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I. INTRODUCTION

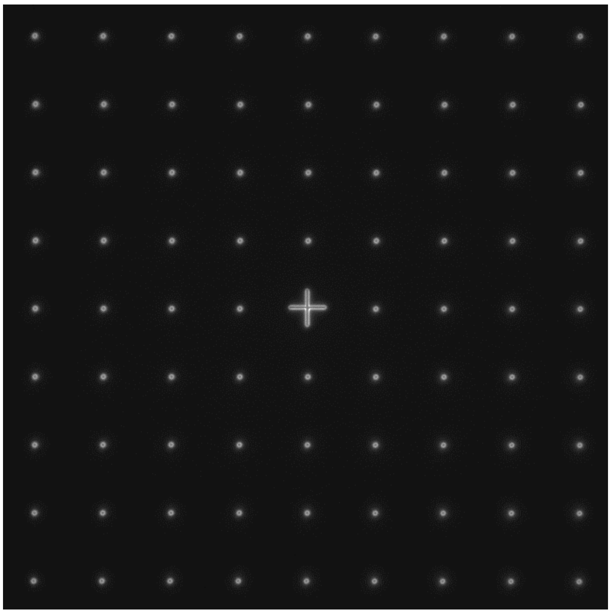
The field distortion is an optical aberration inducing a spatial deformation of the imaged object, usually at the corners of the image. In the presence of field distortion, the magnification is not constant over the field of view; it is dependent on the XY coordinates.

In any fluorescence microscope, the knowledge of the field distortion is important when spatial information in an image is aimed to be measured. For positions, lengths, areas or volumes quantification in images of biological samples, the field distortion shall be known, and eventually corrected, to have access to accurate measurements.

The “field distortion” analysis provides the ***lateral shifts***, due to distortion, within the field of view.

II. IMAGE ACQUISITION PROCEDURE

The ***"field distortion"*** analysis is associated with the ***"field of rings"*** pattern (Pattern family B - see Figure 1).



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

Figure 1: Example of an image 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

- **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 lowest magnification (*e.g.* 20×), then with the highest magnification objective (*e.g.* 100×).

- **Lateral pixel size**
  The lateral pixel size of the Z-stack should be lower or equal to the half of the theoretical lateral resolution limit (Nyquist criterion). However, if possible, we recommend that you adjust the image lateral pixel size to one-third of the theoretical lateral 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) 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 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 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:
The minimum required number of rings in the image is 5×5.
If the number of rings in the image is lower than this value, the algorithm will not work.

III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

a) Select “Field distortion” 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) By default, if one of the rows (or columns) of rings is incomplete or cropped, it will be discarded from the analysis. 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).

