two intensity line profiles, passing through both sides of the center of the cross, both in the horizontal (purple) and the vertical (orange) directions. Each displayed profile is the average of the two profiles along the same direction displayed in the image. These profiles are afterwards concatenated and displayed inside one single graph for visual convenience.
- It applies a smoothing to the intensity line profiles.
- It detects the peaks (in red) in the vertical profiles and the peaks (in blue) in the horizontal profiles.
- It calculates the normalized mean contrast for each cross.
  Note that the offset (background) is not subtracted prior the normalized mean contrast calculation. If you want the offset (background) to be subtracted, you need to acquire a

background image and use the optional setting “Background subtraction” before starting the analysis.

- It eventually rejects the detected peaks for the lines that would have an associated normalized mean contrast lower than 1.5 %. The crosses for which the peaks have been either not detected or rejected are labeled as “Not detected” in the “Measured normalized mean contrast” column of the table “Optical sectioning function values”.
- It displays the normalized mean contrast versus the specified line difference into the “Optical sectioning function” graph.

A more detailed description of the main part of the algorithm, consisting in measuring the normalized mean contrast, is presented below.

Once the crosses have been detected and segmented, and for each cross, the algorithm plots the average profiles along the horizontal and vertical directions. In these profiles, it detects the peaks and measures their intensities (*cf.* Figure 6).

On the one hand, for each cross, the normalized mean contrast is calculated, based on the peaks intensities, according to the following equation:

$$Normalized\ mean\ contrast = 100 \times (1 - Mean\ contrast)$$
$$= 100 \times \left(1 - \frac{\overline{I_{peak\ horizontal}} - \overline{I_{peak\ vertical}}}{\overline{I_{peak\ horizontal}}}\right)$$

Where: $\overline{I_{peak\ horizontal}}$ is the average intensity of the two peaks’ magnitude in the horizontal profile and $\overline{I_{peak\ vertical}}$ is the average intensity of the two peaks’ magnitude in the vertical profile, for one particular cross (*cf.* Figure 6).

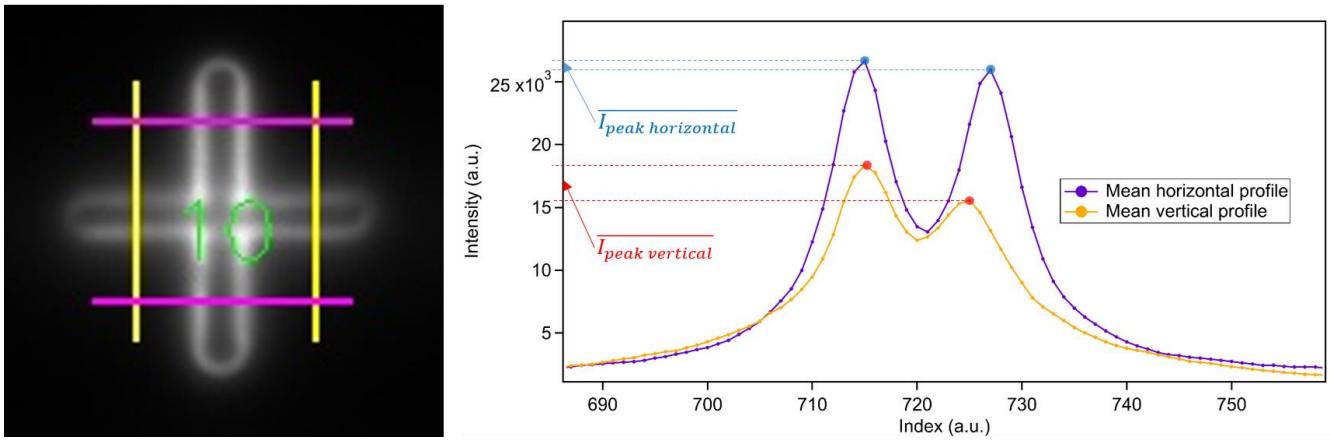


Figure 6: On the left, isolated cross with the horizontal (purple) and vertical (orange) lines along which the profiles are extracted. On the right, mean horizontal (purple) and vertical (orange) profiles, with their respectively blue and red peaks detected. $\overline{I_{peak\ horizontal}}$ is the average intensity of the two blue peaks’ magnitude and $\overline{I_{peak\ vertical}}$ is the average intensity of the two red peaks’ magnitude.

On the other hand, for each cross, the depth difference between the horizontal and vertical lines composing the cross is known from the manufacturing process of the slide.

At the end, the specified line depth difference of each cross is associated to the corresponding contrast values previously measured. The “normalized mean contrast versus line depth” data are plotted in the “Optical sectioning function” graph and fitted with a 5th-order polynomial fit (*cf.* Figure 7).

An “anchor” point is added in the “Optical sectioning function” and included in the fitting; it has a 0 µm value for the specified line depth difference and a 100 % value for the contrast.

