Living up to Life

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reSOLUTION
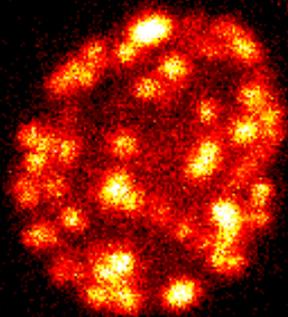
Confocal
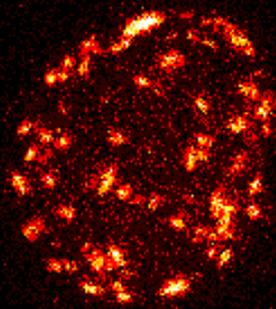
STED
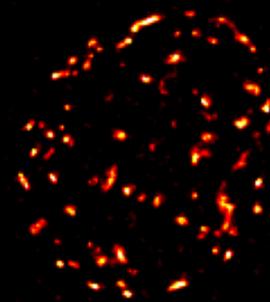
STED
deconvolved

Huygens STED Deconvolution Quick Guide

This document is intended to give Leica STED users a brief introduction to deconvolving images on Huygens Professional using images acquired with the Leica TCS SP8 STED 3X microscope. For a more detailed description of Huygens Professional, including additional features and tools, please visit the *Manuals* section of SVI (www.svi.nl).

The Parameter Editor

Right-click on image thumbnail
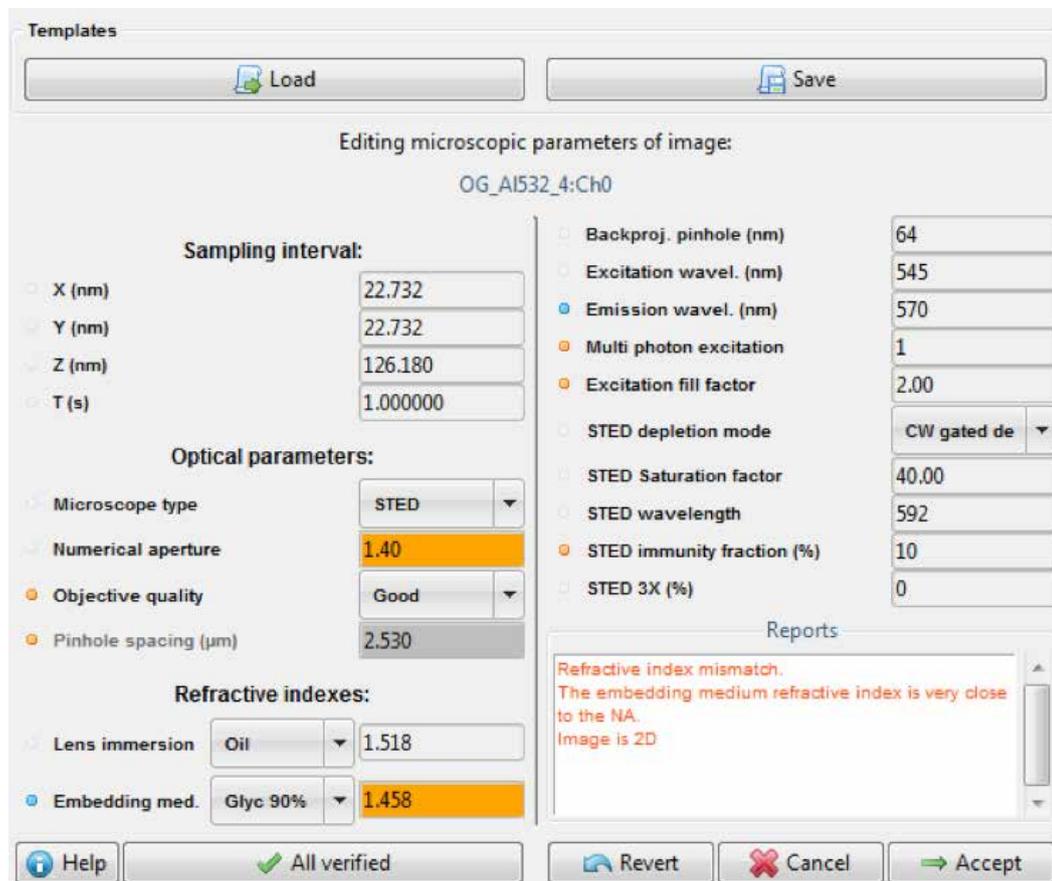
Fig. 1 Overview of the Huygens image parameter editor. This window gives access to all relevant image parameters required for deconvolving STED images. When importing .lif files, the majority of the parameters are automatically extracted from the metadata (see the Huygens Pro User's Guide for more information from SVI).