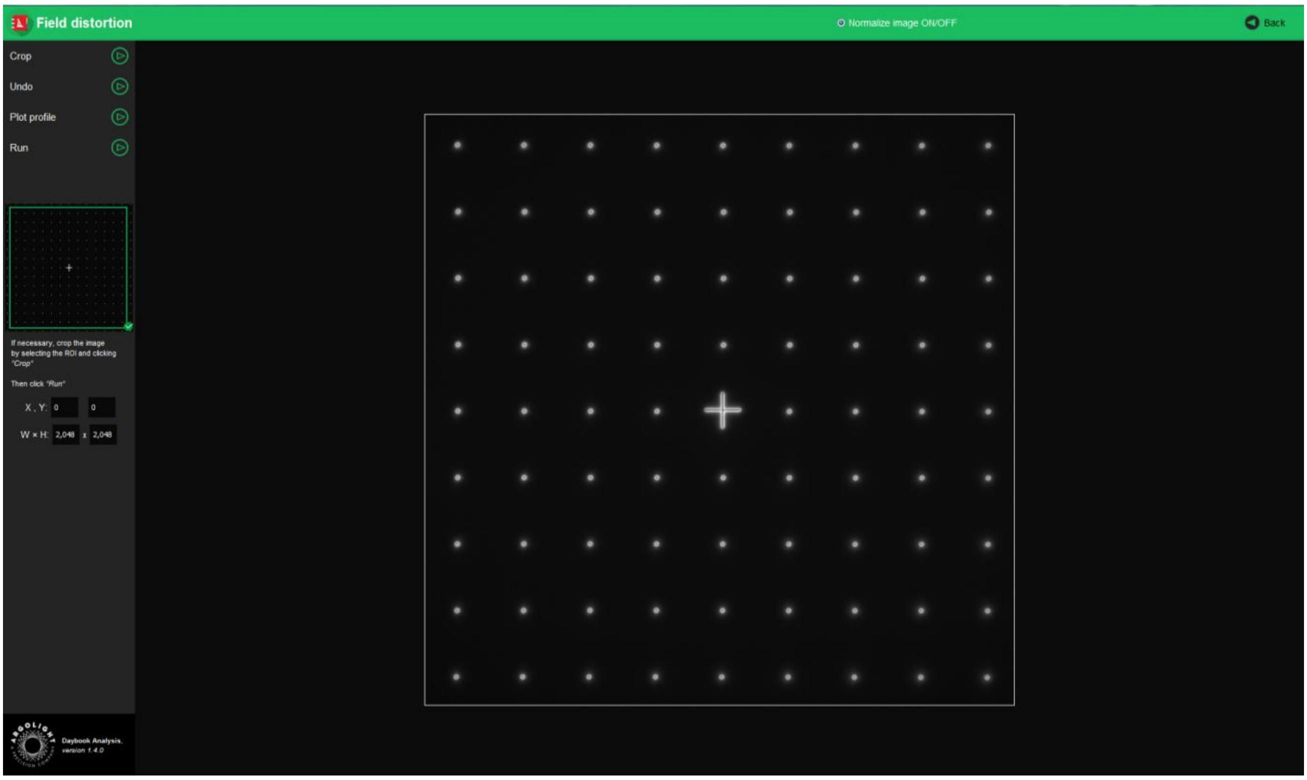
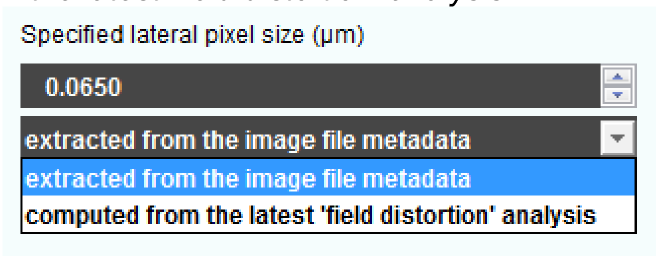


Figure 2: Crop window.

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 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”*.



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 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 algorithm metadata.

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 maps, graphs, and tables.

