Table of contents

I. INTRODUCTION 1
II. IMAGE ACQUISITION PROCEDURE 3
1. ACQUISITION RECOMMENDATIONS 3
2. HOW TO IMAGE THE PATTERN? 4
III. IMAGE ANALYSIS PROCEDURE 6
1. HOW TO LAUNCH AN ANALYSIS? 6
2. ANALYSIS SETTINGS 7
IV. RESULTS PAGE DESCRIPTION 9
1. INTERFACE 9
2. OPTIONS 9
V. ANALYSIS ALGORITHM DESCRIPTION 11
1. DIAGRAM 11
2. DESCRIPTION 11
VI. OUTPUT METRIC DESCRIPTION 12
1. PRIMARY METRICS 12
2. TRANSFORMATION PARAMETERS 12
3. SECONDARY METRICS 13
4. ALGORITHM METADATA 13
5. IMAGE METADATA 14
VII. HOW TO CORRECT A LATERAL CO-REGISTRATION INACCURACY? 16

I. INTRODUCTION

The co-registration accuracy is the ability of a fluorescence microscope to record images of a multi-labeled object without introducing additional shifts that would not be related to the object itself.

In any fluorescence microscope, the knowledge of the co-registration accuracy, both in the lateral and axial directions, is important when color information in an image is aimed to be used. For co-localization quantification in images of biological samples stained with several labels, the co-registration accuracy between the different channels shall be known, and eventually corrected, to prevent from misinterpretation.

The “lateral co-registration accuracy” analysis provides the ***lateral shifts*** between two channels, due to the co-registration inaccuracy of the system, within the entire field of view.

II. IMAGE ACQUISITION PROCEDURE

The ***"lateral co-registration accuracy"*** analysis is associated with the ***"field of rings"*** pattern (Pattern B - see Figure 1).
It requires images of this pattern acquired on different channels (at least on two channels).

