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I. INTRODUCTION

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

During the acquisition of image Z-stacks, the lateral (XY) stage may drift. Therefore, it is important to ensure that the XY stage do not drift too much. Sources of stage drift can origin from the environmental conditions, such as temperature fluctuations and air flow.

The “stage drift during Z-stacking” analysis allows to determine the XY ***stage drift*** during Z-stacking, to ensure it is within the specifications.

II. IMAGE ACQUISITION PROCEDURE

The ***"stage drift during Z-stacking"*** analysis is associated with the ***"3D crossing stairs"*** patterns (Pattern I - See Figure 1).

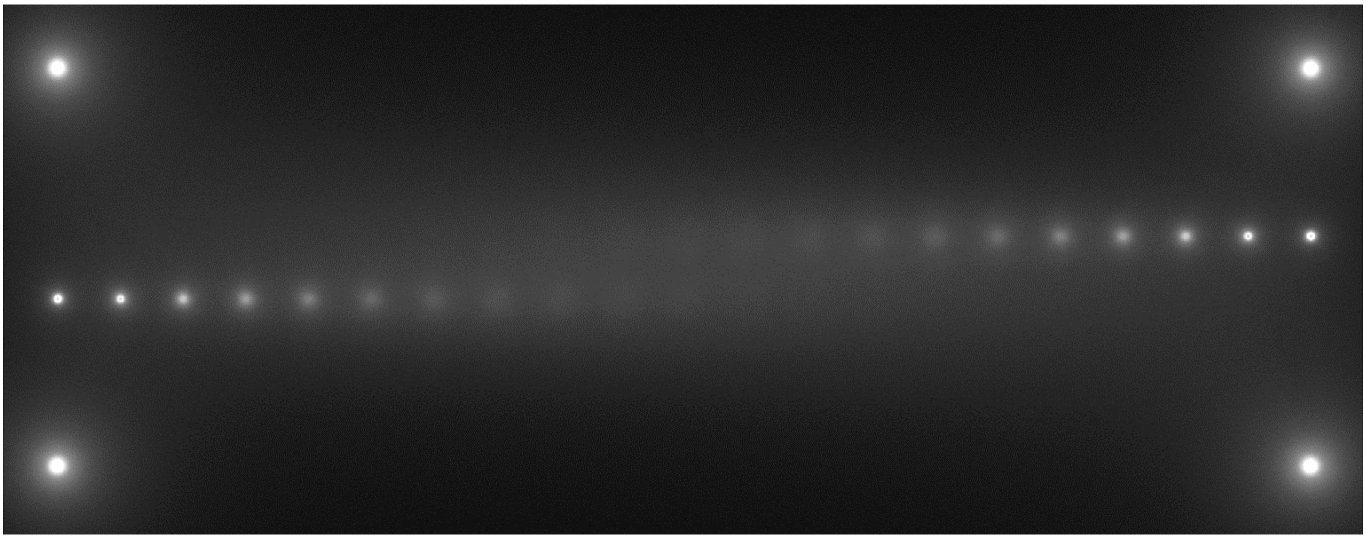


Figure 1: Image example of the "3D crossing stairs" pattern, fulfilling the acquisition recommendations.

1. ACQUISITION RECOMMENDATIONS

- **Recommended image type**

Z stack	Yes
Multi-channel	No
Tiles	No

The single channel Z-stack images should be acquired using a high numerical aperture objective over a Z-range and with a Z-step corresponding to a usual acquisition of 3D biological samples. The channel should be chosen to provide the best signal-to-background and signal-to-noise ratios. 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.

- **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).

- **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.

- **Axial pixel size (interval between each slice)**
  The axial pixel size of the Z-stack should be lower or equal to the half of the step of the imaged pattern. For example, for "3D crossing stairs" having a 1 µm step, the Z-sampling of the Z-stack should be 0.5 µm.

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 your pattern

a) Image the pattern by following the acquisition recommendations.
b) Save images into a raw, non-compressed format (for example, the acquisition software proprietary format) or into a lossless compression format (*e.g.* TIFF). The image file must have a dynamic range of 8 or 16 bits.

Important:
- For the Argo-HM and Argo-SIM slides, the "3D crossing stairs" to be imaged must be those having a ***1 µm step***.
- For the Argo-LM slide, there is only one "3D crossing stairs" pattern to be imaged.
- For the Argo-Z slide, the "3D crossing stairs" to be imaged must be those having a ***5 µm step***.