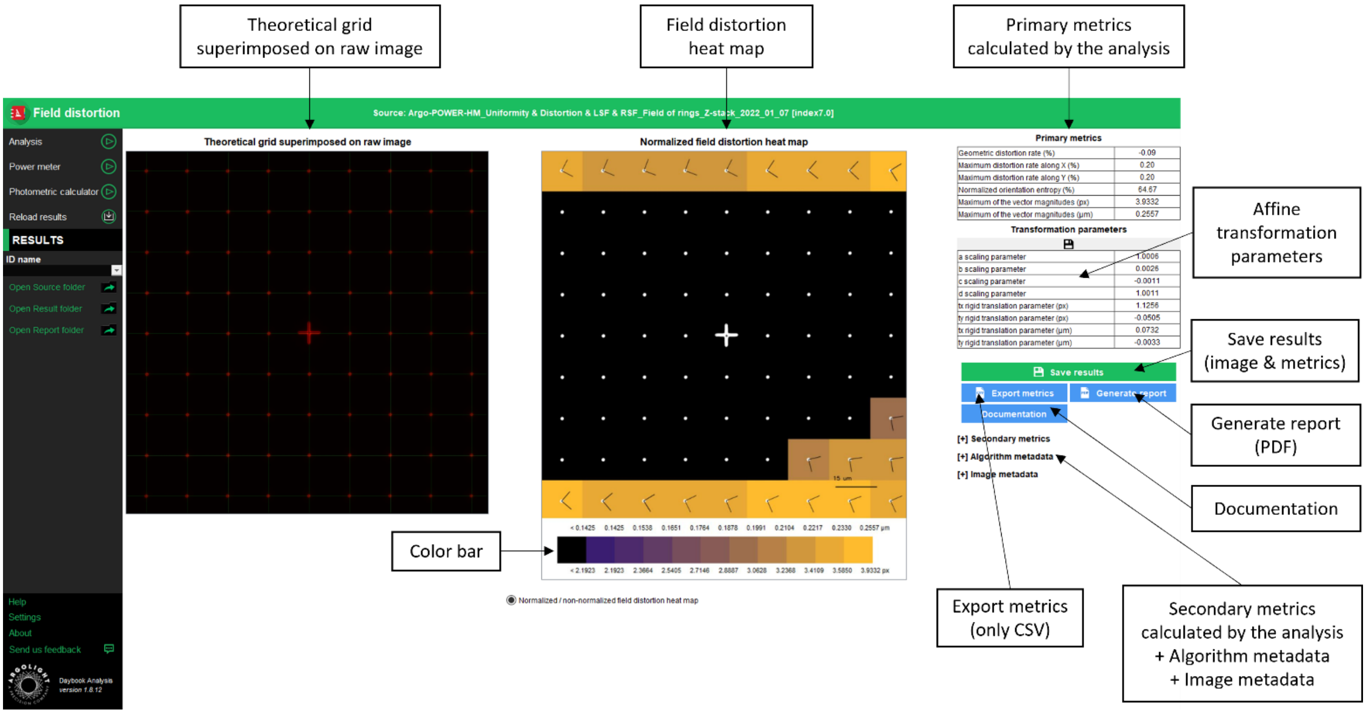


Figure 3: Results page.

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).

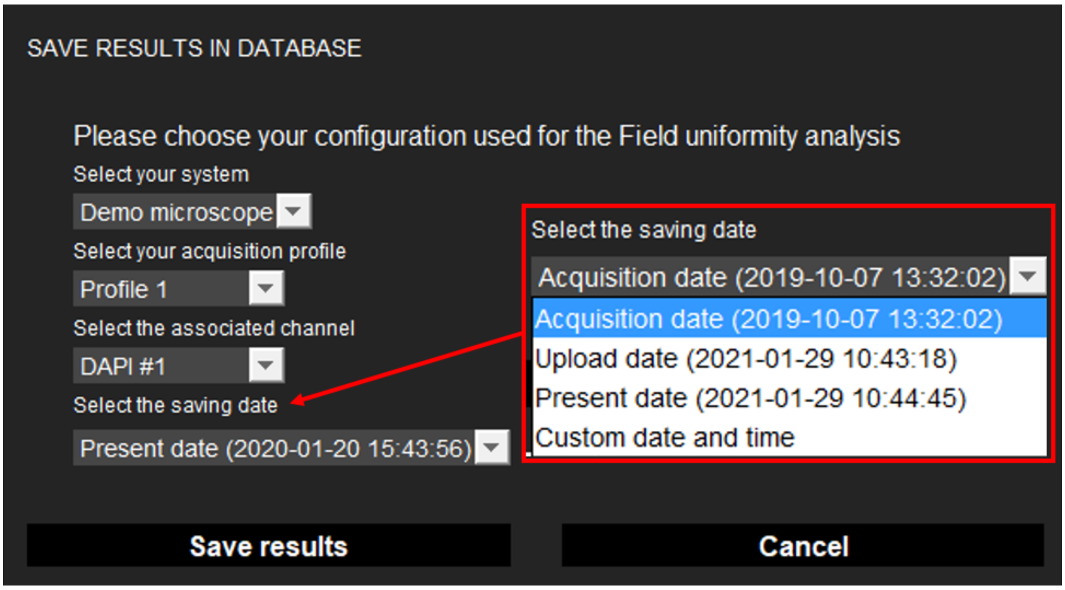


Figure 4: Interface window for saving the results in the database.

