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I. IMAGE ACQUISITION PROCEDURE

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

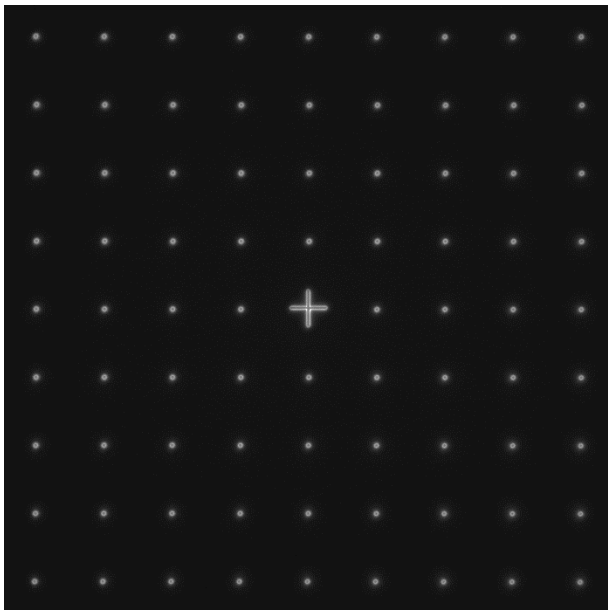


Figure 1: Image example of the field of rings, with a cross at the center, fulfilling the acquisition recommendations.

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.* 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 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>

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- <u>Adjust your setup</u>

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.

Important:
The minimum required number of rings in the image is 3×3.
If the number of rings in the image is lower than this value, the algorithm will not work.

II. 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).

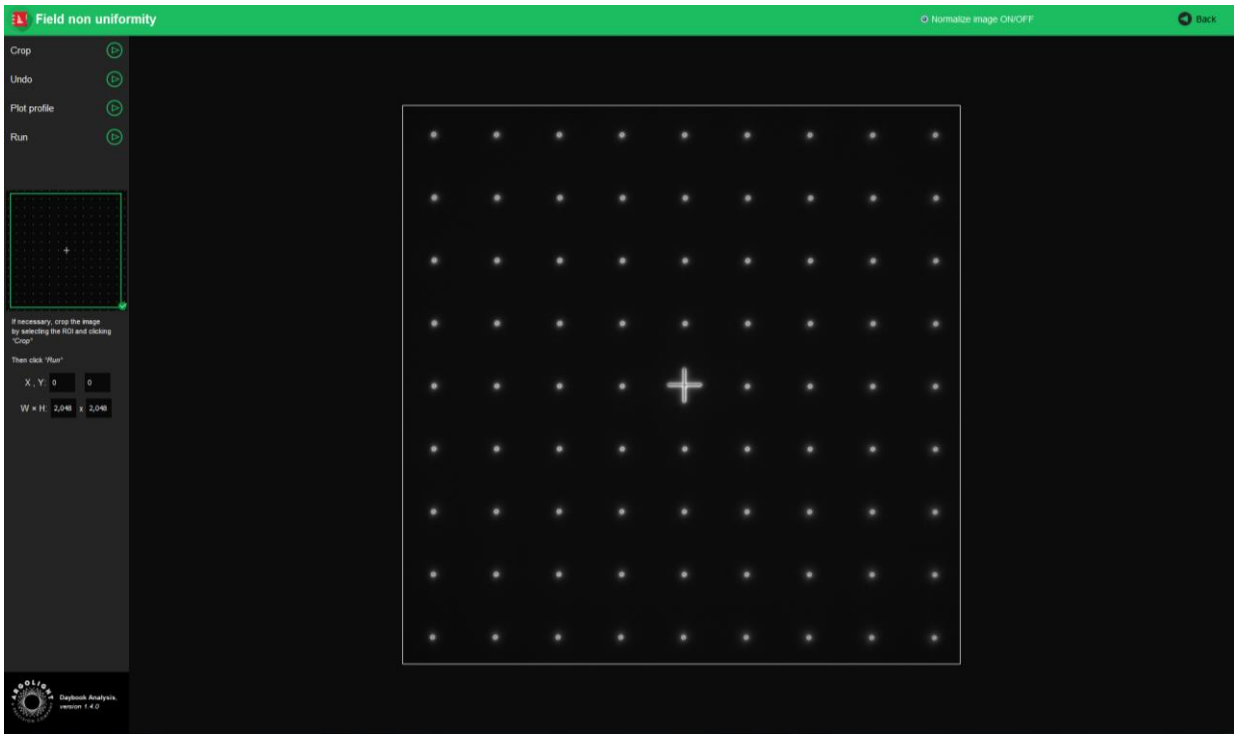


Figure 2: Crop window.

