Huygens Essential

Deconvolution Workshop Guide

Scientific Volume Imaging B.V.

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Cover illustration: Macrophage recorded by Dr. James Evans (White-head Institute, MIT, Boston MA, USA) using widefield microscopy, as deconvolved with Huygens®. Stained for tubulin (yellow/green), actin (red) and the nucleus (DAPI, blue).

Contents

CHAPTER 1

Introduction

Huygens Essential is an image processing software package tailored for restoration, visualization and analysis of microscopic images. Its wizard driven user interface guides through the process of deconvolving images from light microscopes. Huygens Essential is able to deconvolve a wide variety of images ranging from 2D widefield images to 4D multi-channel multi-photon confocal time series. To facilitate comparison of raw and deconvolved data or results from different deconvolution runs Huygens Essential is equipped with a dual 4D slicer tool. Also 3D images and animations can be rendered with its powerful visualization tools. Post-restoration analysis is possible using the interactive analysis tools.

Based on the same image processing engine (the compute engine) as Huygens Professional, Huygens Essential combines the quality and speed of the algorithms available in Huygens Professional with the ease of use of a wizard driven intelligent user interface fortified with a versatile and intuitive batch processor.

Huygens Essential uses cross-platform technology. It is available on Microsoft Windows Vista, Windows 7, and Windows 8 (64 bit), Linux (64 bit), and Mac OS X Lion, Mountain Lion, and Mavericks (X11, XQuartz). IRIX and Itanium distributions are available on demand.

References to SVI Wikipages

In this chapter words/piece of sentence starting with capitals, refer to an SVI Wiki[1] page. For example, the sentence "A mismatch between the Lens Refractive Index and the Medium Refractive Index will cause several serious problems" refers to two SVI Wiki pages, namely `http://www.svi.nl/LensRefractiveIndex` and `http://www.svi.nl/MediumRefractiveIndex`.

1. `http://www.svi.nl/`

CHAPTER 2

Deconvolution Wizard

The Processing Stages in the Wizard

The deconvolution wizard guides you through the process of microscopic image deconvolution (also referred to as *restoration*) in several stages. In Huygens Essential one needs to verify the microscopic parameters before continuing with deconvolution. This is done with either the Microscopic Parameter Editor or with the Parameter wizard, which in turn guides you through the process of parameter verification. The parameter wizard is especially useful for inexperienced users.

Each stage is composed of one or more tasks. While proceeding, each stage is briefly described in the bottom-left *Help* window tab. The stage progress is indicated in the *Wizard status* pane, below the *Help* tab. Additional information can be found in the online help (HELP→ONLINE HELP) as well as by clicking on the highlighted help questions.

The following steps and stages are to be followed:

- Loading an image.
- Verifying microscopic parameters.
- Start Stage: here the possibility exists to load a deconvolution template and a measured PSF. If a deconvolution template is chosen, the steps following the cropping stage can be skipped.
- Preprocessing Stage: this stage goes through all preprocessing steps.
  - Cropping stage
  - Stabilization (STED data)
  - Select channel
  - Inspecting the image histogram.
  - Background Estimation
- Deconvolution Stage:
  - Setting the final deconvolution parameters.
  - Run the deconvolution.
  - Select final result
- Postprocessing Stage: correct for Z-drift if any and accept the result.
- End Stage: Option to Restart, Save deconvolution template and Finish.

The next sections of this chapter will explain the wizard stages in detail.

Loading an Image

Select FILE→OPEN on the Huygens main window to open the file dialog, browse to the directory where the images are stored, and select the image, e.g. faba128.h5. A demo image (`faba128.h5`) is placed in the `Images` subdirectory of the installation path.

Most file formats from microscope vendors are supported, but some of them require a special option in the license to be read. See the SVI support Wiki[1] for updated information.

If the file is opened successfully, select the image and check whether the image dimensions are correct by looking under the tab STATISTICS located at the top-right of the main window. Some tools, such as those to convert image dimensions, are described in more detail in the next subsections

Then either the DECONVOLUTION WIZARD button can be pressed (right lower corner) to begin deconvolving the image or, if a bead image was loaded, one can proceed by selecting PSF DISTILLER in the DECONVOLUTION menu to generate a *point spread function* from measured beads (See Chapter 6 “The PSF Distiller” on page 33). A special license is needed for activating the PSF Distiller option.

