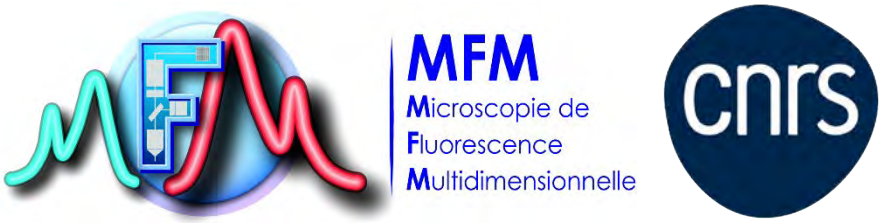
The MetroloJ_QC plugin
Version 1.3.1 Oct 2024

The project:

MetroloJ_QC is a branch of the legacy plugin MetroloJ, developed by Fabrice Cordelières and Cédric Matthews. The main goal of the branch is to allow automation of all Quality Control (QC) tests routinely performed in a light microscopy facility.

The QC plugin, its manual and associated protocols, the Faklaris et al. 2021 work, come as a result of a collective work of the members of the Metrology Working Package (WP) GT3M within the French Technological Network of the Multi-Dimensional Fluorescence Microscopies (RTmfm), supported by the Mission pour les Initiatives Transverses et Interdisciplinaires du CNRS. The WP members associated with this work and the initial MetroloJ plugin are: Suzanne Bolte, Pierre Bourdoncle, Fabrice Cordelière, Aurélien Dauphin, Raphaëlle Desvaux, Alain Dieterlen, Dominique Dumas, Orestis Faklaris, Mickaël Fere, Perrine Frère, Ludovic Galas, David Geny, Jean-François Gilles, Thomas Guilbert, Laurent Héliot, Didier Hentsch, Philippe Legros, France Lam, Camille Lebugle, Ludovic Leconte, Tudor Manoliu, Sébastien Marais, Cedric Matthews, Baptiste Monterroso and Damien Schapman.



The MetroloJ_QC project follows scripts/macros initiated by Julien Cau and David Akbar designed to automatize the initial MetroloJ plugin. MetroloJ_QC main contributors to the previous and current versions are Ioannis Alexopoulos, Leslie Bancel-Vallée, , Julien Cau, Orestis Faklaris, Thomas Guilbert and Baptiste Monterroso. Sandra Ritz, Martin Spitaler & Tomasz Wegierski.

We would specifically like to thank Laurent Gelman for inputs and the square root PSF image idea, Roland Nitschke for fruitful discussions and Sebastian Beer for cameras noise estimation formulas and stimulating discussions.

The plugin is available for download (compiled and full version) here.

This is the plugin companion of a large study described in Faklaris et al. 2021 https://doi.org/10.1101/2021.06.16.448633

Please report any bug you may find while using this plugin to metrologie@groupes.renater.fr

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PLUGIN INSTALLATION & REQUIREMENTS

The project can be downloaded from GitHub here.
https://github.com/MontpellierRessourcesImagerie/MetroloJ_QC/tree/main
Feel free to contribute to further MetroloJ_QC developments.
A .jar version of the plugin can also be downloaded.

QC uses ImageJ options that are only available starting from version 1.53s. We do recommend updating Fiji or ImageJ to the latest version.

First, close ImageJ in case the software is already running. Copy and paste the MetroloJ_QC.jar file into the ImageJ/Plugins folder. Download the version 5.5.13 of *iText library* by following this link. Note that a link is directly available in the "About Window" that can be opened through the "About" button of the main bar (Figure 1, Plugins Option line, right-hand button).

iText library will be used by the plugin to generate pdf reports. Copy and paste it into the ImageJ/Plugins folder. Restart ImageJ. Version 1.3 of the plugin also uses bioformats, make sure a valid version of bioformats is used by your ImageJ install. v1.3 was developed using bioformats package version 6.6.1 and should be working with later/earlier versions.

A *MetroloJ-QC* entry should appear under the ImageJ's plugins menu, that launches the main bar.

THE METROLOJ_QC TOOLS & HELP

The main bar of the MetroloJ_QC plugin gives access to 3 types of tools:
The upper buttons are for analysis of "unique" images. These can be either single- (all tools but co-registration tool) or multichannels stacks.

The middle bar offers automation of the most popular Field Illumination, PSF profiler and coregistration tools. These types of analysis are routinely performed on an imaging facility. As a microscope stand is equipped with several lenses, the number of analyses increases, hence automation eases the quality check procedure.

The lower bar allows (from left to right):

- To toggle the main ImageJ window
- To close the bar
- To customize the bar and remove some above-mentioned tools
- To open this manual

- To get some information about the plugin and have a direct access to the iText plugin download URL.

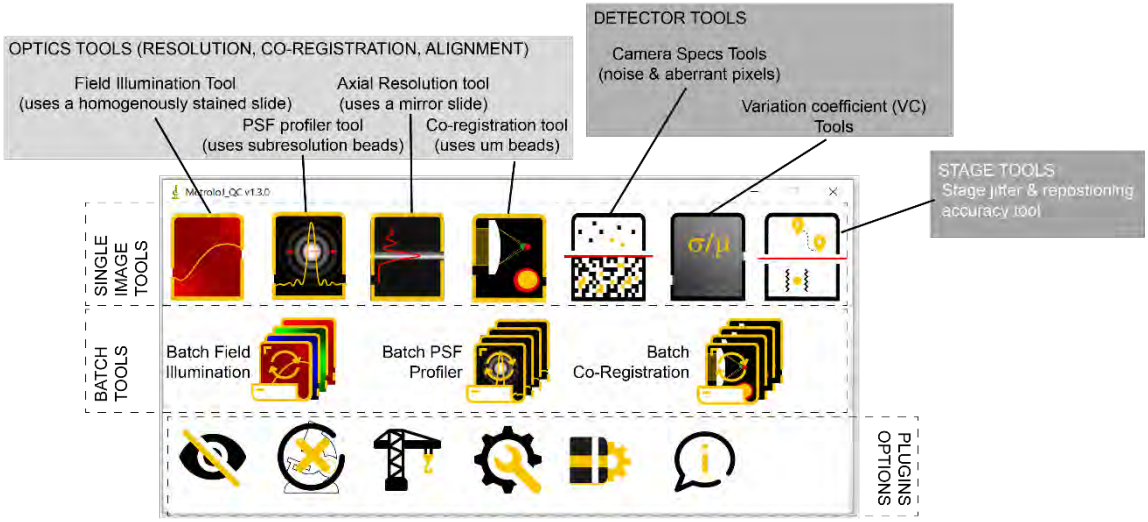
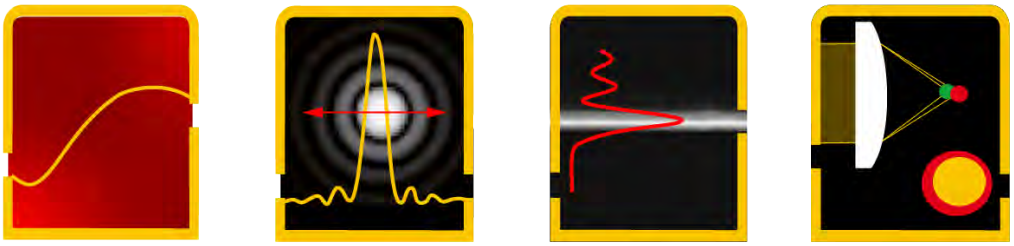


Figure 1.The MetroloJ_QC bar.

Tools are packaged in three groups:

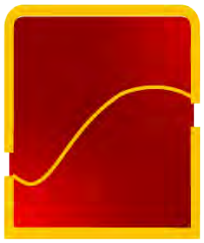
- OPTICS TOOLS: these allow
  - monitoring of the illumination homogeneity across the Field of View (Field Illumination tool).
  - Measurements of axial and lateral resolution of the system. These tools involve the use of either sub resolution beads (e.g., 160 nm fluorescent beads for a widefield setup). This tool, coined PSF Profiler, is quite popular. A second tool (Axial Resolution tool), using Z-scans of a mirror slide may be used to measure the axial resolution.
- DETECTOR TOOLS:
  - The Camera Specs tool allows measurement of camera noise parameters (such as DSNU, rms noise) and identification of aberrant pixels (such as hot/dead, warm and cold pixels).
  - The Variation Coefficient tool is useful to measure single-point detector fluctuations.
- STAGE TOOLS:
  - monitoring 2 or 3D Drift along time
  - Measurement of stage (re-)positioning accuracy

- PLUGINS OPTIONS:
  - Hide/close ImageJ Main bar/QC menu
  - QC menu configuration and general settings
  - Setting of the appropriate bead/annuli segmentation (for PSF Profiler, Co-registration and stage tools).
  - QC info & documentation



OPTICS TOOLS

This series of tools are of interest to make sure the optical system is properly aligned, quantifications over the Field of View are reliable. Resolution tools help to make sure the system performs within a reasonable range compared to theory. Finally, co-registration tools are mandatory to make sure the systems chromatic aberration (lateral and axial aberrations) are kept below what can be considered as acceptable.



FIELD ILLUMINATION TOOL

Accurate Quantification and comparison of fluorescence intensity requires all fluorochromes within the Field of View to be excited with the same illumination intensity, otherwise some corrections have to be applied. The Field Illumination tool checks that the excitation intensity is homogenous enough for reliable quantification. The maximum of excitation illumination intensity is located and compared to key points and key centering/uniformity parameters are computed.

Samples for monitoring proper field illumination and recommended parameters

Any fluorescent homogenous slide may be used. These can be either prepared with a fluorescent dye solution or purchased (e.g., “Chroma” or Thorlabs fluorescent plastic slides, Argolight or Brakenhoff slides.
Images should be acquired following the P02 protocol established by the GT3M working group of the RTmfm network.

The QC Field Illumination tool’s algorithm.

The plugin locates both minimum & maximum intensities within each channel. It also locates the center of intensity (ImageJ Center of Mass parameter). A normalized

intensity image is generated (meaning an image whose pixel intensity is the corresponding pixel intensity of the original image divided by the maximum intensity). The user is prompted to divide the 0 to maximum intensity range into bins. A threshold is then used to either locate all pixels whose intensity is equal to the maximum intensity or the pixels whose relative intensity are within the last bin (e.g., of 10 bins were used, relative intensities from 0.9 to 1). The centroid (ImageJ Centroid parameter) is then located. Distances to the geometrical center of these remarkable points (Center of intensity, maximum intensity, center of the thresholded zone) are computed. Another table is computed, displaying location/intensity of maximum/minimum pixels and the original and normalized intensities of specific points of the edges of the image.
Uniformity and Centering Accuracy as defined in ISO21073:2019 are also computed.

$$Uniformity = \frac{minimum\ intensity}{maximum\ intensity} * 100 \quad (A)$$

$$fUniformity = 100 - 100 * \frac{\sigma_{8\ corners}}{maximum\ intensity} \quad (B)$$

$$Centering\ Accuracy = 100 - 100 * \frac{2}{\sqrt{w^2+h^2}} * \sqrt{(x_{ref} - \frac{w}{2})^2 + (y_{ref} - \frac{h}{2})^2} \quad (C)$$

Were x_{ref} and y_{ref} are the coordinates of the "reference", w the width and height of the image and $\sigma_{8\ corners}$ the standard deviation of the intensities of the eight "corners" of the image.

An iso-intensity map is generated. The normalized intensity image is simplified in n zone (n being the number of bins set by the user) and displayed.
The long version of the report will generate and analyze four intensity profiles along the horizontal and vertical axis and both diagonals passing through the image's center.

Field Illumination Tool parameters:

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).
STEP2. Open an image containing the field illumination to analyze. The image may be a multichannel image or a single channel image.
STEP3. Click on the Field Illumination tool icon.
CRITICAL: The images should be calibrated. If uncalibrated, the algorithm stops.

The plugin's interface should appear (see Figure 2).

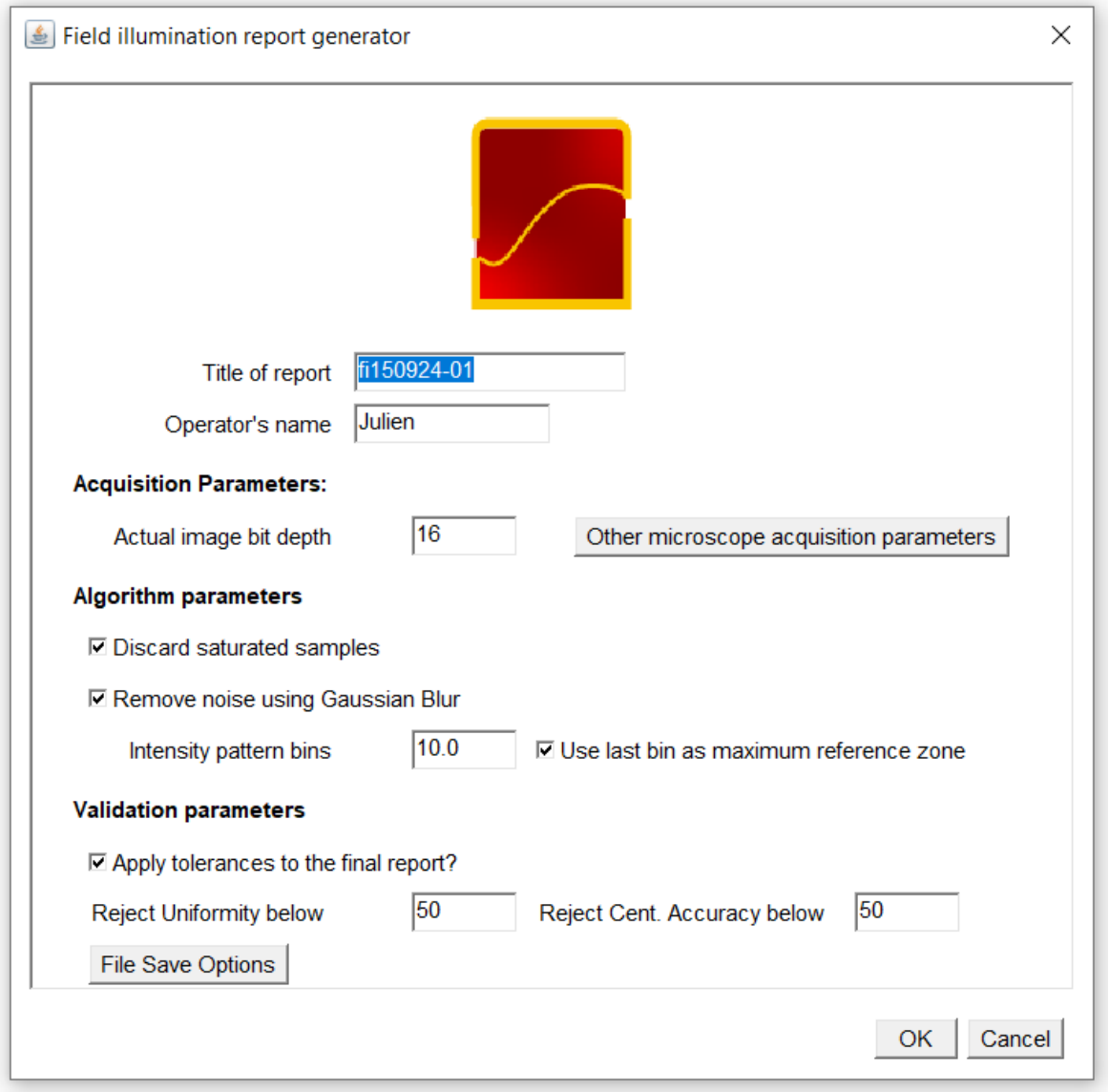


Figure 2. User interface of the Field Illumination tool

STEP4. The plugin analyses the opened image and finds the channel number (1 if single channel image). Mind the plugin was not intended to analyze 3D XYZ images. Enter the microscope's detector output bit depth, as this will be used if the discard saturated samples option is selected. You may trace other acquisition parameters using the dedicated button. This opens the Figure 3 "filter parameters" dialog window. Fill-in these other acquisition parameter fields (filters combination/set name, emission/exc. wavelengths). These parameters are not used by the Field Illumination algorithm per se. Click Ok to go back to the main dialog. If not hidden, some sample information and some comments might also be provided using the appropriate boxes.

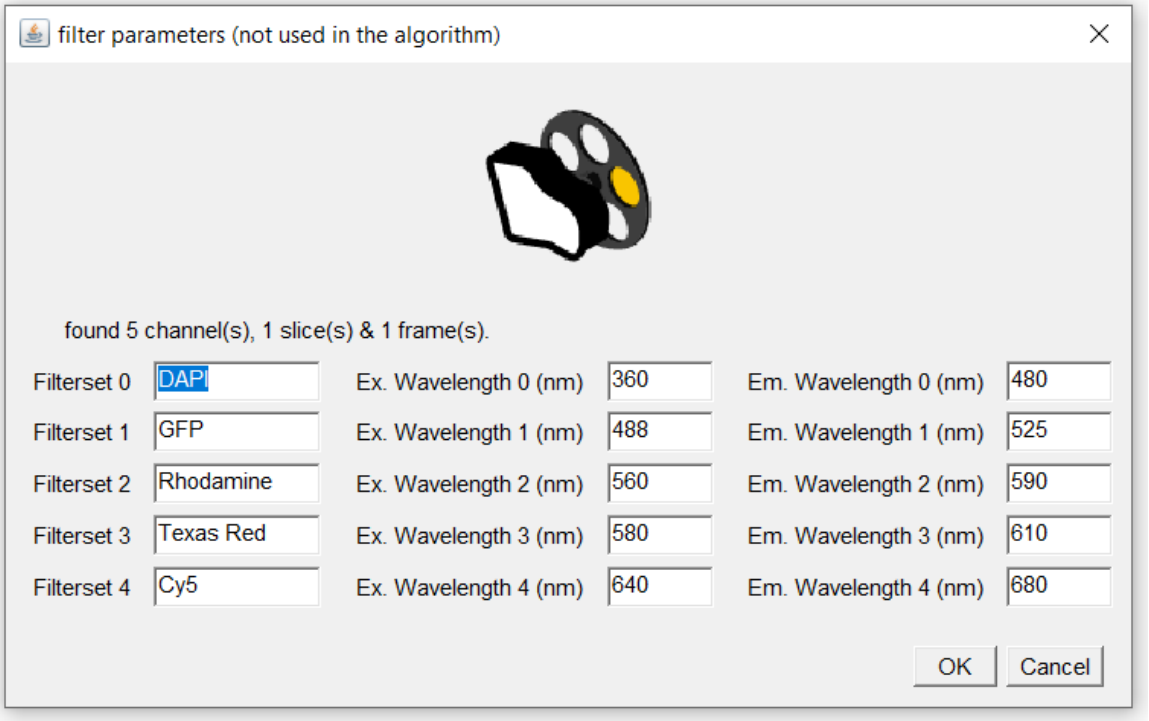


Figure 3.Field illumination tool : optional microscope parameters dialog.

STEP5. Enter the algorithm parameters. Choose to discard or not saturated image. This uses the bit depth acquisition parameters to define the maximum saturated intensity. Whenever saturation occurs in a few isolated pixels, noise may be removed using a Gaussian blur of sigma=2. Note that saturation computation is done after the Gaussian blur step. Hence, whenever aberrant saturated isolated pixels are polluting the channel, if Gaussian blur gets rid of them, the image will no more be considered as saturated as no saturated pixels will be found in the smoothed image. In the case of “clusters” of saturated pixels, the applied Gaussian Blur is not strong enough to get rid of the cluster center saturated pixels and the channel will still be reputed as saturated and skipped if the discard saturated sample option is selected.

STEP6. Type in the intensity patterns bins. This value will be used for computation of the reference zone (see 10) and for generation of the iso-intensity map. A value of 10 will generate iso-intensity steps of 10%.

STEP7. Ticking the next “use last bin as maximum reference zone” will replace the reference zone from the 100% zone (meaning the centroid of all pixel with maximum intensity) to the last bin. If #bins was 10 for instance, the centroid of all pixel with a normalized intensity between 90% of the max and max will be computed. If the option is not used, the reference in the centering accuracy formula used is the maximum intensity pixel. If the option is selected, the reference is the centroid of the reference zone (ie. the last bin zone as defined above). Some further text indicating how the calculation is made is added to the report.

STEP8. Decide whether you want to set validation parameters. Tick the “apply tolerances to the final report” if you wish to highlight within and outside specs in the final pdf report. If ticked, all uniformity values below/above the next field value (“reject uniformity below”) will be highlighted in red/green respectively. All centering accuracy values below/above the field value “Reject Cent. Acc. Below” will be highlighted in red/green.

STEP9. Click the “File Save Option” to set the output options (Figure 4).

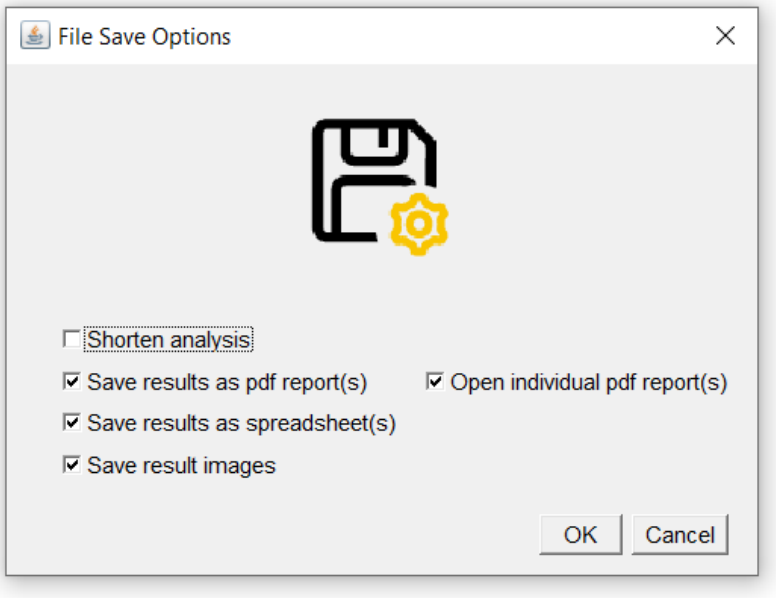


Figure 4.MetroloJ_QC : the file Save Options dialog

Set the file save options:

- You have the possibility to skip some output files. The pdf report, in its long or short version (see 3).
- Decide whether the results should be saved as a pdf file
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls files containing tabulation separated values of all tables of the pdf report (ie. uniformity/centering accuracy for all channels, locations of the maximum intensity, center of intensity and centroid of the reference zone) and coordinates statistics (max, min, remarkable points of the edge). If the long version of the report is chosen, intensity profiles are saved in separated .xls files for each channel.
- You may save the the iso-intensity pattern images and (if long version chosen) the intensity profiles along horizontal/vertical/diagonals (see below in the Field Illumination report description above) as jpg files as well.

All generated files are saved in a subfolder of a “processed” folder located in the same folder of the original image. The subfolder’s name can be changed in the first “title” field of the dialog.

STEP10. Click OK. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 5 (left panel) will appear. In this case, change the title.
MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP4) and the actual file format depth, a different type of error message is triggered (Figure 5, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

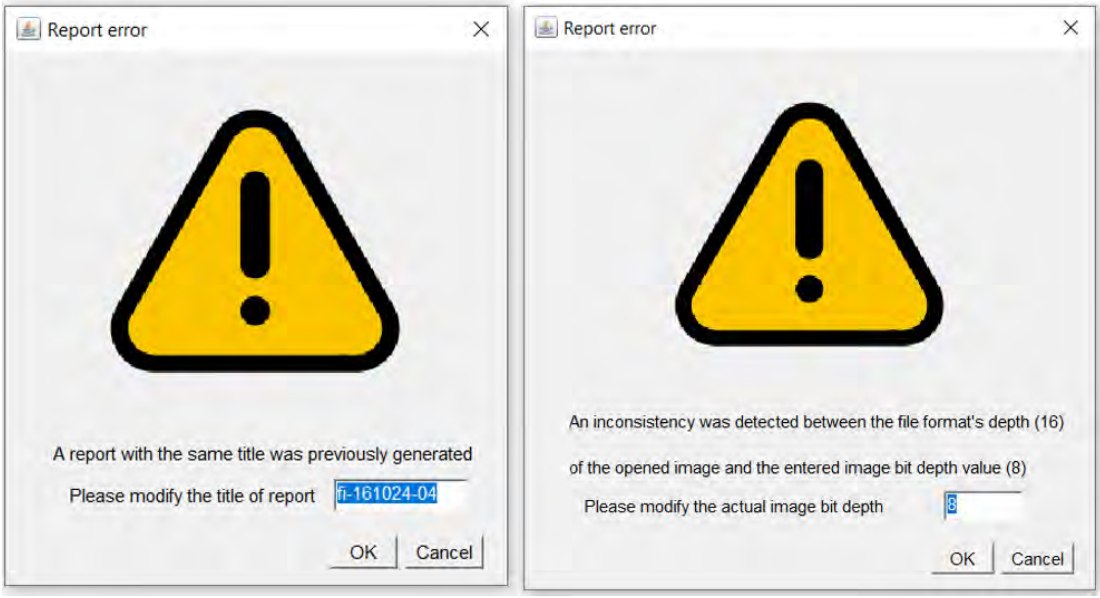


Figure 5.Error dialogs triggered by the Field Illumination tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved!

NB: the user has the possibility to use alternative modes. For this, before starting the Field Illumination analysis click the cog icon and select the “show the debug options” or the “Use (unverified) other tools” checkboxes (Figure 6).

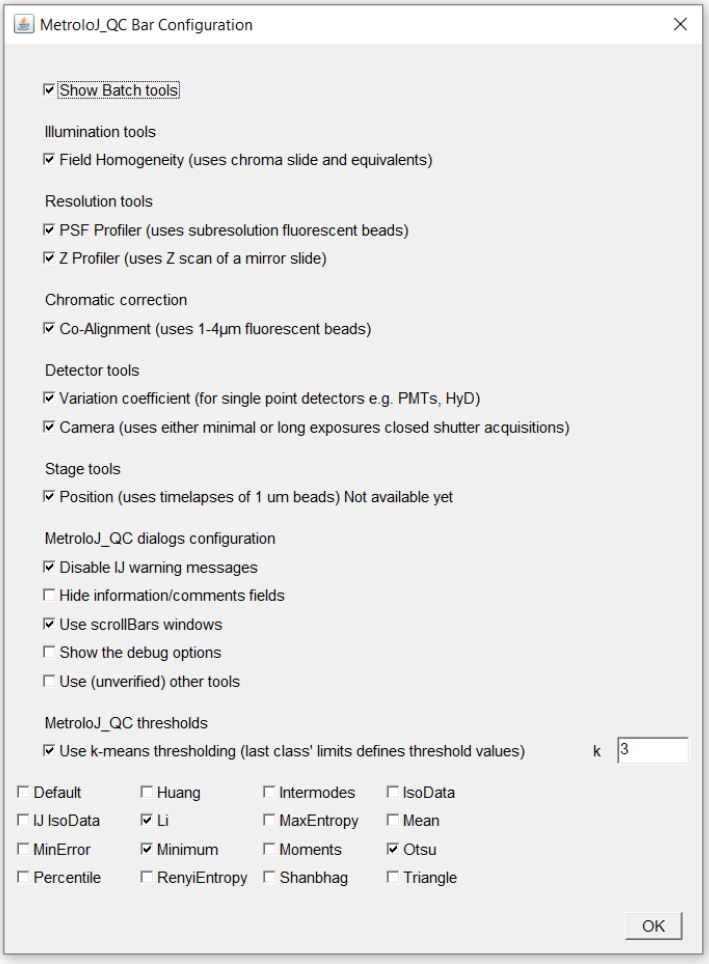


Figure 6.Field Illumination Tool alternative modes

Description of the field illumination tool report.

The field illumination report starts with a summary of the parameters entered by the operator at step 6 (see Figure 7). The main first section is Microscope info (tracing all info provided at STEP4.). Additional information such as the name of the image associated with the report and the image calibration/sampling distance) are added. The proportion of saturation is indicated for each channel. Saturation is computed for the whole image. The next warnings section collects some useful warning to help result interpretation. If the user chose not to discard saturated images, he/she is reminded saturation, if any, may bias results.

fi170924-19 (SHORT)

Microscope info:

Image		5x		
image's creation	date	2024-06-13 09:13:27		
	method used	from file creation date		
Actual image depth		16		
Filter combination(s)		Wavelengths		Saturation
		Ex. (nm)	Em. (nm)	
DAPI		405.0	480.0	none
GFP		488.0	525.0	none
Rhodamine		560.0	590.0	none
Texas Red		580.0	610.0	none
Cy5		640.0	680.0	none

Warnings:

Noise was removed using a gaussian Blur of sigma=2.0. The centering accuracy is computed using the 90.0-100% reference zone.

Figure 7.Field illumination tool report: microscope & warnings sections of the report

The Main Field Illumination parameters section (Figure 8) is a table of all Uniformity & centering accuracy values associated for each channel. Whenever option “use last bin as maximum reference zone” was used, the user is reminded the reference zone used is different from the original ISO21073:2019 formula. If the “use last bin” option is not used, mind that whenever more than one pixel bears the maximum intensity value, the reference zone is the 100%-100% ie. the maximum intensity pixel position is the mean position of all maximum intensity pixels. In case a few pixels have an aberrant high value, which is the maximum value, then the image might look “centered” although those quite difficult-to-detect-visually pixels can be not centered at all, as seen with the CA/Uniformity values.

Uniformity & Centering Accuracy:

Filters combination/set	Uniformity (%)	Centering Accuracy (%)	Image
DAPI	34.4	69.7	C1-5x
GFP	51.4	94.6	C2-5x
Rhodamine	59.8	93.9	C3-5x
Texas Red	58.9	96.4	C4-5x
Cy5	47.9	95.3	C5-5x

Green: within specifications, red: outside specifications (ie. uniformity below 50.0 or centering accuracy below 50.0). Centering accuracy computed using the 90.0%-100% zone as reference rather than the maximum intensity pixel position.

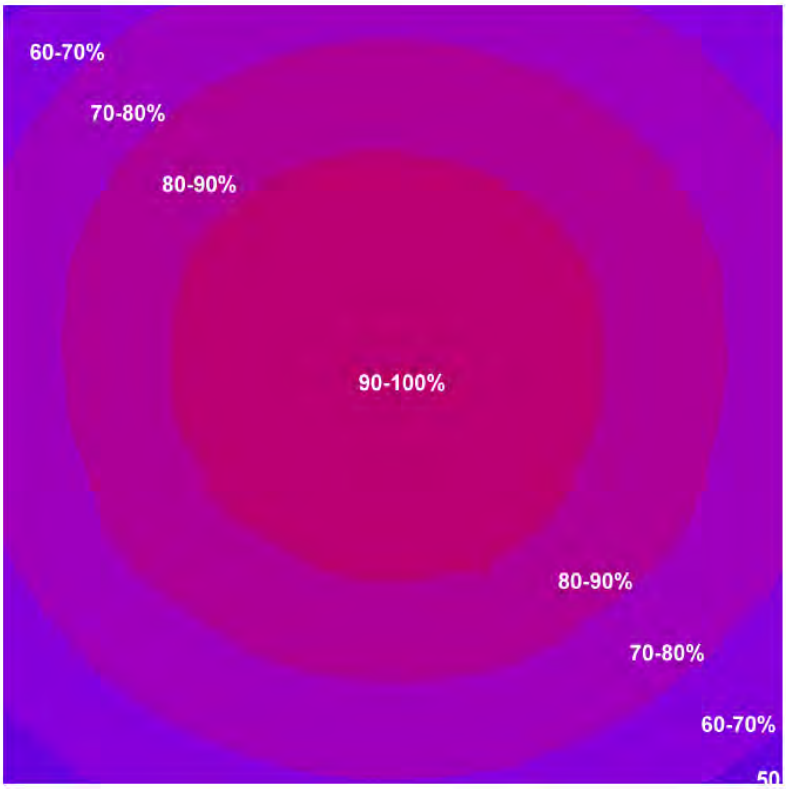
Figure 8.Field Illumination tool report: Uniformity, Field Uniformity & Centering accuracy values.

The following sections are detailed field illumination information per channel (Figure 9 to Figure 11).

- The iso-intensity pseudocolor image of the normalized intensity image is provided in section normalized intensity profile Channel [n] (Figure 9).
- The location of the image center (geometrical center), the center of intensity (Centre of Mass of the page channel), the maximum intensity pixel for the page channel) and the reference zone are provided, along with the distances to the geometrical image center (see section Channel [n] center's locations, Figure 9).
- Long version of the report includes a profile along horizontal/vertical/diagonal lines going through the image geometrical center (channel[n] intensity profiles section, Figure 10).
- A final section provides the intensity of points along the image's edge (Channel[n] coordinates' statistics, Figure 11). The table contains both raw and normalized intensities of the maximum/minimum intensity pixels (+ coordinates), the centers and values for 8 characteristic pixels, corresponding to the 8 intercepts of the lines along which the intensity profiles are retrieved.

DAPI (ex. 488.0 nm, em. 525.0nm)

DAPI normalised intensity profile:



DAPI centres' locations:

Filters combination /set	Type	Image Centre	Centre of intensity/mass	Last maximum intensity pixel	Centre of the 90.0-100% reference zone
DAPI	Coordinates in pixels	(1024.0, 1024.0)	(1024.8, 1008.4)	(930.0, 1022.0)	(1011.6, 947.3)
	Distance to image centre in	-	20.167	121.288	100.187

Figure 9.Field Illumination tool report: the isointensity image & centres locations coordinates/distance to center.

DAPI diagonal & geometrical centre intensity profiles:

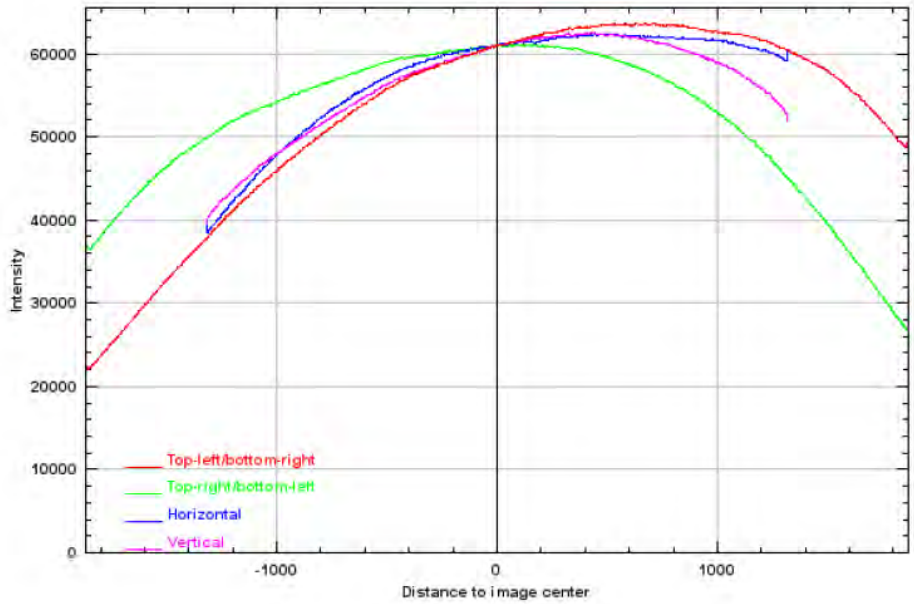


Figure 10. Field Illumination tool report: intensity profile across TL/BR, TR/BL, horizontal & vertical lines.

DAPI coordinates' statistics:

Filters combination/set	Location	Intensity	relative intensity to max
DAPI	Maximum (1344.0,1372.0)	63793	1.0
	Center of Mass(1084.9,1065.9)	61582	0.965
	Reference zone center(1370.9,1293.3)	63562	0.996
	Minimum(9,0)	21966	0.344
	Top-left corner	22979	0.36
	Top-right corner	36385	0.57
	Bottom-left corner	27888	0.437
	Bottom-right corner	48771	0.765
	Upper bound, middle pixel	39561	0.62
	Lower bound, middle pixel	51912	0.814
	Left bound, middle pixel	39397	0.618
	Right bound, middle pixel	60347	0.946

Figure 11. Field illumination tool report: the coordinates' statistics section.

For the sake of either debugging or tracing the analysis parameters, the last sections (Analysis parameters) are a summary of all algorithm parameters used (Figure 12) and the A, B and C formulas used for Field Illumination metrics calculation (Figure 13).

Analysis parameters

Tool & Operator	Tool	Field-Illumination
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	, 28 août 2024 15:34
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Homogeneité\Processed\fi280824-01\
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Gaussian blur noise removal applied		true
Isointensity image steps width		10.0%
Reference zone		90.0%-100%
Tolerance	Applied in this report	true
	Uniformity valid if above	50.0
	CA valid if above	50.0

Analysis log

image name	creation date	saturation	status
5x	2024-06-13 09:13:27	none	analysed

Figure 12. Field Illumination tool report: the analysis parameters.

Formulas used:

$$Uniformity = \frac{minimum\ intensity}{maximum\ intensity} * 100$$

$$Centering\ Accuracy = 100 - 100 * \frac{2}{\sqrt{w^2+h^2}} * \sqrt{(x_{ref} - \frac{w}{2})^2 + (y_{ref} - \frac{h}{2})^2}$$

x_{ref} and y_{ref} are the coordinates of the "reference", w and h the width and height of the image.

Figure 13. Field illumination's tool formula used.

A log file is also added (Figure 14), that summarizes how the input analyzed image was handled. In cases where no analysis is performed, since version 1.3.0, a report is however generated and the reason why the input image was left unanalyzed can be found in this log file.

Analysis log

image name	creation date	saturation	status
5xSatCh0	2024-06-13 09:13:27	Ch.0 saturated	analysed

Analysis log

image name	creation date	saturation	status
saturated5x	2024-06-13 09:13:29	Ch.0,1,2,3,4 saturated	not analysed

Figure 14. The analysis log of a partially saturated image (top) and a “fully” saturated image, with no unsaturated channel left to be analysed.

Unverified Field Illumination tools

In the alternative “use (unverified) other tools” mode, fUniformity values are displayed the following analysis is done:

- Using the identified center (either the 100% zone or the reference area’s center if the option was checked at STEP 7), horizontal and vertical lines are drawn across this position (Figure 15).
- Intensity profiles are drawn across both lines and a second-degree polynomial fit is applied using the following formula:

$$I(x) = \mathrm{a} + \mathrm{bx} + \mathrm{cx}^2$$

The coefficient of variation of the image is shown in the main Uniformity and centering accuracy Table (Figure 16) as well as the mean of the horizontal and vertical absolute c values.

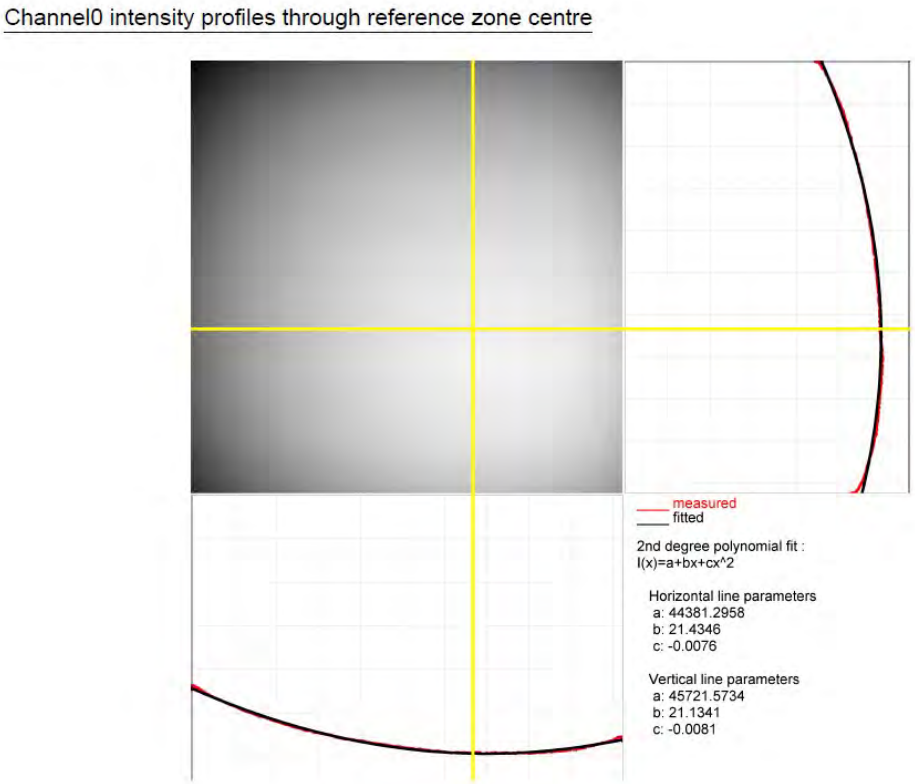


Figure 15. *Intensity profile through the reference zone's center

Uniformity & Centering Accuracy:

Channel	Uniformity (%)	Field Uniformity (%)	Centering Accuracy (%)	Image	Coef. of Variation	Mean c fit value
Channel0	52.5	88.5	72.6	C1-100x	0.1056	0.0079
Channel1	61.0	91.5	89.7	C2-100x	0.0749	0.0082
Channel2	67.7	91.9	90.2	C3-100x	0.0481	0.0039
Channel3	70.1	92.4	96.7	C4-100x	0.0394	0.0037
Channel4	67.1	91.8	92.3	C5-100x	0.0405	0.0034

Figure 16. The Uniformity and Centering Accuracy table in the "use (unverified) other tools" mode

All data is saved in a processed/title subfolder. The pdf report is saved within this subfolder as a title_imageName.pdf file (Figure 17). If option "save report images" is selected, isointensity images and plots (not in the short version of the report) for each channel are saved in a title_imageName_data folder (Figure 18). When the "save all data in spreadsheet" is selected, the main Uniformity and Centering accuracy table and each centers' location tables are saved in a _results.xls file. The intensity profiles plots are saved in a _intensityProfiles.xls file and each channels' coordinates' statistics are saved in a single _stats.xls file.

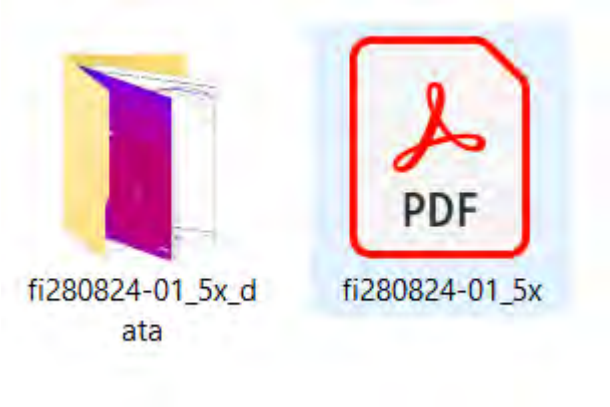


Figure 17. Field Illumination tool: Main files generated

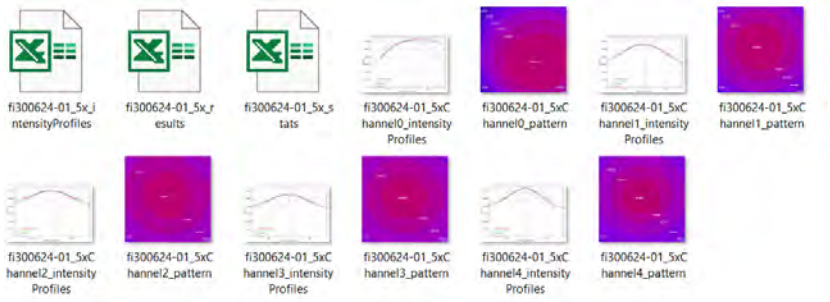


Figure 18. Field Illumination tool: additional generated files.

PSF PROFILER TOOL

The size, shape and symmetry of the Point Spread Function (PSF) are characterized by the objective lens and any other lenses within the optical beam path. The PSF is rarely close to what can be expected from theory. Hence, the recorded image's resolution is rarely as good as one might expect and both image quality and the subsequent quantification can be affected. Evaluating and monitoring the PSF over time is the first key step to determine the performance stability of a microscope.

Samples for Point Spread Function images acquisition & recommended parameters

We recommend to follow the "Sample 01" acquisition protocol established by the GT3M WP of the RTmfm network.

A tutorial video, made by the GT3M WP of the RTmfm, is available here: https://youtu.be/ll4X_e8_mo8

The QC PSF profiler tool's algorithm.

The initial MetroloJ plugin assumes the dataset contains a single bead. If chosen, multibeads images can be first process, to identify single beads. For this purpose, a maximum intensity projection (MIP) along the Z axis of the user-defined channel (or channel #0 is single-channel image) is generated, smoothed (for the purpose of hot pixel removal) and maxima are identified. Square ROIs are drawn around each identified bead. Beads too close to each other or too close to the edge/top or bottom planes of the stack are discarded. If the option to identify multiple beads is not used, the plugin will focus on the highest intensity bead (as in initial MetroloJ plugin).

For each identified bead/highest intensity bead, the plugin will generate a maximum intensity projection of the stack along the z axis. The (x, y) coordinates of the maximum intensity pixel (MIPix) are then collected. A XZ cross-section is generated, along a line passing through the previously determined 2D MIPix. From this image, the z coordinate of the MIPix is defined. The z slice is set to the z MIPix coordinate. The x profile and y profile are collected along the line passing through the MIPix. The z profile is collected on the XZ view, along the line passing through the MIPix.

All three profiles are then fitted to a Gaussian, using the following equation and ImageJ's built-in curve fitting function:

$$y = a + (b - a) * e^{\frac{-(x-c)^2}{2d^2}} \quad \text{(D)}$$

The resolution, i.e., the full-width at half-maximum (FWHM), is calculated as follows for each profile, based on the d parameter retrieved from the fitting:

$$FWHM = 2d\sqrt{2ln\,(2)} \quad \text{(E)}$$

The measured FWHM are compared to theoretical resolutions res_x^o, res_y^o and res_z^o, calculated as follows, depending on the microscope's type (please note a change in the confocal theoretical values used in the original MetroloJ plugin).

- Widefield microscopes

$$res_{x,y}^o = \frac{0.51*\lambda_{em}}{NA} \quad \text{(F)}$$

$$res_z^o = \frac{1{,}77n*\lambda_{em}}{NA^2} \quad \text{(F')}$$

References: Wilhelm, S. Confocal Laser Scanning Microscopy 2011 (Carl Zeiss ed.).

λ_{em} : emission wavelength
NA: numerical aperture

- Confocal microscopes (assuming pinhole ≥1 AU, NA>0.5)

$$res_{x,y}^o = \frac{0.51*\lambda_{ex}}{NA} \quad \text{(G)}$$

$$res_z^o = \frac{0.88*\lambda_{ex}}{n-\sqrt{n^2-NA^2}} \quad \text{(G')}$$

References: Wilhelm, S. Confocal Laser Scanning Microscopy. 2011 (Carl Zeiss ed), Amos, B. *et al,* Confocal Microscopy. in Handbook of Comprehensive Biophysics 2012 3–23 (Elsevier).

λ_{ex} : excitation wavelength
NA: numerical aperture
n: immersion/mounting medium refractive index

- Spinning Disk microscopes

$$res_{x,y}^o = \frac{0.51*\lambda_{em}}{NA} \quad \text{(H)}$$

$$res_z^o = \frac{\lambda_{em}}{n-\sqrt{n^2-NA^2}} \quad \text{(H')}$$

References: Toomre, D. and Pawley J.B. Disk-Scanning Confocal Microscopy. in Handbook Of Biological Confocal Microscopy 2006 221–238 (Springer)

λ_{em} : emission wavelength
NA: numerical aperture
n: immersion/mounting medium refractive index

- Multiphoton microscopes

$$res^{o}_{x,y} = \frac{0.377*\lambda_{ex}}{NA} \quad \text{(I) for NA<0.7}$$

$$res^{o}_{x,y} = \frac{0.383*\lambda_{ex}}{NA^{0.91}} \quad \text{(I') for NA>0.7}$$

$$res^{o}_{z} = \frac{0.626*\lambda_{ex}}{n-\sqrt{n^2-NA^2}} \quad \text{(I")}$$

References: Zipfel, W.R. et al, Nonlinear magic: multiphoton microscopy in the biosciences Nat Biotechnol. 2003 Nov;21(11):1369-77

λ_{ex} : excitation wavelength
NA: numerical aperture
n: immersion/mounting medium refractive index

PSF Profiler Tool parameters:

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).
STEP2. Open a file containing the beads/PSF images to analyze. The file may contain a single or multichannel Z stack.
STEP3. Click on the PSF Profiler tool icon.
CRITICAL: The images should be calibrated. If uncalibrated, the algorithm stops. In case the stack has not been spatially calibrated, a message error pops up: click on Ok. In the calibration dialog box provide the appropriate values, then re-launch the plugin.

The plugin's interface should appear (see Figure 19).

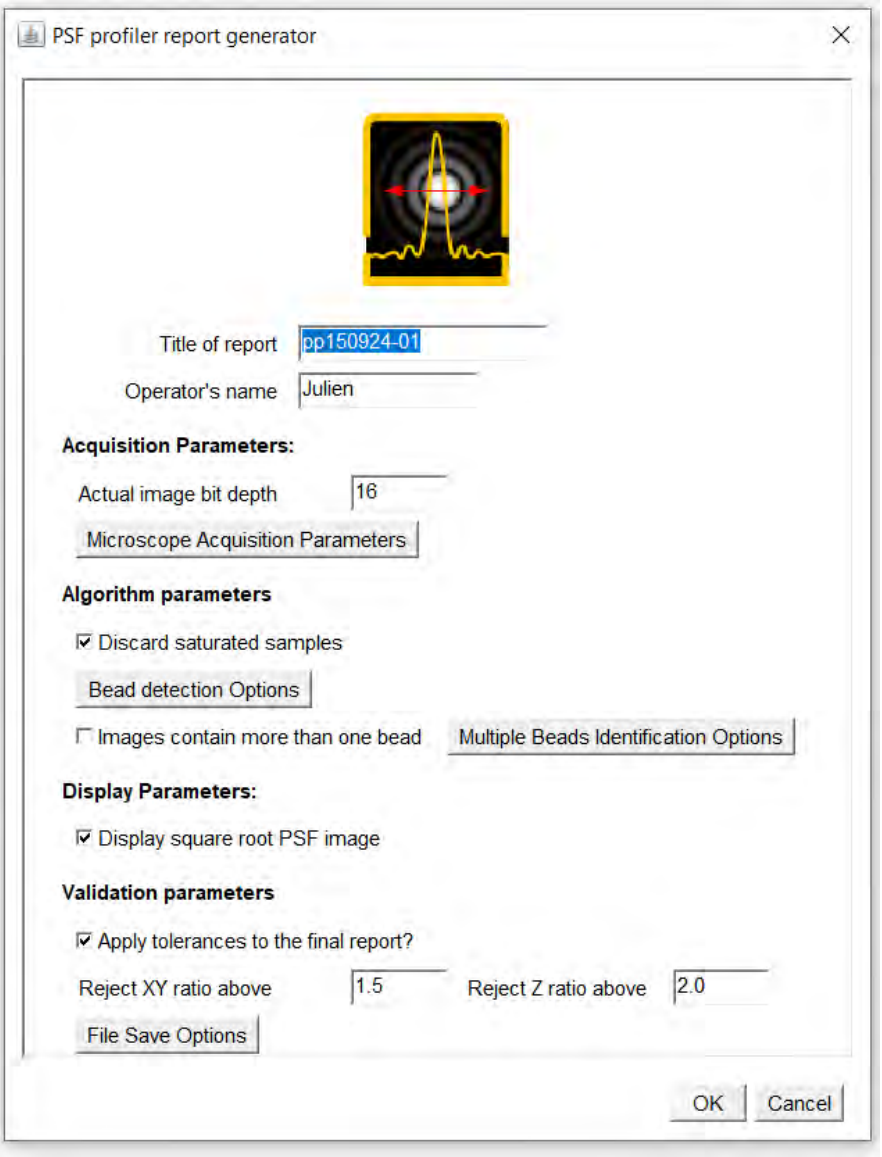


Figure 19. PSF profiler tool : the user's interface.

STEP4. Set the image's depth. Then click the Microscope Acquisition parameters button. These parameters are crucial for the determination of theoretical resolution values. If left unverified, the raw resolution values are still valid, but use of theoretical calculated values is hazardous. In the microscope acquisition parameters Dialog (Figure 20), set the microscope type. In the cases of Widefield and spinning disc confocal microscopes, the theoretical resolution uses the emission wavelength (as well as numerical aperture and immersion medium refraction index, formula F/F' and H/H'). In cases of multiphoton and single point Laser Scanning Microscopes (CLSM), the excitation wavelengths are used (together with NA and refraction index, formula I/I'/I" and G/G'). For CLSM, the pinhole size is also used, while a 2-photon excitation is assumed for multiphoton excitation microscopes.

