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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 family I - See Figure 1).

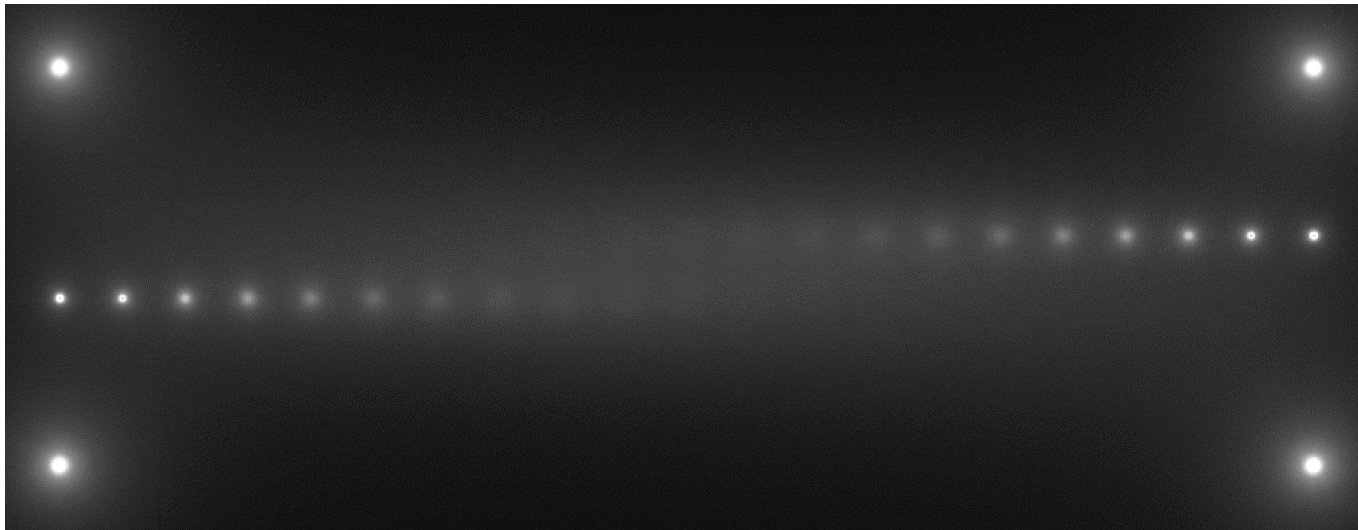


Figure 1: Example of an image of the “3D crossing stairs” pattern, fulfilling the acquisition recommendations.

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.

- 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?



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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) 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:

- 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?

- Select “Stage drift during Z stacking” in the “Select analysis” list.
 - Upload your image(s) using the “Upload file” button.
Select one image of the Z-stack of the “3D crossing stairs” pattern.
 - Set the required and optional settings (see section III.2 “Analysis Settings”).
 - Click on “Start the analysis”.
- Stage drift during Z stacking** **Start the analysis** ☒ Normalized image On/Off
- If needed, select a region of interest (ROI) and click on “Crop” to crop the image (*cf.* Figure 2).
 - Click on “Run”.
Results are displayed and can be saved as CSV, PDF, or transferred into Daybook Data Manager (if available in your package).