Sampling interval (XYZT): values taken from image properties
Microscope type: values taken from image properties
Numerical aperture: values taken from image properties
Objective quality: take default value "good"
Coverslip position: use special tool (icon to the left)
Imaging direction: for DMI microscopes always "Upwards"
Pinhole spacing: irrelevant for confocal/STED microscopy
Lens immersion: use "Oil"
Embedding medium: choose appropriate medium from drop-down menu

Backproj. pinhole: values taken from image properties
Excitation wavelength: values taken from image properties
Emission wavelength: values taken from image properties
Multi photon excitation: values taken from image properties
Excitation fill factor: values taken from image properties
default value = 2, except for TiSaph STED (value = 1.2)
STED depletion mode: values taken from image properties
STED saturation factor: see below for more details
STED wavelength: values taken from image properties
STED immunity fraction: see below for more details
STED 3X: see below for more details

STED saturation factor

The saturation factor originally refers to the term:

$$\frac{I}{I_{SAT}}$$

which is part of the STED resolution formula:

$$\Delta d = \frac{\lambda}{2n\sin\alpha \sqrt{1 + \dfrac{I}{I_{SAT}}}}$$

which defines the full-width-half-maximum (FWHM) of the STED PSF and finally the resolution of the STED image. With the implementation of gated STED and the TCS SP8 STED 3X, the classic understanding of the saturation factor (mainly dependent on the dye's the laser intensity and the depletion efficiency of the chosen dye) was expanded by including additional information about the excitation and depletion laser mode (pulsed or CW) and gated detection. In practice, there are two parameters that influence STED resolution and consequently the Huygens saturation factor: STED depletion laser intensity and gate start values. Keep in mind that, for simplicity's sake and overall practicability, the ISAT value of only one "ideal" dye was used for the calculation of the STED PSF in Huygens. Per default, for the LAS AF version 3.3 and later, a predicted value (derived from the imaging parameters) is automatically set in the image properties and transferred to Huygens. For .lif files that were acquired in previous releases of LAS AF, the saturation factor needs to be entered manually in Huygens. The saturation factor value might require further adjustments depending on the dye's STED efficiency. Keep an eye on the image resolution (line profile measurements) in case some additional changes are needed.

Below, an array is displayed showing the relation between the saturation factor and the FWHM value of several calculated PSFs. It should provide a reference in case the real image resolution differs from the predicted one.

Fig. 2 Compilation of a series of **calculated** PSFs (Operations Window → Theoretical PSF) with different saturation factors (SF) showing an increasing (but not linear!) full-width-half-maximum (FWHM) value. Pixel size: 15 nm

Fig. 3 Effect on image quality with the saturation factor set too high. Arrows indicate possible artifacts (noise amplification and excessive granulation and contrasting of structures).

Fig. 4 Effect on image quality with the saturation factor set too low. Arrows indicate spots that show the loss of obvious substructures, which can be clearly identified in the raw data.

Significant differences between the saturation factor values and reality can be analyzed on deconvolved data:

- Saturation factor too high: the calculated PSF is significantly smaller than the real PSF. This will result in artifacts perceived as strongly granulated structures, which do not correlate with the original image. Additionally, noise can be misinterpreted as signal (arrow, see below). In general, excessively small PSF predictions will have less obvious effects on the data, but small spots and textures could be misjudged as structural details and result in an erroneous interpretation of the imaging data.
- Saturation factor too low: Image will appear convoluted, almost regardless of the SNR chosen in the STED Deconvolution Wizard (see section below). Noise reduction might still be acceptable, but the deconvolved image will have a massive loss of information with possible artifact generation (arrows, see below).

STED 3X

Huygens actually extracts the correct value directly from the image properties of the .lif file. Nonetheless there are a few things to consider. In principal the STED 3D slider regulates the percentage of light that goes either through the "vortex", or the "3D doughnut" beam path, which will have an influence on the ratio between the lateral and axial resolution increase.

Therefore, the shape of the calculated PSF with the same saturation intensity changes according to the position of the 3D slider. The lateral resolution continuously decreases, while the axial resolution increases (see example of calculated PSFs with a saturation value of 30 in Fig. 5).

The resolution increase, the ratio between both dimensions and finally the 3D slider percentage on which isotropic resolution is achieved are again dependent on the STED efficiency of the chosen dye.

The easiest approach for determining the correct saturation factor in 3D STED consists in acquiring one additional control image with 0% STED 3D and adjusting the Huygens saturation factor to the measured FWHM. The same saturation factor can then be used for all 3D STED slider positions as long as the same STED laser intensities and gate start values are used.