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

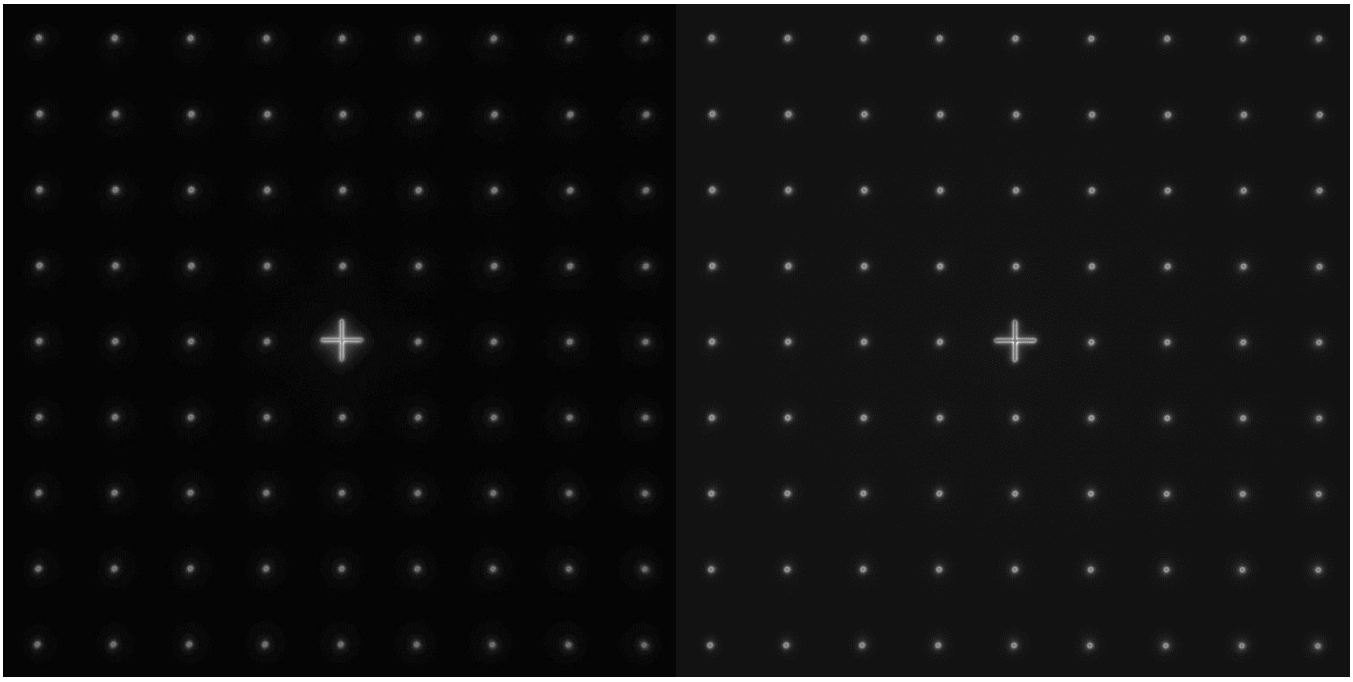


Figure 1: Image examples of the "field of rings" pattern acquired on two different channels. Left: DAPI channel. Right: GFP channel. The image having the best contrast (left) will be used as reference (green channel), in order to improve the detection of rings in the 2nd image (right).

1. ACQUISITION RECOMMENDATIONS

- **Recommended image type**

Z stack	Yes (if your microscope allows to do it)
Multi-channel	Yes
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.

- **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).

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

- **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.

- **Image sampling rate**
  The sampling rate of the image should fulfill the Nyquist criterion, *i.e.* the image pixel size should be at least the half of the theoretical resolution limit. However, if possible, we recommend adjusting the image pixel size to one third of the theoretical 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) 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 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 your pattern

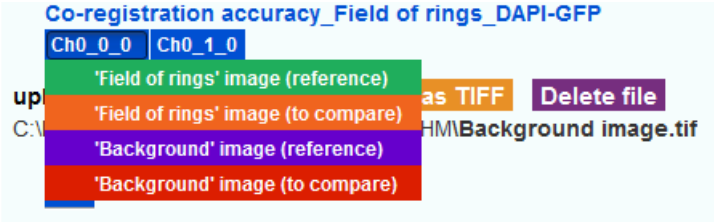
a) Image the pattern by following the acquisition recommendations.
b) Save images into a raw, non-compressed format (for example, the acquisition software proprietary format) or into a lossless compression format (*e.g.* TIFF). The image file must have a dynamic range depth of 8 or 16 bits.

III. IMAGE ANALYSIS PROCEDURE

1. HOW TO LAUNCH AN ANALYSIS?

a) Select “Lateral co-registration accuracy” in the “Select analysis” list.

b) Upload your image(s) using the “Upload file” button.
As “’Field of rings’ image (reference)”, select the “field of rings” image you would like to use as the reference.
As “’Field of rings’ image (to compare)”, select another “field of rings” image you would like to compare with respect to the reference one.