Converting a Dataset

Before applying deconvolution, it is important that the image dimensions are correctly defined. Dimensions can be changed with the conversion tools listed under the TOOL menu. For example, a 3D stack can be converted here into a 3D time series (TOOLS→CONVERT->CONVERT XYZ TO XYZTusing the `convert3D24D` command) or vice versa, or a 3D stack can be converted into a time series of 2D images (TOOLS→CONVERT->XYZ TO XYTusing the `convertZ2T` command) or vice versa. In case of a refractive index mismatch, it is important to set the orientation of the image correctly with respect to the image direction and coverslip position (TOOLS→MIRROR ALONG Zusing the `mirror` command). The MIRROR tab in the converter tool can also be used for that purpose. Read more at “Setting the Coverslip Position” on page 42.

Time Series

A time series is a sequence of images recorded along at uniform time intervals. Each recorded image is a time frame. Huygens Essential is capable of deconvolving 2D-time or 3D-time data automatically. There are some tools that are intended only for time series, as the widefield *bleaching corrector* or the *z-drift corrector.*

Adapting the image

In the TOOLSDECONVOLUTION menu you can find a contrast inverter helpful for the processing of brightfield images (See “Brightfield Images” on page 45.). A Crop tool is also available, but its use is recommended only after properly tuning the image parameters> This tool will be explained in a later stage in this manual.

1. http://www.svi.nl/FileFormats

Verifying Microscopic Parameters

In Huygens Essential it is mandatory to verify your microscopic parameters before deconvolution. If they are not verified yet, you will be asked to do so using either the parameter wizard or the parameter editor.In Huygens Essential you are not obligated to verify your microscopic parameters, but it is recommended to check them either with the parameter wizard or the parameter editor.In Huygens Essential it is mandatory to

TABLE 2.1. Optical parameters explained.

Parameter	Explanation
Microscope type	Select from *widefield*, *confocal*, *spinning disk*, *4Pi or STED.* For a multiphoton microscope insert an *excitation photon count* higher than 1. Select *widefield* if a nondescanned detector was used, and *confocal* in case a pinhole of less than a few Airy disc units was used.
Numerical aperture	The NA of the objective lens.
Objective quality	Select from perfect, poor, or something in between.
Coverslip position	The *position of the glass interface* which is between the lens immersion and embedding medium, relative to the nearest slice of the stack.
Imaging direction	Select from *upward* or *downward*. Upward means that the objective lens is closest to the bottom slice in the stack. Upward stands for an inverted microscope where the objective points upward.
Backprojected pinhole radius	The radius (in nm) of the pinholes in the spinning disk as it appears in the *specimen plane*. This is the physical pinhole radius divided by the total magnification of the detection system.
Backprojected pinhole spacing	The distance (in µm) between the pinholes in the spinning disk as it appears in the *specimen plane*. This is the physical pinhole distance divided by the total magnification of the detection system.
Lens refractive index	The RI of the immersion medium for the objective lens.
Medium refractive index	The RI of the specimen embedding medium.
Excitation wavelength	The wavelength (in nm) of the excitation light (usually a laser line).
Emission wavelength	The wavelength (in nm) of the emitted light.
Excitation photon count	The number of photons used in *multi-photon* microscopy.
Excitation fill factor	The width of the beam relative to the aperture. The default value is 2, meaning that the aperture has a diameter of 2σ, where σ is the standard deviation of the Gaussian distribution in the beam.
STED saturation factor	Factor describing how much the fluorescence is suppressed by the STED beam.[1]
STED wavelength	Wavelength of STED depletion laser beam (nm)
STED immunity fraction	Fraction of fluorophore immune (photoresistant) to the depletion beam.
STED shape coefficients	Depletion doughnut shape parameters: width, height (optical units), center contrast.
STED 3X	Percentage of laser power used for the Z depletion beam, The remaing part is used for the vortex beam
Deviating microscope type	The deviating microscope type for the current channel.

