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I. INTRODUCTION

The field uniformity is a well-known parameter related to the inhomogeneous spatial distribution of the intensity in the image of an even object.

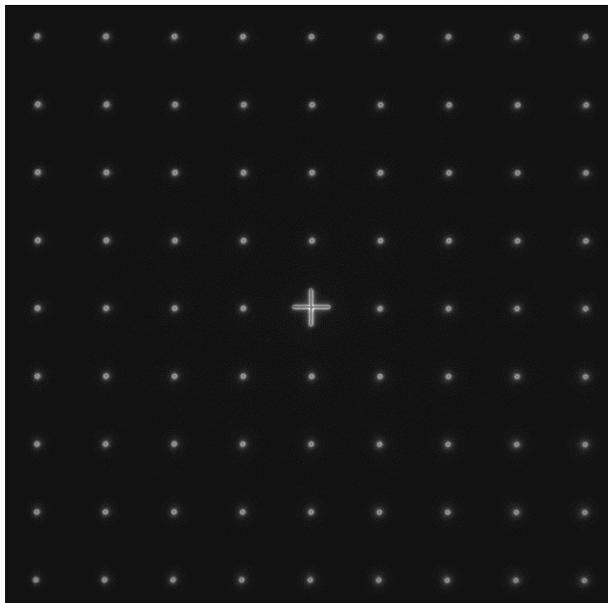
In any fluorescence microscope, the knowledge of the field uniformity is important when the image intensity information is aimed to be measured. For intensity quantification in images of biological samples, the field uniformity shall be known, and eventually corrected, to have access to accurate measurements.

The “field uniformity” analysis provides the ***spatial distribution of the intensity***, due to illumination and detection inhomogeneities, within the field of view.



II. IMAGE ACQUISITION PROCEDURE

The “*field uniformity*” analysis is associated with the “*field of rings*” pattern (Pattern B - see Figure 1).



CAUTION

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

Figure 1: Image example 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

When a multi-channel Z-stack is acquired, the reader in Daybook Analysis 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. 100x) then with the smallest magnification objective (e.g. 20x).





- **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.

2. HOW TO IMAGE THE PATTERN?

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) 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 (the best is the acquisition software proprietary format) or into a lossless compression format (e.g. TIFFs can be lossless, depending on the technique chosen for storing the pixel data). The image file must have a dynamic range of 8 or 16 bits.

Important:

The minimum required number of rings in the image is 3x3.

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 uniformity” 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 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” to run the analysis. Results are displayed and can be saved as CSV, PDF, or transferred into Daybook Data Manager (if available in your package).

