



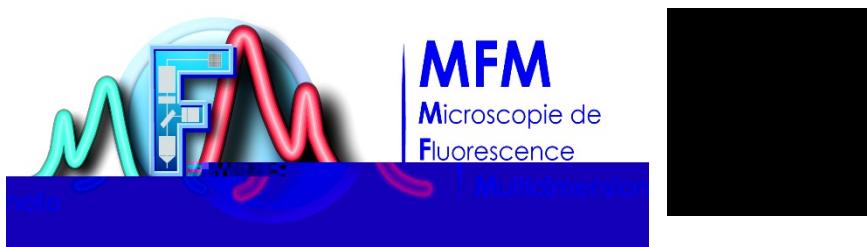
## The MetroloJ\_QC plugin

### Version 1.1 May 2021

#### 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.



This is a sample document.

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## PLUGIN INSTALLATION & REQUIREMENTS

The project can be downloaded from GitHub here.

[https://github.com/MontpellierRessourcesImagerie/MetroloJ\\_QC/tree/main](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 1.53g. 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 (Fig1, 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 1.0 of the plugin also uses bioformats, make sure a valid version of bioformats is used by your ImageJ install. v1.0 was developed using bioformats package version 6.5.1 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 customise the bar and remove some above-mentioned tools
- To open this manual



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

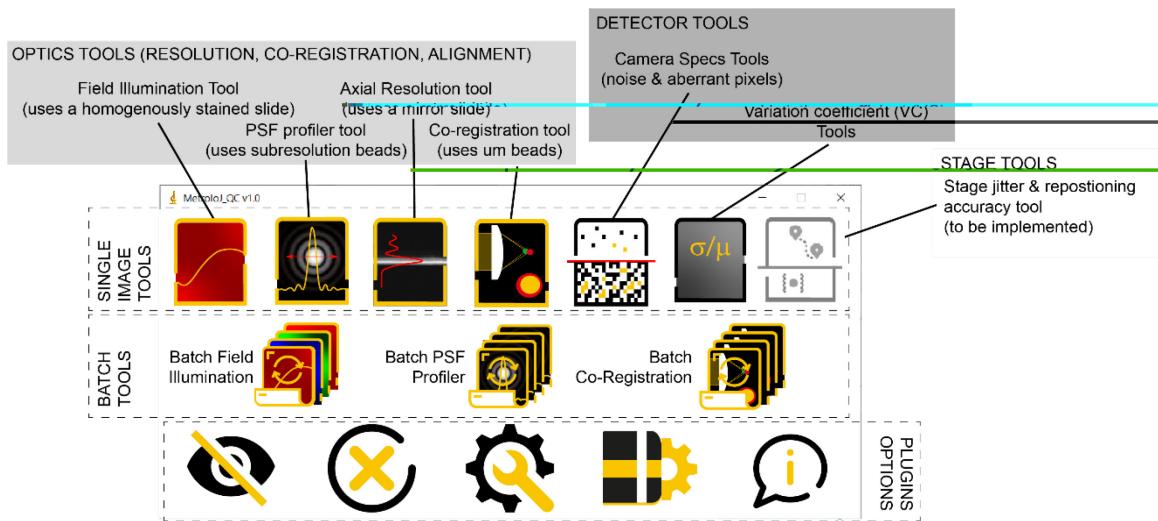
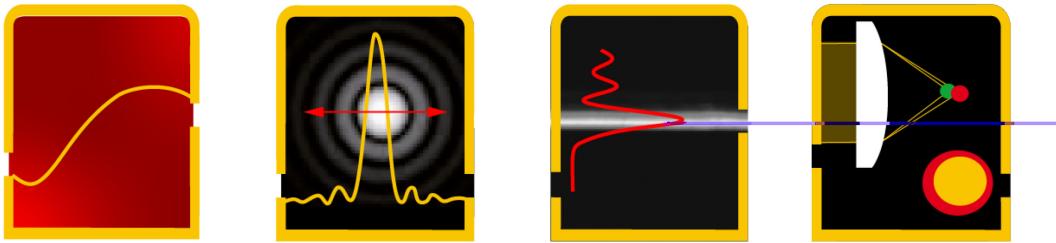


Figure 1. The MetroloJ\_QC bar.

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 subresolution 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** are under development and will eventually measure stage jitter and repositioning accuracy.





## 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 (Image J Center of Mass parameter). A normalized intensity image is generated (meaning an image whose pixel intensity is



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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.

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

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

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

$$\frac{x_{ref} - y_{ref}}{\sigma_{8 \text{ corners}}}$$

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 analyse. 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);



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**Field illumination report generator**

Title of report  Operator's name

Acquisition Parameters (used in the plugin)

Actual image bit depth

Other acquisition parameters (not used for computation)

Microscope type

found 1 channel(s), 1 slice(s) & 1 frame(s).

Em. Wavelength 0 (nm) <input type="text" value="561.0"/>	Ex. Wavelength 0 (nm) <input type="text" value="580.0"/>
Objective NA <input type="text" value="1.40"/>	Pinhole (AU) <input type="text" value="1.00"/>
Objective im. med. refractive index <input type="text" value="1.515"/>	

Add here other useful sample information to trace   
Add here any comments

Scale bar

Plugin parameters

Discard saturated samples  
 Remove noise using Gaussian Blur  
 Save pdf report(s)  
 Shorten analysis  
 Apply tolerances to the final report?

Intensity pattern bins   Use last bin as maximum reference zone  
 Open individual pdf report(s)  
 Save report images  
 Reject Uniformity below   Save all data in a spreadsheet  
 Reject Cent. Accuracy below

**OK** **Cancel**

**Field illumination report generator**

Title of report  Operator's name

Acquisition Parameters (used in the plugin)

Actual image bit depth

Other acquisition parameters (not used for computation)

Microscope type

found 1 channel(s), 1 slice(s) & 1 frame(s).

Em. Wavelength 0 (nm) <input type="text" value="405.0"/>	Ex. Wavelength 0 (nm) <input type="text" value="460.0"/>
Objective NA <input type="text" value="1.40"/>	Pinhole (AU) <input type="text" value="1.00"/>
Objective im. med. refractive index <input type="text" value="1.515"/>	

Sample infos:  Comments:

Scale bar

Plugin parameters

Discard saturated samples  
 Remove noise using Gaussian Blur  
 Save pdf report(s)  
 Shorten analysis  
 Apply tolerances to the final report?

Intensity pattern bins   Use last bin as maximum reference zone  
 Open individual pdf report(s)  
 Save report images  
 Reject Uniformity below   Save all data in a spreadsheet  
 Reject Cent. Accuracy below

**OK** **Cancel**

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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 analyse 3D XYZ images. Enter the microscope's detector output bit depth, as this will be used if the discard saturated samples option is selected. Fill-in the other fields (microscope's type, emission/exc. wavelengths, the objective's numerical aperture and immersion medium refraction index). Sample information and some comments might also be provided using the appropriate boxes. None of these fields values are used in any further computation and user is prompted to fill in these field for the sole sake of tracing the conditions associated with the analysed image only. Fill in the scale bar field (will be only used if the report/images are saved).

**STEP5.** Choose to discard or not saturated image. 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.** Ticking the "Save pdf/image/plots/data" will generate:

- The pdf report, in its long or short version (see 3)
- jpeg images of the iso-intensity pattern and (if long version chosen) the intensity profiles along horizontal/vertical/diagonals (see #3 in the Field Illumination report description above)
- some .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.



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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.** 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.

The report is generated, and appropriate files are saved.

#### 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 3). The main first section is **Microscope info** (tracing all infos 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.

Figure 3. Field illumination tool report: microscope & warnings sections of the report



### QC2906-1

#### Microscope info:

Image	5xCY3		
(found) image's creation date	2015-06-05 10:27:29 (from Metadata)		
Actual image depth	16		
Microscope	Confocal (pinhole 1.0 AU)		
Objective	NA: 1.4 & im. refractive index: 1.515		
Channel	Ex. (nm)	Em. (nm)	saturation
Channel #			
Channel 0	580.0	561.0	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.

The [Main Field Illumination parameters](#) section (Figure 4) 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:

Channel	Uniformity (%)	Field Uniformity (%)	Centering Accuracy (%)	Image
Channel0 (em. 470.0 nm)	65.0	90.3	74.7	C1-25x
Channel1 (em. 550.0 nm)	79.4	95.0	96.6	C2-25x
Channel2 (em. 600.0 nm)	77.3	93.8	89.4	C3-25x
Channel3 (em. 680.0 nm)	81.2	95.1	92.8	C4-25x

Green: within specifications, red: outside specifications (ie., uniformity below 50% or centering accuracy below 50%).  
Centering accuracy computed using the 90.0%-100% zone as reference rather than the maximum intensity pixel's position.

Figure 4. Field Illumination tool report: Uniformity, Field Uniformity & Centering accuracy values.

The following sections are detailed field illumination information per channel (Fig. 5 to 7).

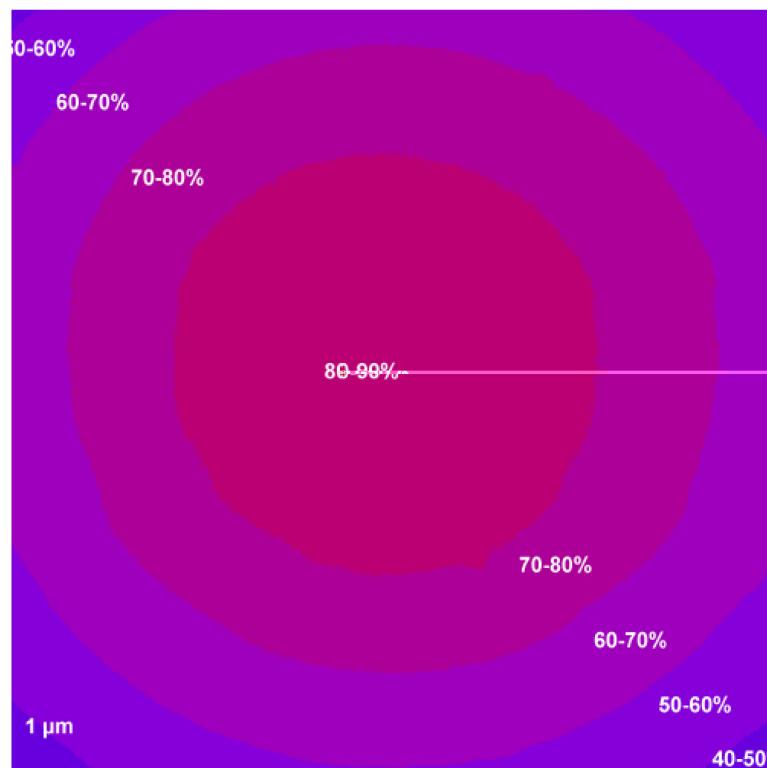
- The iso-intensity pseudocolor image of the normalised intensity image is provided in section [normalised intensity profile Channel \[n\]](#) (Fig. 5).
- The location of the image centre (geometrical centre), the centre of intensity (Centre of Mass of the page channel), the maximum intensity pixel for the page channel and the reference zone are provided, along with the distances to the geometrical image centre (see section [Channel \[n\] centre's locations](#), Fig. 5).
- Long version of the report includes a profile along horizontal/vertical/diagonal lines going through the image geometrical centre ([channel\[n\] intensity profiles](#) section, Fig. 6).
- A final section provides the intensity of points along the image's edge ([Channel\[n\] coordinates' statistics](#), Fig. 7). The table contains both raw and normalized intensities of the maximum/minimum intensity pixels (+ coordinates), the centres and values for 8 characteristic pixels, corresponding to the 8 intercepts of the lines along which the intensity profiles are retrieved.

For the sake of either debugging or tracing the analysis parameters, the last sections ([Analysis parameters](#)) are a summary of all algorithm parameters used (Fig.8) and the [A, B and C formulas used](#) for Field Illumination metrics calculation.



Channel0 (em. 405.0 nm)

Normalised intensity profile Channel0:



Channel0 centres' locations:

Channel	Type	Image Centre	Centre of intensity/mass	Last maximum intensity pixel	Centre of the 90.0-100% reference zone
Channel 0	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	121.287	100.186

Figure 5. Field Illumination tool report: the isointensity image & centres locations coordinates/distance to center.



