

PSFj manual

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1. Introduction

1.1 In brief

PSFj is a software tool that automatically analyses the full field-of-view (FOV) performance of a given fluorescence microscope/objective lens combination with respect to its optical resolution and chromatic aberrations. PSFj provides reporting functions to document the momentary performance of a system, and it allows for the export of the obtained data, e.g. for image restoration purposes.

PSFj is based on ImageJ and JAVA, it runs on Windows, Mac and Linux PCs, as a stand-alone application.

1.2 Applications

PSFj was developed with a number of applications in mind in order to satisfy the needs of

- Microscope users
- Microscope facility managers
- Microscope developers

... as an easy-to-use tool to quantify the performance of fluorescence light microscopes and their optical components for ...

- Routine checks of microscopes and objective lens performance
- Comparison and selection of best-performing objectives/microscopes
- Calibration of microscopes for quantitative applications

1.3 Principle, scope, and deliverables

PSFj is based on the analysis of image stacks of a large number (many hundreds) of fluorescent beads. A quantitative analysis of the fluorescence signals from the beads provides information on the resolution, the flatness of the focal plane and the fidelity of the chromatic correction along the lateral and axial dimensions. All information is retrieved as a function of position in the FOV.

Using 2D fitting algorithms, the analysis measures for each fluorescence channel ...

- the PSFs lateral and axial **full-width-at-half-maximum (FWHM)**. This provides a measure for the resolution.
- the **center-of-mass co-ordinates**. This provides measures of planarity and chromatic aberration correction. It furthermore detects misaligned/tilted stages and sample-holders or cameras.

Upon analysis of the data, the following is delivered:

- The data is visualized using heat maps to represent the information across the FOV.
- The data is compared to the diffraction limited (theoretical) performance of the system.
- A pdf file containing all microscope information and the analysis results is generated to document the measurements.
- Data can be exported using .csv files.
- Interactive inspection and export of the results for specific beads in the image stack is possible

In particular: PSFj outputs a .xls file to be used with bUnwarpJ, an ImageJ plugin (Ignacio Arganda-Carreras, Carlos O. S. Sorzano, Roberto Marabini, Jose M. Carazo, Carlos Ortiz de Solorzano, and Jan Kybic, [“Consistent and Elastic Registration of Histological Sections using Vector-Spline Regularization”](#), Lecture Notes in Computer Science, Springer Berlin / Heidelberg, volume 4241/2006, CVAMIA: *Computer Vision Approaches to Medical Image Analysis*, pages 85-95, 2006. ; <http://biocomp.cnb.csic.es/~iarganda/bUnwarpJ/>) for lateral non-linear (elastic) alignments of images of different channels ('unwarping' the wavelength dependence of chromatic aberrations).

1.4 FAQ

For what types of microscopes can I use PSFj?

Since the analysis is based on image-stacks of fluorescent beads, PSFj can be used to investigate any microscope that can produce such data, e.g. Light sheet-, Confocal-, Widefield-, Spinning Disc-, TIRF-microscopes, etc.

What type of results do I get?

All results are provided as function of position in the FOV and include (see Figure 4.7):

- 1) minimum and maximum lateral FWHM (resolution in the xy-plane)
- 2) the resolution asymmetry (A), i.e., the ratio of the minimal lateral FWHM (FWHM_{\min}) over the maximum lateral FWHM (FWHM_{\max}). (→ Parameter A)
- 3) the orientation of the maximum lateral FWHM, in the case of distorted PSFs.
- 4) the axial FWHM (resolution along the z-axis)
- 5) the z-position of the best focus as a function of the position in the FOV. This provides a measure of the planarity of the focal plane

and in the case of dual-color analysis:

- 6) the lateral and axial chromatic shift throughout the FOV, for different color channels (emission wavelengths). This is a measure of lateral and axial chromatic aberrations.

2. Installation

2.1 System requirements

PSFj runs on Windows 7, Linux, and MacOS 10.9 and requires Java 7

Recommended minimum hardware configuration is:

- 64-bits OS and JAVA version
- Dual-core processor
- 4GB of RAM*

*the amount of RAM required for an analysis is about twice the size of the analyzed data. In other words, to analyze 4GB of data, PSFj requires 8GB of RAM. Note: PSFj can only use 2GB of memory on 32-bits systems, no matter how much memory the computer is equipped with.

2.2 Run PSFj

To run PSFj, extract the zip-file (http://www.knoplabs.de/psfj/dl/psfj_latest.zip for Windows and Linux, http://www.knoplabs.de/psfj/dl/psfj_macos_latest.zip for MacOS). It will extract a folder “PSFj” containing executables (psfj.exe for Windows, psfj for Linux, and PSFj for MacOS) and a library “psfj__lib” folder. Note: PSFj runs only if the “psfj__lib” folder is in the same folder as the executables. For convenience you can generate a shortcut and place it in a place of your choice, e.g. desktop.

3. Acquisition of image stacks of fluorescent beads

In order to obtain optimum performance from your fluorescence microscope it is crucial to realize that microscopes have been designed for a highly specific set of conditions including the refractive index of the immersion medium, coverslip thickness, temperature. Even slight deviations from the specified conditions can have substantial (deteriorating) effects on the performance of the system. It is thus pivotal to adhere to the conditions the microscope was designed for. Caution should thus be used when attributing a bad performance to the microscope – imaging might have just been under conditions for which the microscope was not designed. First step in judging or dealing with an apparent underperformance of a microscope should thus be to check if the used imaging conditions match the conditions the system was designed for. In this context it is of course of particular importance to use a properly prepared sample slide containing the fluorescent beads needed for testing the system.

3.1 Sample preparation

Glass slide and cover slips

Cover slip thickness needs to adhere tightly to the value specified for the objective (usually 170 µm thick – #1.5). In addition, beads should be attached to the cover slip and not to the glass slide since the latter will position the beads a few micrometer away from the cover slip glass surface when imaged through the cover slip.

Fluorescent beads

For direct extraction of PSFs from an image stack of fluorescent beads (use multicolor beads if you intend to measure chromatic aberrations), the beads should ideally be as small as possible. However the smaller the beads the lower the signal-to-noise ratio (for a given exposure time) and the more difficult the quantification becomes. When using fluorescence microscopes with 20 – 100 x magnification lenses, we thus recommend using beads with a diameter of 100 – 200 nm. We achieved good results with 175 nm (green) single-color beads (PS-speck, Kit P-7220, Molecular Probes) for resolution and planarity measurements or 220 nm diameter multicolor beads (TetraSpeck, T-7280, Molecular Probes) for chromatic colocalization measurements. PSFj will automatically correct for the bead size. Also, check that your beads are essentially aggregate free. If necessary, try to disperse these aggregates using a water bath sonicator. The beads should also exhibit a narrow size distribution, i.e., a small coefficient of variation of their diameter (check the specification of the manufacturer). For preparation of the slide, follow the manufacturer's protocol, which usually includes the following steps:

1. Dilute the original bead suspension to a suitable concentration (around 10^9 beads per ml).
Bead density is critical: if the density is too high, this will cause that many beads will be discarded from the analysis. If the density is too low, only few beads are available for analysis and the statistics will not be good. Manually prepared bead slides show heterogenous

distributions of the beads, so that image stacks with higher or lower bead densities can be recorded and analyzed.