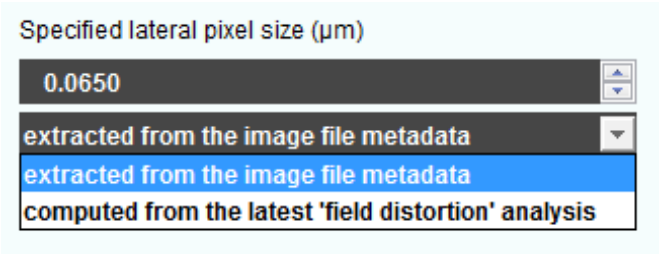
f) Click on “Run” to run the analysis.
Results are displayed and can be saved as “.*csv”, “*.pdf”, or transferred into Daybook Data Manager (if available in your package).

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"*.



- **Feature type**

Daybook can extract the intensity response in two ways:
- By measuring the average intensity in each ROI.
- By measuring the average of the peak intensities from the intensity profile perpendicular to the lines.

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**

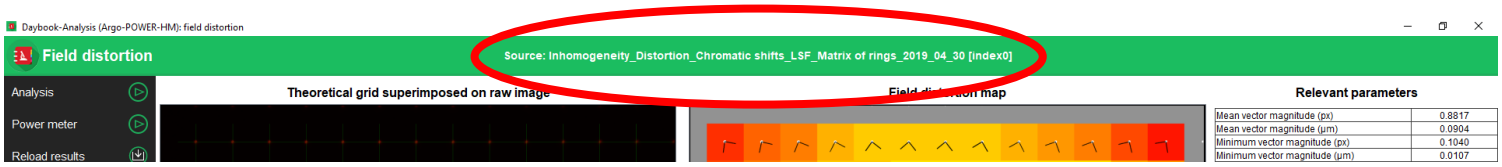
On Z-stacks analysis, the axial pixel size is determined from the proprietary file.

- **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.

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.



- **Maximum intensity projection**

Works only for mono- or multi-channel Z-stacks. It projects onto a 2D image the maximum intensity pixels of the images from the Z-stack.

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.