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Channel0 intensity profiles:

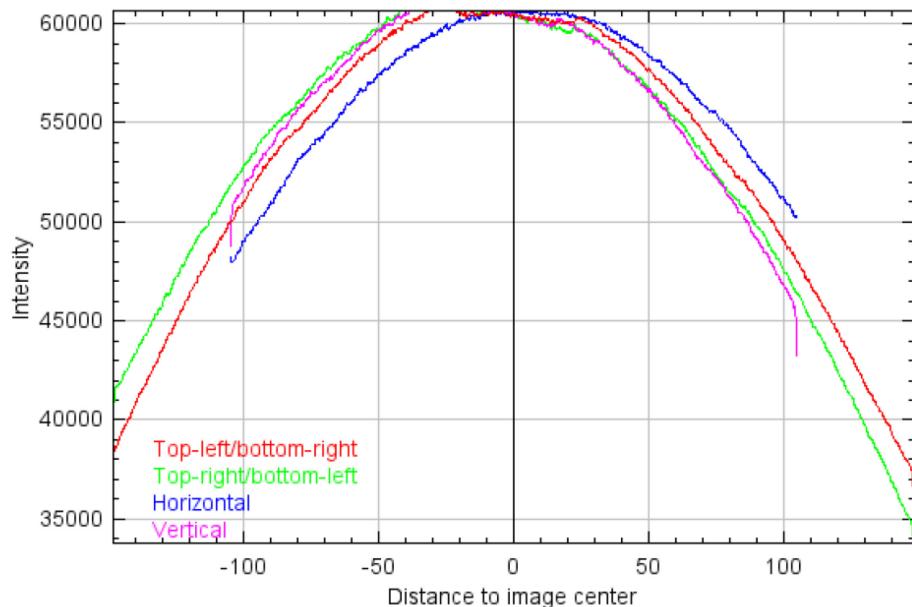


Figure 6. Field Illumination tool report: intensity profile across TL/BR, TR/BL, horizontal & vertical lines.

Channel0 coordinates' statistics:

Channel	Location	Intensity	relative intensity to max
Channel 0	Maximum found at (1296.0,1391.0)	43561	1.0
	Center of Int./Mass found at (1035.7,1063.0)	42201	0.969
	Reference zone center found at (1135.7,1344.3)	43235	0.993
	Minimum found at (17,0)	23870	0.548
	Top-left corner	25245	0.58
	Top-right corner	24910	0.572
	Bottom-left corner	31273	0.718
	Bottom-right corner	33686	0.773
	Upper bound, middle pixel	30447	0.699
	Lower bound, middle pixel	38748	0.89
	Left bound, middle pixel	34878	0.801
	Right bound, middle pixel	37563	0.862

Figure 7. Field illumination tool report: the coordinates' statistics section.



## Analysis parameters

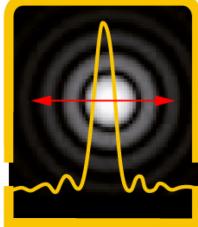
Tool & Operator	Tool	Field Illumination
	Versions	MetroloJ QC v1.1.2, ImageJ v2.1.0/1.53j, Java v1.8.0_172, OS Windows 10
	Operator & date	Julien, 21 juin 2021 17:10
data	result folder	D:\Users\Julien Cau\Desktop\MetroloJ QC Test\Homogénéité\Processed\QC2106-30\5xCY3
	type of saved data	.pdf, .jpg, .xls
	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%
Tolerance	Reference zone	90.0%-100%
	applied in this report	true
	Uniformity valid if above	50.0
	CA valid if above	50.0

Figure 8. Field Illumination tool report: the analysis parameters.



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## 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/lI4X\\_e8\\_m08](https://youtu.be/lI4X_e8_m08)

### 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:

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



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:

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

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

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

$$res_z^o = \frac{1.77n * \lambda_{em}}{NA^2} \quad (\text{F}')$$

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

$\lambda_{em}$ : emission wavelength

$NA$ : numerical aperture

- Confocal microscopes (assuming pinhole  $\geq 1$  AU,  $NA > 0.5$ )

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

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

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).

$\lambda_{ex}$ : excitation wavelength

$NA$ : numerical aperture

$n$ : immersion/mounting medium refractive index

- Spinning Disk microscopes

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

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

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

$\lambda_{em}$ : emission wavelength

$NA$ : numerical aperture

$n$ : immersion/mounting medium refractive index

- Multiphoton microscopes



$$res_{xy}^o = \frac{0.377 * \lambda_{ex}}{NA} \quad (\text{I}) \text{ for } NA < 0.7$$

$$res_{xy}^o = \frac{0.383 * \lambda_{ex}}{NA^{0.91}} \quad (\text{I}') \text{ for } NA > 0.7$$

$$res_z^o = \frac{0.626 * \lambda_{ex}}{n - \sqrt{n^2 - NA^2}} \quad (\text{I}'')$$

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

$\lambda_{ex}$ : 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 analyse. 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 9).

**STEP4.** The number of channels/slices is found and the user is prompted to fill-in some microscope information. Whenever the file order is different from the usual XY planes Z-stacks, the plugin will yield false results. Emission wavelengths, objective immersion medium refraction index & NA are used for theoretical formulas in the widefield set-up (formulas F and F'). Excitation wavelengths is also used for computation of theoretical resolution with confocal set-ups (formulas G and G'). Mind these formulas consider the image to be noise-free. Some more sample information and/or comments might also be provided using the appropriate boxes, for the sake of traceability.

**STEP5.** The user may discard saturated beads. 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, an Otsu automatic threshold value is calculated and applied to highlight bead sections. 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.



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close to the image's edges, the identified beads list is filtered to remove unwanted cases:

- Close neighbouring bead rejection criterion.
- Bead too close from lateral edges criterion
- Bead too close from the top/bottom of the stack criterion. This is quite useful as Gaussian fit of Z-profiles of beads that are too close are usually of bad quality.

A jpg “beadOverlay” image (found in a processed/subfolder, see below) is generated (see Fig. 10). This image will highlight the identified beads and why the bead was analysed or discarded from the analysis list.

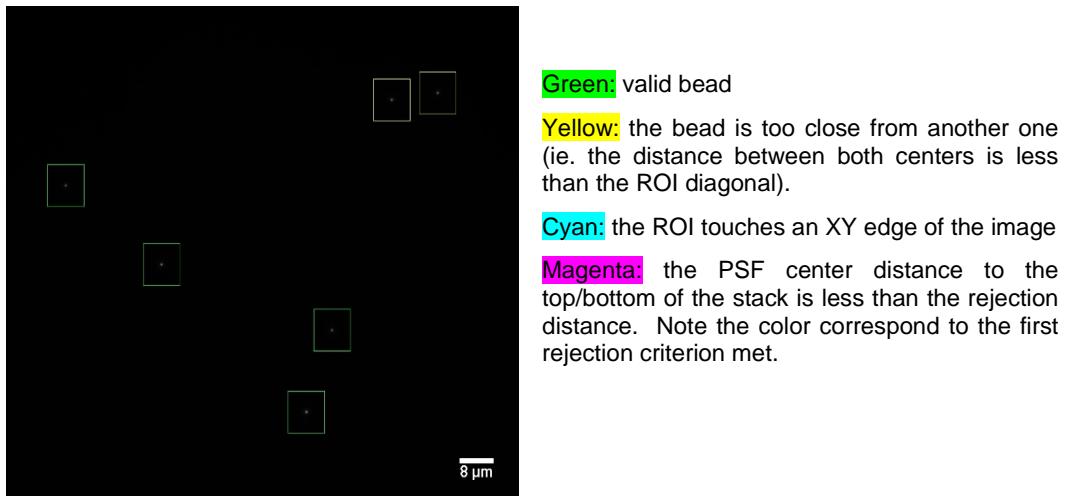


Figure 10. PSF Profiler Tool : bead identification overlay image.

**STEP8.** 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 section mean intensity is measured (signal). The background is estimated in an annulus around the bead sections. The thickness of this annulus can be changed. This thickness (in um) is converted in pixels and annuli are drawn. If the annuli reach the edges of the image, the algorithm will draw new annuli using half of the thickness. If necessary, this will be repeated until the annuli are not touching the edges of the image. Note that whenever the filled-in thickness has to be halved this means the crop value is way too small and this is likely to yield poor estimations. The ratio of bead sections mean intensity (signal) to annuli (background) mean intensity is calculated. Mind this is no signal to noise ratio evaluation.

**STEP9.** 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.

**STEP10.** The user has the possibility to i) generate a pdf report (mind all reports will be automatically opened if the open thickbox is selected: the number of generated pdf files may exceed the pdfReader capacity), ii) save jpg images of the bead side views (XY, YZ, XZ views)/fit profiles and iii) save all measured values in a tab separated



values spreadsheet. The user has the possibility to get a longer/shorter version (the fitting X, Y, Z profiles are in the long version only).

**STEP11.** 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 coloured, whatever the fitting ratio (and the quality of the FWHM approximation) might be.

**STEP12.** Click on Ok. The report is generated! 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 PSF profiler tool report.

The first main section **Microscope info** (Fig. 11) 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. If not available, the creation date will be the file's creation date (ie. when the file was created on the computer used to analyse 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.

The image calibration and sampling distance are also added. The Shannon-Nyquist  $\Delta_{xy}$  and  $\Delta_z$  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'.



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$$\propto \arcsin \frac{NA}{n} \quad (J)$$

$\alpha$ : angular aperture

$NA$ : numerical aperture

$n$ : immersion/mounting medium refractive index

- Widefield microscopes:

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

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

- Confocal microscopes:

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

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

- Multiphoton microscope (k-photon)

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

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

#### 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](#) (Fig. 12) section provides all channel resolution tables (Fig. 4). 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.





**QC2906-1**

Microscope info:

Image	63x-03_bead3					
(found) image's creation date	2021-03-30 15:43:52 (from file creation date)					
Actual image depth	16					
Microscope	Confocal (pinhole 1.0 AU)					
Objective	NA: 1.4 & im. refractive index: 1.515					
Channel	sampling (X,Y,Z)				saturation	
Channel	Ex. (nm)	Em. (nm)	Nyquist ( $\mu\text{m}$ )	Found ( $\mu\text{m}$ )		Nyquist/found ratio
Channel 0	450.0	405.0	0.04x0.04x0.12	0.071x0.071x0.063	1.8, 1.8, 3.0	none
Channel 1	488.0	525.0	0.044x0.044x0.13		1.6, 1.6, 2.8	none
Channel 2	561.0	600.0	0.05x0.05x0.15		1.4, 1.4, 2.4	none
Channel 3	638.0	680.0	0.057x0.057x0.17		1.2, 1.2, 2.1	none

Warnings:

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

Figure 11. PSF Profiler Tool report: microscope info and warnings sections.

Resolution table:

Channel	Sig/Backgnd ratio	Dimension	Measured FWHM ( $\mu\text{m}$ )	theory ( $\mu\text{m}$ )	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 12. 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.

$$LAR = \frac{\min FWHM_x FWHM_y}{\max FWHM_x FWHM_y} (N)$$

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<sup>2</sup>, Sig/Backgnd ratio or LAR values. In Fig. 13, the PSF has a LAR of 1 and a R<sup>2</sup> of 1



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Figure 13. PSF Profiler Tool report. Profile views and LAR table.

Finally, 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, Fig. 14). The user may find the actual fitting parameter of each dimension on the left hand side of the plot:

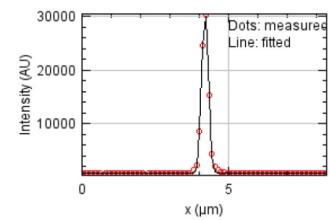
- equation against which the profile is fitted (formula D)
- the number of iterations used before reaching the final parameters
- the sum of residuals squared (ie. sum of the differences between the original intensity values and the fitted ones, squared)
- standard deviation: standard deviation of the 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 centre along the dimension axis



---

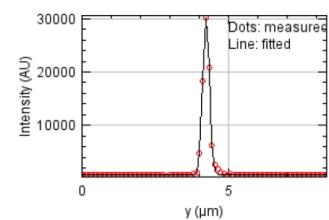
X profile & fitting parameters:  
 Fitted on  $x = a + (b-a) \cdot \exp(-(x-c)^2/(2 \cdot d^2))$   
 Sum of residuals squared: 2567064.73  
 Standard deviation: 178.02291  
 $R^2$ : 0.99844  
 Parameters:  
 a = 763.86017  
 b = 30961.1500  
 c = 4.20364  
 d = 0.10967

---



Y profile & fitting parameters:  
 Fitted on  $y = a + (b-a) \cdot \exp(-(y-c)^2/(2 \cdot d^2))$   
 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  $z = a + (b-a) \cdot \exp(-(z-c)^2/(2 \cdot d^2))$   
 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

---

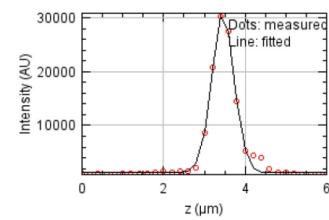


Figure 14. PSF Profiler Tool report: long version of Detailed channel detection info section.

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](#) (Fig. 15) and [formulas used](#).