The number of channels/slices is found and the user is prompted to fill-in the relevant information. Whenever the file order is different from the usual XY planes Z-stacks, the plugin will yield false results. Mind these formulas consider the image to be noise-free. Click the OK button to go back to the main dialog.
If not hidden, some more sample information and/or comments fields might also be used to trace additional information.

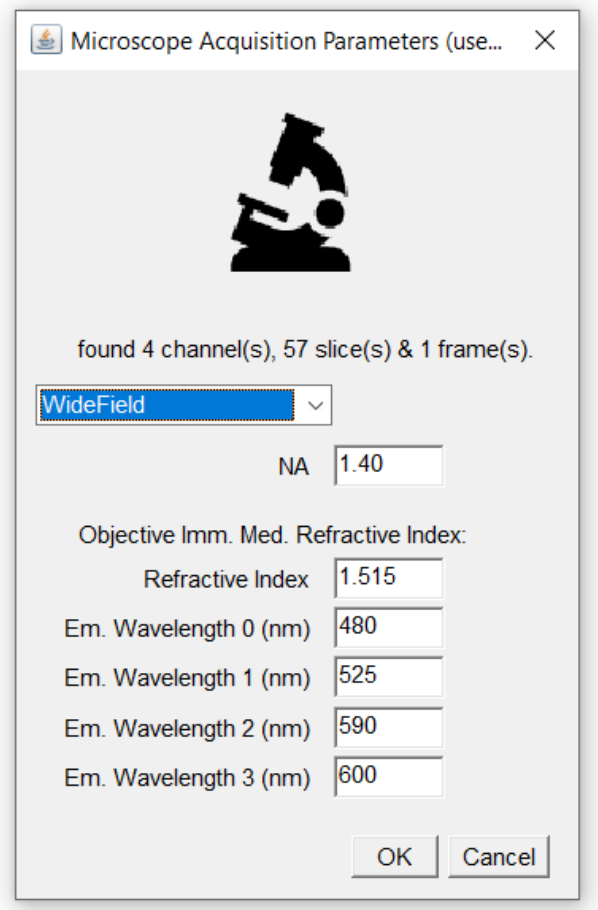


Figure 20. MetroloJ_QC : the microscope acquisition parameters options.

STEP5. The user may discard saturated beads (and uses the bit depth value provided by the user in the previous field to detect saturation). This option is used on the original image (or the cropped image beads option when multiple beads option is used). Briefly, whatever the option status is, all slices of a given channel are montaged in a single 2D image, a bead detection threshold is calculated and applied to highlight bead sections. Select the bead detection threshold with the rolling menu. See the "TESTS SECTION" to find the appropriate threshold. Then, the bead sections are masked and the proportion of saturated pixels within the mask is calculated. When all channels are saturated, the image is skipped if the option is chosen. If a subset of channels is unsaturated, the analysis proceeds with those channels.

STEP6. Click the bead detection options button. This opens the “Bead Detection options” window (Figure 21).

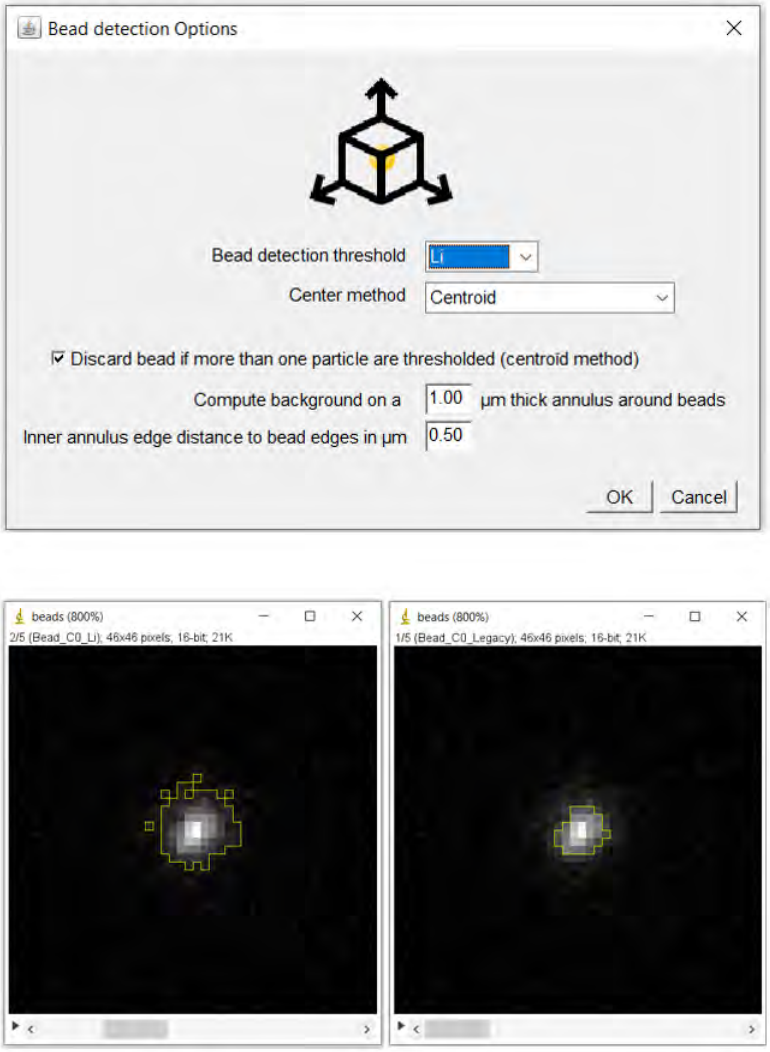


Figure 21. MetrologJ_QC : the bead detection Options dialog.

For a moment forget the first parameter (bead detection threshold) and focus on the center method option. Two bead center detection modes are provided in version 1.3.0. Select the appropriate one:

- the legacy MetroloJ plugin uses maximum intensities to identify the bead center’s coordinates. A maximum intensity projection image is calculated and the maximum intensity pixel is identified (X and Y bead center’s coordinates in pixels). Then, for this given (X, Y) coordinates pixel, the Z slice that has the highest intensity is found (Z bead center’s coordinate).
- The centroid method uses thresholded, sum intensity projections in the 3 dimensions. The X and Y center’s coordinates are given by the centroid of the thresholded XY sum projection. If XY coordinates are found, the Z center’s coordinate is the average of the centroids’ Z coordinates of both XZ and YZ

projections. See the “TESTS SECTION” to find the appropriate threshold. Set the first “bead detection threshold” field. When using the centroid method, an additional option is available: “Discard bead if more than one particle is thresholded.” If this option is enabled, the center coordinate detection will fail whenever two beads in the input image are thresholded. In cases where beads have a low signal-to-background ratio, the thresholded region may primarily encompass the bead, while a few outer pixels may also get thresholded (as shown in the bottom-left panel of Figure 21). If this option is selected, the analysis of such beads will fail, and "Center detection failed" messages will appear in the output tables. If necessary, change the detection threshold (ex. from Li, bottom-left panel to “legacy” bottom-right panel).

STEP7. A hint for bad quality beads is the signal to background ratio. The Signal to Background ratio is computed during the process of saturation ratio evaluation (step 5). The identified bead sections (green in Figure 22) mean intensity is measured (signal). The background is estimated in an annulus around the bead sections (yellow in Figure 22). The thickness and bead’s edge distance to inner annulus’ edge (see Figure 23, top-right panel). can be changed in the last two field of the bead detection options dialog. Both thicknesses (in um) are converted in pixels (rounded-up to the higher pixel). Annuli are drawn around each bead sections The ratio of bead sections mean intensity (signal) to annuli (background) mean intensity is calculated. Mind this is no signal to noise ratio evaluation.

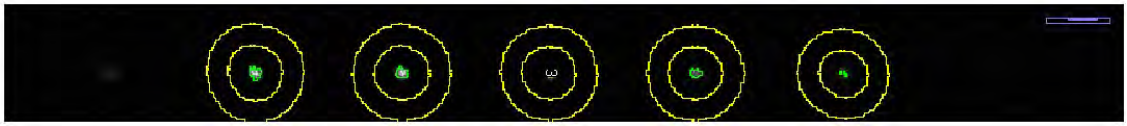


Figure 22. An example of the montage of the bead sections. PSF beads are identified using a threshold (see above), then the background annuli are drawn around each section.

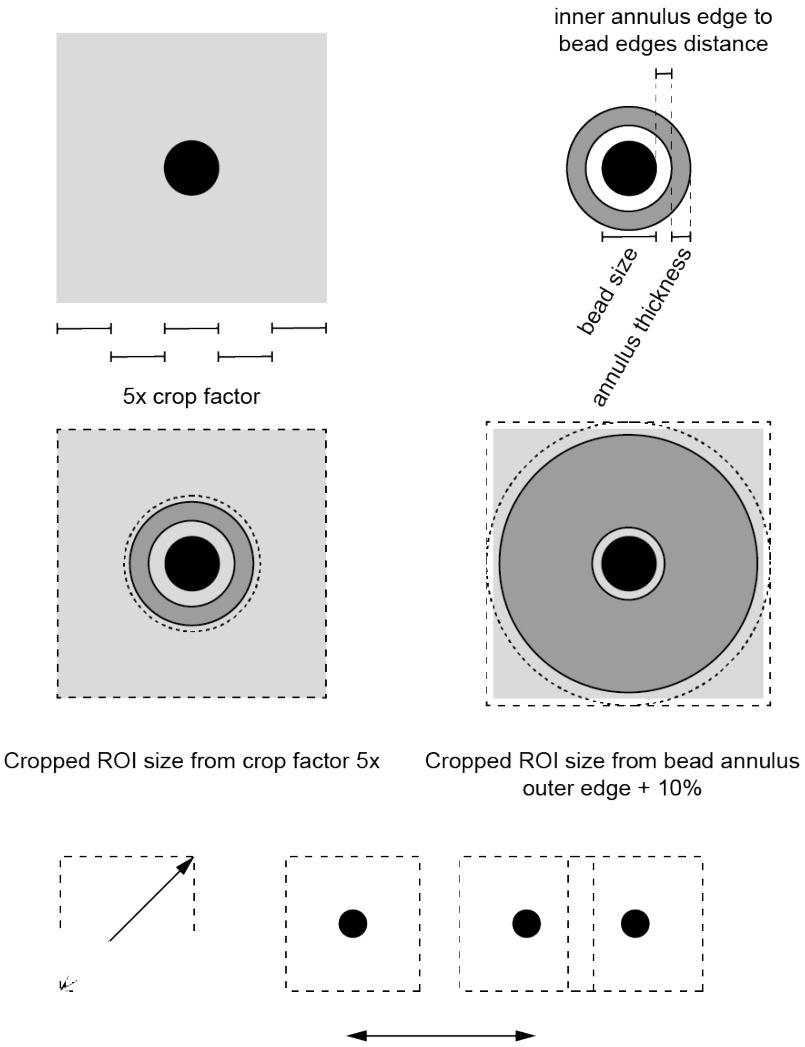


Figure 23. Crop factor, bead size, and annulus geometry (top panels). If the crop factor-derived ROI width (grey square) exceeds the “annulus + 10%” diameter (small dashed circle), it is used as the effective crop ROI to create the cropped bead image (medium left panel). Conversely, if the “annulus + 10%” diameter (small dashed circle) is larger than the crop factor-derived ROI width, then the “annulus + 10%” is used as the effective crop ROI size to create the cropped bead image (medium right panel). Rejection of neighbouring beads is based on measuring the intercentre distance and comparing it to the effective crop ROI diagonal length (bottom left panel). Hence, neighbouring beads are both rejected if their respective effective crop ROIs overlap (righthandside beads, bottom right panel). But they can also be rejected although crop ROIs are not overlapping, because their intercentres distance is smaller than the diagonal.

Click on OK to go back to the main dialog, if not using multiple beads-containing images, all algorithm parameters are set and profiles are drawn in each dimension through the identified bead center. A gaussian fit is applied and FWHM and R2 values are calculated.

MULTIPLE BEADS IMAGES (STEPS 11 & 12)

STEP8. When multiple beads-containing stacks are used, the user may tick “images contain more than one bead”. The process of finding multiple beads within an image is called “bead identification” (while “bead detection refers to finding a single bead and calculating its center coordinates). Click the “multiple beads identification button”, this opens the corresponding dialog (Figure 24).

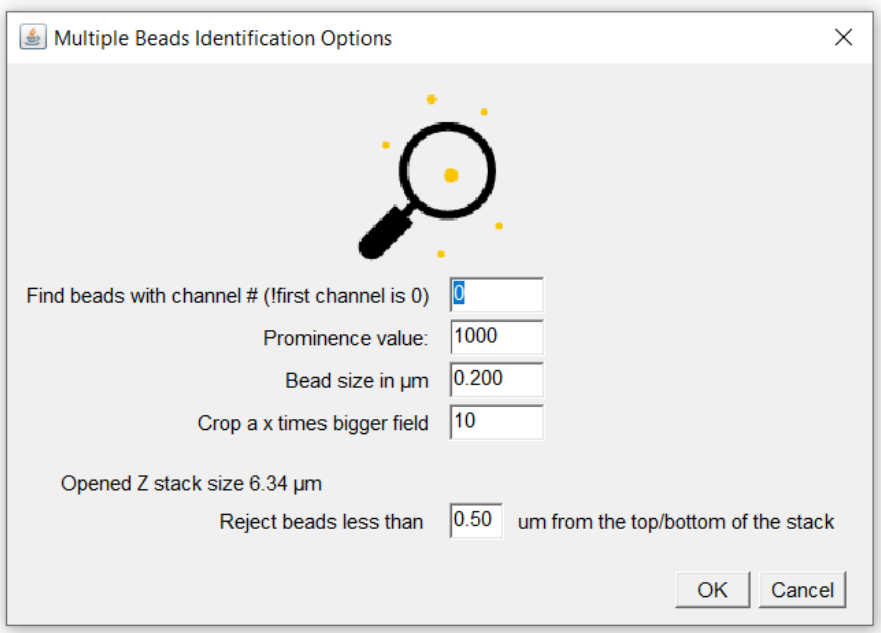


Figure 24. MetroloJ-QC multiple beads identification options (PSF Profiler case).

- In cases of multichannel images, beads are identified using one channel. A sum intensity projection is calculated for this channel and smoothed (to remove false beads triggered by noise).
- Bead identification relies on the ImageJ find maxima algorithm. Hence, the user is requested to type in the prominence value. This prominence is the difference of intensity of the bead center w/ the bead edges. Mind the appropriate prominence value may vary using different depth images (8, 12, 16 bits) or images with a poor/good signal to noise ratio. The user may find the appropriate value by using ImageJ and MIP the bead detection channel, run Process>smooth (noise removal) and run Process>Find Maxima. Ticking the “preview point selection” option will help find the right prominence (formerly noise) value. Back to the original (multichannel) Z stack, the appropriate prominence value can be used. Note that the smoothing is only used for the purpose of bead identification and the cropped, analyzed image beads are not smoothed.
- Once beads X and Y coordinates are identified, back to the original image, the Z coordinate is determined by locating the plane where the pixel at these XY coordinates has the highest intensity.

STEP9. For the purpose of cropping, the user is prompted to type-in the bead size and the crop factor. A bead size of 0.17µm and a crop factor of 50 will yield a cent red square ROI of box size of 0.17*50=8,5µm (Figure 25, top left panel). Enter the distance below which beads should be considered too close from the top and bottom of the Z stack (Figure 25).

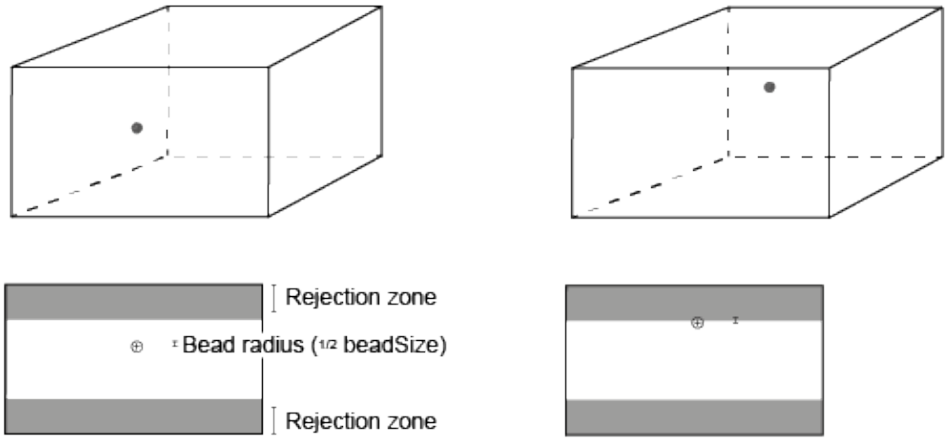


Figure 25. PSF beads identified as being too close to the bottom or top of the Z stack are rejected. The edges of the beads, determined by their size and center position, must be outside the user-defined rejection zone (see left panel). Beads with edges falling within this rejection zone are rejected, even if their centers are outside the rejection zone (right panel).

To avoid errors in PSF fitting caused by nearby beads or beads close to the image edges, the identified beads list is filtered to remove unwanted cases:

- Beads with close neighboring beads (with a center-to-center distance smaller than the crop ROI diagonal)
- Beads too close to the lateral edges.
- Beads too close to the top or bottom of the stack, as Gaussian fitting of Z-profiles for these beads tends to be of poor quality.

When the multiple beads mode is activated (and the “save pdf report” option is selected), a "multiple bead image summary" report is generated and saved as a title_imageName_identifiedBeads.pdf file. This contains no analysis results.

The last page of this report shows a maximum intensity projection of the input image, highlighting all detected beads and their respective "crop" ROIs (Figure 26). The ROIs of valid beads are displayed in green. Beads that have close neighboring beads are shown in yellow. If a bead's crop ROI touches the (XY) edges of the image, it is colored cyan. Finally, if the PSF center’s distance to the top or bottom of the stack is less than the rejection distance+the bead radius, its ROI is labelled magenta. Note that the displayed color corresponds to the first rejection criterion met. The overlay image can be saved if the “save report images” option is selected.

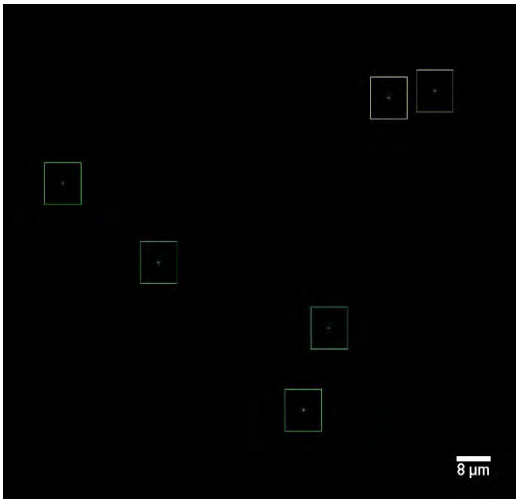


Figure 26. PSF Profiler Tool : bead identification overlay image.

If the “save all data in a spreadsheet” or “save report images” options are selected, additional files are generated (Figure 27) that contains an xls file version of the report (_identifiedBeads.xls), the beads coordinates (_identifiedBeadsCoordinates.xls) and the overlay image (identifiedBeadsOverlay.jpg).

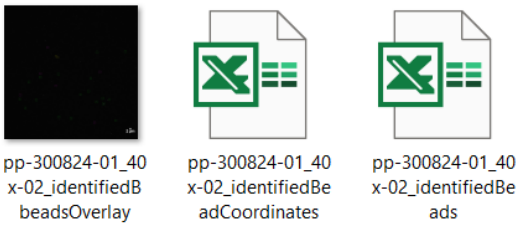


Figure 27. PSFProfiler tool: additional files generated in the multiple beads images mode

Once beads are identified, “crop” ROI around bead images are further duplicated and processed as if they were single-bead images (ie. as when the option is not ticked). Click on OK to go back to the main dialog window.

STEP10. Set Display parameters. The user may find it useful to display XY, XZ and YZ maximum intensity projection views using the square root of the signal. This is achievable using the Display square root PSF image option. In this case, an inverted LUT is applied so that spherical aberration tails for instance are easier to detect.

STEP11. Decide whether to use validation parameters. For the purpose of easy identification of within specs/outsides specs values, some Tolerance values may be applied to the final pdf report. When selected, this option will highlight in green/red all resolution values below/above n times the theoretical resolution values. For instance,

if the measured FWHM is 246nm and the theoretical expected value was 220 nm, and the corresponding tolerance ratio 1.5, the measured/theory ratio is 246/220=1.2, below the tolerance ratio and the value will be considered within specs (green). Mind that the FWHM measurement (and subsequent ratio value) will be colored, whatever the fitting ratio (and the quality of the FWHM approximation) might be.

STEP12. Set the file Save Options. Click the “File Save Option” to set the output options (Figure 28).

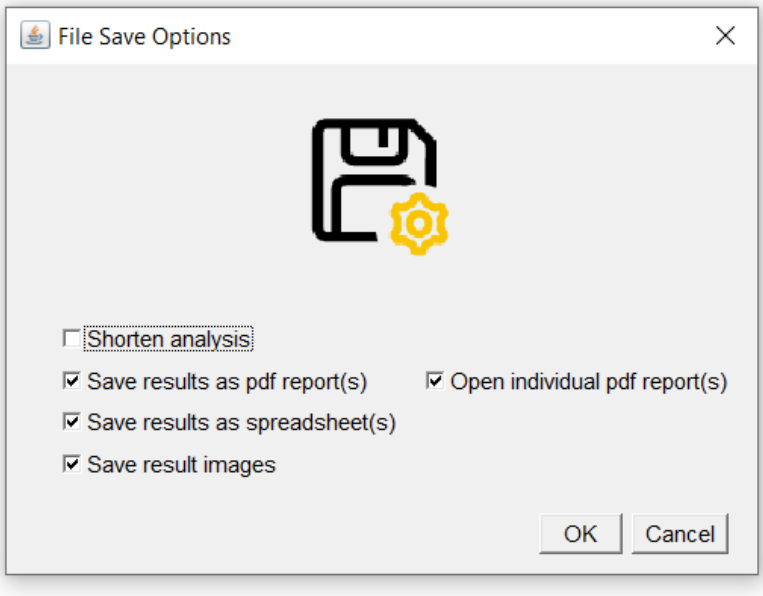


Figure 28. MetroloJ_QC : the file Save Options dialog

Set the file save options:

- You have the possibility to skip some output files (long or short versions, see below).
- Decide whether the results should be saved as a pdf file (mind all reports will be automatically opened if the open thickbox is selected: the number of generated pdf files may exceed the pdfReader capacity),
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls files containing tabulation separated values of all tables of the pdf report. If the long version of the report is chosen, intensity profiles are saved in separated .xls files for each channel.
- You may save the side view images and (if long version chosen) the intensity profiles profiles (see below in the PSF Profiler report description above) as jpg files as well.

All generated files are saved in a subfolder of a “processed” folder located in the same folder of the original image. The subfolder’s name can be changed in the first “title” field of the dialog.

STEP13. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 29 (left panel) will appear. In this case, change the title.
MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP4) and the actual file format depth, a different type of error message is triggered (Figure 29, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

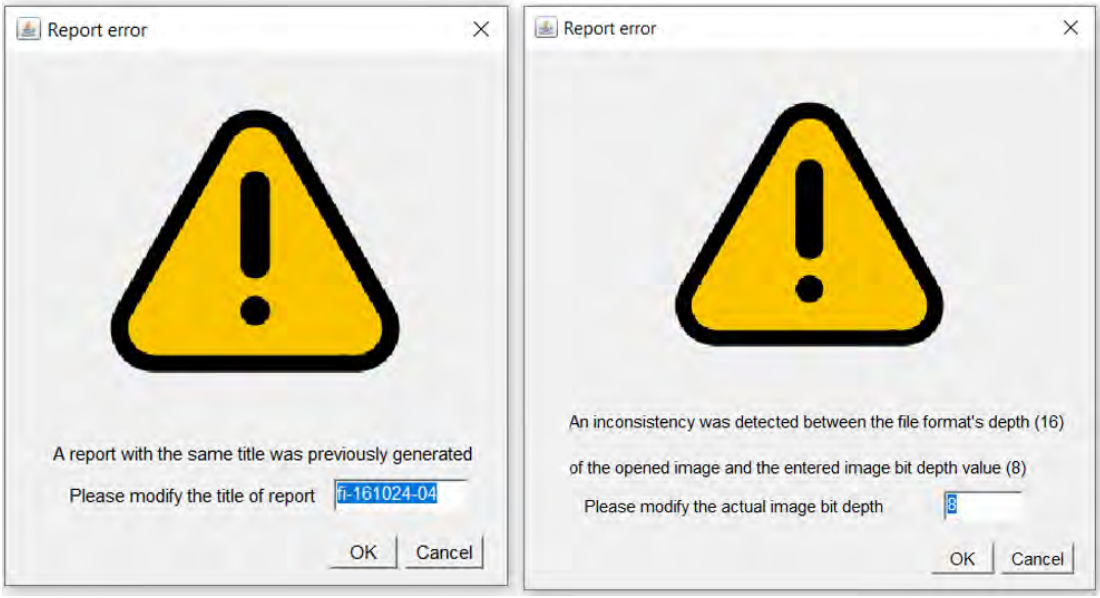


Figure 29. Error dialogs triggered by the PSF Profiler tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved!

Description of the PSF profiler tool report.

The first main section Microscope info (Figure 30) summarizes all meaningful information such as wavelength, etc. As mentioned above, in the confocal case, although the pinhole value is not used for any calculation, it is reported here to remind the user the acquisition conditions may differ from the conditions of theoretical resolution formula application. Please, mind all formulas from F to I suppose both a perfect match of the refractive indices of the objective immersion & mounting media and the use of the appropriate coverslip thickness (as indicated on the objective specifications). Moreover, in confocal set-up, noise may have detrimental effects. Additional information such as the name of the image associated with the report, its

creation date is provided. The creation date will first be the file's ImageAcquisitionDate OME metadata. Il not available, the creation date will be the file's creation date (ie. when the file was created on the computer used to analyze the data). This may differ from the original creation date! Finally, if the file is an unsaved byproduct of a precedent ImageJ analysis, there is no associated creation date.

pp-240924-01

Microscope info:

Image		40x-01_bead2					
image's creation	date	2024-06-13 09:14:22					
	method used	from file creation date					
Actual image depth		16					
Microscope type		WideField					
Objective	NA	1.4					
	im. refractive index	1.515					
Channel(s)		Wavelengths		Saturation	sampling (X,Y,Z)		
		Ex. (nm)	Em. (nm)		Nyquist (µm)	Found (µm)	Nyquist/found ratio
Channel 0			480.0	none	0.086x0.086x0.256	0.076x0.076x0.422	0.9, 0.9, 1.6
Channel 1			525.0	none	0.094x0.094x0.28		0.8, 0.8, 1.5
Channel 2			590.0	none	0.105x0.105x0.315		0.7, 0.7, 1.3
Channel 3			610.0	none	0.109x0.109x0.326		0.7, 0.7, 1.3
Bead original coordinates(in pixels)		385.0, 514.0					

Warnings:

(No saturated pixels detected). The highlighted undersampled channels may alter the result interpretation. (A subresolution bead is used for all channels).

Figure 30. PSF Profiler Tool report: microscope info and warnings sections.

The image calibration and sampling distance are also added. The Shannon-Nyquist $\Delta_{x,y}$ and Δ_z sampling distances are given using the formulas K to M. Note that in the multiphoton case, the plugin is assuming k=2. Correct sampling is quite a key issue for the measurement of FWHM. Inappropriate sampling will yield values higher than the theoretical resolution. Closer values may be achieved using the correct sampling density. Note that the computed Shannon-Nyquist correct sampling values are highly

stringent and higher values are usually used/found (such as 2.3 times the theoretical resolution value). Finally, whenever a dimension is undersampled, the whole cell is highlighted in red (this does not mean undersampling affects all dimensions).

All above formulas can be found at https://svi.nl/NyquistRate. Spinning Disc Shannon-Nyquist criterion used in the plugin follows K and K'.

$$\propto = arcsin\left(\frac{NA}{n}\right) \quad \text{(J)}$$

α: angular aperture
NA: numerical aperture
n: immersion/mounting medium refractive index

- Widefield microscopes:

$$\Delta_{x,y} = \frac{\lambda_{em}}{4.NA} \quad \text{(K)}$$

$$\Delta_z = \frac{\lambda_{em}}{2.n.(1-cos\ (\propto))} \quad \text{(K')}$$

- Confocal microscopes:

$$\Delta_{x,y} = \frac{\lambda_{ex}}{8.NA} \quad \text{(L)}$$

$$\Delta_z = \frac{\lambda_{ex}}{4.n.(1-cos\ (\propto))} \quad \text{(L')}$$

- Multiphoton microscope (k-photons)

$$\Delta_{x,y} = \frac{\lambda_{ex}}{4.k.NA} \quad \text{(M)}$$

$$\Delta_z = \frac{\lambda_{ex}}{2.k.n.(1-cos\ (\propto))} \quad \text{(M')}$$

References:
Wilson, T. and Tan J.B. Three-dimensional image reconstruction in conventional and confocal microscopy. BioImaging 1993 1:176-184.
Sheppard, C.J.R. *et al.* Electromagnetic field near the focus of wide-angular lens and mirror systems. IEE J. 1977 Microwaves, Opt. Acoust. 1, 129-132.
Sheppard, C.J.R. The spatial frequency cut-off in three-dimensional imaging. Optik 1986 72:131-133.
Sheppard, C.J.R. The spatial frequency cut-off in three-dimensional imaging II. Optik 1986 74:128-129.

The Warnings section contains all image & bead related warnings that might be useful to interpret the report.

The next Resolution table (Figure 31) section provides all channel resolution tables. The measured FWHM are provided for each dimension (X, Y, Z), along with the theoretical values (theory) derived from formulas 2.3 to 2.6. The fit goodness (ie. the

correlation coefficient R^2 associated with the dimension profile's fit) is reported. The user is invited to check the bead image in the corresponding channel (as reported in a later section). The measured FWHM/theoretical resolution ratio is also provided. If the "Apply tolerances to the final report" is selected, within specs and outside specs values are highlighted in green and red respectively.

Resolution table:

Channel	Sig/Backgnd ratio	Dimension	Measured FWHM (µm)	theory (µm)	Fit Goodness	Mes./theory ratio
Channel 0 (em. 525.0nm)	8.7	X	0.258	0.191	1.0	1.35
		Y	0.257	0.191	1.0	1.35
		Z	0.647	0.716	0.99	0.9

Green: within specifications, red: outside specifications (ie. XY ratios above 1.5 or Z ratio above 1.5)

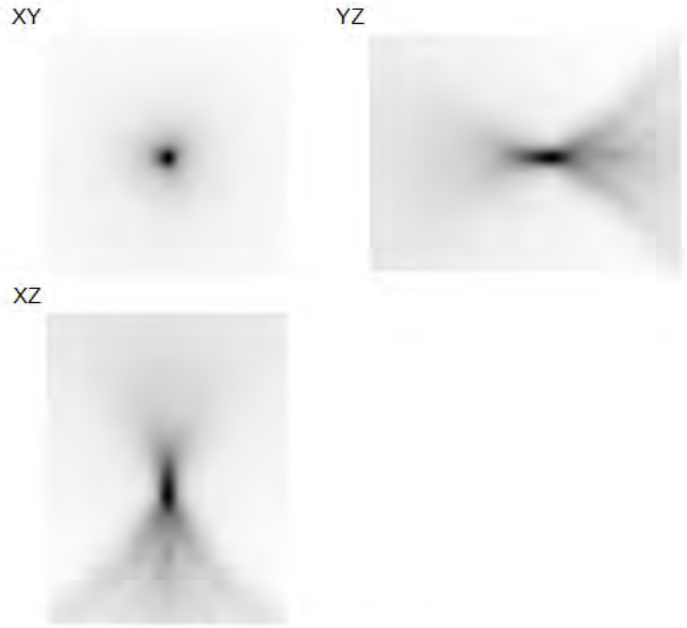
Figure 31. PSF Profiler Tool report : Resolution table.

Potential XY asymmetry can be monitored using the Lateral Asymmetry Ratios (LAR) section. For each channel, the LAR is computed following the N formula. LAR values diverging from 1 suggest a lateral asymmetry as may be found with strong astigmatism.

$$LAR = \frac{min\,[FWHM_x, FWHM_y]}{max\,[FWHM_x, FWHM_y]} \text{ (N)}$$

The following Detailed channel detection info section displays XY, XZ and YZ profile views of the beads. It is always worth looking at the beads and comparing them with the R^2, Sig/Backgnd ratio or LAR values. In Figure 32, the PSF has a LAR of 1 and a R^2 of 1

Channel #0



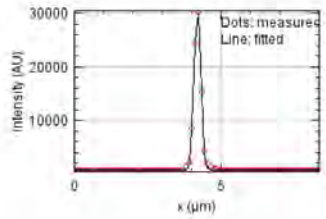
Channel 0 (em. 480.0nm)				
Sig./Backgnd ratio	LAR	Dimension	FWHM	Fit goodness
5.0	0.97	X	0.255	1.0
		Y	0.248	1.0
		Z	0.664	0.95

Figure 32. PSF Profiler Tool report. Profile views and LAR table.

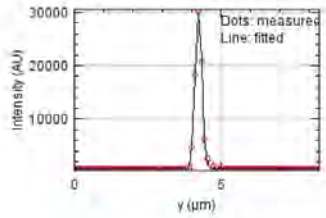
Long versions of the Detailed channel detection info include a table for each channel reporting the fit parameters and a plot comparing intensities (red circles) and fitted profile (black, Figure 33). The user may find the actual fitting parameter of each dimension on the left-hand side of the plot:

- equation against which the profile is fitted (formula D)
- the number of iterations used before reaching the final parameters
- the sum of residuals squared (ie. sum of the differences between the original intensity values and the fitted ones, squared)
- standard deviation: standard deviation of the residuals
- the correlation coefficient R^2 (gives indication on the fitting goodness)
- the Gaussian's constants a to d (see formula D). c is the position of the bead center along the dimension axis

X profile & fitting parameters:
Fitted on x = a + (b-a)*exp(-(x-c)^2/(2*d^2))
Sum of residuals squared: 2567064.73
Standard deviation: 178.02291
R^2: 0.99844
Parameters:
a = 763.86017
b = 30961.1500
c = 4.20364
d = 0.10967



Y profile & fitting parameters:
Fitted on y = a + (b-a)*exp(-(x-c)^2/(2*d^2))
Sum of residuals squared: 3075218.66
Standard deviation: 194.84779
R^2: 0.99803
Parameters:
a = 755.58866
b = 30241.4353
c = 4.23962
d = 0.10926



Z profile & fitting parameters:
Fitted on z = a + (b-a)*exp(-(x-c)^2/(2*d^2))
Sum of residuals squared: 15681428.1
Standard deviation: 722.98981
R^2: 0.99145
Parameters:
a = 1295.94833
b = 31078.3632
c = 3.45668
d = 0.27455

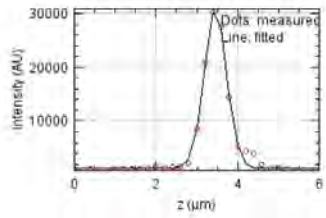


Figure 33. PSF Profiler Tool report: long version of Detailed channel detection info section.

Finally, user-provided Sample info and Comments, if any, are reported on the last page of the report, as are reported the user-defined analysis parameters (Figure 34).

Analysis parameters

Tool & Operator	Tool	PSF Profiler
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	Julien, 24 septembre 2024 10:44
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\psf\40\Processed\pp-240924-03\
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Beads	Bead detection threshold	Legacy
	Center detection method	Centroid
	Discard bead if more than one particle are thresholded	true
	Background annulus thickness in µm	1.0
	Background annulus distance to bead edges in µm	0.5
	Multiple beads in image	false
Square Root PSF Image displayed		true
Tolerance	Applied in this report	true
	X & Y FWHM ratios valid if below	1.5
	Z FWHM ratio valid if below	2.0

Figure 34. PSF Profiler Tool report: analysis parameters section.

A summary “log” table indicates how the input image was handled (Figure 35). From version 1.3.0, even though no analysis is performed (e.g., because the input image is saturated in all channels), a report is generated and the log table (Figure 36) indicates why no analysis was done.

Analysis log

image name	creation date	saturation	sampling density	status
40x-single	2024-06-28 16:54:07	none	Ch.0,1,2,3 undersampled	analysed

Figure 35. PSF Profiler Tool report: summary “log” table summarizing how the file was handled. Example of a valid input image

Analysis log

image name	creation date	saturation	sampling density	status
sat-40x-single	2024-06-28 17:35:36	Ch.0,1,2,3 saturated	Ch.0,1,2,3 undersampled	not analysed

Figure 36. PSF Profiler Tool report: summary “log” table summarizing how the file was handled. Example of a non-valid input image

Formulas used are also displayed at the end of the pdf file (Figure 37).

Formulas used:

Lateral ($res^o_{x,y}$) and axial (res^o_z) theoretical resolution values used for widefield microscopes are calculated as defined in Wilhelm, S. Confocal Laser Scanning Microscopy, 2011:

$$res^o_{x,y} = \frac{0.51*\lambda_{em}}{NA} \qquad res^o_z = \frac{1,77n*\lambda_{em}}{NA^2}$$

NA: numerical aperture, λ_{em}: emission wavelength, n: refractive index of the lens immersion & mounting media.

Axis profiles are fitted using ImageJ Gaussian Curve Fitter and the following formula $y = a + (b-a) * e^{\frac{-(x-c)^2}{2d^2}}$ (Gaussian fitting).

Measured lateral and axial resolution (Full Width at Half Maximum, FWHM) values are derived using $\mathrm{FWHM} = 2d\sqrt{2ln(2)}$

Compliance with the Shannon-Nyquist criterion uses the following formulas for Shannon-Nyquist distances calculation:

$$\alpha = \arcsin(\frac{\mathrm{NA}}{\mathrm{n}})$$

$$\Delta_{x,y} = \frac{\lambda_{em}}{4.NA} \qquad \Delta_z = \frac{\lambda_{em}}{2.n.(1-cos(\alpha))}$$

Figure 37. PSF Profiler Tool report: the formula section (example of a widefield microscope).

When single bead images are selected, all data is saved in a processed/title subfolder. If options are selected, side views and profile plots (not in the short version of the report) for each channel are saved in a title_imageName_data subfolder (Figure 38, top panel).

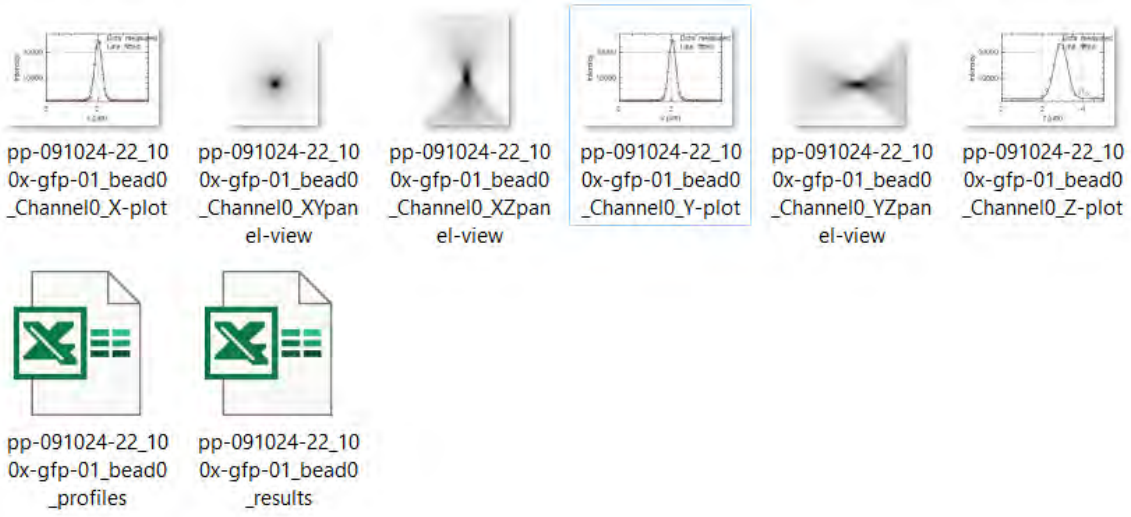


Figure 38. Files generated when save report images are saved (long version of the report). Top panel the main processed/title_imageName folder, bottom panel : the content of the title_imageName_data subfolder.

The .xls files of the tables of the pdf report are saved within the _data subfolder as a _results.xls file. If the long version of the report was chosen, the profiles for all channels/dimensions are stored in an _profiles.xls file (Figure 38).

When multiple bead images option is selected, files derived from each bead are saved in a processed/title/bead# (Figure 40). If the save pdf reports option is selected, a title_imageName_identifiedBeads.pdf document is generated that contains the microscope info, the analysis parameters, a log file that summarizes how the beads were identified and analyzed (Figure 39).

Analysis log

image name	creation date	sampling density	identified raw beads	valid beads	saturation	status
40x-02	2024-06-13 09:14:22	Ch.0,1,2,3 undersampled	26	18	none	valid beads found
				bead0	none	analysed
				bead1	none	analysed
				bead2	none	analysed
				bead3	none	analysed
				bead4	none	analysed
				bead5	none	analysed
				bead6	none	analysed
				bead7	none	analysed
				bead8	none	analysed
				bead9	none	analysed
				bead10	none	analysed
				bead11	none	analysed
				bead12	none	analysed
				bead13	none	analysed
				bead14	none	analysed
				bead15	none	analysed
				bead16	none	analysed
				bead17	none	analysed

Figure 39. PSFProfiler tool: the analysis log table when images contain multiple beads.

Other “identifiedBeads” files mentioned above are stored in a title_imageName_beadData subfolder (Figure 40).

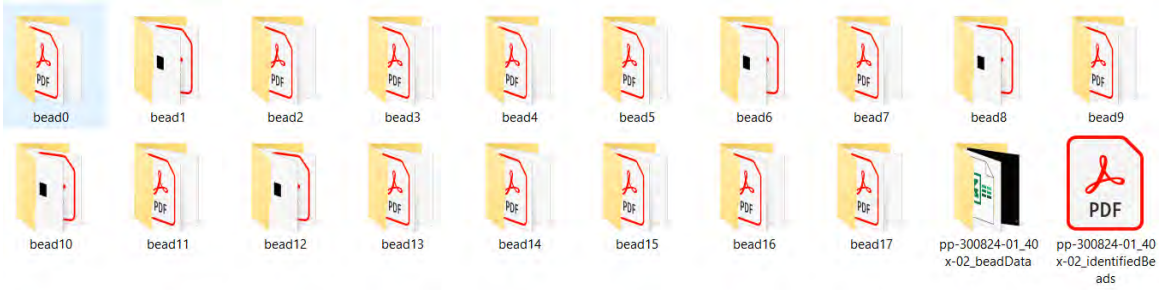


Figure 40. Files and folders generated when images contain multiple beads.

Axial Resolution Tool

Axial resolution is a key parameter for monitoring along time the microscope performances. An easy way to measure this is to image a Z-stack of a mirror slide.

Sample for measuring axial resolution and recommended parameters

Fix a reflective surface on a slide, overlaid by a mounting medium, topped by a coverslip. The mirror surface can be any single metal coated reflector mirror (Edmund optics' 4-6 Wave Mirror 20mm x 20mm Enhanced AluminumSilver, Ref. 36044NT43-872., Thorlabs PFR10-P01). Prepare the slide as follow:

- Clean a type 1.5 (i.e., 0.17mm ± 20µm) thick coverslip. If available, either use a micrometer to check the thickness or use 1.5H grade (0.17mm ± 5µm). Carefully clean the mirror.
- Glue the glass side of the mirror to the coverslip. You may use mounting medium to seal the mirror on the slide. Wait for the glue to set.
- Mount the coverslip on the slide/mirror by using either immersion oil or appropriate refractive index mounting medium. Remove excess of solution by pressing firmly on the coverslip.

Acquire a 3D stack of the mirror surface. Guidelines for confocal setup are the following:

- Use the reflection mode if any
- set zoom to maximum, open the pinhole and adjust the laser intensity to minimum
- Focus onto the mirror surface then close the pinhole to an adequate value (1AU or less). Adjust excitation and detection parameters.
- Acquire a 3D stack, respecting the Shannon-Nyquist criterion.

The QC Axial resolution tool's algorithm.

After the user has defined a rectangular ROI, the plugin will generate an average intensity projection of the image along the y axis.
The resulting 1D intensity profile is then fitted to a Gaussian, using equation D and ImageJ's built-in curve fitting function. The axial resolution, i.e., the full-width at half-maximum (FWHM), is calculated using formula E, based on the parameters retrieved

from the fitting. The measured FWHM is then compared to theoretical axial resolution (formulas F', G', H' and I").

Tool parameters:

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).

STEP2. Open a file containing the single channel/multichannel 3D stack.

STEP3. Click on the Axial resolution tool icon.

STEP4. In case the image has not been spatially calibrated, a message error will pop up. Click on Ok, provide the appropriate values in the calibration dialog box, then re-launch the plugin.

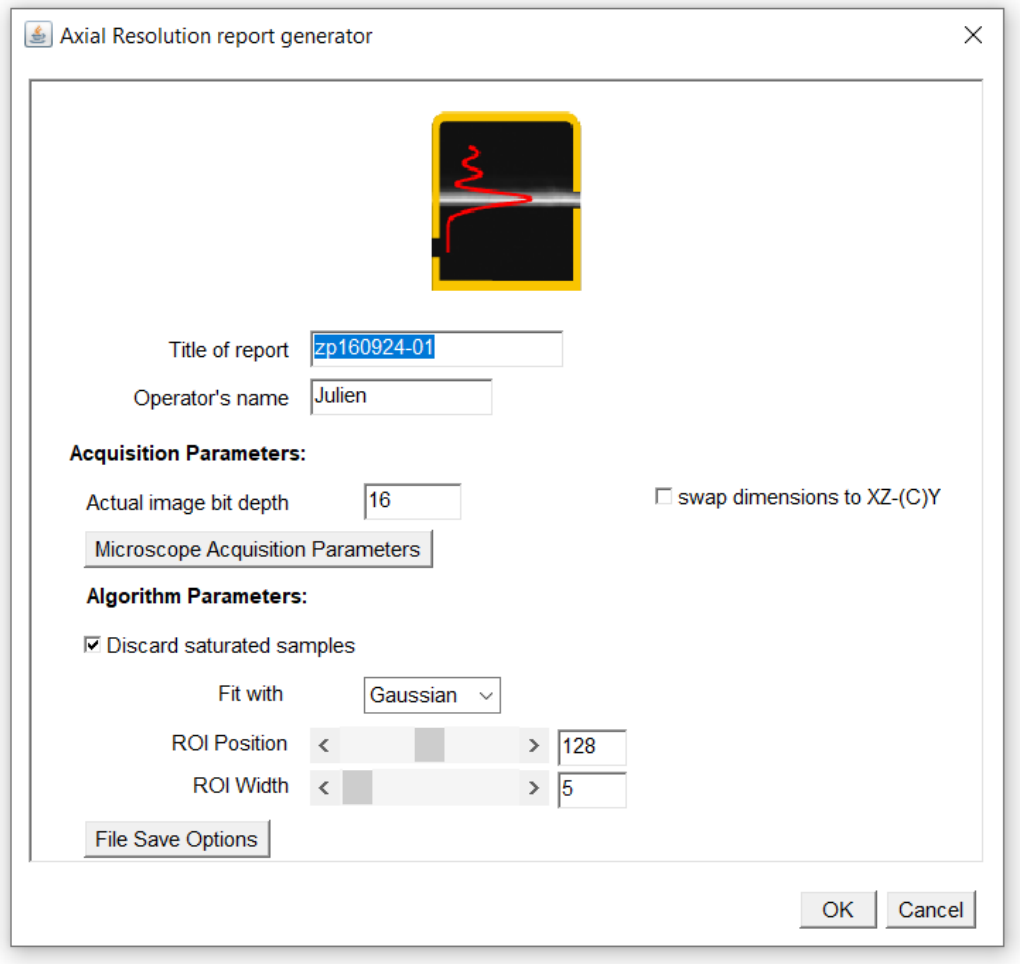


Figure 41. Axial Resolution Tool : the User's interface.

STEP5. The plugin's interface should appear (see Figure 41). The plugin is intended to be used with any format (XY-Z stack, XZ-Y stack or YZ-X stack). However, any XY-Z input stack should be transformed into a XZ-Y. If a XYZ Stack was open, tick the swap dimensions to XZ-(C)Y checkbox to generate the correct view (Figure 42). This uses the ImageJ Image>Stack>Reslice[/]…(note that calibration follows this swap).

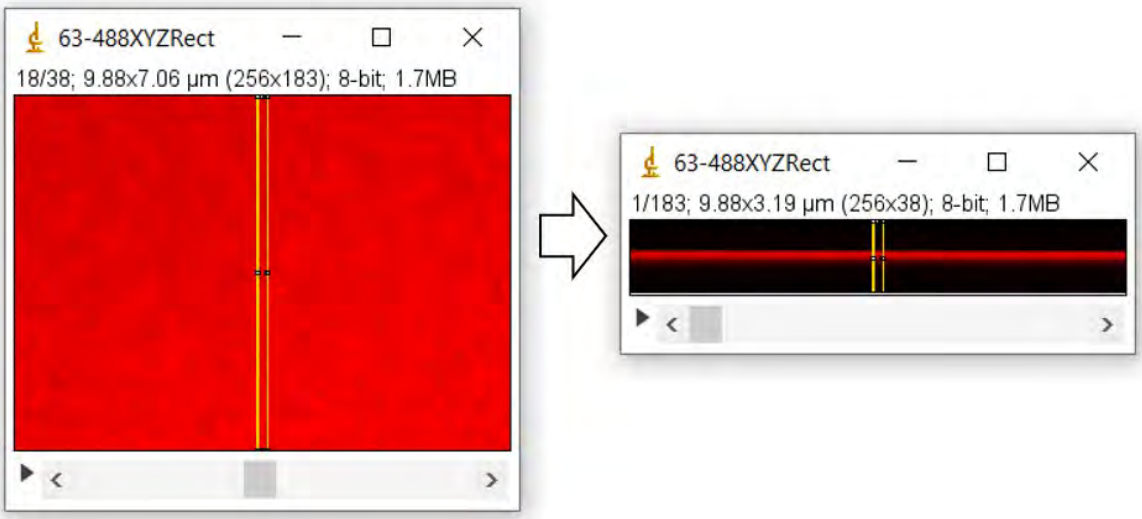


Figure 42. Axial Resolution tool: the swap option result (a XYZ stack, left, is changed to the expected XZY or YZX stack format, right).

STEP6. Set the image's depth. This parameter is used to compute the maximum, saturated intensity (when saturated samples should be discarded). Click the Microscope Acquisition parameter button. This opens the microscope acquisition dialog (Figure 43). These parameters are crucial for the determination of theoretical axial resolution values. If left unverified, the raw resolution values are still valid, but use of theoretical calculated values is hazardous. In the microscope acquisition parameters Dialog (Figure 43), set the microscope type. In the cases of Widefield and spinning disc confocal microscopes, the theoretical resolution uses the emission wavelength (as well as numerical aperture and immersion medium refraction index, formula F' and H'). In cases of multiphoton and single point Laser Scanning Microscopes (CLSM), the excitation wavelengths are used (together with NA and refraction index, formula I" and G'). For CLSM, the pinhole size is also used, while a 2-photon excitation is assumed for multiphoton excitation microscopes.
The number of channels/slices is found and the user is prompted to fill-in the relevant information. Mind these formulas consider the image to be noise-free.
Click the OK button to go back to the main dialog.
If not hidden, some more sample information and/or comments fields might also be used to trace additional information.

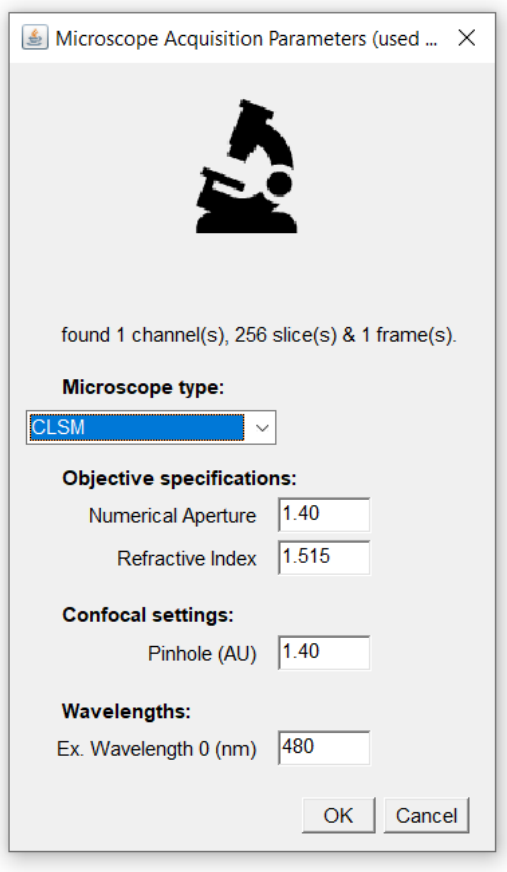


Figure 43. MetroloJ_QC : the microscope acquisition parameters dialog (CLSM case).

