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I. 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.

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

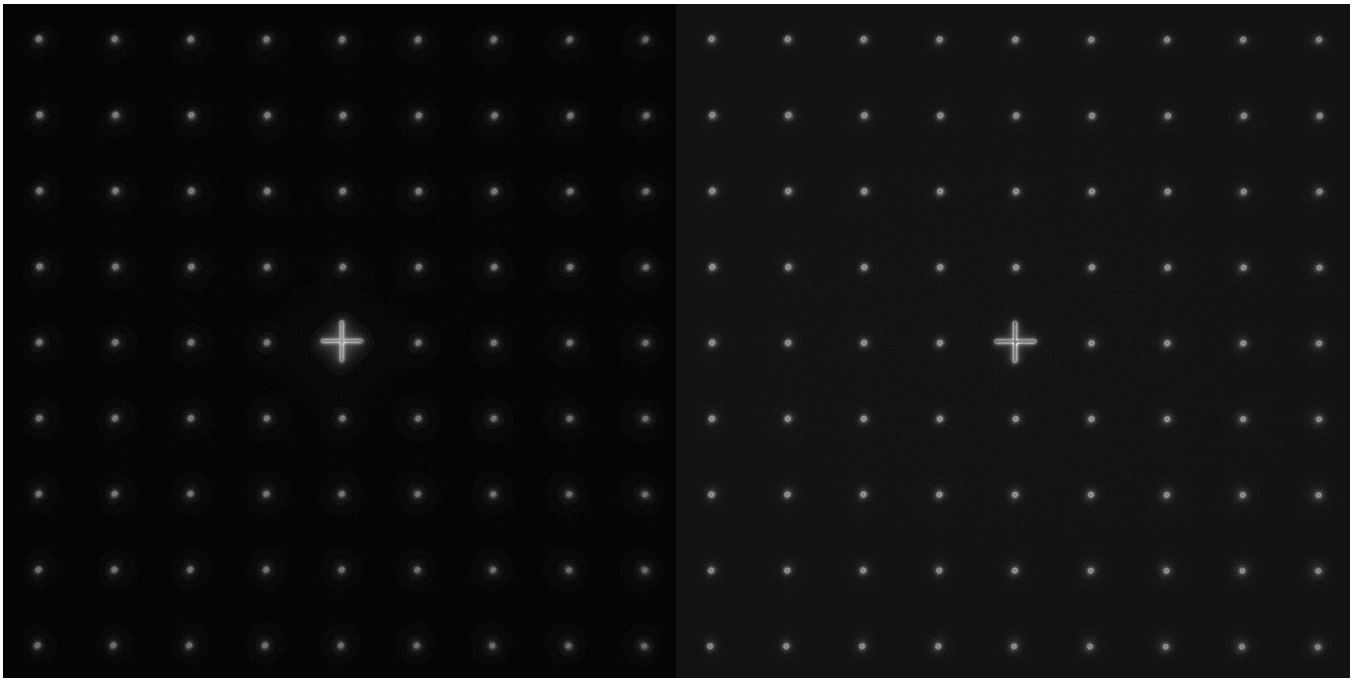

Figure 1: Image examples of the field of rings acquired on two different channels. Left: DAPI channel. Right: GFP channel. The image having the best contrast (left) will be used as reference (green channel), in order to improve the detection of rings in the 2nd image (right).

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).

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- **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 dynamic 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 dynamic range of 8 or 16 bits, the allowed size for computers (1-byte and 2-byte chunks, respectively). If the detector captures raw data with a dynamic range different from 8 or 16 bits, convert the images into 8- or 16-bit depth 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.

Image examples acquired following the acquisition recommendations can be found in your Daybook folder, located here: C:\Program Files\Daybook\Daybook-Analysis\trial images

We encourage you to process these images to have an idea of the image quality required to perform the analysis, and to start being familiar with the use of the software.

2. HOW TO IMAGE THE PATTERN?

1- <u>Find the patterns</u>

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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 bit depth of 8 or 16 bits.

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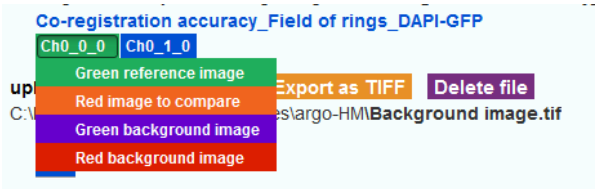
II. IMAGE ANALYSIS PROCEDURE

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 “Green reference image”, select the field of rings image that has the best signal-to-background ratio.
As “Red image to compare”, select another field of rings image.



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).