## Analysis parameters

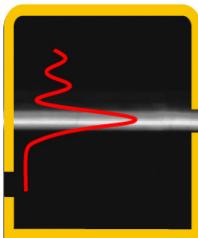
Tool & Operator	Tool	PSF Profiler
	Versions	MetroloJ_QC v1.1.3, ImageJ v1.53g, Java v14.0.2, OS Windows 10
	Operator & date	Julien, 30 juin 2021 18:39
data	result folder	D:\users\julien\cauv\desktop\MetroloJ_QC\Test\psfsubset\Processed\QC3006-12\bead6\63x-02_bead6
	type of saved data	.pdf, .jpg, .xls
	input data bit depth	16
	dimension order	XY-(C)Z
discard saturated samples		true
beads	Background annulus thickness in µm	0.99 (all channels)
	multiple beads in image	true
	bead detection channel	0
	bead size (µm)	0.2
	bead crop Factor	10.0
	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	1.5
	Z FWHM ratio valid if below	2.0

Figure 15. PSF Profiler Tool report: analysis parameters section.

When single bead images are selected, all data is saved in a processed/title subfolder. If selected, side views and profile plots (not in the short version of the report) for each channel are saved in a “image name” folder. The .xls files of the tables of the pdf report are saved in an imagename\_summary.xls file. If a long version was chosen, the profiles for all channels/dimensions are stored in an imagename\_profiles.xls file.

When multiple bead images option is selected, files derived from each bead are saved in a processed/title/bead#.





## 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.  $0.17\text{mm} \pm 20\mu\text{m}$ ) thick coverslip. If available, either use a micrometer to check the thickness or use 1.5H grade ( $0.17\text{mm} \pm 5\mu\text{m}$ ). 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.

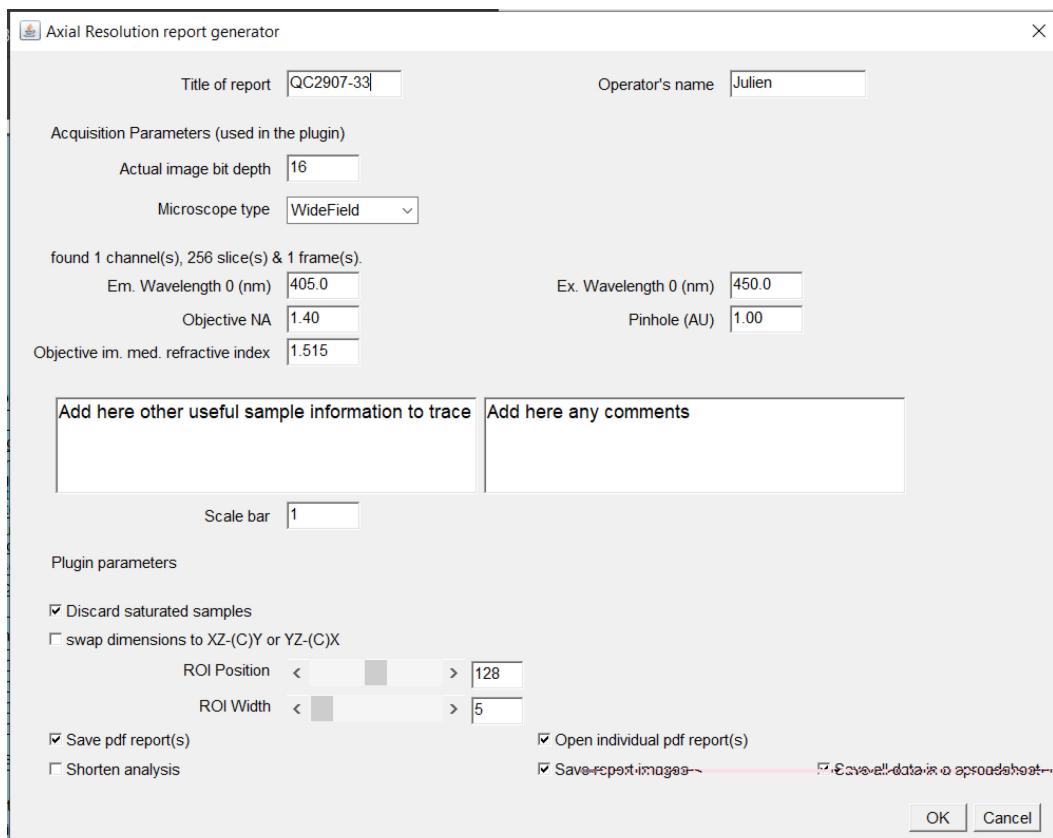
The resulting 1D intensity profile is then fitted to a Gaussian, using equation D and ImageJ's built-in curve fitting function. The axial resolution, i.e. the full-width at half-maximum (FWHM), is calculated using formula E, based on the parameters retrieved



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
- STEP5.** The plugin's interface should appear (see figure 16). Choose the appropriate channel dimension. The plugin is intended to be used with any format (XY-Z stack, XZ-Y stack or YZ-X stack). Changing the dimension order to the actual real one will yield accurate sampling and sampling ratio values for each dimension.



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Figure 16. Axial Resolution Tool : the User's interface.

**STEP6.** Choose the microscope's type, enter the emission/excitation wavelengths, the numerical aperture of the objective, its immersion medium refractive index and, for single-point scanning confocal, the pinhole aperture. These specs are used to compute the theoretical resolution values.



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**STEP7.** The Stack format should be XZY or YZX. If a XYZ Stack was open, tick the swap dimensions to XZ-(C)Y or YZ-(C)X checkbox to generate the correct view (figure 17). This uses the ImageJ Image>Stack>Reslice[...]...

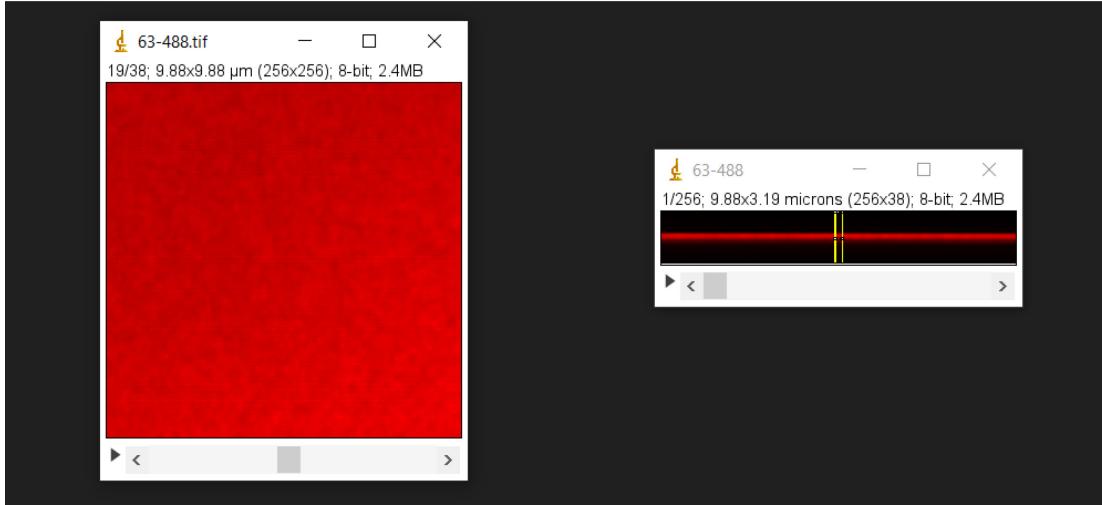


Figure 17. Axial Resolution tool: the swap option result (a XYZ stack, left, is changed to the expected XZY or YZX stack format, right).

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

**STEP9.** Sample information and some comments might also be provided using the appropriate boxes. Ticking the save pdf report will create a pdf report that can be further stored for traceability. The jpg option will create images of the fitted profiles. Finally, “save all data in spreadsheet” option will create excel files storing the results.

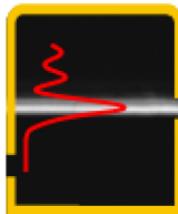
**STEP10.** Click on Ok. The report is generated, and the appropriate files are saved. If the original file folder is not found, a new dialog box will appear, inviting the user to choose a folder where all data should be saved.

#### Description of the Axial Resolution tool report.

Microscope info and warnings sections (figure 18) summarize all setup-related parameters and contains useful information to help result interpretation.



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## QC2506-7

### Microscope infos:

Image	63-488					
(found) image's creation date	original file info & metadata could not be found					
Actual image depth	16					
Microscope	Confocal (pinhole 1.0 AU)					
Objective	NA: 1.4 & im. refractive index: 1.515					
Channel	sampling (X,Y,Z)					saturation
Channel	Ex. (nm)	Em. (nm)	Nyquist ( $\mu\text{m}$ )	Found ( $\mu\text{m}$ )	Nyquist/Found ratio	
Channel 0	580.0	561.0	0.052x0.052x0.155	0.039x0.039x0.084	0.7, 0.7, 0.5	none

### Warnings:

(All channels sampled following Shannon-Nyquist criterion).

Figure 18. 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 19). The coordinates of the ROI are given. Please note, whenever applies, that the ROI is a 2D roi within the selected plane (eg. In the example of figure 17, plane 1 out of 256).

### Resolution table:

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

Channel	FWHM	Theoretical resolution
Channel 0	0.388 $\mu\text{m}$	0.554 $\mu\text{m}$

Figure 19. 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 20). 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;



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- 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 centre along the first dimension axis;

Channel0 (em. 561.0 nm)

Profile view Channel:



Z profiles

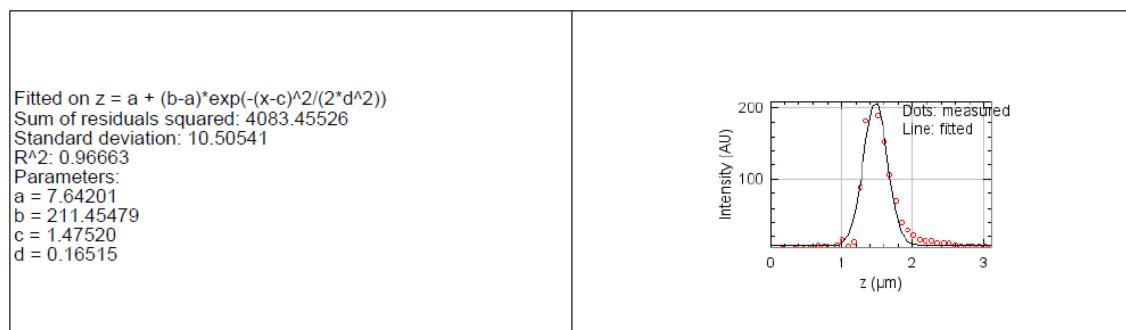


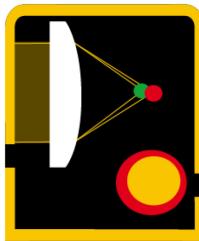
Figure 20. Axial Resolution tool report : profiles views (long versions of the report only).

Sample info (optional), Comments (optional), analysis parameters (figure 21) & formulas used are summarized in the next page of the report.

Figure 21. Axial resolution tool report: analysis parameters



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## 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 localisation, co-expression or colocalisation 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 4 $\mu$ m 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, 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". An ellipse is fitted to those pixels (i.e. to the bead's outline), and the coordinates of its centre of mass are determined. Once all coordinates have been retrieved for each channel, distance between the centre from channel A and centre from channel B is calculated:

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

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 centre of the structure on image A, a structure of image B will be co-localized if it lies within a circle, centred on centre 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 Fig. 24). 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 more easy to calculate the reference distance. The two characteristic angles, the azimuth  $\Phi$  and the zenith  $\Theta$  (see formulas 5.2 and 5.3) are first calculated, based on the coordinates of the two centres to analyse. Knowing this orientation, as well as the x, y and z theoretical resolutions ( $res_x^o$ ,  $res_y^o$  and  $res_z^o$  respectively, as calculated using formulas 5.5 to 5.13), the distance from the reference centre to the border of the ovoid shape  $r_{ref}$  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 Fig. 24C) or not (see Fig. 24B).

$$\Phi = \frac{x_B - x_A}{\sqrt{x_B - x_A^2 + y_B - y_A^2}} \quad (P)$$

$$\Theta = \frac{z_B - z_A}{\sqrt{x_B - x_A^2 + y_B - y_A^2 + z_B - z_A^2}} \quad (Q)$$

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



Figure 23. Calculation of the reference distance  $r_{ref}$ . Left: Centers of objects A and B are drawn as a red and green spheres, respectively. The PSF is schematized in light yellow, while the first Airy volume appears in dark yellow. The former width, height and depth define the resolution along the 3 axis. Middle: A and B are not co-localized as  $r > r_{ref}$ . Right: A and B are co-localized as  $r \leq r_{ref}$ . Illustration 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) intercentre distances for all combinations and compare it to their respective reference distance. A ratio of the measured intercentre distance to the reference distance is also calculated.