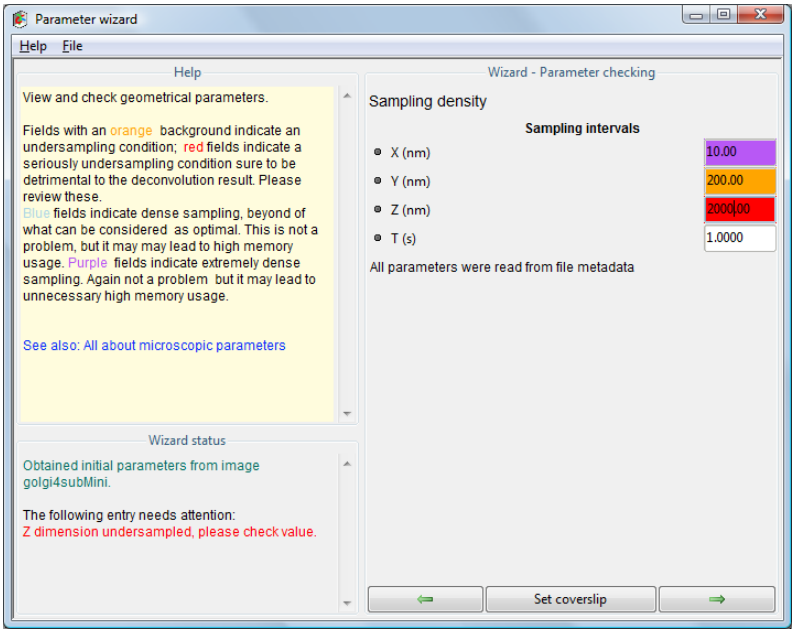


FIGURE 2.1. Parameter wizard: check sampling density. Red coloring indicates a suspicious value, and orange a non-optimal value.

1. Chapter 4 on page 23

verify your microscopic parameters before deconvolution. If they are not verified yet, you will be asked to do so using either the parameter wizard or the parameter editor.In Huygens Essential you are not obligated to verify your microscopic parameters, but it is recommended to check them either with the parameter wizard or the parameter editor.In Huygens Essential it is mandatory to verify your microscopic parameters before deconvolution. If they are not verified yet, you will be asked to do so using either the parameter wizard or the parameter editor.In Huygens Essential you are not obligated to verify your microscopic parameters, but it is recommended to check them either with the parameter wizard or the parameter editor.

By using the parameter wizard or the parameter editor, one can modify the parameters that are explained in Table 2.1.

If values are displayed in a *red* background, they are highly suspicious. An *orange* background indicates a non-optimal situation (See Figure 2.1). Oversampling is also indicated with a *cyan* background, that becomes *violet* when it is significant.

The parameter editor and parameter wizard can be found by right-clicking on the image thumbnail and selecting EDIT PARAMETERSPARAMETER EDITOR or PARAMETER WIZARD. The parameter wizard can also be found in the DECONVOLUTION menu. Figure 2.1 shows the parameter wizard. The parameter editor is shown in Figure 2.2.

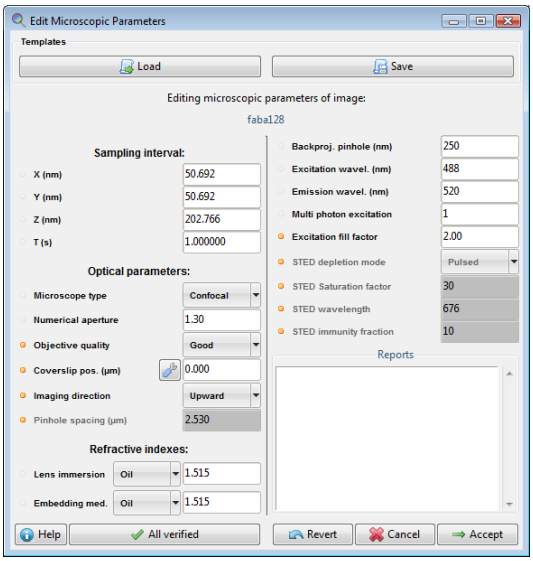


FIGURE 2.2. The microscopic parameter editor. This window can be opened by right-clicking on the thumbnail and selecting EDIT PARAMETERSPARAMETER EDITOR.