- **Image options:**
  - Zoom in and out. The images can be zoomed in and out by using the mouse roller.

- **Graph options:**
  - 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.

  - 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.

V. ANALYSIS ALGORITHM DESCRIPTION

1. DIAGRAM

The diagram below describes the algorithm that allows the extraction of the field distortion from the “field of rings” image (*cf.* Figure 5).

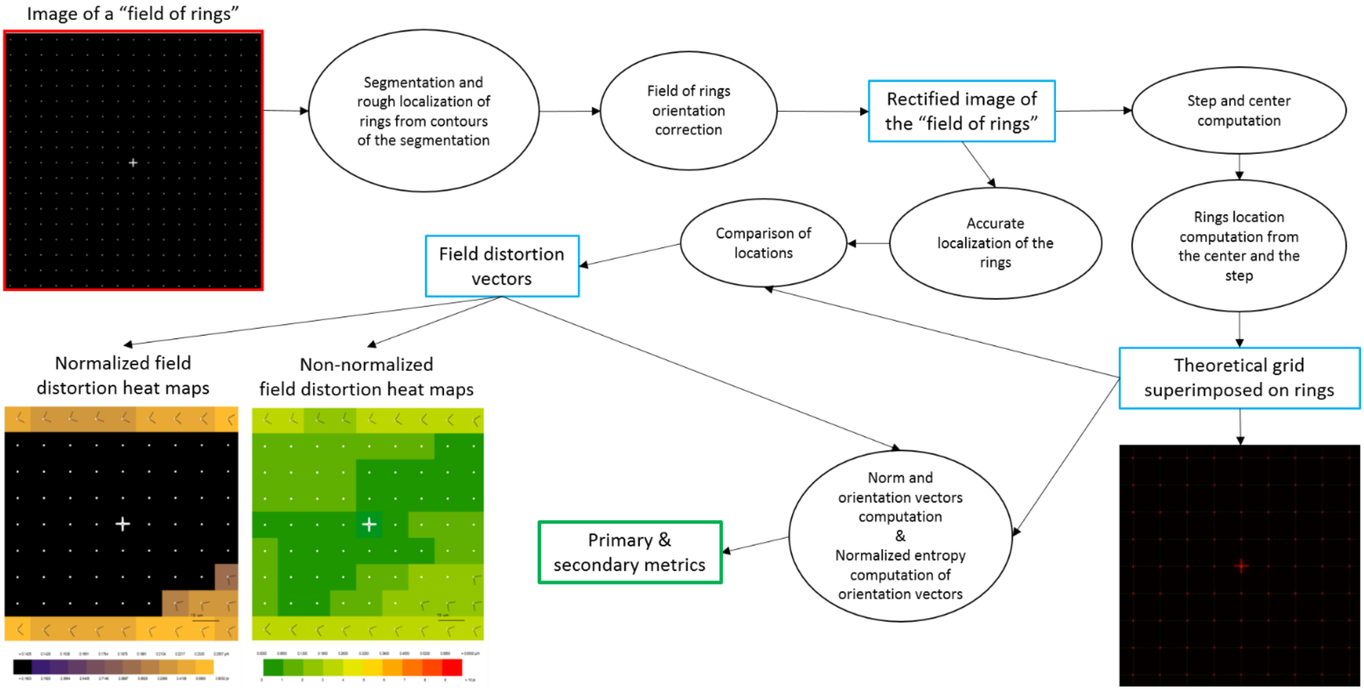


Figure 5: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It applies a smoothing filter to detect the rings in the image.
- It segments the rings.
- It applies an automatic orientation correction.
- It determines the XY coordinates of the centroid of each ring.
- It measures the shift between these coordinates and the coordinates of the nodes from a superimposed theoretical (*i.e.* perfect) grid.
- It displays these shifts into a field distortion heatmap, in which the arrows and the colors indicate respectively the direction and the magnitude of the distortion.