STEP7. Consider rejecting saturated sample. Saturation is calculated for each channel within the ROI and along the Y axis (i.e., for each channel, saturation within the ROI for each slice of the XZ(C)-Y is measured and the average saturation is calculated).

STEP8. Select the position of the ROI and adjust its width; Note that the ROI's display might not be updated.

STEP9. Set the file Save Options. Click the "File Save Option" to set the output options (Figure 44).

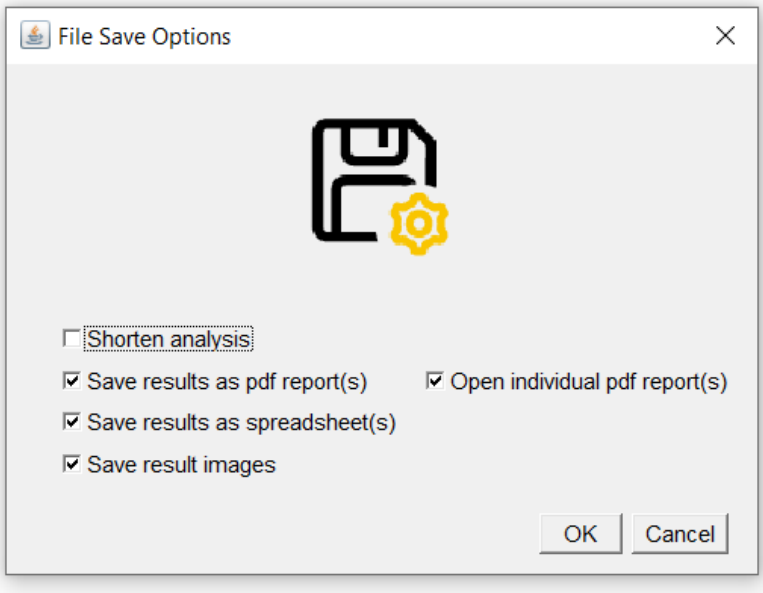


Figure 44. MetroloJ_QC : the file Save Options dialog

Set the file save options:

- Select the shorten analysis checkbox if you don't want to have the Z profile's plot and fitting parameters.
- Decide whether the results should be saved as a pdf file and whether the pdf should be automatically displayed.
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls file containing tabulation separated values of all tables of the pdf report. The values of the intensity profiles across Z are saved in separated .xls.
- You may save the input image+ ROI, together with the fitted ROI's profiles as jpg files as well.

All generated files are saved in a subfolder of a "processed" folder located in the same folder of the original image. The subfolder's name can be changed in the first "title" field of the dialog.

STEP10. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 45 (left panel) will appear. In this case, change the title.

MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP6) and the actual file format depth, a different type of error message is triggered Figure 45, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

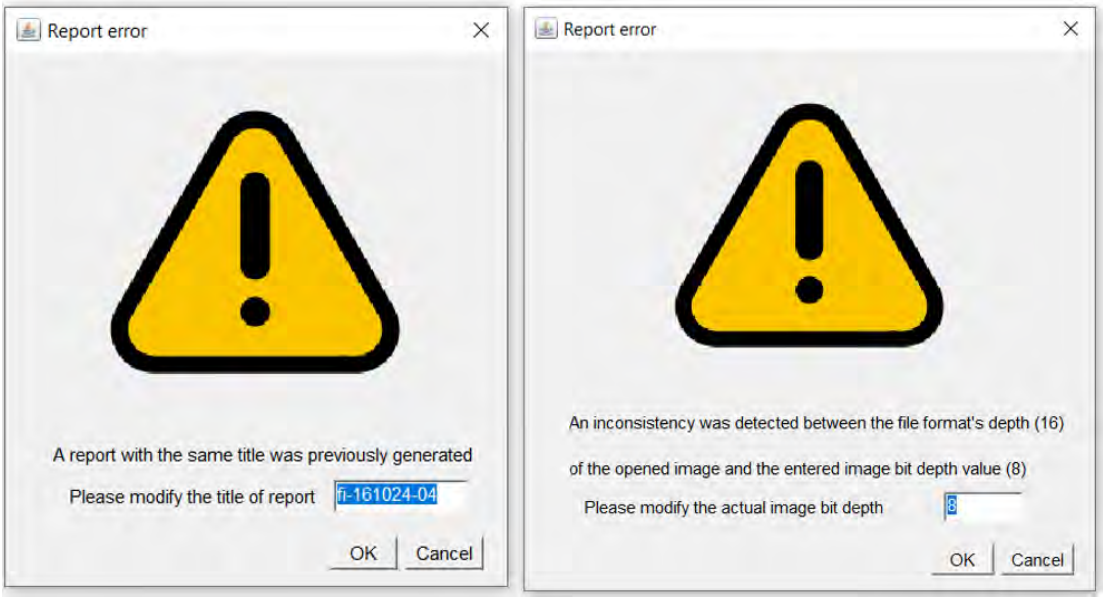


Figure 45. Error dialogs triggered by Axial Resolution tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved!

Description of the Axial Resolution tool report.

Microscope info and warnings sections (Figure 46) summarize all setup-related parameters and contains useful information to help result interpretation.

zp160924-02

Microscope info:

Image		63-488XYZRect				
image's creation	date	2024-07-10 11:27:23				
	method used	from file creation date				
Actual image depth		16				
Microscope type		Confocal				
Pinhole		1.0 AU)				
Objective	NA	1.4				
	im. refractive index	1.515				
Channel(s)		Wavelengths		Saturation	sampling (X,Y,Z)		
		Ex. (nm)	Em. (nm)		Nyquist (µm)	Found (µm)	Nyquist/found ratio
Channel 0		488.0	525.0	none	0.044x0.044x0.13	0.039x0.039x0.084	0.9, 0.9, 0.6

Warnings:

(All channels sampled following Shannon-Nyquist criterion).

Figure 46. Axial resolution Tool Report: microscope info and warnings sections.

The measured axial resolution values, along with theoretical values are summarized in the Resolution table (Figure 47).

Resolution table:

ROI: from (125, 0) to (130, 38)

Channel	FWHM	Theoretical resolution
Channel 0	0.359 µm	0.459 µm

Figure 47. Axial Resolution tool report : the resolution table.

Long versions of the report include the profile view and Z profiles, including the ROI's outlines and the fitting parameters (Figure 48). The red dots are the measured intensities while the black line is the fitted Gaussian. Fitting parameters are indicated:

- equation against which the profile is fitted (Gaussian)
- number of fitting iterations

- sum of residuals squared: sum of the differences between the original intensity values and the fitted ones, squared;
- standard deviation: standard deviation of the residuals;
- the correlation coefficient R^2 (gives indication on the fitting goodness). It is recommended to discard poor fitting results (e.g., R^2 value lower than 0.95).
- the Gaussian's constants a to d (see formula 4.1), c being the position of the beads center along the first-dimension axis.

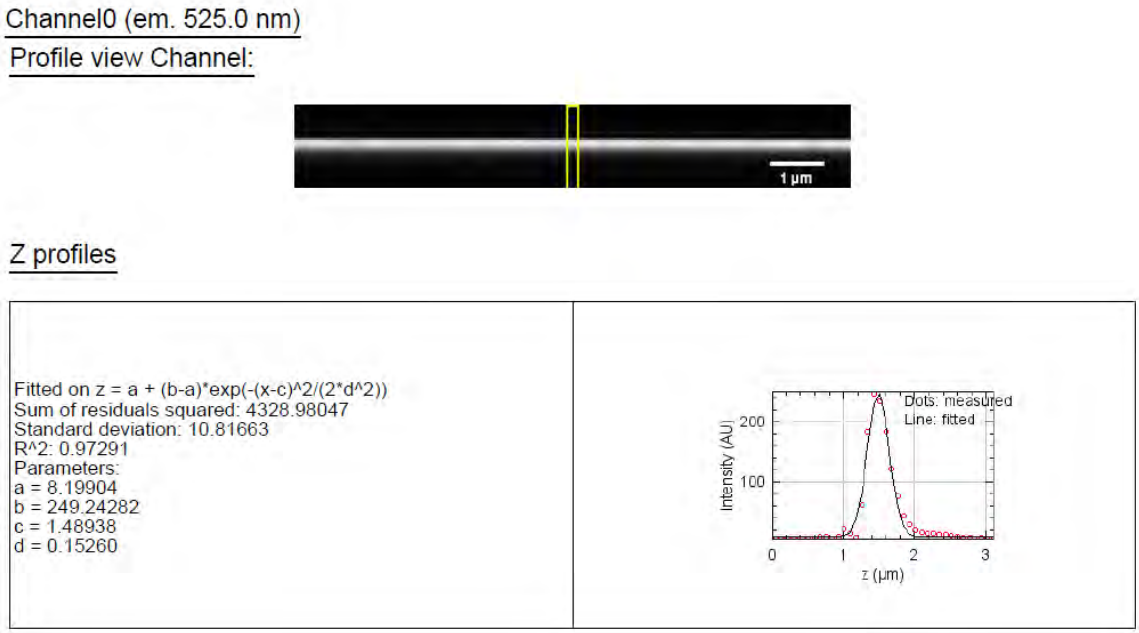


Figure 48. Axial Resolution tool report : profiles views (long versions of the report only).

Analysis parameters (Figure 49, top panel) are summarized in the analysis parameters section. How the input file was handled is explicated in the analysis log (Figure 49, bottom panel). An example of a saturated non-analyzed input file is given (Figure 50). Formulas used are summarized in the next page of the report (Figure 51).

Analysis parameters

Tool & Operator	Tool	Axial Resolution
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	Julien, 16 septembre 2024 09:59
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\mirroir\Processed\zp160924-02\
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XZ-(C)Y or YZ-(C)X
Discard saturated samples		true

Analysis log

image name	creation date	saturation	sampling density	status
63-488XYZRect	2024-07-10 11:27:23	none	correct	analysed

Figure 49. Axial resolution tool report: analysis parameters

Analysis log

image name	creation date	saturation	sampling density	status
63-2CH-XYZsat	2024-07-10 10:43:30	Ch.1 saturated	correct	analysed

Figure 50. Axial resolution tool report: analysis parameters

Formulas used:

Axial (res_z^o) theoretical resolution value used for widefield microscopes is calculated as defined in Wilhelm, S. Confocal Laser Scanning Microscopy, 2011:

$$res_z^o = \frac{1{,}77n*\lambda_{em}}{NA^2}$$

NA: numerical aperture, λ_{em}: emission wavelengths, n: refractive index of the lens immersion & mounting media

Z axis profiles is fitted using ImageJ Gaussian Curve Fitter and the following formula $y = a + (b - a) * e^{\frac{-(x-c)^2}{2d^2}}$ (Gaussian fitting).

Measured axial resolution (Full Width at Half Maximum, FWHM) value is derived using $FWHM = 2d\sqrt{2\ln(2)}$

Compliance with the Shannon-Nyquist criterion uses the following formulas for Shannon-Nyquist distances calculation:

$$\alpha = \arcsin(\frac{NA}{n})$$

$$\Delta_z = \frac{\lambda_{em}}{2.n.(1-\cos(\alpha))}$$

Figure 51. Z profiler tool: formulas used (confocal microscope case).

All associated data is saved in a processed/title/title_imageName_data subfolder (Figure 52, top panel). If the “save report images” option is selected ROI view and profile plots (not in the short version of the report) for each channel are saved. If the “save all data in a spreadsheet” option is selected, a _results.xls file is generated that contains all data of the pdf version of the report. The profiles plot’s coordinates (long version of the report) are saved in a _profiles.xls file. These files are saved in a title_imageName_data subfolder (Figure 52, bottom panel).

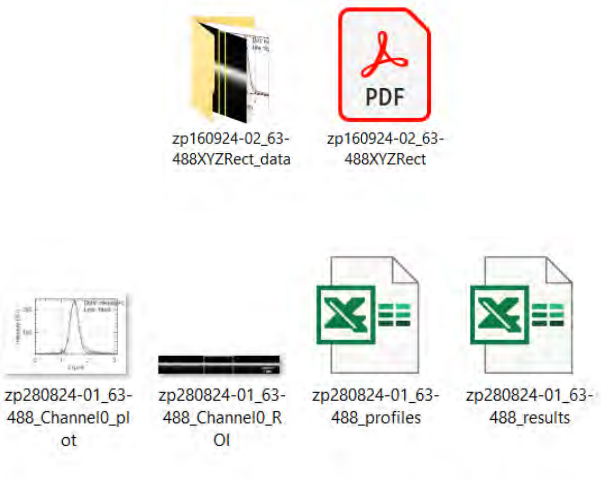
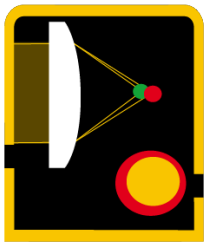


Figure 52. Files generated when save report images are saved (long version of the report).



Co-Registration Resolution Tool

Relative location of a given staining with respect to other channels requires that all chromatic (ie. both lateral and longitudinal) aberrations are corrected. The objective's design allows more or less precise corrections (from no correction, achromat/fluotar, apochromat and superapo corrections). The correction's performance may be affected as well. Hence, measuring accurate co-registration is of major importance whenever experiments such as localization, co-expression or colocalization studies are carried-out.

Acquisition sample for monitoring co-registration and recommended parameters

We recommend using multi-labelled fluorescent beads (e.g. Molecular Probes' 1 or 4µm TetraSpeck or FocalCheck beads). Smaller beads (such as 160 or 100nm diameter beads) should be avoided.
As objectives performances are associated with mounting conditions (such as immersion refractive index and coverslip thickness), attention should be paid so that the mounting medium refractive index and coverslip thickness are appropriate. Moreover, to assess proper co-registration in depth within the sample, a proper slide/coverslip configuration should be used to mimic real samples.

The user may refer to the GT3M WP of the RTmfm network and its P04 protocol.

The QC co-registration tool's algorithm.

Whenever the option is chosen, the plugin will first identify individual beads. Starting from these individual images, the plugin will proceed as in the original MetroloJ co-alignment analysis. For any channel combination, the plugin will generate two summed intensity projection of the stack along the y and z axes. For each projection, a histogram segmentation is done on the log of intensities, aiming at separating two populations of intensities (background and signal). Each projection is then thresholded in order to highlight the "signal pixels' population" and the center is determined using different methods.
Once all coordinates have been retrieved for each channel, distance between the center from channel A and center from channel B is calculated:

$$r = distance_{A-B} = \sqrt{(x_B - x_A)^2 + \ (y_B - y_A)^2 + (z_B - z_A)^2} \quad \text{(O)}$$

For each couple of coordinates, a reference distance r_{ref} is calculated. This distance is quite easy to determine in 2D as it corresponds to the xy resolution. While considering the center of the structure on image A, a structure of image B will be co-localized if it lies within a circle, centered on center A, with a radius equal to the minimum lateral resolution distance (calculated using one of the channel wavelengths, the longest wavelength in the co-registration algorithm).

Due to the disparate resolutions over the three dimensions, this distance is not so easy to calculate in 3D. However, the answer might come from the observation of the factor limiting the resolution: the PSF (Point Spread Function) and more precisely the first Airy disc which might be approximated in 3D as having an ovoid shape (see Figure 53 right panel). Therefore, in 3D, the reference distance is calculated by considering a reference point and fitting a 3D ellipse around it for which the two characteristic radii correspond to x/y and z resolutions. In this matter changing from Cartesian coordinates to Polar coordinates make it easier to calculate the reference distance. The two characteristic angles, the azimuth Φ and the zenith Θ (see formulas 5.2 and 5.3) are first calculated, based on the coordinates of the two centers to analyze. Knowing this orientation, as well as the x, y and z theoretical resolutions ($\mathrm{res}_{\mathrm{x}}^{\mathrm{o}}$, $\mathrm{res}_{\mathrm{y}}^{\mathrm{o}}and\mathrm{res}_{\mathrm{z}}^{\mathrm{o}}$ respectively, as calculated using formulas 5.5 to 5.13), the distance from the reference centre to the border of the ovoid shape rref is calculated (see expression 5.4). The inter-centre distance r is then compared to this reference distance to assess if co-localization occurs (see Figure 53, left panel) or not (see Figure 53, medium panel).

$$\varphi = \arccos \frac{x_B - x_A}{\sqrt{(x_B - x_{A)}{}^2 + (y_B - y_{A)}{}^2}} \quad \text{(P)}$$

$$\theta = \arccos \frac{z_B - z_A}{\sqrt{(x_B - x_{A)}{}^2 + (y_B - y_{A)}{}^2 + (z_B - z_{A)}{}^2}} \quad \text{(Q)}$$

$$res_{\theta,\varphi}^{o} * = \frac{res_x^o * res_y^o * res_z^o}{\sqrt{(res_y^o * res_z^o * cos\Phi * sin\Theta)^2 + (res_x^o * res_z^o * sin\Phi * sin\Theta)^2 + (res_x^o * res_y^o * cos\Theta)^2}} \quad \text{(R)}$$

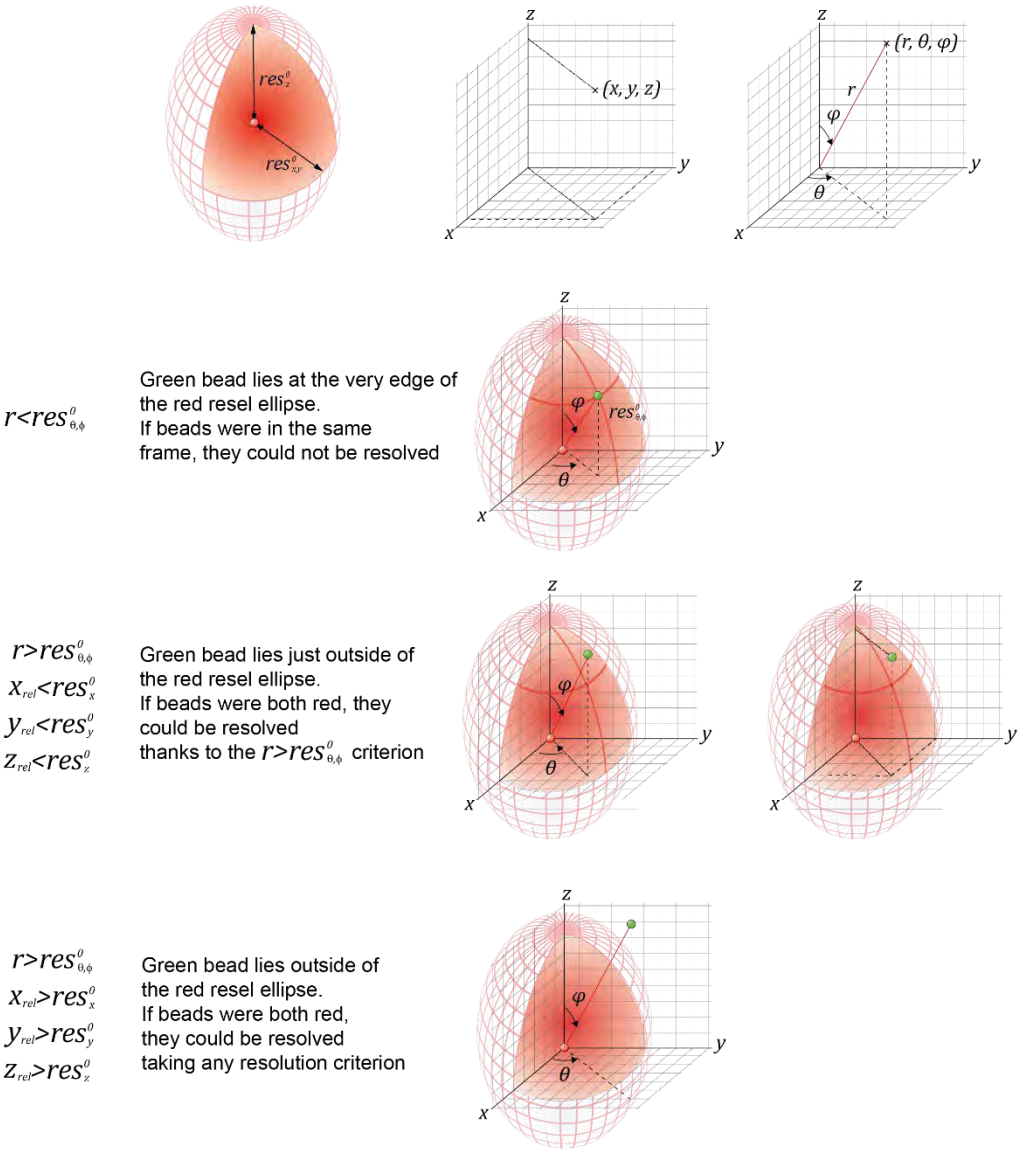


Figure 53. Calculation of the reference distance $res^{o}_{\theta,\varphi}$. Left: Centers of objects A and B are drawn as a red and green spheres, respectively. The resolution element centered on the red bead's center (the first Airy volume) appears as an ellipsoid (top panel left). Cartesian coordinates and polar coordinates are displayed in the middle and right top panel. For a pixel of the ellipsoid with polar coordinates θ and φ, $res^{o}_{\theta,\varphi}$ is its r coordinate. If the green bead's center lies within the red ellipsoid, then it can be considered as colocalized because if both beads' centers were of the same color they could not be resolved (second panel from top). However if the green bead's center lies just outside or further away from the red ellipsoid, red and green beads centers could be resolved (bottoms panels). The third panel from the top shows that it is important to compare the intercenter distance r with the resolution reference distance $res^{o}_{\theta,\varphi}$ for the same θ and φ angles that are observed between the red and green beads' centers. Adapted from Cordelières and Bolte, ImageJ User and Developer Conference Proceedings, 2008, Luxembourg.

The plugin will generate all channel combinations possible, measure all dimensions' pixel shifts, the (calibrated/uncalibrated) center-to-center distances for all combinations and compare it to their respective reference distance. A ratio of the measured center-to-center distance to the reference distance is also calculated.

Co-registration Tool parameters:

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).

STEP2. Open a file containing the multichannel 3D stack.

STEP3. Click on the co-registration tool icon. The plugin's interface should appear (see Figure 54).

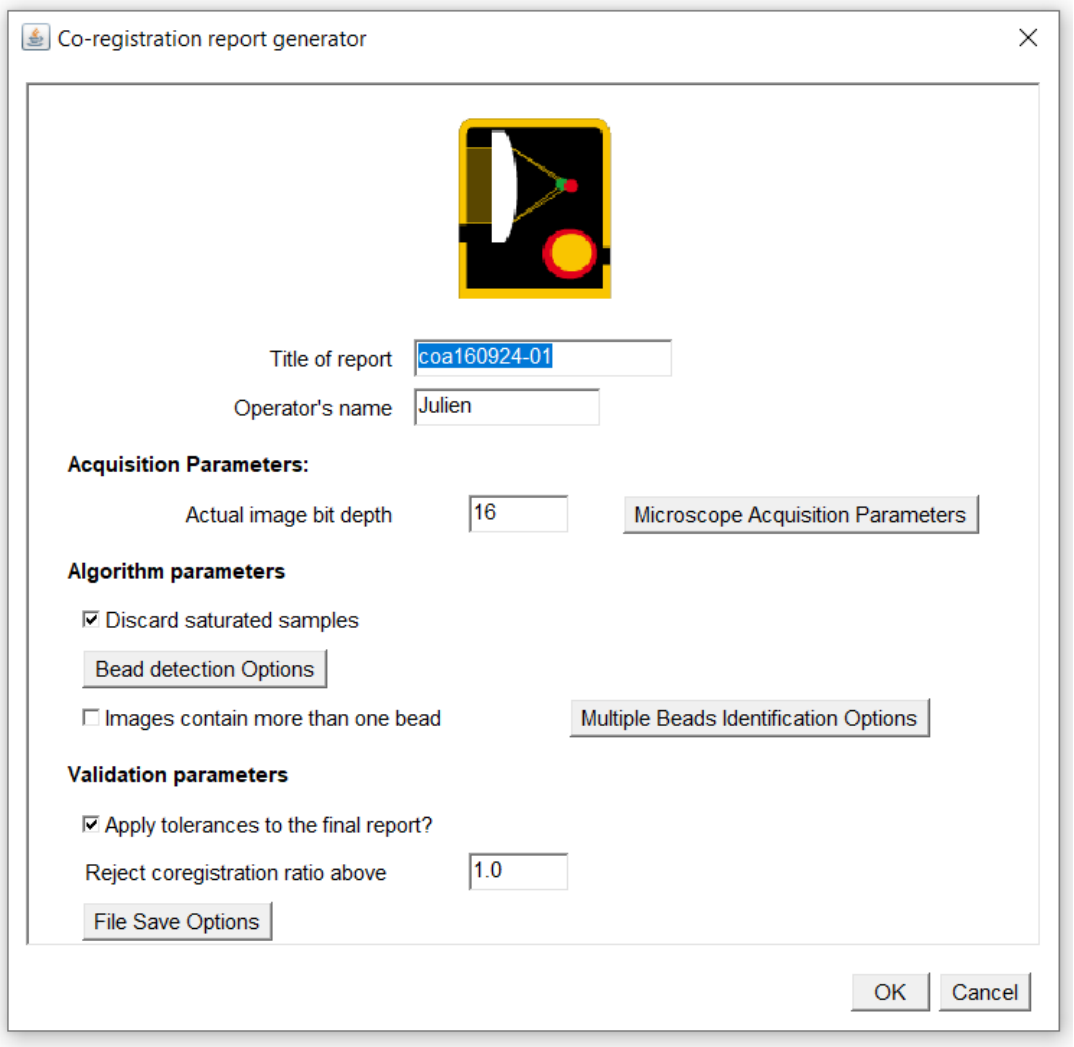


Figure 54. Co-registration tool: the user's interface.

STEP4. In case the selected multichannel z stack has not been spatially calibrated, a message error pops up: click on Ok. In the calibration dialog box provide the appropriate values, then re-launch the plugin.

STEP5. Enter a title for the report. All generated data will be stored in a processed/title subfolder located in the same folder containing the original opened image. If such a folder was previously generated (ie. a previous report with the same name was generated using an image that is within the same folder as the stack), the analysis stops.

STEP6. Enter the image's depth. This field is used to compute the maximum saturated intensity, whenever the user chooses to discard saturated samples. Click the "Microscope Acquisition Parameters". This new dialog window (Figure 55) is crucial for the plugin, as these parameters will allow the calculation of the reference distance. Select the appropriate microscope type. In the unlikely case the 3D (+Channels C) data is not order as a XY stack along Z, the analysis will yield wrong results. In this case,

the user is invited to rotate the dataset so that XY-planes Z stacks are provided. This has to be changed whenever, say, a confocal XZ scan across Y is performed. If the dimension order is wrong, the references values will be wrong. The user can check that Channel versus slices are correctly detected. If not, click cancel and change dimensionality using for instance image>Properties.

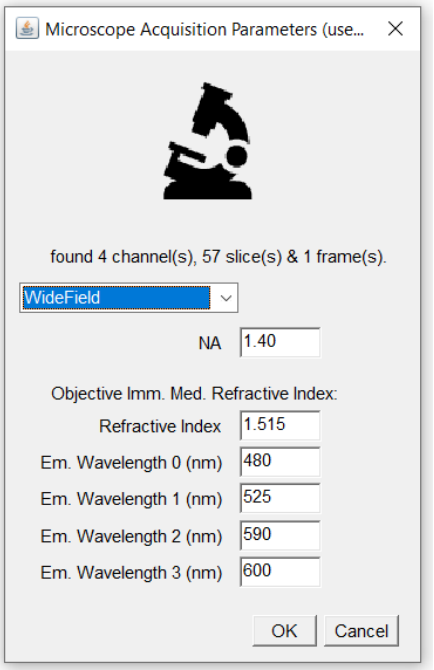


Figure 55. MetroloJ_QC: the microscope's interface (widefield case)

STEP7. The user is prompted to fill-in some microscope information. Emission wavelengths and objective immersion medium refraction index & NA are used for theoretical lateral/axial resolution formulas with widefield and spinning disc confocal set-ups (formulas F/F' and H/H'). Excitation wavelength, objective NA and immersion medium refractive medium are used for computation of theoretical resolution with single-point scanning confocal set-up and multiphoton microscope (formulas G/G' and I/I'/I"). Note that multiphoton resolution assumes a 2-photon excitation. For confocal setups, the entered pinhole size is actually not used in the formula.
The actual formulas used are further reminded in the pdf report. Click OK to go back to the main dialog window. For the sake of traceability, some more sample information and/or comments might also be provided using the appropriate boxes.

STEP8. Set the algorithm's parameters. As saturation alters the bead center detection, the user has the possibility to detect bead saturation. The user may discard saturated beads. Saturation is measured on the original image (or the cropped image beads option when multiple beads option is used). Briefly, whatever the option status is, all slices of a given channel are montaged in a single 2D image, a bead detection threshold is calculated and applied to highlight bead sections. Select the bead detection threshold with the rolling-menu in the "Bead detection parameters". See the "TESTS SECTION" to find the appropriate threshold. Then, the bead sections are masked and the proportion of saturated pixels within the mask is calculated. When all

channels are saturated, the image is excluded from channel-to-channel combinations. If a minimum of two channels are unsaturated, the analysis proceeds with those channels, otherwise analysis is skipped. To set the bead detection threshold, click the “Bead Detection option button”. This opens a new “bead detection option” (Figure 56). Set the bead detection threshold :

- Legacy Threshold: This method operates similarly to k-means clustering of the intensity histogram with a k-value of 2 classes. The 32-bits projection is converted into a 16-bit image, and the 16-bit histogram is displayed in log mode (intensity versus log count). Whenever some processing was applied to the 32-bits projection, the display is reset (in order to avoid clipping of the dynamic range). The histogram is divided into 2 classes: the first class ranges from the minimum value (min) to an initial midpoint, and the second class ranges from this midpoint to the maximum value (max). The midpoint is initially set to (max - min) / 2 and is adjusted through an iterative process. In each iteration, the previous limit is stored and compared to the new limit, then the mean values of the two classes are calculated. The new limit is set as the mean of these class means. The legacy algorithm performs 100 iterations, using the resulting limit as the threshold between the two classes.
- Built-in ImageJ Histogram Segmentation Algorithm: This method uses one of the built-in segmentation algorithms in ImageJ. The list of available algorithms can be modified in the “CONFIGURATION SECTION”.

- “k-Means” Threshold: This method also uses k-Means clustering for segmentation but with a configurable k-value. Instead of two classes, it divides the histogram into k classes. The initial class widths are set to (max - min) / k. These limits are refined through an iterative process similar to the legacy threshold method. The intensities of the last (k^{th}) class are used to threshold the image. The k-value can be adjusted in the “CONFIGURATION SECTION”.

You can test the accuracy of bead detection using the “TESTS SECTION”.
An additional option is available to exclude partially thresholded beads or input images containing two beads. To prevent these cases, select the "Discard bead if more than one particle is thresholded" option.

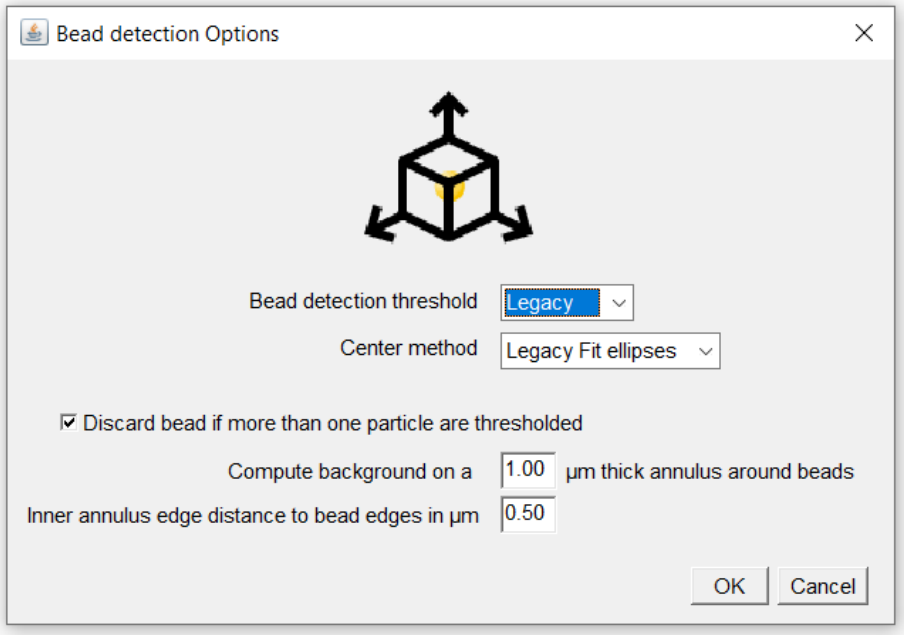


Figure 56. MetrologJ_QC : the bead detection Options dialog.

STEP9. A clue for identifying poor quality beads is the signal-to-background ratio. This ratio is calculated during the saturation ratio evaluation process (STEP8). It involves measuring the mean intensity of the identified bead section (signal) and estimating the background intensity in an annulus around the bead section. The annulus thickness can be adjusted, and it is converted from micrometers to pixels for drawing. When using a single-bead input image, there is no warning if the annulus boundaries extend beyond the image. The ratio is then calculated by dividing the mean intensity of the bead section (signal) by the mean intensity of the annulus (background). Note that this is not an evaluation of the signal-to-noise ratio. Set the annulus thickness and distance to bead's edges. You can have an idea of the bead and annulus detection using the "TESTS SECTION".

STEP10. Set the method for detecting the bead's center. To achieve this, XY, XZ, and YZ sum projections are computed and thresholded. The X and Y coordinates of the center are obtained from the XY projection. The Z coordinate is determined by averaging the Z coordinates identified from the XZ and YZ projections. Note that the original MetroloJ plugin uses only the Z coordinate derived from the XZ projection.

STEP10b. Set the center detection method :

- The "Legacy fit-ellipse" method takes the thresholded projection and fits an ellipse. This method is the original one, as implemented in MetroloJ and earlier versions of MetroloJ-QC. The corresponding X, Y coordinates are derived from the centers of the ellipses of the XY projection, while the Z coordinates is the average of the Z coordinates of the center of the ellipses defined using the XZ

and YZ projections (note that the original MetroloJ plugin only uses the XZ projection). The method starts with an ellipse centered on the center of mass of the thresholded area. It continuously fits an ellipse to the object in the segmented image, refining the position of the ellipse's center until the minor axis of the ellipse is sufficiently large.
The found ellipse, as well as the thresholded bead projection was originally not displayed. This is now changed in the sideViews panels (see Figure 66). In cases the thresholded bead's mask spans the entire stack depth (left panels Figure 57), the Z coordinate might be biased. Whenever the fitted ellipse looks cropped in sideview panels (see corresponding bottom left panel), it is recommended to change the threshold method or switch to the centroid detection method.

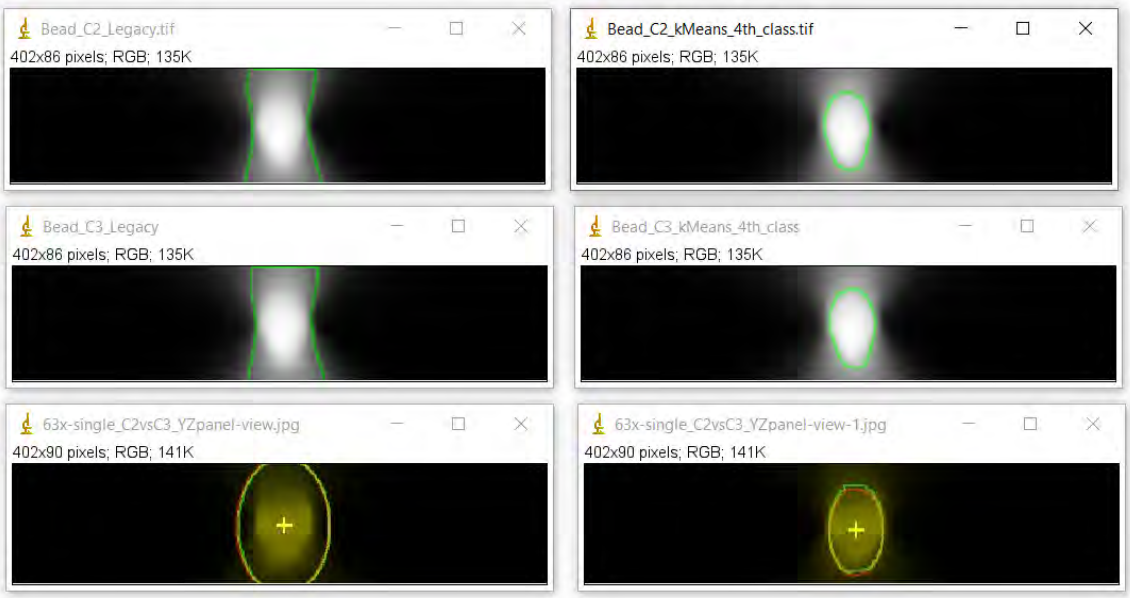


Figure 57. Potential fit-ellipses bias. When the bead is thresholded using some threshold (here legacy threshold) that selects all slices (top and medium-left panels for channel C2 and C3 respectively), the resulting ellipses (bottom-left panel green for C2 and red for C3) appear as cropped and the analysis concludes that there is no misalignment between C2 and C3 (no Z pixel shift). When the thresholded areas doe not span the entire stack's depth (top-rignt panel for C2 and medium-right panel for C3), the ellipses are not cropped and the analysis concludes that channels are misaligned (0.5 pixel shift).

- The centroid method skips the process of fitting the ellipse and the bead's XY coordinates are the center of Mass (centroid) of the XY sum projection, while the bead's Z coordinate is the average of the Z coordinates of the XZ and YZ sum projections.

Once all bead detection parameters are set, click ok to go back to the main dialog's window.

MULTIPLE BEADS IMAGES (STEPS 11 & 12)

STEP11. When using stacks containing multiple beads, users can select the "images contain more than one bead" option. The process of finding multiple beads within an image is referred to as "multiple bead identification" (while bead detection refers to

finding single beads' coordinates). Click the "multiple beads identification parameters" button. The corresponding dialog window opens (Figure 58). In the case of multichannel images, bead identification is performed using a single specified channel. To do this, enter the appropriate channel number in the "Find bead with Channel#" field (note that channel 0 is the first channel). Bead identification of X and Y coordinates is done on a Z sum intensity projection of the chosen channel. To aid in detection, background is removed using a rolling background algorithm (as found in Process > Subtract Background with a rolling ball radius of 50 pixels, leaving all options unselected). Any noise is eliminated with a Gaussian blur of 2 pixels (Process > Filter > Gaussian Blur). The selected bead detection threshold is then applied, and beads are identified using ImageJ's particle analysis tool. A filter is used to exclude objects with an area less than 50% or more than 400% of the expected bead area, based on the bead diameter field (see next step). Z coordinates are derived from Y sum intensity projection of the chosen channel (XZ image), processed like the Z sum projection that was used to get the X and Y coordinates. Vertical lines across each bead center are drawn and plotted. A gaussian fit is used to get the center of the bead.

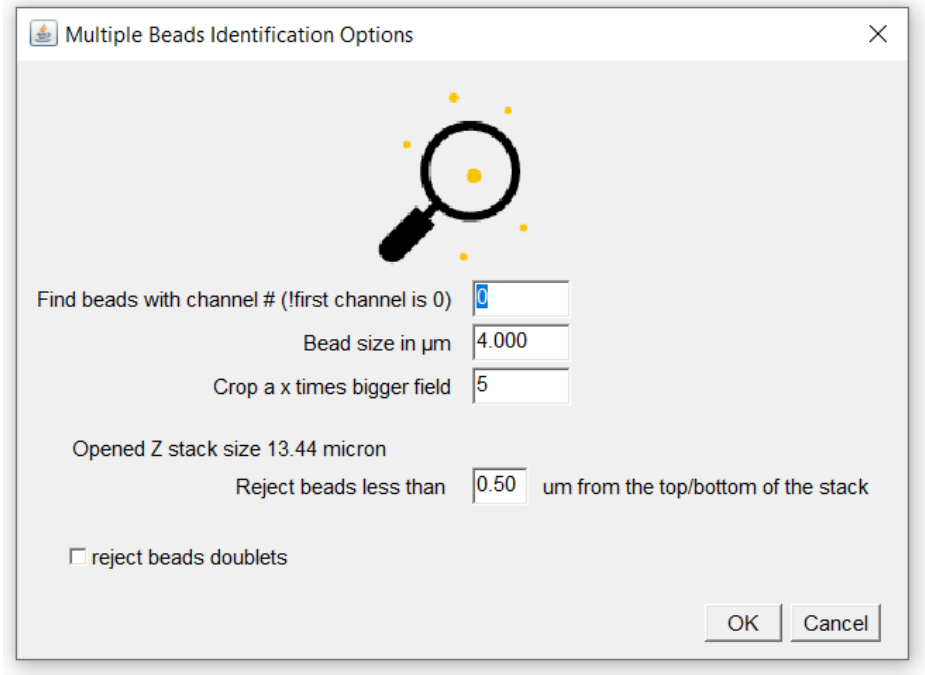


Figure 58. MetroloJ-QC multiple beads identification options (Co-Registration case).

STEP12. For cropping, users are prompted to enter the bead size (also used for bead detection at STEP11) and the crop factor. For example, a bead size of 4 µm and a crop factor of 5 will result in a centered square ROI with a size of 4 * 5 = 20 µm. If this ROI size is smaller than the background annulus plus 10%, the actual ROI size will be defined by the annulus plus 10% (see Figure 23).

As further co-registration analysis may be either polluted by close beads or erroneous because some information was lost as the bead was too close to the image's edges, the identified beads list is filtered to remove unwanted cases. To reject beads that lie

too close to the top or bottom of the stack (Figure 59), enter the rejection distance ("reject beads less than xx um from top/bottom of the stack, see Figure 58).

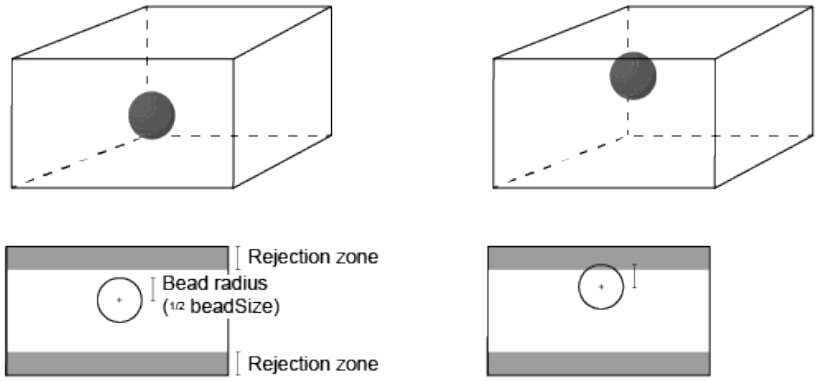
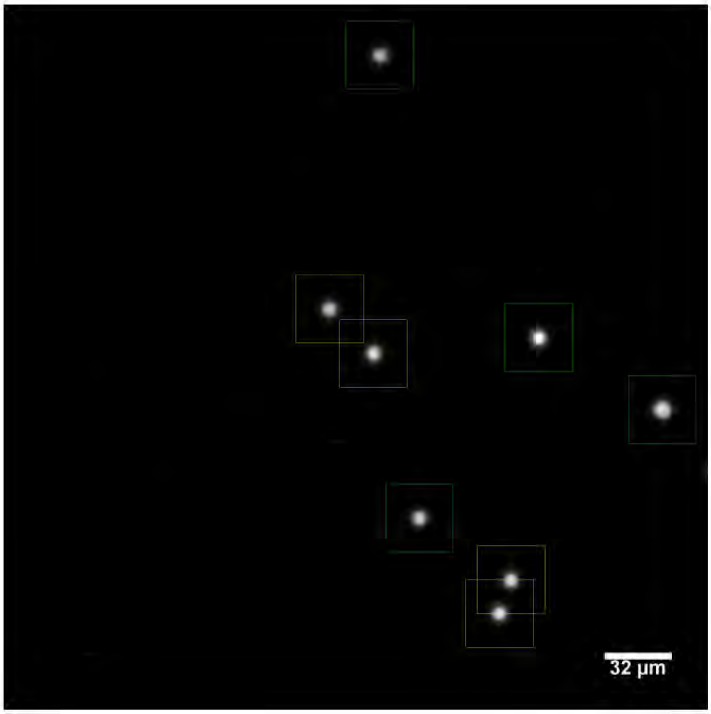


Figure 59. beads identified as being too close to the bottom or top of the Z stack are rejected. The edges of the beads, determined by their size and center position, must be outside the user-defined rejection zone (see left panel). Beads with edges falling within this rejection zone are rejected, even if their centers are outside the rejection zone (right panel).

The identified beads Overlay image is displayed in the summary pdf file (if the "save pdf report" option is selected) or saved as a jpg file if the save report images option is used. This image will highlight the selected beads (see Figure 60, green, the bead# is overlaid on the bead), beads removed as they were too close to the edge (Cyan) or too close from each other (yellow), or too close from the top/bottom of the stack (magenta). Finally, beads doublet can be excluded based on the circularity of the identified thresholded ROI. If the circ. value of the object is smaller than a minimal Circ. Value of 0.9, the bead is excluded. Note that this 0.9 value cannot be changed. Doublets will appear in white in the overlay image.



Green: valid bead

Yellow: the bead is too close from another one (ie. the distance between both centers is less than the ROI diagonal).

Cyan: the ROI touches an XY edge of the image

Magenta: the bead's center distance to the top/bottom of the stack is less than the rejection distance. Note the color correspond to the first rejection criterion met.

White : suspected doublet (none here)

Figure 60. Co-registration tool: bead identification overlay image. (note smaller PSF beads are not taken into account)

The original image is then cropped and generated single-bead containing images are processed as if they contained only a single bead (i.e., the option is not selected).
Click OK in the "multiple beads identification" window to go back to the original dialog.

STEP13. Set validation parameters. For the purpose of easy identification of within specs/outsides specs values, some Tolerance value may be applied to the final pdf report. When selected, the option "Apply tolerance to the final report" will highlight in green/red all combination ratio values above the value typed-in the field "Reject coalignment if ratio >".

STEP14. Set the file Save Options. Click the "File Save Option" to set the output options (Figure 61).

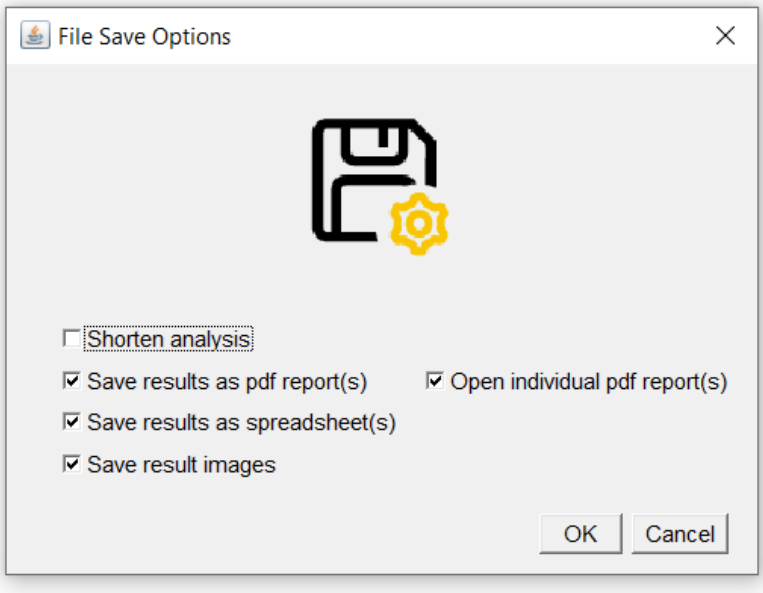


Figure 61. MetroloJ_QC : the file Save Options dialog

Set the file save options:

- Select the shorten analysis checkbox if you don't want to have pixel shifts, ISO21073 co-registration and calibrated/uncalibrated distances tables.
- Decide whether the results should be saved as a pdf file and whether the pdf should be automatically displayed (keep in mind if dozens of beads are to be analyzed, you may reach the pdf reader opened windows capacity).
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls file containing tabulation separated values of all tables of the pdf report.
- You may save the images of the beads combination sideviews (XY, YZ, XZ views) as jpg files as well.

All generated files are saved in a subfolder of a "processed" folder located in the same folder of the original image. The subfolder's name can be changed in the first "title" field of the dialog.

STEP14. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 62 (left panel) will appear. In this case, change the title.

MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP6) and the actual file format depth, a different type of error message is triggered (Figure 62, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

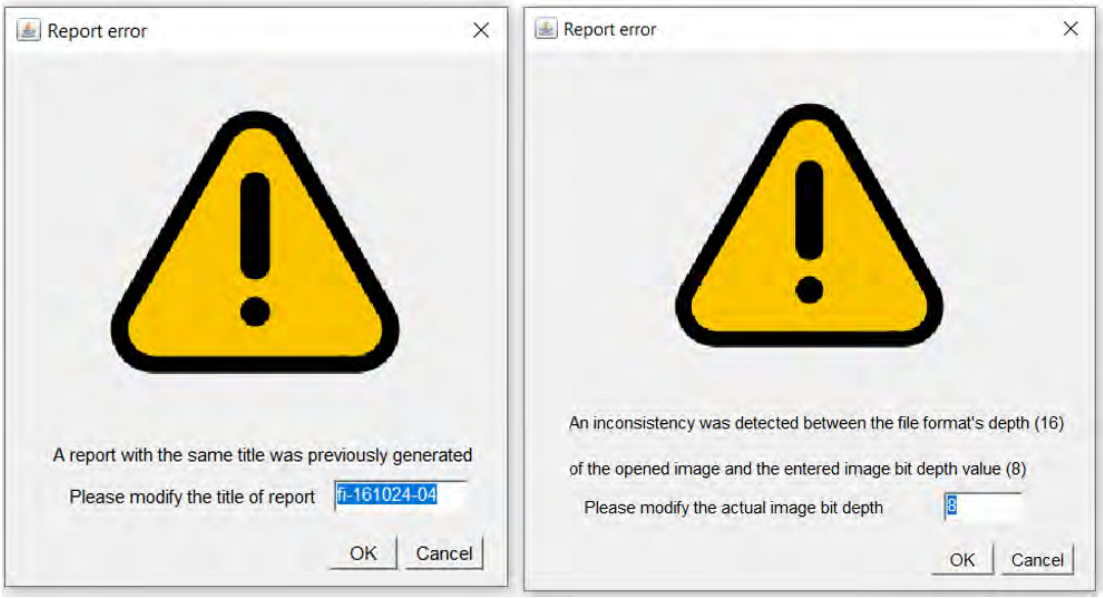


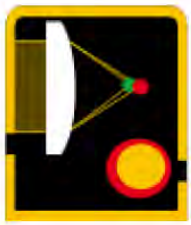
Figure 62. Error dialogs triggered by the co-registration tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved!

. The original image file location is used to create a “processed” folder. Files will be further saved within a “title” folder (as provided by the user in the first “title of report” field of the dialog box).

Description of the co-registration tool report.

The first sections of the co-registration tool report (Figure 63) is a summary of the image’s associated microscope info/parameters used to generate the coregistration report (or a bead image if the multibeads image option was selected in STEPS11-12). Shannon-Nyquist sampling distances are given using the formulas J to M. The Warnings section provides the users with some warnings that might be useful to interpret the report or compare it with previous results. Figure 63 is derived from a multiple beads-containing image, the beads coordinates within the original image are indicated in last row of the microscope table.



coa240924-01

Microscope info:

Image		40x-01-1						
image's creation	date	2024-09-02 17:09:10						
	method used	from file creation date						
Actual image depth		16						
Microscope type		WideField						
Objective	NA	1.4						
	im. refractive index	1.515						
Channel(s)		Wavelengths		Saturation	sampling (X,Y,Z)			
		Ex. (nm)	Em. (nm)		Nyquist (µm)	Found (µm)	Nyquist/found ratio	
Channel 0			480.0	0.5%	0.086x0.086x 0.256	0.163x0.163x 0.24	1.9, 1.9, 0.9	
Channel 1			525.0	none	0.094x0.094x 0.28		1.7, 1.7, 0.9	
Channel 2			590.0	none	0.105x0.105x 0.315		1.5, 1.5, 0.8	
Channel 3			610.0	none	0.109x0.109x 0.326		1.5, 1.5, 0.7	

Warnings:

The highlighted undersampled channels may alter the result interpretation.
(The bead size is appropriate for this coalignment analysis).

