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I. INTRODUCTION

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

Confocal fluorescence microscopes allow to perform 3D imaging, thanks to their ability to remove the out-of-focus light. 3D imaging is achieved by reconstructing a Z-stack of images, which accuracy may be altered by different issues. Sources of 3D reconstruction inaccuracy can origin from the Z-stage, from a wrong adjustment of the objective correction collar for coverslip thickness or from the environmental conditions, such as temperature fluctuations and air flow.

The “accuracy of 3D reconstruction” analysis allows to check the ability of confocal fluorescence microscopes to acquire Z-stacks of images correctly, so that their reconstruction into 3D images is accurate, *i.e.* representative of the reality.

II. IMAGE ACQUISITION PROCEDURE

The ***“accuracy of 3D reconstruction”*** analysis is associated with the ***“sphere”*** pattern (Pattern G - See Figure 1).

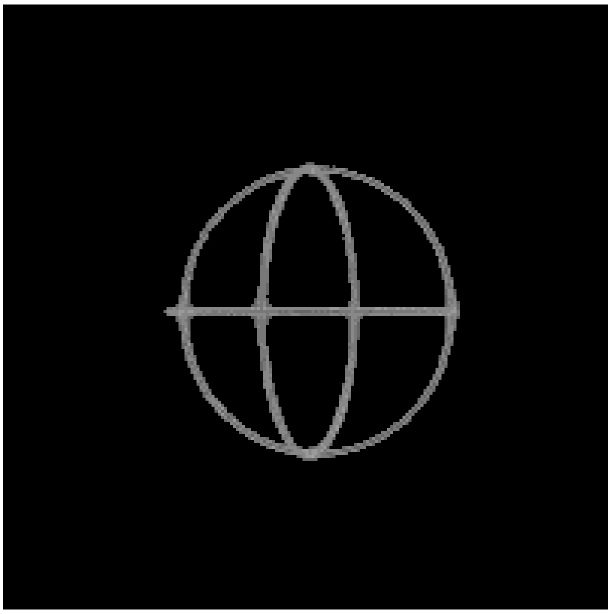


Figure 1: Image example of the “sphere” pattern, fulfilling the acquisition recommendations.

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 using a high numerical aperture objective over a Z-range covering a distance larger than the length of the sphere meridians.
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 adjusting the image axial pixel size to one-third of the theoretical axial resolution limit.

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 central cross 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 “Accuracy of 3D reconstruction” in the “Select analysis” list.

b) Upload your image(s) using the “Upload file” button.
Select one image of the Z-stack of the “sphere” pattern.

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).

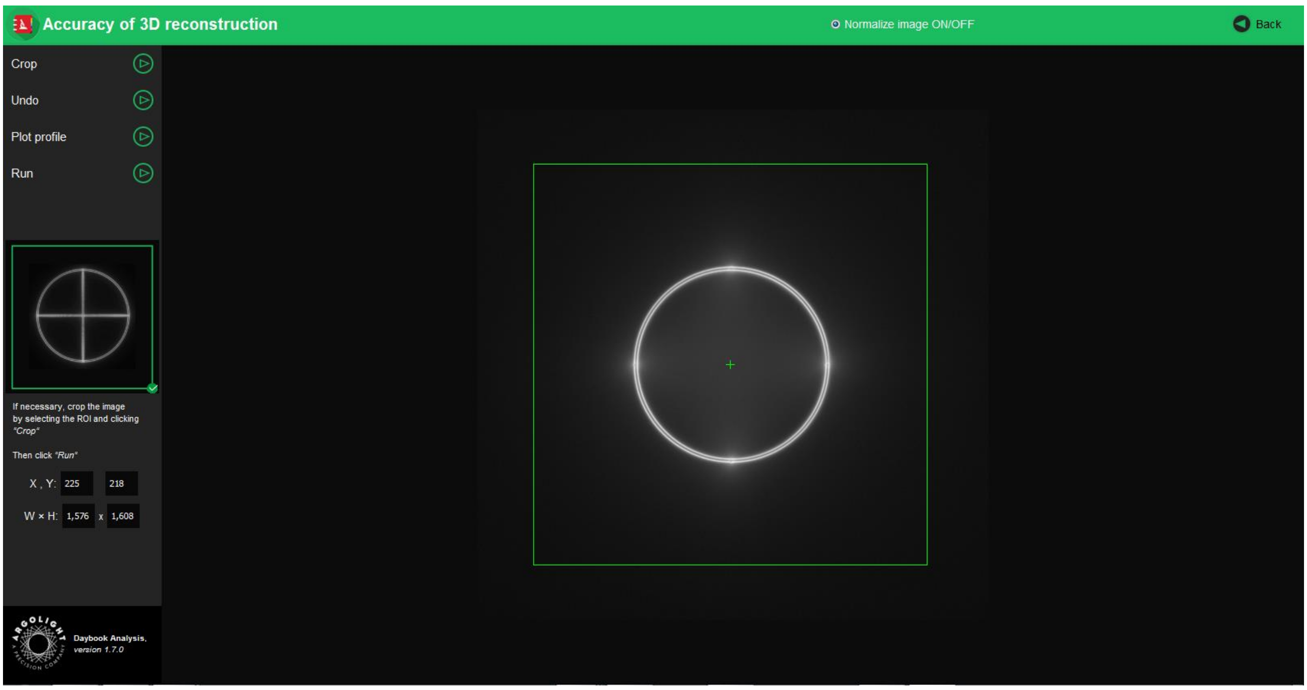
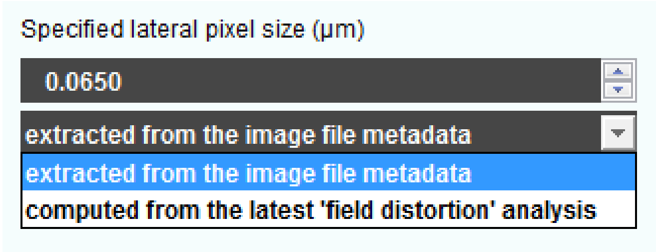