#### Co-registration Tool parameters:



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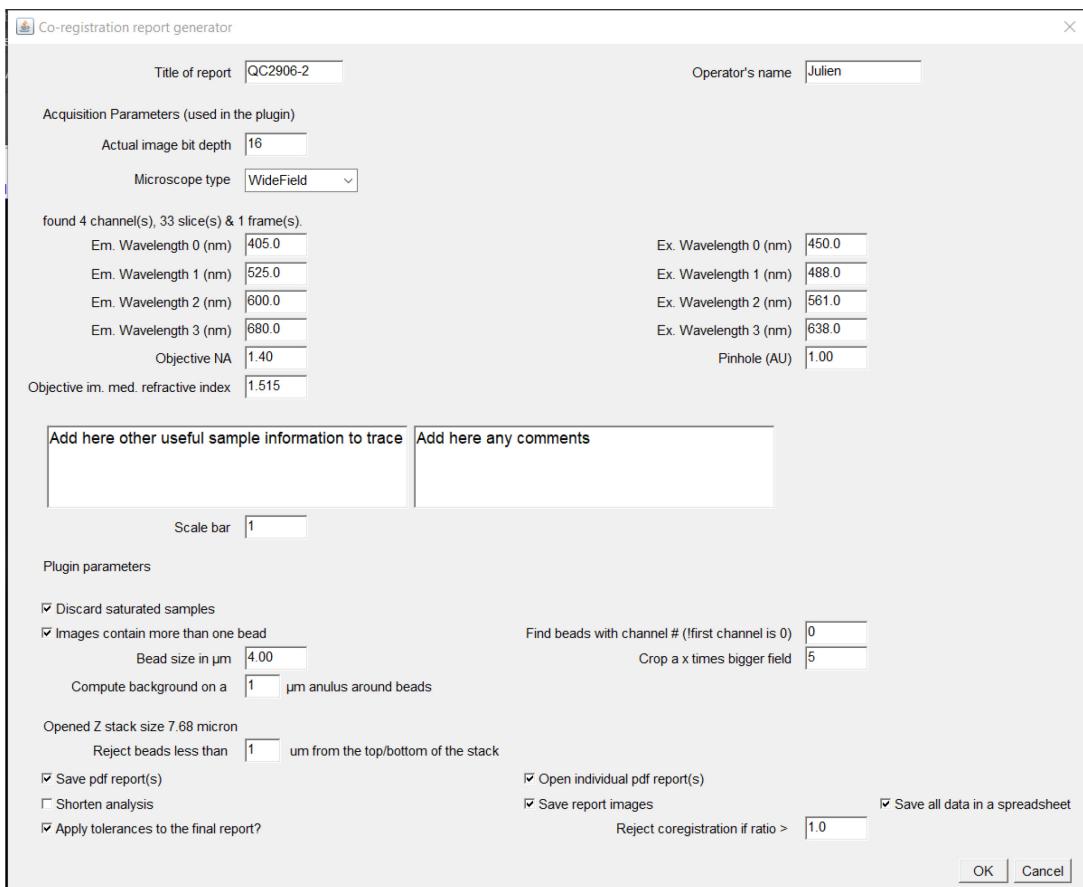


Figure 24. Co-registration tool: the user's interface.

**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.** 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. 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.

**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 set-up. Excitation wavelength, objective NA and immersion medium refractive medium are used for computation of theoretical resolution with single-point scanning confocal set-up. Note that multiphoton resolution assumes a 2-photon excitation. All formulas used (F to I)



are further reminded in the pdf report). For the sake of traceability, some more sample information and/or comments might also be provided using the appropriate boxes.

**STEP8.** As saturation alters the bead centre detection, the user has the possibility to detect bead saturation. The saturation is computed as follow. A montage 2D image of all slices is used to compute a “whole bead” automatic Otsu threshold value calculation. The value is then used to measure the area of the bead on each slice. The saturated fraction of this area is then calculated. Selecting “discard saturated sample” will automatically skip saturated channels. If there are no unsaturated channel combination to analyse, the image is skipped.

**STEP9.** When multiple beads-containing stacks are used, the user may tick “images contain more than one bead”. In cases of multichannel images, beads are not identified per channel. A single channel is rather used for the purpose of bead identification (fill-in the Find bead with Channel# field with the appropriate channel number, keeping in mind channel 0 is the first channel). Bead images are further cropped and processed as if they were single-bead images (ie. option not ticked). Bead identification is performed on a Z maximum intensity projection of the given user-defined channel. An Otsu automatic threshold is applied and beads are identified using ImageJ’s particle analysis tool. A filter is applied to remove object whose area is below 50% and above 400% of the expected bead area, as computed from the bead diameter field.

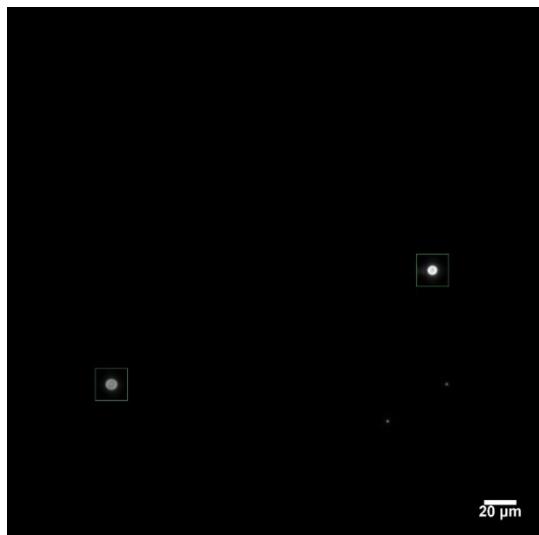
**STEP10.** For the purpose of cropping, the user is prompted to type-in the bead size (also used above for bead detection) and the crop factor. A bead size of 4um and a crop factor of 5 will yield a centered square ROI of box size of  $4*5=20\text{um}$ . As further co-registration analysis may be either 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. A jpg “beadOverlay” image will be generated (found in a processed/subfolder, see below) is generated (see Figure 24). This image will highlight the selected beads (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, no occurrence in figure 24). As bead identification uses a Z-projection, it may happen that the bead image is too close to the top or bottom of the Z-stack. These clipped beads will yield wrong results.

**STEP11.** 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 8). The identified bead section mean intensity is measured (signal). The background is estimated in an annulus around the bead sections. The thickness of this annulus can be changed. This thickness (in um) is converted in pixels and annuli are drawn. If the annuli reach the edges of the image, the algorithm will draw new annuli using half of the thickness. If necessary, this will be repeated until the annuli are not touching the edges of the image. Note that whenever the filled-in thickness has to be halved this means the crop value is way too small and this is likely to yield poor estimations. The ratio of bead sections mean intensity (signal) to annuli (background) mean intensity is calculated. Mind this is no signal to noise ratio evaluation.

**STEP12.** The user has the possibility to i) generate a pdf report/open it (keep in mind if dozens of beads are to be analysed, you may reach the pdf reader opened windows capacity), ii) save jpg images of the beads combination sideviews (XY, YZ, XZ views)/fit profiles and iii) save all measured values in a tab separated values spreadsheet. The user has the possibility to get a longer/shorter version (pixel shifts, calibrated/uncalibrated distances tables are in the long version only).

**STEP13.** 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 coalignement if ratio >”.

**STEP14.** Click on Ok. The report is generated. 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).



**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

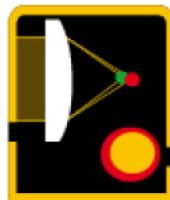
**Magenta:** the PSF center distance to the top/bottom of the stack is less than the rejection distance. Note the color correspond to the first rejection criterion met.

Figure 25. Co-registration tool: bead identification overlay image. (note smaller PSF beads are not taken into account)

### Description of the co-registration tool report.

The first sections of the co-registration tool report (Figure 25) 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 step 9). 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.





QC2906-1

Microscope info:

Image	63x-02_bead1					
(found) image's creation date	2020-08-21 10:33:28 (from Metadata)					
Actual image depth	16					
Microscope	WideField					
Objective	NA: 1.4 & im. refractive index: 1.515					
Channel	sampling (X,Y,Z)				saturation	
Channel	Ex. (nm)	Em. (nm)	Nyquist ( $\mu\text{m}$ )	Found ( $\mu\text{m}$ )		
Channel 0	450.0	405.0	0.072x0.072x0.216	0.103x0.103x0.24		1.4, 1.4, 1.1
Channel 1	488.0	525.0	0.094x0.094x0.28			1.1, 1.1, 0.9
Channel 2	561.0	600.0	0.107x0.107x0.321			1.0, 1.0, 0.7
Channel 3	638.0	680.0	0.121x0.121x0.363		0.8, 0.8, 0.7	

Warnings:

The highlighted undersampled channels may alter the result interpretation.

(The bead size is appropriate for this coalignment analysis).

Figure 26. Co-registration tool : microscope info and warnings sections

For each channel combination, ratios of the measured intercentre distances to reference distance ratio are displayed in the [ratios table](#) (Figure 26). 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 centre’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  $res_z^o$ values 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 step 8) are computed (referred to as signal). Then anuli of x um are drawn around each bead section and the mean intensity of all anuli is calculated (referred to as background). “x” can be changed at step 8. A signal to background ratio is calculated. Mind this is in no way a measure of the SNR ratio.



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Ratios table:

	Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0	0.915	0.613	0.721	0.721	0.721
Channel 1	0.915	0.34	0.38	0.17	0.38
Channel 2	0.613	0.34	0.17	0.17	0.17
Channel 3	0.721	0.38	0.17	0.0	0.0
Channel 4	0.721	0.38	0.17	0.0	0.0
Resolutions ( $\mu\text{m}$ )	0.148 0.148 0.554	0.191 0.191 0.710	0.219 0.219 0.021	0.248 0.248 0.038	0.248 0.248 0.038
Bead centres' coord. ( $\mu\text{m}$ )	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

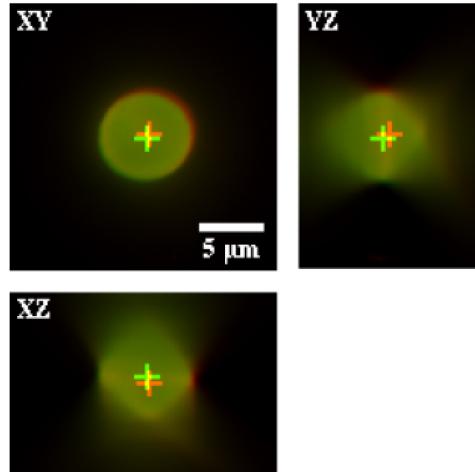
Green: within specifications, red: outside specifications (ie. ratio above 1.0)

Figure 27. Co-registration tool report: An example of the ratios table.

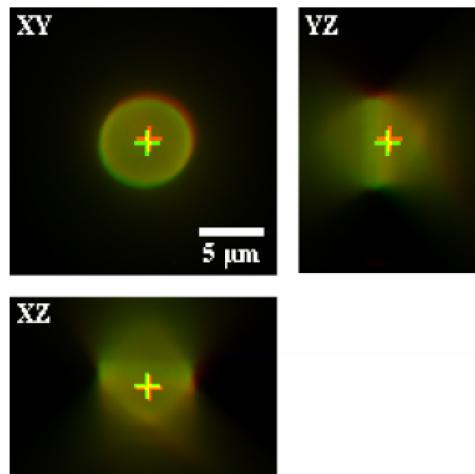
For each combination, Profile view images composed of three maximum intensity projections, XY, XZ and YZ (side views) are generated. Crosses indicate the respective position of the green channel (first channel using the stack order) and the red channel. In Figure 27, the upper sideview compares channel 0 in green and Channel 1 in red.



Profile view:



Channel 0 (Em. Wavelength 470.0 nm) vs channel 1 (Em. Wavelength 525.0 nm)



Channel 0 (Em. Wavelength 470.0 nm) vs channel 2 (Em. Wavelength 600.0 nm)

Figure 28. Co-registration tool report: part of a profile view section.

Long versions of the report include an ISO21073 co-registration accuracy table (Figure 28). These metrics are defined in the ISO21073 norm. The lateral  $\Delta_{x,y}$  and axial  $\Delta_z$  co-registration accuracy between channel i and j are given using formula S & T and the coordinates of the centre 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 29. 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 29). The reference channel is the channel in the first cell of each column. Each row shows how much pixels separate one channel from the reference along x, y and z axis. This information might be useful to compensate for chromatic aberration using image processing softwares (provided the shift is the same w/ bead position within the field of view). On each column, resolutions (as expressed in pixels) and centre's coordinates (in pixels) are given for the reference channel.

Long versions also contain Distance tables (uncalibrated & calibrated) (Figure 30). The table displays distances calculated between the centres of both channels for all channel combinations.



Pixel shift table:

	Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0		-2.5 1.0 0.0	-1.5 1.0 0.0	-1.5 1.5 0.0	-1.5 1.5 0.0
Channel 1	2.5 -1.0 0.0		1.0 0.0 0.0	1.0 0.5 0.0	1.0 0.5 0.0
Channel 2	1.5 -1.0 0.0	-1.0 0.0 0.0		0.0 0.5 0.0	0.0 0.5 0.0
Channel 3	1.5 -1.5 0.0	-1.0 -0.5 0.0	0.0 -0.5 0.0		0.0 0.0 0.0
Channel 4	1.5 -1.5 0.0	-1.0 -0.5 0.0	0.0 -0.5 0.0	0.0 0.0 0.0	
Resolutions (pix.)	2.27 2.27 2.519	2.942 2.942 3.265	3.363 3.363 3.731	3.811 3.811 4.229	3.811 3.811 4.229
Centres'coord.	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
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

DISTANCES table (calibrated).