Figure 63. Co-registration tool : microscope info and warnings sections

For each channel combination, ratios of the measured center-to-center distances to reference distance ratio are displayed in the ratios table (Figure 64). If the “Apply Tolerances to the final report was selected”, values below/above the reference coregistration ratio are highlighted in green/red respectively. The bead center’s coordinates and theoretical resolutions (x, y and z for each channel and res_x^o, res_y^o and res_z^o for each channel) are also reported. These are the values used to compute the combinations reference distances used in formulas P to R. res_x^o, res_y^o and res_z^ovalues of the longest wavelength of the channel combination is chosen. The bead signal to background ratio is computed to help the user to discard aberrant values. Briefly, mean channel intensity values of the bead sections (as identified for saturation calculation purpose at STEP8) are computed (referred to as signal). Then annuli of x um are drawn around each bead section and the mean intensity of all annuli is calculated (referred to

as background). “x” can be changed at STEP8. A signal to background ratio is calculated. Mind this is in no way a measure of the SNR ratio.

Ratios table:

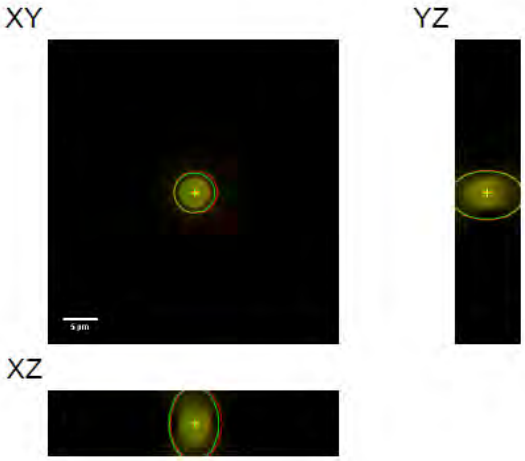
	Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0		0.915	0.613	0.721	0.721
Channel 1	0.915		0.34	0.38	0.38
Channel 2	0.613	0.34		0.17	0.17
Channel 3	0.721	0.38	0.17		0.0
Channel 4	0.721	0.38	0.17	0.0	
Resolutions (µm)	0.148 0.148 0.554	0.191 0.191 0.718	0.219 0.219 0.821	0.248 0.248 0.93	0.248 0.248 0.93
Bead centres'coord.(µm)	153.5 154.0 17.0	151.0 155.0 17.0	152.0 155.0 17.0	152.0 155.5 17.0	152.0 155.5 17.0
Bead quality (SB Ratio)	3.1	3.4	3.3	3.3	3.1
Title	C1-100x-02_bead1.tif	C2-100x-02_bead1.tif	C3-100x-02_bead1.tif	C4-100x-02_bead1.tif	C5-100x-02_bead1.tif

Green: within specifications, red: outside specifications (ie. ratio above 1.0)

Figure 64. Co-registration tool report: An example of the ratios table.

For each valid combination, Profile view images composed of three maximum intensity projections, XY, XZ and YZ (side views) are generated. Crosses indicate the respective position of the green channel (first channel using the stack order) and the red channel. Figure 65 compares channel 0 in green and Channel 1 in red. When using the Legacy fit-ellipse center detection method, the found green/red ellipses are drawn. If using the Centroïd detection method, the outlines of the thresholded bead area are drawn.

Profile view:



Channel 0 (Em. Wavelength 410.0 nm) vs channel 1 (Em. Wavelength 450.0 nm)

Figure 65. Co-registration tool report: part of a profile view section (Fit-ellipse example)

Long versions of the report include an ISO21073 co-registration accuracy table (Figure 66). These metrics are defined in the ISO21073 norm. The lateral $\Delta_{x,y}$ and axial Δ_z co-registration accuracy between channel i and j are given using formula S & T and the coordinates of the center of the bead in channel i (x_i, y_i, z_i) and channel j (x_j, y_j, z_j).

$$\Delta_{x,y} = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2} \quad \text{(S)}$$

$$\Delta_{x,y} = \sqrt{(z_i - z_j)^2} \quad \text{(T)}$$

ISO 21073 co-registration accuracy:

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		Lateral: 0.363 Axial:0.36	Lateral: 0.438 Axial:0.12	Lateral: 0.514 Axial:0.12
Channel 1	Lateral: 0.363 Axial:0.36		Lateral: 0.081 Axial:0.24	Lateral: 0.163 Axial:0.24
Channel 2	Lateral: 0.438 Axial:0.12	Lateral: 0.081 Axial:0.24		Lateral: 0.081 Axial:0.0
Channel 3	Lateral: 0.514 Axial:0.12	Lateral: 0.163 Axial:0.24	Lateral: 0.081 Axial:0.0	
Resolutions (µm)	0.176 0.176 0.313	0.213 0.213 0.377	0.244 0.244 0.434	0.278 0.278 0.493
Centres'coord.(µm)	10.0 10.2 6.7	9.8 10.5 6.4	9.8 10.6 6.6	9.8 10.6 6.6
Title	C1-40x-01_bead1.tif	C2-40x-01_bead1.tif	C3-40x-01_bead1.tif	C4-40x-01_bead1.tif

Figure 66. Co-registration tool report: an example of the ISO21073 co-registration accuracy table (long version of the report only).

Long versions of the report include a Pixel shift table (Figure 67). Each row shows how much pixels separate one channel from the reference along x, y and z axis. For instance, X shift of -2.5 between channel 0 (row) and channel 1 (column) is the difference between the X coordinate of the bead center in Channel 1 (151) and the X coordinate of the bead center in Channel 0 (153.5). This information might be useful to compensate for chromatic aberration using image processing software (provided the shift is the same w/ bead position within the field of view). On each column, resolutions (as expressed in pixels) and center's coordinates (in pixels) are given for the reference channel.

Long versions also contain Distance tables (uncalibrated & calibrated) (Figure 68). The table displays distances calculated between the centers of both channels for all channel combinations.

Pixel shift table:

		Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	X shift		-0.029	-0.704	-0.704	-0.995
	Y shift		0.591	-0.28	1.339	1.339
	Z shift		-1.449	-2.302	-3.095	-3.78
Channel 1	X shift	0.029		-0.675	-0.675	-0.966
	Y shift	-0.591		-0.872	0.747	0.747
	Z shift	1.449		-0.853	-1.646	-2.331
Channel 2	X shift	0.704	0.675		0.0	-0.291
	Y shift	0.28	0.872		1.619	1.619
	Z shift	2.302	0.853		-0.792	-1.478
Channel 3	X shift	0.704	0.675	0.0		-0.291
	Y shift	-1.339	-0.747	-1.619		0.0
	Z shift	3.095	1.646	0.792		-0.685
Channel 4	X shift	0.995	0.966	0.291	0.291	
	Y shift	-1.339	-0.747	-1.619	0.0	
	Z shift	3.78	2.331	1.478	0.685	
X, Y & Z theoretical resolutions (in pix.)		1.695, 1.695 & 3.284	1.854, 1.854 & 3.591	2.048, 2.048 & 3.968	2.154, 2.154 & 4.173	2.401, 2.401 & 4.652
Centres'coord. (X, Y & Z)		24.7, 24.7 & 29.1	24.7, 25.3 & 27.7	24.0, 24.4 & 26.8	24.0, 26.0 & 26.0	23.7, 26.0 & 25.3
Title		C1-63x-02_bead5.tif	C2-63x-02_bead5.tif	C3-63x-02_bead5.tif	C4-63x-02_bead5.tif	C5-63x-02_bead5.tif

Figure 67. Co-registration tool report: pixel shift table (long versions only).

Distances table (uncalibrated):

	Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0		1.565	2.424	3.444	4.131
Channel 1	1.565		1.394	1.93	2.632
Channel 2	2.424	1.394		1.803	2.211
Channel 3	3.444	1.93	1.803		0.744
Channel 4	4.131	2.632	2.211	0.744	
X, Y & Z resolutions (in pix.)	1.695, 1.695 & 3.284	1.854, 1.854 & 3.591	2.048, 2.048 & 3.968	2.154, 2.154 & 4.173	2.401, 2.401 & 4.652
Centres'coord.(X, Y & Z in pix.)	24.7, 24.7 & 29.1	24.7, 25.3 & 27.7	24.0, 24.4 & 26.8	24.0, 26.0 & 26.0	23.7, 26.0 & 25.3
Title	C1-63x-02_bead5.tif	C2-63x-02_bead5.tif	C3-63x-02_bead5.tif	C4-63x-02_bead5.tif	C5-63x-02_bead5.tif

Distances table (calibrated):

		Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	Measured distance in µm)		0.296	0.467	0.638	0.775
	Reference distance in µm)		0.576	0.614	0.538	0.56
Channel 1	Measured distance in µm)	0.296		0.205	0.345	0.483
	Reference distance in µm)	0.576		0.32	0.486	0.522
Channel 2	Measured distance in µm)	0.467	0.205		0.23	0.341
	Reference distance in µm)	0.614	0.32		0.256	0.348
Channel 3	Measured distance in µm)	0.638	0.345	0.23		0.14
	Reference distance in µm)	0.538	0.486	0.256		0.568
Channel 4	Measured distance in µm)	0.775	0.483	0.341	0.14	
	Reference distance in µm)	0.56	0.522	0.348	0.568	
X, Y & Z resolutions (in µm)		0.175, 0.175 & 0.657	0.191, 0.191 & 0.718	0.211, 0.211 & 0.794	0.222, 0.222 & 0.835	0.248, 0.248 & 0.93
Centres'coord.(X, Y & Z, in µm)		2.5, 2.5 & 5.8	2.5, 2.6 & 5.5	2.5, 2.5 & 5.4	2.5, 2.7 & 5.2	2.4, 2.7 & 5.1
Title		C1-63x-02_bead5.tif	C2-63x-02_bead5.tif	C3-63x-02_bead5.tif	C4-63x-02_bead5.tif	C5-63x-02_bead5.tif

Figure 68. Coregistration tool report: uncalibrated/calibrated distances tables (long versions only)

Finally, if any, user-provided Sample info & Comments are reported in the final page of the report, as are reported the user-defined analysis parameters used to generate the report (Figure 69), a log file that indicates how the input image was handled (Figure 70) and the formulas used (Figure 71).

Analysis parameters

Tool & Operator	Tool	Co-registration
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	Julien, 24 septembre 2024 10:43
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Coalignement\40X\Processed\coa240924-02\
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Beads	Bead detection threshold	Legacy
	Center detection method	Centroid
	Discard bead if more than one particle are thresholded	true
	Background annulus thickness in µm	1.0
	Background annulus distance to bead edges in µm	0.5
	Multiple beads in image	false
Tolerance	Applied in this report	true
	Ratio valid if below	1.0

Figure 69. Co-registration tool report: analysis parameters table.

Analysis log

image name	creation date	saturation	sampling density	status
63x-single	2024-07-11 10:20:33	none	Ch.0,1,2,3 undersampled	analysed

Figure 70. Co-registration tool report: log table

Formulas used:

The reference distance is calculated as follows:

$$r_{ref} = \frac{res_x^o * res_y^o * res_z^o}{\sqrt{(res_y^o * res_z^o * cos\Phi * sin\Theta)^2 + (res_x^o * res_z^o * sin\Phi * sin\Theta)^2 + (res_x^o * res_y^o * cos\Theta)^2}}$$

$$\Phi = \arccos \frac{x_B - x_A}{\sqrt{(x_B - x_{A)}{}^2 + (y_B - y_{A)}{}^2}}$$

$$\Theta = \arccos \frac{z_B - z_A}{\sqrt{(x_B - x_{A)}{}^2 + (y_B - y_{A)}{}^2 + (z_B - z_{A)}{}^2}}$$

$res_{x,y}^o = \frac{0.51*\lambda_{em}}{NA}$ $res_z^o = \frac{\lambda_{em}}{n-\sqrt{n^2-NA^2}}$

x_A, y_A, z_A and x_B, y_B, z_B are the bead coordinates in channel A and B respectively, NA: numerical aperture, λ_{em}: emission wavelength, n: refractive index of the lens immersion & mounting media.

Lateral ($res_{x,y}^o$) and axial (res_z^o) theoretical resolution values used for widefield microscopes are calculated as defined in Wilhelm, S. Confocal Laser Scanning Microscopy, 2011.

Compliance with the Shannon-Nyquist criterion uses the following formulas for Shannon-Nyquist distances calculation:

$$\alpha = \arcsin(\frac{NA}{n})$$

$\Delta_{x,y} = \frac{\lambda_{em}}{4.NA}$ $\Delta_z = \frac{\lambda_{em}}{2.n.(1-cos(\alpha))}$

Figure 71. Co-registration tool: formulas used in the report.

When single bead images are selected, all data is saved in a processed/title subfolder (Figure 72, top panel). If selected, side views and profile plots (not in the short version of the report) for each channel and dimension are saved in a title_imageName_data subfolder (Figure 72, bottom panel). The .xls files of the tables of the pdf report are saved in an title_imagename_results.xls file within this subfolder.

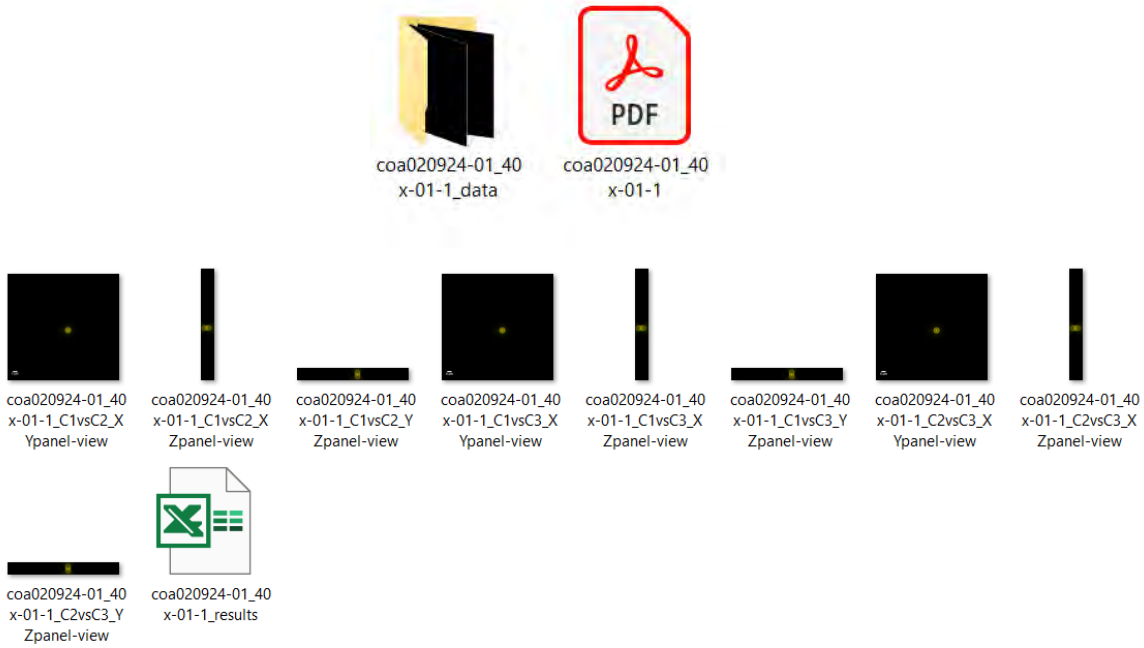


Figure 72. Coregistration tool: Files generated when save report images are saved (long version of the report).

When multiple bead images option is selected, files derived from each bead are saved in a processed/title/bead# (Figure 73). If the save pdf reports option is selected, a summary pdf document is generated (title_imageName_identifiedBeads.pdf, Figure 72) that contains the microscope info, the analysis parameters, a log file that summarizes how the beads were identified and analyzed (Figure 74). This latter figure highlights that saturation analysis is performed per bead: while the whole image is saturated for 2 channels, fully-unsaturated beads can be found. If the “save report images” option is selected, a “imagename_summary_beadsOverlay.jpg” file is also saved (Figure 75). If the “save all data in a spreadsheet” option is selected, the tables in the summary pdf document are saved title_imageName_beadData (Figure 73 and Figure 75).

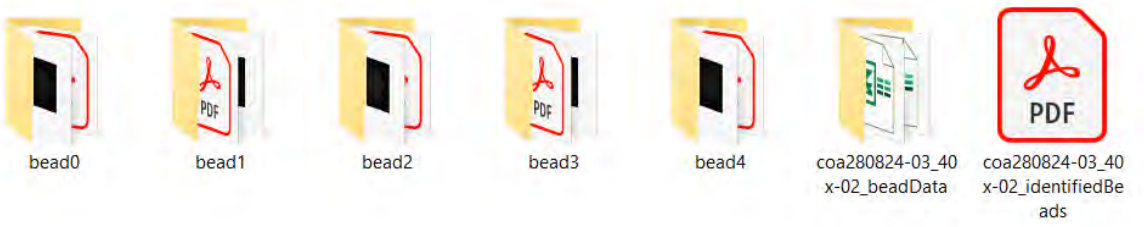


Figure 73. Coregistration tool: Files and folders generated when images contain multiple beads.

Analysis log

image name	creation date	sampling density	identified raw beads	valid beads	saturation	status
63x-01	2020-08-21 10:29:02	Ch.0,1,2,3 undersampled	6	4	Ch.1,3 saturated	valid beads found
				bead0	Ch.3 saturated	analysed
				bead1	Ch.3 saturated	analysed
				bead2	none	analysed
				bead3	none	analysed

Figure 74. Coregistration tool: The log table of the summary pdf file in multiple beads-containing image mode

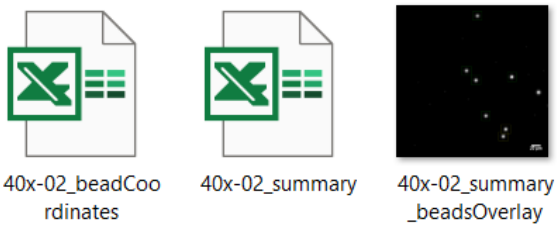
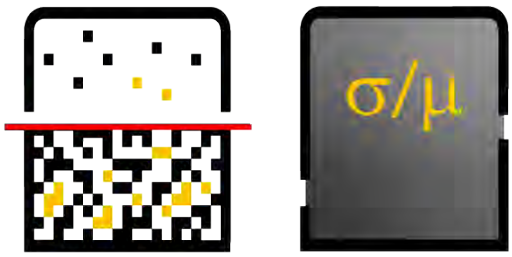
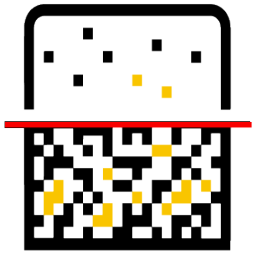


Figure 75. Coregistration tool: additional files generated in the multiple beads-containing images mode.



DETECTOR TOOLS

This series of tools is aimed at characterizing some of the detector's properties. The Camera Tool will allow measurements of noise and identification of abnormal pixels within a detector array (such as a sCMOS camera). The Variation Coefficient Tool indicates how the measurement of a same signal fluctuates. This is quite useful for single "point" detectors (such as HyD or PMTs).



Camera Tool

Accurate fluorescence quantification, such as in low-light challenging conditions, assumes readout noise is constant and evenly distributed. Noise specifications as well as abnormal pixel behavior (warm, cold or hot pixel) can be measured/identified on a regular basis to qualify an array detector (such as a CCD, EM-CCD or sCMOS camera).

Image acquisition

The user should refer to acquisition protocol P06B protocol of the GT3M WP of the RTmfm network.

The QC camera tool's algorithm.

The plugin is intended to:

- Measure noise-associated specifications (read noise, dark offset, DSNU)
- Locate and quantify warm, cold and hot pixels.

As for noise specifications measurements, the plugin will generate an average intensity projection image and a standard deviation of intensity projection image of a timelapse stack of dark images.
The average intensity projection image (averageProj) is used to calculate the offset and Dark Signal Noise Uniformity (DSNU):

- Offset is the mean average intensity across all pixels of the average intensity projection (ie. mean intensity of averageProj).
- The DSNU is the standard deviation of the mean intensity projection image, multiplied by the gain (e-/ADU). Hence, DSNU is the standard deviation to the mean of the averageProj image, corrected by the gain factor.

The standard deviation projection image (SDProj) is used for computation of the rms and median noises.

- the median noise is the median value of the standard deviation projection image (ie. median intensity of SDProj).
- For each pixel of SDProj of x and y coordinates, the intensity (Standard Deviation) is first multiplied by the gain to get the intensity in e- counts. The pixel intensity $SD_{x,y}$ is squared and the sum S of all pixels within the projection is calculated.

$$S = \sum_{x,y} SD_{x,y}^2 \text{ (U)}$$

The rms noise specification is derived using formula V.

$$rms = \sqrt{\frac{S}{\text{width x height}}} \text{ (V)}$$

Where width & height are the number of rows and columns of the standard deviation projection SDProj.

The same dataset can be used to monitor hot/cold pixels. For each time frame, the plugin will measure the mean intensity and look for pixel whose value is a percentage more (warm pixels) or less (cold pixels) than this mean “noise” value. This percentage is user-defined. Dead, maximum-intensity pixels (hot pixels) are also identified. Then, the average number of warm/cold/hot pixels per frame is computed. If along the dark timelapse a pixel was once reported as warm/cold/hot, then it will be considered as abnormal and will be part of a mask, that can be further used to correct for these abnormal pixels. Long version of the report finely analyses the frequency of abnormal behavior of each warm/cold/hot pixels of the mask.

Camera Tool parameters:

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).

STEP2. Open a file containing the single/multichannel t-stack. Check image>properties to make sure the frames are not considered as z slices.

STEP3. Click on the camera tool icon. The plugin's interface should appear (see Figure 76).

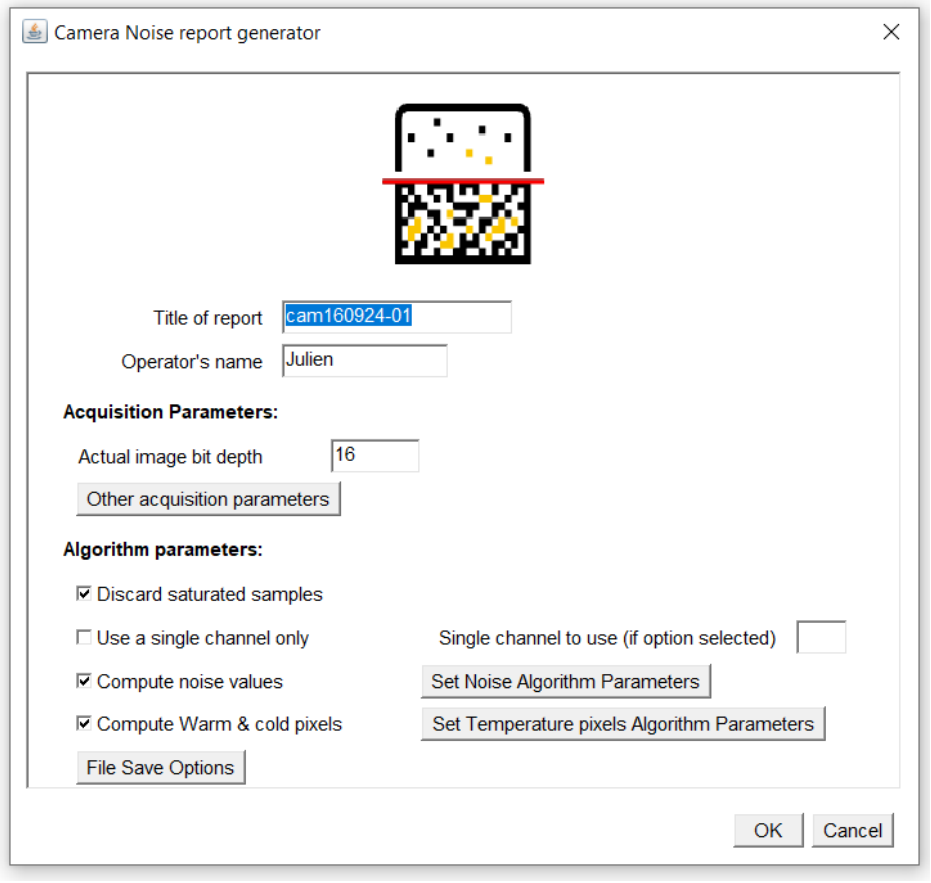


Figure 76. Camera Tool: the user's interface.

STEP4. Enter a title for the report. All generated data will be stored in a processed/title subfolder located in the same folder containing the original opened image.

STEP5. Select acquisition parameters. Enter the image's bit Depth. As 12 or 14bits images are genuinely saved as 16 bits files, for the sake of simplicity the user is requested to indicate the detector dynamic range in bits, rather than rely on more or less accurate metadata. This parameter is only used to monitor saturation (and discard any saturated image if the algorithm's option is selected. Click on the "other acquisition parameters" button. This displays the corresponding dialog (Figure 77). These parameters are only here for the purpose of tracing. Enter the detector type. The plugin is intended to adapt to any type of microscope setup. Hence, multichannel images (as acquired with multicamera setups for instance) can be used. Input files are stacks containing time frames. Z-stacks can't be used (even though they were Z-T stacks for

instance). The user has some indications of how the stack is taken into account (below the detector type: in Figure 77, the input file has 2 channels, 1 Z slice and 100-time frames). Whenever more than one channels are detected, the camera names can be provided.
Click on OK to go back to the main dialog window.

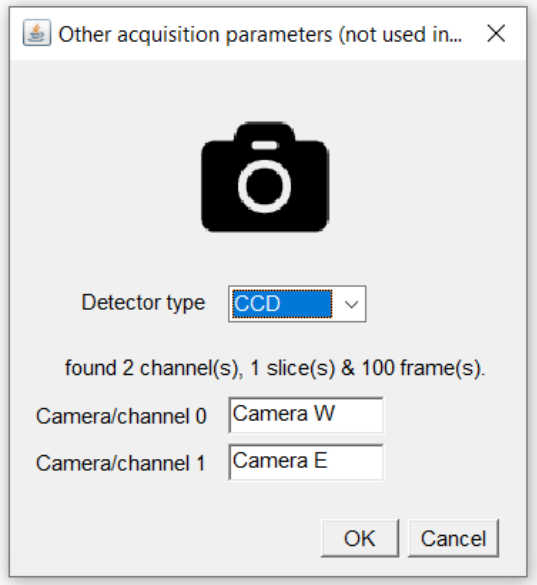


Figure 77. MetroloJ_QC: the detector dialog.

STEP6. Set the algorithm parameters. The user may discard saturated images from the analysis. However, this option is probably pointless as the dark images should not be saturated. Whenever hot pixels are looked for, the option is not taken into account (and hot pixels are identified).

STEP6. Multichannels files can be however used as a single channel input by clicking the “use a single channel only” and entering the channel number (please mind the first channel is #0, the second #1, etc.…).

STEP7. The next block are noise parameters. Tick the “Compute Noise Values” option to activate the noise-related tools (such as offset, DSNU and median noise calculation). Click the “set noise algorithm parameters”, this opens the corresponding dialog window (Figure 78).

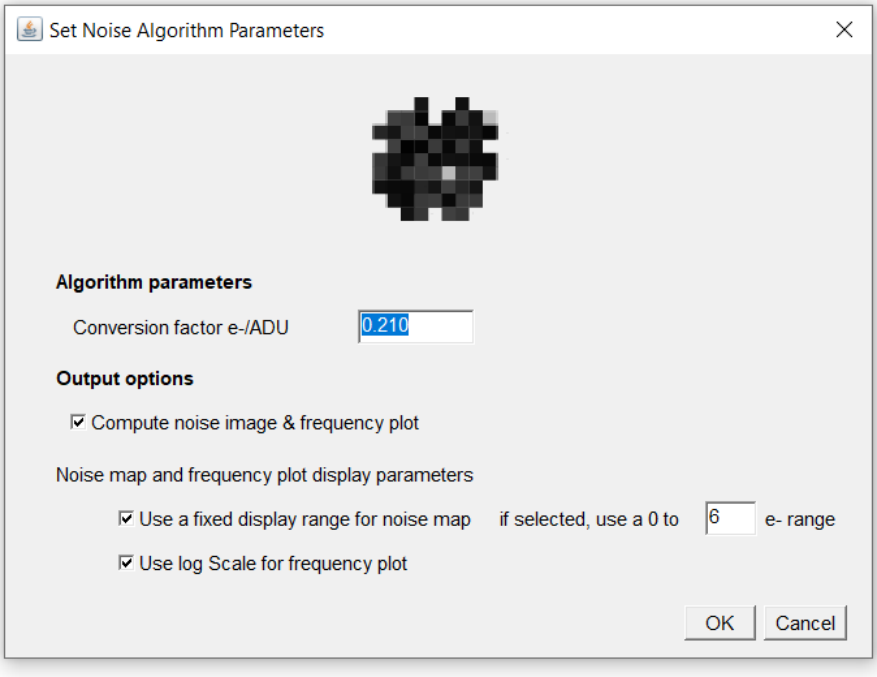


Figure 78. Camera tool: the noise parameters.

For the purpose of conversion from ADU to e-, the conversion factor (that can be retrieved from the detector's datasheet) has to be set. Note that for setups with two cameras, the cameras should be of the same brand and model (i.e., same conversion factor).
Noise specs can be derived from minimal exposure closed-shutter images. The "noise" distribution across the image(aka standard deviation of each pixel intensity) can be analyzed and frequency plots generated. For advanced noise analysis, select the "Compute noise Image and Frequency Plot" option. The noise map (calibrated standard deviation projection) is displayed using a Fire LUT. Default display uses min and max calibrated standard deviation values found in the noise Map. If a few pixels show an abnormal high standard deviation, it may be useful to restrict the noise map dynamic range to lower values. Hence, to ease the analysis, it is recommended to select the "Use a fixed display range for noise Map" and set the maximum displayed value accordingly (a value of 6e- is suggested).
The noise frequency plot can be displayed using either a normal scale (read noise/count) or a logarithmic scale (read noise/log(count)). To choose the logarithmic display option for frequency plots, tick the "Use Log Scale for Frequency Plot" checkbox. Click on OK to go back to the main dialog window.

STEP8. Warm, cold, and hot pixels can be monitored using a long exposure time in dark conditions (e.g., 30 seconds with the shutter off). To compute warm, cold, or hot pixels, select the appropriate checkboxes. If the "Compute Warm and Cold Pixels" checkbox is ticked, click the "set temperature pixels algorithm parameters". This opens the corresponding window (Figure 79).

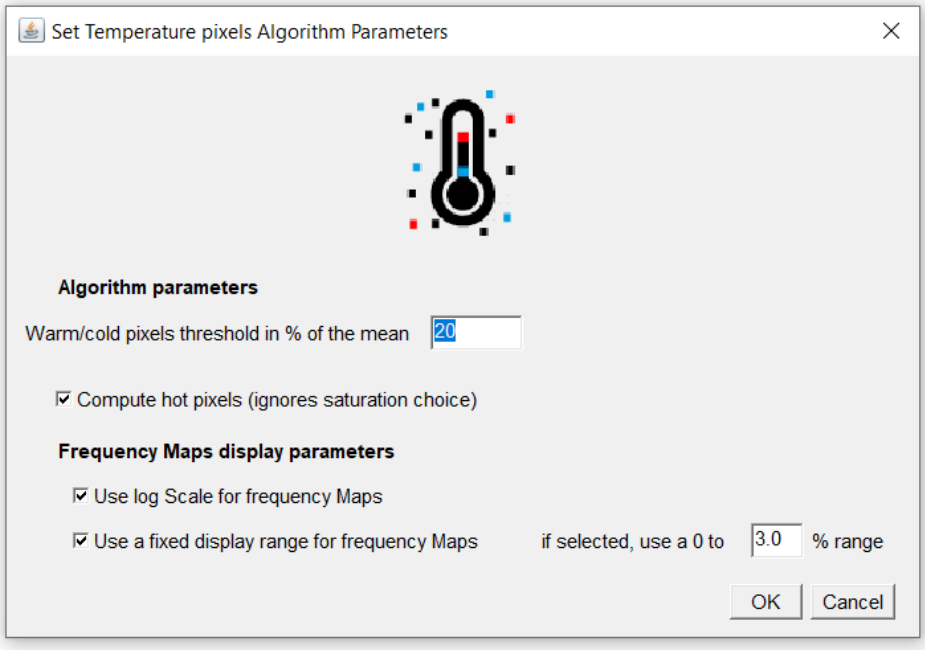


Figure 79. Camera tool: the temperature pixels parameters.

Enter the threshold value (thr) that defines warm and cold pixels. Warm pixels in a frame are those whose intensity is thr% or more above the average frame value, while cold pixels are those whose intensity is thr% or more below the average frame value.

Note that a pixel's behavior can vary across frames, so it can be useful to analyze how frequently a given pixel behaves as a warm pixel, for example. This analysis is available in the LONG version of the report.
Use the 3 Frequency Maps parameters to set the appropriate display:

- "Use Log Scale for Frequency Image" will display the image with a LogFire LUT (as if the frequency image was transformed into a log(frequency) image).
- The default display range is the min/max value of the frequency image. If most warm pixels occur in only, say, 3 out of 100 frames, but one extremely warm pixel appears in 39 out of 100 frames, the max value will be 39%. This could cause the display to lose pixels that are warm in just 3% of the frames. To see these, tick the "Use a Fixed Display Range for Frequency Maps" checkbox and set the "Use Range (in %) from 0 to" field to 3 to set the appropriate display.

Click on ok to go back to the main dialog.

STEP9. Set the file Save Options. Click the “File Save Option” to set the output options (Figure 80).

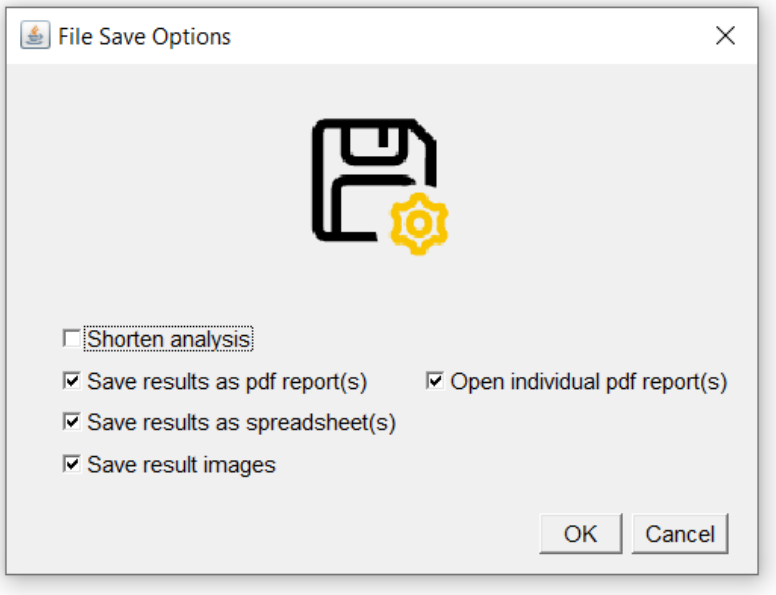


Figure 80. MetroloJ_QC : the file Save Options dialog

Set the file save options:

- Select the shorten analysis checkbox to skip the warm, cold & hot pixels distribution analysis and frequency maps (see below).
- Decide whether the results should be saved as a pdf file and whether the pdf should be automatically displayed.
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls file containing tabulation separated values of all tables of the pdf report.
- You may save the images warm/cold/hot .tif mask (if applies) for each channel.

All generated files are saved in a subfolder of a “processed” folder located in the same folder of the original image. The subfolder’s name can be changed in the first “title” field of the dialog.

STEP10. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 81 (left panel) will appear. In this case, change the title.
MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP5) and the actual file format depth, a different type of error message is triggered (Figure 81, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images

- 32-bits files format images are not declared as 32-bits images

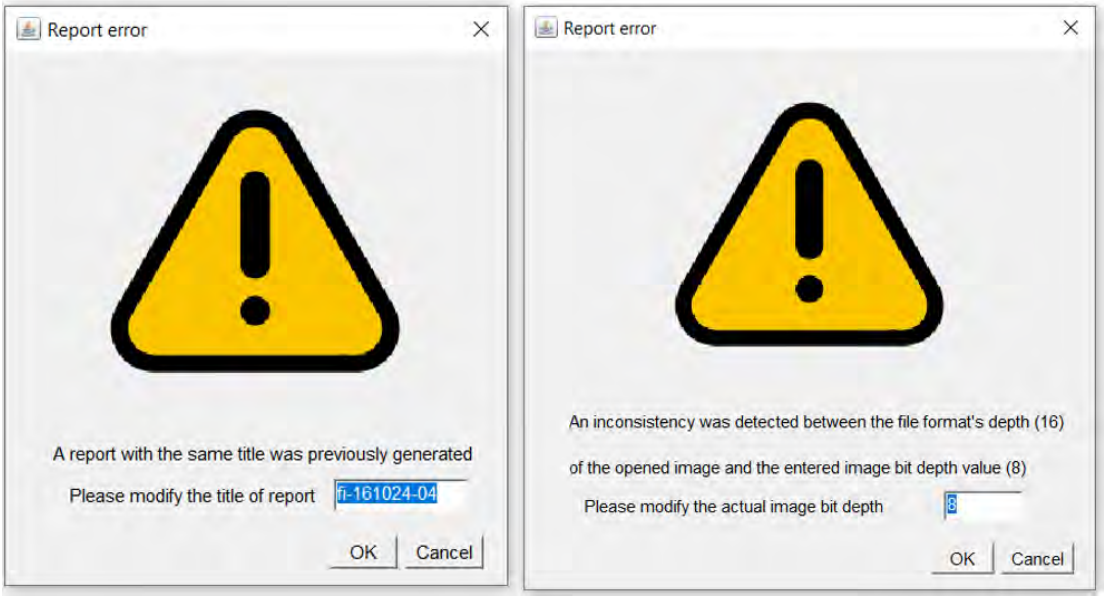


Figure 81. Error dialogs triggered by the camera tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved! Mind that warm/cold/hot pixels analysis is time-consuming (even though the plugin uses multiple threads). A typical 100 frame stack may be analyzed in 25 seconds. The original image file location is used to create a “processed” folder. Files will be further saved within a “title” folder (as provided by the user in the first “title of report” field of the dialog box).

Description of the camera tool report.

The Microscope info (Figure 82) is a summary of the image used to generate the camera tool report. The Warnings section provides the users with some warnings that might be useful to interpret the report or compare it with previous results.

cam18072024-16

Detectors info:

Image		temp
image's creation	date	2024-06-13 09:11:19
	method used	from file creation date
Detector type		CCD
Detector output bit depth		16
Conversion Factor (e-/ADU)		0.21
Saturation		none

Warnings:

(No saturated pixels detected).

Figure 82. Camera Tool report: the microscope info and warnings sections.

When the compute noise values option in STEP7 was selected, the next Offset and Noise Specifications table (Figure 83) contains all computed specifications. A noise map image is generated (Figure 84, top panel). The noise map image is the standard deviation projection of the t-stack where pixel intensity values (SD) is expressed in electrons. Whenever, for the sake of quick visual evaluation of noise across the detector, a fixed 0 to 6 e- dynamic range was chosen, a warning message is displayed (as in Figure 84). A frequency distribution plot of this projection is also displayed (Noise distribution, Figure 84, bottom panel).

Offset & Noise Specifications

Offset value (ADU)		98.4
Noise	rms (e-)	0.533
	median (e-)	0.374
	DSNU (e-)	0.115

Figure 83. Camera tool report: the Offset and Noise specifications section

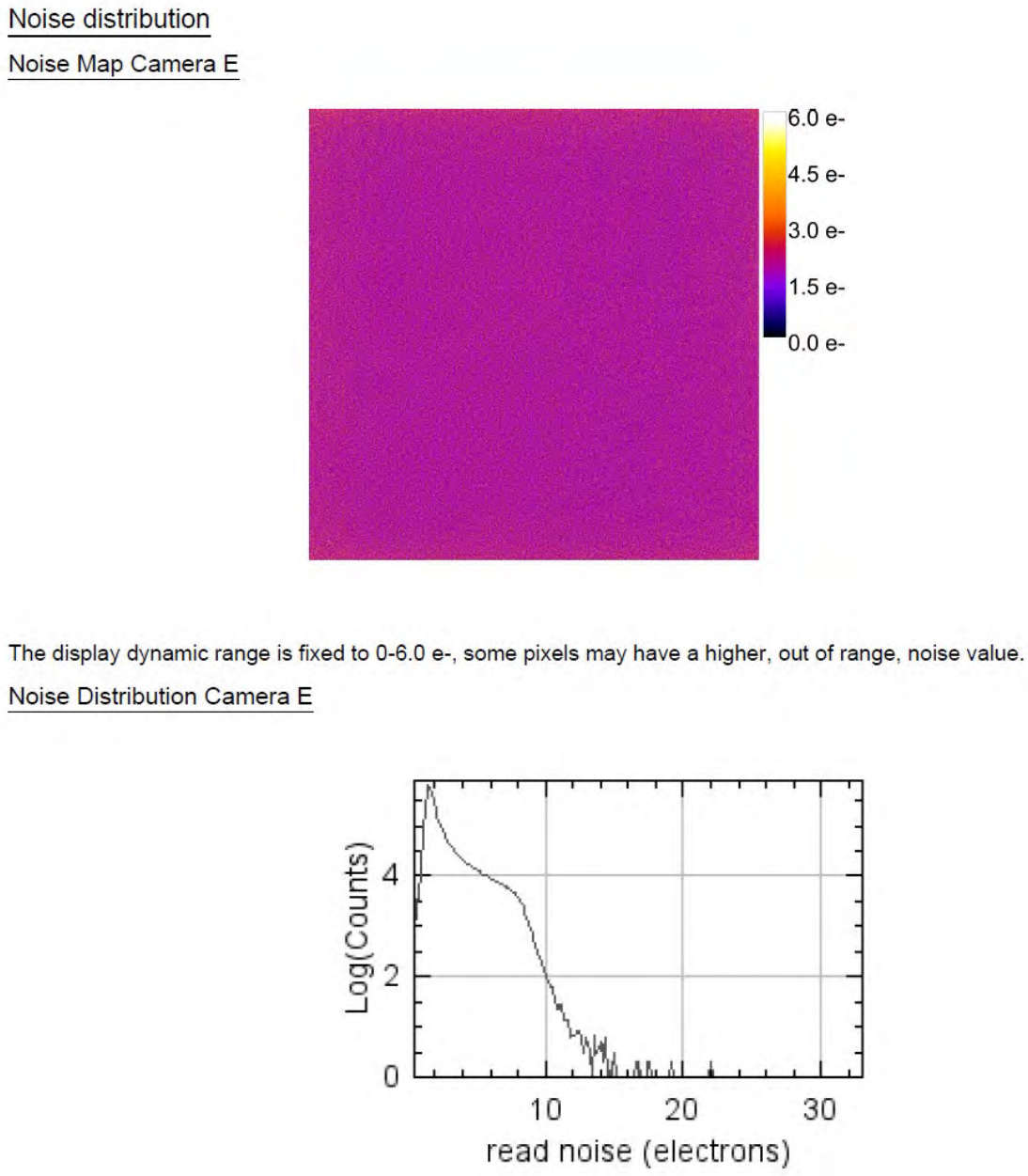


Figure 84. Camera tool report: the noise map and noise distribution plot

If chosen at STEP8, identification of warm, cold and hot pixels if any is performed. The Hot, Warm and Cold pixels summary table (Figure 85) indicates, for each type of abnormal pixels, the average number (across all timepoints) of abnormal pixels and the proportion (relative to the whole image). In Figure 85, the average number of warm pixels found across the frames is 1211.2 (which accounts for 0.029% of the 2048x2048= 4 194 304 pixels of the image.

Hot, warm & cold pixels

Summary

	Warm pixels		Cold pixels		Hot pixels	
	average number/frame	% total	average number/frame	% total	average number/frame	% total
Camera E	1211.2	0.029	261.1	0.006	0.0	0.0

Figure 85. Camera tool report: the abnormal pixels summary table.

Long versions of the report include further analyses. Since a given pixel may behave abnormally in only a few frames of the entire t-stack, it is useful to analyze abnormal behavior at the pixel level:

- Abnormal behavior frequency information: In the example shown in Figure 86, the 'warmest' pixel in the dataset appeared as 'warm' in 39 out of 100 frames. On average, pixels identified as 'warm' appear as such in 2.14 frames out of 100. The median number of frames where pixels are considered 'warm' is 2 across the entire warm frequency distribution, while most pixels are never warm (modal frequency of 0).

Camera E

Warm pixel behavior frequency summary:

Warm pixels Camera E	
Max. frequency	39.0/100 frames
Mean frequency	2.14/100 frames
Modal frequency	0.0/100 frames
Median frequency	2.0/100 frames

Figure 86. Camera Tool report: abnormal pixels frequency table.

- Warm/cold/hot pixels map (Figure 87): when relevant (i.e., some aberrant pixels have been found), a frequency Map is shown. It displays, for each pixel, the frequency (i.e., the number of frames a pixel is, say, warm divided by the total number of frames and multiplied by 100) of abnormal behavior. If the image has a large width/height, zoom in to see the warm/cold/hot pixels clearly. When the 'save images' option is selected, masks of the warm, cold, and hot pixels can be used to correct images and remove those aberrant pixels (e.g., using convolution)."

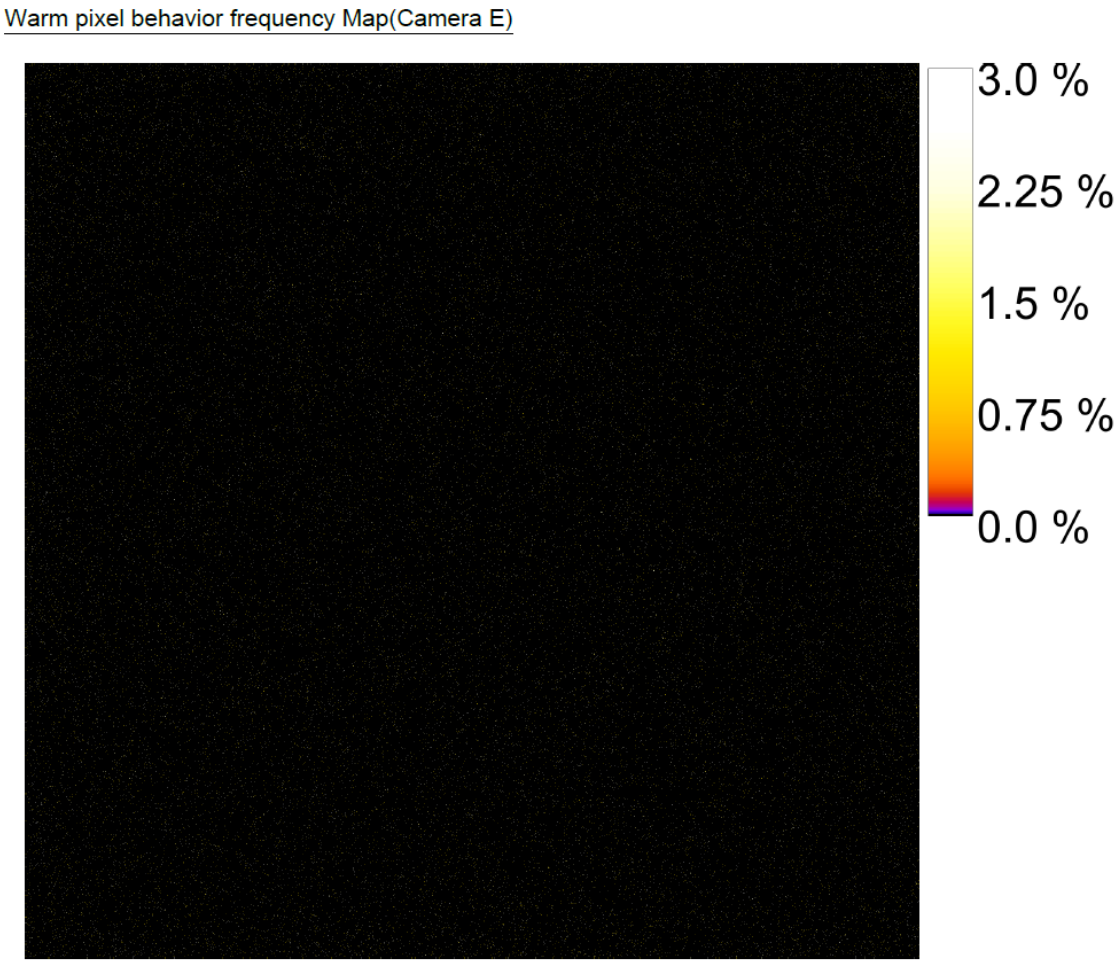


Figure 87. Camera Tool report: abnormal pixels frequency Map.

The final Analysis parameters section reports all parameters used for the analysis (Figure 88).

Analysis parameters

Tool & Operator	Tool	Camera Noise
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	, 7 août 2024 15:28
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Detecteurs\Processed\cam070824-73\tempFirstFrameSatCh1
	Type of saved data	.pdf, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Channels	Use one channel only	true
	channel used	1
Noise	Compute	true
	Create noise map and frequency histogram	true - log scale histogram - fixed range map
Warm, Cold & hot pixels	Compute Warm & Cold pixels	true
	Pixels are considered warm or cold if their intensity deviates by more than	20.0 % from the image's mean intensity.
	Compute Hot pixels	true
	Create frequencies maps	true - log LUT - fixed range map

Figure 88. Camera noise report: the plugin's parameters table.

A log file is also added, to trace how the file was handled (Figure 89).

Analysis log

image name	creation date	saturation	status
tempFirstFrameSatCh1	2024-08-07 14:19:23	none	analysed

Figure 89. Camera noise report: log table.

If requested, all additional data/images are saved in a "title_imageName_data" subfolder (Fig. 85, top panel). The .xls files of the tables of the pdf report are saved :

- noise_results.xls file contains the noise values.
- STDev_distribution.xls file contains the noise distribution plot values.
- Warm_Cold_Hot_results.xls contains the average frequency table (see Figure 85
- Warm_Cold_Hot_Frequencies.xls contains all frequency tables associated with frequency Maps (see an example in Figure 86).

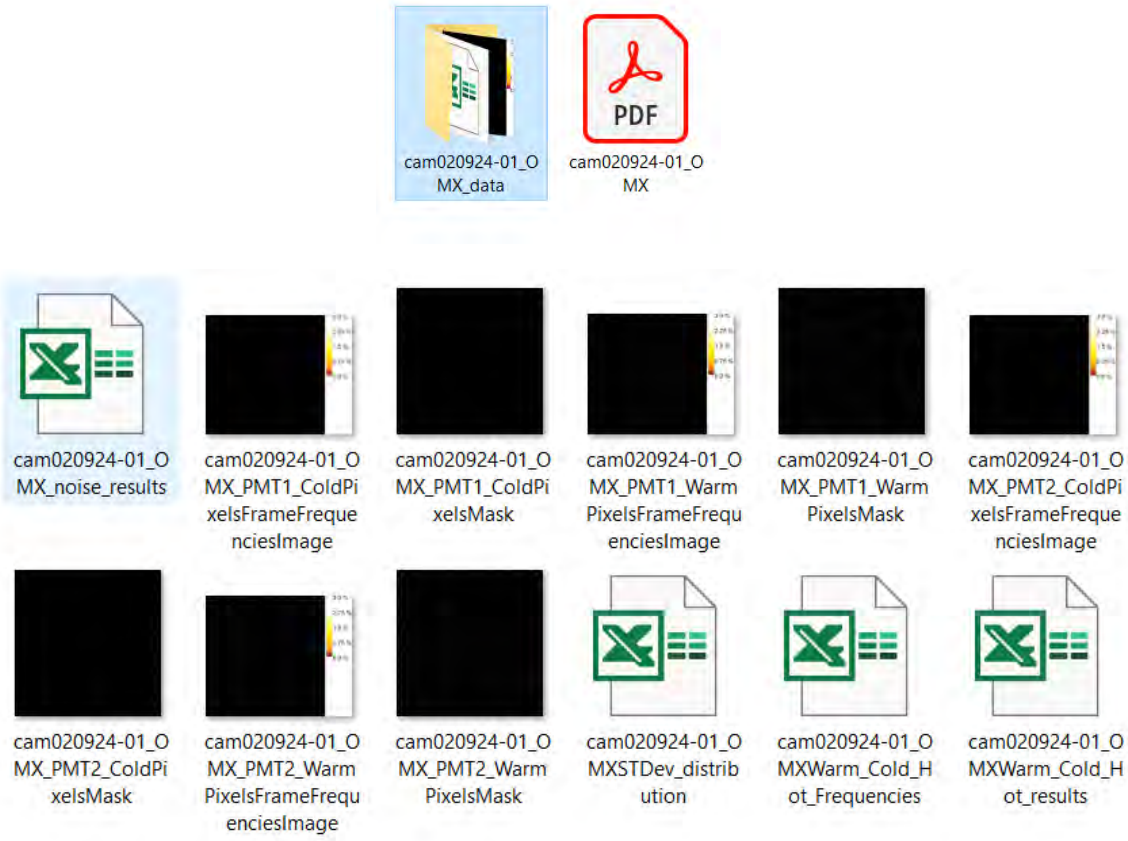


Figure 90. Files generated when save report images are saved (long version of the report).

Variation Coefficient Tool

Accurate fluorescence quantification using a single point detector assumes, for a shot-noise free given amount of received light, that the readout values are similar. The variation coefficient tool measures the measurement spread.