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

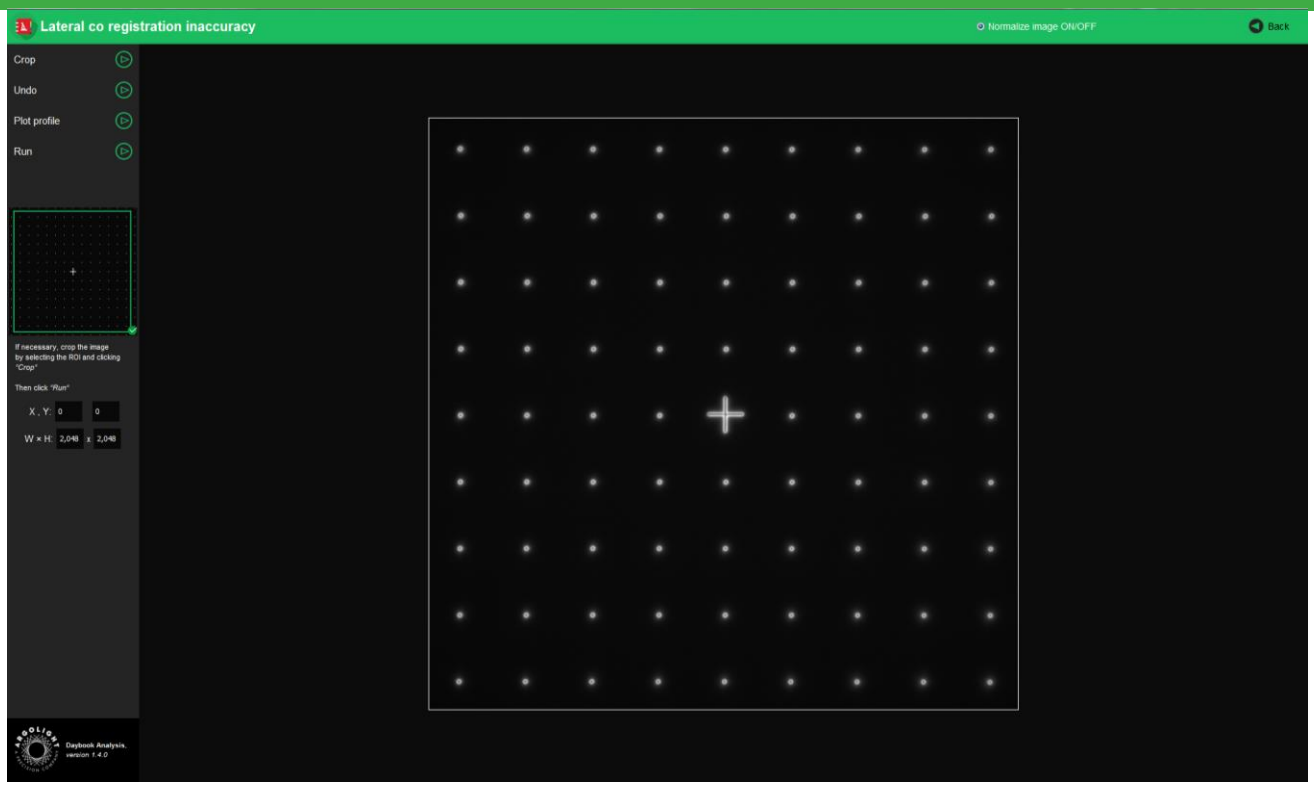
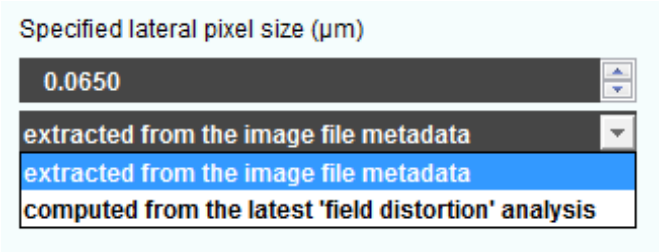


Figure 2: Crop window.

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 proprietary file:
  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**
  Subtracts the background in images where the signal-to-background ratio (SBR) is too low to be analyzed by Daybook Analysis.

It requires to acquire 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**
  Removes 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 proprietary file.

- **Best focus selection**
  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 metrics and reports.

Note: In the particular case of the “Lateral co-registration accuracy” analysis, the “Best focus selection” option applies to both images to be analyzed.

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.