Note: the origins of the distortion vectors are the nodes of the theoretical (*i.e.* perfect) grid.

VI. OUTPUT METRIC DESCRIPTION

1. PRIMARY METRICS

- *Geometric distortion rate* is a parameter providing information about the average amount of distortion in an image. It can be positive of negative, whether the distortion has a pincushion or barrel shape, respectively. It is expressed in %, according to the following formula:

$$Geometric\ distortion\ rate = 100 \times \frac{H' - H}{H}\ (averaged\ over\ the\ four\ diagonals)$$

  Where H' and H are the distances from the center of the distorted and undistorted rings, respectively, in each corner.

- *Maximum distortion rate along X* is the maximum distortion rate along the horizontal direction, corresponding to the row of rings presenting the maximum amount of distortion in the image. It is expressed in %, according to the following formula:

$$Maximum\ distortion\ rate\ along\ X = \left|100 \times \frac{\Delta x}{L}\right|$$

  Where Δx is the maximum amount of distortion along X, and L the length of the distorted line.

- *Maximum distortion rate along Y* is the maximum distortion rate along the vertical direction, corresponding to the column of rings presenting the maximum amount of distortion in the image. It is expressed in %, according to the following formula:

$$Maximum\ distortion\ rate\ along\ Y = \left|100 \times \frac{\Delta y}{L}\right|$$

  Where Δy is the maximum amount of distortion along Y, and L the length of the distorted line.

- *Normalized orientation entropy* provides information on the orientation disparity of the distortion vectors, normalized with respect to the maximum entropy. It is expressed in %, according to the following formula:

$$H_{normalized} = -\frac{100}{H_{maximum}} \sum_{i=1}^{360} P_i \ln(P_i)$$

  Where P_i is the presence probability of the vectors orientation found among 360 possible orientations (one probability per degree).

  The maximum entropy is calculated for a uniform distribution of orientations going from 0° to 360° with an increment of 1°, as follows:

$$H_{maximum} = -\sum_{j=1}^{360} P_j \ln(P_j)$$

  Where $P_j = \frac{1}{360}$ is the equally distributed probability, according to a uniform law (1 vector for any of the 360 possible orientations).

To provide numbers, if all the distortion vectors are oriented along the same direction, the normalized orientation entropy is zero. If for example 360 distortion vectors are radially oriented with an increment of 1° (*i.e.* a first vector has an orientation of 1°, a second vector has an orientation of 2°, and so on until 360°), the normalized orientation entropy is 100%.

- *Maximum of the vector magnitudes* is the magnitude of the vector showing the highest amount of distortion. It is expressed both in pixels and µm.

2. TRANSFORMATION PARAMETERS

- The image transformation required to overlay the location of each ring (determined from the raw image) to one of each node of a perfect theoretical grid is expressed as follows:

$$[x_{ring} \quad y_{ring}] = \begin{bmatrix} a & b & tx \\ c & d & ty \end{bmatrix} \times [x_{grid} \quad y_{grid} \quad 1]^T$$

  Where $\{x_{ring} \,;\, y_{ring}\}$ and $\{x_{grid} \,;\, y_{grid}\}$ are the coordinates of the rings and of the nodes of a perfect theoretical grid, respectively.
  This transformation is limited to combinations of translation, uniform scaling (zoom) and rotation only.

- *a, b, c and d* are the uniform scaling (zoom) and rotation parameters. They are unitless.

- *tx and ty* are the rigid translation parameters. They are expressed both in pixels and in µm.

3. SECONDARY METRICS

- *Mean of the vector magnitudes* is the mean of all the distortion vector magnitudes. It is expressed both in pixels and µm.