III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

a) Select “Stage drift during Z stacking” in the “Select analysis” list.

b) Upload your image(s) using the “Upload file” button.
Select one image of the Z-stack of the “3D crossing stairs” pattern.

c) Set the required and optional settings (see chapter 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).

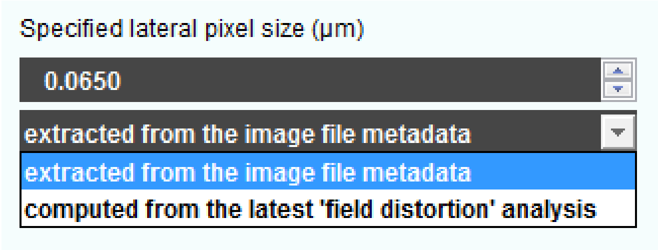
f) Click on “Run”.
Results are displayed and can be saved as CSV, PDF, or transferred into Daybook Data Manager (if available in your package).

Figure 2: Crop window.

2. ANALYSIS SETTINGS

1- 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"*.



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

IV. RESULTS PAGE DESCRIPTION

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.

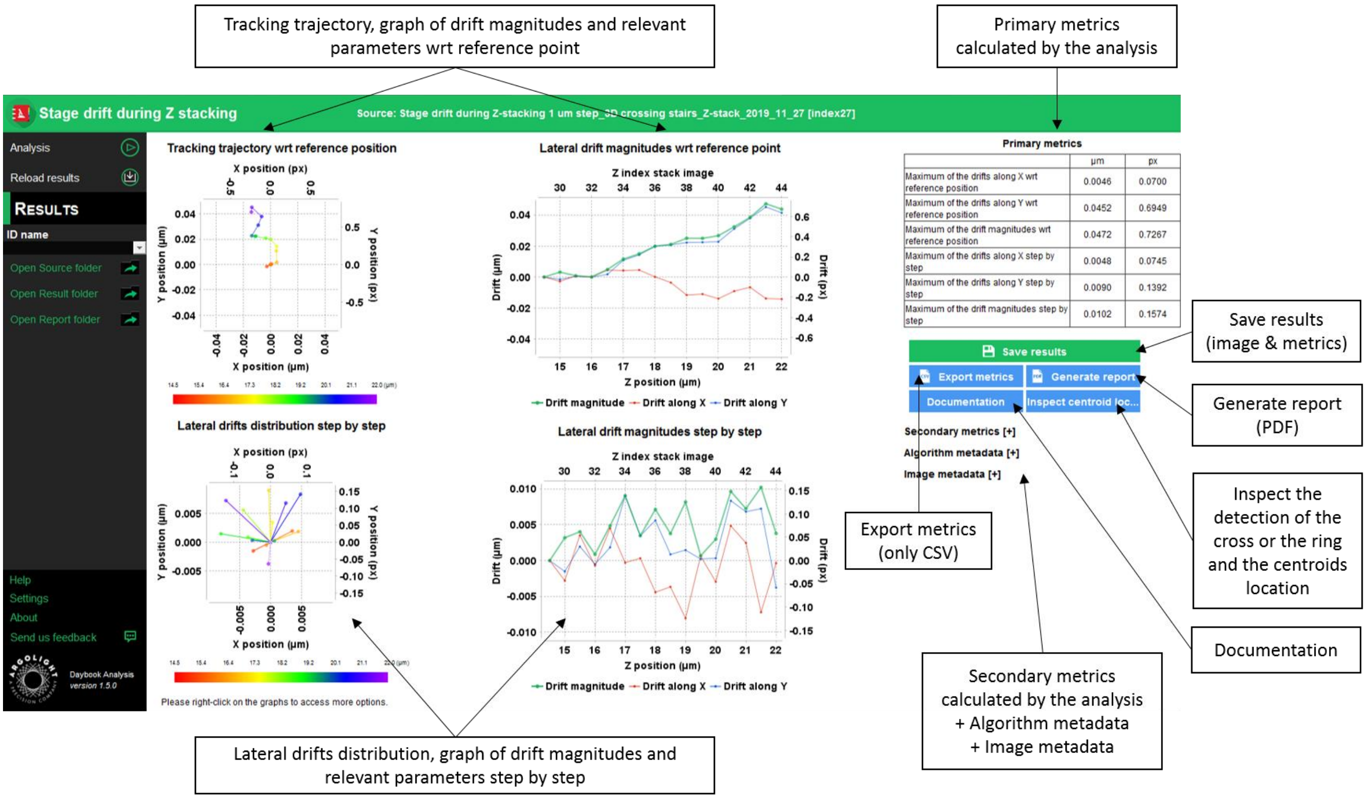


Figure 3: Results page.