Samples and image acquisition parameters needed for Variation Coefficient Tool

To measure the Variation Coefficient of a detector, use any homogenous fluorescent slide (such as chroma slides). As indicated in the original MetroloJ manual, large diameter uniformly labelled beads might also be used and prepared as for the co-registration tool. The images should be close to saturation. It is not recommended to use (confocal) averaging as this is reducing the variations.

Variation Coefficient Tool parameters.

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).
STEP2. Open a file containing the single/multichannel images. Draw Region of Interest on the image and store them in the Roi Manager (hit the appropriate shortcut key or use edit>Selection>Add to Manager).
STEP3. Click on the Variation Coefficient tool icon. The plugin's interface should appear (see Figure 91).

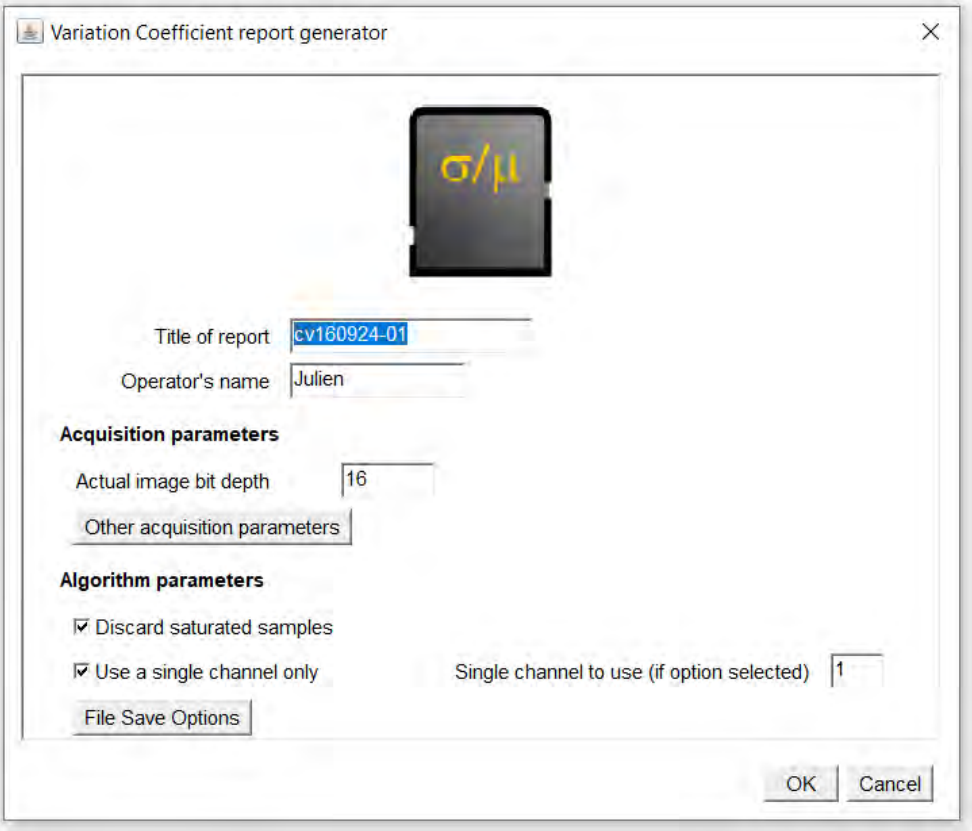


Figure 91. Variation Coefficient Tool: the user's interface.

STEP4. Enter a title for the report. All generated data will be stored in a processed/title subfolder located in the same folder containing the original opened image.

STEP5. Set the acquisition parameters. Enter the image's depth. This will be only used to define the maximum saturated intensity whenever the "Discard saturated samples" option is selected. Set additional acquisition parameters by clicking on the "Other acquisition parameters" button. This opens the corresponding detector dialog (Figure 92).

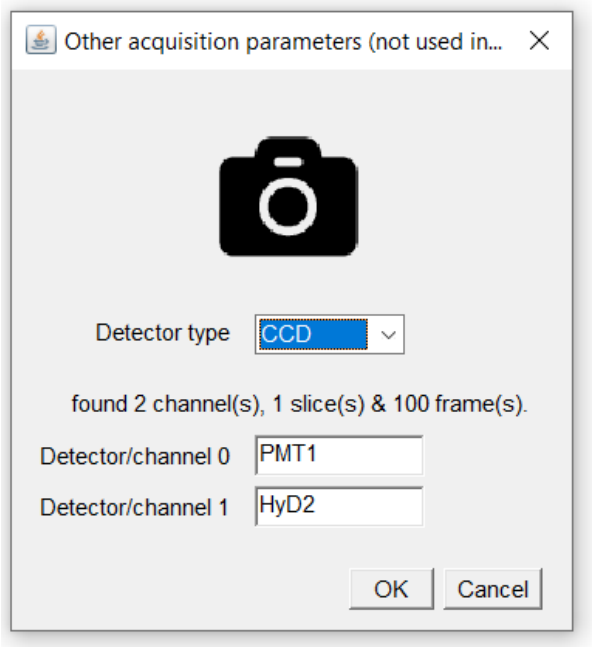


Figure 92. MetroloJ_QC: the detector dialog.

Set the detector(s) type. The file structure is analyzed and the number of channels identified. Fill-in the names of each channel/detector and click OK to go back to the main dialog.

STEP6. Since the Variation Coefficient of saturated ROIs will be biased, users have the option to exclude any saturated images. Please note that if any part of an image is saturated, the analysis will be halted, even if the user-defined ROIs are not saturated.

STEP7. Choose whether all channels/detectors should be analyzed or restrict the analysis to a given channel using the “use a single channel only”. Mind that the first channel of the dataset is #0.

STEP8. Set the file Save Options. Click the “File Save Option” to set the output options (Figure 93).

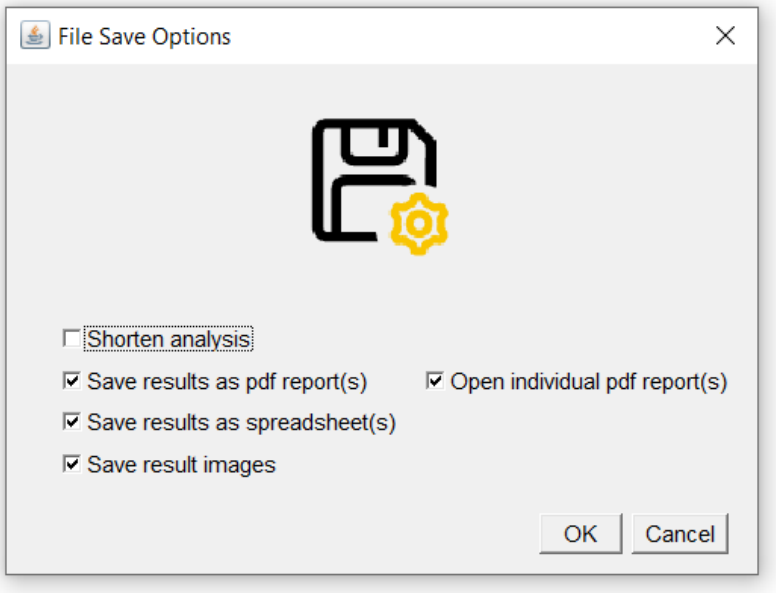


Figure 93. MetroloJ_QC : the file Save Options dialog

Set the file save options:

- Select the shorten analysis checkbox to skip the distribution histogram(s) generation.
- Decide whether the results should be saved as a pdf file and whether the pdf should be automatically displayed.
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls file containing tabulation separated values of all tables of the pdf report. LONG versions of the report also include the values of the distribution histogram(s). ROIs are saved in a zip folder.
- You may save the ROI-overlaid images channel montage as a .jpg file, as well as the distribution histogram(s) plots (LONG versions).

All generated files are saved in a subfolder of a “processed” folder located in the same folder of the original image. The subfolder’s name can be changed in the first “title” field of the dialog.

STEP9. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 94 (left panel) will appear. In this case, change the title.
MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP5) and the actual file format depth, a different type of error message is triggered (Figure 94, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images

- 32-bits files format images are not declared as 32-bits images

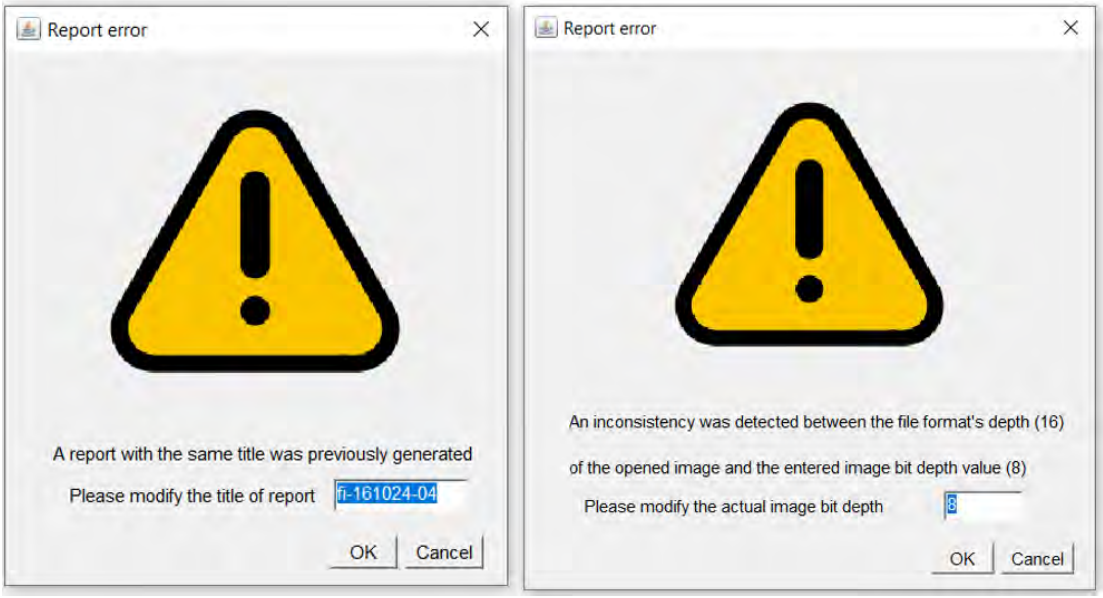


Figure 94. Error dialogs triggered by the Variation Coefficient tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved! The analysis can be quite time consuming. The original image file location is used to create a “processed” folder. Files will be further saved within a “title” folder (as provided by the user in the first “title of report” field of the dialog box).

Description of the Variation Coefficient Tool report

The report starts with information and warnings on the input image (Figure 95).

cv070824-01

Detectors info:

Image		CV
image's creation	date	2024-06-13 09:11:04
	method used	from file creation date
Detector type		CCD
Detector output bit depth		16
Saturation	Camera W	none
	Camera E	none

Figure 95. Variation Coefficient Tool report: microscope info and warnings sections.

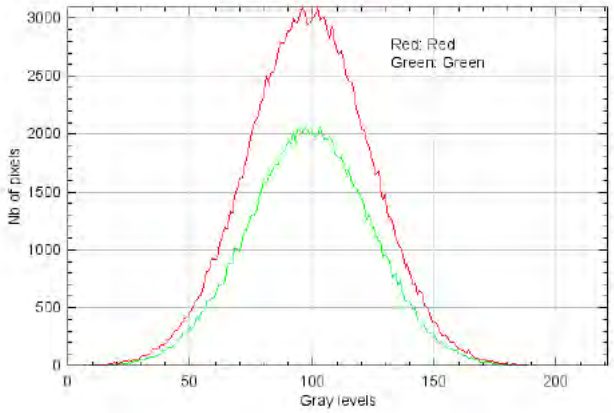
The next ROIs used for measurements section shows the location of each color-coded ROI (Figure 96).

ROIs used for measurements:

ROI	Upper Left corner's coordinates	Lower Right corner's coordinates
Red	(330, 222)	(720, 714)
Green	(870, 1074)	(1158, 1518)

Figure 96. Variation Coefficient Tool report: locations of ROIs used for the analysis

Then, the intensity distribution profiles for each channel/detector are displayed (Figure 97, top panel). A color code indicates the ROIs.



	Red	Green
Standard deviation	25.106	25.116
Average	98.666	98.745
Nb pixels	191880	127872
VC	0.254	0.254
VCs relative to min VC value	1.0	1.0

Figure 97. Variation Coefficient Tool report: the VC table and ROI histograms.

The Figure 97 bottom panel table displays:

- The standard deviation σ and average intensity μ of the channel intensity within each ROI (column).
- The variation coefficient VC is calculated using formula W.

$$VC = \frac{\sigma}{\mu} \text{ (W)}$$

- Whenever multiple ROIs are selected, the best (ie. lowest) measurements is compared to the VC values found in the other ROIs.

Finally, a summary table of all analysis parameters used for analysis is provided (Figure 98) together with a log table that traces how the file was handled.

Analysis parameters

Tool & Operator	Tool	Variation Coefficient
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	, 7 août 2024 15:58
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Detecteurs\Processed\cv070824-01\CV
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Channels	Use one channel only	false

Analysis log

image name	creation date	saturation	status
CV	2024-06-13 09:11:04	none	analysed

Figure 98. Variation Coefficient Tool report: the analysis parameters and log tables.

When the corresponding options have been selected, images and data can be saved individually (Fig. 93) in a title_imageName_data subfolder, together with the analyzed Regions Of Interest. The .xls version of the pdf report can be found in the _results.xls file. The coordinates of the intensity distribution histogram can be found in _histogram.xls file.

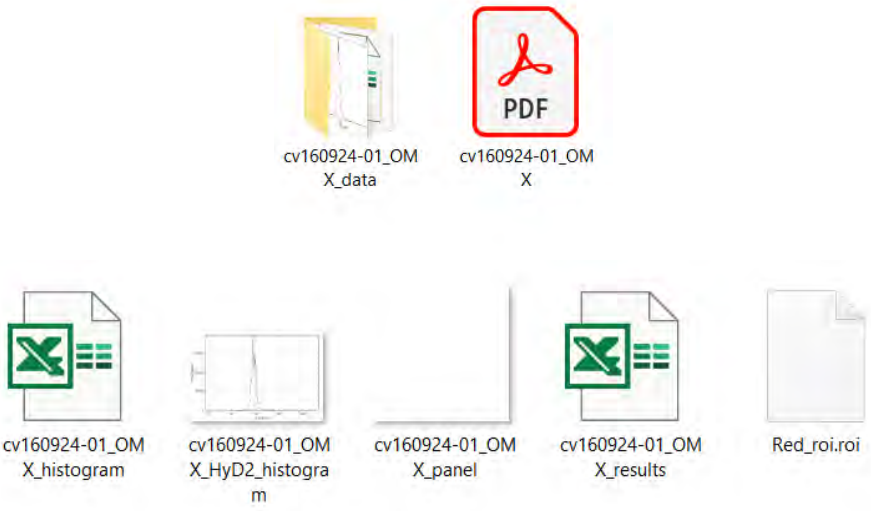


Figure 99. Variation Coefficient Tool report: the generated files..

Stage jitter & repositioning accuracy tool

Accurate motion analysis relies on maintaining a constant relative position between the stage and objective throughout the timelapse acquisition, with any potential stage drift kept to a minimum. When acquiring timelapses at multiple positions, precise stage repositioning is also crucial for ensuring

Image acquisition

The user should refer to acquisition protocol

The QC camera tool's algorithm.

The plugin processes bead images, which can be either single-channel 2D or 3D images captured over time. Each image may contain a single bead or multiple beads. Since the plugin detects beads using intensity thresholding, it's important that the bead diameter corresponds to the pixel size. It is recommended to use beads that span at least ~10 pixels.

The first frame is expected to contain at least one bead. Saturation is monitored in this initial frame (based on the assumption that if the first frame is not saturated, subsequent frames are unlikely to be saturated). The plugin does not calculate background intensity around the bead(s), as this process can be time-consuming.

If the image contains multiple beads, the plugin identifies them and assumes that the relative positions of the beads remain constant (i.e., the beads are firmly fixed in place, eliminating the need for tracking bead trajectories). Once identified, the input image is cropped into timelapses containing only a single bead, ensuring that one timelapse is not affected by another bead passing by. This is however checked at every timepoint and the algorithm makes sure one single particle gets thresholded.

Each single-bead timelapse is then analyzed for:

- The bead's XY(Z) coordinates at each timepoint
- Movement analysis, including mean drift velocity, axis movement, and mean squared displacement (MSD).

Drift Profiler Tool parameters.

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).
STEP2. Open a file containing the single channel 2 or 3D timelapse images.
STEP3. Click on the Drift profiler tool icon. The plugin's interface should appear (see Figure 100).

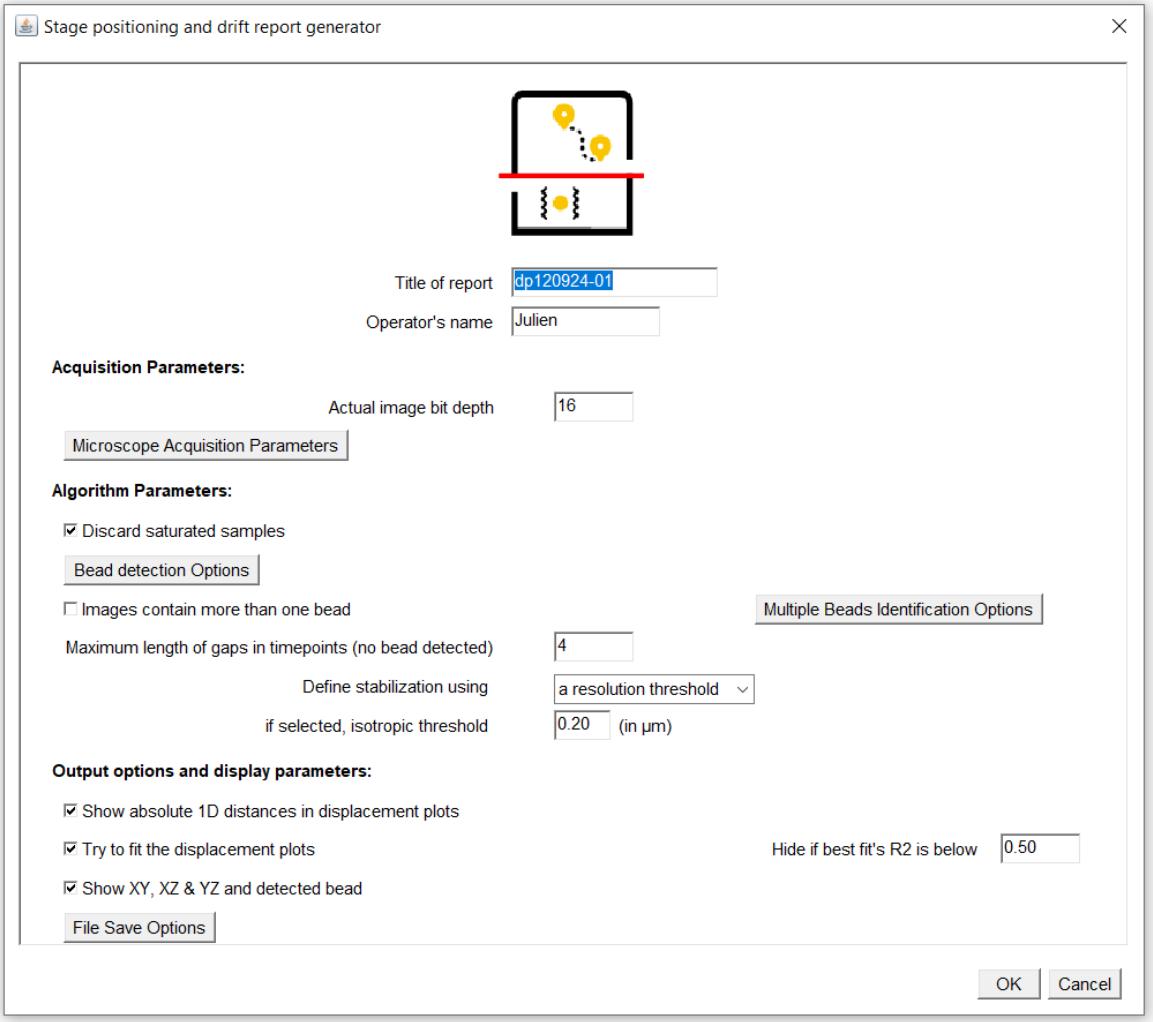


Figure 100. Drift Profiler Tool: the user's interface.

STEP4. Set acquisition parameters. Enter the image's depth. This parameter is only used whenever the "Discard saturated samples" is selected (and allows computation of the maximum, saturated, intensity). Set the microscope Acquisition Parameters by clicking on the dedicated button. This opens the corresponding dialog (Figure 101). This new dialog window is only important for the plugin for stabilization time calculation when the a "resolution (displacement) threshold is used, as these parameters will allow the calculation of the resolution and reference distances. Select the appropriate microscope type. If having a (C-)3D+t stack, each slice is expected to be a XY image

(ie. not a YZ or XZ image). If the 3D dimension order is wrong, the references values will be wrong.

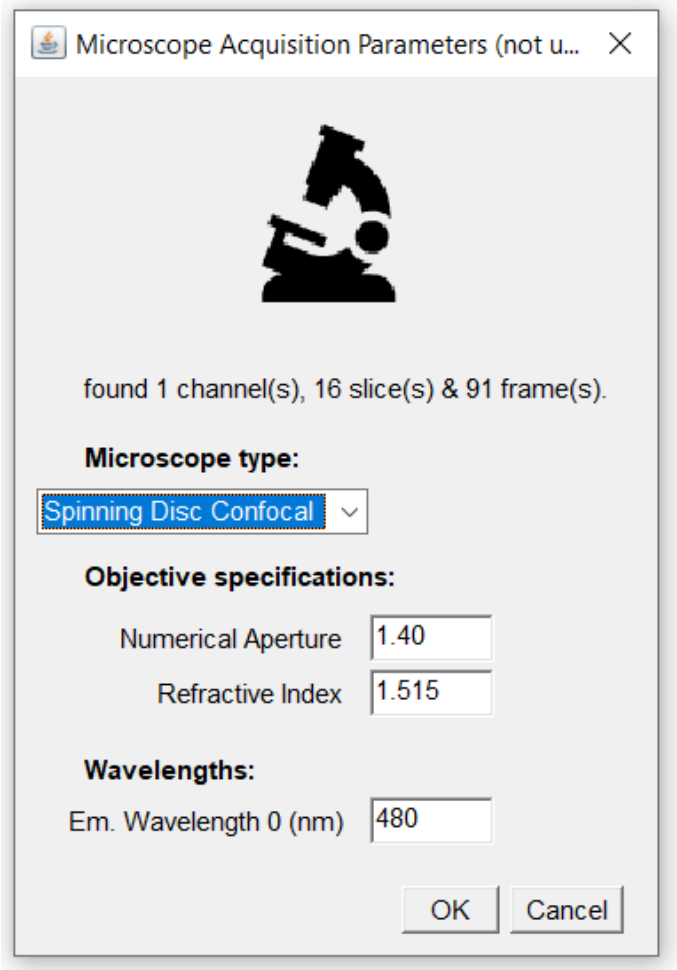


Figure 101. MetroloJ_QC: the microscope's interface (spinning disc case)

The user is prompted to fill-in some microscope information. Emission wavelengths and objective immersion medium refraction index & NA are used for theoretical lateral/axial resolution and reference distances formulas with widefield and spinning disc confocal set-ups (formulas F/F' and H/H'). Excitation wavelength, objective NA and immersion medium refractive medium are used for computation of these distances with single-point scanning confocal set-up and multiphoton microscope (formulas G/G' and I/I'/I"). Note that multiphoton resolution assumes a 2-photon excitation. The actual formulas used are further reminded in the pdf report. Click OK to go back to the main dialog window.

For the sake of traceability, some more sample information and/or comments might also be provided using the appropriate boxes (provided these fields are displayed, see the configuration section.

STEP5. Set the algorithm's parameters. As saturation alters the bead center detection, the user has the possibility to detect bead saturation. The user may discard saturated beads. Saturation is measured on the first frame of the original image (or the cropped image beads option when multiple beads option is used). Briefly, whatever the option status is, all slices are montaged in a single 2D image, a bead detection threshold is calculated and applied to highlight bead sections. Select the bead detection threshold with the rolling-menu in the "Bead detection parameters". See the "TESTS SECTION" to find the appropriate threshold. Then, the bead sections are masked and the proportion of saturated pixels within the mask is calculated. If the first frame detected bead is saturated and the option "discard saturated samples" is selected, the analysis stops. To set the bead detection threshold, click the "Bead Detection option button". This opens a new "bead detection option" (Figure 102) :

- Legacy Threshold: This method operates similarly to k-means clustering of the intensity histogram with a k-value of 2 classes. The 32-bits projection is converted into a 16-bit image, and the 16-bit histogram is displayed in log mode (intensity versus log count). Whenever some processing was applied to the 32-bits projection, the display is reset (in order to avoid clipping of the dynamic range). The histogram is divided into 2 classes: the first class ranges from the minimum value (min) to an initial midpoint, and the second class ranges from this midpoint to the maximum value (max). The midpoint is initially set to (max - min) / 2 and is adjusted through an iterative process. In each iteration, the previous limit is stored and compared to the new limit, then the mean values of the two classes are calculated. The new limit is set as the mean of these class means. The legacy algorithm performs 100 iterations, using the resulting limit as the threshold between the two classes.
- Built-in ImageJ Histogram Segmentation Algorithm: This method uses one of the built-in segmentation algorithms in ImageJ. The list of available algorithms can be modified in the "CONFIGURATION SECTION".
- "k-Means" Threshold: This method also uses k-Means clustering for segmentation but with a configurable k-value. Instead of two classes, it divides the histogram into k classes. The initial class widths are set to (max - min) / k. These limits are refined through an iterative process similar to the legacy threshold method. The intensities of the last (k^{th}) class are used to threshold the image. The k-value can be adjusted in the "CONFIGURATION SECTION".

You can test the accuracy of bead detection using the "TESTS SECTION".

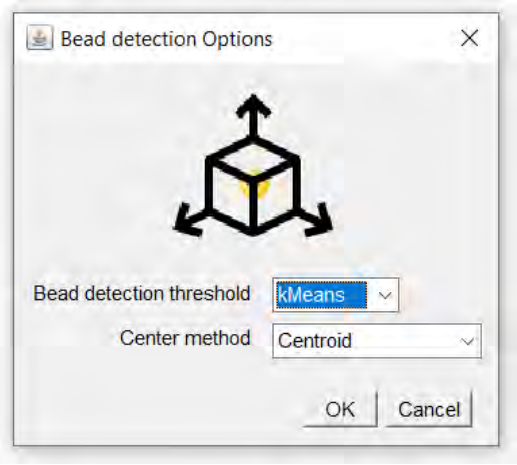


Figure 102. MetrologJ_QC : the bead detection Options dialog.

NB. The Drift Profiler tool does not measure bead signal/background ratio.

STEP6. Set the method for detecting the bead's center. To achieve this, XY, XZ, and YZ sum projections are computed and thresholded. The X and Y coordinates of the center are obtained from the XY projection. The Z coordinate is determined by averaging the Z coordinates identified from the XZ and YZ projections. Note that the original MetroloJ plugin uses only the Z coordinate derived from the XZ projection.

STEP6b. Set the center detection method :

- The "Legacy fit-ellipse" method takes the thresholded projection and fits an ellipse. This method is the original one, as implemented in MetroloJ and earlier versions of MetroloJ-QC. The corresponding X, Y coordinates are derived from the centers of the ellipses of the XY projection, while the Z coordinates is the average of the Z coordinates of the center of the ellipses defined using the XZ and YZ projections (note that the original MetroloJ plugin only uses the XZ projection). The method starts with an ellipse centered on the center of mass of the thresholded area. It continuously fits an ellipse to the object in the segmented image, refining the position of the ellipse's center until the minor axis of the ellipse is sufficiently large.

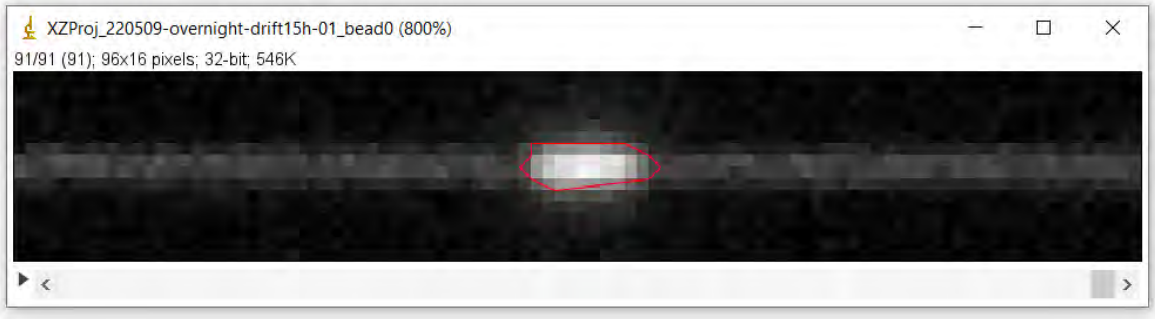


Figure 103. Potential fit-ellipses bias. When the bead is thresholded using some threshold (here legacy threshold) that selects all slices (top and medium-left panels for channel C2 and C3 respectively), the resulting ellipses (bottom-left panel green for C2 and red for C3) appear as cropped and the analysis

concludes that there is no misalignment between C2 and C3 (no Z pixel shift). When the thresholded areas doe not span the entire stack's depth (top-rignt panel for C2 and medium-right panel for C3), the ellipses are not cropped and the analysis concludes that channels are misaligned (0.5 pixel shift).

- The centroid method skips the process of fitting the ellipse and the bead's XY coordinates are the center of Mass (centroid) of the XY sum projection, while the bead's Z coordinate is the average of the Z coordinates of the XZ and YZ sum projections.

To make sure the bead positions are properly identified, it is recommended to select the output option "show XY, XZ & YZ views and detected bead". Provided the ROI is not selected, the outlines' color of the bead will change from blue to red across the frames. In cases the bead's outlines are poorly detected, the coordinates might be biased (see Figure 103). In this case, it is recommended to change the threshold method or switch to the centroid detection method.

Once all bead detection parameters are set, click ok to go back to the main dialog's window.

MULTIPLE BEADS IMAGES (STEPS 7 & 8)

STEP7. When using stacks containing multiple beads, users can select the "images contain more than one bead" option. The process of finding multiple beads within an image is referred to as "multiple bead identification" (while bead detection refers to finding single beads' coordinates). Click the "multiple beads identification parameters" button. The corresponding dialog window opens (Figure 104). Bead identification of X and Y coordinates is done on a Z sum intensity projection of the chosen channel. To aid in detection, background is removed using a rolling background algorithm (as found in Process > Subtract Background with a rolling ball radius of 50 pixels, leaving all options unselected). Any noise is eliminated with a Gaussian blur of 2 pixels (Process > Filter > Gaussian Blur). The selected bead detection threshold is then applied, and beads are identified using ImageJ's particle analysis tool. A filter is used to exclude objects with an area less than 50% or more than 400% of the expected bead area, based on the bead diameter field (see next step). Z coordinates are derived from Y sum intensity projection of the chosen channel (XZ image), processed like the Z sum projection that was used to get the X and Y coordinates. Vertical lines across each bead center are drawn and plotted. A gaussian fit is used to get the center of the bead.

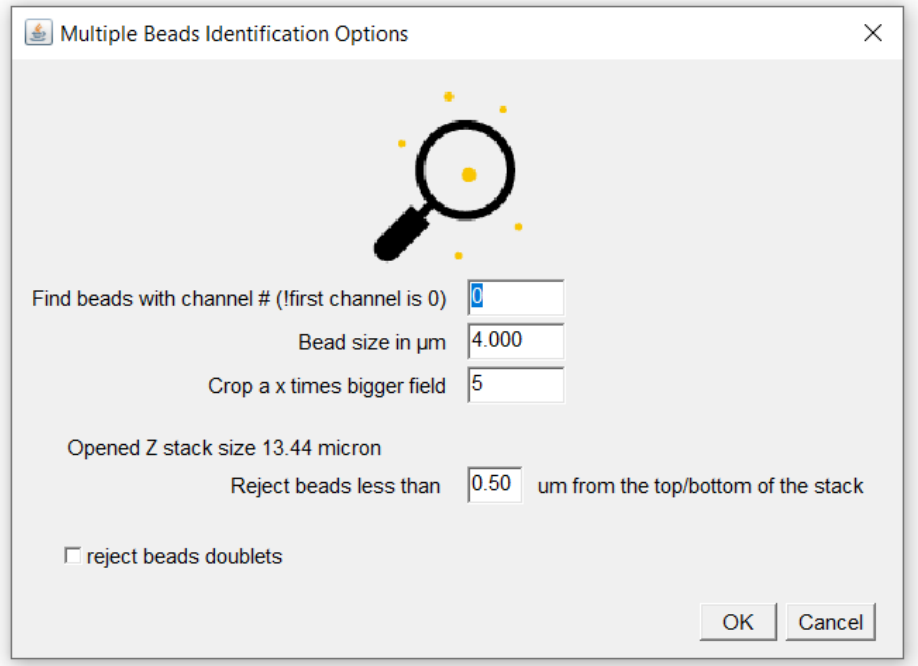


Figure 104. MetroloJ-QC multiple beads identification options (Co-Registration case).

STEP8. For cropping, users are prompted to enter the bead size (also used for bead detection at STEP7) and the crop factor. For example, a bead size of 4 µm and a crop factor of 5 will result in a centered square ROI with a size of 4 * 5 = 20 µm.

As further co-registration analysis may be either polluted by close beads or erroneous because some information was lost as the bead was too close to the image's edges, the identified beads list is filtered to remove unwanted cases. To reject beads that lie too close to the top or bottom of the stack (Figure 105), enter the rejection distance ("reject beads less than xx um from top/bottom of the stack, see Figure 104).

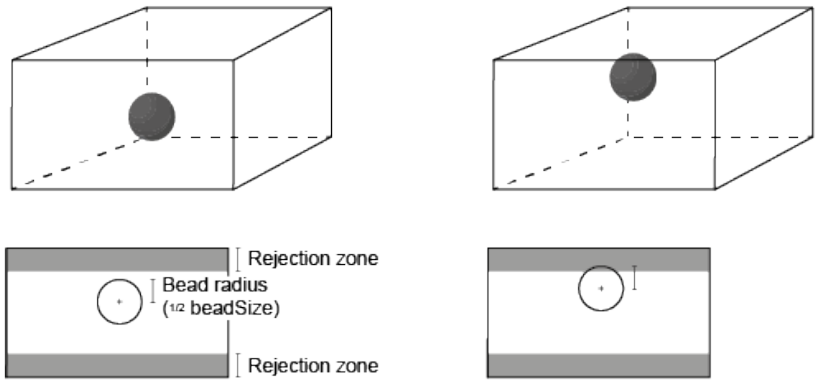
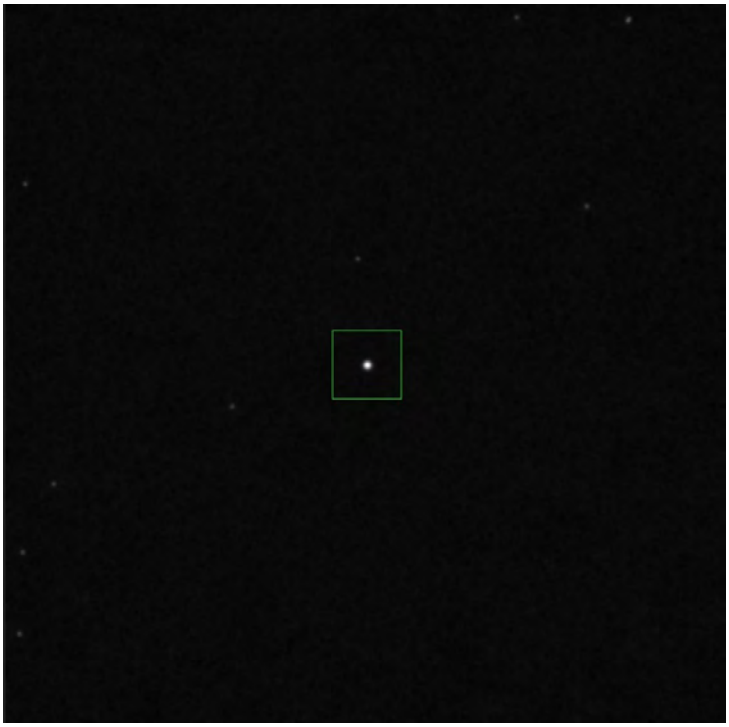


Figure 105. beads identified as being too close to the bottom or top of the Z stack are rejected. The edges of the beads, determined by their size and center position, must be outside the user-defined rejection zone

(see left panel). Beads with edges falling within this rejection zone are rejected, even if their centers are outside the rejection zone (right panel).

The identified beads Overlay image is displayed in the summary pdf file (if the “save pdf report” option is selected) or saved as a jpg file if the save report images option is used. This image will highlight the selected beads (see Figure 106, green, the bead# is overlaid on the bead), beads removed as they were too close to the edge (Cyan) or too close from each other (yellow), or too close from the top/bottom of the stack (magenta).



Green: valid bead

Yellow: the bead is too close from another one (ie. the distance between both centers is less than the ROI diagonal).

Cyan: the ROI touches an XY edge of the image

Magenta: the bead’s center distance to the top/bottom of the stack is less than the rejection distance. Note the color correspond to the first rejection criterion met.

Figure 106. Co-registration tool: bead identification overlay image. (note smaller PSF beads are not taken into account)

The original image is then cropped and generated single-bead containing images are processed as if they contained only a single bead (i.e., the option is not selected).
Click OK in the “multiple beads identification” window to go back to the original dialog.

NB. Even though the image contains a single bead, if the width of the field of view is much larger (>10x) than the bead's size, it is recommended to tick the multiple bead option, to lower the computation time.

STEP9. Set the other drift profiler algorithm's parameters :

- Gap Length refers to the maximum number of frames during which the bead's coordinates are missing. In each frame, the coordinates of the bead are determined, but if none are identified (for example, due to interference from another bead in the field of view, causing the calculation to fail), the coordinates will be marked as invalid (NaN). The algorithm allows these invalid coordinates to be corrected. The user can define how long such corrections should be applied. If the maximum gap length is set to 4, any gaps of fewer than 4 frames will be corrected by interpolating between the last known and next valid coordinates. However, if the gap exceeds 4 frames, the analysis of the timelapse will stop at that gap.
- To compute the (potential) stabilization time, stabilization has to be defined. If using "a resolution threshold", this means that stabilization is achieved in a given X, Y, Z direction whenever the remaining frames display a movement that is less than the lateral (X, Y) or axial (Z), resolution distance. For 3D displacement, stabilization will be achieved if the 3D distance is less than the reference distance (see Figure 107). Alternatively, choose "isotropic threshold" and set the threshold value. This will apply the same threshold in all direction (X, Y, Z and 3D).

STEP10. Set output options and display parameters :

- The output 1D displacements plots compute the overall distance between two timepoints. For instance, for the X dimension :

$$displacement_X(t) = x_t - x_{t-1}$$

  If $x_t < x_{t-1}$, the displacement will be negative. If selecting the "show absolute 1D distances in displacement plot", the absolute value of $displacement_X(t)$ will be displayed on the 1D-displacement plot and each individual 1D-X, Y and Z (if any) displacement plots.
- You have the option to fit the displacement plots (both 3D and individual 1D displacement plots). Select this option to fit the displacement between frame 1 and the last frame. The algorithm will test different fitting formulas (as provided by the CurveFitter plugin) and retain the one that gives the best R^2 value. To avoid displaying poor fits, make sure to set a sufficiently high R^2 threshold value.
- You may want to inspect the outlines of the detected beads in 2D (XY projection) and in 3D (if the input image is a 3D stack+t) using XZ and YZ projections. To enable this, select the "Show XY (XZ & YZ) projections and detected bead" option.

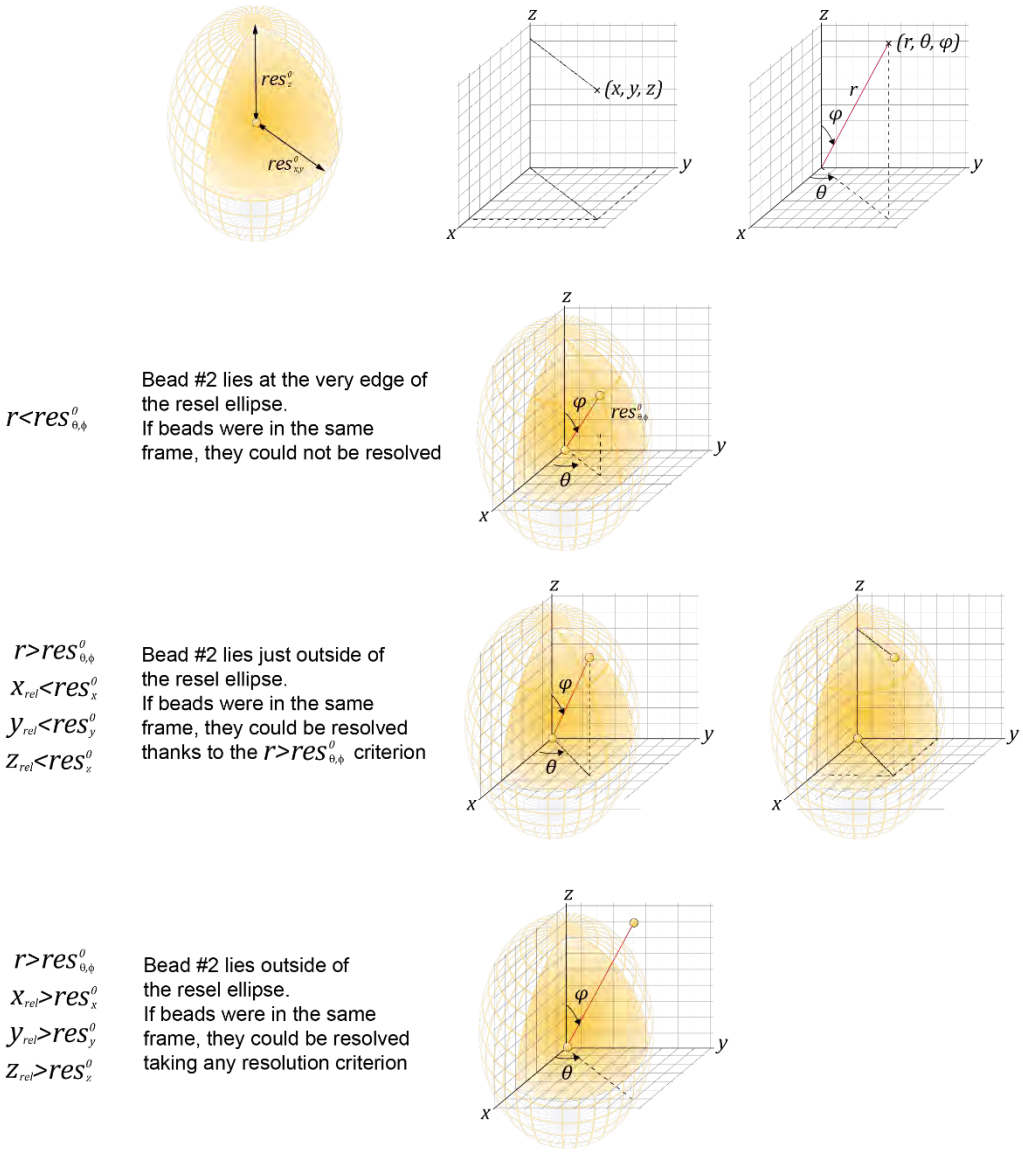


Figure 107. Calculation of the reference distance $res^{o}_{\theta,\varphi}$. Left: The bead's center at frame t and t+1 are drawn as a yellow spheres. The resolution element centered on the one frame's bead's center (the first Airy volume) appears as an ellipsoid (top panel left). Cartesian coordinates and polar coordinates are displayed in the middle and right top panel. For a pixel of the ellipsoid with polar coordinates θ and φ, $res^{o}_{\theta,\varphi}$ is its r coordinate. If the bead's center of frame t+1 lies within the ellipsoid centered on the bead's center at frame t, then the t to t+1 sequence can be considered as stabilized, because if both beads' centers were in the same frame they could not be resolved (second panel from top). However if the bead's center at frame t+1 lies just outside or further away from the ellipsoid centered on t frame's bead's center, both centers could be resolved (bottoms panels). The third panel from the top shows that it is important to compare the intercenter distance r with the resolution reference distance $res^{o}_{\theta,\varphi}$ for the same θ and φ angles that are observed between bead's centers of frame t and t+1..

STEP11. Set the file Save Options. Click the “File Save Option” to set the output options (Figure 108).

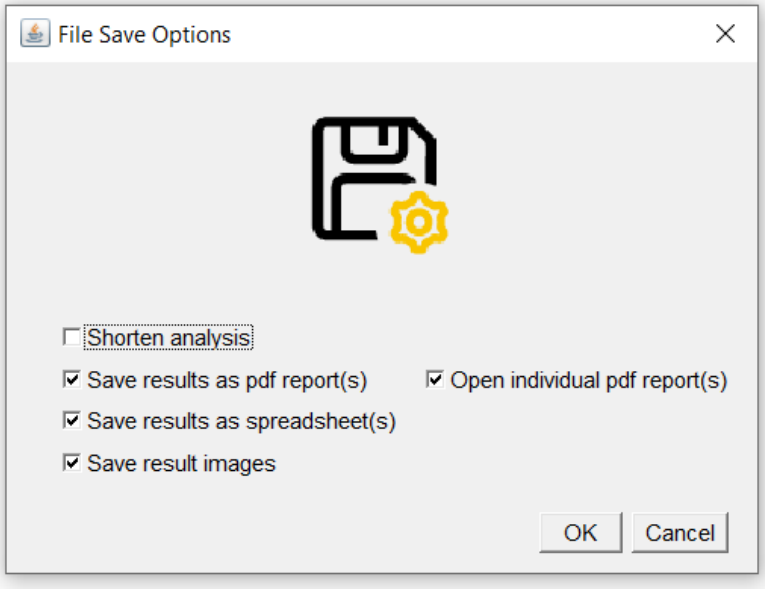


Figure 108. MetroloJ_QC : the file Save Options dialog

Set the file save options:

- Select the shorten analysis checkbox if you don’t want to have the normalized relative coordinates plot and the MSD analysis.
- Decide whether the results should be saved as a pdf file and whether the pdf should be automatically displayed (keep in mind if dozens of beads are to be analyzed, you may reach the pdf reader opened windows capacity).
- Would you like to get the data as spreadsheet files, tick the corresponding option. This generates a .xls file containing tabulation separated values of all tables of the pdf report.
- You may save the images of the displacements (MSD, 1D and 3D displacement plots) as individual images.

All generated files are saved in a subfolder of a “processed” folder located in the same folder of the original image. The subfolder’s name can be changed in the first “title” field of the dialog.

STEP12. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 109 (left panel) will appear. In this case, change the title.
MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP4) and the actual file format depth, a different type of error message is triggered (Figure 109, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images

- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

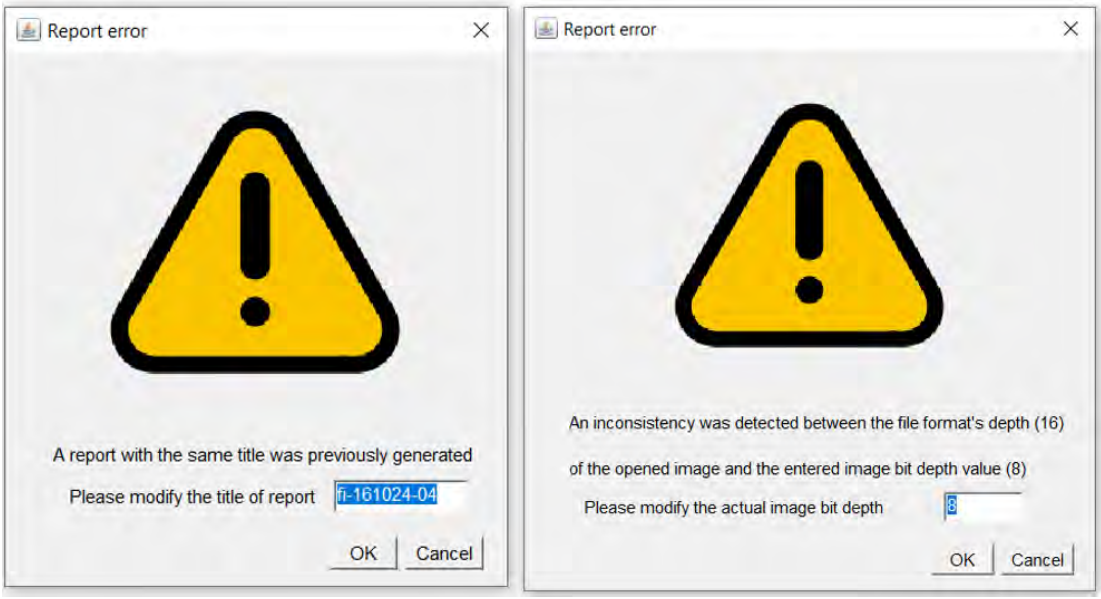
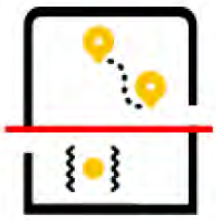


Figure 109. Error dialogs triggered by the Drift Profiler tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved! The original image file location is used to create a “processed” folder. Files will be further saved within a “title” folder (as provided by the user in the first “title of report” field of the dialog box).

Description of the Drift Profiler tool report.

The first sections of the co-registration tool report (Figure 110) is a summary of the image’s associated microscope info/parameters used to generate the Drift Profiler report (or a bead image if the multibeads image option was selected in STEPS7-8). The Warnings section provides the users with some warnings that might be useful to interpret the report. When using the multiple beads option, since beads are identified in the first frame, the first successful bead detection will always occur at timepoint 0. If not using this option, the first timepoint can have a value like 10. The analysis will begin from timepoint 10, meaning the elapsed time will start from frame 10.



dp240924-07

Microscope info:

Image		220509-overnight-drift15h-01-crop					
image's creation	date	2024-09-04 13:26:40					
	method used	from file creation date					
Actual image depth		16					
Microscope type		WideField					
Objective	NA	1.4					
	im. refractive index	1.515					
Channel(s)		Wavelengths		Saturation	sampling (X,Y,Z)		
		Ex. (nm)	Em. (nm)		Nyquist (µm)	Found (µm)	Nyquist/found ratio
Channel 0			480.0	none	0.086x0.086x0.256	0.414x0.414x1.0	4.8, 4.8, 3.9

Warnings:

(No saturated pixels detected).

First timepoint with a successfully detected bead: 0

Figure 110. Drift Profiler tool : microscope info and warnings sections

The next image (Figure 111) shows, for the bead outlines at the first analyzed frame, then the position of the XY coordinates of the bead's center as detected for each frame. A continuum from blue to red displays each frame's bead position. Figure 111 directly show some biased drift in the Y direction (towards the top of the image).

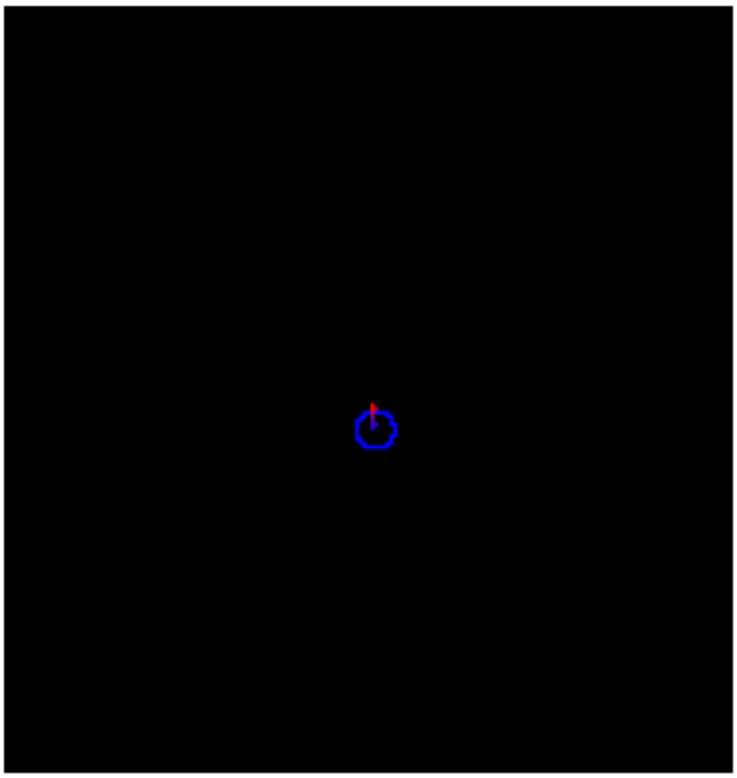
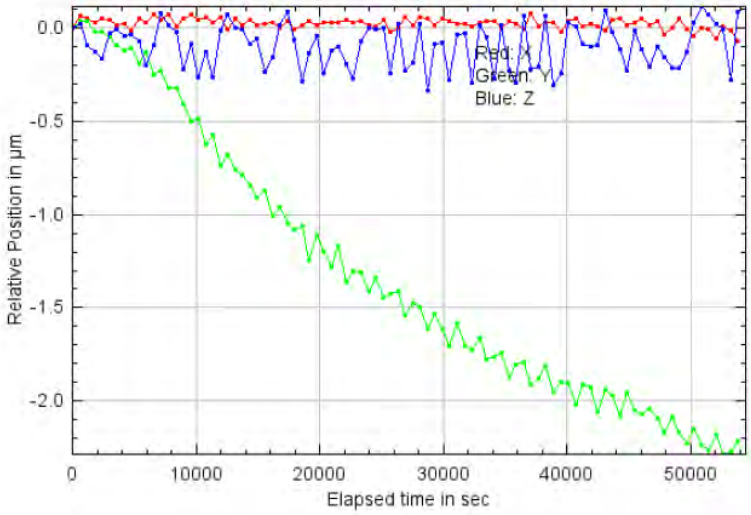


Figure 111. Drift Profiler tool : the overall XY displacement figure.