- *Standard deviation of the vector magnitudes* is the standard deviation of all the distortion vector magnitudes It is expressed both in pixels and µm.

- *Minimum of the vector magnitudes* is the magnitude of the vector showing the lowest amount of distortion. It is expressed both in pixels and µm.

- *Measured lateral pixel size* is the size of one pixel, measured from the average ring spacing in the “field of rings” pattern. It can eventually be compared to the specified lateral pixel size provided by the metadata in the proprietary files. It is expressed in µm.

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

$$Lateral\ pixel\ size\ difference = 100 \times \frac{Specified\ lateral\ pixel\ size - Measured\ lateral\ pixel\ size}{Specified\ lateral\ pixel\ size}$$

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 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.
- *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.
- *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.
- *Number of detected rings* is the number of rings from the “field of rings” pattern that are detected by the algorithm and used in the analysis.
- *Number of rejected rings* is the number of rings from the “field of rings” pattern that are detected by the algorithm but not used in the analysis. Rejected rings often are rings cut on the rim of the image.
- *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.
- *Distance between the rings from the center of the field of view (FOV)* is the average distance of the first rings surrounding the center of the theoretical grid. It is expressed in

pixel.

- *X coordinate of the theoretical grid center* is the coordinate along X (starting from the top left corner) of the center of the theoretical grid. It is expressed in pixel.

- *Y coordinate of the theoretical grid center* is the coordinate along Y (starting from the top left corner) of the center of the theoretical grid. 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.

VII. HOW TO CORRECT A DISTORTED FIELD?

To correct a distorted field in an image of a biological sample, one can save the transformation parameters into a TXT file and then use for instance the TransformJ Affine plugin in ImageJ (https://imagescience.org/meijering/software/transformj/) to carry out the correction. If using the TransformJ plugin, make sure all the different options are unchecked, as illustrated in Figure 6.

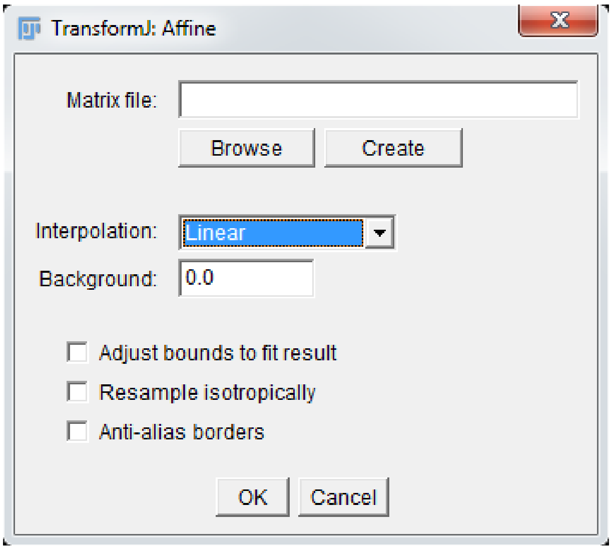


Figure 6: Interface window of the TransformJ plugin in ImageJ, showing that all the different options are unchecked.

The transformation parameters provided by Daybook Analysis allow an affine (*i.e.* linear) correction, that is a correction for any combination of rotation, translation and non-uniform scaling. If the correction to be applied is both linear and nonlinear, then applying an affine transformation will not be sufficient to perfectly correct a distorted field.

The TXT file should be organized as follows:

a,b,0,tx
c,d,0,ty
0,0,1,0
0,0,0,1

a, *b*, *c* & *d* are unitless.
tx and *ty* must be expressed in µm if the image has an associated lateral pixel size, or in pixel if not.

Such a TXT file can directly be generated by clicking on the floppy disk button, close to the “Transformation parameters” table (*cf.* Figure 7).