	Channel 0	Channel 1	Channel 2	Channel 3	Channel 4
Channel 0		0.175 (0.191) (using 525.0nm for calculation)	0.117 (0.191) (using 600.0nm for calculation)	0.138 (0.191) (using 680.0nm for calculation)	0.138 (0.191) (using 680.0nm for calculation)
Channel 1	0.175 (0.191) (using 525.0nm for calculation)		0.065 (0.191) (using 600.0nm for calculation)	0.073 (0.191) (using 680.0nm for calculation)	0.073 (0.191) (using 680.0nm for calculation)
Channel 2	0.117 (0.191) (using 600.0nm for calculation)	0.065 (0.191) (using 600.0nm for calculation)		0.033 (0.191) (using 680.0nm for calculation)	0.033 (0.191) (using 680.0nm for calculation)
Channel 3	0.138 (0.191) (using 680.0nm for calculation)	0.073 (0.191) (using 680.0nm for calculation)	0.033 (0.191), Using 525.0nm for calculation	0.0 (0.554), Using 525.0nm for calculation	0.0 (0.554), Using 525.0nm for calculation
Channel 4	0.138 (0.191) (using 680.0nm for calculation)	0.073 (0.191) (using 680.0nm for calculation)	0.033 (0.191) (using 680.0nm for calculation)	0.0 (0.554) (using 680.0nm for calculation)	
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
Centres'coord.(μm)	10.0 10.0 3.7	9.8 10.1 3.7	9.9 10.1 3.7	9.9 10.1 3.7	9.9 10.1 3.7
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

Figure 30. Co-registration tool report: pixel shift table (long versions only).

Figure 31. 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 31) and the **formulas** used.



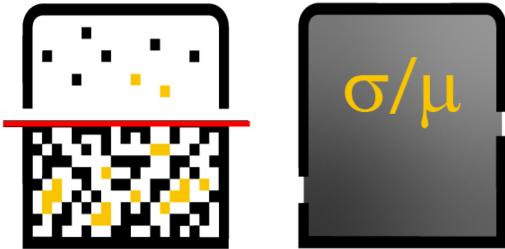
## Analysis parameters

Tool & Operator	Tool	Co-registration
	Versions	MetroloJ_QC v1.1.3, ImageJ v1.53g, Java v14.0.2, OS Windows 10
	Operator & date	Julien, 30 juin 2021 18:55
data	result folder	D:\Users\Julien Cau\Desktop\MetroloJ_QC Test\Coalignement\Processed\QC3006-1\bead1\100x-01_bead1
	type of saved data	.pdf, .jpg, .xls
	input data bit depth	16
	dimension order	XY-(C)Z
	discard saturated samples	true
beads	Background anulus thickness in µm	0.98 (all channels)
	multiple beads in image	true
	bead detection channel	0
	bead size (µm)	4.0
	bead crop Factor	5.0
	bead rejection distance to top/bottom	0.5 µm
Tolerance	applied in this report	true
	ratio valid if below	1.0

Figure 32. Co-registration tool report: analysis parameters table.

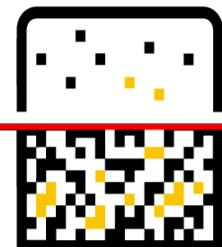


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## DETECTOR TOOLS

This series of tools is aimed at characterising 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 behaviour (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 intenedt to:

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



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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  $SD_{xy}$  is squared and the sum  $S$  of all pixels within the projection is calculated.

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

The rms noise specification is derived using formula V.

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

Where  $S$  &  $V$  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 behaviour 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 32).

**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 the appropriate detector type and image 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.

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 32, 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. 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...).

**STEP6.** The user may discard saturated images from the analysis. However, this option is pointless as the dark images should not be saturated. Whenever hot pixels are looked for, the option is not taken into account.



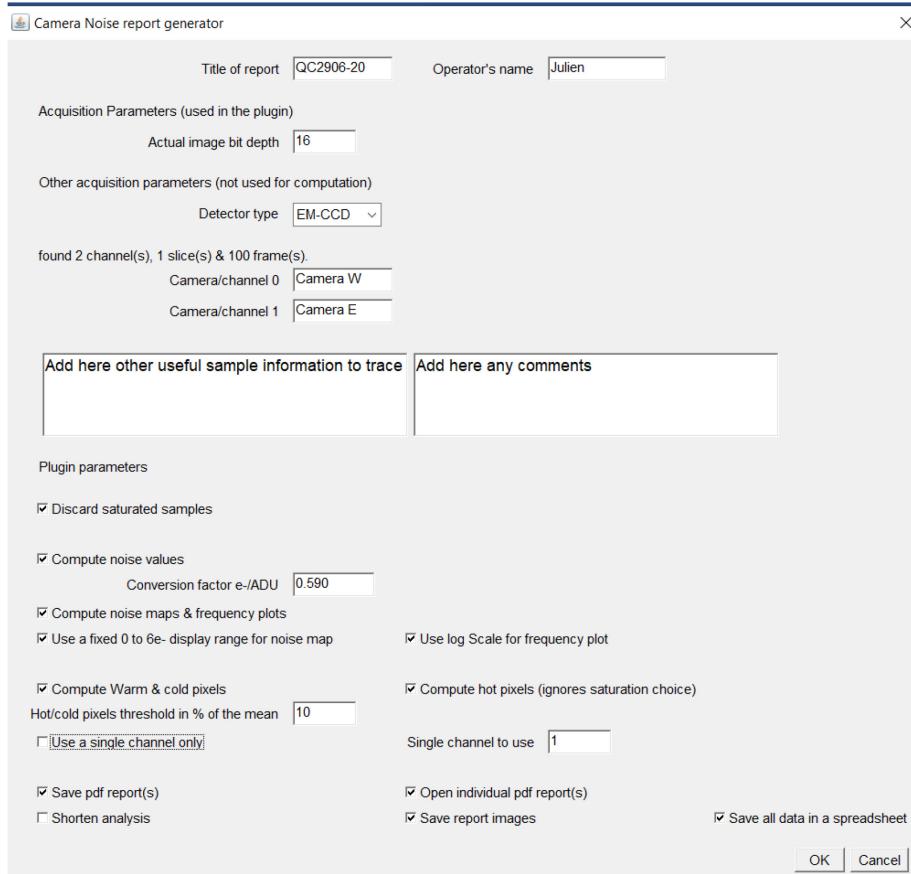


Figure 33. Camera Tool: the user's interface.

**STEP7.** The next block are noise parameters. Tick if offset, DSNU and median noise should be calculated. 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. Noise specs can be derived from minimal exposure closed-shutter images. The “noise” (aka standard deviation of each pixel intensity) can be analysed and frequency plots generated. As for 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 0-6 e-. The frequency distribution of the noise map can be generated. It may help to use a log scale to display the count (Y axis) for each standard deviation/noise value in the image (X-Axis).

**STEP8.** Warm, cold and hot pixels can be monitored. For this purpose, long exposure time in dark conditions (say 30s, shutter off) may be used. To compute warm & cold pixel or hot pixels, click the appropriate checkbox. If “Compute warm and cold pixels” checkbox is ticked, enter the threshold value (thr). Warm pixels in a frame are pixels whose intensity is thr% and more than the average frame value. Cold pixels in a frame are pixels whose intensity is thr% and less than the average frame value.



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**STEP9.** The “shorten” report option will skip the hot & cold pixel distribution analysis (see below).

**STEP10.** The user has the possibility to i) generate a pdf report/open it, ii) save jpg images of the warm/cold/hot mask (if applies) for each channel and iii) save all measured values in a tab separated values spreadsheet. The user has the possibility to get a longer/shorter version.

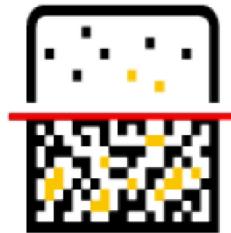
**STEP11.** Click on Ok. The report is generated. Mind that warm/cold/hot pixels analysis is time-consuming (even though the plugin uses multiple threads). A typical 100 frame stack may be analysed in 25 seconds. The original image file location is used to create a “processed” folder. Files will be further 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 33) 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.

When the compute noise values option in [STEP7](#) was selected, the next [specifications table](#) (Figure 34) contains all computed specifications. A [noise map](#) image is generated (Figure 35). 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 35). A frequency distribution plot of this projection is also displayed (Noise distribution).





**QC2906-20**

Microscope info:

Image	OMX
(found) image's creation date	2021-06-29 22:52:20 (from file creation date)
Detector	EM-CCD
Detector output bit depth	16
Conversion Factor (e-/ADU)	0.59
Saturation	Camera W
	none
Saturation	Camera E
	none

Warnings:

(No saturated pixels detected).

Figure 34. Camera Tool report: the microscope info and warnings sections.

Offset & Noise Specifications

Field	Value
Offset value (ADU)	99.7
Noise	rms (e-)
	median (e-)
	DSNU (e-)

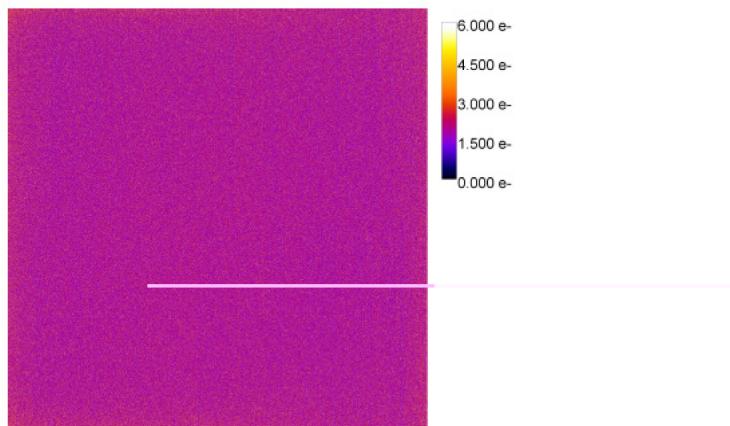
Figure 35. Camera tool report: the specification section



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### Noise distribution

#### Noise Map Channel 0



The display dynamic range is set to 0-6 e-, some pixels may have a higher, out of range, standard deviation.

#### Noise Distribution Channel 0

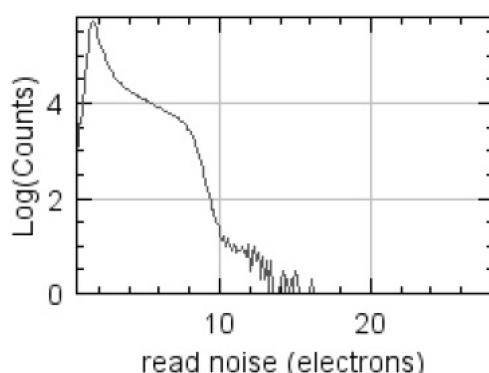


Figure 36. Camera tool report: the noise map and noise distribution plot

#### Hot, warm & cold pixels summary

Warm pixels		Cold pixels		Hot pixels	
average number/frame	% total	average number/frame	% total	average number/frame	% total
28249.2	0.674	19079.3	0.455	0.0	0.0

Figure 37. Camera tool report: the abnormal pixels summary table.

If chosen at **STEP7**, identification of warm (green), cold (blue) and hot pixels if any (red) is performed. The [Hot, Warm and Cold pixels summary](#) table (Figure 36) indicates, for each type of abnormal pixels, the average number (across all timepoints) of abnormal pixels and the proportion (relative to the whole image).



As a given pixel may behave abnormally in a few frames of the whole t-stack, it is quite interesting to analyse the abnormal behaviour at the pixel scale. The next Hot, Warm & Cold pixels behaviors section displays :

- Abnormal behaviour frequency informations (figure 37). In the example of figure 37, the “warmest” pixel of the dataset was found as “warm” in 37 of the 100 frames. Pixels that show-up as “warm” do so, on average, only in 4.555 frames out of the 100 frames of the dataset. Across the frequency distribution of all pixels identifies in some frames as warm, the median number of frames where they are considered as “warm” is 3.
- The warm/cold/hot pixels map (figure 38). Whenever no warm/cold/hot pixels are found (as is the case in figure 38 for hot pixels), they are of course not displayed in the map). The frequency is displayed as % (ie. the number of frames a given pixel is –say- warm divided by the total number of frames and multiplied by 100). If the image as large width/height, zoom in to see the warm/cold/hot

When the “save images” option is selected, mask of the warm, cold and hot pixels can be further used to correct images and remove those aberrant pixels (using for instance a convolution).