XY-view

XZ-view

| **3D Slider: 0%** | **3D Slider: 20%** | **3D Slider: 40%** | **3D Slider: 60%** | **3D Slider: 80%** | **3D Slider: 100%** |
| **L/A ratio: 1:10** | **L/A ratio: 1:4,5** | **L/A ratio: 1:2,8** | **L/A ratio: 1:2** | **L/A ratio: 1:1,4** | **L/A ratio: 1:0,8** |
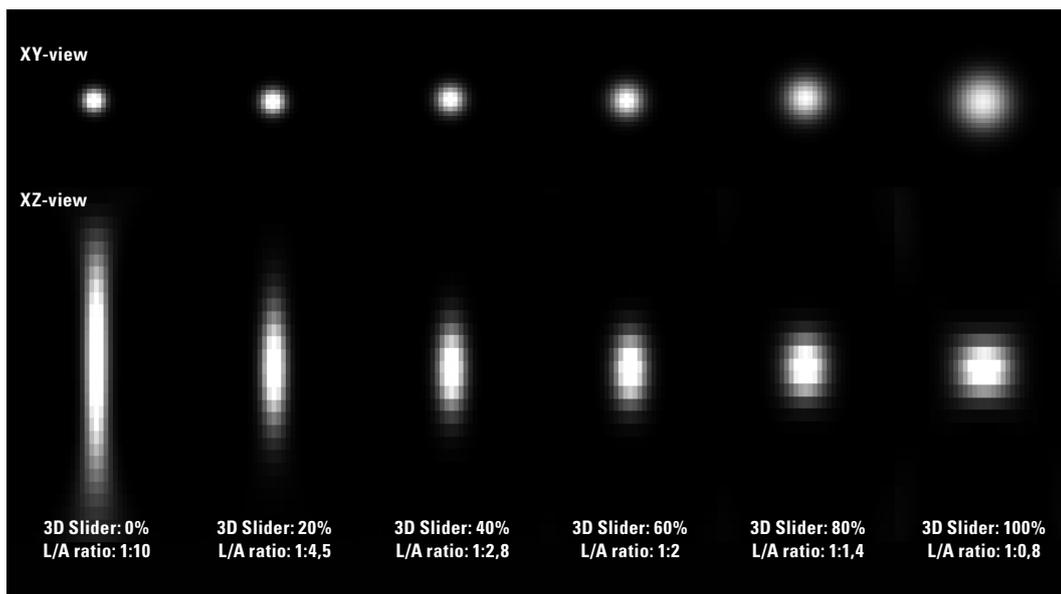
Fig. 5 Compilation of different calculated PSFs generated in Huygens (Operations Window → Theoretical PSF) with increasing 3D STED values. With the redistribution of light between both light paths the ratio between the axial and lateral FWHM changes significantly. Pixel size: 15 nm x 40 nm (xz).

STED immunity fraction

The Huygens definition of "immunity fraction" is given by the population of fluorophores, which are immune to the STED depletion laser and can be described as an additional confocal PSF component that is added to the pure STED PSF.
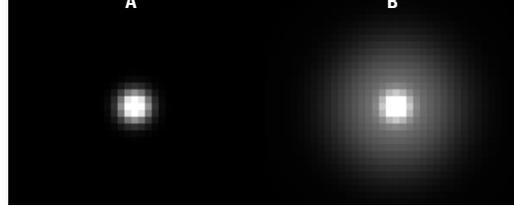
Fig. 6 Comparison of two STED PSFs. On the left the "pure" STED PSF can be seen with the immunity fraction set to 0%. On the right the same PSF was generated with an immunity fraction of 15%. Pixel size 15 nm.

Here, the immunity fraction is a broad definition that is intended to cover three different STED effects.

1. Anti-Stokes excitation with the STED depletion laser:
   In some instances the STED depletion laser can also excite the dye. This is always dependent on the dye/laser combination. Leica's recommended dye/laser combinations usually have none to only very little anti-Stokes excitation, but in some cases (multi-color experiments) it might be necessary to use dyes, which are spectrally located closer to the STED depletion laser. For deconvolution this additional increase of the PSF footprint can be mimicked by increasing the immunity fraction. Note that the STED depletion laser has a different PSF shape (donut) to that of a regular excitation laser (spot), which is not taken into account in the computation of the immunity fraction.

2. Short lifetime component in STED CW and gated STED:
   CW STED depletion adds an significant contribution of fluorescence lifetime into the STED PSF, which biases resolution towards emission with longer lifetimes. Short-lived fluorescence, on the other hand, has low STED efficiency and poorer resolution. The fluorescent signal is always comprised by a whole range of different lifetime components which all add up in the final STED CW PSF. Short-lived fluorescence is therefore "immune" to the STED light, which results in similar PSFs to those seen in Fig. 6B. The more gate is used during the detection the less prominent this pedestal will be. As a rule of thumb, STED CW uses up to 10% immunity fraction, whereas gated STED images are in the range of 3% to 5%.

3. Poor depletion efficiency in pulsed STED:
   In most cases the pulsed STED solution gives you the cleanest form of the STED PSF. Nonetheless due to synchronization issues, intrinsic dye photo-physics, and width of the STED laser pulse, a population of dyes might not be effectively depleted and is per definition immune to the depletion laser.

Overall, the immunity fraction has only little influence on the quality of deconvolved images. In cases where the real STED PSF pedestal is rather broad (strong anti-Stoke's excitation and STED CW) adjusting the immunity fraction of the calculated PSF to a proper level might enable lower background threshold levels. Lower background estimates on their side increase the amount of information that can be extracted/enhanced from the original image.