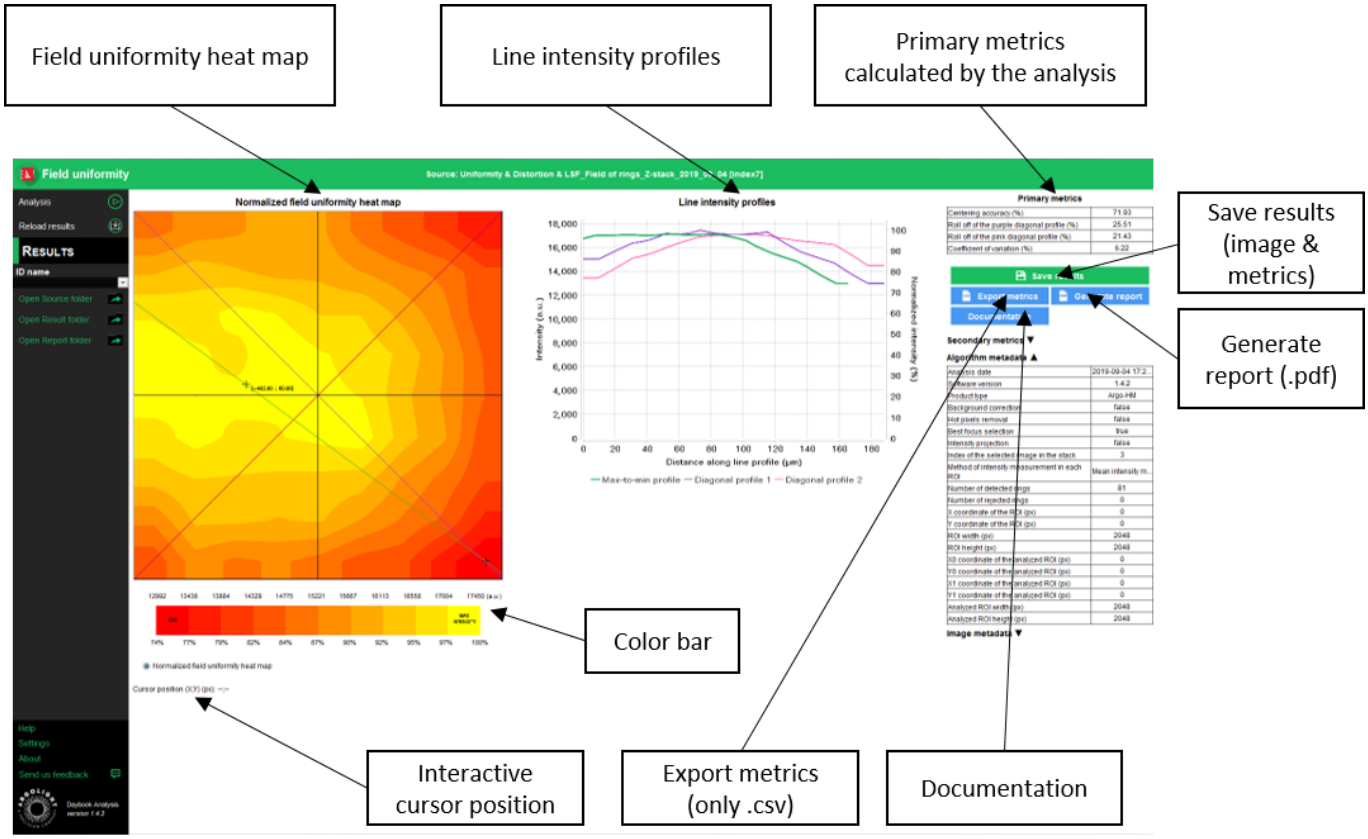


Figure 3: Results page.

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:**
  - 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.

IV. ANALYSIS ALGORITHM DESCRIPTION

The diagram below describes the algorithm that allows the extraction of the field uniformity from the field of rings image (*cf.* Figure 4).

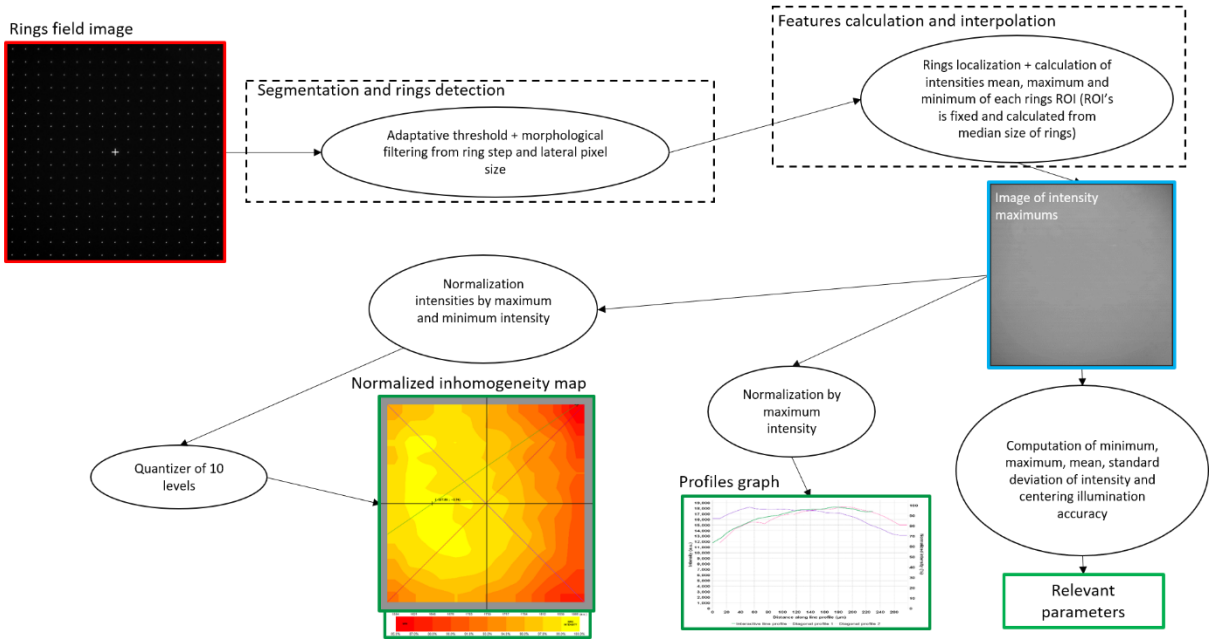


Figure 4: Schematic description of the different steps of the analysis algorithm.