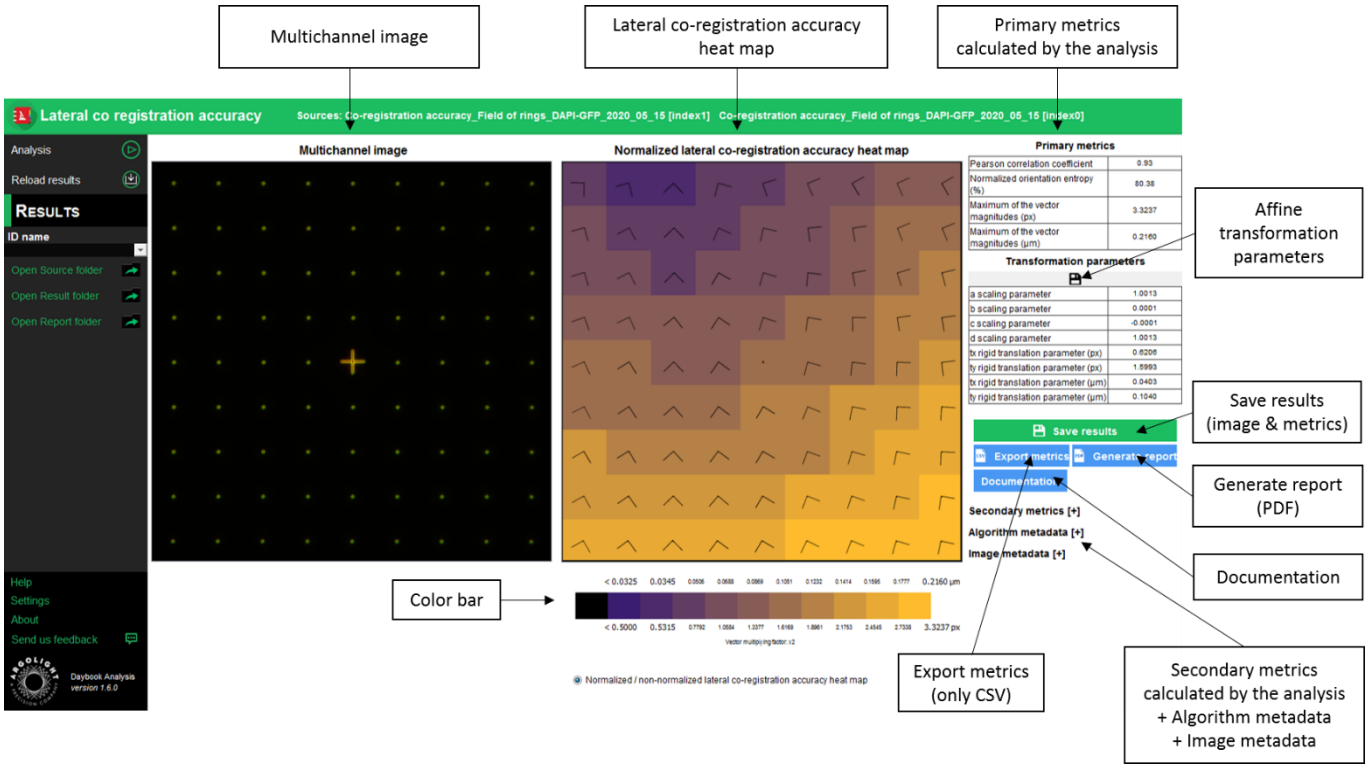


Figure 3: Results page.

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 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).