Figure 2: Crop window.

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 proprietary file:
  Select *“extracted from the image file metadata”*.
  - Or from a previous *“field distortion”* analysis:
  Select *“computed from the latest ‘field distortion’ analysis”*.



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

- **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.

IV. RESULTS PAGE DESCRIPTION

1. INTERFACE

The picture below shows the results interface for this analysis (*cf.* Figure 3).
Useful information is found in the displayed images, maps, graphs and tables.

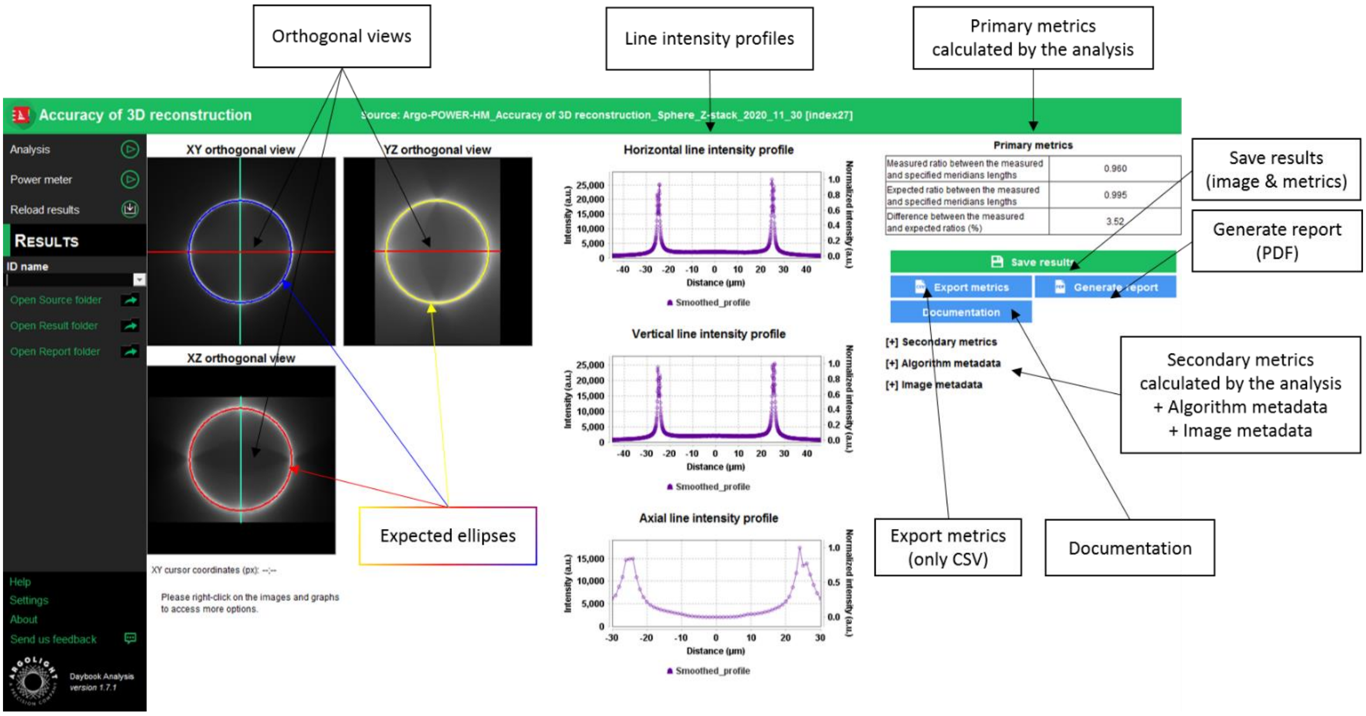


Figure 3: Results page.