2. Sonicate the bead solution (using a water bath sonicator) in order to dissociate possible aggregates
3. Wash a #1.5 (170 µm thick) coverslip using 70% ethanol (please note: some objective lenses require coverslips of different thickness. In the case of water-dipping objectives, no coverslip is required; in this case the beads are adsorbed directly on the surface of the glass slide, as described in the next step.)
4. Place a small amount (around 5-10 µl) of the bead solution on the coverslip and let it dry.
5. Place a small amount (depending on your coverslip size, around 10 µl for 24x24 mm coverslips) of mounting medium (e.g. the mounting medium included in the PS-speck Kit P-7220, Molecular Probes) on the coverslip and place the slide upside down onto a microscope slide.
6. Seal the gap between coverslip and microscope slide, e.g. using non-fluorescent nail polish.

(**Please note:** Dust/dirt particles, but also too much or too little mounting medium can cause that the coverslip is not aligned parallel to the surface of the glass slide. If this is the case, the coverslip surface with the beads may not be orthogonal to the optical axis of the microscope. Consequently PSFj will show a linear gradient (along the direction of the tilt) in the heat map that visualizes the planarity of the system.)

3.2 Image acquisition

For recording a z-stack of fluorescence images of the beads, the following has to be taken into consideration.

Number of planes and spacing

The stack should cover a range of about 6 to 10 times the expected FWHM, i.e., $\approx 2\lambda n / (NA)^2$ centered on the plane of best focus, where λ is the wavelength of the fluorescence emission (center wavelength of the used emission filter), n is the refractive index of the used immersion medium (e.g. 1 for air, 1.33 for water, 1.52 for oil), and NA is the numerical aperture of the used objective lens. The spacing between planes should be about a third (or less) of the expected FWHM, i.e., $\approx 2\lambda n / (NA)^2 / 3$ (Nyquist sampling). Exposure times should be adjusted to yield a signal-to-noise-ratio (SNR) of at least 50.

(Note: PSFj assumes, that the camera pixel size is small enough to provide sufficient sampling (at least Nyquist) of the lateral extent of the PSF, i.e., that the pixel dimension is about a third (or less) than the expected lateral FWHM ($M \cdot 0.61\lambda / (NA) / 3$ where M is the overall magnification of the microscope. If this is not the case it might be possible to increase the magnification through the use of a different tube lens (e.g. by 1.5x) as is provided by most microscopes.

For the analysis of the chromatic aberrations of the system, two stacks of multi-color beads (see section 3.1) of the same FOV but at different excitation/emission wavelengths need to be acquired.

Typically, these stacks are acquired sequentially, however, (e.g. in the case of imprecise mechanics of the stage) interleaved acquisition of images at both wavelengths on a given plane is preferred. (Please note: Aberration between different color channels are not necessarily caused by chromatic aberrations, since also mechanical or thermal drift will influence the results. In order to identify such artifacts and to estimate how much of the detected aberrations are of ‘chromatic’ origin, it is possible to compare two independently recorded stacks of the same position in the sample and using the same wavelength settings.)

3.3 Image- and metadata import and export

Image file formats

PSFj requires image stacks of 16bit multipage tif format. Most proprietary microscope acquisition software packages are able to export the files in this format. In all other cases, ImageJ and the Bio-Formats plugin (<http://www.openmicroscopy.org/site/support/bio-formats5/users/imagej/>) can be used to convert files formats to tif.

Import of metadata

Due to the large heterogeneity in the metadata file formats PSFj does not import such data. Instead, the relevant data has to be entered by hand. However, once entered, this data is stored in the same folder with the images using the name of the image and the extension “.ini”. When opening an image stack in PSFj, any corresponding .ini file in the same folder will be loaded automatically.

The parameters required to calibrate the analysis are:

- The magnification (M) and the numerical aperture (NA) of the used objective lens (e.g. 60x, NA=1.40 – usually specified on the body of the objective) Note: most microscopes provide options to increase the magnification e.g. by 1.5x. If such option is used the relevant magnification parameter which needs to be entered in PSFj is the total magnification e.g. $60x \cdot 1.5x = 90x$.
- The refractive index (n) of the used immersion fluid (e.g. n=1 for air, n=1.33 for water, n=1.52 for oil – check the oil container)
- The diameter (e.g. 150 nm) of the fluorescent beads
- The emission wavelength (center wavelength of the used emission filter)
- The voxel size, i.e., the size of the volume elements. If this is not known, one can alternatively specify the size of the pixel on the camera chip (check the datasheet of your camera – Google will find it for you) and the spacing between the planes in the image stack. PSFj will then calculate the voxel size for you.

(Please note: it is easy to forget to note for example the position of the magnification knob (e.g. 1x or 1.5x) available with most microscopes. So, particular care should be taken to ensure proper documentation of the parameter.)

4. Image analysis

4.1 Image stack import

PSFj requires a multi-page tiff-file as input. Converting stacks of different formats or single images into a multi-page tiff-file can be done using ImageJ or Fiji (see also section 3.3). For chromatic aberration analysis, two stacks of the same (multi-color) beads are required, recorded using the wavelengths/filter you want to analyze (see also section 3.2). Currently, maximally two wavelengths can be compared. Hence, for comparison of 3 or more wavelengths, sequential analysis is required.

Just click on “Add image stack”. You can also drag and drop your image stack(s) onto the PSFj window (see Fig. 4.1).