#### Hot, warm & cold pixels behaviors

Pixel type channel0	Warm Pixels	Cold Pixels	Hot Pixels
Max. frequency	37.0/100 frames	78.0/100 frames	None found
Mean frequency	4.555/100 frames	3.84/100 frames	None found
Modal frequency	0.0/100 frames	0.0/100 frames	None found
Median frequency	3.0/100 frames	2.0/100 frames	None found

Figure 38. Camera Tool report: abnormal pixels frequency table.



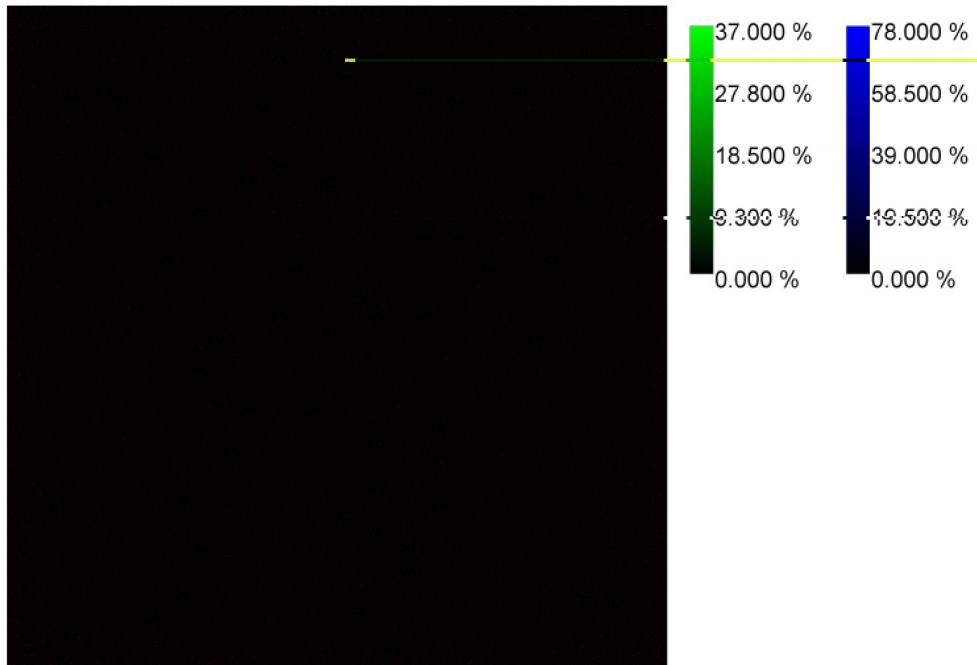


Figure 39. Camera Tool Report (long version). The warm/cold/hot pixels map.

The final [Analysis parameters](#) section reports all parameters used for the analysis (Figure 39).

#### Analysis parameters

Tool & Operator	Tool	Camera Noise
	Versions	MetroloJ_QC v1.1.2, ImageJ v2.1.0/1.53j, Java v1.8.0_172, OS Windows 10
	Operator & date	Julien, 21 juin 2021 16:44
data	result folder	D:\Users\Julien Cau\Desktop\MetroloJ QC Test\Detecteurs\CCD\Processed\QC2106-32\Lecture_1_w1Cy5camera_t1
	type of saved data	.pdf, .jpg, .xls
	input data bit depth	16
Noise	Compute	true
	Create noise map and frequency histogram.	true - log scale histogram - fixed ranged map
Warm and Cold pixels	Compute	true
	warm/cold if differs from more than	10.0 % from the image mean
Hot pixels	true	
Channels	Use one channel only	true
	channel used if true	0



Figure 40. Camera noise report: the plugin's parameters table.



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## 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 40).

**STEP4.** The file structure is analysed and the number of channels identified. Fill-in the names of each channel/detector and choose whether all channels/detectors should be analysed 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.

**STEP5.** As Variation Coefficient of saturated Rois will be biased, the user has the possibility to discard any saturated image.

**STEP6.** The user has the possibility to i) generate a pdf report/open it, ii) save jpg images of the channels+ROIs/intensity distribution profiles in each Roi and iii) save all measured values in a tab separated values spreadsheet and ROIs in a zip folder. The user has the possibility to get a longer/shorter version.

**STEP7.** Click on Ok. The report is generated. 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).



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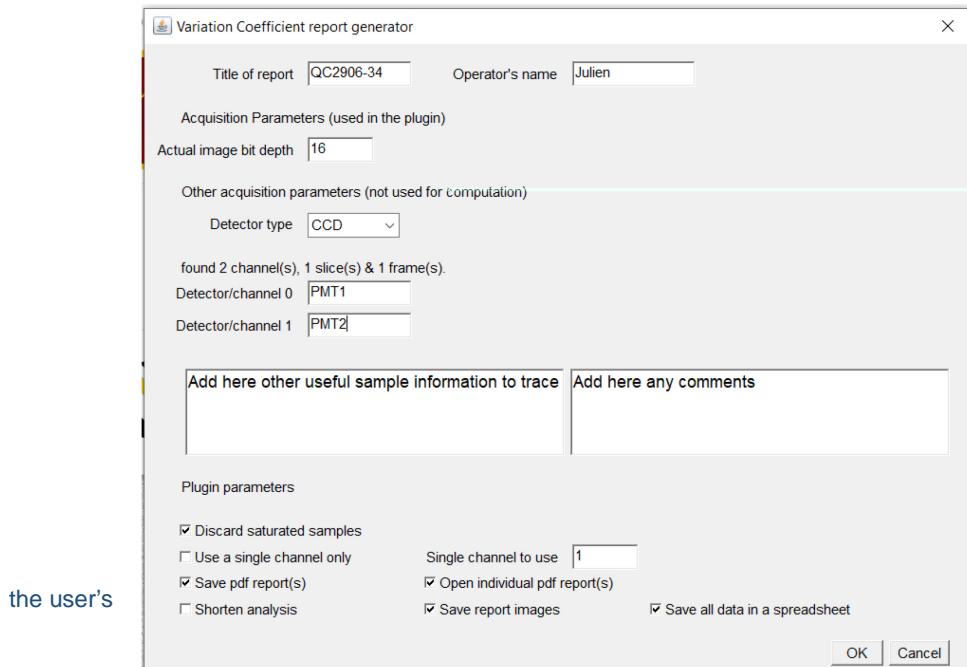


Figure 41. Variation Coefficient Tool: interface.

the user's

## Description of the Variation Coefficient Tool report

The report starts with information and warnings on the input image (figure 42).



**QC2906-34**

### Microscope info:

Image	CV	
(found) image's creation date	2021-06-30 12:39:27 (from file creation date)	
Detector	CCD	
Detector output bit depth	16	
Saturation	PMT1 PMT2	none none

### Warnings:

(No saturated pixels detected).

Figure 42. Variation Coefficient Tool report: microscope info and warnings sections.



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The next [ROIs used for measurements](#) section shows the location of each color-coded ROI (figure

### ROIs used for measurements:

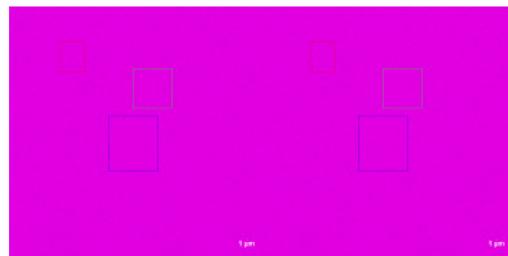


Figure 43. Variation Coefficient Tool report: locations of ROIs used for the analysis

Then, the intensity distribution profiles for each channel/detector are displayed (figure 44). A color code indicates the ROIs. The following tables displays:

The standard deviation  $\sigma$  and average intensity  $\mu$  of the channel intensity within each ROI (column). The variation coefficient  $VC$  is calculated using formula W.

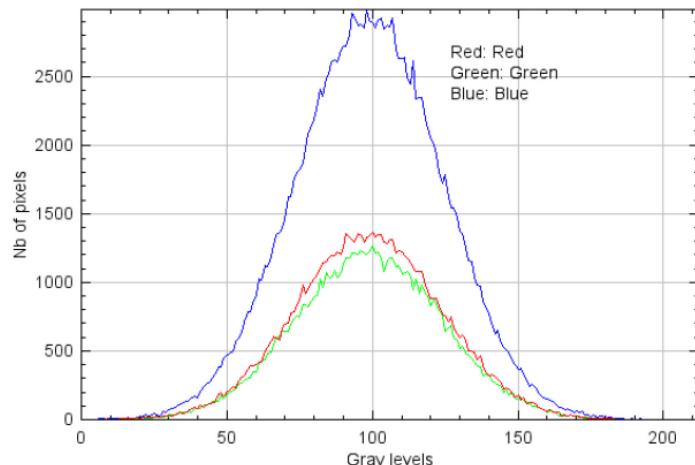
$$VC = \frac{\sigma}{\mu} (W)$$

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 45).



## PMT1



	Red	Green	Blue
Standard deviation	25.127	25.17	25.187
Average	98.61	98.697	98.842
Nb pixels	84645	76500	185040
CV	0.255	0.255	0.255
CVs relative to min CV value	1.0	1.001	1.0

Figure 44. Variation Coefficient Tool report: the VC table and ROI histograms.

## Analysis parameters

Tool & Operator	Tool	Variation Coefficient
	Versions	MetroloJ_QC v1.1.3, ImageJ v1.53g, Java v14.0.2, OS Windows 10
data	Operator & date	Julien, 30 juin 2021 13:07
	result folder	D:\Users\Julien Cau\Desktop\MetroloJ QC Test\Detector\Processed\QC2000-33HDV
	type of saved data	.pdf, .jpg, .xls
	input data bit-depth	16
	Use one channel only	false
	channel used if true	-

Figure 45. Variation Coefficient Tool report: the analysis parameters table.



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## 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 analysed. 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

**STEP1.** To use the plugin, Start ImageJ, launch the MetroloJ\_QC bar (plugins>MetroloJ\_QC).



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**STEP2.** Click on the batch field illumination tool icon. The user is prompted to select the folder containing the images. The plugin will open the first bioformat-compatible image of the folder. The plugin's interface should appear (see Figure 46).

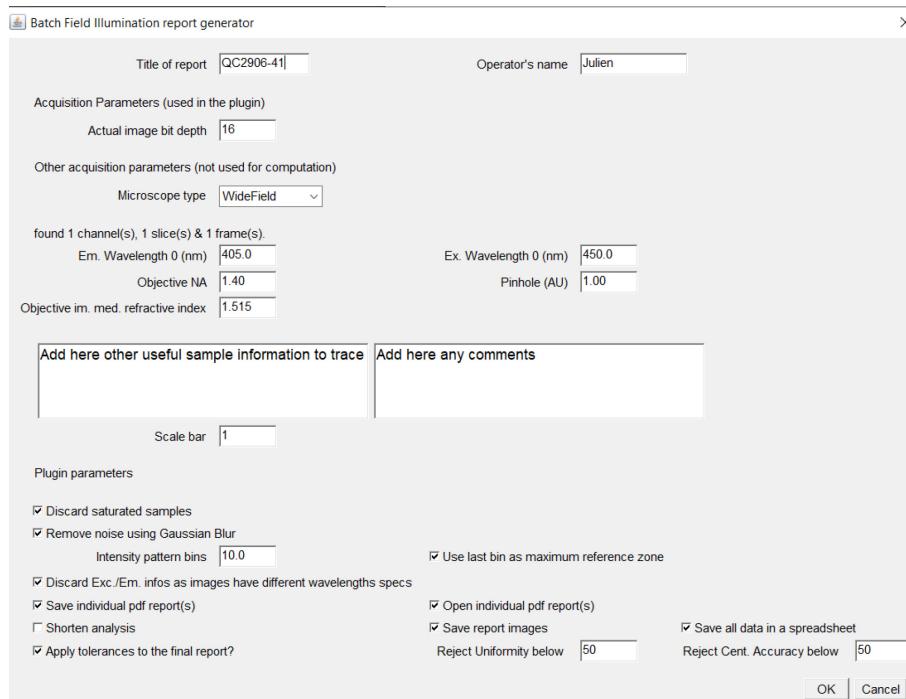


Figure 46. Batch Field Illumination Tool: the user's interface.

**STEP3.** The user will find the familiar interface of 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 infos as images have different wavelengths specs”. This will erase from any individual report this information, that is anyhow not useful for any of the plugin calculation.

**STEP4.** 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 step 4, 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).



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The [microscope info](#) section (Fig. 47) indicates how many images are analysed (unsaturated images if discard saturated samples is selected, total images if not). If the **STEP3** “discard Exc/Em infos as images have different wavelengths specs” option was chosen, these specs are greyed out in the microscope info table.



## QC2906-41 - SUMMARY

### Microscope info:

data	29 analysed images		
Actual image depth	16		
Microscope	WideField		
Objective	NA: 1.4 & im. refractive index: 1.515		
Channel	Channel		unsaturated/total images
Channel	Ex. (nm)	Em. (nm)	
Channel 0			29/29

### Warnings:

(no saturation issue detected)

Figure 47. Batch Field Illumination Report: microscope info and warnings sections.