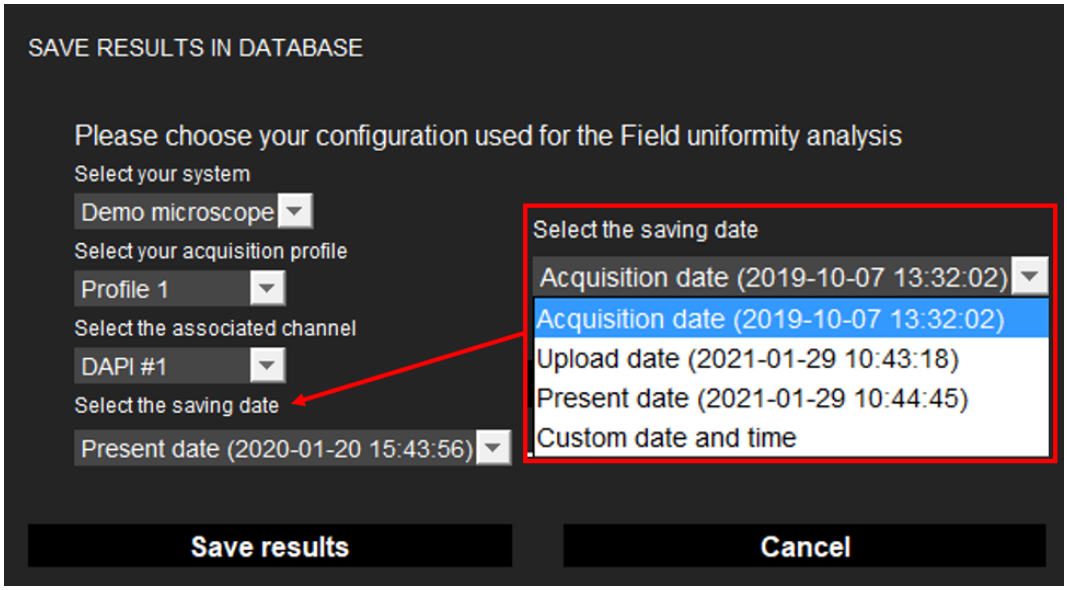


Figure 4: Interface window for saving the results in the database.

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.

- **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 lateral co-registration accuracy from two images of the “field of rings” pattern (*cf.* Figure 5).

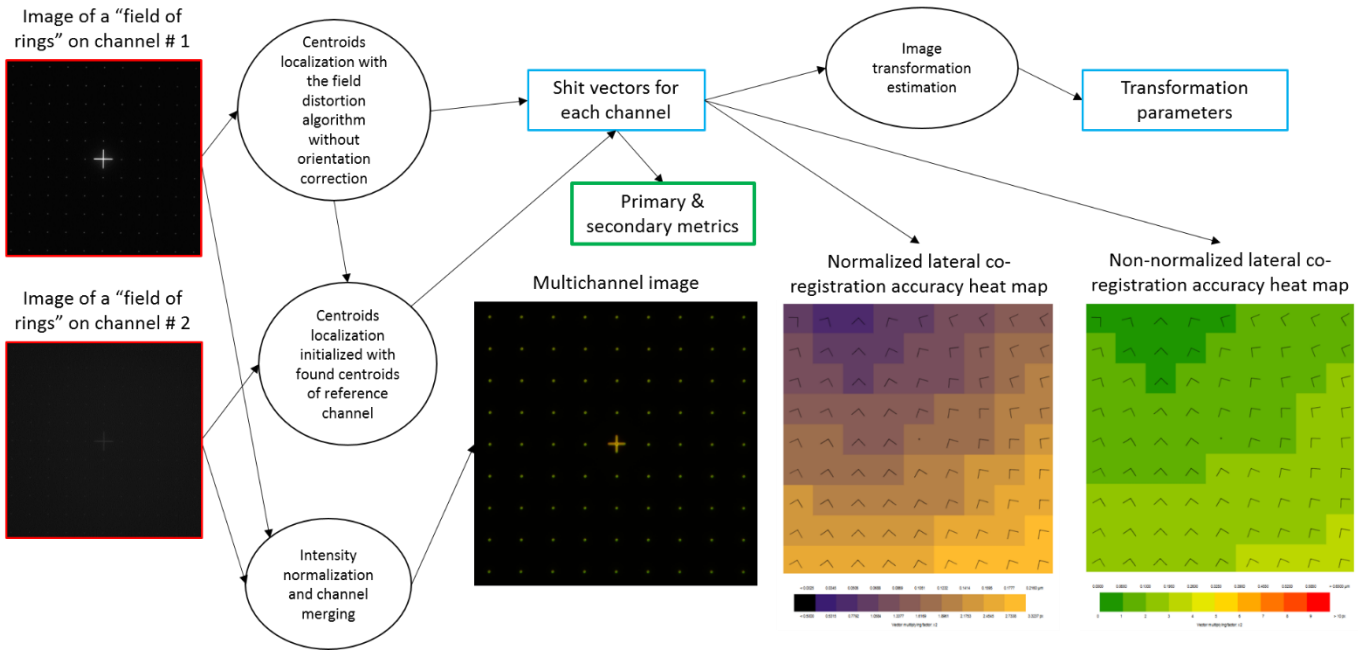


Figure 5: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It detects and segments the rings in the image.
- It determines the XY coordinates of the centroid of each ring.
- It measures the shift between these coordinates for each channel.
- It displays these shifts into a lateral co-registration accuracy heatmap, in which the arrows and the colors indicate respectively the direction and the magnitude of the shifts between the two channels.