Exceedingly low levels of immunity fraction in the theoretical PSF would make the algorithm disregard the pedestal of the real PSF, which could be finally misjudged by the software as a real signal and would eventually lead to artifacts, like strong signal amplification on the edges of small structures in images.

Conclusion

Matching the calculated, theoretical PSF to the "real" one can be time-consuming and requires a few tries. But once determined, the variations in the PSF shape between experiments with similar settings are minimal. Additionally, with increasing experience, these adjustments become easier with time.

There are tools in Huygens that can help with the estimation of the PSFs FWHM values, which is helpful in deconvolution. The "*PSF FWHM Estimator*" located in the "*Analysis*" section automatically generates a FWHM estimation in a given image with a single PSF visible. This can be used to guess an approximate value for the resolution of the microscope either on very small fluorescent beads (< 35 nm) or even on small structures, or fluorescent background inside the actual sample. These values can then be used to adjust the image parameters for the calculated PSF.

A more elegant, but also more advanced solution is offered by the "*PSF Distiller Wizard*" found in the "*Deconvolution*" section. Here the real PSF is measured and converted into a mathematical model. Deconvolving the data with distilled PSF will eventually give the best results, since it takes account of system specific imperfections and asymmetries. The PSF Distiller requires images with very good signal-to-noise levels to properly extract the required information.

The Deconvolution Wizard

Right-click on image thumbnail

With all image properties now properly set, the next step consists in using the Deconvolution Wizard for the final image processing. In this step the user is guided through a series of steps which will define the deconvolution parameters and ultimately perform the deconvolution. A full description of the deconvolution steps and image processing tools in the Huygens software can be found in the *Professional User Guide of the Download → Manuals* section on the website of SVI (www.svi.nl). In this quick guide only the most relevant steps will be included to generate high quality images and avoid the most common artifacts.

Choose a PSF / Load deconvolution template
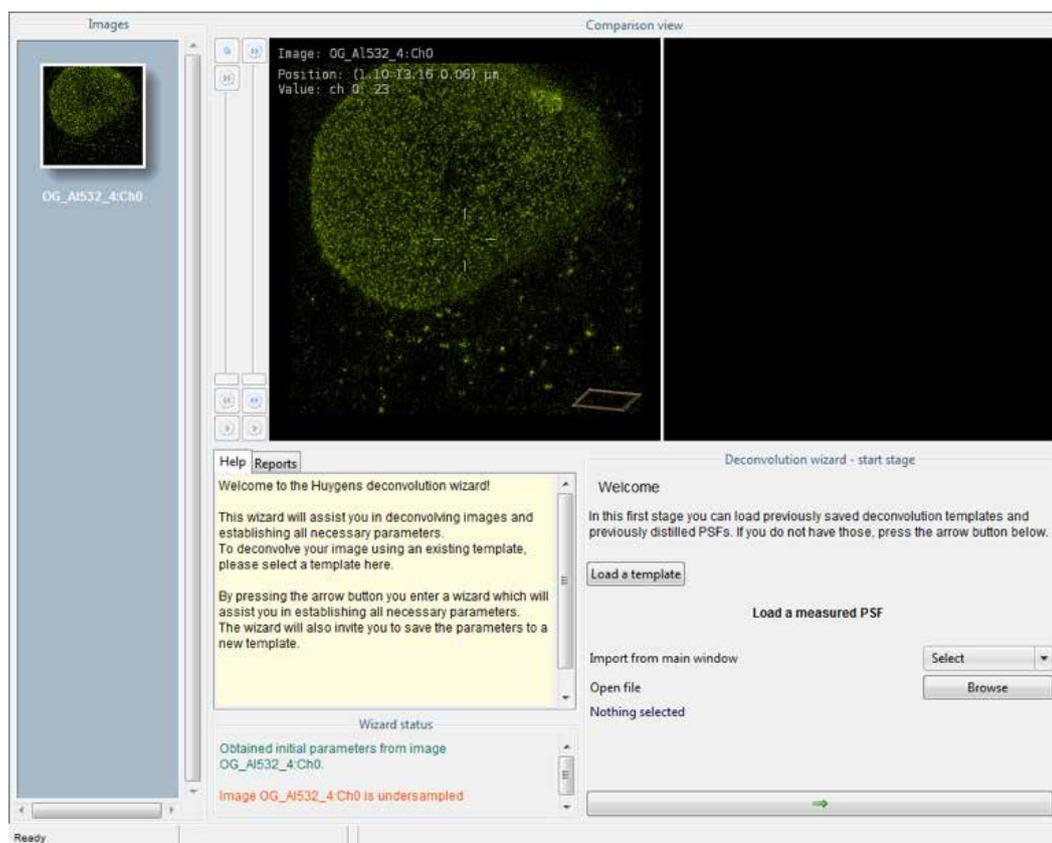
Fig. 7 Loading deconvolution templates and special PSFs from files

In this step the user is asked to load previously saved deconvolution templates and import previously calculated, or distilled PSF representations. If no values or references are loaded in this section the software will automatically generate a calculated PSF using the image parameters of the deconvolved image as a reference (also see Parameter Editor above). Note that the deconvolution wizard is also provided with extended help files (see yellow text box in Fig. 7, 8, 9 and 11) and direct links to the SVI webpage. For quick deconvolution experiments this step can be disregarded.