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 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 4).

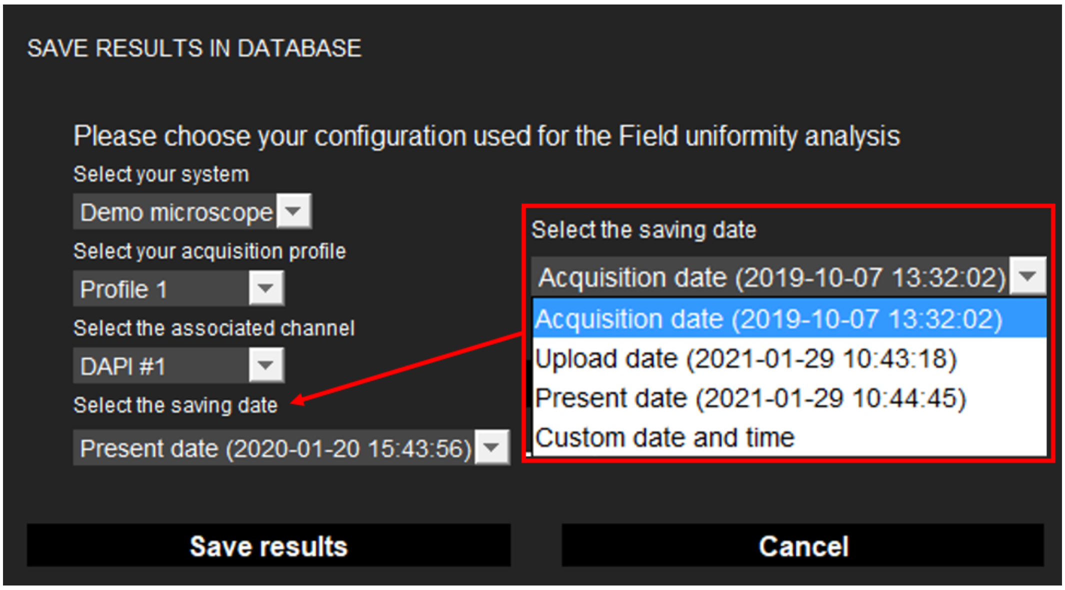


Figure 4: Interface window for saving the results in the database.

- **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 accuracy of the 3D reconstruction from a Z-stack of the "sphere" (*cf.* Figure 5).