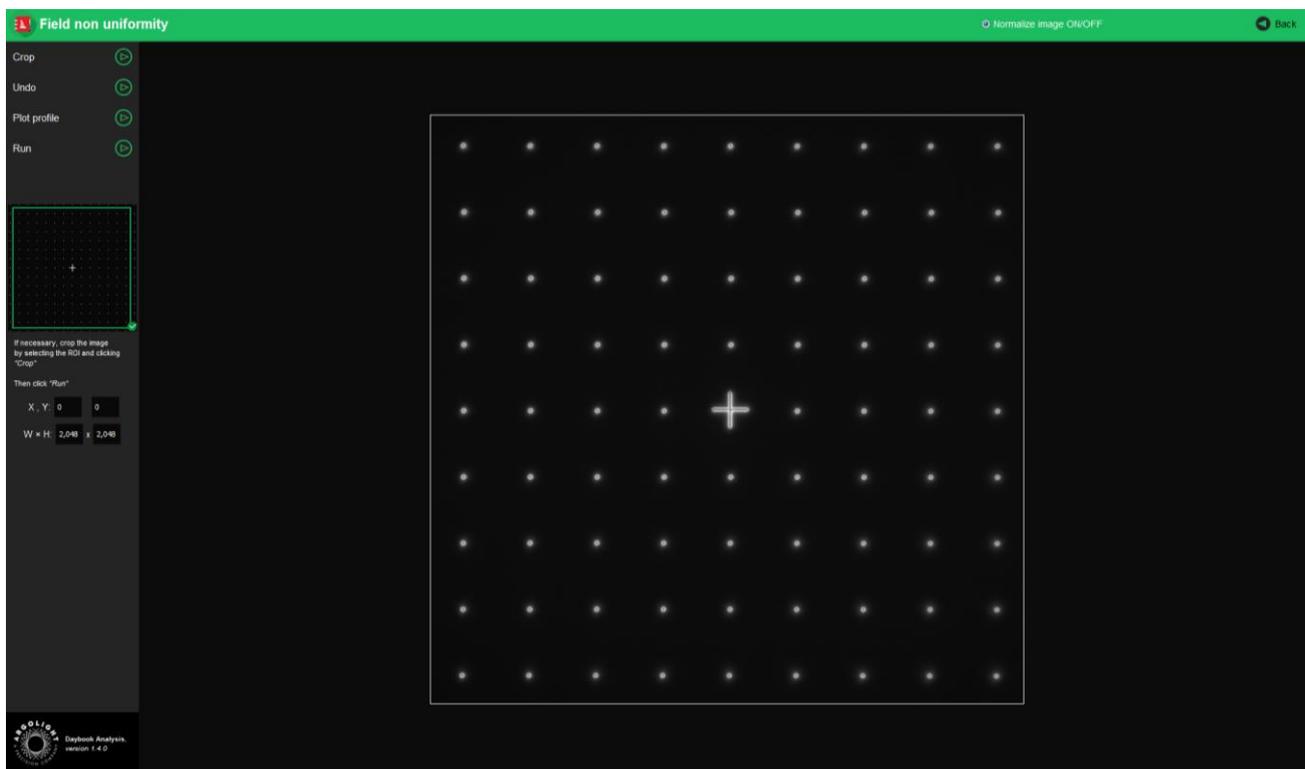


Figure 2: Crop window.



2. ANALYSIS SETTINGS

1- Required settings

- **Specified lateral pixel size**

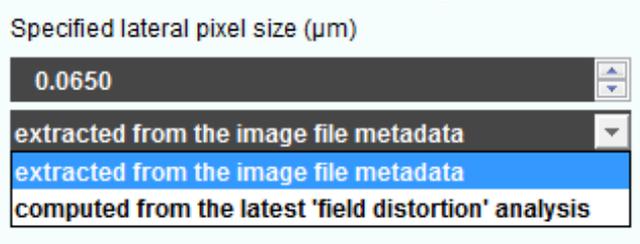
There are two ways to get the lateral pixel size of the image to be analyzed:

- 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**

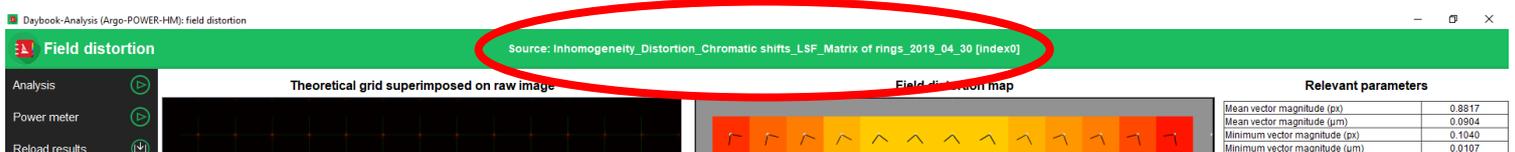
On Z-stacks analysis, the axial pixel size is determined from the proprietary file.

- **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 algorithm metadata.





- **Intensity projection**

Works only for mono- or multi-channel Z-stacks. It projects onto a 2D image the maximum or the mean intensity pixels of the images from the Z-stack. It is advised to use this option to prevent from any tilt that could be introduced if the lateral stage and/or the sample holder are not perfectly horizontal.



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.