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 present date (date of the analysis) or at a custom date (*cf.* Figure 4).

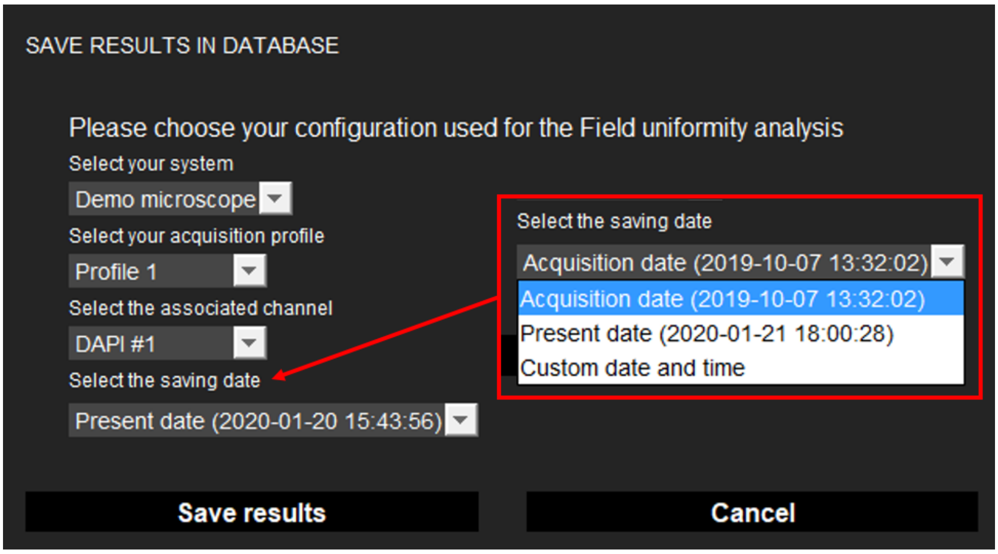


Figure 4: Interface window for saving the results in the database.

- **Detection of the pillar centroids:**
  Click on the "Inspect centroid locations" button to inspect the detection of the pillar as well as the centroids location in the Z-stack.

- **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

The diagram below describes the algorithm that allows the extraction of the stage drift during a Z-stacking from a Z-stack of the “3D crossing stairs” (*cf.* Figure 5).