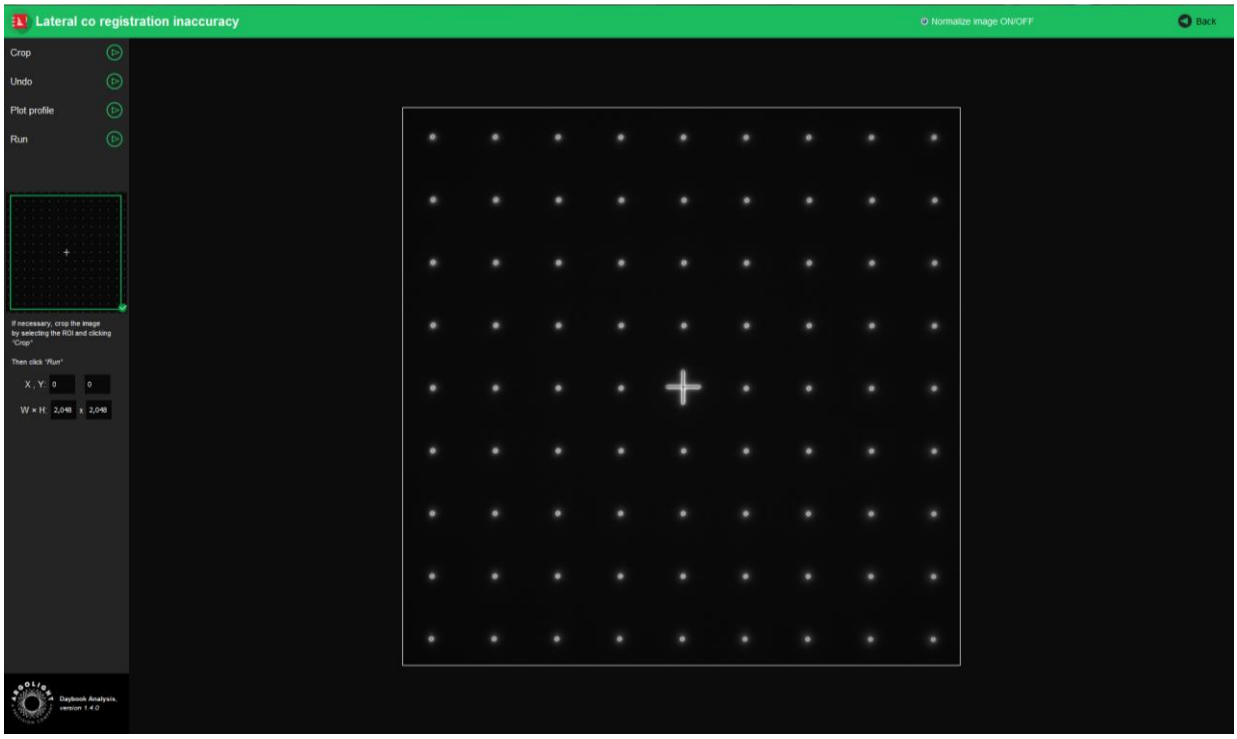


Figure 2: Crop window.

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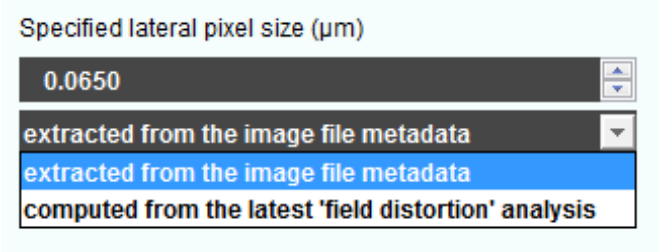
f) Click on “Run». Results are displayed and can be saved as “*.csv”, “*.pdf”, or transferred into Daybook Data Manager (if available in your package).

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 correction**

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.

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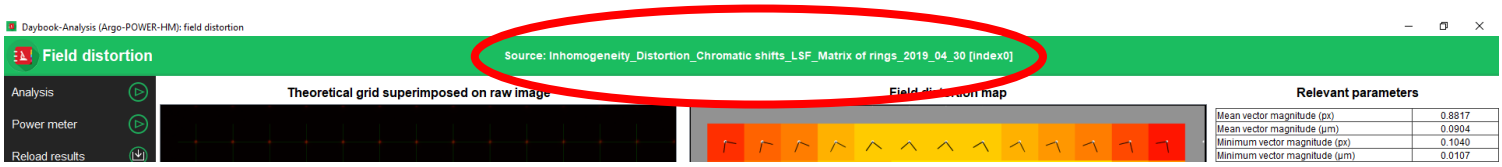
The index of the selected image is displayed in the middle top of the results page (see figure below). Information about the selected image can also be found in the metrics and reports.