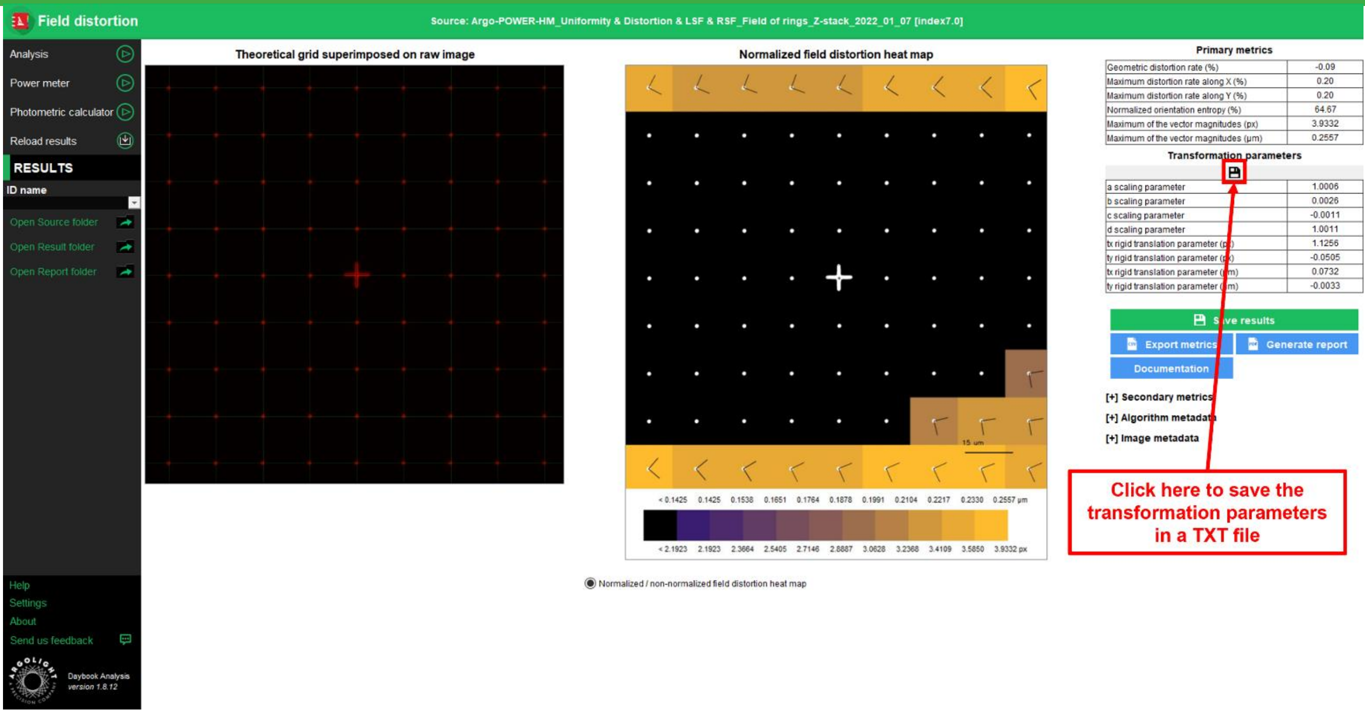


Figure 7: How to save the image transformation parameters in a TXT file.

Warning:

The image to be corrected should be acquired with the same conditions as the one of the "field of rings" pattern, which is located (170 ± 5) µm below the top surface, within a glass that has a refractive index of 1.5244 ± 0.0007 at 570 nm.

The imaging conditions of the biological sample that need to be met are the following:

- The biological sample must be mounted just after a #1.5 coverslip. According to ISO 8255-1:2017, the #1.5 coverslip has the following properties: thickness of (170 ± 5) µm, refractive index of 1.5255 ± 0.0015 at 570 nm, Abbe number of 56 ± 2.

Otherwise, optical aberrations, such as spherical or chromatic aberrations, could degrade the correction quality.

Deviating from these requirements may lead to a wrong correction, or at worst to an increase of the field distortion amount in the corrected image. We highly encourage users to try the correction and evaluate how relevant it is to perform such a correction, with respect to their own system.

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