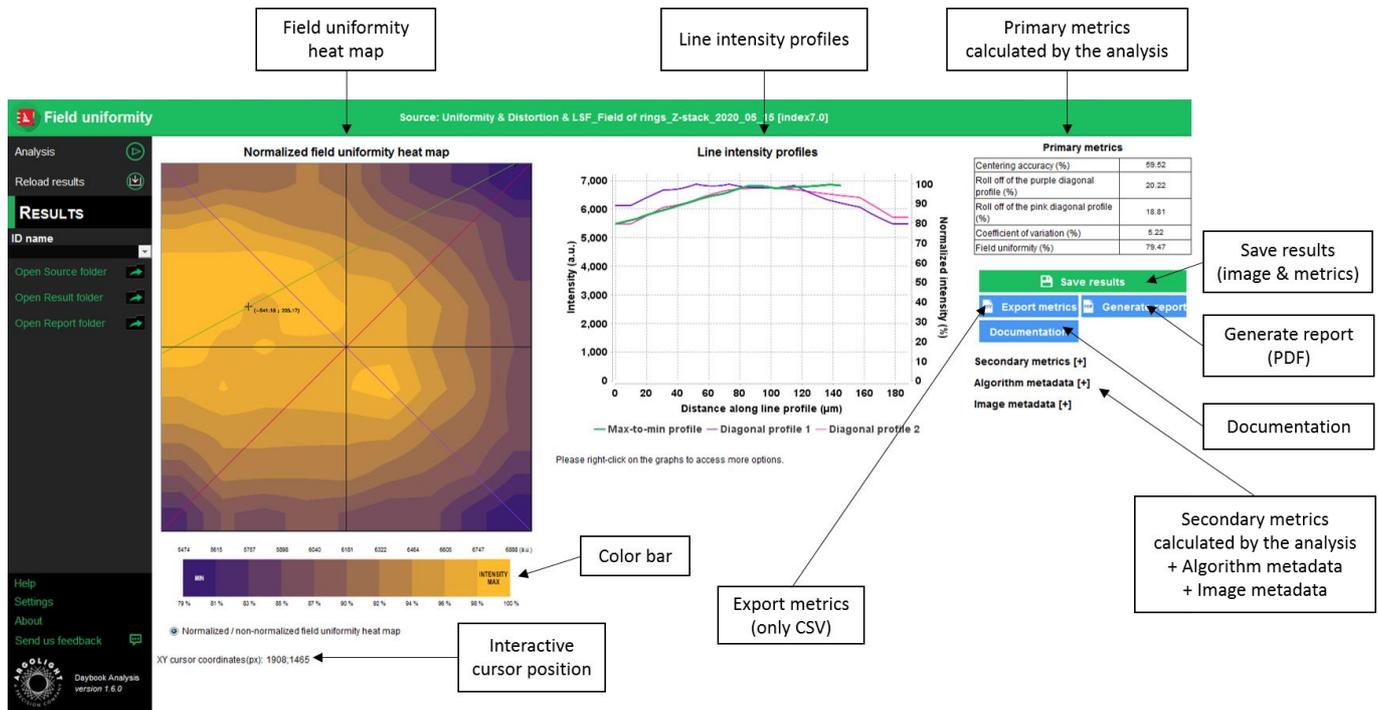


Figure 3: Results page.

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).

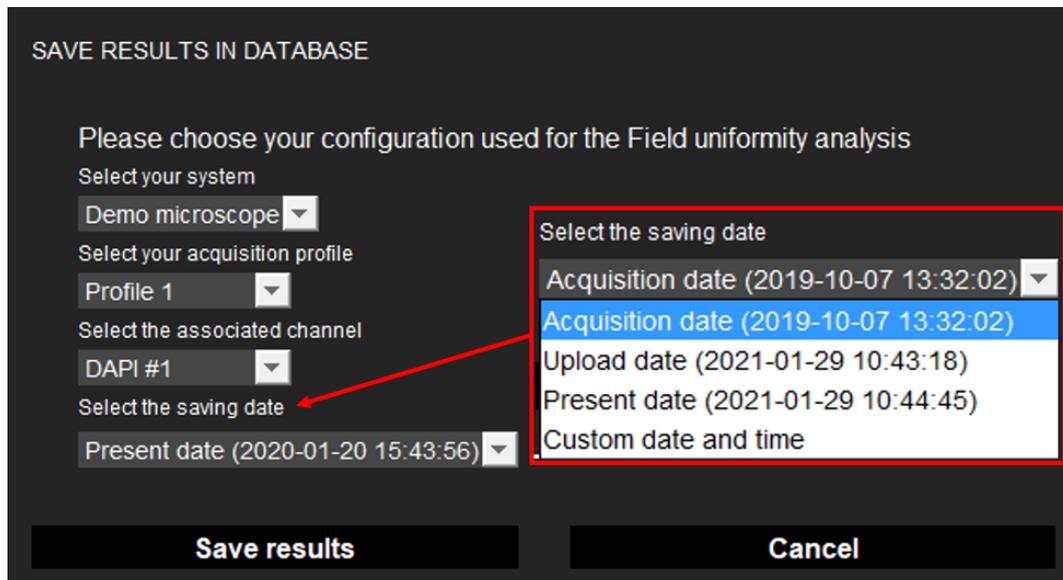


Figure 4: Interface window for saving the results in the database.

- **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 field uniformity from the “field of rings” image (cf. Figure 5).