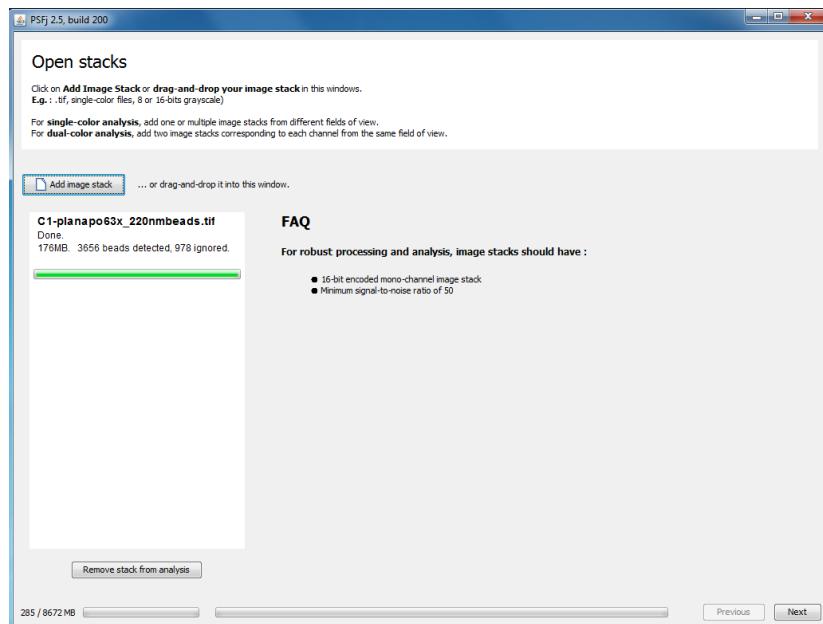


Fig. 4.1 Image-stack import for single-color analysis of a single image stack

For the analysis of one wavelength, multiple stacks acquired using the same settings but from different FOVs can be loaded and analyzed. This leads to improved statistics (see Fig. 4.2): the stacks will be analyzed together and the results will be combined.

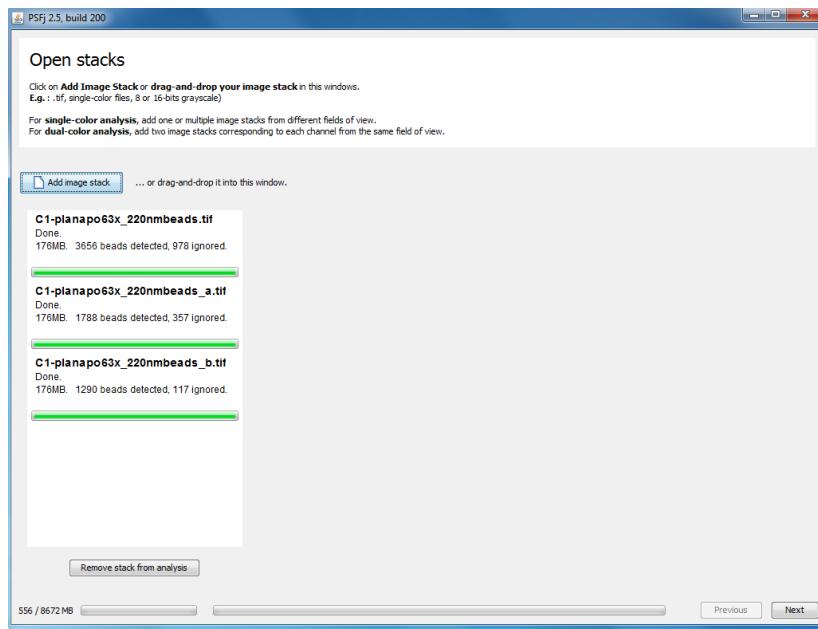


Fig. 4.2 Image-stack import for single-color analysis of multiple image stacks

You can add two image stacks acquired at different emission wavelengths for chromatic aberration (dual-color) analysis. In this case the center emission wavelength for each stack is required (see Fig. 4.3), and the performed analysis will automatically include chromatic aberrations.

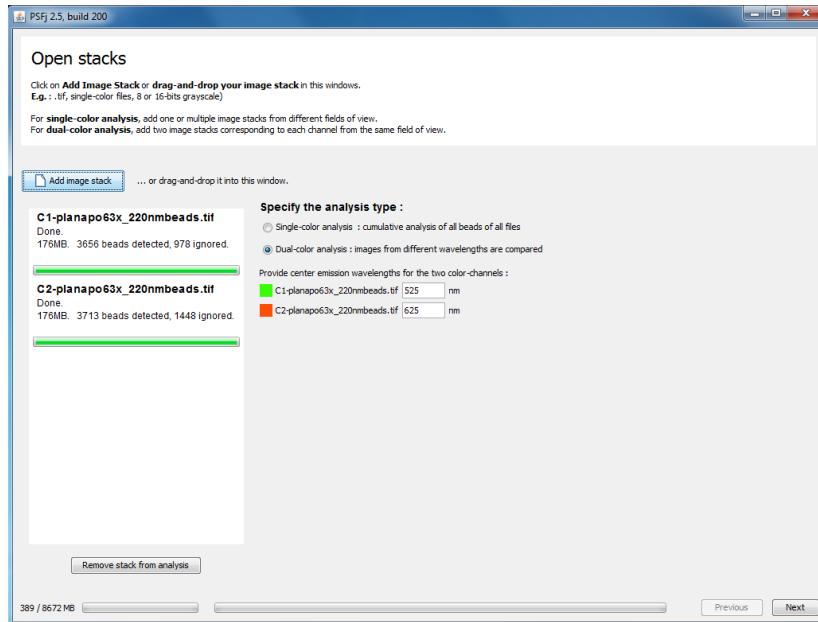


Fig. 4.3 Image-stack import for dual-color analysis. Only one stack per color can be loaded. Please specify the wavelengths.

To continue to the next step (calibration of the images) please click on ‘Next’ button.

4.2 Calibration

To calibrate the images (see Fig. 4.4) you need to provide the system specifications and the parameters for acquisition of the image stacks (see also section 3.3.). Once entered, the information will be saved in a separate file with the ending “.ini” into the folder containing your image stack(s). Subsequent import of the image stack in PSFj will automatically import all relevant parameters from this file and no manual input is required. In the case of dual color analysis, the information on the emission wavelengths of the two color image stacks has been entered in the previous (image-stack import) window or imported automatically from the “.ini” files.

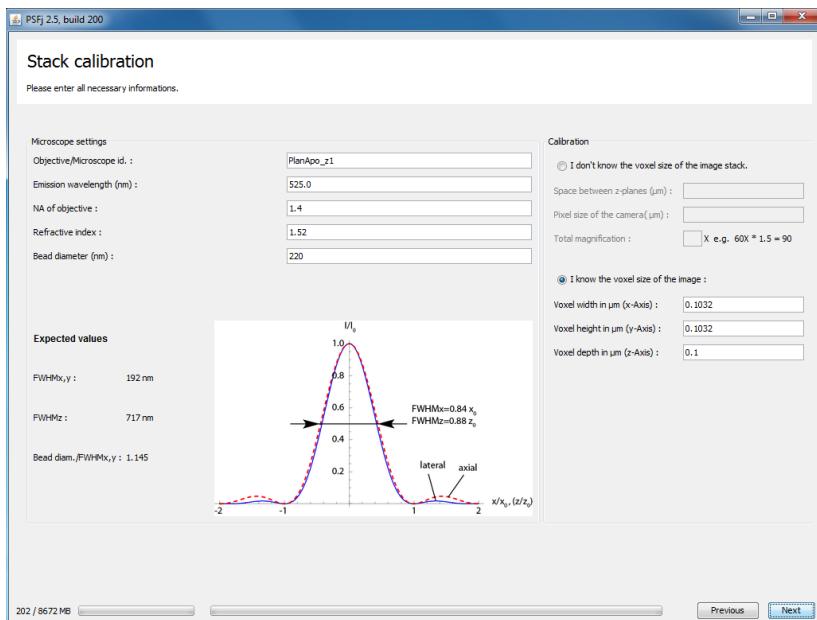


Fig. 4.4 Image-stack calibration window

To start the analysis click on the ‘Next’ button at the bottom right.