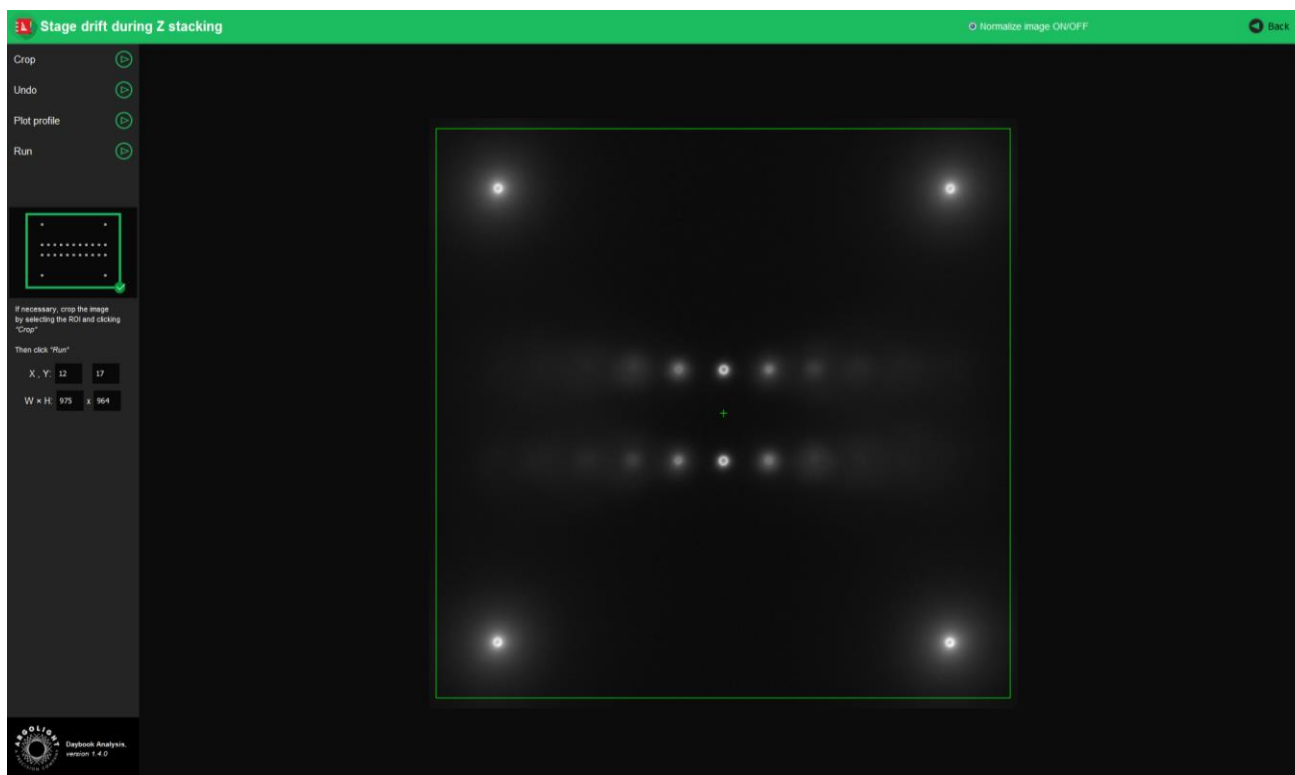


Figure 2: Crop window.

2. ANALYSIS SETTINGS

1- Required settings





- **Specified lateral pixel size**

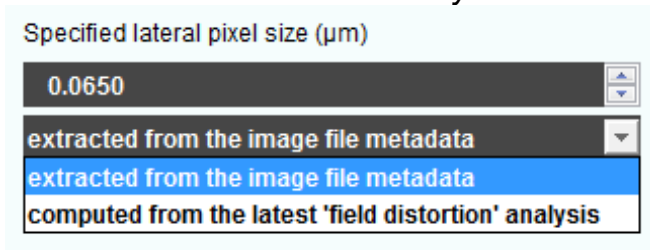
There are two ways to get the lateral pixel size of the image to be analyzed:

- Either from the 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*”.



- **Specified axial pixel size**

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

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.





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.