Cropping Tool
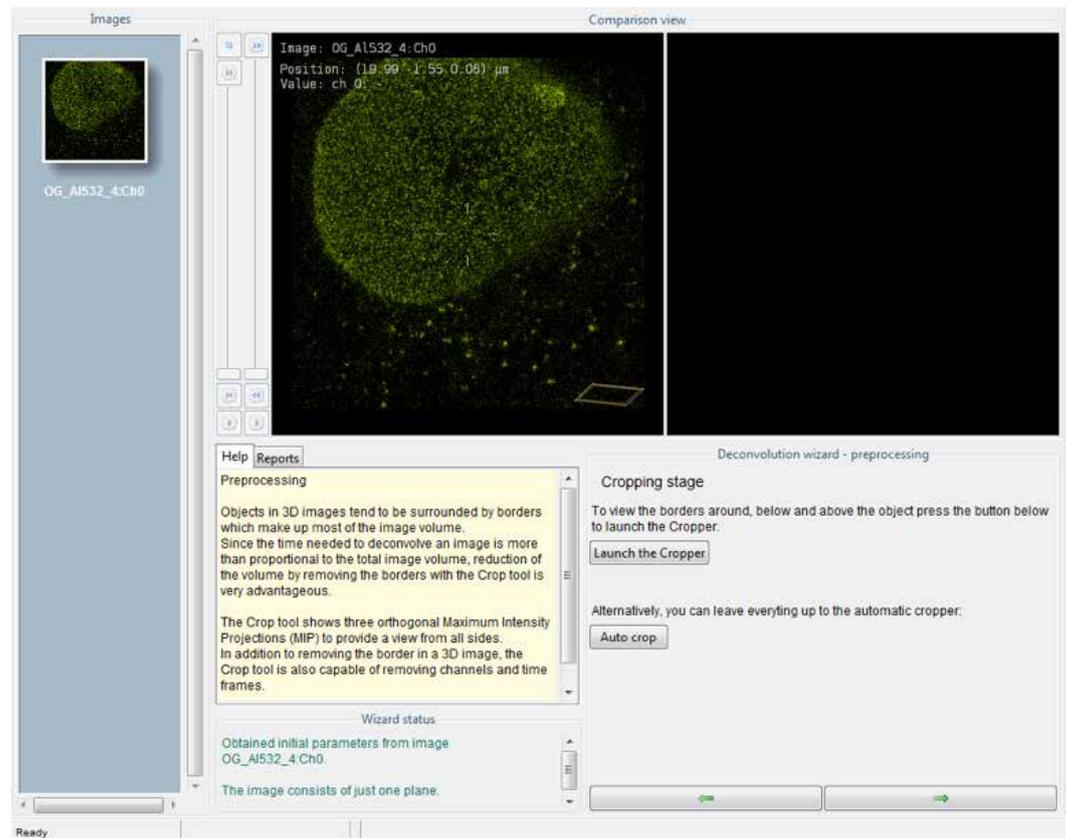
Fig. 8 Cropping images to reduce data size and increase deconvolution speed

In this second step the user is given the opportunity to remove empty regions of the image to enable a more efficient and faster deconvolution process. The "Auto crop" option automatically generates suggestions for cropping borders, which further increases the ease of use.