4.3 Analysis workflow

4.3.1 PSF detection and centroid estimate

In the first step of the analysis, the plane of best focus in the stack is determined by finding the plane (image) at which the image standard deviation attains a maximum. Next, approximate center positions of PSFs in that plane are determined by image segmentation using a simple threshold operation and a connected-component labeling algorithm provided by the library IJBlob¹⁰ whereby the threshold is set automatically to $\bar{I}_B + 10\sigma_B$, where \bar{I}_B is the average intensity and σ_B is its standard deviation of the first image (at the bottom) of the stack, i.e., at the plane farthest away from the plane of best focus at the center of the stack. The automatic settings should provide good results for most stacks but of course can be adjusted (in the case of multiple channels, for each stack

separately – select the stack in the left panel by clicking on it) by the user (results can be monitored in a preview window – release slide for review) by moving the focus and threshold slider (see Fig. 4.5 and below). The auto buttons bring the settings back to the automatic settings (see above). The mouse wheel can be used to zoom the image. Approximate selection of the best plane of focus is sufficient, as this will not influence the exact analysis of the individual beads. Don't use too stringent threshold settings, as this may lead to the detection of only the brightest beads. These may consist of bead aggregates, and will therefore bias the results.

4.3.2 Sub-stack size

In the next step, sub-stacks with square cross-sections centered around the PSF centroids are extracted. Their width is automatically set to $\text{Round} \left[10 \cdot \lambda \cdot M / A_{NA} / d_{pixel} \right]$ (about 10 times the expected FWHM), where M is the magnification and d_{pixel} is the camera pixel pitch, but can be adjusted using the sub-stack size slider (release slide for review). The sub-stack cross-sections are shown in the preview as green squares (see Fig. 4.5). The auto button brings the setting back to the automatic setting (see above). Image contrast and brightness can be adjusted using the double slider at the bottom of the image. Note: at high bead densities, a single PSF may show up in multiple sub-stacks. In order to preclude multiple inclusion of such a PSF into the results, only the sub-stack containing the brightest (average over the segment) centrally located PSF is retained for further analysis. All sub-stacks are processed in the next step (clicking on the next button at the bottom right).

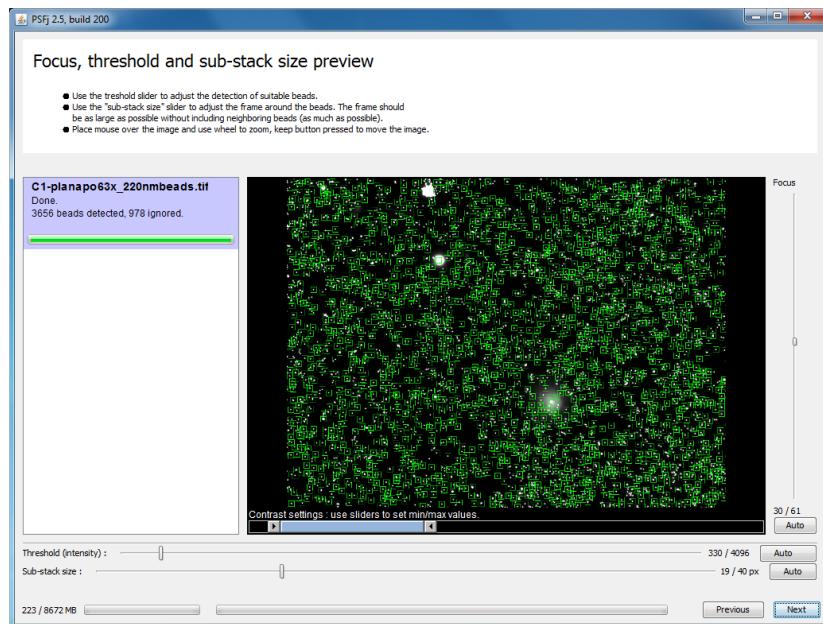


Fig. 4.5 focus, threshold, and sub-stack size preview window

4.3.3 PSF-analysis

During the sub-stack processing (see Fig. 4.6), individual PSFs are fitted using a generalized elliptic Gaussian 2D fitting routine provided by micromanager (Arthur Edelstein, Nenad Amodaj, Karl Hoover, Ron Vale, and Nico Stuurman (2010), Computer Control of Microscopes Using μ Manager. Current Protocols in Molecular Biology 14.20.1-14.20.17) based on Levenberg-Marquardt optimization. Estimates of the centroid required by the fitting routine are provided by intensity weighted BSF center positions (center of gravity) determined for each sub-stack using the 3D Object Counter ImageJ plugin (Wagner, T. & Lipinski, H.-G. IJBlob: An ImageJ Library for Connected Component Analysis and Shape Analysis. *Journal of Open Research Software* 1, e6 (2013).) with the input parameters ‘threshold’ and ‘voxel-range’ set to $\bar{I}_B + 10\sigma_B$, and 1-10000, respectively. From the Gaussian fit, the principle (smallest) FWHM, the asymmetry (smallest over largest FWHM), the orientation (angle between the x-axis and the major axis (largest FWHM) of the Gaussian ellipse and the centroid position (x_0, y_0)) are determined. The axial FWHM is determined from a fit with Gaussian profile along the z axes through the determined centroid position using the fitting algorithm provided by Metroloj (Cordelières, F. P. & Matthews, C. Metroloj: an ImageJ plugin to help monitor microscopes’ health. *imagejdocu.tudor.lu* at http://imagejdocu.tudor.lu/lib/exe/fetch.php?media=plugin:analysis.metroloj:matthews_cordeliere_s_-imagej_user_developer_conference_-_2010.pdf). As a quality check, only BSFs fitted with regression coefficients $R^2 > 0.9$ are retained. The determined FWHM are then corrected for bead size (see supplement of the paper).