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III. RESULTS PAGE DESCRIPTION

The picture below shows the results page for this analysis (*cf.* Figure 3).
Results are displayed in the form of maps, graphs, and tables.

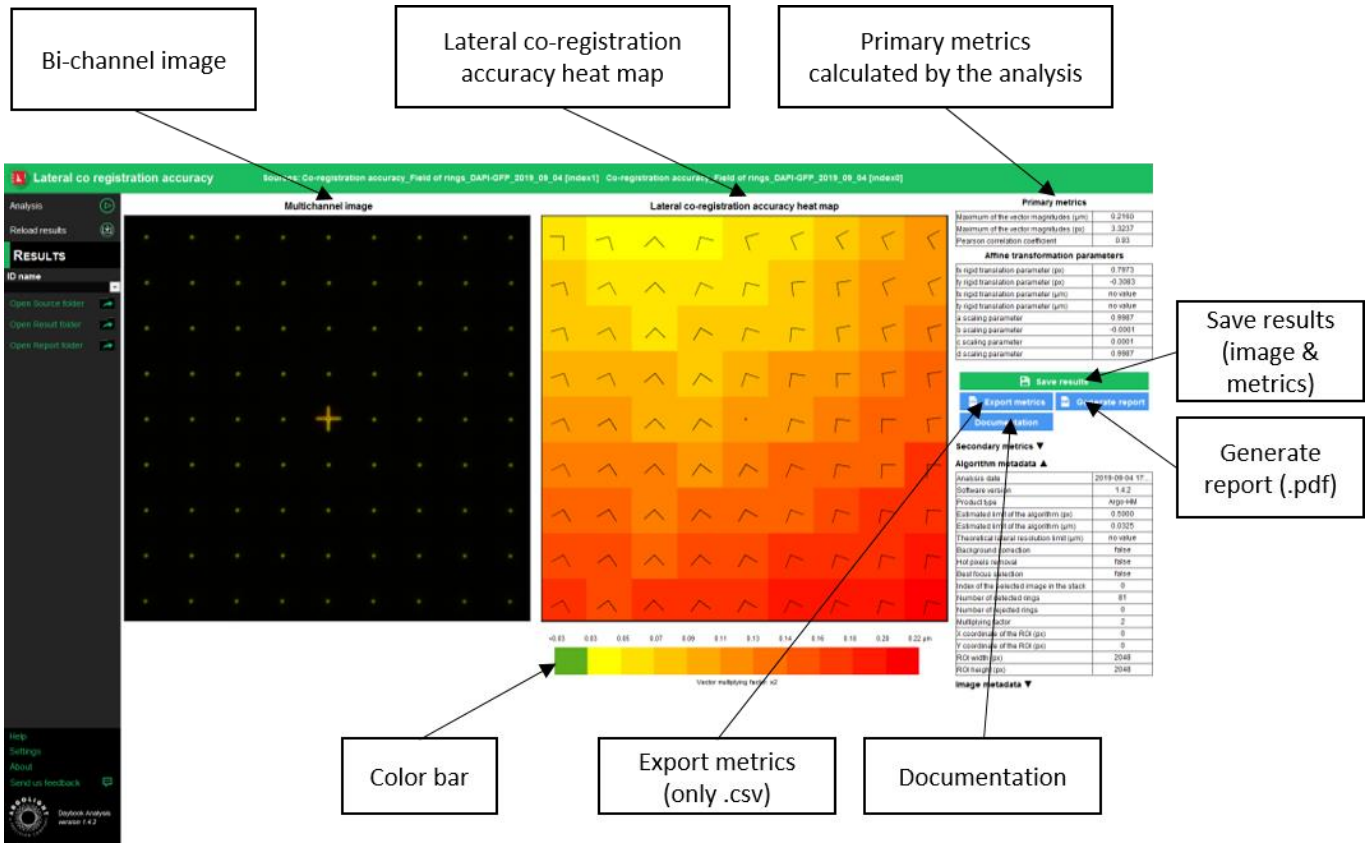

Figure 3: Results page.

When Daybook Data Manager is disabled, the results can be saved into a "*.csv" file thanks to the "Save results" or "Export metrics" buttons.

When a valid Daybook Data Manager license is registered, the results are transferred into Daybook Data Manager thanks to the "Save in Data Manager" button.

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 9).

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.

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

- **Graph options:**

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- 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" file, or the graph values into a "*.txt" file.
  - "AutoRange": Adjust automatically the ranges of the axes.

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IV. ANALYSIS ALGORITHM DESCRIPTION

The diagram below describes the algorithm that allows the extraction of the lateral co-registration accuracy from two images of the field of rings (*cf.* Figure 4).