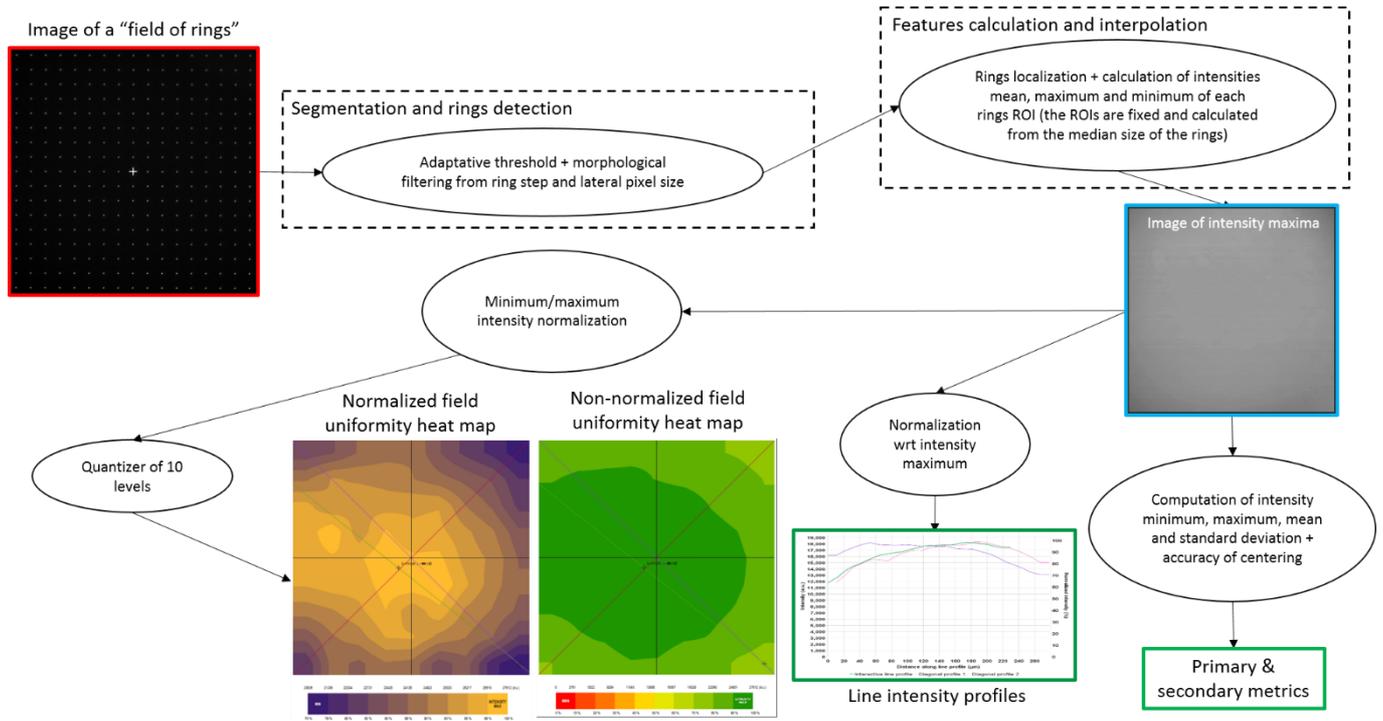


Figure 5: Schematic description of the different steps of the analysis algorithm.

2. DESCRIPTION

In short, the algorithm works as follows:

- It detects and segments the rings in the image.
- It finds the XY coordinates of the centroid of each ring.
- It measures the maximum intensity in the ROI of each detected ring.
- It interpolates linearly the intensity values of the blanks between the rings.
- It displays these intensities into a field uniformity heat map, either normalized or non-normalized, in which the colors indicate the magnitude of the field uniformity.



VI. OUTPUT METRIC DESCRIPTION

Many parameters and ways to display them can be found in the literature or can be invented to describe the spatial distribution of the intensity uniformity in the entire field of view. No consensus has arisen from the community to determine which of these parameters are the most suitable. That is why we propose what we think to be an exhaustive list of all the possible parameters that can be used to describe the field uniformity of a microscope.