Histogram Tool and Background Estimation
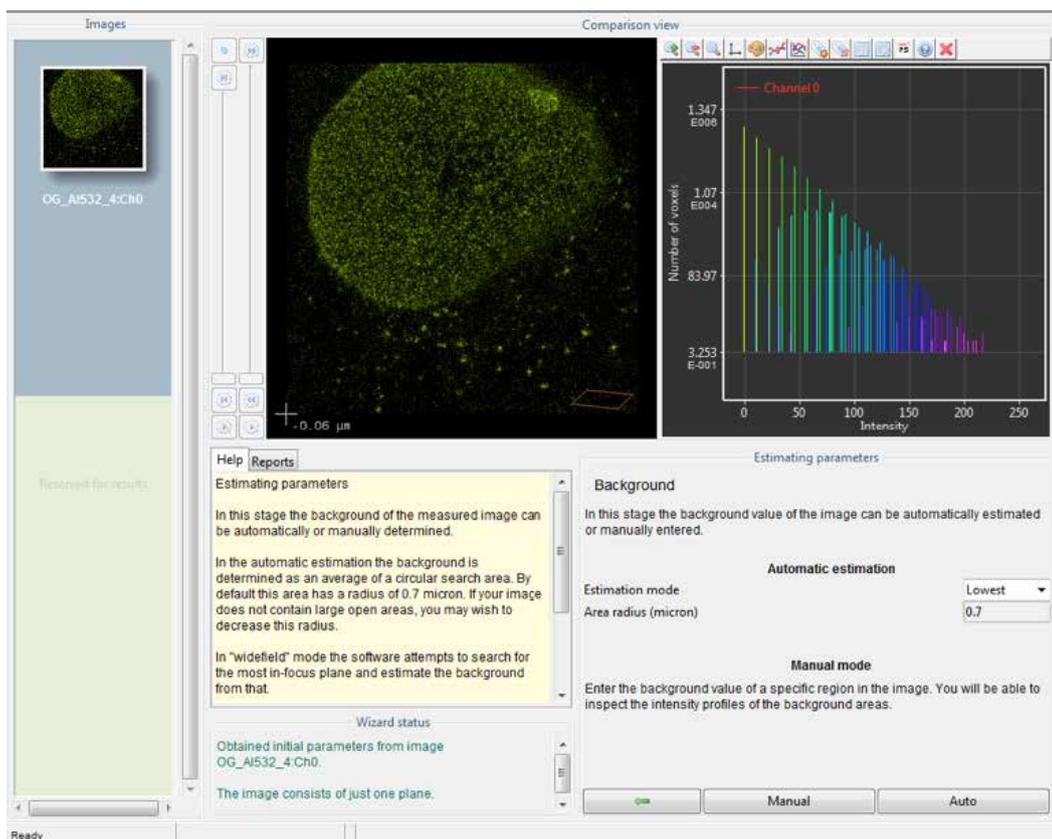
Fig. 9 Clipping of bright signal and background estimation are crucial parameters for deconvolution

Inspecting the image histogram and defining the background levels is of crucial importance for the quality of the result. In the histogram inspection the software warns the user of very bright images which contain regions where the intensity of pixels reaches the maximum value (in 8-bit images, 255). In these "saturated" regions the structural information given by the intensity profile is lost (clipping) and this will lead to artifact generation. For STED data it is very hard to recover the lost information of clipped regions via image reconstruction, so it is important to avoid clipping.

The background estimation stage allows the user to set a relative or absolute background value. If the background is set too low, the software may interpret higher background levels and noise as signal and erroneously amplify them. If the background level is set too high, real signal may be ignored and suppressed, which can lead to loss of information.

The automatic value estimation can be done with two different algorithms. "Lowest" searches the image for flat regions with the lowest pixel values, and "in/near object" searches for object and determines the background in direct proximity of these objects. The "lowest" algorithm will therefore generally produce lower background levels than the "in/near object". If necessary, there is an option which allows the user to set a custom value in the "Manual" mode.
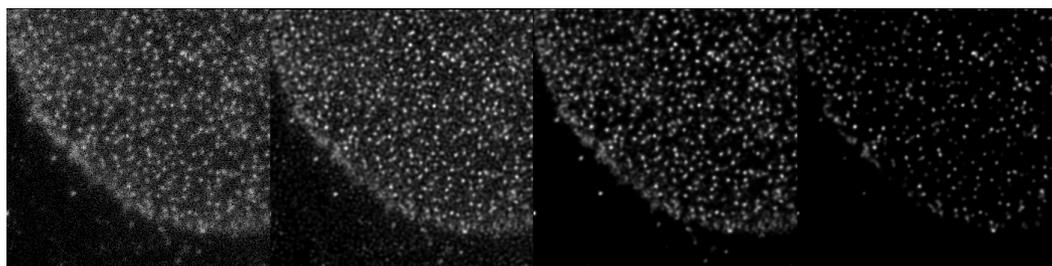
Fig. 10 Deconvolved images with different absolute background level settings (raw image; background=0; background=15; background=30). Different background values show the effects of improper estimation. At too low levels (0) the noise is interpreted as signal which leads to strong artifact generation, whereas high background estimation (30) structural information is lost.