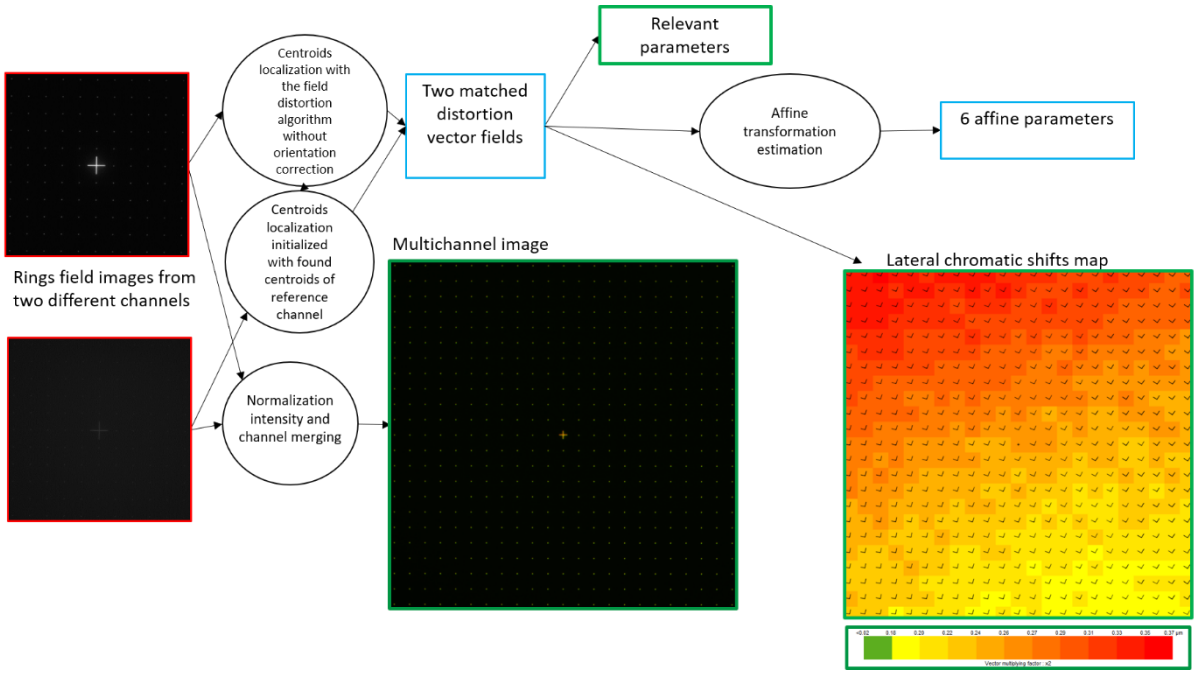

Figure 4: Schematic description of the different steps of the analysis algorithm.

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.

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V. OUTPUT METRIC DESCRIPTION

1. PRIMARY METRICS

- *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.

- *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.

2. AFFINE TRANSFORMATION PARAMETERS

- The affine 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, rotation, and uniform scaling.

- *a, b, c and d* are the rotation and uniform scaling parameters.

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

3. SECONDARY METRICS

- *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).

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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.

- *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 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.

- *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.

- *Theoretical lateral resolution limit* is the theoretical lateral resolution limit (corresponding to the PSF FWHM criterion) the user may have entered before executing the analysis. It is expressed in µm. This parameter defines a low limit below which the lateral co-registration accuracy is

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considered to be within the manufacturer's specifications, for instance for chromatic aberration (Monochromat, Achromat, Semi-apochromat or Apochromat).

- *Background correction* indicates if the "Background correction" 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 in the pattern "field of rings" detected by the algorithm.
- *Number of rejected rings* is the number of rings in the pattern "field of rings" rejected by the algorithm, due to a non-detection or because some rings are cut in the image.

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.
- *Detector dynamic range* is the dynamic 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.
- *Bit depth* is the size of the image, provided by the metadata associated to the raw image. It is expressed in bits (8 or 16 bits).
- *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

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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.

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

To correct the lateral co-registration inaccuracy between two images of a biological sample, one can copy the affine transformation parameters into a "*.txt" file, and then use for instance the TransformJ Affine plugin in ImageJ (https://imagescience.org/meijering/software/transformj/) to process the correction.

The "*.txt" file should be organized as follow:

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

Here, *tx* and *ty* must be expressed in pixel.

Warning:
The image to be corrected must be acquired with the same conditions as the one of the field of rings.
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.
Deviating from these requirements will lead to a wrong correction, eventually to an increase of the lateral co-registration inaccuracy amount in the corrected image.

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Encountered an issue or a question when running this analysis?
Please send a screenshot and your issue description at
customer@argolight.com

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