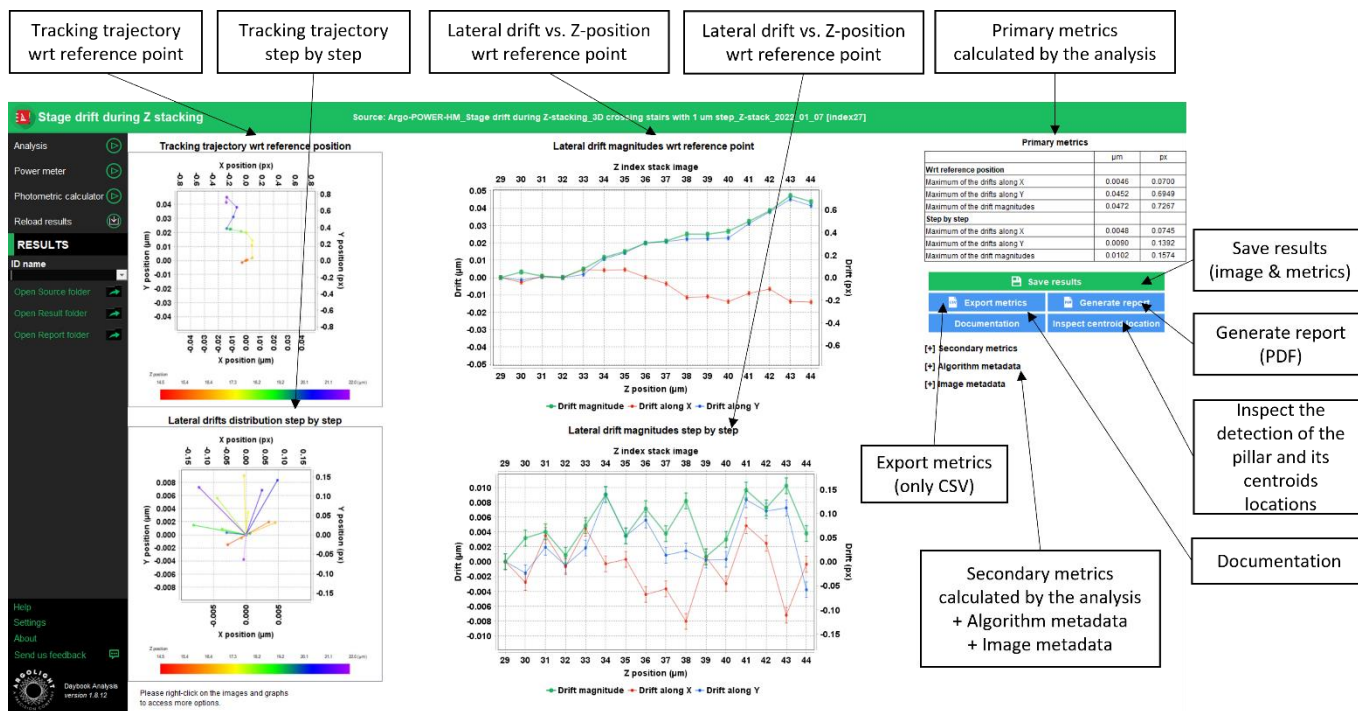


Figure 3: Results page.

2. OPTIONS

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).



SAVE RESULTS IN DATABASE

Please choose your configuration used for the Field uniformity analysis

Select your system
Demo microscope ▼

Select your acquisition profile
Profile 1 ▼

Select the associated channel
DAPI #1 ▼

Select the saving date
Present date (2020-01-20 15:43:56) ▼

Select the saving date

- Acquisition date (2019-10-07 13:32:02) ▼
- Acquisition date (2019-10-07 13:32:02)
- Upload date (2021-01-29 10:43:18)
- Present date (2021-01-29 10:44:45)
- Custom date and time

Save results Cancel

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

1. DIAGRAM

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).

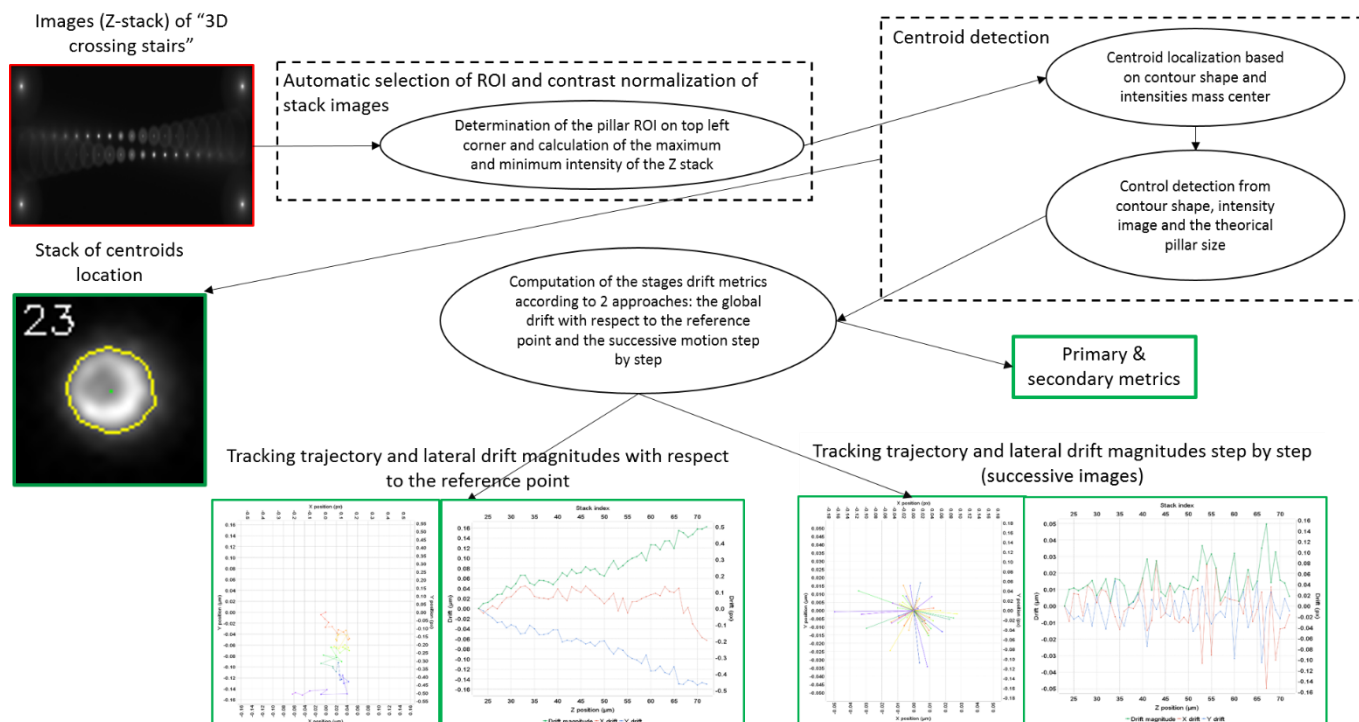


Figure 5: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

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:

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

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:

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

- *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 problem description to:
customer@argolight.com