1. PRIMARY METRICS

- **Centering accuracy** is a measure of how well the intensity is centered with respect to the field of view. It is expressed in % and is given by the following equation:

$$\text{Centering accuracy} = 100 \times \left(1 - 2 \frac{\sqrt{(x_{\max} - x_{\text{center}})^2 + (y_{\max} - y_{\text{center}})^2}}{\sqrt{w^2 + h^2}} \right)$$

Where $\{x_{\max}; y_{\max}\}$ are the coordinates of the intensity maximum centroid; $\{x_{\text{center}}; y_{\text{center}}\}$ are the coordinates of the center of the field of view; w and h are the width and height of the field of view, before any eventual ROI selection (crop).

- **Roll-off** is a measure of the intensity variation along a line intensity profile. It is expressed in %, and is given by the following equation:

$$\text{Roll-off} = 100 \times \frac{I_{\max} - I_{\min}}{I_{\max}}$$

Where I_{\max} and I_{\min} are the maximum and minimum intensities, respectively, along a line intensity profile. A roll-off of 0 % denotes a system with a perfect field uniformity.

- **Roll-off of the purple diagonal profile** is the roll-off value along the purple diagonal in the field uniformity heat map.
- **Roll-off of the pink diagonal profile** is the roll-off value along the pink diagonal in the field uniformity heat map.
- **Coefficient of variation** is a measure of the fluctuations of intensity in the image compared to the mean intensity. It is expressed in % and is given by the following equation:

$$\text{Coefficient of variation} = 100 \times \frac{\text{Standard deviation}}{I_{\text{mean}}}$$

- **Field uniformity** is a measure of the uniformity in the image. It is expressed in %, and is given by the following equation:

$$\text{Field uniformity} = 100 \times \frac{I_{\min}}{I_{\max}}$$

Where I_{\max} and I_{\min} are the maximum and minimum intensities, respectively, in the



image.

2. SECONDARY METRICS

- *Roll-off of the green line profile* is the roll-off value along the green profile in the field uniformity heat map between the maximum and the minimum intensity values in the image. It is expressed in %.
- *Intensity mean* is the mean intensity calculated from the field uniformity raw image. It is expressed in arbitrary unit.
- *Intensity standard deviation* is the standard deviation calculated from the raw image. It is expressed in arbitrary unit.

- *Contrast* is a measure of the contrast of intensity in the image. It is expressed in % and is given by the following equation:

$$Contrast = 100 \times \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$

Where I_{max} and I_{min} are the maximum and minimum intensities, respectively, in the image.

- *Intensity maximum* is the maximum intensity in the raw image. It is expressed in arbitrary unit.
- *Intensity minimum* is the minimum intensity in the raw image. It is expressed in arbitrary unit.
- *X or Y coordinate of the intensity minimum* is the coordinate of the minimum intensity along X or Y, the origin of the coordinate system being the center of the image. It is expressed both in pixel and in μm .
- *X or Y coordinate of the centroid* is the coordinate of the centroid (or center of mass) along X or Y, the origin of the coordinate system being the center of the image. It is expressed both in pixel and in μm .
- *Standard deviation normalized wrt detector bit depth* is the standard deviation calculated from the raw image, normalized with respect to the detector bit depth. It is expressed in % and is given by the following equation:

$$Std \text{ normalized wrt the detector bit depth} = 100 \times \frac{Standard \ deviation}{2^{Detector \ bit \ depth}}$$

Where *Standard deviation* is the standard deviation of the image for all the pixels, and



Detector bit depth is the bit depth of the detector.

- *Standard deviation normalized wrt intensity maximum* is the standard deviation calculated from the raw image, normalized with respect to the intensity maximum in the image. It is expressed in % and is given by the following equation:

$$\text{Std normalized wrt intensity maximum} = 100 \times \frac{\text{Standard deviation}}{I_{max}}$$

- *Level factor normalized wrt detector bit depth* represents one graduation of the field uniformity heat map color bar, compared to the detector bit depth. It is expressed in % and is given by the following equation:

$$\begin{aligned} &\text{Level factor normalized wrt image dynamic range} \\ &= 100 \times \frac{I_{max} - I_{min}}{2^{\text{Detector bit depth}} \times \text{Number of color bar divisions (i. e. 10)}} \end{aligned}$$

- *Level factor normalized wrt intensity maximum* represents one graduation of the field uniformity heat map color bar. It is expressed in % and is given by the following equation:

$$\begin{aligned} &\text{Level factor normalized wrt intensity maximum} \\ &= 100 \times \frac{I_{max} - I_{min}}{I_{max} \times \text{Number of color bar divisions (i. e. 10)}} \end{aligned}$$

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.
- *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.
- *Intensity projection* indicates if the “Intensity projection” 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.