Note: the origins of the co-registration vectors are the centroids of the rings in the reference image (*i.e.* the image displayed in green).

VI. OUTPUT METRIC DESCRIPTION

1. PRIMARY METRICS

- *Pearson correlation coefficient (r)* is the correlation coefficient computed from the comparison function of the intensity levels of each pixel for the two images. This is one of the classic parameters to assess colocalization. It can have values between +1 and −1, where +1 corresponds to a total positive correlation (perfect colocalization), 0 to no correlation at all (total non-colocalization), and −1 to a total negative correlation. It is unitless, and is given by the following formula:

$$r = \frac{mean(I_{green} \times I_{red}) - mean(I_{green}) \times mean(I_{red})}{\sigma(I_{green}) \times \sigma(I_{red})}$$

Where *mean* denotes the mean (average) value and *σ* the standard deviation.

- *Normalized orientation entropy* provides information on the orientation disparity of the lateral shift 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 lateral shift vectors are oriented along the same direction, the normalized orientation entropy is zero. This is usually the case when the lateral shifts come only from a filter set cube. If for example 360 lateral shift 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%. This is usually the case when the lateral shifts come only from a refractive optics with a circular symmetry, for example a lens.

- *Maximum of the vector magnitudes* is the magnitude of the vector showing the highest amount of lateral shift. It is expressed both in pixel and µm.

2. TRANSFORMATION PARAMETERS

- The image transformation required to overlay the location of each red ring to one of each green ring is expressed as follows:

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

Where $\{x_{red}\ ;\ y_{red}\}$ and $\{x_{green}\ ;\ y_{green}\}$ are the coordinates of the red and the green rings, respectively.
This transformation is limited only to combinations of translation, uniform scaling (zoom) and rotation.

- *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 pixel and in µm.

3. SECONDARY METRICS

- *Mean of the vector magnitudes* is the mean magnitude of all the lateral shift vectors. It is expressed both in pixel and µm.

- *Standard deviation of the vector magnitudes* is the standard deviation computed from the magnitude of all the lateral shift vectors. It is expressed both in pixel and µm.

- *Minimum of the vector magnitudes* is the magnitude of the vector showing the lowest amount of lateral shift. It is expressed both in pixel and µm.

- *Mean of the tx and ty rigid translation parameters* is the mean magnitude of the *tx* and *ty* rigid translation parameters. It is expressed both in pixel and µm.

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.

- *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.

- *(Vector) multiplying factor* is the multiplying coefficient applied to the magnitude of the heat map vectors. It allows a better display of the vector arrows.

- *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 central ring. 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.

- *Normalized green channel* is the name of the image file which is displayed (in a normalized way) in green in the multichannel image in the results page.

- *Normalized red channel* is the name of the image file which is displayed (in a normalized way) in red in the multichannel image in the results page.

VII. HOW TO CORRECT A LATERAL CO-REGISTRATION INACCURACY?

To correct the lateral co-registration inaccuracy between two images 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.

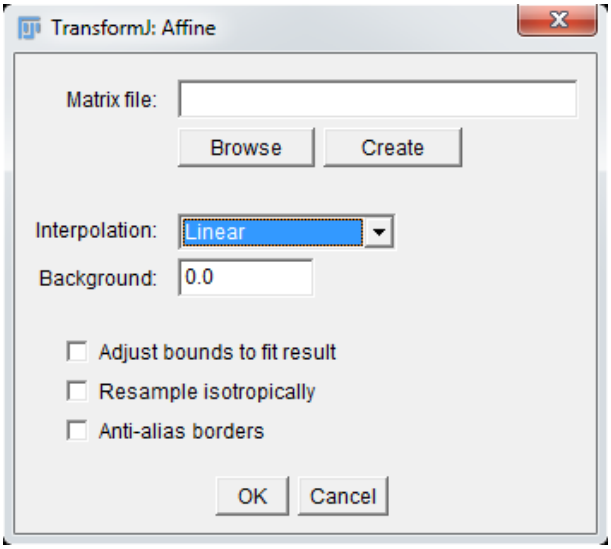


Figure 6: Interface window of the TransformJ plugin in ImageJ, showing that all the different options are unchecked.