In short, the algorithm works as follows:

- It detects and segments the rings in the image.
- It finds the XY coordinates of the centroid of each ring.
- It measures the mean or maximum intensity of each ring (depending on the one selected in the required settings).
- It interpolates the intensity values of the blanks between the rings.
- It displays these intensities into a field uniformity heatmap, either normalized or non-normalized, in which the colors indicate the magnitude of the field uniformity.

V. 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 inhomogeneity 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:

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

  Where $\{x_{max}\ ;\ y_{max}\}$ are the coordinates of the intensity maximum centroid; $\{x_{center}\ ;\ y_{center}\}$ 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 an intensity profile. It is expressed in %, and is given by the following equation:

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

  Where $Intensity_{max}$ and $Intensity_{min}$ are the maximum and minimum intensities, respectively, along an intensity profile.

  Roll-off of the pink diagonal profile is the roll-off value along the pink diagonal in the homogeneity heatmap.

  Roll-off of the purple diagonal profile is the roll-off value along the purple diagonal in the homogeneity heatmap.

- *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:

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

2. SECONDARY METRICS

Roll-off of the green line profile is the roll-off value along the green profile in the homogeneity heatmap between the maximum and the minimum intensity values. It is expressed in %.

- *Intensity mean* is the mean intensity calculated from the 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:

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

- *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 coordinate of the centroid* is the location of the maximum intensity in the horizontal X direction. It is expressed both in pixel and in µm.

- *Y coordinate of the centroid* is the location of the maximum intensity in the vertical Y direction. It is expressed both in pixel and in µm.

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

$$Std\ normalized\ by\ the\ sensor\ dynamic = 100 \times \frac{Standard\ deviation}{Sensor\ dynamic}$$

Where $Standard\ deviation$ is the standard deviation of the image for all the pixels, and $Sensor\ dynamic$ is the dynamic of the sensor (for example, a 16-bits dynamic sensor has a dynamic of 2^{16} = 65536).

- *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:

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

- *Level factor normalized wrt sensor dynamic range* represents one graduation of the homogeneity

heatmap color bar, compared to the full sensor dynamic range. It is expressed in % and is given by the following equation:

$$Level\ factor\ normalized\ wrt\ sensor\ dynamic\ range = 100 \times \frac{I_{max} - I_{min}}{Sensor\ dynamic\ range \times Number\ of\ color\ bar\ divisions\ (i.e.\ 10)}$$

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

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

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 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.
- *Intensity projection* indicates if the “Intensity projection” option has been activated or not.
- *Intensity projection type* indicates the type (mean or maximum) of intensity projection when activating the “Intensity projection” option.
- *Method of intensity measurement in each ROI* indicates the method (mean or maximum) used for the intensity measurement in each ROI.
- *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.

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.
- *Detector dynamic range* is the dynamic range of the detector, expressed in bits. For example, a 16-bit detector can capture 2^{16} = 65536 intensity levels.
- *Bit depth* is the size of the image, expressed.in bits (8 or 16 bits).
- *Image width* is the width of the image, expressed.in pixel.
- *Image height* is the height of the image, expressed.in pixel.

VI. HOW TO CORRECT A NON-UNIFORM FIELD?

To correct the field non-uniformity in an image of a biological sample, one can use the field uniformity raw image. This image is named “homogeneity_raw_map.png” and can be found in the "Temp folder" if Daybook Data Manager is activated or in the "Result folder" if not.

The correction is performed using the following mathematical operation:

$$Corrected\ image = \frac{Image\ to\ correct - Background\ image}{Field\ non\ uniformity\ raw\ image}$$

Where $Corrected\ image$ is the image corrected for a non-uniform field;
$Image\ to\ correct$ 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;
$Field\ non\ uniformity\ raw\ image$ the image generated by Daybook Analysis.
This image is named “homogeneity_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 correction” option (for more information, see “Background correction” in the “Optional settings” section).

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.
The intensity histograms of field of rings image and the biological sample image must be similar.
Deviating from these requirements will lead to a wrong correction, eventually to an increase of the field non-uniformity amount in the corrected image.

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