- *Intensity projection type* indicates the type (mean or maximum) of intensity projection when activating the “Intensity projection” 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 image to be analyzed, considering whether it was cropped or not. It is expressed in pixel.
- *Y coordinate of the ROI* is the coordinate along Y (starting from the top left corner) of the image to be analyzed, considering whether it was cropped or not. It is expressed in pixel.
- *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 .
- *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. It is expressed in pixel.
- *Image height* is the height of the image. It is expressed in pixel.



VII. HOW TO CORRECT A NON-UNIFORM FIELD?

NB: the following will not apply if you or the algorithm cropped the “field of rings” image for the analysis.

To correct the field non-uniformity in an image of a biological sample, one can use the field uniformity raw image and then use for instance the “Calculator Plus” plugin (in the “Process” menu) in ImageJ (<https://imagej.nih.gov/ij/plugins/calculator-plus.html>) to carry out the correction.

The correction is performed using the following mathematical operation:

$$\begin{aligned}
 & \textit{Corrected image} \\
 &= \textit{Intensity mean}["\textit{Field_uniformity_raw_map"} \textit{ image}] \\
 &\times \frac{\textit{Image to correct} - \textit{Background image}}{\textit{"Field_uniformity_raw_map"} \textit{ image}}
 \end{aligned}$$

Where *Corrected image* is the image corrected for a non-uniform field;
Intensity mean[Field uniformity raw image] the mean intensity calculated from the “field_uniformity_raw_map” image;
Image to correct the image of a biological sample to correct for a non-uniform field;
Background image an image of the biological sample where there is no signal of interest;
Field uniformity raw image the image generated by Daybook Analysis.
 This image is named “field_uniformity_raw_map.png”. It can be found in the "Temp folder" if Daybook Data Manager is activated or in the "Result folder" if not.
 To generate this image, we recommend using a background image of an area where there is no fluorescent pattern and ticking the “Background subtraction” option (for more information, see “Background subtraction” in the “Optional settings” section).

If using the “Calculator Plus” plugin, make sure you select the “Divide” operation, and fill-in the mean intensity in the K1 box, as illustrated in Figure 6.

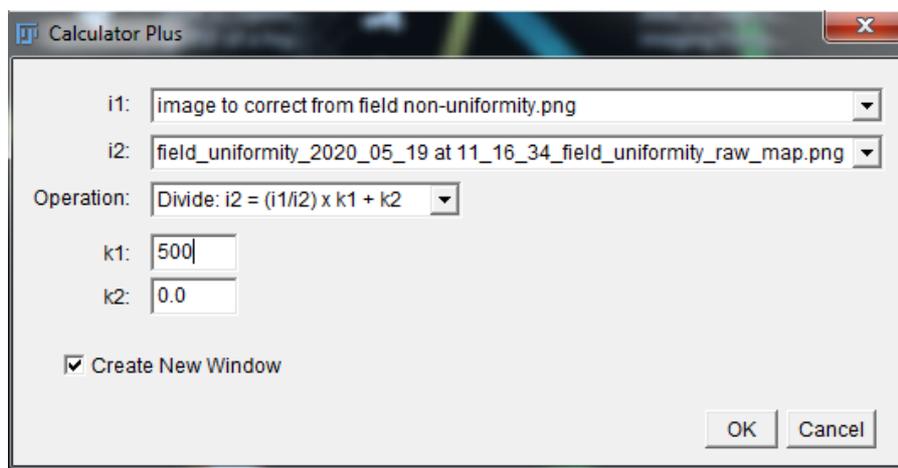


Figure 6: Interface window of the Calculator Plus plugin in ImageJ, showing the selection of the “Divide operation” and the filling-in of a value in the K1 box.



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 $(170 \pm 5) \mu\text{m}$ 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 should be mounted just after a #1.5 coverslip. According to ISO 8255-1:2017, the #1.5 coverslip has the following properties: thickness of $(170 \pm 5) \mu\text{m}$, refractive index of 1.5255 ± 0.0015 at 570 nm, Abbe number of 56 ± 2 .
- Images acquisition settings should be chosen so that the intensity histograms of the biological sample image and the “field of rings” image are similar.

Otherwise, optical aberrations, such as spherical or chromatic aberrations, and/or intensity-related issues, could degrade the correction quality.

Deviating from these requirements may lead to a wrong correction, or at worst to an increase of the field non-uniformity 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**