Microscopic Template Files

Once the proper parameters have been set and verified, they can be saved to a Huygens template file (`.hgst`). These templates can be applied at the start of the parameter wizard or in the parameter editor.

The LOAD A TEMPLATE button will allow the selection of a template from a list of saved template files which reside both in the common templates directory and in the user's personal template directory. The Huygens common templates directory is named `Templates`, and resides in the Huygens installation directory. The user's personal templates directory is called `SVI/Templates` and can be found in the user's home directory[1].

Using a Measured PSF

Measured PSF's improve the deconvolution results and may also serve as a quality test for the microscope. If the measured PSF contains less channels than the image, a theoretical PSF will be generated for the channels of which no PSF is available. See Chapter 6 “The PSF Distiller” on page 33 and “The Point Spread Function” on page 39 for more information.

Once the deconvolution wizard is opened, a previously created deconvolution template and/or a measured Point Spread Function (PSF) can be loaded. A measured PSF should only be used for deconvolution if the image and the bead(s) were recorded with the same microscope at the same parameter settings.

The Intelligent Cropper

The time needed to deconvolve an image increases more than proportionally with its volume. Therefore, the deconvolution can be accelerated considerably by *cropping* the image.

Huygens Essential is equipped with an intelligent cropper which automatically surveys the image to find a reasonable proposal for the crop region (See Figure 2.3). In computing this initial proposal the microscopical parameters are taken into account, making

1. The user home directories are usually located in `C:\Users` on Windows 7 and Vista and in `C:\Documents and Settings` on Windows XP and lower. On Mac OS X they are usually in `/Users` and on Linux in `/home`.

sure that cropping will not have a negative impact on the deconvolution result. Because the survey depends on accurate microscopical parameters it is recommended to use the cropper as final step in the preprocessing stage (press YES when the wizard asks to launch the cropper), but it can also be launched from outside the wizard through the menu TOOLS→CROPEDIT→CROP.

Cropping in *X*, *Y*, and *Z*.

The borders of the proposed cropping region are indicated by a colored contour. The initial position is computed from the image content and the microscopic parameters at launch time of the cropper.

The three views shown are *maximum intensity projections* (MIPs) along the main axes. By default the entire volume (including all time frames) is projected. The red, yellow, and blue triangles can be dragged to restrict the projected volume.

The cropper allows manual adjustment of the proposed crop region. To adjust the crop region, drag the corners or sides of the cropbox to the desired position or use the entries in the *Specifications* panel. To crop the original image press the CROP button. To create a new cropped image, press the EXTRACT button. The EXTRACT button is not visible if the the cropper is used within the deconvolution wizard. Do not crop the object too tightly, because that would remove blur information relevant for deconvolution, and background area for the efficient estimation of the background level.

The three views shown are Maximum Intensity Projections (MIP's) along the main axes. The projections are computed by tracing parallel rays perpendicular to the projection plane through the data volume, each ray ending in a pixel of the projection image. The maximum intensity value found in each ray path is projected. For example, each pixel in the xy projection image corresponds with the maximum value in the vertical column of voxels above it.