Deconvolution Stage
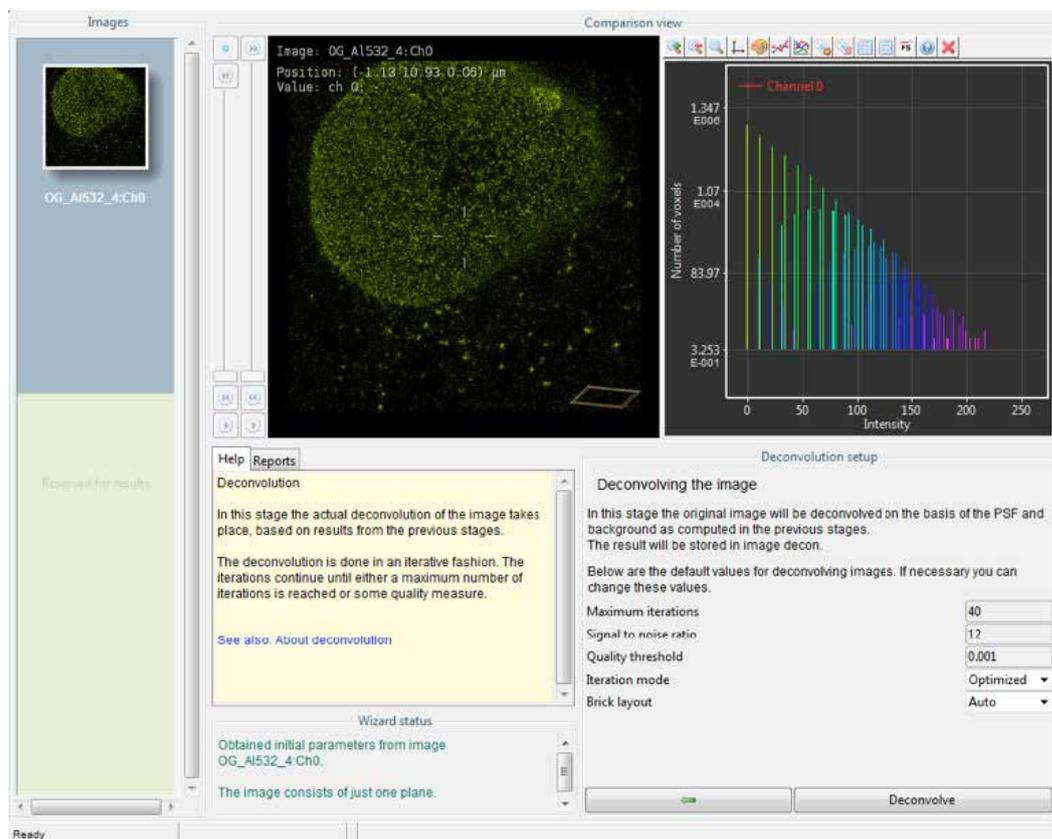
Fig. 11 Setting up the deconvolution parameters

Maximum iterations:
In the default CMLE algorithm in Huygens, as in many other deconvolution solutions, the evaluation of the ideal image is done over several iterations. The more iterations the better the image will be deconvolved. The "deconvolution strength" can be characterized by the contrast enhancement and increased resolutions in comparison with the raw images. Using too many iterations in combination with a too high SNR setting may lead to artifacts generation. The default iteration number is set to 40 for confocal and STED images.

Signal-to-noise ratio (SNR):
The signal-to-noise ratio setting controls the maximum achievable resolution after many iterations and therefore has an influence on the object's sharpness.

Findig the correct SNR values is sometimes difficult. Standard values for STED range between 10 and 15, but it is advisable to check if these values really fit. For this, a line profile can be created across the image (click and drag on the image) in order to estimate the different intensity and background levels. For SNR estimation it is recommended to use similar background values to the ones entered in the background estimation step. Default SNR is set to 7 for STED images and 20 for confocal images.
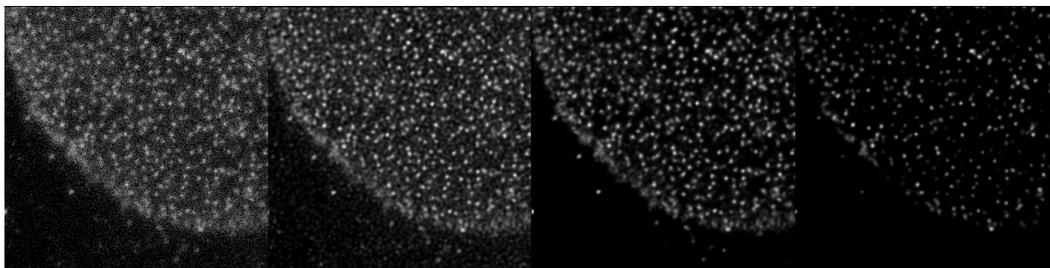
Fig. 12 Deconvolved images with different signal-to-noise level settings (raw image; SNR=2; SNR=12; SNR=40). SNR values have an effect on the object sharpness. At low levels (2) the structures appear slightly larger than in the raw image. With high SNR values (40) the objects become excessively sharp. Here, no structures disappear with higher values, unlike in Fig. 10.

Quality threshold:
Measures the difference of the quality index between iterations and serves as a stop criterion. If the quality index shows no significant change between iterations, the deconvolution process is stopped. This value can be used to avoid unnecessary iterations. Default is set to 0.05

Iteration mode:
It is advisable to keep this at "*Optimized*". For more details, please refer to the SVI user's guide.

Brick layout:
It is advisable to keep this at "*Auto*". For more details, please refer to the SVI user's guide.