The report then proceeds with the analysis of relative coordinates. The first section (top panel of Figure 112) presents a plot of the relative positions, comparing the x, y, or z coordinates to those at the firstTimepoint frame. Figure 112 highlights fluctuations in the relative x coordinates (red), with greater fluctuations in the z coordinates (blue), while the y coordinate exhibits a general "downward" trend. This indicates that the absolute y coordinate decreases, meaning the bead is moving upward toward the top of the image (where x=0, y=0 represents the upper left corner). The average relative coordinate is shown in the bottom panel of Figure 112. The further the average value deviates from 0, the greater the drift. Additionally, the standard deviation of the relative position is displayed, along with how this standard deviation compares to the absolute mean coordinate, expressed as a percentage.

Relative X, Y and Z coordinates



Relative Positions table:

Dimension	X	Y	Z
average position in µm	0.03	-1.32	-0.09
Standard deviation in µm (%)	0.03 (107.8%)	0.71 (54.1%)	0.12 (124.3%)

Figure 112. Drift Profiler tool : the relative coordinates section.

.LONG versions of the report then display the normalized coordinates plots (Figure 113). These coordinates are calculated following equations :

$$x_{norm}(t) = \frac{x_{rel}(t) - mean_x}{\sigma_x} \text{ (X)}$$

$$y_{norm}(t) = \frac{y_{rel}(t) - mean_y}{\sigma_y} \text{ (Y)}$$

$$z_{norm}(t) = \frac{z_{rel}(t) - mean_z}{\sigma_z} \text{ (Z)}$$

with $mean_x$ and σ_x being the mean and standard deviation of the x relative coordinates across the timelapse, $mean_y$ and σ_y being the mean and standard deviation of the y relative coordinates across the timelapse, and $mean_z$ and σ_z being the mean and standard deviation of the z relative coordinates across the timelapse.

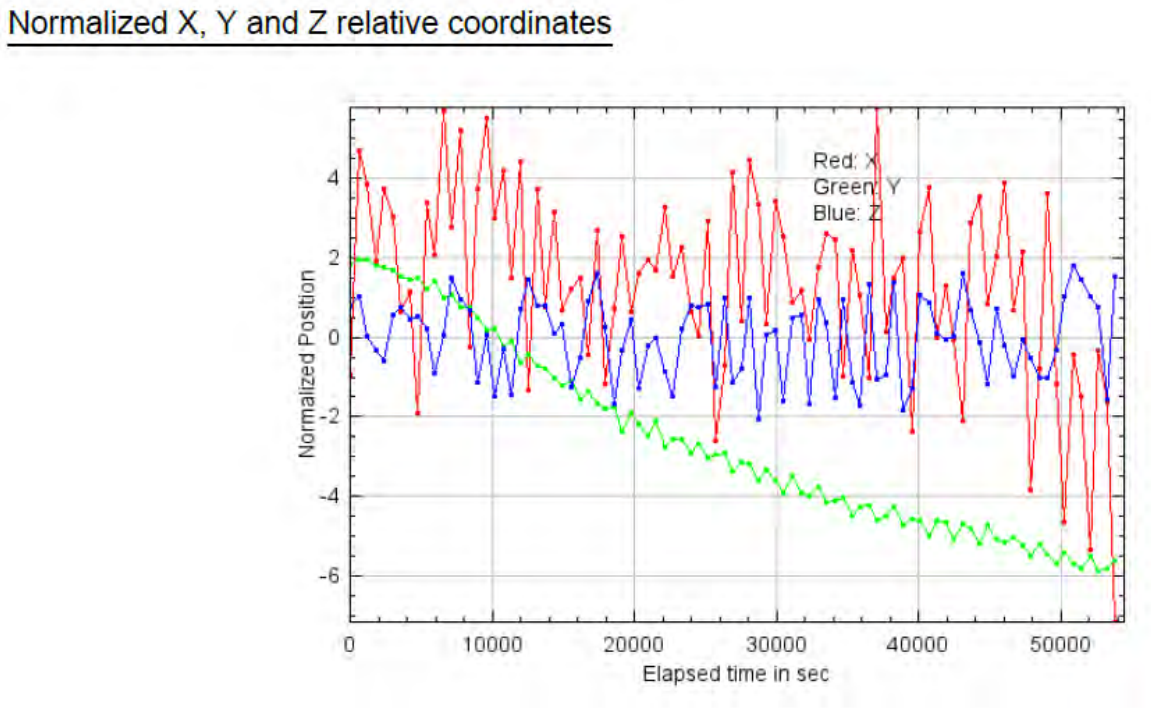


Figure 113. Drift Profiler tool report: An example of the normalized relative coordinates plot.

The report then displays the displacement section. Displacement is defined as the distance between the bead centers of two consecutive timepoints (formula AA to AD).

$$displacement_X(t) = x_t - x_{t-1}$$ (AA)

$$displacement_Y(t) = y_t - y_{t-1}$$ (AB)

$$displacement_Z(t) = z_t - z_{t-1}$$ (AC)

$$displacement_{3D}(t) = \sqrt{displacement_X(t)^2 + displacement_Y(t)^2 + displacement_Z(t)^2}$$ (AD)

3D displacement is shown in Figure 114, while 1D displacements are shown in a single plot in Figure 115. The top panel shows the plot when the “show absolute 1D distances in displacement plot” is selected, while the bottom panel shows the plot if the option is left unselected.

3D Displacement

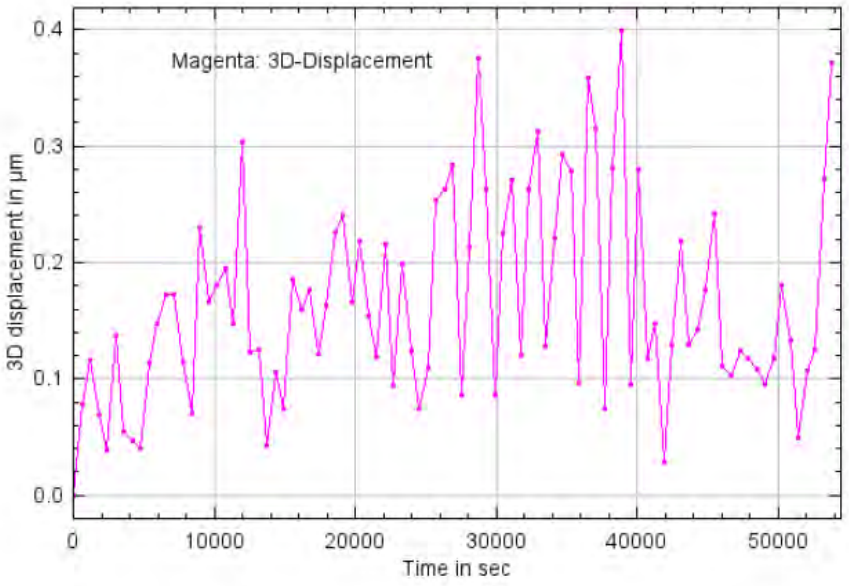


Figure 114. Drift Profiler tool report: An example of the 3D displacement plot.

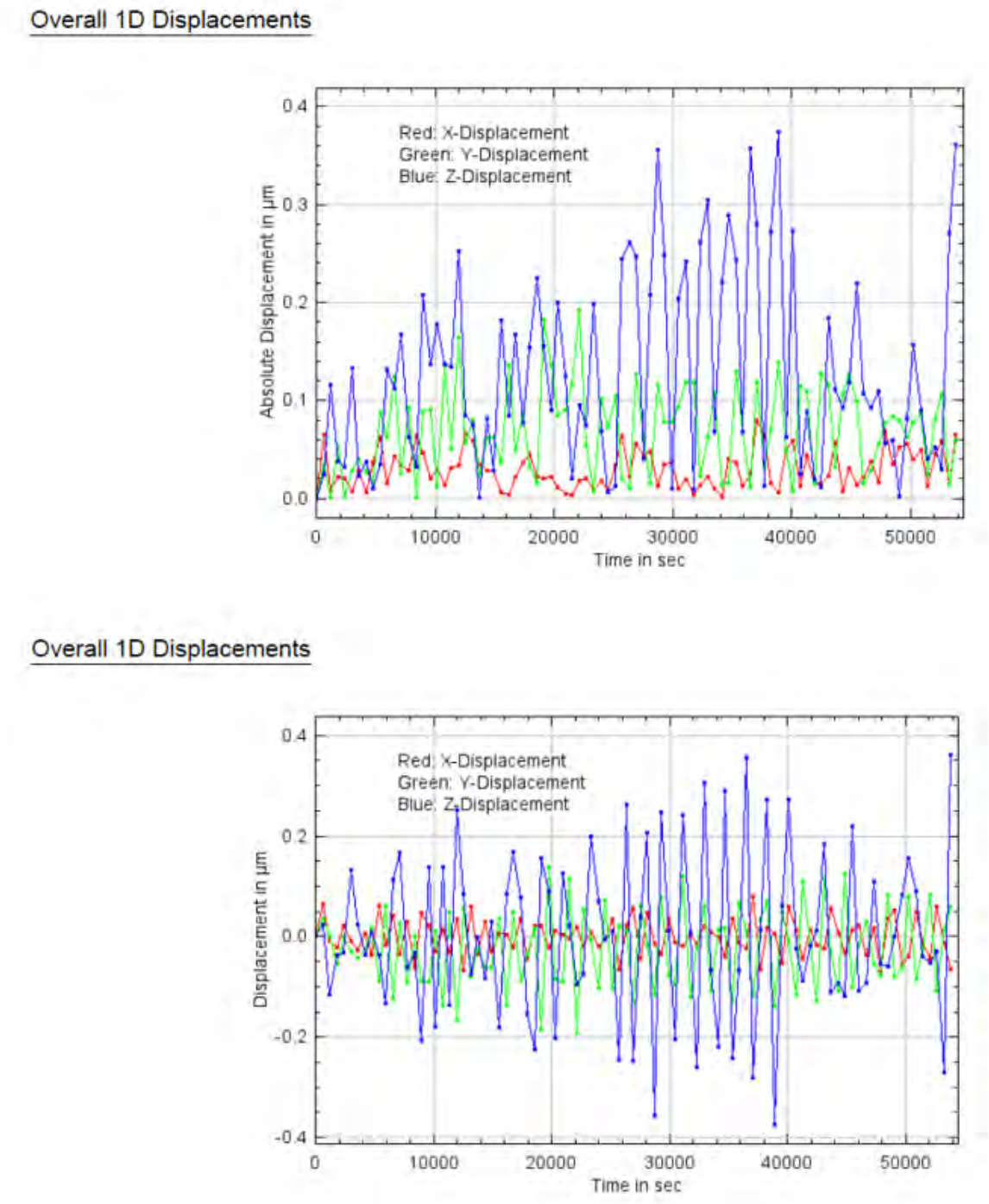


Figure 115. Drift Profiler tool report: Example of X, TY and Z displacements plots. Top panel : absolute displacement, bottom panel : relative displacement.

The next table (Figure 116) shows the average and standard deviation of the velocity for each dimension. Velocity is defined as in formula AE.

$$velocity\ (t) = \frac{displacement\ \ (t)}{frame\ interval} \text{ (AE)}$$

Velocity table:

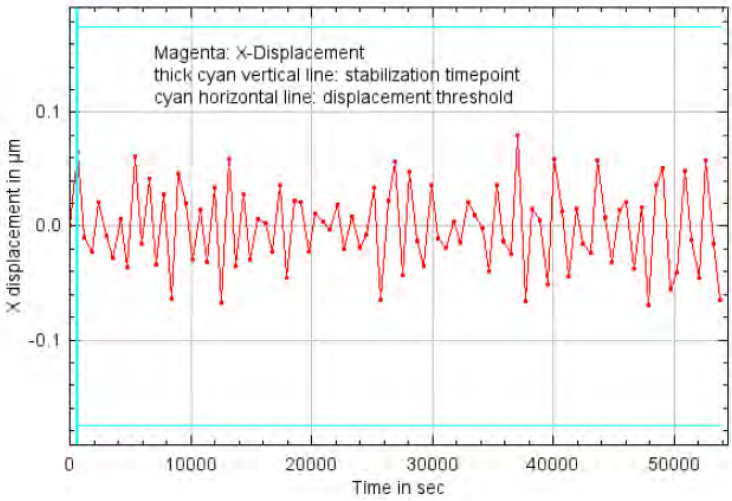
Dimension	X	Y	Z	3D
Average velocity in nm/sec	0.0	-0.04	0.0	0.28
Standard deviation in nm/sec	0.06	0.13	0.27	0.14

Figure 116. Drif Profiler tool report: the overall velocity table.

Then the stabilization timepoint is measured. Stabilization is defined as the firstTimepoint upon which each frame displacement value is below a threshold (either a resolution threshold or an isotropic threshold, see STEP9). Figure 117 shows an example of stage (X dimension) that is stabilized from start. The cyan line in the 1D displacement plot highlights the stabilization timepoint (here 1).

Stabilization periods and velocities tables:

X-Displacement:



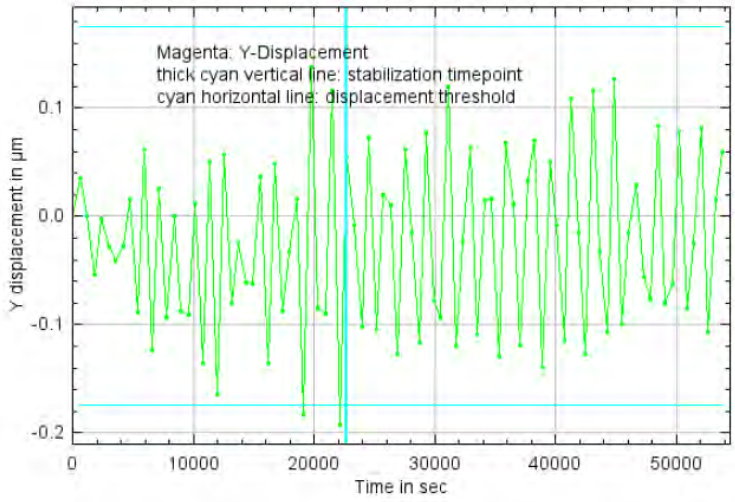
X-Displacement	Stabilization timepoint	Stabilization time (in sec)
Value	stabilized from start	

X-Displacement	Overall velocity	Velocity before stabilization	Velocity after stabilization
Average velocity in nm/sec	0.0		0.0
Standard deviation in nm/sec	0.06		0.06

Figure 117. Drift profiler tool report: an example of stable stage.

Figure 118 shows a stage (Y-dimension) that displays some drift getting below the threshold at timepoint 38. Mean velocities before and after stabilization are shown in the bottom table. Although values are more or less similar, one can see that before stabilization (left-hand side of the vertical Cyan line) fluctuations in the Y-displacement are higher and above the threshold.

Y-Displacement:



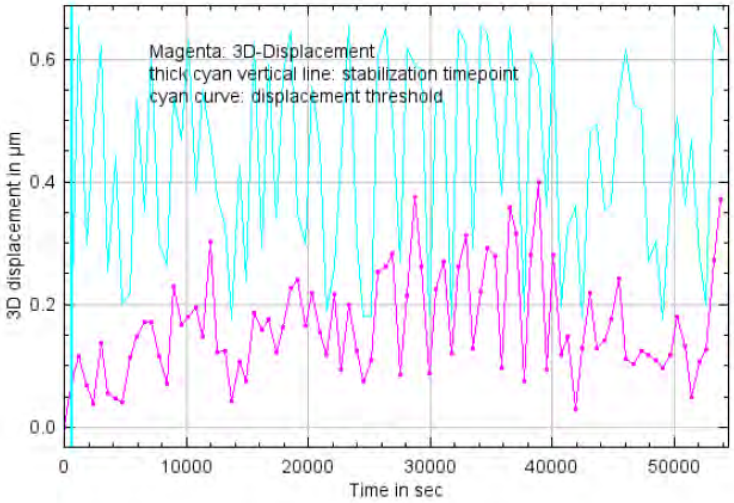
Y-Displacement	Stabilization timepoint	Stabilization time (in sec)
Value	38	22746.0

Y-Displacement	Overall velocity	Velocity before stabilization	Velocity after stabilization
Average velocity in nm/sec	-0.04	-0.06	-0.03
Standard deviation in nm/sec	0.13	0.13	0.13

Figure 118. Drift profiler tool report: an example of stabilizing stage.

Finally, when the resolution threshold is used, each frame's 3D displacement is compared to reference distance $res^{o}_{\theta,\varphi}$, hence the threshold values are not constant along frames but rather depend on the θ and φ angles between the bead's centers at frame t and t+1 (Figure 119).

3D-Displacement:



3D-Displacement	Stabilization timepoint	Stabilization time (in sec)
Value	stabilized from start	

3D-Displacement	Overall velocity	Velocity before stabilization	Velocity after stabilization
Average velocity in nm/sec	0.28		0.28
Standard deviation in nm/sec	0.14		0.14

Figure 119. Drift profiler tool report: an example of 3D displacements compared with the reference resolution distance $res^{o}_{\theta,\varphi}$.

LONG versions also contain a MSD plot (Figure 120) for each dimension. MSD values are given by formula AF to AI.

$$MSD_X\ (frame) = x^2_{rel}(t)\ \text{(AF)}$$

$$MSD_Y\ (frame) = y^2_{rel}(t)\ \text{(AG)}$$

$$MSD_Z\ (frame) = z^2_{rel}(t)\ \ \text{(AH)}$$

$$MSD_{3D}\ (frame) = x^2_{rel}(t) + y^2_{rel}(t) + z^2_{rel}(t)\ \text{(AI)}$$

Mean Square Displacement:

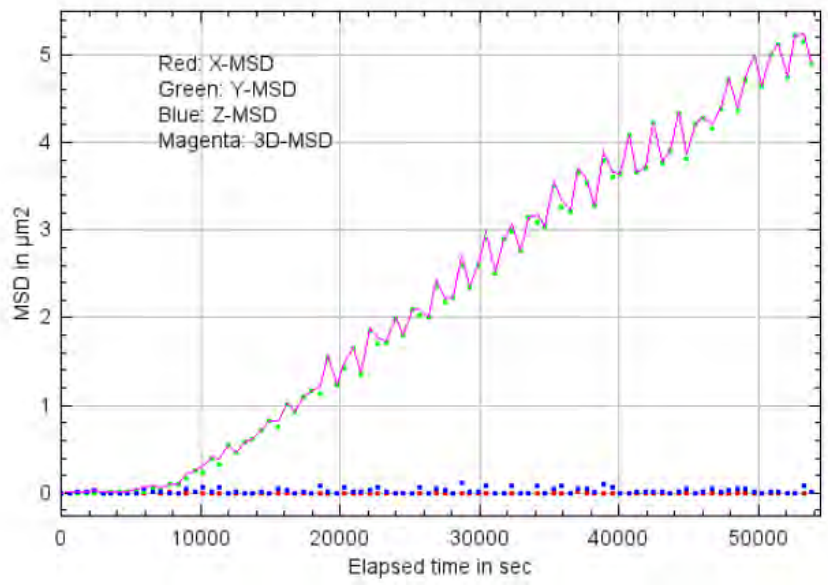


Figure 120. Drift profiler tool report: an example of MSD Plot of the LONG version of the report.

Finally, if any, user-provided Sample info & Comments are reported in the final page of the report, as are reported the user-defined analysis parameters used to generate the report (Figure 121), a log file that indicates how the input image was handled (Figure 122) and the formulas used (Figure 123).

Analysis parameters

Tool & Operator	Tool	Stage positioning and drift
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	Julien, 24 septembre 2024 14:20
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\drift\Processed\dp240924-27\
	Type of saved data	.pdf, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Beads	Bead detection threshold	Legacy
	Center detection method	Centroid
	Discard bead if more than one particle are thresholded	true
	Multiple beads in image	false
Other algorithm parameters	Maximum gap length	4
	Stabilization definition threshold	uses theoretical resolution
Output and display parameters	Show absolute 1D distances	false
	Show displacement fit on plots	yes (discard if R2 is below0.15)
	Show detected beads on projections	yes

Figure 121. Co-registration tool report: analysis parameters table.

Analysis log

image name	creation date	saturation	status
220509-overnight-drift15h-01-crop	2024-09-04 13:26:40	none	analysed

Figure 122. Co-registration tool report: log table

Formulas used:

Relative coordinates x_{rel}, y_{rel} and z_{rel}:

$$x_{rel}(t) = x(t) - x(ref)$$

$$y_{rel}(t) = y(t) - y(ref)$$

$$z_{rel}(t) = z(t) - z(ref)$$

$x(t)$, $y(t)$ and $z(t)$, are the bead's coordinates at frame interval t, $x(ref)$, $y(ref)$ and $z(ref)$ is the bead position at the reference frame (currently the first frame t=0).

Normalized coordinates x_{norm}, y_{norm} and z_{norm}:

$x_{norm}(t) = \frac{x_{rel}(t) - mean_x}{\sigma_x}$ with $mean_x$ and σ_x being the mean and standard deviation of the x relative coordinates across the timelapse.

$y_{norm}(t) = \frac{y_{rel}(t) - mean_y}{\sigma_y}$ with $mean_y$ and σ_y being the mean and standard deviation of the y relative coordinates across the timelapse.

$z_{norm}(t) = \frac{z_{rel}(t) - mean_z}{\sigma_z}$ with $mean_z$ and σ_z being the mean and standard deviation of the z relative coordinates across the timelapse.

Mean Squared Displacement:

$$MSD_X\ (frame) = x_{rel}^2(t)$$

$$MSD_Y\ (frame) = y_{rel}^2(t)$$

$$MSD_Z\ (frame) = z_{rel}^2(t)$$

$$MSD_{3D}\ (frame) = x_{rel}^2(t) + y_{rel}^2(t) + z_{rel}^2(t)$$

Elapsed time:

$$elapsed\ time\ (frame) = frame * frame\ interval$$

Figure 123. Co-registration tool: formulas used in the report.

When single bead images are selected, all data is saved in a processed/title subfolder (Figure 124, top panel). If selected, profile plots for each dimension are saved in a title_imageName_data subfolder (Figure 124, bottom panel). The .xls files of the tables of the pdf report are saved in an title_imagename_results.xls file within this subfolder, while the coordinates are saved in a title_imagename_position.xls file. The coordinates of the Displacement (distance) plots values are saved in a title_imagename_displacementPlot.xls file. LONG versions of the report include a title_imagename_MSDPlot.xls file containing the MSD plots values.

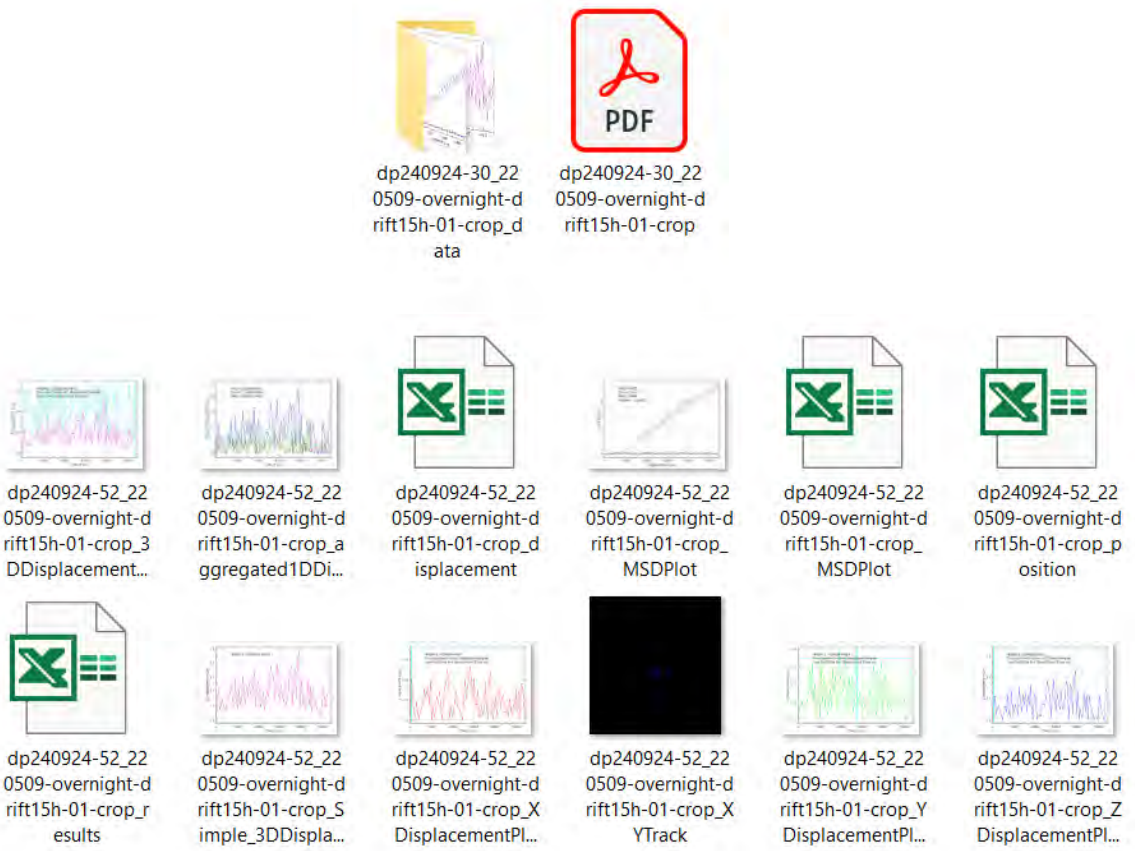


Figure 124. Drift profiler tool: Files generated when save report images are saved (long version of the report).

When multiple bead images option is selected, files derived from each bead are saved in a processed/title/bead# (Figure 125). If the save pdf reports option is selected, a summary pdf document is generated (title_imageName_identifiedBeads.pdf, Figure 125) that contains the microscope info, the analysis parameters, a log file that summarizes how the beads were identified and analyzed (Figure 126).

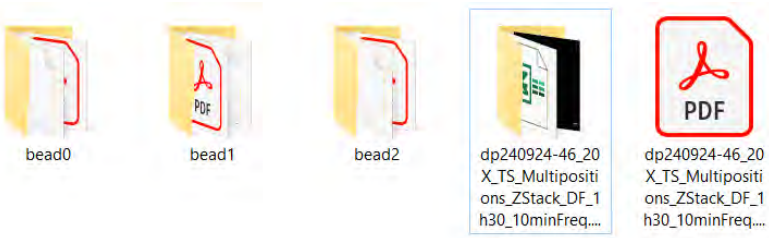


Figure 125. Drift Profiler tool: Files and folders generated when images contain multiple beads.

Analysis log

image name	creation date	identified raw beads	valid beads	saturation	status
20X_TS_Multipositions_ZStack_DF_1h30_10minFreq.czi-20X_TS_Multipositions_ZStack_DF_1h30_10minFreq	2022-05-18 10:31:50	4	3	none	valid beads found
			bead0	none	analysed
			bead1	none	analysed
			bead2	none	analysed

Figure 126. Drift profiler tool: The log table of the summary pdf file in multiple beads-containing image mode

BATCH TOOLS

Acquisition of datasets for Quality Control can be time-consuming. Once the QC tests frequency is defined (say every month), QC images acquisition and analysis can become a real burden. The facility manager should investigate any way of automating acquisition. Image analysis should, ideally, be performed “on the fly” (ie. a few minutes after acquisition) as to avoid any unnecessary delay. Direct analysis allows identification of problems/misalignments that can be tackled immediately. Rapid analysis of the correction effects optimizes the QC process.
We provide here with batch tools of the main three most-frequent QC procedures.

BATCH FIELD ILLUMINATION TOOL

Analysis dataset requirements for Batch Field Illumination Tool

This plugin is a batch version of the Field Illumination Tool. As for any batch version provided in MetroloJ_QC, a first image of the dataset is opened and analyzed. The structure of this image has to be the same for any other image of the dataset.
By structure, in the case of the Field Illumination Tool, we basically mean here the number of channels/file (as the Field Illumination Tool is adapted to either single

channel images or multiple channel images). Hence, if a 3 channels file is opened, any other images within the folder are supposed to be 3 channels images.

Batch Field Illumination parameters

STEP0. It is recommended to hide ImageJ's warning messages triggered by the field homogeneity tool.

In the Main MetroloJQC bar, click the cog icon to configure the menus. In the bottom "MetroloJQC dialogs configuration", then click the "Configure general settings" button (Figure 127, left panel). click Disable IJ warning Messages (Figure 128, right panel), then click OK to go back to the MetroloJ_QC Bar configuration dialog, and click OK again to exit this dialog.

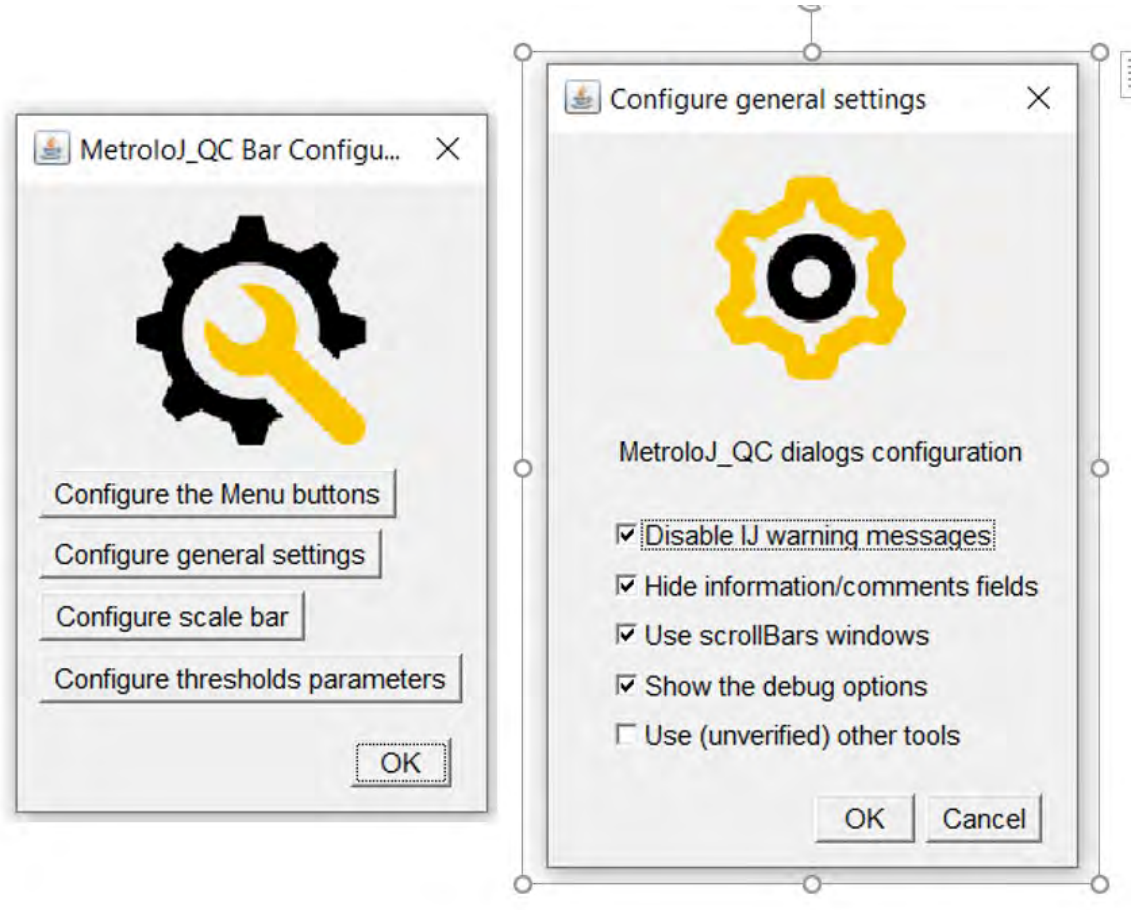


Figure 127. MetroloJ QC's configuration tool

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).
STEP2. Click on the batch field illumination tool icon. The user is prompted to select the folder containing the images (Figure 128). Select the directory and click select. The plugin will open the first bioformat-compatible image of the folder. The plugin's interface should appear (see Figure 129).

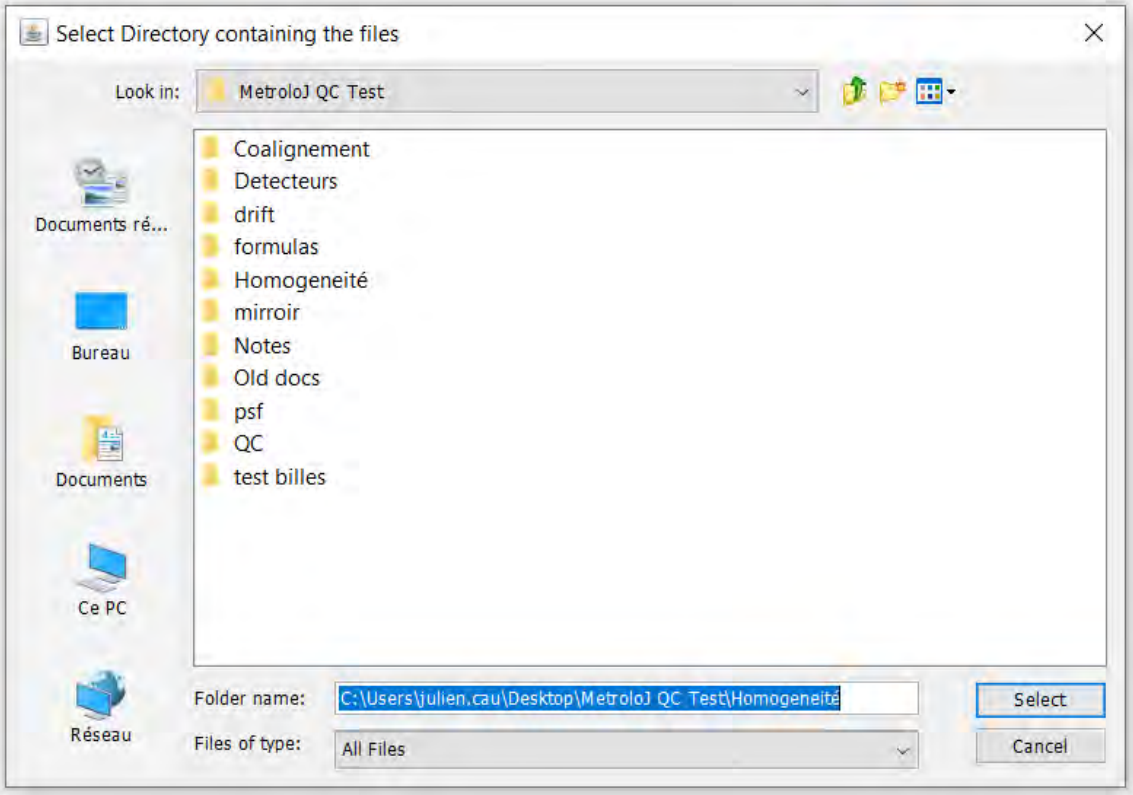


Figure 128. Batch Field Illumination tool: the first dialog window.

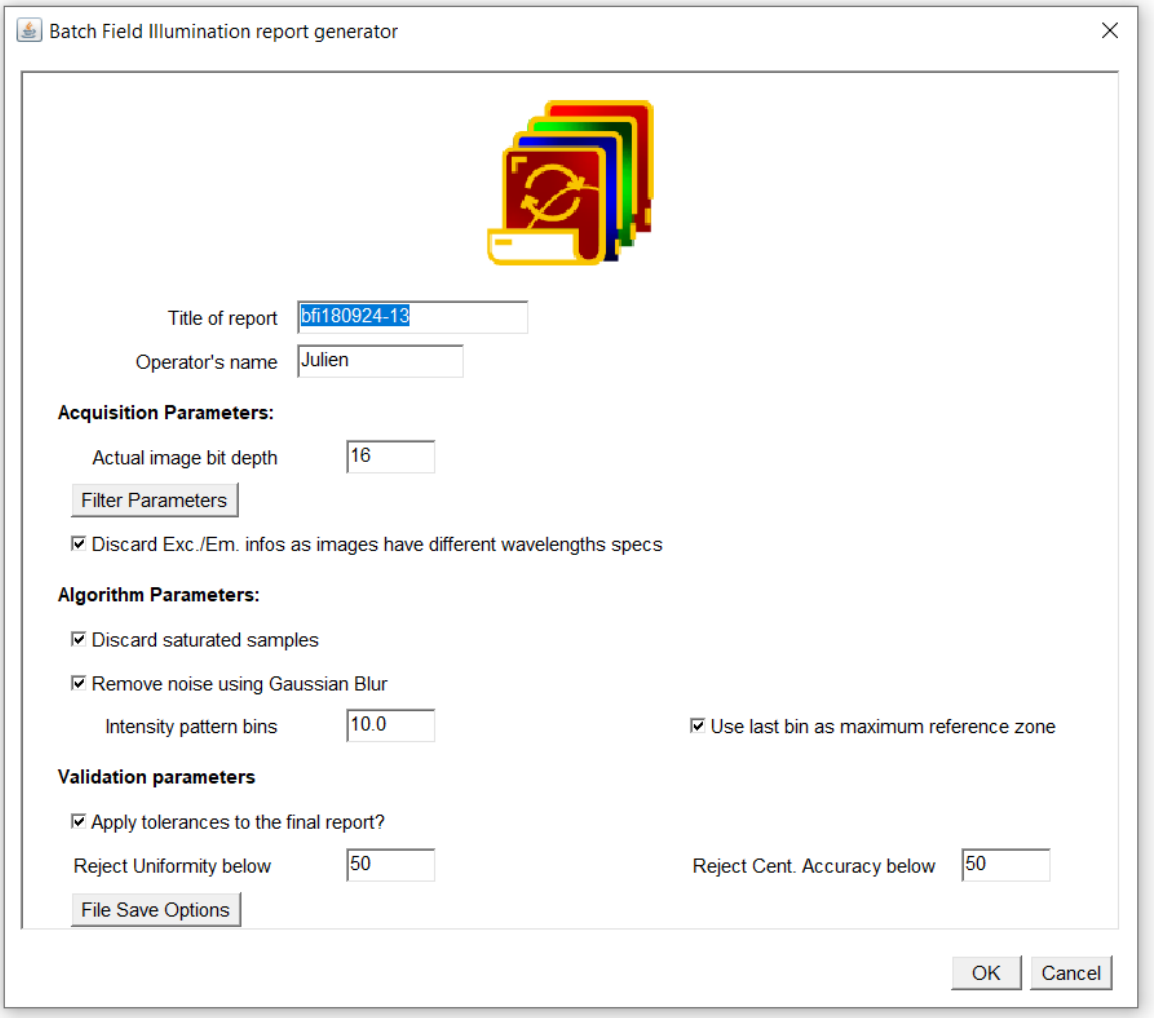


Figure 129. Batch Field Illumination Tool: the user's interface.

STEP3. The user will find the familiar interface of the Field Illumination tool. As images may be of different channels, in this case it is recommended for the sake of traceability to select the additional "discard Exc/Em info as images have different wavelengths specs" (in the "acquisition parameters" section). This will erase from any individual report this information, that is anyhow not useful for any of the plugin calculation.

STEP4. In the File Save Options, decide whether pdf report should be open (Figure 130).

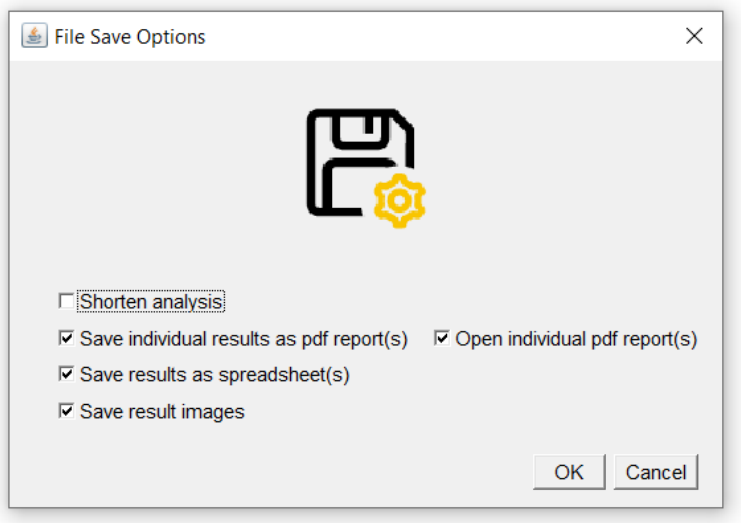


Figure 130. Batch Field Illumination Tool: the file save options dialog

STEP5. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 131 (left panel) will appear. In this case, change the title.

MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP3) and the actual file format depth, a different type of error message is triggered (Figure 131, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

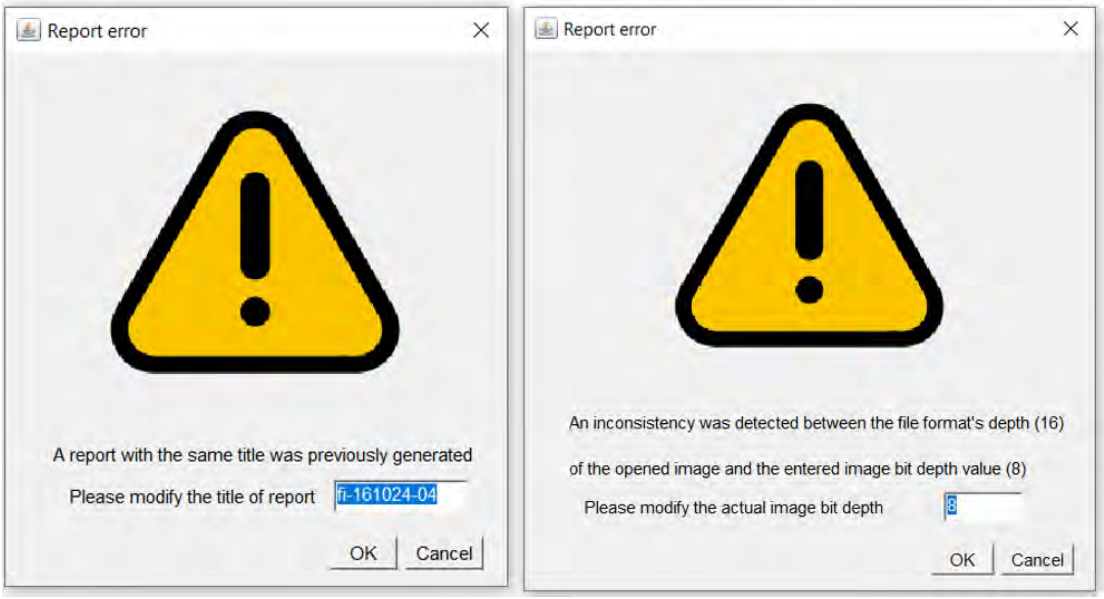


Figure 131. Error dialogs triggered by the Batch Field Illumination tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved! A summary pdf report will be generated, even though the save individual pdf report(s) is left unselected. If this latter option is chosen, each analysed file will generate an individual report.

The Batch Field Illumination Tool report

The report structure is more or less the same compared to the Field Illumination Tool. The plugin will, upon request, generate individual pdf (with the “save individual pdf option”, excel files and jpg images). It is recommended, at STEP4, to unselect the open individual pdf report(s) (so that, if any, these reports won’t be opened.

A summary pdf file is produced (even though the save individual pdf option is not selected).
The microscope info section (Figure 132) indicates how many images are analyzed (unsaturated images if discard saturated samples is selected, total images if not).

bfi180924-22 - Batch Summary

Microscope info:

data	8 analysed images		
images location	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Homogeneité\		
Actual image depth	16		
Filter combination(s)	Wavelengths		unsaturated/total images
	Ex. (nm)	Em. (nm)	
DAPI	480.0	480.0	7/8
GFP	488.0	525.0	all ok
Rhodamine	560.0	590.0	7/8
Texas Red	580.0	610.0	all ok
Cy5	640.0	680.0	7/8

Warnings:

saturation issues reported for one or more files (see Analysed images section below)

Figure 132. Batch Field Illumination Report: microscope info and warnings sections

If the STEP3 discard Exc/Em info as images have different wavelengths specs" option was chosen, these specs are removed from the microscope info table of the batch Field Illumination report (Figure 133) as well as in any individual report generated (Figure 134).

bfi180924-15 - Batch Summary

Microscope info:

data	8 analysed images
images location	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Homogeneité\
Actual image depth	16
Channel(s)	unsaturated/total images
Channel 0	7/8
Channel 1	all ok
Channel 2	7/8
Channel 3	all ok
Channel 4	7/8

Warnings:

saturation issues reported for one or more files (see Analysed images section below)

Figure 133. Batch Field Illumination Report: microscope info and warnings sections (option discard Ex/Em. Infos as images have different wavelengths specs).

bfi-090824-07

Microscope info:

Image		5x
image's creation	date	2024-06-13 09:13:27
	method used	from file creation date
Actual image depth		16
Microscope type		WideField
Objective	NA	1.4
	im. refractive index	1.515
Channel(s)		Saturation
Channel 0		none
Channel 1		none
Channel 2		none
Channel 3		none
Channel 4		none

Figure 134. Individual Field Illumination Report when generated by Batch Field Illumination Report microscope info and warnings sections (option discard Ex/Em. Infos as images have different wavelengths specs).

The next main field illumination parameters table is a summary of all results (Figure 135). As in the regular Field Illumination Tool, within and outside specs can be highlighted using the appropriate option.

Main Field Illumination parameters:

image	Channel	Uniformity	Centering Accuracy
100x	Channel 0	52.5	72.6
	Channel 1	61.0	89.7
	Channel 2	67.7	90.2
	Channel 3	70.1	96.7
	Channel 4	67.1	92.3
10x	Channel 0	32.4	81.7
	Channel 1	37.5	94.5
	Channel 2	44.5	94.2
	Channel 3	44.2	95.7
	Channel 4	42.7	93.1
20x	Channel 0	41.4	71.1
	Channel 1	58.8	90.3
	Channel 2	65.2	86.7
	Channel 3	66.6	92.0
	Channel 4	60.6	87.7
40x	Channel 0	44.7	73.5
	Channel 1	50.9	92.3
	Channel 2	52.1	93.7
	Channel 3	53.9	96.8
	Channel 4	59.5	87.0
5x	Channel 0	34.4	69.7
	Channel 1	51.4	94.6
	Channel 2	59.8	93.9
	Channel 3	58.9	96.4
	Channel 4	47.9	95.3
5xSatCh0	Channel 0	Saturated channel	
	Channel 1	51.4	94.6
	Channel 2	59.8	93.9
	Channel 3	58.9	96.4
	Channel 4	47.9	95.3
sat20x-2	Channel 0	41.7	71.2
	Channel 1	60.3	90.3
	Channel 2	Saturated channel	
	Channel 3	69.5	92.1
	Channel 4	67.7	86.9
sat20x	Channel 0	41.6	71.1
	Channel 1	60.0	90.3
	Channel 2	67.2	86.8
	Channel 3	69.0	92.1
	Channel 4	Saturated channel	

Centering accuracy computed using the 90.0%-100% reference zone. Green: within specifications, red: outside specifications (ie. uniformity below 50.0 or centering accuracy below 50.0)

Figure 135. Batch Field Illumination Tool Report: the Main Field Illumination section.

Finally, for the sake of keeping track of the parameters used for analysis, the Analysis parameters table (Figure 136) is displayed. Note that individual, generated analysis parameter tables display in their first row “Batch Field-Illumination” as well.

A log table that summarizes how files were handled is also shown (Figure 137). If the "discard saturated sample" option was chosen, then any image that was not analyzed because saturated will still be listed here, so that the user knows the image was open but analysis stopped as saturation was found. Figure 137 also shows that files with an unexpected number of channels (as compared to the first opened image that sets the MetroloJ Dialog Menu) are not analyzed. At the end of the report, formulas used are displayed (Figure 138).

Analysis parameters

Tool & Operator	Tool	Batch Field-Illumination
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	, 9 août 2024 10:08
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Homogeneité\Processed\bfi-090824-06\
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Gaussian blur noise removal applied		true
Isointensity image steps width		10.0%
Reference zone		90.0%-100%
Tolerance	Applied in this report	true
	Uniformity valid if above	50.0
	CA valid if above	50.0

Figure 136. Batch Field Illumination Tool report: analysis parameters section.

Analysis log

image name	creation date	saturation	status
100x	2024-06-13 09:13:25	none	analysed
10x	2024-06-13 09:13:25	none	analysed
20x	2024-06-13 09:13:26	none	analysed
40x	2024-06-13 09:13:26	none	analysed
5x	2024-06-13 09:13:27	none	analysed
5xSatCh0	2024-06-13 09:13:27	Ch.0 saturated	analysed
63x	2024-06-13 09:13:27		unexpected number of channels (4 vs 5)
sat20x-2	2024-06-13 09:13:28	Ch.2 saturated	analysed
sat20x	2024-06-13 09:13:28	Ch.4 saturated	analysed
saturated5x	2024-06-13 09:13:29	Ch.0,1,2,3,4 saturated	not analysed

Figure 137. Batch Field Illumination Tool report: the list of all analysed images

Formulas used:

$$Uniformity = \frac{minimum\ intensity}{maximum\ intensity} * 100$$

$$Centering\ Accuracy = 100 - 100 * \frac{2}{\sqrt{w^2+h^2}} * \sqrt{(x_{ref} - \frac{w}{2})^2 + (y_{ref} - \frac{h}{2})^2}$$

x_{ref} and y_{ref} are the coordinates of the "reference", w and h the width and height of the image.

Figure 138. Formulas used in the Batch Field Illumination Tool.

Whenever the "Unverified Field Illumination tools" mode is used (see Field Illumination Tool section), the table of Figure 135 is modified as to include the (unverified) new metrics (Figure 139).

Main Field Illumination parameters:

image	Channel	Uniformity	field Uniformity	Centering Accuracy	Coef. of Variation	Mean c fit value
100x	Channel 0	52.5	88.5	72.6	0.1056	0.0079
	Channel 1	61.0	91.5	89.7	0.0749	0.0082
	Channel 2	67.7	91.9	90.2	0.0481	0.0039
	Channel 3	70.1	92.4	96.7	0.0394	0.0037
	Channel 4	67.1	91.8	92.3	0.0405	0.0034
10x	Channel 0	32.4	83.7	81.7	0.1617	0.0152
	Channel 1	37.5	87.9	94.5	0.167	0.0196
	Channel 2	44.5	89.4	94.2	0.1274	0.0172
	Channel 3	44.2	89.2	95.7	0.128	0.0175
	Channel 4	42.7	88.8	93.1	0.1246	0.0158
20x	Channel 0	41.4	84.8	71.1	0.1424	0.0105
	Channel 1	58.8	90.9	90.3	0.0823	0.0096
	Channel 2	65.2	91.8	86.7	0.0593	0.0061
	Channel 3	66.6	92.4	92.0	0.0525	0.0058
	Channel 4	60.6	90.7	87.7	0.064	0.0067
40x	Channel 0	44.7	87.0	73.5	0.1277	0.0103
	Channel 1	50.9	90.5	92.3	0.1131	0.0122
	Channel 2	52.1	90.2	93.7	0.0992	0.0125
	Channel 3	53.9	90.4	96.8	0.095	0.0122
	Channel 4	59.5	90.8	87.0	0.0634	0.0072
5x	Channel 0	34.4	81.8	69.7	0.1671	0.0123
	Channel 1	51.4	90.7	94.6	0.1197	0.0142
	Channel 2	59.8	92.7	93.9	0.0822	0.0114
	Channel 3	58.9	92.7	96.4	0.088	0.0117
	Channel 4	47.9	92.1	95.3	0.1229	0.0186
5xSatCh0	Channel 0	Saturated channel				
	Channel 1	51.4	90.7	94.6	0.1197	0.0142
	Channel 2	59.8	92.7	93.9	0.0822	0.0114
	Channel 3	58.9	92.7	96.4	0.088	0.0117
	Channel 4	47.9	92.1	95.3	0.1229	0.0186
sat20x-2	Channel 0	41.7	84.7	71.2	0.1423	0.0105
	Channel 1	60.3	91.2	90.3	0.0823	0.0096
	Channel 2	Saturated channel				
	Channel 3	69.5	93.2	92.1	0.0525	0.0058
	Channel 4	67.7	92.5	86.9	0.0593	0.0061
sat20x	Channel 0	41.6	84.7	71.1	0.1423	0.0105
	Channel 1	60.0	91.2	90.3	0.0823	0.0096
	Channel 2	67.2	92.4	86.8	0.0593	0.0061
	Channel 3	69.0	93.1	92.1	0.0525	0.0058
	Channel 4	Saturated channel				

Centering accuracy computed using the 90.0%-100% reference zone. Green: within specifications, red: outside specifications (ie. uniformity below 50.0 or centering accuracy below 50.0)

Figure 139. Batch Field Illumination Tool Report: the Main Field Illumination section when (unverified) additional tools are used.