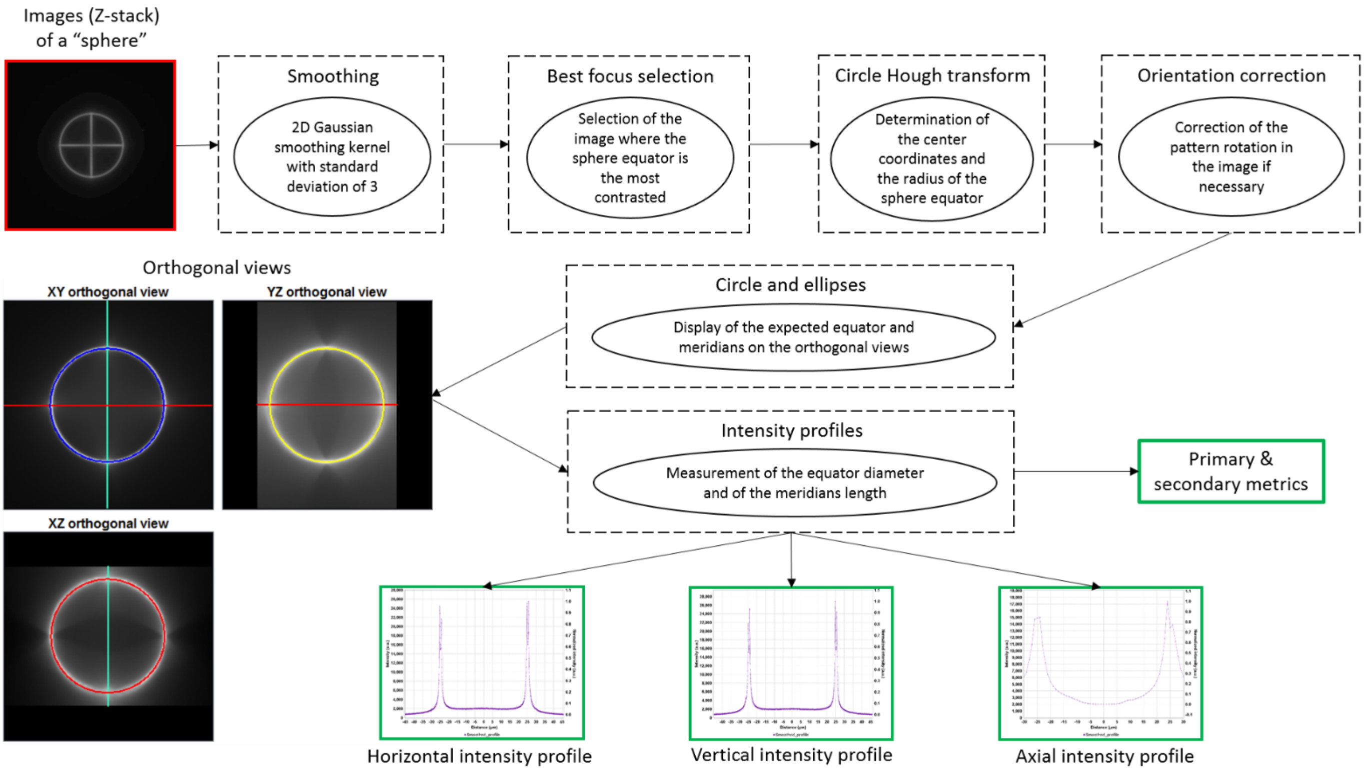


Figure 5: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It processes each image of the Z-stack by applying a 2D Gaussian smoothing kernel with standard deviation of 3.
- It analyzes each image of the Z-stack to find the image where the sphere equator is the most contrasted.
- In this image, it applies a circle Hough transform to determine both the center coordinates and the radius of the sphere equator.
- It applies an orientation correction if the pattern is rotated by more than 0.2° in the image.
- It displays the XY orthogonal view containing the sphere equator and draws a blue circle on top of it.
- It displays the XZ and YZ orthogonal views passing from the center of the equator,

containing the two sphere meridians, and draws a red ellipse and a yellow ellipse on top of them. These ellipses have the expected dimensions, depending on the Argolight product type and the refractive index of the immersion medium.

- It plots the intensity profiles passing from the center of the equator in the X, Y and Z directions.
- From these graphs, it measures the equator diameter and the meridians length.
- It calculates the *"measured ratio between the measured and specified meridians length"*, which aims to be compared to the *"expected ratio between the measured and specified meridians length"*. The *"difference between the measured and expected ratios"* is a metric of the accuracy of 3D reconstruction.

Note that depending on the immersion medium used to image the sphere, it may be normal to observe shrunk or elongated meridians, as illustrated in Figure 6.
When the refractive index of the immersion medium is about 1.518 (the refractive index of the immersion medium used to engrave the sphere in the Argoglass®), the imaged pattern looks like a ball (*cf.* Figure 6.a).
When the refractive index of the immersion medium is lower 1.518, the imaged pattern looks like a compressed ball (*cf.* Figure 6.b).
When the refractive index of the immersion medium is higher than 1.518, the imaged pattern looks like a dilated ball (*cf.* Figure 6.c).