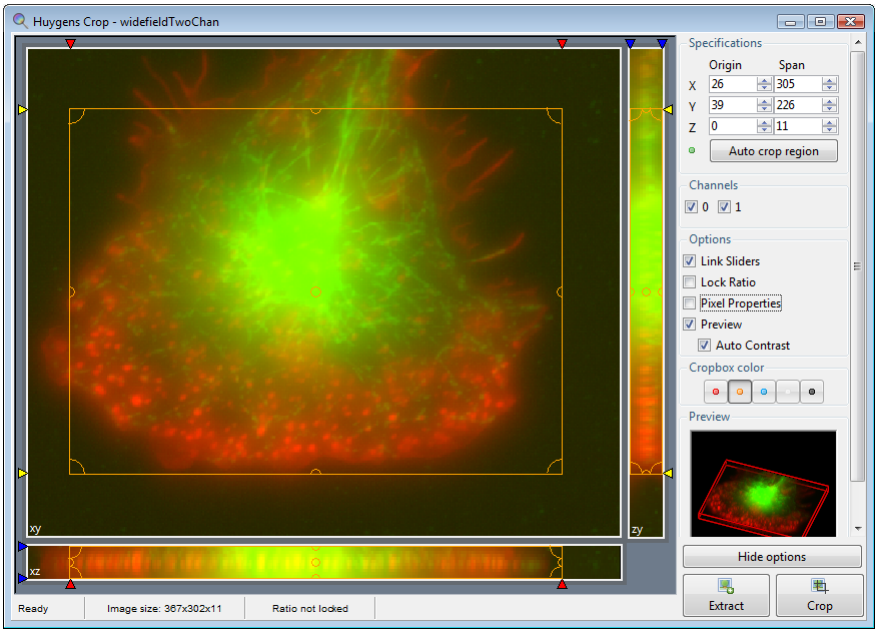


FIGURE 2.3. The crop tool in Huygens Essential.

Cropping in Time and removing channels

Select the `from` and `to` frame under *Specifications* to crop in time. There are entries for the time-selection, if the image is a time series. Only a continuous range can be selected to crop in time.

All available channels are listed under *Channels* in the operation window and can be removed individually. By using the right-click on a channel checkbox, all channels can be turned on or off, except for the selected channel. In the main window you can separte the image into individual channels using *Spli*t, which can be found under the *Tools* menu or as an icon in the taskbar.

Cropper Customizations and Options

The cropper is extended with extra visualization options, which are available under *Options*. LOCK RATIO fixes the aspect ratio of the cropping box for each projection separately. PIXEL PROPERTIES shows the pixel intensities and position of the mouse when you hover over the image. The intensities however are only shown when one time frame and one channel are selected. If the PREVIEW box is checked, a small renderer shows the resulting image after cropping.

Stabilizing STED data

STED image acquisition is often subjective to drift. Thus, it is strongly recommended to stabilize the STED images before deconvolving them. This can be done easily within the Deconvolution Wizard, after the cropping stage.

The option to stabilize the data along the z direction is present in the Deconvolution Wizard when working with STED data. If stabilization is actually applied depends on the percentage level of STED 3X. The stabilization will automatically correct the raw data for drifts and misalignments along the z direction. The deconvolution will be performed on the corrected, stabilized dataset. Once run, the stabilization cannot be undone.

This stage can be skipped at the Deconvolution Wizard if the image has already been stabilized by other means, for example, the Huygens Object Stabilizer.

To stabilize the image in the Deconvolution Wizard just click on the "AUTO STABILIZE" button at the "STABILIZATION" stage (See Figure 2.4).