The next [main field illumination parameters](#) table is a summary of all results (Figure 48). As in the regular Field Illumination Tool, within and outside specs can be highlighted using the appropriate option.



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**Main Field Illumination parameters:**

image	Channel	Uniformity	field Uniformity	Centering Accuracy
100X-CY5	Channel 0	67.8	92.7	96.7
100X-dapi	Channel 0	54.8	88.9	76.6
100X-dsred	Channel 0	57.5	91.0	88.2
100X-gfp	Channel 0	55.6	89.7	88.9
100X-TR	Channel 0	57.8	91.1	84.3
10X-CY5	Channel 0	46.4	89.3	91.8
10X-dapi	Channel 0	41.8	87.0	80.3
10X-dsred	Channel 0	44.4	89.8	96.8
10X-gfp	Channel 0	43.1	88.8	95.3
10X-TR	Channel 0	46.5	89.8	98.3
20X-CY5	Channel 0	56.5	90.4	94.5
20X-dapi	Channel 0	59.5	88.5	70.7
20X-dsred	Channel 0	62.7	91.6	96.7
20X-gfp	Channel 0	63.1	92.3	92.0
20X-TR	Channel 0	59.2	91.1	99.1
40X-CY5	Channel 0	58.6	91.1	97.1
40X-dapi	Channel 0	49.2	88.7	77.8
40X-dsred	Channel 0	50.2	90.2	97.2
40X-gfp	Channel 0	55.4	90.5	85.3
40X-TR	Channel 0	50.6	90.4	96.9
5X-405	Channel 0	41.6	87.0	80.5
5X-CY5	Channel 0	57.0	92.5	94.8
5X-dapi	Channel 0	46.6	66.9	75.6
5X-dsred	Channel 0	65.1	93.4	95.7
5X-gfp	Channel 0	31.0	92.9	94.0
5X-TR	Channel 0	63.0	93.3	98.1
63X-CY5	Channel 0	64.1	94.6	93.1
63X-dapi	Channel 0	44.2	87.0	76.4
63X-dsred	Channel 0	66.9	92.5	95.3
63X-gfp	Channel 0	51.2	88.8	83.0
63X-TR	Channel 0	64.9	92.3	95.5

Centering accuracy computed using the 300% / 700% reference zone. Images with one or more channels failing to meet the specifications (ie. uniformity below 50.0 or centering accuracy below 50.0)

Figure 48. Batch Field Illumination Tool Report: the MFI section.

Finally, for the sake of keeping track of the parameters used for analysis, the **Analysis parameters** table (Figure 49) is added at the end of the report, along with the **formulas used** and the **analysed image** list (see figure 50). If the “discard saturated sample” option was chosen, then any image that was not analysed because saturated will still be listed here, so that the user knows the image was open but analysis stopped as saturation was found.



## Analysis parameters

Tool & Operator	Tool	Batch Field-Illumination
	Versions	MetroloJ_QC v1.1.3, ImageJ v1.53g, Java v14.0.2, OS Windows 10
	Operator & date	Julien, 30 juin 2021 14:10
data	result folder	D:\Users\Julien Cau\Desktop\MetroloJ_QC Test\Homogénéité\Processed\QC2906-41\
	type of saved data	.pdf, .jpg, .xls
	input data bit depth	16
dimension order		
discard saturated samples		
Gaussian blur noise removal applied		
isointensity image steps width		
Reference zone		
Tolerance	applied in this report	true
	Uniformity valid if above	50.0
	CA valid if above	50.0 ..

Figure 49. Batch Field Illumination Tool report: analysis parameters section.

## Analysed images:

- 100xCY3: analysed
- 100xCY5: analysed
- 100xDAPI: analysed
- 100xGFP: analysed
- 100xTR: analysed
- 10xCY3: analysed
- 10xCY5: analysed
- 10xDAPI: analysed

Figure 50. Batch Field Illumination Tool report: the list of all analysed images





## 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 analyse 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).

### 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 analyse (Figure 51). 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



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found bioformats-compatible image will be opened and the plugin's main dialog interface should appear (see Figure 52).

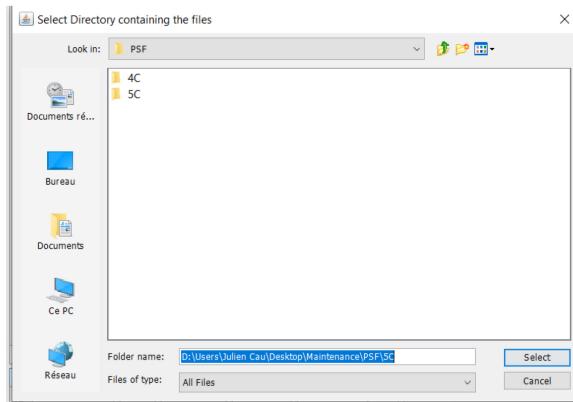


Figure 51. Batch PSF Profilertool: the first dialog window.

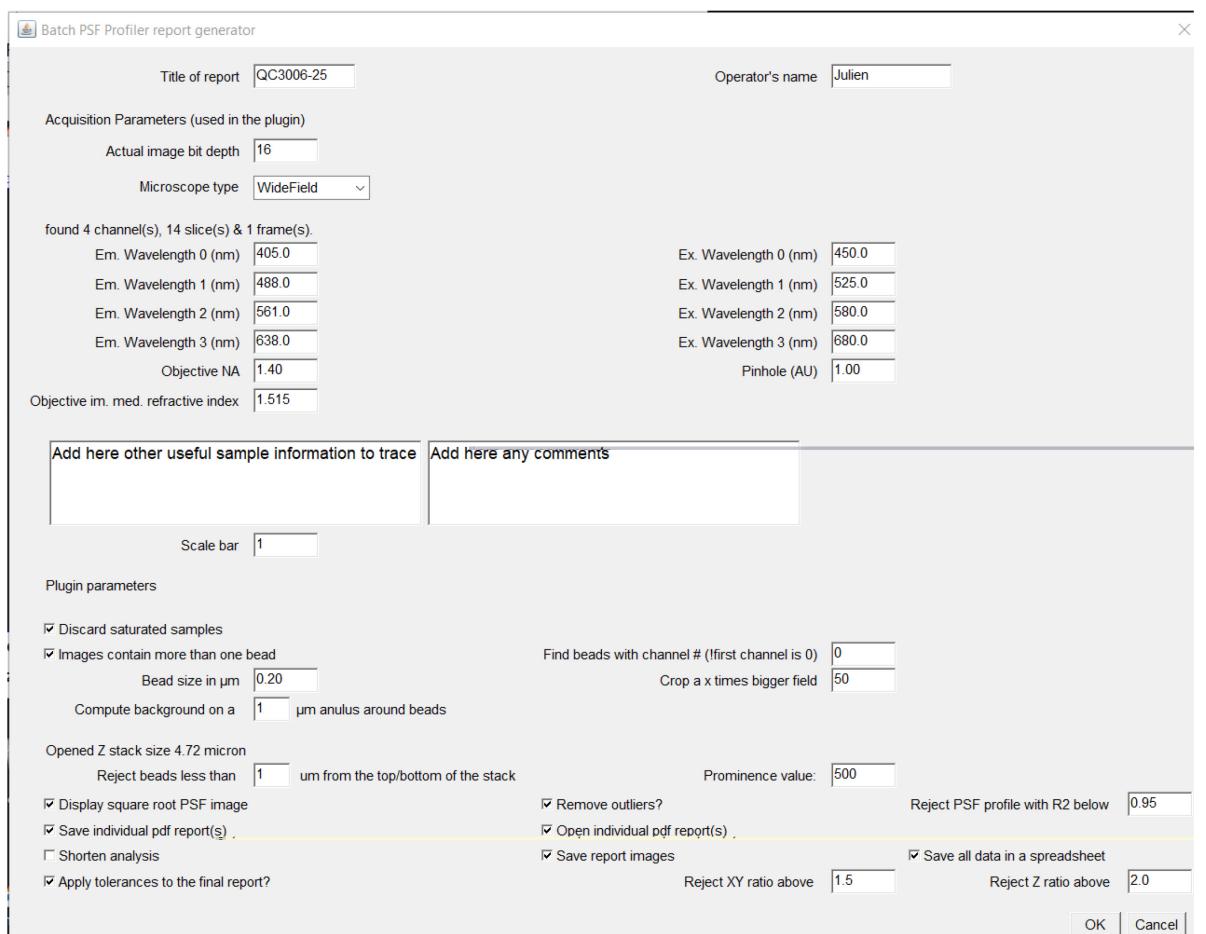


Figure 52. Batch PSF Profiler tool: the user's interface/Main dialog window.



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**STEP3.** Most of the field/parameters are similar to the PSF Profiler tool. Two additional fields allow aggregation/filtering/compilation of the results:

- “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.
- “Remove outliers”: 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. All values below  $Q1 - 1.5 * IQR$  (lower fence) and above  $Q3 + 1.5 * IQR$  are discarded. When checked, outliers will be removed. Mind the number of total beads analysed (“Microscope section” of the main report) is not the number of total beads used at the end to compute each mean FWHM.

**STEP4.** If selected, each individual bead image pdf report/jpg image/datasheet will be saved. If none of them are selected, the whole process of PSF profiler report goes on and the main pdf report will be produced together with summary spreadsheets. If selected, each individual report (if any) and the main report may be processed using the above mentioned tolerances.

#### Description of the Batch PSF Profiler report

The SUMMARY report has similar [Microscope info & warnings](#) sections (Figure 53) this will report all acquisition parameters as in Generate PSF Profiler report for each channel is reported for each section (unsaturated samples if the discard saturation option is used, total images if not). The warnings are provided to help result interpretation.



Microscope info:

data	17 analysed images				
Actual image depth	16				
Microscope	WideField				
Objective	NA: 1.4 & im. refractive index: 1.515				
Channel	Channel		sampling (X,Y,Z)		unsaturated/total images
Channel	Ex. (nm)	Em. (nm)	Nyquist ( $\mu\text{m}$ )	correctly sampled/total images	
Channel 0	450.0	405.0	0.072x0.072x0.216	(17/17, 17/17, 0/17)	17/17
Channel 1	525.0	488.0	0.087x0.087x0.261	(17/17, 17/17, 0/17)	17/17
Channel 2	580.0	561.0	0.1x0.1x0.3	(17/17, 17/17, 0/17)	17/17
Channel 3	680.0	638.0	0.114x0.114x0.341	(17/17, 17/17, 0/17)	17/17

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

The resolution table (Figure 54) 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).

Resolution table:

Channel	X	Y	Z
Channel 0	0.253 +/- 0.05 $\mu\text{m}$ 16.0 beads (0.148 $\mu\text{m}$ ) mean $R^2$ : 0.89 mean SBR: 11.71	0.243 +/- 0.038 $\mu\text{m}$ 14.0 beads (0.148 $\mu\text{m}$ ) mean $R^2$ : 0.9 mean SBR: 11.71	0.902 +/- 0.21 $\mu\text{m}$ 17.0 beads (0.554 $\mu\text{m}$ ) mean $R^2$ : 0.91 mean SBR: 11.71
Channel 1	0.295 +/- 0.024 $\mu\text{m}$ 16.0 beads (0.178 $\mu\text{m}$ ) mean $R^2$ : 0.96 mean SBR: 13.2	0.325 +/- 0.035 $\mu\text{m}$ 16.0 beads (0.178 $\mu\text{m}$ ) mean $R^2$ : 0.94 mean SBR: 13.2	1.254 +/- 0.121 $\mu\text{m}$ 17.0 beads (0.668 $\mu\text{m}$ ) mean $R^2$ : 0.95 mean SBR: 13.2
Channel 2	0.293 +/- 0.02 $\mu\text{m}$ 16.0 beads (0.204 $\mu\text{m}$ ) mean $R^2$ : 0.98 mean SBR: 13.13	0.317 +/- 0.024 $\mu\text{m}$ 16.0 beads (0.204 $\mu\text{m}$ ) mean $R^2$ : 0.94 mean SBR: 13.13	1.349 +/- 0.066 $\mu\text{m}$ 17.0 beads (0.768 $\mu\text{m}$ ) mean $R^2$ : 0.96 mean SBR: 13.13
Channel 3	0.297 +/- 0.04 $\mu\text{m}$ 16.0 beads (0.232 $\mu\text{m}$ ) mean $R^2$ : 0.93 mean SBR: 8.13	0.365 +/- 0.038 $\mu\text{m}$ 16.0 beads (0.232 $\mu\text{m}$ ) mean $R^2$ : 0.91 mean SBR: 8.13	1.432 +/- 0.108 $\mu\text{m}$ 17.0 beads (0.873 $\mu\text{m}$ ) mean $R^2$ : 0.93 mean SBR: 8.13

Figure 54. Batch PSF Profiler Tool Report: resolution info section.



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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 55).