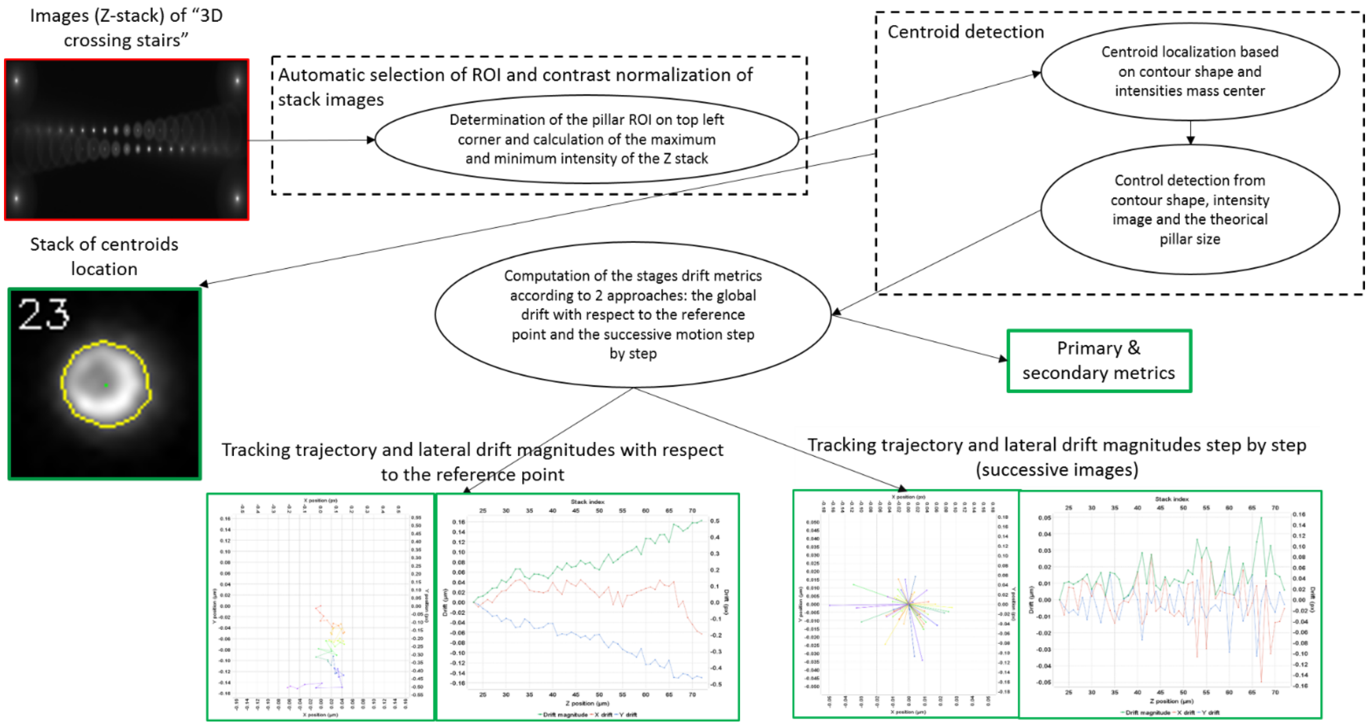


Figure 5: Schematic description of the different steps of the analysis algorithm.

In short, the algorithm works as follows:

- It analyzes each image of the Z-stack to find the image that has the largest intensity gradient. This image is considered as the reference image.
- In this reference image, it detects the pattern of interest, *i.e.* the pillar in the top left corner of the “3D crossing stairs” pattern.
- It normalizes the images of the Z-stack, only in the ROI of the detected pillar.
- In the ROI of each Z-stack image, it detects the centroid in each slice of the pillar and determines the XY coordinates of the centroids.
- It calculates the trajectory relative to the starting position, considered as the origin (0;0), as well as the trajectory step by step (*i.e.* between images).

  Note: The analysis of the circularity of the pillar contour might sometimes fail in some images. The circularity is defined as follows:

  $$Circularity = \frac{4 \times \pi \times Contour\ area}{Contour\ perimeter^2}$$

  The detection feature can be accessed through the “Inspect centroid location” button. An error message appears if the detection rate is below 70 %.

- It calculates the length of the pillar and evaluates a confidence range, corresponding to 80 % of the pillar length. The images of the Z-stack out of the confidence range are discarded.
- It calculates and displays the statistics (minimum, maximum, average, standard deviation, magnitudes, drifts in X and Y) into graphs and tables.

VI. OUTPUT METRIC DESCRIPTION

The images, graphs and metrics provided by this analysis are gathered in two groups: *"wrt reference position"* and *"step by step"*:
- *"wrt reference position"* means that any drift is measured with respect to a reference position. This reference position is the initial position, *i.e.* the coordinates of the centroid of the pillar in the ***first detected image***.
- *"step by step"* means that any drift is measured with respect to the drift value preceding it.

- *Drift magnitude* is expressed both in pixel and in µm, and is given by the following equation:

$$Drift\ magnitude = \sqrt{(Drift\ along\ X)^2 + (Drift\ along\ Y)^2}$$

1. PRIMARY METRICS

- *Maximum of the drifts along X or Y* is the maximum value of the drifts along X or Y. It is expressed both in pixel and in µm.
- *Maximum of the drift magnitudes* is the maximum value of the drift magnitudes. It is expressed both in pixel and in µm.

2. SECONDARY METRICS

- *Minimum of the drifts along X or Y* is the minimum value of the drifts along X or Y. It is expressed both in pixel and in µm.
- *Mean of the drifts along X or Y* is the mean (average) value of the drifts along X or Y. It is expressed both in pixel and in µm.
- *Standard deviation of the drifts along X or Y* is the standard deviation of the drifts along X or Y. It is expressed both in pixel and in µm.
- *Minimum of the drift magnitudes* is the minimum value of the drift magnitudes. It is expressed both in pixel and in µm.
- *Mean of the drift magnitudes* is the mean (average) value of the drift magnitudes. It is expressed both in pixel and in µm.
- *Standard deviation of the drift magnitudes* is the standard deviation of the drift magnitudes. It is

expressed both in pixel and in µm.

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.

- *Estimated limit of the algorithm* is the evaluated practical limit of the algorithm on the measurement of the shifts. It is expressed both in pixel and µm.

- *Detection rate* is the ratio between the number of images in which the pillar has been correctly detected (referred as “detected images”), and the total number of “useful images” in the Z-stack.

  Useful images are the ones in which the pillar has a detectable shape. The detection rate is expressed in %, according to the following formula:

$$Detection\ rate = 100 \times \frac{Number\ of\ "detected\ images"}{Number\ of\ "useful\ images"}$$

- *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