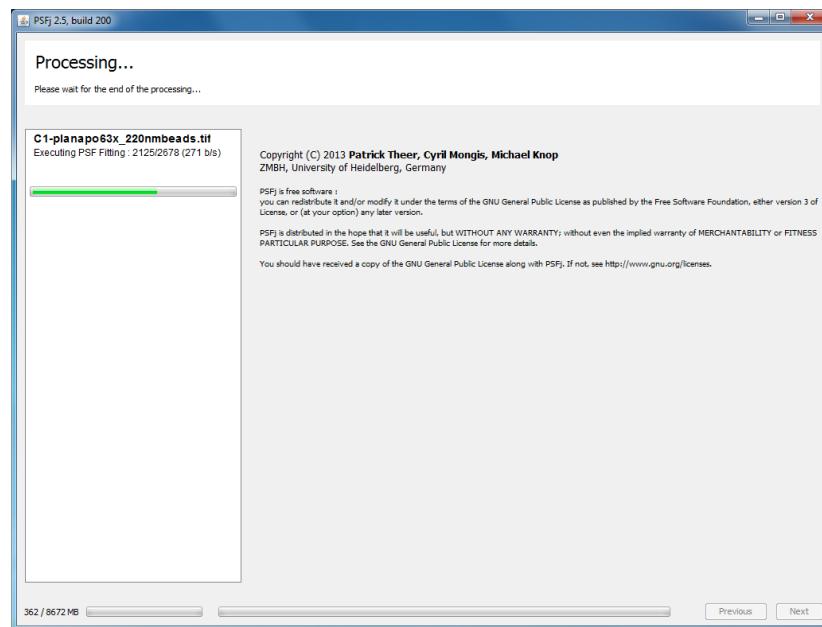


Fig. 4.6 sub-stack analysis window

4.3.4 Chromatic aberration analysis

Dual color image stacks are processed and analyzed individually as described above yielding resolution and planarity heat maps for each color channel separately. Second, a nearest neighbor analysis of BSFs between the two channels yielding the distance (shift) between corresponding BSFs (nearest neighbors) is performed and heat maps showing the lateral (

$\Delta\rho(\lambda_1, \lambda_2) = \sqrt{\Delta x(\lambda_1, \lambda_2)^2 + \Delta y(\lambda_1, \lambda_2)^2}$) and axial ($\Delta z(\lambda_1, \lambda_2)$) chromatic shifts as a function of BSF center position are generated. Heat maps are pseudo colored and scaled to display a range from $-FWHM$ (blue) to $FWHM$ (red). In cases where a BSF is found to be nearest neighbor of two or more BSFs, only the closest neighborhood is retained. In addition, nearest neighbors further apart than $2FWHM_z$ are excluded from the analysis.

4.4 Results

After the analysis has been finished the results will be displayed automatically. The screen will show an overview about the results, and

- Overview panel

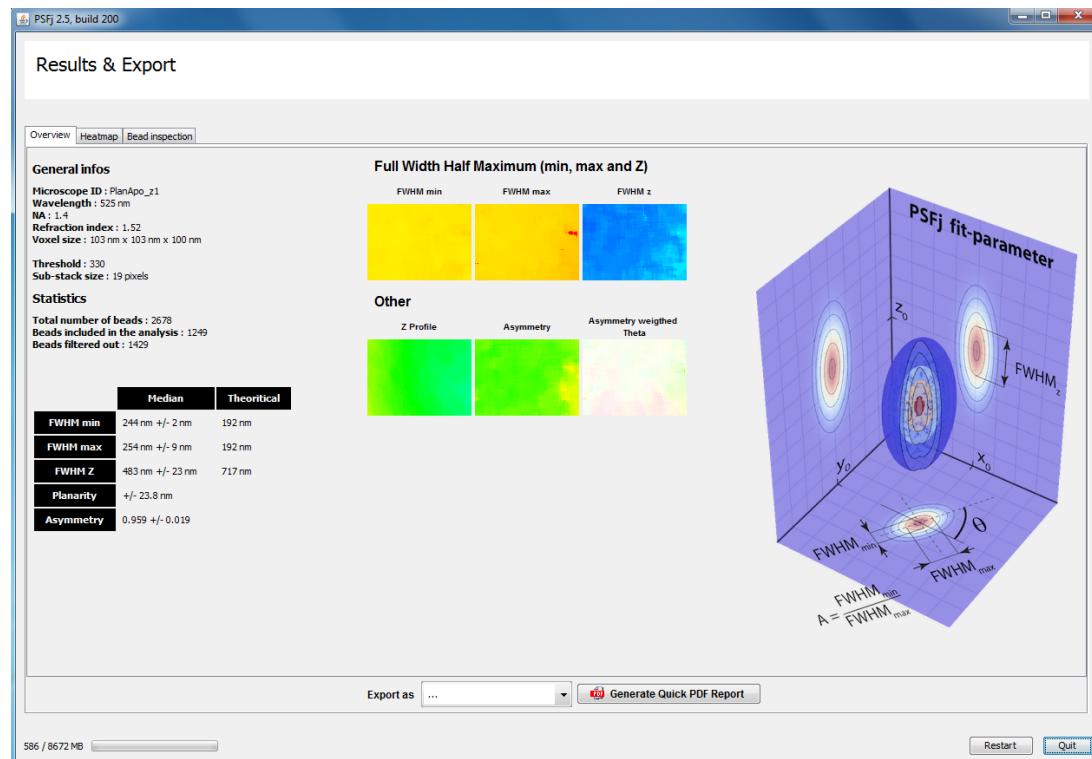


Fig. 4.7 overview panel of the results and export window showing general image stack information as well as the number of detected and analyzed beads and provides an overview of the results, i.e., showing the different heatmaps and a table comparing the average full FOV median with the values expected from theory and export options via a drop down menu (see below).