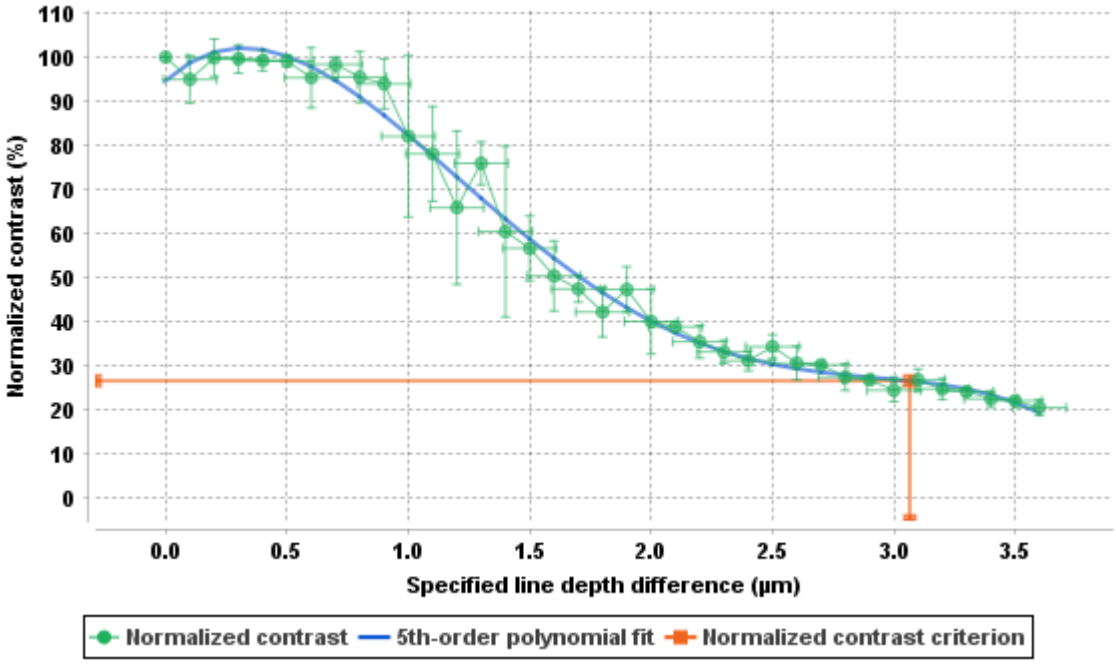


Figure 7: Optical sectioning function, *i.e.* the normalized mean contrast versus the specified line depth difference.

VI. OUTPUT METRIC DESCRIPTION

1. PRIMARY METRICS

- *Normalized contrast criterion* is the value of the contrast set by the user to determine the corresponding optical sectioning strength. It is expressed in %.
  For instance, in analogy with the lateral resolution, the well-known Schuster, Rayleigh and Sparrow criteria correspond to contrast thresholds of 99.9 %, 26.5 %, and 0.1 %, respectively.

- *Optical sectioning strength* is the value of the optical sectioning strength determined for the contrast criterion set by the user. It is obtained from the intercept between the horizontal line at the set contrast and the 5^{th}-order polynomial fitting function of the optical sectioning function. It is expressed in µm.

- *Optical sectioning strength at 50 % normalized contrast* is the value of the optical sectioning strength determined for a 50 % contrast criterion. It is obtained from the intercept between the horizontal line at 50 % contrast and the 5^{th}-order polynomial fitting function of the optical sectioning function. It is expressed in µm.

2. OPTICAL SECTIONING FUNCTION VALUES

The values of both the specified line depth difference and the measured normalized mean contrasts, that are plotted in the “Optical sectioning function” graph, are displayed in the “Optical sectioning function values” table.

3. SECONDARY METRICS

- *Maximum detectable line depth difference* is the maximum line depth difference in each cross that can be detectable, *i.e.* from which the contrast can be measured. It is expressed in µm.

- *Minimum detectable line depth difference* is the minimum line depth difference (higher than zero) in each cross that can be detectable, *i.e.* from which the contrast can be measured. It is expressed in µm.

- *Intensity maximum* is the maximum intensity value in the mean intensity profile. It is expressed in arbitrary unit.

- *Intensity minimum* is the minimum intensity value in the mean intensity profile. 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.
- *Measurement uncertainty of the optical sectioning strength* is the uncertainty of the algorithm on the measurement of the optical sectioning strength from the analyzed image. It is equal to the *specified line depth difference increment*. It is expressed in µm.
- *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.
- *Objective immersion medium* is the immersion medium selected in the analysis settings. The theoretical refractive index (RI) of the immersion medium is used to calculate the expected metrics along the Z axis.
- *Detected number of crosses* is the detected number of crosses. For the analysis to provide correct results, it must be the same as the *expected number of crosses*.
- *Expected number of crosses* is the expected number of crosses, for a given slide type: 36 for Argo-HM, 16 for Argo-SIM.
- *Specified minimum line depth difference* is the minimum line depth difference specified by Argolight. It is expressed in µm and is given by the following equation, for both Argo-HM and Argo-SIM:

$$0.1 \times Refractive\ index\ of\ the\ immersion\ medium/1.518$$

- *Specified maximum line depth difference* is the maximum line depth difference specified by Argolight. It is expressed in µm and is given by the following equation:

$3.6 \times Refractive\ index\ of\ the\ immersion\ medium/1.518$ for Argo-HM
$1.6 \times Refractive\ index\ of\ the\ immersion\ medium/1.518$ for Argo-SIM

- *Specified line depth difference increment* is the line depth difference increment specified by Argolight. It is expressed in µm and is given by the following equation, for both Argo-HM and Argo-SIM:

$$0.1 \times Refractive\ index\ of\ the\ immersion\ medium/1.518$$

- *RMSE for the fitting function of the OSF* is the root mean square error between the 5th-order polynomial fit and the experimental data in the optical sectioning function. It is a measurement of the goodness of the fit. It is expressed in % and is given by the following equation:

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

Where *n* is the number of experimental points, $C_{measured\_i}$ the measured contrast value of the *i*th experimental point and C_{fit_i} the contrast value of the *i*th point from the 5th-order polynomial fit.

- *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 problem description to:
customer@argolight.com