All generated files can be found in the processed>title subfolder (Figure 140). Each individual image with the expected number of channels is analyzed and triggers a report (even though analysis doesn't proceed as saturation is found in each channel for instance). The pdf summary report can be found in the processed/title/title-

BatchSummary.pdf (top panel). The spreadsheet version of the report is saved in a title_data folder as a title_BatchSummary.xls file.

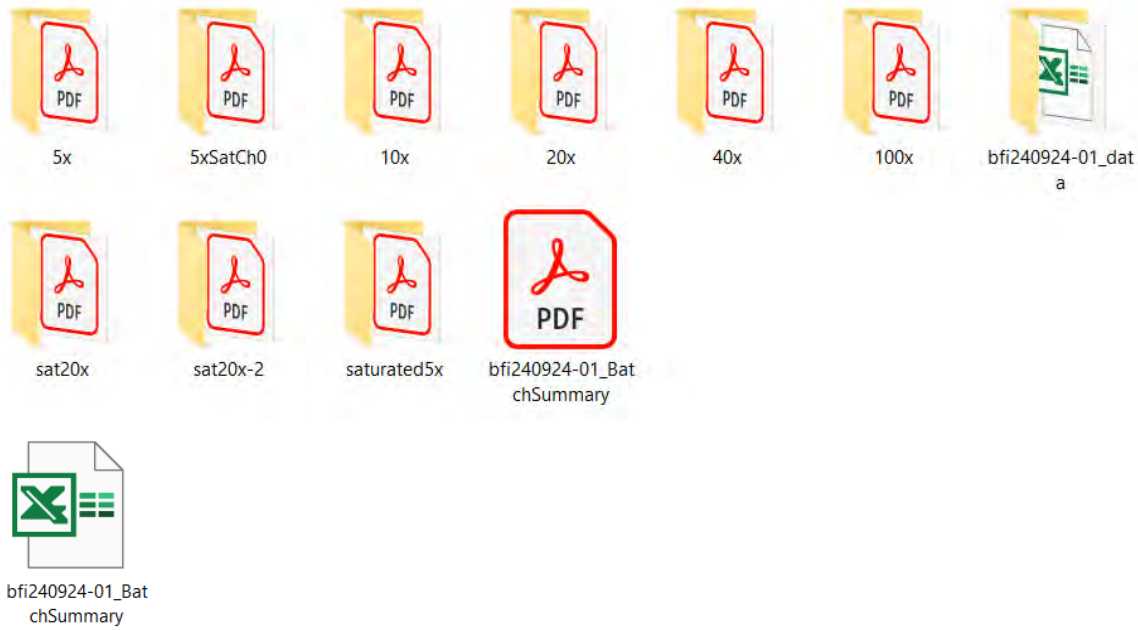


Figure 140. An example of files generated by the Batch Field Illumination Tool

BATCH PSF PROFILER TOOL

Analysis dataset requirements for Batch PSF Profiler Tool

The plugin is a batch version of the PSF profiler tool. It will analyze any bioformat-compatible files within a folder and aggregate values. Note that:

- this tool can be used to aggregate psf measurements from a single file containing multiple beads.
- Files in subfolder will not be investigated.

A final pdf report aggregates all values, provided they meet a quality criterion (ie. fits are of good quality).

Mind that this analysis is made per channel and per dimension. For instance, let us consider 10 beads and 2 channels. All criteria are met for the first eight beads in both channels. Then:

- The fits' qualities of bead 9 channel 0 is good enough only for X and Y dimensions and does not meet the criterion for Z. The fits' qualities for all dimensions in channel 1 are OK.
- The fits' qualities of bead 10 is poor for dimension X and ok for the others in both channels.

Values that will be aggregated will be:

- Channel 0: 9 beads for dimension X (1-9), 10 beads for dimensions Y (1-10), 9 beads (1-8 & 10) for Z.
- Channel 1: 9 beads for dimension X (1-9), 10 beads for dimensions Y (1-10), 10 beads for Z (1-10).

Whenever the spreadsheet option is selected, raw values before of all beads are saved in a specific file.

Batch PSF Profiler Tool Parameters

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).

STEP2. Select the Batch PSF Profiler tool then select the directory containing the images to analyze (Figure 141). Mind no subfolder will be investigated and all files should have the same # of channels (and wavelengths are supposed to be all identical). The images may have different pixel sizes, number of slices or width/height. The first found bioformats-compatible image will be opened and the plugin's main dialog interface should appear (see Figure 142).

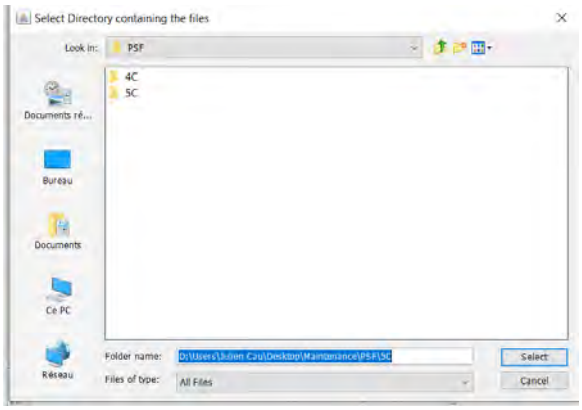


Figure 141. Batch PSF Profiler tool: the first dialog window.

STEP3. Most of the field/parameters are similar to the PSF Profiler tool. Two additional fields allow aggregation/filtering/compilation of the results (Validation parameters section, Figure 142):

- "Remove outliers": the list of values to be averaged is ordered, the median and Q1/Q3 values are calculated (Q1: 25% of data are below this quartile, Q2 is the median with 50% of data are below this quartile and 75% of data are below the Q3 quartile, while Q4 is the maximum value of the data series). The interquartile range IQR=Q3-Q1 is calculated. All values below Q1 - 1.5 * IQR (lower fence) and above Q3+1,5*IQR are discarded. When checked, outliers will be removed. The title_BatchRawData .xls file (in processed/title/title_data folder) includes a table of the values of the lower and upper fences. Mind the number of total beads analyzed ("Microscope section" of the main report) is not the number of total beads used at the end to compute each mean FWHM.
- "Reject PSF profile with R^2 below": this option is to discard all "suspect" values. Poor signal/noise ratio images or image acquisition issues (such as the stage hurt during the acquisition) will lead to poor profile fitting. There is no way not to go through the rejection process and, if necessary, a threshold value of 0 may be used to include all reports.

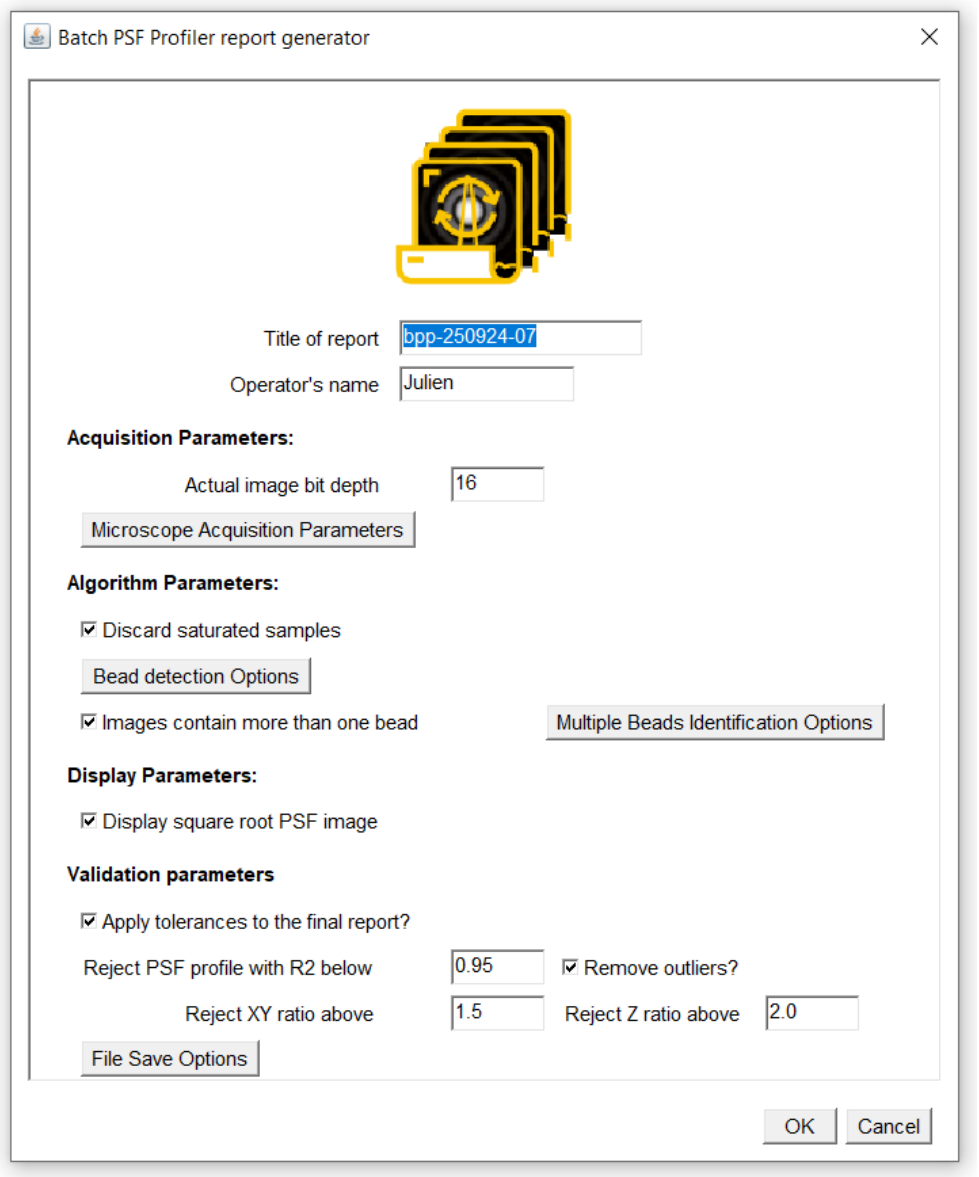


Figure 142. Batch PSF Profiler tool: the user's interface/Main dialog window.

STEP4. In the File Save Options, decide whether pdf report should be open (Figure 143).

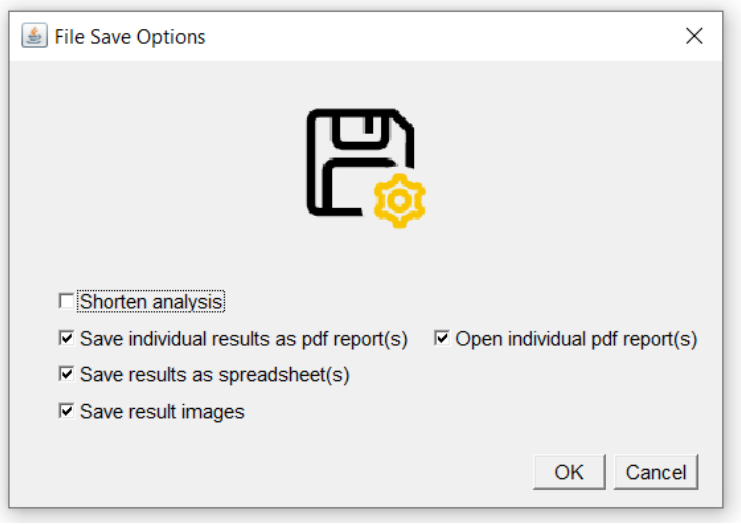


Figure 143. Batch Field Illumination Tool: the file save options dialog

STEP5. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 144 (left panel) will appear. In this case, change the title.

MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP3) and the actual file format depth, a different type of error message is triggered (Figure 144, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

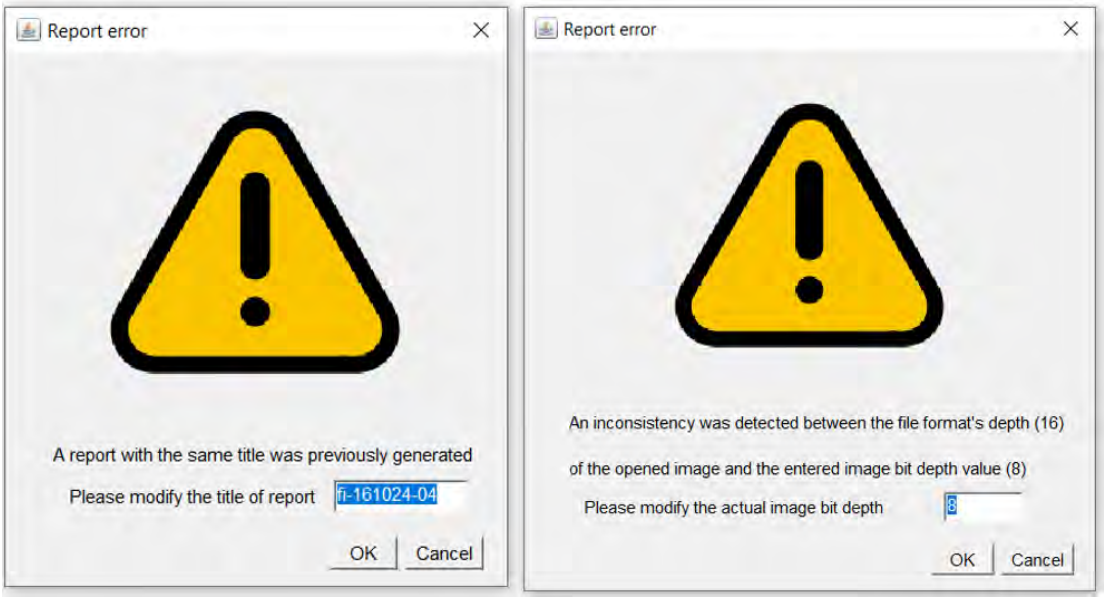


Figure 144. Error dialogs triggered by the Batch PSF Profiler tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved! A summary pdf report will be generated, even though the save individual pdf report(s) is left unselected. If this latter option is chosen, each analysed file will generate an individual report.

Description of the Batch PSF Profiler report

The Batch Summary report has similar Microscope info & warnings sections (Figure 145) as compared to the PSF Profiler tool's report. However, while the non-batch version of the report refers to the input image, the batch version only refers to analyzed, individual bead images. For instance, Figure 145 shows the analysis of 7 multiple beads containing images located in the input folder. Out of those, 79 single-bead images were generated. Although one input image was saturated for one channel, no individual valid bead was found and thus this saturated image is not included in the Figure 157 table).

Warnings are provided to help result interpretation.

bpp-250924-08 - Batch Summary

Microscope info:

data		79 analysed images				
images location		C:\Users\julien.cau\Desktop\MetroloJ QC Test\psf\40\				
Actual image depth		16				
Microscope type		WideField				
Objective	NA	1.4				
	im. refractive index	1.515				
Channel(s)		Wavelengths		unsaturated/total images	sampling (X,Y,Z)	
		Ex. (nm)	Em. (nm)		Nyquist (µm)	correctly sampled/total images
Channel 0			480.0	all ok	0.086x0.086x0.256	(all ok, all ok, 0/79)
Channel 1			525.0	all ok	0.094x0.094x0.28	(all ok, all ok, 0/79)
Channel 2			590.0	all ok	0.105x0.105x0.315	(all ok, all ok, 0/79)
Channel 3			610.0	all ok	0.109x0.109x0.326	(all ok, all ok, 0/79)

Warnings:

(no saturation issue detected)
Undersampling issues reported for one or more files (see Analysed images & beads section below)
(A subresolution bead is used for all channels).

Figure 145. Batch PSF Profiler Tool Report: microscope info and warnings sections.

The resolution table (Figure 146) aggregates all “valid” measurements (ie. with a R^2 value higher than the threshold). Average FWHM values +/- standard deviation are indicated. The number of beads used to compute the average value is indicated (ie. after rejection of poor fitting values & outliers removal if any). The theoretical expected value is indicated within brackets. Mind that the values are averaged whatever their lateral position in the field of view might be (supposing translation invariant PSF).
While the PSF Profiler tool does not calculate any mean values, the batch PSF Profiler tool does. At this point, bead identification is key to get accurate average values. It is recommended, when using “images contain more than one bead” option, to set the best bead identification parameters. Beads are identified using the Find Maxima

algorithm, thus choosing the appropriate identification channel and prominence values is important. Figure 146 shows the batch Profiler Tool's analysis of various images with bead identification channel set to channel0 and a prominence value of 3000. Figure 147 shows the corresponding raw results of lateral (X) resolution of the identification channel 0 (ie. prominence value of 3000). The R2 threshold was set to 0.95, excluding 11 out of 21 measurements. One outlier value (0.152um) was removed (and was actually very close to the theoretical 0.149um). The corresponding average value is 280 nm, with 9 measurements and a standard deviation of 27 nm (10%). If now the prominence is set to 1000, one would think more beads are identified and analyzed. Figure 148 and Figure 149 show that this is the case (43 found beads, 14 valid images for Channel 0/X and 32 below R2-threshold excluded measurements). However, 3 measurements are aberrant, below-resolution values is high and those are not detected as outliers. Moreover, 2 of them are quite close to the theoretical 0.149um. Lowering the prominence value results in an average resolution value of 212 nm but a high standard deviation (99 nm, 47%), 5 new "valid" measurements including 3 aberrant values and two measurements corresponding to a value that was previously considered as outlier when prominence was set to 3000.

Channel 0	average FWHM (µm)	0.28	0.288	1.112
	FWHM std dev (µm)	0.027	0.025	0.416
	theoretical value (µm)	0.149	0.149	0.561
	number of beads	9	7	5
	mean R2 value	0.98	0.96	0.97
	mean SBR value		39.37	

Figure 146. Batch PSF Profiler Tool Report: average resolutions section (prominence 3000, rejection distance 2um)

Channel0, X

image	Bead X coordinate	Bead Y coordinate	Status	FWHM (µm)	R2	SBR
40x-02_bead6	448	2146	R2 below R2 Threshold	0,092627475	0,877309229	40,22366342
40x-02_bead9	2201	1971	R2 below R2 Threshold	0,113702564	0,87418925	35,58609873
40x-02_bead8	2063	1948	R2 below R2 Threshold	0,125716591	0,863329542	34,46664942
40x-02_bead1	1815	1525	R2 below R2 Threshold	0,185515343	0,919586081	45,81903121
40x-02_bead5	760	1507	R2 below R2 Threshold	0,187428162	0,815312819	41,0177374
40x-02_bead10	2538	972	R2 below R2 Threshold	0,187505821	0,895497646	33,82678376
40x-02_bead0	1600	1872	R2 below R2 Threshold	0,204797768	0,888849169	43,56844262
40x-02_bead3	1402	2371	R2 below R2 Threshold	0,219668048	0,78650936	42,81408586
40x-02_bead4	1769	1796	R2 below R2 Threshold	0,264172012	0,720805363	36,23093671
40x-03_bead4	366	244	R2 below R2 Threshold	0,266146678	0,916175284	36,46568707
40x-03_bead0	247	165	R2 below R2 Threshold	0,316050491	0,945629793	46,99143584
40x-02_bead7	522	1606	outlier (below lower fence of 0,163 µm)	0,152524294	0,961645567	32,71648045
40x-02_bead2	1152	2338	valid	0,229519623	0,9902983	43,74554376
40x-03_bead1	334	476	valid	0,248231977	0,967006606	40,61583523
40x-03_bead5	113	423	valid	0,257946739	0,95066149	36,43041676
40x-03_bead2	34	367	valid	0,27184835	0,98817661	42,4998319
40x-01_bead1	82	111	valid	0,295723277	0,979969266	32,36114535
40x-01_bead0	519	258	valid	0,298154858	0,986220087	57,54848292
40x-03_bead3	478	413	valid	0,305011712	0,975804185	37,34877083
40x-01_bead2	306	460	valid	0,305519534	0,990701867	31,86820314
40x-single_bead0	43	44	valid	0,305519545	0,990701867	31,86820314

Prominence 3000

	Xres Lower Fence (µm)	Xres Upper Fence (µm)
Channel0	0,163	0,39

Figure 147. Selection of raw Res_x values for channel 0 (those which R2 is below the 0.95 threshold value are hidden) from the Figure 146 analysis (prominence 3000, rejection distance 2um)

Channel 0	average FWHM (µm)	0.212	0.191	0.682
	FWHM std dev (µm)	0.099	0.117	0.49
	theoretical value (µm)	0.149	0.149	0.561
	number of beads	14	13	16
	mean R2 value	0.98	0.96	0.98
	mean SBR value		36.23	

Figure 148. Batch PSF Profiler Tool Report: average resolutions section (prominence 1000, rejection distance 2um)

Channel0, X

image	Bead X coordinate	Bead Y coordinate	Status	FWHM (µm)	R2	SBR
40x-02_bead25	1630	1506	R2 below R2 Threshold	0,035985849	0,908396874	61,77799914
40x-02_bead30	340	693	R2 below R2 Threshold	0,03658324	0,866550012	26,72104704
40x-02_bead31	1253	96	R2 below R2 Threshold	0,037075378	0,891781634	68,33580981
40x-02_bead18	2305	2215	R2 below R2 Threshold	0,038574295	0,762331482	34,89058734
40x-02_bead29	1942	31	R2 below R2 Threshold	0,039373179	0,745282003	28,22306376
40x-02_bead13	2236	2682	R2 below R2 Threshold	0,086269098	0,748385492	34,13232184
40x-02_bead14	1667	699	R2 below R2 Threshold	0,091785747	0,925698511	31,2283444
40x-02_bead5	448	2146	R2 below R2 Threshold	0,092627475	0,877309229	40,22366342
40x-02_bead12	1678	1786	R2 below R2 Threshold	0,093168358	0,89023325	32,05353854
40x-02_bead33	381	1188	R2 below R2 Threshold	0,095510583	0,766423031	31,66985195
40x-02_bead23	683	753	R2 below R2 Threshold	0,096092909	0,891358721	26,76643326
40x-02_bead17	1568	488	R2 below R2 Threshold	0,110413625	0,942530709	33,53255695
40x-02_bead7	2063	1948	R2 below R2 Threshold	0,125716591	0,863329542	34,46664942
40x-02_bead16	1873	1595	R2 below R2 Threshold	0,131327528	0,833069684	37,58206327
40x-02_bead24	1792	1997	R2 below R2 Threshold	0,151411257	0,929775332	32,16151101
40x-02_bead9	829	1875	R2 below R2 Threshold	0,169568344	0,925252552	36,95361051
40x-02_bead1	1815	1525	R2 below R2 Threshold	0,185515343	0,919586081	45,81903121
40x-02_bead4	760	1507	R2 below R2 Threshold	0,187428162	0,815312819	41,0177374
40x-02_bead8	2538	972	R2 below R2 Threshold	0,187505821	0,895497646	33,82678376
40x-02_bead0	1600	1872	R2 below R2 Threshold	0,204797768	0,888849169	43,56844262
40x-02_bead21	2191	1106	R2 below R2 Threshold	0,215181794	0,879076587	30,47155445
40x-02_bead2	1402	2371	R2 below R2 Threshold	0,219668048	0,78650936	42,81408586
40x-02_bead10	987	1018	R2 below R2 Threshold	0,250202838	0,688966924	31,940182
40x-02_bead20	1003	1592	R2 below R2 Threshold	0,256897984	0,457913266	30,14051078
40x-02_bead34	2634	2537	R2 below R2 Threshold	0,260763109	0,605074748	25,61972875
40x-02_bead3	1769	1796	R2 below R2 Threshold	0,264172012	0,720805363	36,23093671
40x-03_bead4	366	244	R2 below R2 Threshold	0,266146678	0,916175284	36,46568707
40x-02_bead35	1777	1602	R2 below R2 Threshold	0,309925272	0,865539784	28,14249496
40x-03_bead0	247	165	R2 below R2 Threshold	0,316050491	0,945629793	46,99143584
40x-02_bead15	2213	1672	R2 below R2 Threshold	0,332931648	0,778906274	33,34933212
40x-02_bead26	2454	704	R2 below R2 Threshold	0,439111645	0,478210573	30,80848807
40x-02_bead27	1444	941	R2 below R2 Threshold	0,457605894	0,415623196	27,726714
40x-02_bead11	2760	1315	valid	0,028185345	0,999827353	34,72839418
40x-02_bead28	1233	762	valid	0,030784739	0,999290948	26,8726556
40x-02_bead32	2224	33	valid	0,076072357	0,989902924	26,42428135
40x-02_bead6	522	1606	valid	0,152524294	0,961645567	32,71648045
40x-02_bead22	2059	126	valid	0,167127097	0,977052412	27,99738234
40x-02_bead19	2508	1741	valid	0,220723373	0,957155609	47,92725117
40x-03_bead1	334	476	valid	0,248231977	0,967006606	40,61583523
40x-03_bead5	113	423	valid	0,257946739	0,95066149	36,43041676
40x-03_bead2	34	367	valid	0,27184835	0,98817661	42,4998319
40x-01_bead1	82	111	valid	0,295723277	0,979969266	32,36114535
40x-01_bead0	519	258	valid	0,298154858	0,986220087	57,54848292
40x-03_bead3	478	413	valid	0,305011712	0,975804185	37,34877083
40x-01_bead2	306	460	valid	0,305519534	0,990701867	31,86820314
40x-single_bead0	43	44	valid	0,305519545	0,990701867	31,86820314

Prominence 1000

	Xres Lower Fence (µm)	Xres Upper Fence (µm)
Channel0	-0,066	0,517

Figure 149. Batch PSF Profiler Tool: Selection of raw Res_x values for channel 0 (those which R2 is below the 0.95 threshold value are hidden) from the Figure 148 analysis (prominence 1000, rejection distance 2um)

Another aspect is how the analyzed bead are centered within the stack. Using the same input dataset, with a prominence value of 3000, if identified beads that lie too close to the top/bottom of the stack are kept (rejection distance of 0.5um in Figure 150), then more beads are analyzed (30, Figure 151), however 16 are rejected because of low R2 gaussian fit values. One outlier value (well below theoretical resolution) is discarded and 13 valid measurements are averaged. The average

resolution is 261nm, while the standard deviation is 43nm (16%). 4 more beads are taken into consideration, with FWHM of 153, 228, 232 and 261nm respectively. The first two additional beads are clearly off-centered and yield the lowest FWHM of the group.

Channel 0	average FWHM (µm)	0.261	0.235	1.231
	FWHM std dev (µm)	0.043	0.091	0.43
	theoretical value (µm)	0.149	0.149	0.561
	number of beads	13	11	8
	mean R2 value	0.97	0.97	0.97
	mean SBR value		39.04	

Figure 150. Batch PSF Profiler Tool: Batch PSF Profiler Tool Report: average resolutions for Channel 0 (prominence 3000, rejection distance of 0.5um)

Channel0, X

image	Bead X coordinate	Bead Y coordinate	Status	FWHM (µm)	R2	SBR
40x-02_bead10	448	2146	R2 below R2 Threshold	0,092627475	0,877309229	40,22366342
40x-02_bead16	2201	1971	R2 below R2 Threshold	0,113702564	0,87418925	35,58609873
40x-02_bead12	1934	2019	R2 below R2 Threshold	0,121289175	0,846610625	32,52128192
40x-02_bead14	2063	1948	R2 below R2 Threshold	0,125716591	0,863329542	34,46664942
40x-02_bead2	1815	1525	R2 below R2 Threshold	0,185515343	0,919586081	45,81903121
40x-02_bead8	760	1507	R2 below R2 Threshold	0,187428162	0,815312819	41,0177374
40x-02_bead17	2538	972	R2 below R2 Threshold	0,187505821	0,895497646	33,82678376
40x-02_bead1	1600	1872	R2 below R2 Threshold	0,204797768	0,888849169	43,56844262
40x-02_bead4	1402	2371	R2 below R2 Threshold	0,219668048	0,78650936	42,81408586
40x-02_bead7	1769	1796	R2 below R2 Threshold	0,264172012	0,720805363	36,23093671
40x-03_bead6	366	244	R2 below R2 Threshold	0,266146678	0,916175284	36,46568707
40x-03_bead5	164	497	R2 below R2 Threshold	0,271852243	0,911960508	37,47792547
40x-02_bead15	327	1963	R2 below R2 Threshold	0,295588328	0,590967874	33,41613858
40x-02_bead9	1137	1437	R2 below R2 Threshold	0,314740904	0,701106436	35,7924987
40x-03_bead0	247	165	R2 below R2 Threshold	0,316050491	0,945629793	46,99143584
40x-02_bead0	2082	2058	R2 below R2 Threshold	0,397680246	0,835150282	59,72597965
40x-02_bead11	1946	982	outlier (below lower fence of 0,127µm)	0,030816768	0,999952045	43,5198347
40x-02_bead13	522	1606	valid	0,152524294	0,961645567	32,71648045
40x-02_bead6	2338	2408	valid	0,227507269	0,96870168	46,93025613
40x-02_bead3	1152	2338	valid	0,229519623	0,9902983	43,74554376
40x-02_bead5	675	1982	valid	0,231683085	0,956473914	38,97544909
40x-03_bead1	334	476	valid	0,248231977	0,967006606	40,61583523
40x-03_bead7	113	423	valid	0,257946739	0,95066149	36,43041676
40x-03_bead3	478	51	valid	0,261736728	0,958385509	34,58705078
40x-03_bead2	34	367	valid	0,27184835	0,98817661	42,4998319
40x-01_bead1	82	111	valid	0,295723277	0,979969266	32,36114535
40x-01_bead0	519	258	valid	0,298154858	0,986220087	57,54848292
40x-03_bead4	478	413	valid	0,305011712	0,975804185	37,34877083
40x-01_bead2	306	460	valid	0,305519534	0,990701867	31,86820314
40x-single_bead0	43	44	valid	0,305519545	0,990701867	31,86820314

Prominence 3000

	Xres Lower Fence (µm)	Xres Upper Fence (µm)
Channel0	0.127	0.401

Figure 151. Batch PSF Profiler Tool: Batch PSF Profiler Tool Report: Selection of raw Res_x values for channel 0 (those which R2 is below the 0.95 threshold value are hidden) from the Figure 150 analysis (prominence 3000, rejection distance of 0.5um)

It is also wise to have a look at raw values. This can be done using the processed/title/title_BatchRawData.xls file. Figure 152 show how Status is displayed in the _BatchRawData.xls file. Whenever the outliers option is selected, a lower and upper fences table is added to the _BatchRawData.xls file (Figure 154).

Channel0, X	image	40x-01_bead0	40x-02_bead7	40x-02_bead8	40x-02_bead9	40x-02_bead10	40x-03_bead0
	Bead X coord	519.0	522.0	2063.0	2201.0	2538.0	247.0
	Bead Y coord	258.0	1606.0	1948.0	1971.0	972.0	165.0
	Status	valid	outlier (below lower fence of 0.163µm	R2 below R2 Threshold	R2 below R2 Threshold	R2 below R2 Threshold	R2 below R2 Threshold
	FWHM (Âµm)	0.2981548582303591	0.15252429351748056	0.12571659098717686	0.11370256404676836	0.1875058213314181	0.31605049074448377
	R2	0.9862200867278583	0.9616455669316629	0.8633295422548682	0.8741892499032499	0.8954976463067221	0.9456297928840298
	SBR	57.548482917147545	32.716480447279764	34.46664941754257	35.58609873122386	33.82678375788315	46.99143584453527

Figure 152. Batch PSF Profiler Tool: An example of raw Res_x values for channel 1 with valid, below R2 Threshold and outlier statuses.

Outliers values

	Xres Lower Fence (µm)	Xres Upper Fence (µm)	Yres Lower Fence (µm)	Yres Upper Fence (µm)	Zres Lower Fence (µm)	Zres Upper Fence (µm)
Channel0	0.163	0.39	0.18	0.372	-0.828	3.183
Channel1	0.206	0.381	0.22	0.391	0.029	1.685
Channel2	0.189	0.387	0.212	0.406	-0.299	2.118
Channel3	0.19	0.415	0.266	0.486	-0.974	2.742

Figure 153. Batch PSF Profiler Tool: the outliers fences values.

The Measured/theoretical resolution ratios and lateral asymmetry ratios table allows quick monitoring on how the objective/microscope performs. If the “Apply tolerances in the final report” was ticked, values below/above XY ratio tolerance or Z ratio tolerance are highlighted in green/red respectively. The mean asymmetry ratios are calculated as PSF Profiler Tool, using average X and Y FWHM (Figure 154).

Measured/theoretical resolution ratios and lateral asymmetry ratios:

Channel	X ratio	Y ratio	Z ratio	Lateral Asymmetry
Channel 0	1.75	1.57	2.19	0.9
Channel 1	1.77	1.87	1.38	0.95
Channel 2	1.53	1.63	1.34	0.94
Channel 3	1.53	1.54	0.96	1.0

Green: within specifications, red: outside specifications (ie. XY ratios above 1.0 or Z ratio above 2.0)

Figure 154. Batch PSF Profiler tool Report. Ratio section.

Finally, a table containing all used analysis parameters is provided (Figure 155). All generated files can be found in the processed>title subfolder (Figure 156). Each individual image is analyzed and triggers a report (even though analysis doesn’t proceed as saturation is found in each channel for instance). The summary reports can be found in the processed/title/title_BatchSummary.pdf. Spreadsheet files are saved as well: processed/title/title_data/title_BatchSummary.xls files contains all tables of the pdf file, while the title_BatchRawData.xls contains all raw data. If using the multiple beads-containing option, this file includes the original bead coordinates. If using outliers exclusion option, the BatchRawData file includes values of respective lower and upper fences of the outliers analyses.

Analysis parameters

Tool & Operator	Tool	Batch PSF Profiler
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	, 9 août 2024 18:14
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\psf\40\\Processed\bpp090824-28\bpp090824-28
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Beads	Bead detection threshold	Legacy
	Center detection method	Legacy Maximum Intensity
	Background annulus thickness in µm	1.0
	Background annulus distance to bead edges in µm	0.5
	Multiple beads in image	true
	Bead identification method	Using Find Maxima (prominence of 3000.0)
	Bead identification channel	0
	Bead size (µm)	0.2
	Bead crop Factor	10.0
	Cropped ROI size in µm	3.52x3.52 (using bead size & background annulus parameters)
	Bead rejection distance to top/bottom	0.5 µm
Square Root PSF Image displayed		true
Tolerance	Applied in this report	true
	X & Y FWHM ratios valid if below	1.0
	Z FWHM ratio valid if below	2.0
Measurement rejected	Outliers	true
	R2 ratio below	0.95

Figure 155. Batch PSF Profiler tool Report: the analysis parameters section.

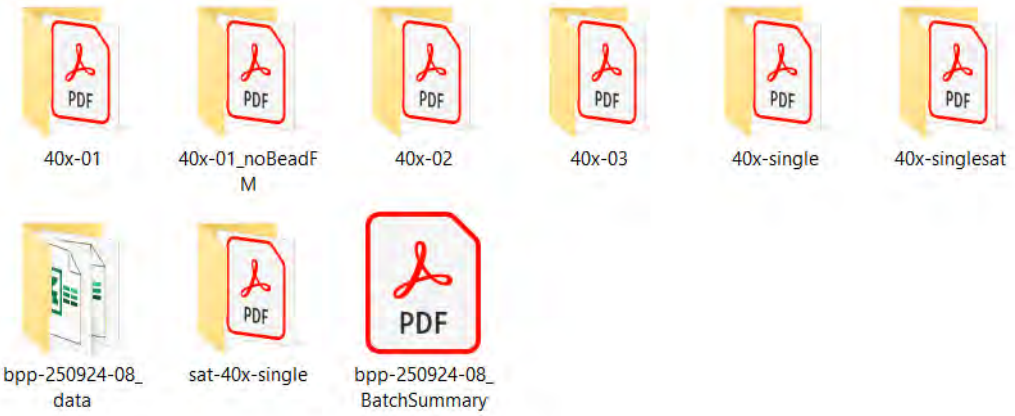


Figure 156. Batch PSF Profiler tool Report: the list of analysed images/beads.

Following the analysis parameter table, a log file provides a summary of how each input file was processed (see Figure 157). In this example, the first input file, 40x-01, had 43 beads identified by the findMaxima algorithm, but only 3 were considered "valid" beads (i.e., sufficiently distant from other identified beads, edges, or the top/bottom of the stack). The third image opened (40x-02) resulted in a significantly higher number of analyses.

Analysis log

image name	creation date	sampling density	identified raw beads	valid beads	saturation	status
40x-01	2024-06-13 09:14:22	Ch.0,1,2,3 undersampled	43	3	none	valid beads found
				bead0	none	analysed
				bead1	none	analysed
				bead2	none	analysed
40x-01_noBeadFM	2024-07-17 08:15:21	Ch.0,1,2,3 undersampled	18	0	none	no valid beads found, not analysed
40x-02	2024-06-13 09:14:22	Ch.0,1,2,3 undersampled	75	51	none	valid beads found
				bead0	none	analysed
				bead1	none	analysed
				bead2	none	analysed
				bead3	none	analysed
				bead4	none	analysed
				bead5	none	analysed
				bead6	none	analysed
				bead7	none	analysed
				bead8	none	analysed
				bead9	none	analysed
				bead10	none	analysed
				bead11	none	analysed
				bead12	none	analysed
				bead13	none	analysed
				bead14	none	analysed
				bead15	none	analysed
				bead16	none	analysed
				bead17	none	analysed
				bead18	none	analysed
				bead19	none	analysed
				bead20	none	analysed
				bead21	none	analysed
				bead22	none	analysed

Figure 157. Batch PSF Profiler Tool: a (truncated) log file.

BATCH CO-REGISTATION TOOL

Analysis dataset requirements for Batch Co-registration Tool

Images to analyze should be stored in the same folder (if images are in subfolders, they will not be analyzed). As bead values will be averaged, the key point is to store similar (multichannel) z-stack (e.g., 5 4-channels Z stacks of the same objective). The stack dimensions (width, height or slices) can be different, as can be their calibrations. However, it does not make so much sense to compare values derived from different voxel sizes. The number of channels and the excitation/emission values have to be the same.

The plugin is a batch version of the Co-registration tool. It will scan all files in a user-defined folder and check whether these are bioformats compatible. Subfolder will not be investigated. Then, each bioformats-compatible file is processed as with the co-registration tool.

A final SUMMARY pdf report aggregates all values.

Batch Co-registration Tool Parameters

STEP1. To use the plugin, Start ImageJ, launch the MetroloJ_QC bar (plugins>MetroloJ_QC).
STEP2. Select the Batch co-registration tool then select the directory containing the images to analyze (Figure 158). Mind no subfolder will be investigated and all files should have the same # of channels (and wavelengths are supposed to be all identical). The images may have different pixel sizes, number of slices, width or height. All files should have the same # of channels (and wavelengths are supposed to be all identical). The first found bioformats-compatible image will be opened and the plugin's main dialog interface should appear (see Figure 159).

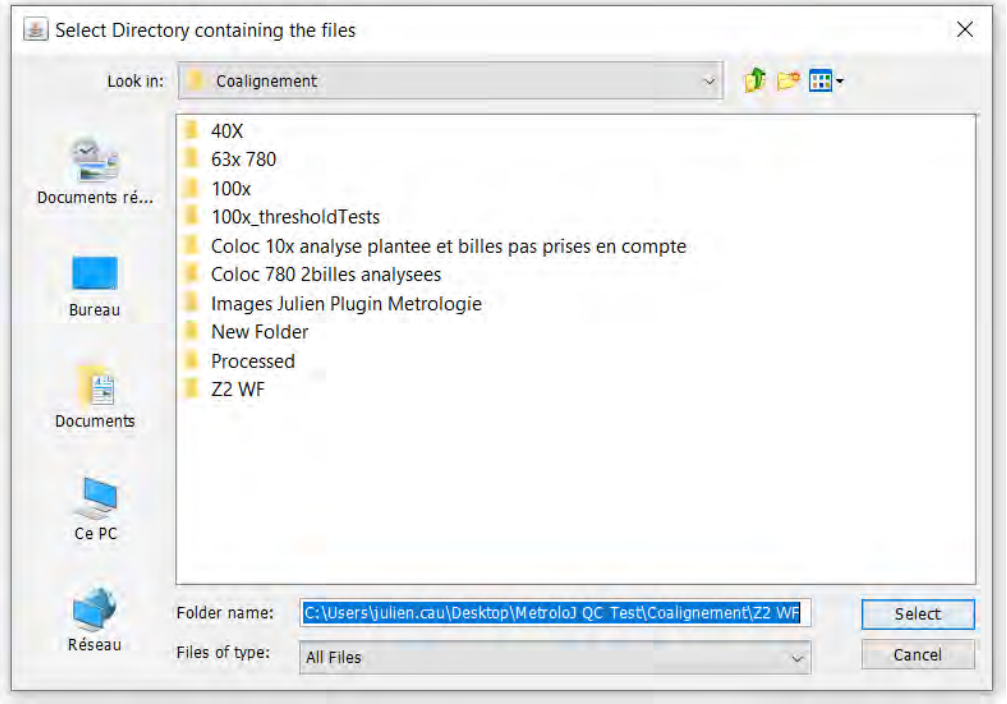


Figure 158. Batch Co-registration tool: first dialog window.

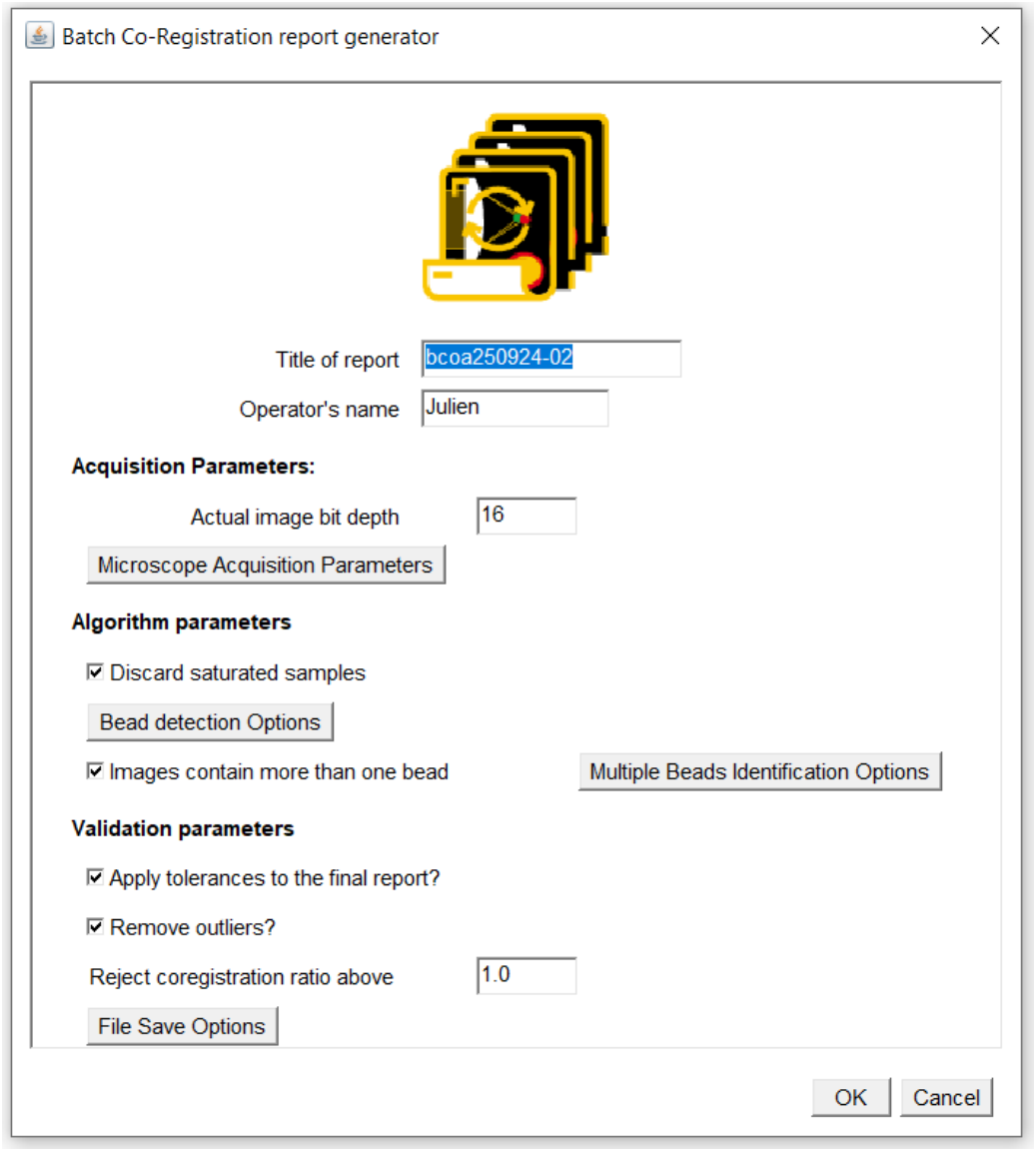


Figure 159. Batch Co-registration Tool: the user's interface/main dialog window

STEP3. Fields should be filled-in as for the co-registration tool. An additional "Remove outliers" field allows the removal of aberrant values (Validation parameters section). The list of values to be averaged is ordered, the median and Q1/Q3 values are calculated. The interquartile range IQR=Q3-Q1 is calculated. If values are different (IQR is not 0), then outliers are removed as follow. All values below Q1 - 1.5 * IQR (lower fence) and above Q3+1,5*IQR are discarded. When checked, outliers will be removed if the sample size is bigger than 5 analyzed beads. The title_BatchRawData .xls file (in processed/title/title_data folder) includes a table of the values of the lower and upper fences. Mind the number of total beads analyzed ("Microscope section" of the main report) is not the number of total beads used at the end to compute each mean ratio.

STEP4. In the File Save Options, decide whether pdf report should be open (Figure 160).

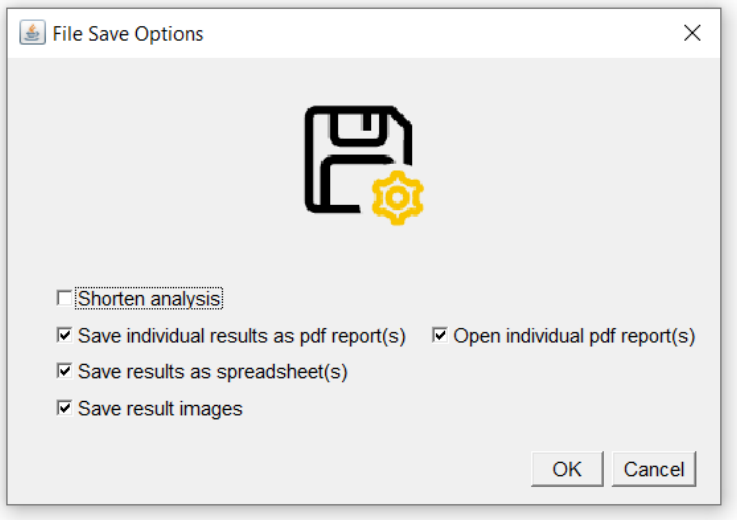


Figure 160. Batch Field Illumination Tool: the file save options dialog

STEP4. In the File Save Options, decide whether pdf report should be open (Figure 130).

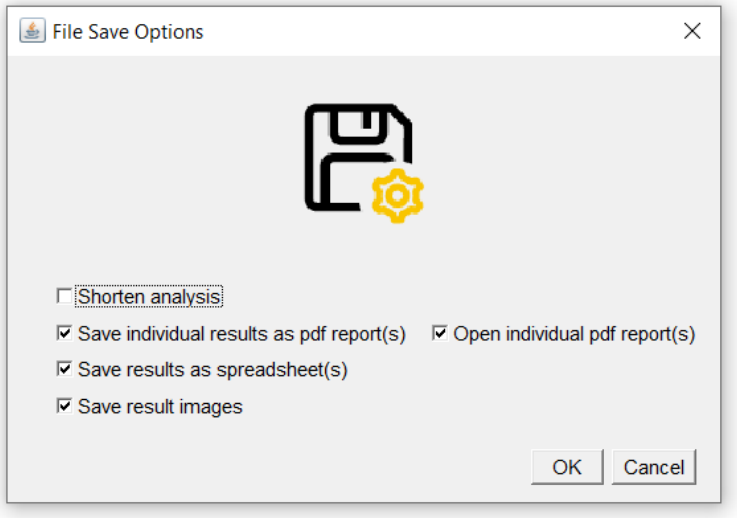


Figure 161. Batch Field Illumination Tool: the file save options dialog

STEP5. You may encounter various error messages. If a previous report was generated, the error dialog shown in Figure 162 (left panel) will appear. In this case, change the title.
MetroloJ_QC is intended for 8-bit and 16-bit file format images. When inconsistencies are detected between the declared bit depth (at STEP3) and the actual file format

depth, a different type of error message is triggered (Figure 162, right panel). These inconsistencies occur when:

- 8-bits files format images are declared as more than 8-bits images
- 16 bits file format images are declared as 8 or 32-bits images or when declared 10-, 12- and 14 bits images are not 16-bits file format images
- 32-bits files format images are not declared as 32-bits images

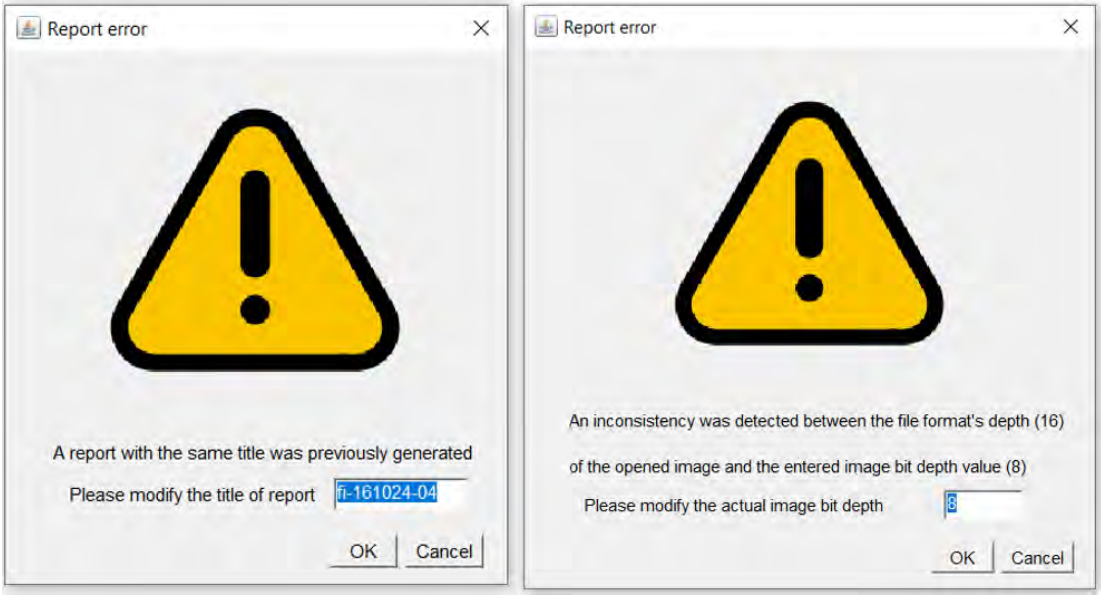


Figure 162. Error dialogs triggered by the Batch co-registration tool

Correct this and declare an appropriate. If there is no more error message, the report is generated, and appropriate files are saved! A summary pdf report will be generated, even though the save individual pdf report(s) is left unselected. If this latter option is chosen, each analysed file will generate an individual report.

Description of the Batch Co-registration tool report.