- Heatmap panel

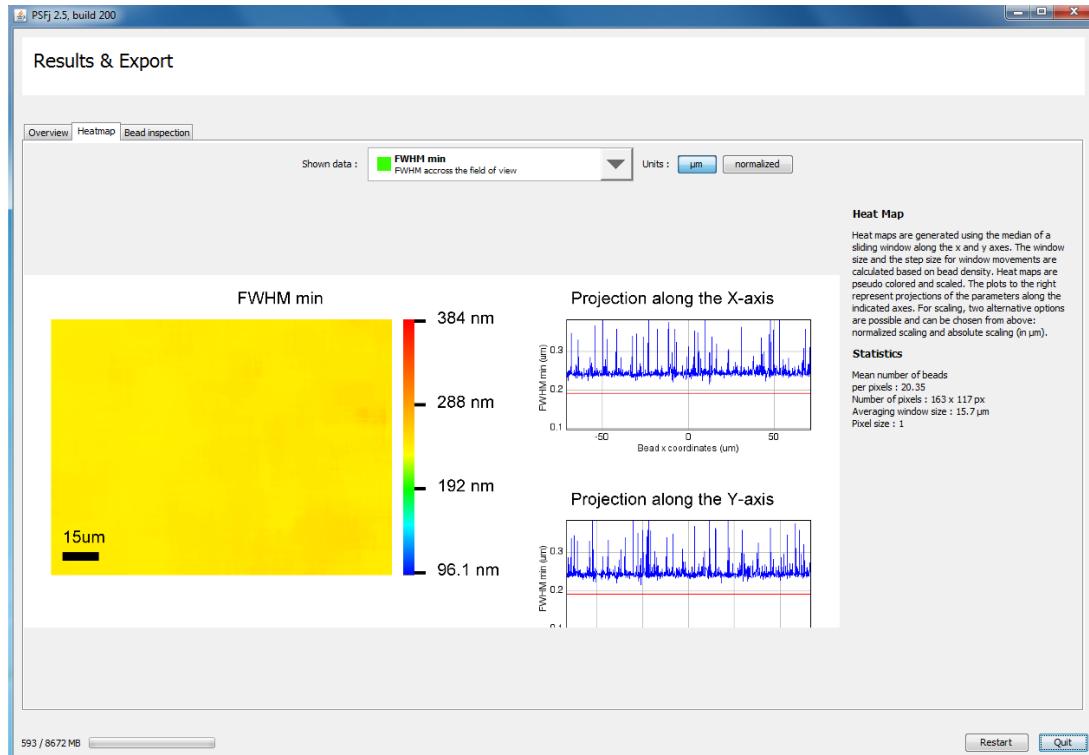


Fig. 4.8 heatmap panel of the results and export window showing the different median heatmaps and their projected profiles (selected via drop down menu) as well as heat-map parameters. Heat maps are pseudo colored and automatically scaled to display a range from $FWHM / 2$ (blue) to $2FWHM$ (red) or $-FWHM_z$ (blue) to $-FWHM_z$ (red) for the resolution and planarity heat maps, respectively. Units can be switched between relative or absolute by clicking the appropriate buttons on top of the heat map. Asymmetry maps are scaled from zero (red) to unity (green). Theta maps are scaled from -90° (violet) to 90° (violet) and color saturation is weighted by the asymmetry between zero for asymmetry 1 (theta is insignificant and the heatmap will appear white) and unity for asymmetry ≤ 0.5 .

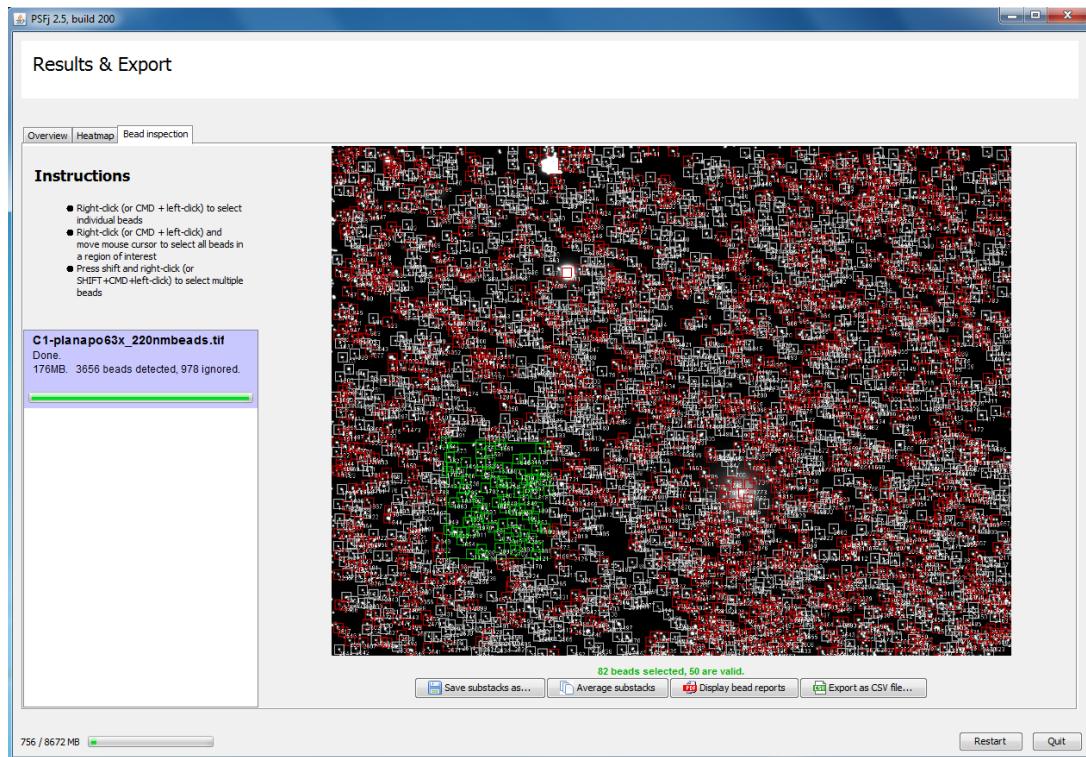
- bead inspection panel

Fig. 4.9 bead inspection panel of the results and export window showing outlines of all excluded (red) and used (white) sub-stacks. Single or multiple sub-stacks can be selected (by command-clicking or command-shift-clicking on single sub-stacks or by command-selection of the region of interest using the mouse). Selected sub-stacks can be exported as individual multipage .tif files, their bead reports can be displayed and an average image stack can be generated and saved as a multipage .tif file. In addition, sub-stack analysis results can be exported as a csv file.

5. Reporting and exporting

The following sections describe all reporting options, the output file formats and the purpose and possible applications of specific output files.

5.1 Summary report

The summary report provides a quick overview of the results and is intended for routine documentation of the daily microscope performance e.g. in a microscope facility. The report can be generated by simply clicking the “generate quick pdf report” button at the bottom of the overview panel (see Fig. 4.7) and will be exported into the folder that contains the image stack(s). For single channel analysis, it contains three pages showing the heat-maps and their projected profiles as well as the resolution table and imaging parameters (see Fig. 5.1a). In the case of dual-color analysis, the report contains a three-page summary report for each color channel as well as three additional pages reporting on the chromatic aberration performance showing lateral as well as axial chromatic shift heat-maps and exemplary overlay images from different representative areas of the FOV (see Fig. 5.1b).