Measured/theoretical resolution ratios and lateral asymmetry ratios:

Channel	X ratio	Y ratio	Z ratio	Lateral Asymmetry
Channel 0	1.72	1.65	1.64	0.96
Channel 1	1.66	1.83	1.88	0.91
Channel 2	1.43	1.55	1.76	0.93
Channel 3	1.28	1.57	1.64	0.81

Green: within specifications, red: outside specifications (ie. XY ratios above 1.5 or Z ratio above 2.0)

Figure 55. Batch PSF Profiler tool Report. Ratio section.

Finally, a table containing all used analysis parameters is provided (Figure 56).



## Analysis parameters

Tool & Operator	Tool	Batch PSF Profiler
	Versions	MetroloJ_QC v1.1.3, ImageJ v1.53g, Java v14.0.2, OS Windows 10
	Operator & date	Julien, 30 juin 2021 18:58
data	result folder	D:\Users\Julien.Cau\Desktop\Metrolo.LOC_Test\psfsubset\Processed\QC3006-25
	type of saved data	.pdf, .jpg, .xls
	input data bit depth	16
dimension order		XY-(C)Z
discard saturated samples		true
beads	Background anulus thickness in µm	1.0 (theoretical, see individual reports for real, used values)
	multiple beads in image	true
	bead detection channel	0
	bead size (µm)	0.2
	bead crop Factor	10.0
	bead rejection distance to top/bottom	0.5 µm
outliers removed		true
discard R2 ratio below		0.95
Tolerance	applied in this report	true
	X & Y FWHM ratios valid if below	1.5
	Z FWHM ratio valid if below	2.0

Bead4: analysed

Bead5: analysed

Bead6: analysed

Figure 56. Batch PSF Profiler tool Report: the analysis parameters section.

Figure 57. Batch PSF Profiler tool Report: the list of analysed images/beads.





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## BATCH CO-REGISTRATION TOOL

### Analysis dataset requirements for Batch Co-registration Tool

Images to analyse should be stored in the same folder (if images are in subfolders, they will not be analysed). 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 analyse (Figure 58). 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 59).



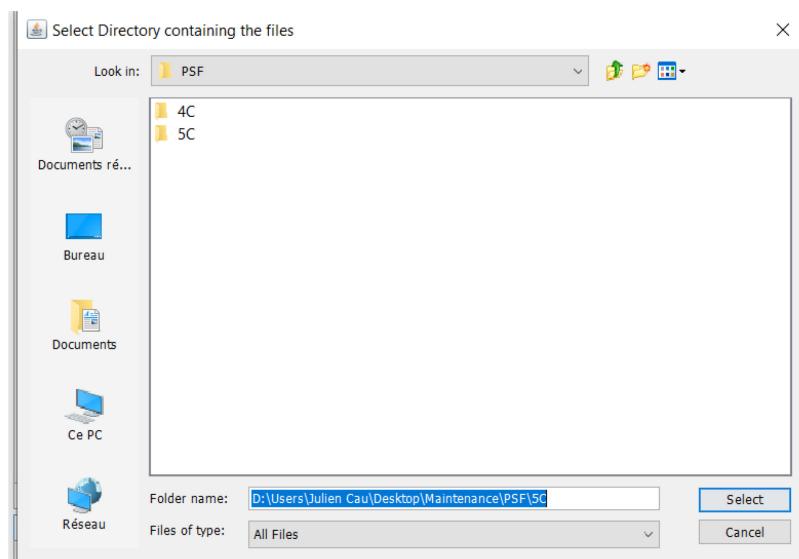


Figure 58. Batch Co-registration tool: first dialog window.

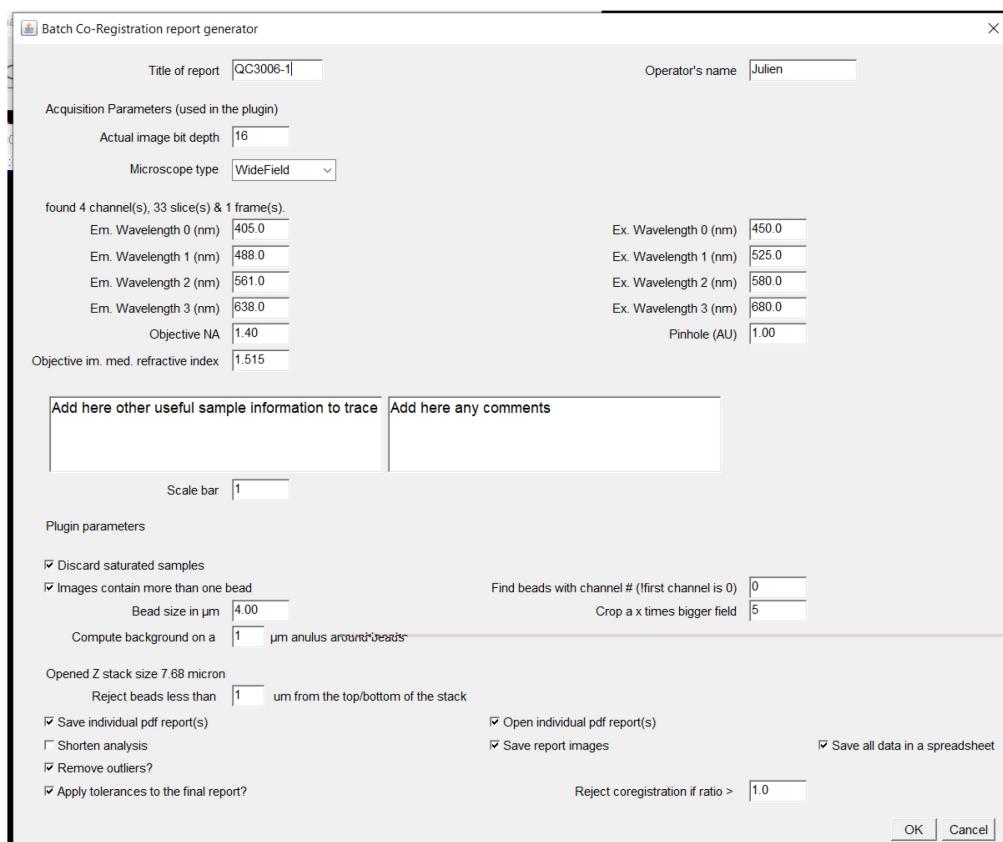


Figure 59. Batch Co-registration Tool: the user's interface/main dialog window

**STEP3.** Fields should be filled-in as for the co-registration tool. An additional “Remove outliers” field allows the removal of aberrant values. The list of values to be averaged is ordered, the median and Q1/Q3 values are calculated. The interquartile range



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$IQR=Q3-Q1$  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 analysed beads. Mind the number of total beads analysed ("Microscope section" of the main report) is not the number of total beads used at the end to compute each mean ratio.

**STEP4.** If selected, each individual bead image pdf report/jpg image/datasheet will be saved. If none of them are selected, the whole process of co-registration report goes on and the main pdf report will be produced together with summary spreadsheets. If selected, each individual report (if any) and the main report may be processed using the above mentioned tolerances.

#### Description of the Batch Co-registration tool report.

The main SUMMARY report starts with the [Microscope info & QC3006-1](#) (Figure 60) report a summary of all acquisition parameters as in the co-registration tool report. The number of total beads analysed is reported at the end of the section. The number of saturated channels analysed and correctly sampled channels is also given.

The Ratio table (Figure 61) 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 summary.xls spreadsheet. The raw ratio table is provided for each bead image/combination.





## QC3006-55 - SUMMARY

### Microscope info:

data	2 analysed images				
Actual image depth	16				
Microscope	WideField				
Objective	NA: 1.4 & im. refractive index: 1.515				
Channel	sampling (X,Y,Z)			unsaturated/total images	
Channel	Ex. (nm)	Em. (nm)	Nyquist ( $\mu$ m)	correctly sampled/total images	
Channel 0	450.0	405.0	0.072x0.072x0.216	(2/2, 2/2, 0/2)	2/2
Channel 1	525.0	488.0	0.087x0.087x0.261	(2/2, 2/2, 2/2)	2/2
Channel 2	580.0	561.0	0.1x0.1x0.3	(2/2, 2/2, 2/2)	2/2
Channel 3	680.0	638.0	0.114x0.114x0.341	(2/2, 2/2, 2/2)	2/2

### Warnings:

(no saturation issue detected)

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

### Ratios table:

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		0.374 +/- 0.171 (n=9.0)	0.877 +/- 0.175 (n=9.0, 33.0% failed)	0.794 +/- 0.302 (n=9.0, 33.0% failed)
Channel 1	0.374 +/- 0.171 (n=9.0)		0.835 +/- 0.34 (n=9.0, 44.0% failed)	0.881 +/- 0.339 (n=9.0, 44.0% failed)
Channel 2	0.877 +/- 0.175 (n=9.0, 33.0% failed)	0.835 +/- 0.34 (n=9.0, 44.0% failed)		0.256 +/- 0.044 (n=6.0)
Channel 3	0.794 +/- 0.302 (n=9.0, 33.0% failed)	0.881 +/- 0.339 (n=9.0, 44.0% failed)	0.256 +/- 0.044 (n=6.0)	

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

Figure 61. Batch Co-registration report: an example of the main report.

Long version of the report include average pixel shift tables, uncalibrated and calibrated distances (Figure 62).



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Analytical

Pixel shift table:

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		-0.444 +/- 0.831 (n=9.0) 1.333 +/- 0.972 (n=9.0) -0.833 +/- 0.236 (n=9.0)	-0.5 +/- 0.408 (n=9.0) 2.889 +/- 0.567 (n=9.0) -1.944 +/- 0.157 (n=9.0)	-0.556 +/- 3.363 (n=3.3) 2.889 +/- 0.458 (n=9.0) -1.944 +/- 0.157 (n=9.0)
Channel 1	0.444 +/- 0.831 (n=9.0) -1.333 +/- 0.972 (n=9.0) 0.833 +/- 0.236 (n=9.0)		-0.056 +/- 0.497 (n=9.0) 1.556 +/- 0.497 (n=9.0) -1.111 +/- 0.208 (n=9.0)	-0.111 +/- 0.567 (n=9.0) 1.556 +/- 0.598 (n=9.0) -1.111 +/- 0.208 (n=9.0)
Channel 2	0.5 +/- 0.408 (n=9.0) -2.889 +/- 0.567 (n=9.0) 1.944 +/- 0.157 (n=9.0)	0.056 +/- 0.497 (n=9.0) -1.556 +/- 0.497 (n=9.0) 1.111 +/- 0.208 (n=9.0)		-0.056 +/- 0.283 (n=9.0) 0.0 +/- 0.236 (n=9.0) 0.0 +/- 0.0 (n=9.0)
Channel 3	0.556 +/- 0.369 (n=9.0) -2.889 +/- 0.458 (n=9.0) 1.944 +/- 0.157 (n=9.0)	0.111 +/- 0.567 (n=9.0) -1.556 +/- 0.598 (n=9.0) 1.111 +/- 0.208 (n=9.0)	0.056 +/- 0.283 (n=9.0) 0.0 +/- 0.236 (n=9.0) 0.0 +/- 0.0 (n=9.0)	
Resolutions (pix.)	1.086 1.086 1.304	1.308 1.308 1.572	1.504 1.504 1.807	1.711 1.711 2.055

Distances table (uncalibrated):

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		2.216 +/- 0.079 (n=6.0)	3.556 +/- 0.497 (n=9.0)	3.556 +/- 0.405 (n=9.0)
Channel 1	2.216 +/- 0.079 (n=6.0)		1.998 +/- 0.451 (n=9.0)	2.034 +/- 0.502 (n=9.0)
Channel 2	3.556 +/- 0.497 (n=9.0)	1.998 +/- 0.451 (n=9.0)		0.278 +/- 0.248 (n=9.0)
Channel 3	3.556 +/- 0.405 (n=9.0)	2.034 +/- 0.502 (n=9.0)	0.278 +/- 0.248 (n=9.0)	
Resolutions (pix.)	1.086 1.086 1.304	1.308 1.308 1.572	1.504 1.504 1.807	1.711 1.711 2.055

Distances table (calibrated):

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		0.381 +/- 0.061 µm (n=8.0)	0.673 +/- 0.079 µm (n=9.0)	0.673 +/- 0.063 µm (n=9.0)
Channel 1	0.381 +/- 0.061 µm (n=8.0)		0.382 +/- 0.07 µm (n=9.0)	0.388 +/- 0.074 µm (n=9.0)
Channel 2	0.673 +/- 0.079 µm (n=9.0)	0.382 +/- 0.07 µm (n=9.0)		0.045 +/- 0.04 µm (n=9.0)
Channel 3	0.673 +/- 0.063 µm (n=9.0)	0.388 +/- 0.074 µm (n=9.0)	0.045 +/- 0.04 µm (n=9.0)	
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

Figure 62. Batch Co-registration tool Report: the pixel shift and distance tables (long version only)

Figure 63. Batch Coalignment tool Report. the analysis parameters table.



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