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 the lateral co-registration inaccuracy.

The TXT file should be organized as follow:

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).

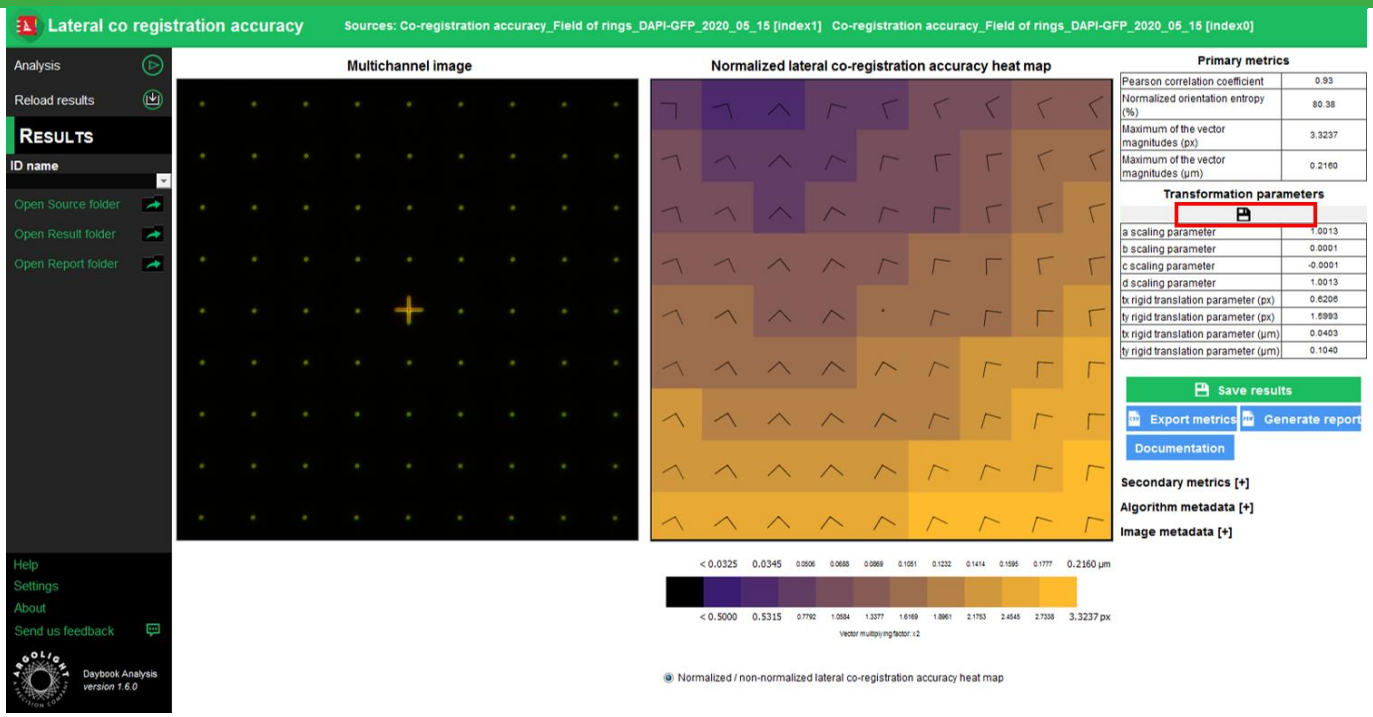


Figure 7: Button to save the image transformation parameters in a TXT file.

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 lateral co-registration inaccuracy 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 issue description at:
customer@argolight.com