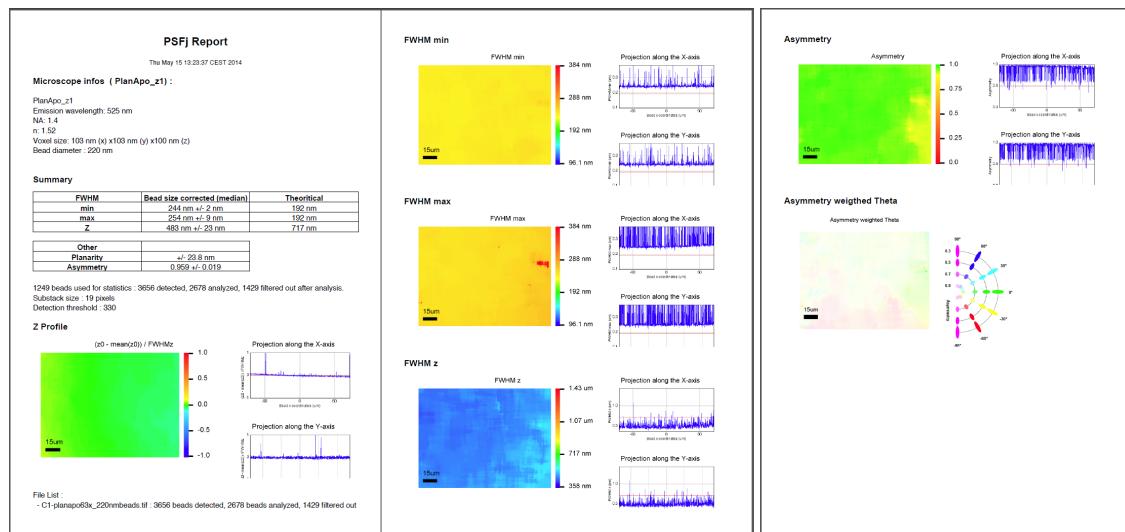


Fig. 5.1a Summary report - single channel analysis

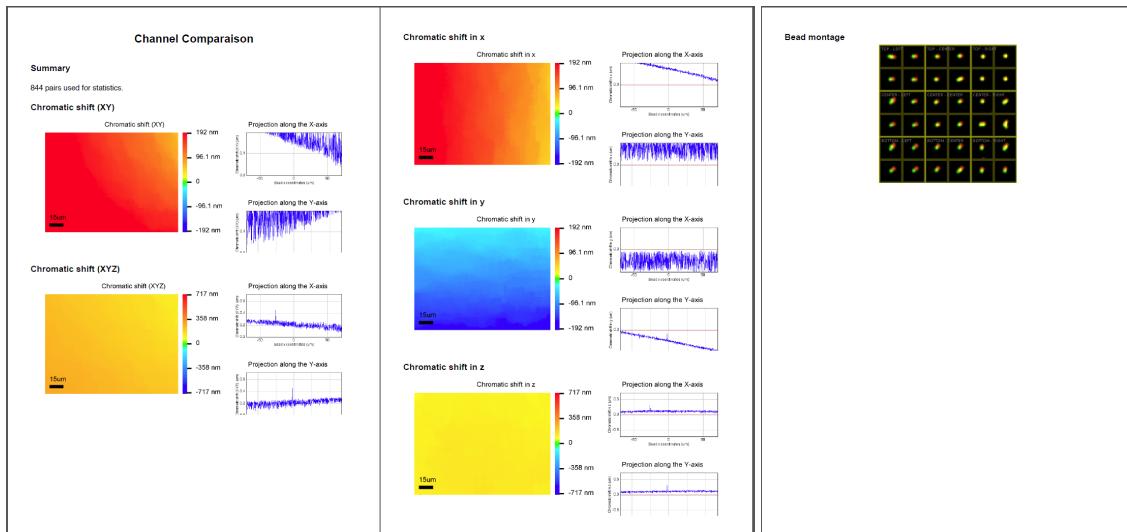


Fig. 5.1b Summary report - chromatic aberration analysis

5.2 Results csv file

The results csv file provides the complete set of results for each individual substack (see Fig. 5.2) and could be used for further analysis and post-processing. It can be generated by selecting the “results csv file” option in the “export as” pull down menu at the bottom of the overview panel (see Fig. 4.7) and will be exported to a user selectable folder. The csv file contains info about each beads position (x_0, y_0, z_0), measured FWHM, resolution asymmetry and orientation (θ) as well as the fit regression coefficients for each (detected) PSF (see table 5.2.1). In addition it contains all imaging parameters, the number of detected and analyzed beads, and the mean \pm stdev of all heat maps.

Table 5.2.1 Results csv file entries

Bead Id	x_0 (um)	y_0 (um)	z_0 (um)	FWHMmin (um)	FWHMmax (um)	FWHMz (um)	$z_0 - \text{mean}(z_0)$ (um)	Asymmetry	Theta (degrees)	$R^2, FWHM_{\min, \max}$	$R^2, FWHM_z$	Fit valid
1	-51,3	-52,3	2,907	0,239	0,251	0,455	0,039	0,953	-51,774	0,985	0,994	1
2	-19,6	-52,2	2,891	0,237	0,25	0,457	0,016	0,95	-19,82	0,76	0,995	0
3	-13,3	-52,3	2,883	0,237	0,248	0,469	0,004	0,955	-29,892	0,992	0,995	1
4	15,45	-52,3	2,866	0,244	0,251	0,525	-0,018	0,97	-49,826	0,991	0,995	1
5	43,55	-52,3	2,875	0,261	0,263	0,522	-0,006	0,993	3,891	0,959	0,995	1
6	50,59	-52,3	2,859	0,223	0,225	0,474	-0,029	0,993	-11,467	0,309	0,985	0
7	46,74	-52	2,858	0,252	0,262	0,524	-0,03	0,963	-32,102	0,989	0,995	1

...

In the case of dual channel analysis a total of three csv files are saved, one for each channel (see Table 5.2.1) and an additional one for the comparison of the channels, containing the corresponding bead IDs from the two channels, the bead positions (x_0, y_0, z_0), and their position differences (chromatic shifts, $\Delta x_0, \Delta y_0, \Delta z_0$) in the two channels (see Table 5.2.2).

Table 5.2.2 Dual-channel comparison results csv file entries

Bead Id ch1	Bead Id ch2	x0 ch1 (um)	x0 ch2 (um)	delta x0 (um)	y0 ch1 (um)	y0 ch2 (um)	delta y0 (um)	z0 ch1 (um)	z0 ch2 (um)	delta z0 (um)
9	8	30,051	30,149	0,098	-52,883	-52,901	-0,018	985,193	986,139	0,107
28	28	-13,327	-13,194	0,132	-52,343	-52,365	-0,022	564,867	566,146	0,094
29	32	15,449	15,541	0,091	-52,301	-52,281	0,02	843,704	844,588	0,101
32	35	46,735	46,802	0,067	-52,047	-52,063	-0,016	1146,858	1147,508	0,066
36	38	59,445	59,487	0,042	-51,852	-51,859	-0,007	1270,018	1270,428	0,109
38	36	18,398	18,507	0,11	-51,737	-51,745	-0,008	872,27	873,332	0,075
39	39	69,614	69,66	0,046	-51,8	-51,806	-0,006	1368,556	1369	0,09

...