Finishing Steps
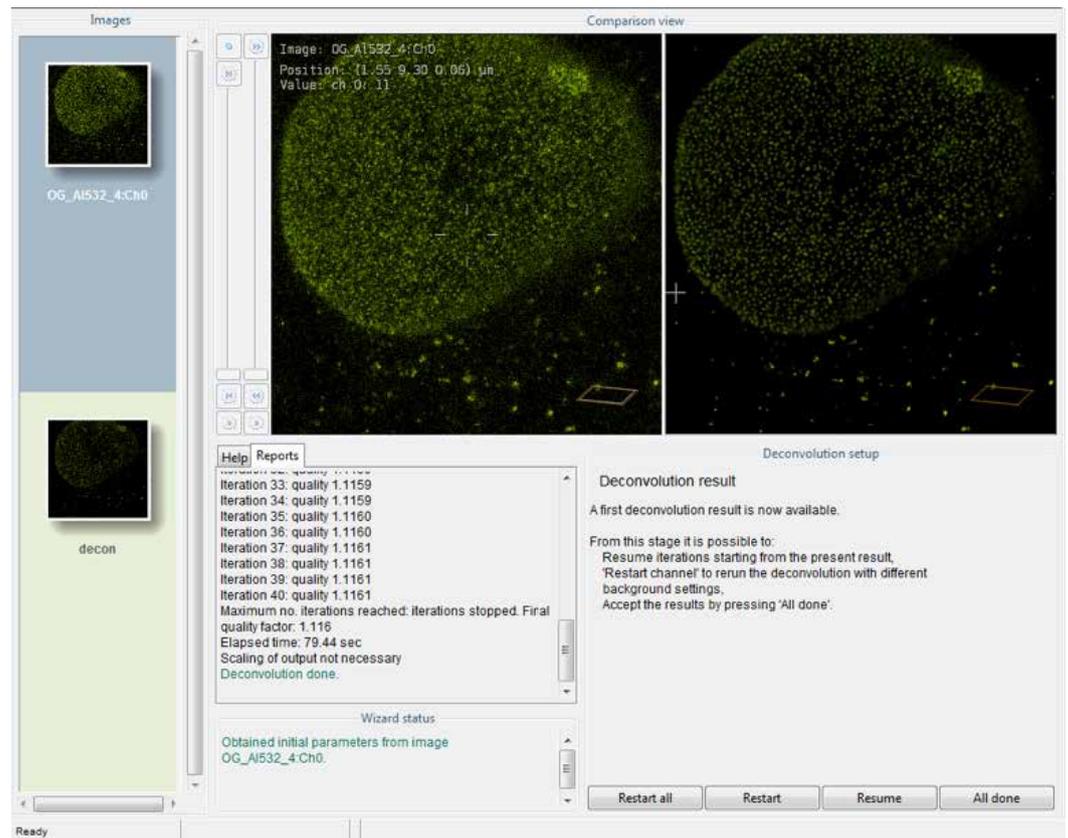
Fig. 13 After the deconvolution process is finished, the software offers the possibility to inspect the deconvolved image and restart the process, if required.

In these final steps, the user has the opportunity to inspect the processed image and compare it with the original one. If necessary, the deconvolution process can be restarted with different parameters and all images can be finally exported back to the "*Thumbnail overview*" window.

Conclusion

Now the meaning of the parameters involved in the process has been elucidated, you should find it easier to set the correct values. Still, there are some basic rules which will ensure the best possible results:

- Before running the deconvolution wizard always check the microscope parameters in the image properties.
- If possible, generate a PSF (*Operations window*) and compare the shape with small structures in your image and match the saturation factor and immunity fraction accordingly.
- If possible, prefer small stacks (at least 3 slices) to single frames. 3D data generates significantly better deconvolved images than 2D data.
- Always compare the deconvolved image with the raw data in order to rule out possible artifacts during the process. Caution is advised if structures in the deconvolved image do not appear in the raw data, or if objects are as small as single pixels.
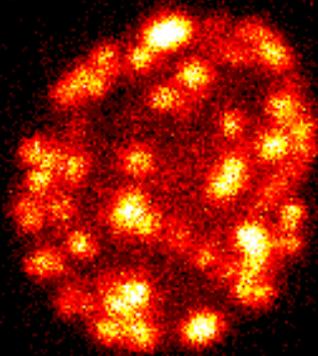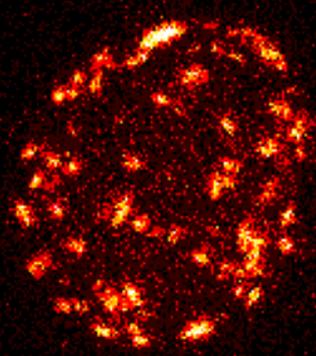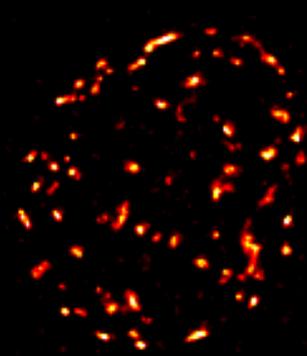
Confocal STED STED deconvolved

PIL1 Eisosome Core Protein tagged with GFP in living yeast. 3D projections. Courtesy of Marko Kaksonen, EMBL, Heidelberg

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Sweden · Kista	+46	8 625 45 45	8 625 45 10
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