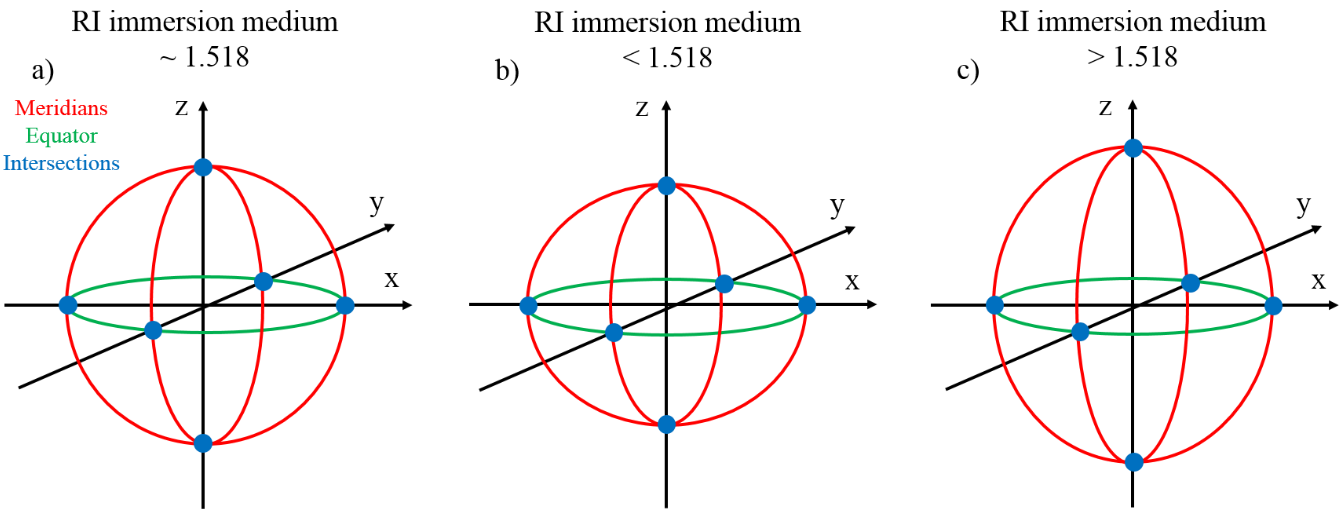


Figure 6: Schematic description of the way the meridians of the sphere appear depending on the refractive index of the immersion medium.

VI. OUTPUT METRIC DESCRIPTION

1. PRIMARY METRICS

- *Measured ratio between the measured and specified meridians lengths* is the ratio between the measured length and the specified length of the two meridians. It is unitless and is given by the following equation:

$$Measured\ ratio = \frac{Measured\ meridians\ length}{Specified\ meridians\ length}$$

- *Expected ratio between the measured and specified meridians lengths* is equivalent to the ratio between the refractive indices of the imaging immersion medium and the engraving immersion medium, quantifying the Z-distances distortion due to the refractive index mismatch between these two media. It is unitless and is given by the following equation:

$$Expected\ ratio = \frac{Refractive\ index\ of\ the\ imaging\ immersion\ medium}{Refractive\ index\ of\ the\ engraving\ immersion\ medium\ (i.e. 1.518)}$$

- *Difference between the measured and expected ratios* is the relative difference between the measured and expected ratios. It is expressed in %, according to the following formula:

$$Difference\ between\ the\ measured\ and\ expected\ ratios = 100 \times \left|\frac{Measured\ ratio - Expected\ ratio}{Expected\ ratio}\right|$$

2. SECONDARY METRICS

- *Measured meridians length* is the measured length of the two sphere meridians. It is expressed in µm.

- *Expected meridians length* is the expected length of the two sphere meridians. It is expressed in µm and is given by the following equation:

$$Expected\ meridians\ length = Specified\ meridians\ length \times \frac{Refractive\ index\ of\ the\ imaging\ immersion\ medium}{Refractive\ index\ of\ the\ engraving\ immersion\ medium\ (i.e. 1.518)}$$

- *Measured equator diameter* is the measured diameter of the sphere equator. It is expressed in µm.

- *Measured axial pixel size* is the interval between two slices, measured from the meridians length. It can eventually be compared to the specified axial pixel size provided by the metadata in the proprietary files. It is expressed in µm.

- *Axial pixel size difference* is the relative difference between the axial pixel size specified in the image metadata and the axial pixel size measured by the algorithm. It is expressed in %, according to the following formula:

$$Axial\ pixel\ size\ difference = 100 \times \frac{Specified\ axial\ pixel\ size - Measured\ axial\ pixel\ size}{Specified\ axial\ pixel\ size}$$

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.

- *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.

- *Angle value calculated for the orientation correction* is the angle value calculated by the algorithm, that can be applied later to analyze images that would require a low orientation correction, usually due to camera or laser scanning misalignment in microscopes. It is expressed in degree.

- *Measurement uncertainty of the measured ratio* is the measurement uncertainty associated to the measured ratio. It is unitless and is given by the following equation:

$$Measurement\ uncertainty = \frac{2 \times Specified\ axial\ pixel\ size}{Specified\ meridians\ length}$$

- *Calibration uncertainty of the expected ratio* is the calibration uncertainty associated to the expected ratio. It is unitless and is constant:

$$Calibration\ uncertainty = 0.010$$

- *Specified meridians length* is the length of the two meridians specified by Argolight: 50 for Argo-HM, 25 for Argo-SIM and 50 for Argo-POWER^{HM}. It is expressed in µm.

- *Specified equator diameter* is the diameter of the equator specified by Argolight: 50 for Argo-HM, 25 for Argo-SIM and 50 for Argo-POWER^{HM}. It is expressed in µm.

- *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 issue description at:
customer@argolight.com