5.3 Heat maps

Heat maps are intended as a graphical representation of the results. They are generated using the median of a sliding window along the x and y axes. The size of the sliding window (square) is set to $s = \sqrt{20/\eta}$, where η is the bead density. The step size is set to $s/20$. Raw gray scale and pseudo colored versions of the heatmaps as provided in the PSFj report (see Fig. 5.1) are generated by selecting the “heatmap” option in the “export as” pull down menu at the bottom of the overview panel (see Fig. 4.7) and exported to a user selectable folder. Heat maps are pseudo colored and automatically scaled to display a range from $FWHM/2$ (blue) to $2FWHM$ (red) or $-FWHM_z$ (blue) to $-FWHM_z$ (red) for the resolution and planarity heat maps, respectively. Units can be switched between relative or absolute by clicking the appropriate buttons on top of the heat map. Asymmetry maps are scaled from zero (red) to unity (green). Theta maps are scaled from -90° (violet) to 90° (violet) and color saturation is weighted by the asymmetry between zero for asymmetry 1 (theta is insignificant and the heatmap will appear white) and unity for asymmetry ≤ 0.5 .

5.4 Landmark XLS

The landmark xls file is an output option for dual channel analysis and contains the beads lateral center-of-mass positions $x_0(\lambda_1), y_0(\lambda_1)$ (Target) and $x_0(\lambda_2), y_0(\lambda_2)$ (Source) in the two channels (see Fig. 5.4). This list is intended to be used for image registration, i.e., correction of lateral chromatic shifts and is in a format accepted by **bUnwarpJ** a plugin for ImageJ (Ignacio Arganda-Carreras, Carlos O. S. Sorzano, Roberto Marabini, Jose M. Carazo, Carlos Ortiz de Solorzano, and Jan Kybík, [“Consistent and Elastic Registration of Histological Sections using Vector-Spline Regularization”](#), Lecture Notes in Computer Science, Springer Berlin / Heidelberg, volume 4241/2006, CVAMIA: *Computer Vision Approaches to Medical Image Analysis*, pages 85-95, 2006. ; <http://biocomp.cnb.csic.es/~iarganda/bUnwarpJ/>), for image registration using elastic deformations. For further reading, please refer to the documentation of the bUnwarpJ Plug In.

	A	B	C	D	E	F	G
1	Index	xSource	ySource	xTarget	yTarget		
2	0	985	8	986	7		
3	1	565	13	566	13		
4	2	844	13	845	13		
5	3	1147	16	1148	16		
6	4	1270	18	1270	17		
7	5	872	19	873	19		
8	6	1369	18	1369	18		
9	7	369	20	371	20		

Fig. 5.4 Landmark xls file entries

5.5 Bead reports

Bead reports provide results and fitting parameters for each individual substack/bead (see Fig. 5.5) and are intended as a visual and quantitative check of the fitting results. They can be generated (one PDF files for every 100 detected PSFs) by selecting the “bead reports PDF file” option in the “export as” pull down menu at the bottom of the overview panel (see Fig. 4.7) and will be exported to a user selectable folder.

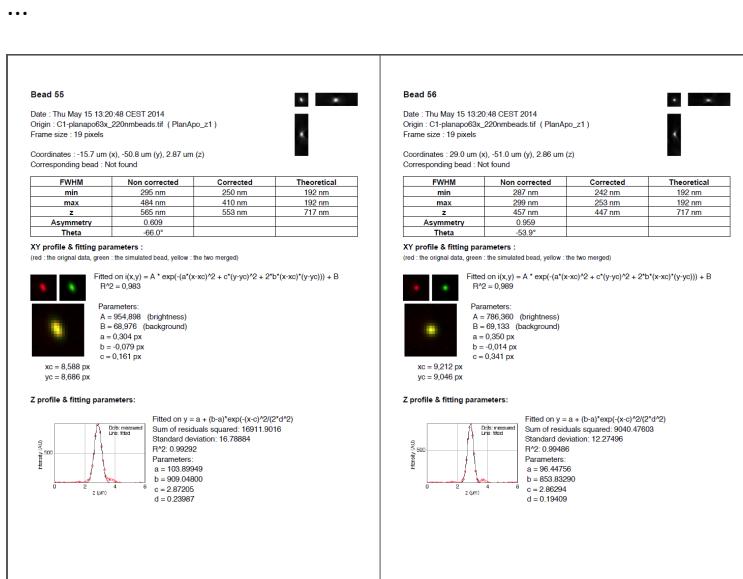


Fig. 5.5 bead-reports pdf file entries

6. Interpretation of results

When is the performance of my system in its technical optimum, and no further improvement is likely to be possible?

In general, the best performance can be expected when adhering to the imaging conditions it has been designed for. Particular care should be taken to use the correct immersion medium and cover slip thickness (if applicable) and work at the specified temperature. Nevertheless, the performance will likely vary between different days and instruments and generally be worse than can be expected from theory or marketing. In our experience, the best objectives will provide a resolution which is about 10-20% below that expected for a diffraction limited system but deviations up to 50% can be considered standard. Further good objectives can be expected to provide a planarity substantially smaller (<10%) than the axial resolution and lateral and axial chromatic shifts substantially smaller (<20%) than their lateral and axial resolutions, respectively. This of course will depends on the level of correction but also on the imaged FOV and should be tested using PSFj.

I get a crappy systems performance, and what now?

1. check that you have entered the proper values for your systems and imaging specifications
2. identify the parameter that exhibits low/bad performance: is it only one parameter, or all parameters? Parameters related to resolution: vibrations, drift, etc? Possible sources: thermal fluctuations: Vibrations from the ventilators of the camera (this is a frequent and super annoying problem with cameras from one particular supplier: consider to make a big fuss). One way to detect significant vibrations is to use the iPhone App 'iSeismometer'.
3. Check the experimental conditions – correct immersion medium?, correct cover-slip thickness and/or adjustment of the objectives thickness correction collar?, correct temperature and/or adjustment of the objectives temperature correction collar?, correct bead size?, correct magnification, i.e., voxel size? – this can be easily tested by comparing the bead positions in two images one taken before and one after moving the bead sample a given distance (say 50 µm) in x and/or y and/or z.

I get a stunning performance of my system, much better than its theoretical values.

Check your parameters.