The main Batch Summary report starts with the Microscope info & Warnings sections (Figure 163) report a summary of all acquisition parameters as in the co-registration tool report. The number of total beads analyzed is reported at the end of the section. The number of saturated channels analyzed and correctly sampled channels is also given.

bcoa250924-14 - Batch Summary

Microscope info:

data		6 analysed images				
images location		C:\Users\julien.cau\Desktop\MetroloJ QC Test\Coalignement\40X\				
Actual image depth		16				
Microscope type		WideField				
Objective	NA	1.4				
	im. refractive index	1.515				
Channel(s)		Wavelengths		unsaturated/total images	sampling (X,Y,Z)	
		Ex. (nm)	Em. (nm)		Nyquist (µm)	correctly sampled/total images
Channel 0			480.0	4/6	0.086x0.086x0.256	(0/6, 0/6, all ok)
Channel 1			525.0	3/6	0.094x0.094x0.28	(0/6, 0/6, all ok)
Channel 2			590.0	all ok	0.105x0.105x0.315	(0/6, 0/6, all ok)
Channel 3			610.0	all ok	0.109x0.109x0.326	(0/6, 0/6, all ok)

Warnings:

Saturation issues reported for one or more files (see analysis log section below)
Undersampling issues reported for one or more files (see Analysed images & beads section below)
(The bead size is appropriate for this coalignment analysis).
Outlier values were removed whenever the sample is 5 and more measurements.

Figure 163. Batch Coregistration tool Report: microscope info and warnings report sections.

The Ratio table (Figure 164) summarizes all generated co-registration reports. The mean ratio +/- standard deviation of the ratio is displayed (the standard deviation is computed if n>3). Whenever the option was chosen, the within and outside specs values are highlighted in green/red. The number of outside specs beads is also indicated, for each combination. It remains up to the user to investigate why some bead ratios are outside specs. For this purpose, the user may refer to the title_BatchSummary.xls spreadsheet and the title_BatchRawData.xls files where raw ratio table is provided for each bead image/combination. A mean ratio can be valid even though some beads have an out-of-bounds ratio (here >1) as shown in Figure 164 with channel 1 vs channel 3 combination.

Mean Ratios table:

		Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	ratio		0.455 +/- 0.099	0.672 +/- 0.063	1.296 +/- 0.19	1.548 +/- 0.265
	beads analysed		11.0	11.0	11.0	11.0
	out of bounds beads (%)		0.0 (0.0%)	0.0 (0.0%)	11.0 (100.0%)	11.0 (100.0%)
Channel 1	ratio	0.455 +/- 0.099		0.508 +/- 0.157	0.943 +/- 0.293	1.226 +/- 0.343
	beads analysed	11.0		12.0	12.0	12.0
	out of bounds beads (%)	0.0 (0.0%)		0.0 (0.0%)	5.0 (41.67%)	8.0 (66.67%)
Channel 2	ratio	0.672 +/- 0.063	0.508 +/- 0.157		0.944 +/- 0.148	1.144 +/- 0.255
	beads analysed	11.0	12.0		12.0	12.0
	out of bounds beads (%)	0.0 (0.0%)	0.0 (0.0%)		6.0 (50.0%)	7.0 (58.33%)
Channel 3	ratio	1.296 +/- 0.19	0.943 +/- 0.293	0.944 +/- 0.148		0.328 +/- 0.071
	beads analysed	11.0	12.0	12.0		12.0
	out of bounds beads (%)	11.0 (100.0%)	5.0 (41.67%)	6.0 (50.0%)		0.0 (0.0%)
Channel 4	ratio	1.548 +/- 0.265	1.226 +/- 0.343	1.144 +/- 0.255	0.328 +/- 0.071	
	beads analysed	11.0	12.0	12.0	12.0	
	out of bounds beads (%)	11.0 (100.0%)	8.0 (66.67%)	7.0 (58.33%)	0.0 (0.0%)	

Green: within specifications, red: outside specifications (ie. ratio above 1.0)

Figure 164. Batch Co-registration report: an example of the mean ratio table.

Long version of the report include average pixel shift tables, uncalibrated and calibrated distances (Figure 165, Figure 166 and Figure 167).

Mean Pixel shift table:

		Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	X shift		-0.364 +/- 0.223 (n=11.0)	-0.5 +/- 0.477 (n=11.0)	-0.409 +/- 0.557 (n=11.0)	-0.227 +/- 0.719 (n=11.0)
	Y shift		0.455 +/- 0.334 (n=11.0)	-0.045 +/- 0.334 (n=11.0)	1.727 +/- 0.617 (n=11.0)	2.045 +/- 0.916 (n=11.0)
	Z shift		-1.727 +/- 0.225 (n=11.0)	-2.955 +/- 0.179 (n=11.0)	-3.477 +/- 0.345 (n=11.0)	-4.227 +/- 0.291 (n=11.0)
Channel 1	X shift	0.364 +/- 0.223 (n=11.0)		-0.208 +/- 0.594 (n=12.0)	-0.125 +/- 0.65 (n=12.0)	0.042 +/- 0.853 (n=12.0)
	Y shift	-0.455 +/- 0.334 (n=11.0)		-0.542 +/- 0.477 (n=12.0)	1.208 +/- 0.776 (n=12.0)	1.458 +/- 1.163 (n=12.0)
	Z shift	1.727 +/- 0.225 (n=11.0)		-1.208 +/- 0.2 (n=12.0)	-1.75 +/- 0.25 (n=12.0)	-2.521 +/- 0.19 (n=12.0)
Channel 2	X shift	0.5 +/- 0.477 (n=11.0)	0.208 +/- 0.594 (n=12.0)		0.083 +/- 0.276 (n=12.0)	0.25 +/- 0.382 (n=12.0)
	Y shift	0.045 +/- 0.334 (n=11.0)	0.542 +/- 0.477 (n=12.0)		1.75 +/- 0.382 (n=12.0)	2.0 +/- 0.736 (n=12.0)
	Z shift	2.955 +/- 0.179 (n=11.0)	1.208 +/- 0.2 (n=12.0)		-0.542 +/- 0.224 (n=12.0)	-1.312 +/- 0.272 (n=12.0)
Channel 3	X shift	0.409 +/- 0.557 (n=11.0)	0.125 +/- 0.65 (n=12.0)	-0.083 +/- 0.276 (n=12.0)		0.167 +/- 0.312 (n=12.0)
	Y shift	-1.727 +/- 0.617 (n=11.0)	-1.208 +/- 0.776 (n=12.0)	-1.75 +/- 0.382 (n=12.0)		0.25 +/- 0.52 (n=12.0)
	Z shift	3.477 +/- 0.345 (n=11.0)	1.75 +/- 0.25 (n=12.0)	0.542 +/- 0.224 (n=12.0)		-0.771 +/- 0.279 (n=12.0)
Channel 4	X shift	0.227 +/- 0.719 (n=11.0)	-0.042 +/- 0.853 (n=12.0)	-0.25 +/- 0.382 (n=12.0)	-0.167 +/- 0.312 (n=12.0)	
	Y shift	-2.045 +/- 0.916 (n=11.0)	-1.458 +/- 1.163 (n=12.0)	-2.0 +/- 0.736 (n=12.0)	-0.25 +/- 0.52 (n=12.0)	
	Z shift	4.227 +/- 0.291 (n=11.0)	2.521 +/- 0.19 (n=12.0)	1.312 +/- 0.272 (n=12.0)	0.771 +/- 0.279 (n=12.0)	
Resolutions (pix.)	X	1.695	1.854	2.048	2.118	2.295
	Y	1.695	1.854	2.048	2.118	2.295
	Z	2.985	3.265	3.607	3.731	4.042

Figure 165. Batch Coalignement tool Report. Mean Pixel shift table.

Mean Distances table (calibrated):

		Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	distance (µm)		0.367 +/- 0.04 µm	0.622 +/- 0.041 µm	0.754 +/- 0.072 µm	0.915 +/- 0.061 µm
	beads analysed		11.0	11.0	11.0	11.0
Channel 1	distance (µm)	0.367 +/- 0.04 µm		0.271 +/- 0.046 µm	0.399 +/- 0.059 µm	0.564 +/- 0.054 µm
	beads analysed	11.0		12.0	12.0	12.0
Channel 2	distance (µm)	0.622 +/- 0.041 µm	0.271 +/- 0.046 µm		0.221 +/- 0.033 µm	0.352 +/- 0.059 µm
	beads analysed	11.0	12.0		12.0	12.0
Channel 3	distance (µm)	0.754 +/- 0.072 µm	0.399 +/- 0.059 µm	0.221 +/- 0.033 µm		0.176 +/- 0.05 µm
	beads analysed	11.0	12.0	12.0		12.0
Channel 4	distance (µm)	0.915 +/- 0.061 µm	0.564 +/- 0.054 µm	0.352 +/- 0.059 µm	0.176 +/- 0.05 µm	
	beads analysed	11.0	12.0	12.0	12.0	
Resolution (in µm)	X	0.175	0.191	0.211	0.219	0.237
	Y	0.175	0.191	0.211	0.219	0.237
	Z	0.657	0.718	0.794	0.821	0.889

Figure 166. Batch Coalignement tool Report. the Mean calibrated distance table.

Mean Distances table (uncalibrated):

		Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	distance (pixels)		1.231 +/- 0.212	2.139 +/- 0.199	3.326 +/- 0.31	4.027 +/- 0.398
	beads analysed		11.0	11.0	11.0	11.0
Channel 1	distance (pixels)	1.231 +/- 0.212		1.302 +/- 0.238	2.274 +/- 0.439	2.993 +/- 0.524
	beads analysed	11.0		12.0	12.0	12.0
Channel 2	distance (pixels)	2.139 +/- 0.199	1.302 +/- 0.238		1.844 +/- 0.246	2.377 +/- 0.434
	beads analysed	11.0	12.0		12.0	12.0
Channel 3	distance (pixels)	3.326 +/- 0.31	2.274 +/- 0.439	1.844 +/- 0.246		0.794 +/- 0.16
	beads analysed	11.0	12.0	12.0		12.0
Channel 4	distance (pixels)	4.027 +/- 0.398	2.993 +/- 0.524	2.377 +/- 0.434	0.794 +/- 0.16	
	beads analysed	11.0	12.0	12.0	12.0	
Resolutions (pix.)	X	1.695	1.854	2.048	2.154	2.401
	Y	1.695	1.854	2.048	2.154	2.401
	Z	2.985	3.265	3.607	3.793	4.229

Figure 167. Batch Co-registration tool Report: the mean uncalibrated distance tables (long version only)

Finally, a table containing all used analysis parameters is provided (Figure 168). All generated files can be found in the processed>title subfolder (Figure 169). Each individual image is analyzed and triggers a report (even though analysis doesn't proceed as saturation is found in each channel for instance). The summary reports can be found in the processed/title/title_BatchSummary.pdf. spreadsheet files are saved as well (even though the "save all data in a spreadsheet" option was left unselected) processed/title/title_BatchSummary.xls files contains all tables of the pdf file, while the title_BatchRawData.xls (in title_data folder) contains all raw data. If using the multiple beads-containing option, this file includes the original bead coordinates. If using outliers exclusion option, the BatchRawData file includes values of respective lower and upper fences of the outliers analyses.

Analysis parameters

Tool & Operator	Tool	Batch Co-Registration
	Versions	MetroloJ_QC v1.3.0, ImageJ v1.53s, Java v22.0.1, OS Windows 10
	Operator & date	, 22 août 2024 16:31
data	result folder	C:\Users\julien.cau\Desktop\MetroloJ QC Test\Coalignement\Z2 WF\Processed\bcoa220824-31\bcoa220824-31
	Type of saved data	.pdf, .jpg, .xls
	Input data bit depth	16
Dimension order		XY-(C)Z
Discard saturated samples		true
Beads	Bead detection threshold	Legacy
	Center detection method	Legacy Fit Ellipses
	Background annulus thickness in µm	1.0
	Background annulus distance to bead edges in µm	0.5
	Multiple beads in image	true
	Bead identification method	Using the bead detection threshold
	Bead identification channel	0
	Bead size (µm)	1.0
	Bead crop Factor	5.0
	Cropped ROI size in µm	5.0x5.0 (using bead size & crop factor parameters)
	Bead rejection distance to top/bottom	0.5 µm
	Reject doublets	false
Tolerance	Applied in this report	true
	Ratio valid if below	1.0
Measurement rejected	Outliers	true

Figure 168. Batch CoAlignement tool Report: the analysis parameters section.

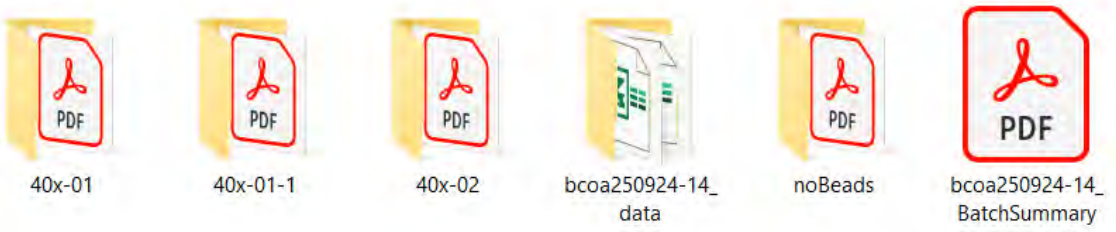


Figure 169. Batch CoAlignement tool Report: files generated.

Following the analysis parameter table, a log file provides a summary of how each input file was processed (Figure 170).

Analysis log

image name	creation date	sampling density	identified raw beads	valid beads	saturation	status
63x-01	2024-08-22 15:33:32	Ch.0,1 undersampled	5	5	none	valid beads found
				bead0	none	analysed
				bead1	none	analysed
				bead2	none	analysed
				bead3	none	analysed
				bead4	none	analysed
63x-02	2024-08-22 15:33:14	Ch.0,1 undersampled	7	5	Ch.0 saturated	valid beads found
				bead0	none	analysed
				bead1	none	analysed
				bead2	none	analysed
				bead3	none	analysed
				bead4	Ch.0 saturated	analysed

Figure 170. Batch CoAlignement Tool: a log file.

As with the Multiple Bead Image Summary of the CoAlignement tool, when images contain more than one bead, the beads detection overlays are displayed at the end of the report as in Figure 171 for each analyzed image.

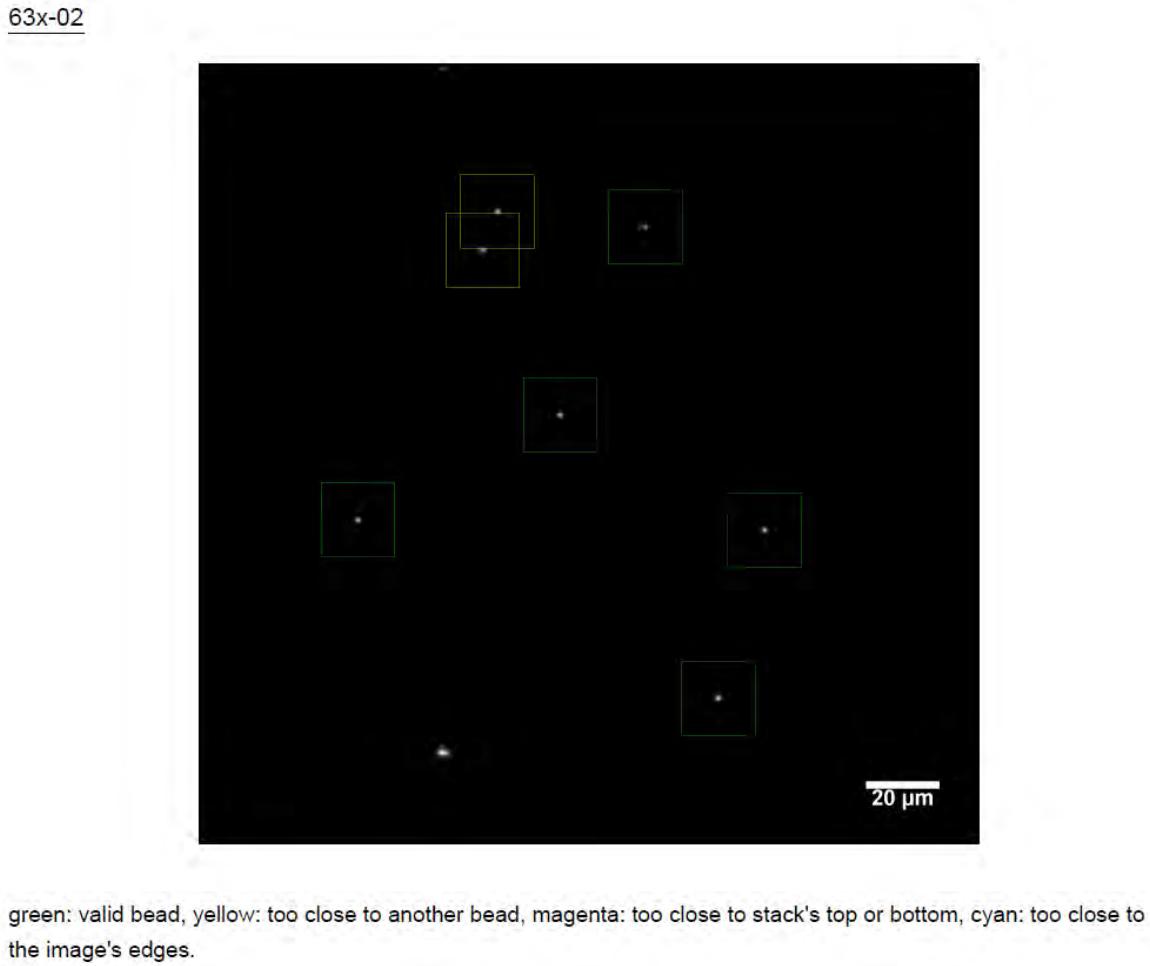


Figure 171. Batch CoAlignement tool: an example of the image overlays displayed for each analyzed image with the “images contain more than one bead” option

PLUGIN'S OPTIONS TOOL

The options' buttons:

Figure 172 shows the available options :

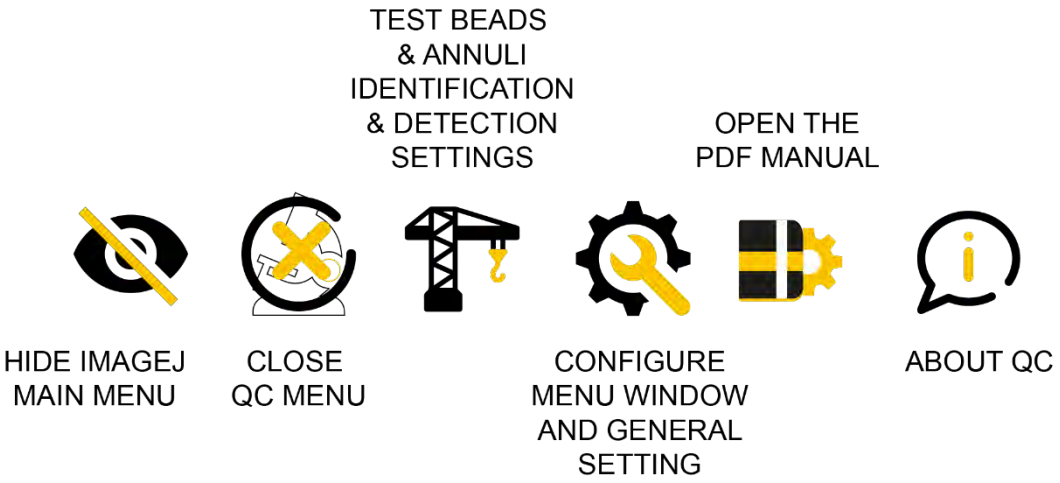


Figure 172. The plugin's options

Below is a short description of the buttons:

Hides the main ImageJ's bar/menu

Closes the MetroloJ QC menu

Opens dialogs to set the best bead/annuli identification (multibeads images) or detection (single bead images) parameters (see below)

Opens a dialog to i) control the visibility of the MetroloJ QC tools on the MetroloJ QC Window and ii) set general parameters (see below)

Open the pdf manual of MetroloJ QC plugin

Display general information about the plugin (including a button to download iText plugin).

CONFIGURATION SECTION (MetroloJ QC Menu configuration & general settings):

Figure 173 illustrates the dialog that appears when you click the configuration button in the MetroloJQC menu/

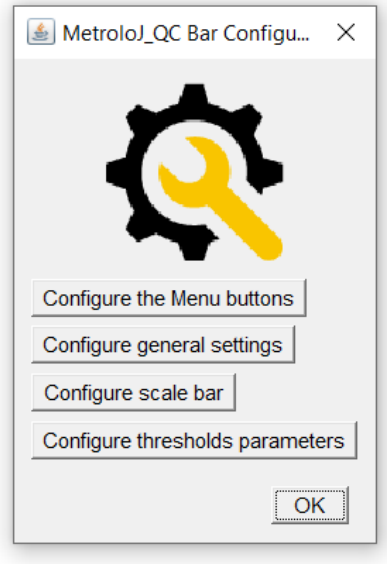


Figure 173. MetroloJ QC configuration dialog

- Click the “Configure the menu buttons” to display or hide various tools in the main MetroloJ_QC bar. This opens the configure the menu buttons Dialog (Figure 174). Select/unselect each individual tool to hide them if needed. The first show batch tool option will hide/display Batch Field Illumination, Batch PSF Profiler Tool & Batch Coregistration tools.

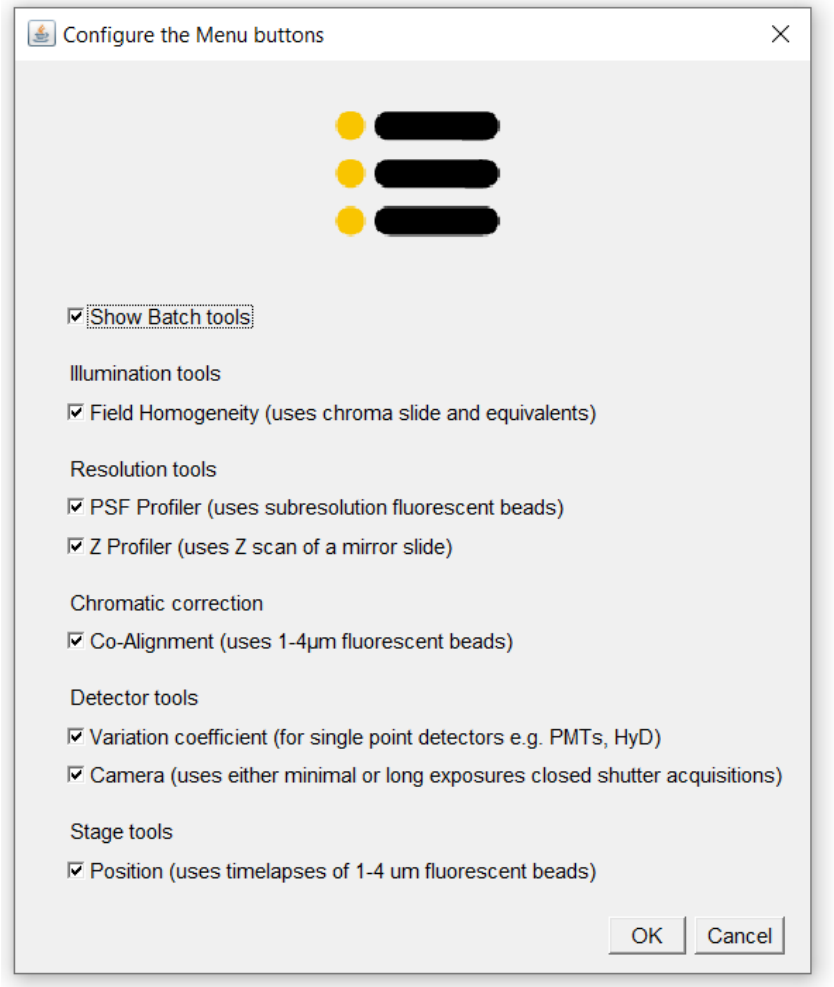


Figure 174. MetroloJ QC “configure the Menu Buttons” dialog

- Click the “Configure general settings button” to change settings that are common to all tools. This opens the Configure general settings Dialog (Figure 175).

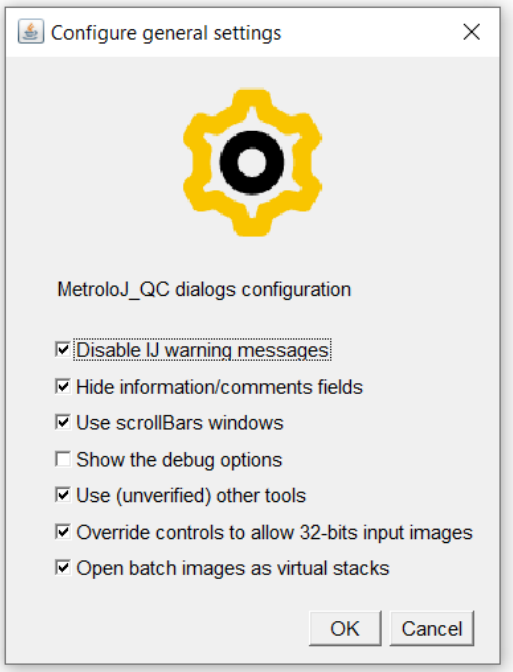


Figure 175. MetroloJ QC “configure general settings” dialog

- Disable IJ's error messages: While it’s generally not recommended if you are using standalone tools, it is advisable to disable these messages when working with batch tools to ensure that the analysis continues uninterrupted (errors will still be logged in the tool’s log table).
- Restore hidden information/comments fields: The original MetroloJ plugin’s dialogs included information and comments that are now hidden. To restore these fields in the dialogs, uncheck the "Hide information/comments fields" option.
- Scrollable dialog windows: The plugin uses scrollable dialog windows by default. If this causes issues, you can switch back to classic window menus.
- Show debug options: This is a developer-specific mode that displays messages in ImageJ’s log window and shows some analysis images that are typically hidden and closed during the analysis. This option is only useful if you have access to the plugin’s Java code.
- Use (unverified) other tools: This option enables additional analyses that are not yet part of the main MetroloJ QC plugin. In version 1.3, this includes extra Field Homogeneity analyses for the QUAREP-LIMI WG3.
- Override controls to allow 32-bits input images: This option removes the initial check that restricts analysis of images saved as 8 or 16 bits files (! a 14-bits images is saved as a 16-bits file format file). PLEASE NOTE THAT THE PROVIDED TOOLS ARE NOT DESIGNED TO WORK WITH 32-BIT IMAGES, AND RESULTS GENERATED FROM SUCH FILES ARE PROVIDED "AS IS."

- Open Batch images as Virtual Stacks: This option enables the “Use Virtual Stack” of the BioFormat importer that is used in batch tools.

Click on OK to go back to the previous Dialog.

- From version 1.3.0 is introduced the possibility to change the scale bar in any generated images. Click the “Configure scale bar” button, this opens the corresponding dialog (Figure 176).

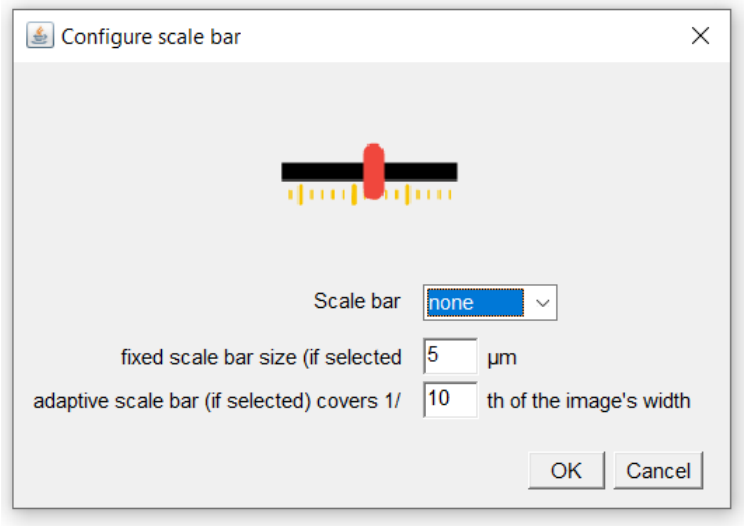


Figure 176. MetroloJ QC “Configure scale bar” dialog

  - To hide the scale bar select “none” in the first rolling menu.
  - Would you like to set a fixed scale bar length, use “Fixed size” and set the appropriate fixed scale bar size in the next numeric field.
  - To use a scale bar that fits a given proportion of the image’s width, use “adaptive” and set the relative size in the last numeric field.

- Additionally, the MetroloJ QC threshold configuration checkboxes allow you to limit the intensity thresholds available in the tools’ dialogs. Click on the “Configure threshold parameters” to open the corresponding dialog (Figure 177).

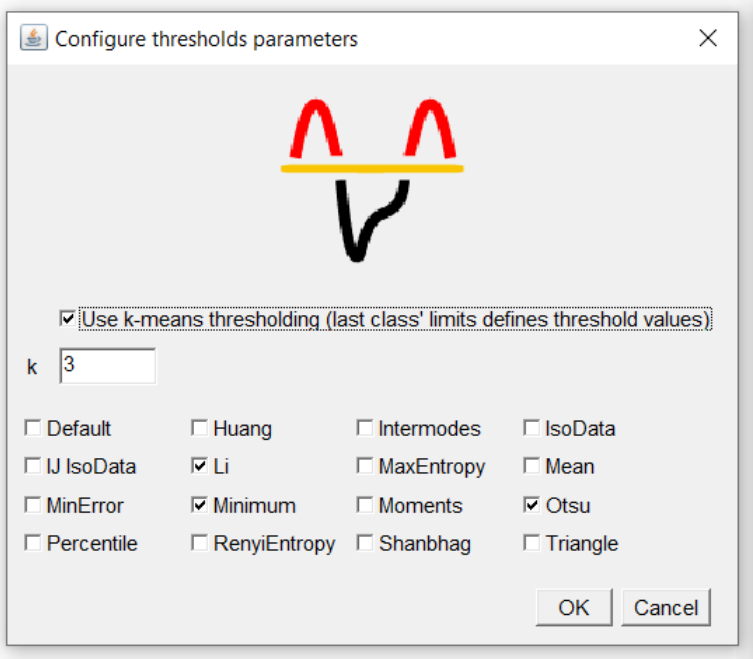


Figure 177. MetroloJ QC "Configure threshold parameters" dialog

- To extend the "legacy threshold histogram segmentation" beyond the 2-class "legacy" threshold, select "use k-means thresholding." As mentioned earlier, the threshold value corresponds to the lower limit of the n^{th} class. This "extended legacy threshold" will be referred to as "k-Means" in the thresholds list. If selected, set the number of classes k.
- If you prefer to replace the legacy histogram segmentation tool with ImageJ's built-in automatic threshold methods, select the desired thresholds from the available list.

Click on OK to go back to the previous Dialog.

TESTS SECTIONS

To ease the process of bead identification (i.e., determining the X, Y, and Z coordinates of beads within an image containing multiple beads, in order to crop the input image into single-bead images) or bead detection (i.e., precisely locating a bead within an image containing just one bead), several additional test tools are available. To access these tools, click the tests crane button.

SEGMENT BEADS TOOL

This tool is helpful for:

- Ensuring that beads are properly detected (e.g., verifying if the identified bead's height exceeds the stack's thickness, as shown in Figure 57).

- Understanding why some beads remain unidentified in an image with multiple beads (i.e., why they are not boxed in the identified beads summary overlay).

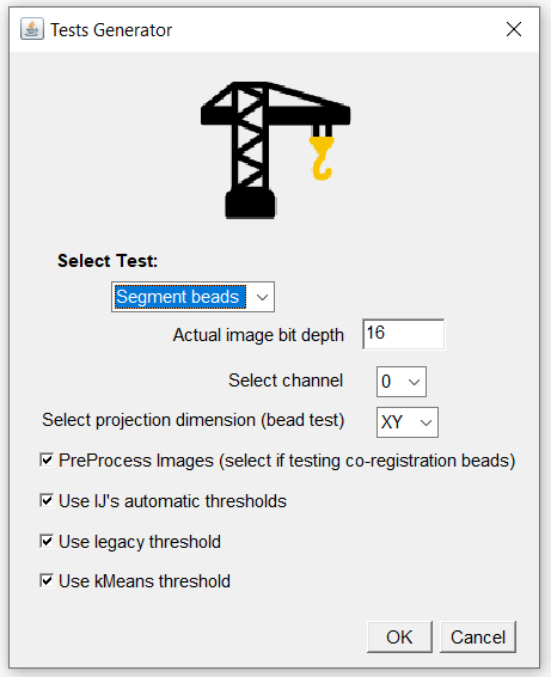


Figure 178. The “segment beads” test tool

STEP A: in the “select test” rolling menu, choose “Segment beads” (Figure 178).

STEP B: Set the image’s bit depth (this parameter is used for the purpose of the legacy/kMeans thresholds calculation. Choose the channel of interest and the projection dimension. If you’re focusing on co-registration bead identification, it’s recommended to select the "preprocess images" option to fully replicate the bead identification process.

STEP C: Choose the threshold methods you want to apply. If you opt for the "use ImageJ’s automatic thresholds" option, the selected built-in ImageJ automatic thresholds (list configured via the "CONFIGURE MENU WINDOW & GENERAL SETTINGS" button) will be applied. Selecting the "k-Means threshold" option will include an additional threshold analysis, using the class value defined in the "CONFIGURE MENU WINDOW & GENERAL SETTINGS" section.

BEAD IDENTIFICATION: The key step in this process is the segmentation of the XY projection. Figure 179 illustrates an example with a mixture of 0.2 µm and 4 µm beads. When using the co-registration tool, it's likely that only the 0.2 µm beads will be retained and boxed, as the segmentation of larger beads may result in objects that are too large compared to the expected 4 µm size. Although the smaller beads should not be considered, they may still be included. The right panel shows a successful

segmentation of the 4 µm beads. The slice title, displayed just above the image in the window, indicates the threshold applied (e.g., "C2_Percentile" in the left panel of Figure 179). If no particles are detected, this will be indicated in the slice title as well.

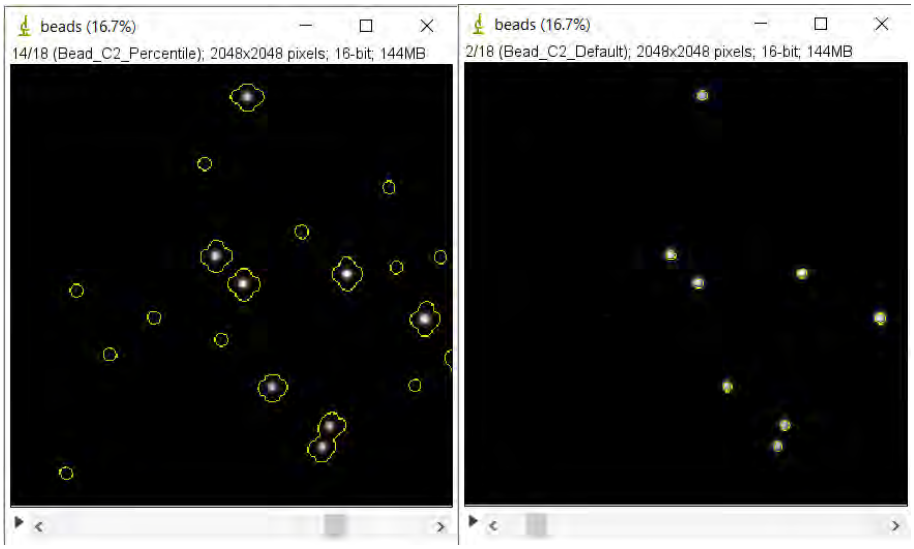


Figure 179. Tests section: an example of segmented multiple beads-containing images.

BEAD DETECTION: In the example of Figure 180, the bead is better detected with the k-Means 3 threshold that with the legacy (k-Means 2) threshold.

Figure 180. Test section : an example of segmented single bead (yellow) (XZ projection)

SEGMENT ANNULI TOOL

This tool will display the montage of the Z slices of a single bead containing 3D stack.

STEP A: in the “select test” rolling menu, choose “Segment Annuli” (Figure 181). Set the image’s bit depth and select the channel of interest.

STEP B: set the bead and annulus size (annulus thickness and inner annulus edge distance).

STEP C: Choose the threshold methods you want to apply (see the SEGMENT BEADS TOOL).

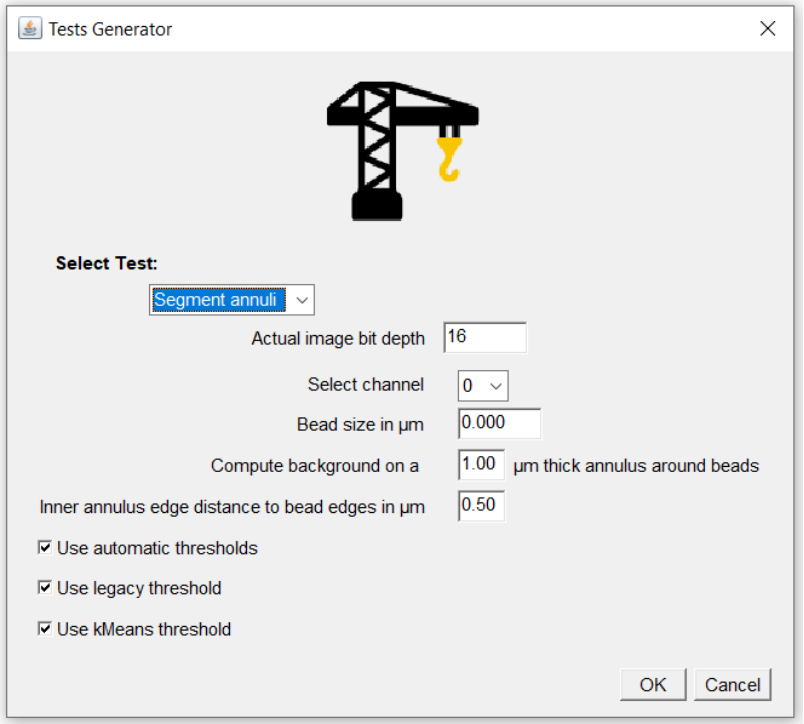


Figure 181. Test section: the segment annuli tool

Figure 182 shows an example of proper segmentation of the annuli of a co-registration 4um bead.

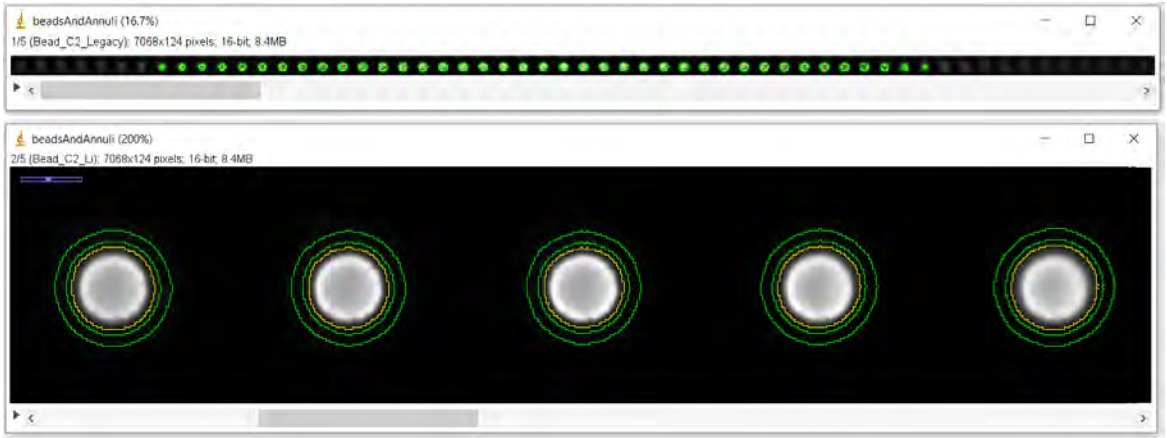


Figure 182. Test section: an example of the annuli detection output. The bead is segmented in yellow (lower panel) and the each sections' annulus is segmented in green (bead size 4um, inner annulus distance to bead edges 0.5um and annulus thickness of 1um). Upper panel: whole stack montage. Lower panel: zoom of 5 central slices.

Figure 183 presents an example of segmenting the annuli of a subresolution 0.2 µm bead. The minimum threshold method struggles to accurately detect all sections of the subresolution bead (upper panel), whereas the legacy threshold method successfully detects all sections, including the bead itself (central panel). It's crucial to adjust the annulus geometry to match the bead size, ensuring the thickness is similar to the bead's diameter.

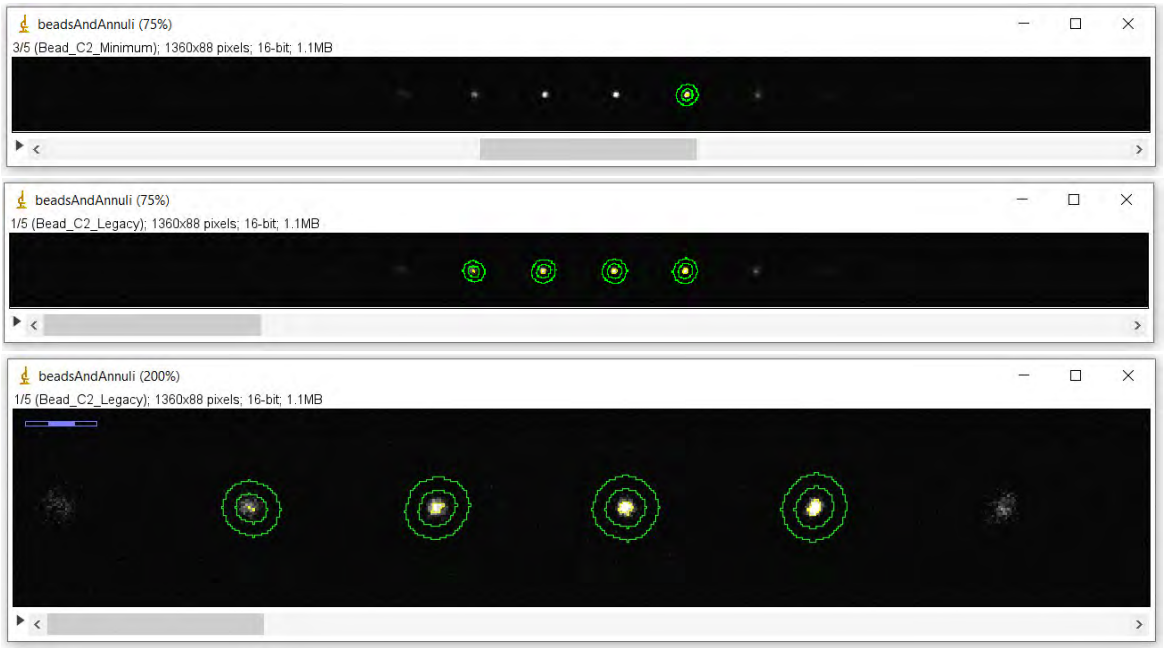


Figure 183. Test section: an example of the annuli detection output. The subresolution bead is segmented in yellow (lower panel) and the each sections' annulus is segmented in green (bead size 0.2um, inner annulus distance to bead edges 0.5um and annulus thickness of 0.5um). Upper panel: whole stack montage with Minimum threshold method. Central panel: whole stack montage with the legacy threshold. Lower panel: zoom of 6 central slices.

FIND MAXIMA TOOL

This tool will display how single resolution beads can be identified.

STEP A: in the "select test" rolling menu, choose "Find Maxima" (Figure 184). Set the image's bit depth and select the channel of interest.

STEP B: To mimic how subresolution beads are identified, check the preprocess images.

STEP C: Enter a starting prominence value. Would you like to only test this value, set the iterations (see below) value to 0.

STEP D: Starting from an initial prominence value, the tool enables users to identify a range of prominence values that result in the expected number of detected subresolution beads. First, set the "target" number of beads, or the expected Find

Maxima Count. The process begins with the initial prominence value, applying the Maximum Finder plugin (Process > Find Maxima) to determine the number of identified maxima. This count is compared to the expected value.

From this starting value, the algorithm will first try to find a prominence value that yields more than expected maxima ("lower bound prominence"). Then, the tool identifies a prominence value that yields less than expected maxima ("upper bound prominence"). To achieve this, the algorithms divides or multiplies the starting prominence value by a given factor (set to 5).

Then using this first "rough" range, the algorithm looks for the "minimum" prominence that yields the expected count using iterations. The first iteration uses the average of lower and upper bound prominence. This yields a given count that is compared to the expected count.

For instance, the expected count is 43. The starting prominence is 150. 150 yields 43 counts. To find the upper bound prominence, the algorithm tries 150*5=750. 750 yields again 43. The next tested value 3750, that yields 40. The upper bound value is set to 3750. To find the lower bound value, 150 is divided by 5 (30). This yields 2597 maxima. The lower bound value is set to 30. Then the refining iterations start with (3750+30)/2=1890, that yields 43 counts (iteration 1). 1890 now defines the upper range value while the lower range value stays at 30. The next tested value is (30+1890)/2=960, that gives 43 counts (iteration 2). The next tested value is (30+960)/2=495, that still gives 43 (iteration 4), as does (30+495)/2=262.5 (iteration 5) and (30+262.5)/2=146.25 (iteration 6). However (30+146.25)/2=88.125 yields 74 counts (iteration 7). This means the tested prominence is too low and 88.125 will set the new lower range value. The next iteration (8) tests (88.125+146.25)/2=117.1875 that gives 46 maxima as compared to the expected 43 count. 117.1875 set the new lower range value and the next (iteration 9) tested value is (117,1875+146.25)/2, etc…

While finding the lower bound, minimum and upper bound prominences, each tested prominence value is stored (as well as its associated count). Once the minimum prominence value is found, the maximum prominence value is searched for starting with the average value of the highest so far tested prominence value that gives the expected value and the lowest prominence value that gives less than expected count. The process of finding the maximum prominence is similar.

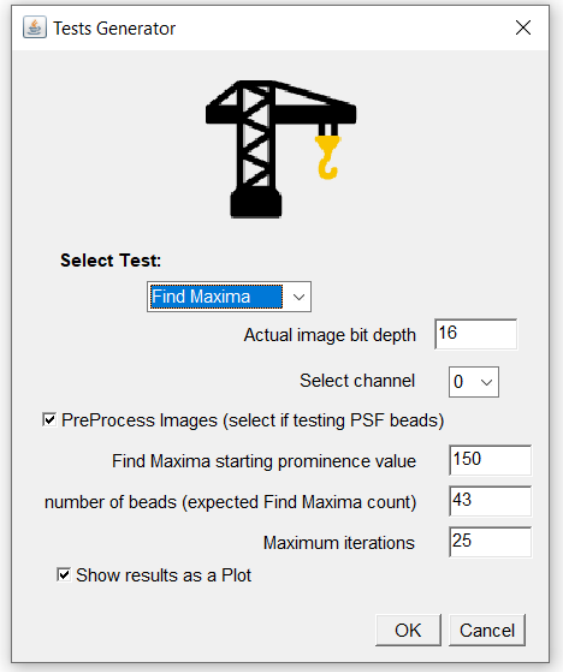


Figure 184. Test section: the Find Maxima tool

STEP E: Each prominence value and associated maxima are displayed in a stack. The user has the possibility to summarize the results as a plot. Figure 185 shows an example the tool's result. Each "slice" displays the maxima found with a given prominence value. This value is displayed in the slice's title (bottom panel Figure 185).

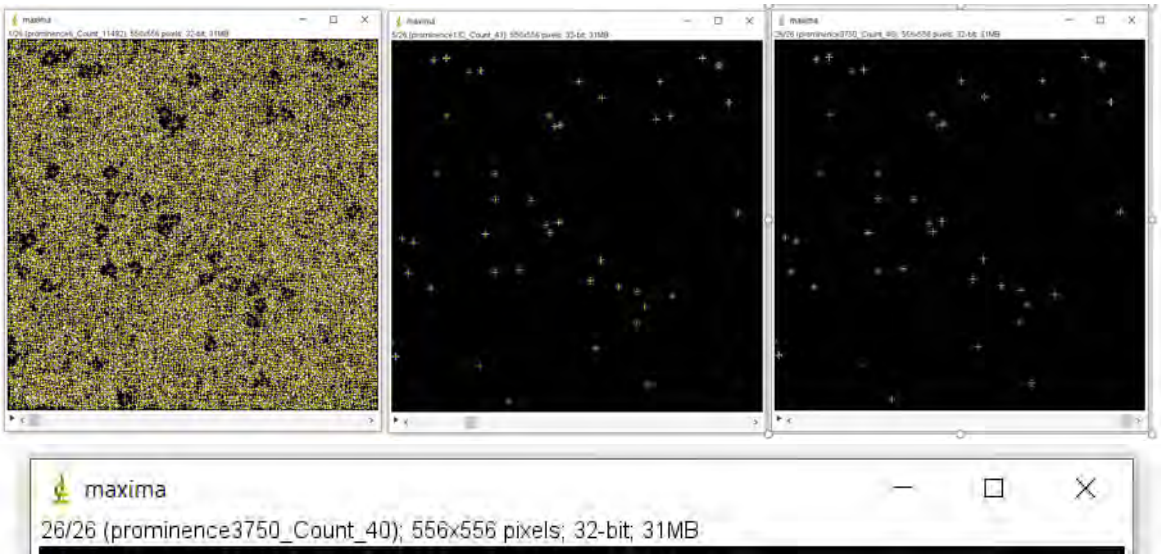


Figure 185. Test section: an example of the find maxima output. Different prominence values are tested. Each slice is associated with a prominence value. The upper left panel yields too much maxima, while the upper right panel fail to identify some of the 43 expected beads. Lower panel: the slice's title of the upper right panel.

Figure 186 presents an example of the Find Maxima output plot. Whenever the lowest count value is more than 100 times less the highest count, the ordinates axis is displayed using a log scale. If the lowest tested prominence value is 100 times less the maximum tested prominence value, then the abscissa axis is displayed using a log scale as well. The expected target maxima range is indicated (132 to 2638 in this case).

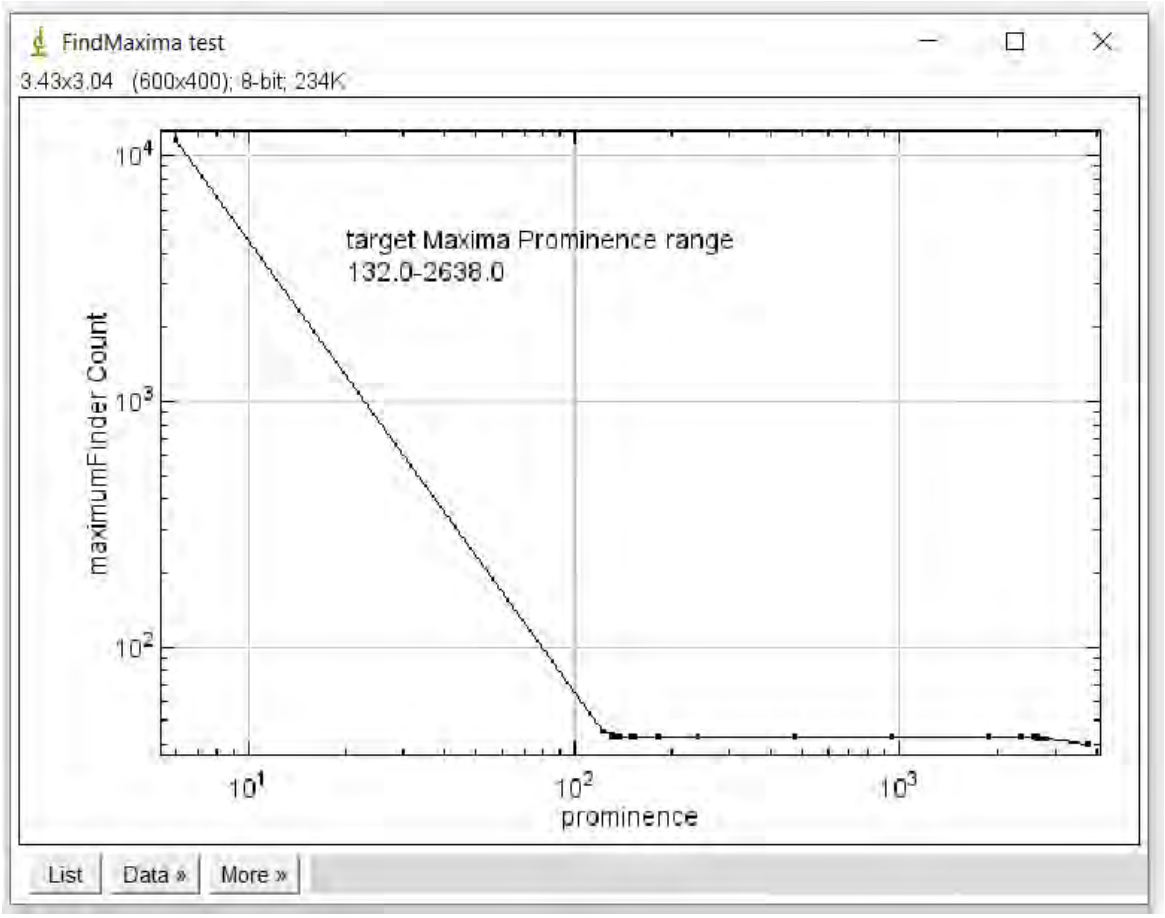


Figure 186. Test section: an example of find Maxima output plot.