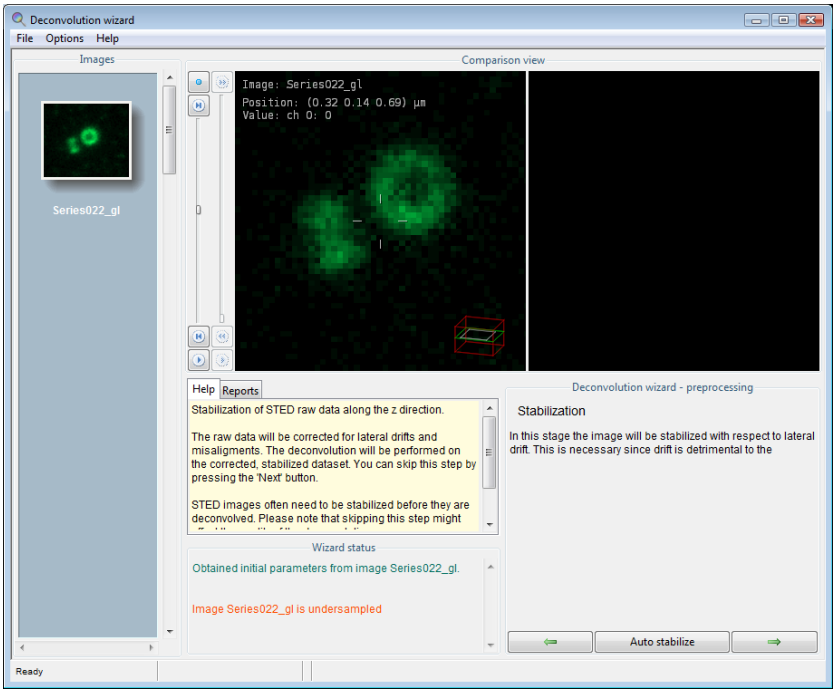


FIGURE 2.4. Stabilization stage for STED data in the Deconvolution Wizard.

The Image Histogram

The histogram is an important statistical tool for inspecting the image. It is included in the deconvolution wizard to be able to spot problems that might have occurred *during the recording*.

The histogram shows the number of pixels as a function of the intensity (gray value) or groups of intensities. If the image is an 8 bit image, gray values vary between 0 and 255. The *x*-axis is the gray value and the *y*-axis is the number of pixels in the image with that gray value. If the image is more than 8 bit, then gray values are collected to form a *bin*. For example, gray values in the range 0-9 are collected in bin 0, values in the range 10-19 in bin 1, etc. The histogram plot now shows the number of pixels in every bin.

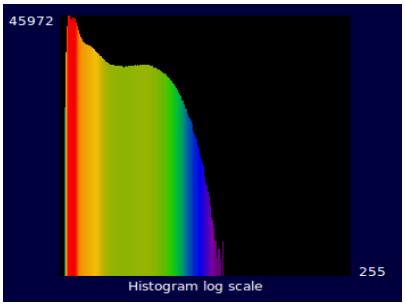


FIGURE 2.5. The image histogram. The vertical mapping mode can be selected from linear, logarithmic or sigmoid.

The histogram in Figure 2.5 shows that the intensity distribution in the demo image is of reasonable quality. The narrow peak shown at the left represents the background pixels, all with similar values. The height of the peak represents the amount of background pixels (note that the vertical axes uses *logarithmic* scaling).

In this case there is also a small black gap at the left of the histogram. This indicates an electronic offset, often referred to as *black level*, in the signal recording chain of the microscope.

If a peak is visible at the extreme right hand side of the histogram it indicates *saturation* or *clipping*. Clipping is caused by intensities above the maximum digital value available in the microscope. Usually, all values above the maximum value are replaced by the maximum value. On rare occasions they are replaced by zeros. Clipping will have a negative effect on the results of deconvolution, especially with widefield images.

The histogram stage is included in the deconvolution wizard for examining purposes only. It does not affect the deconvolution process that follows[1].

Estimating the Average Background

In this stage the *average* background in a volume image is estimated. The average background corresponds with the noise-free equivalent of the background in the measured (noisy) image. It is important for the search strategy that the microscopic parameters of the image are correct, especially the sampling distance and the microscope type.

In this stage the background value of the image (channel) can be automatically estimated or manually entered. For the automatic estimation you can choose the estimation mode and the area radius. The following estimation strategies are available:

- **Lowest value** (default): The image is searched for a 3D region with the *lowest average value*. The axial size of the region is about 0.3 µm; the lateral size is controlled by the radius parameter which is by default set to 0.7 µm.
- **In/near object**: The *neighborhood around the voxel with the highest value* is searched for a planar region with the lowest average value. The size of the region is controlled by the radius parameter.
- **Widefield**: First the image is searched for a 3D region with the lowest values to ensure that the region with the least amount of blur contributions is found. Subsequently the background is determined by searching this region for the planar region with radius *r* that has the lowest value.

Press the AUTO button in the wizard to continue with the automatic estimation with the options entered in this stage. Press the MANUAL button, if you want to skip the automatic estimation step and estimate it yourself.

In the next step the estimated value can be adapted either by altering the value in the *Absolute background* field or in the *Relative background* field. Setting the latter to -10, for example, lowers the estimated background by 10%. You can also choose how the background is stored in the template. If you plan to use this template also for other images that have been acquired with the same settings, we advise to store the relative value.

In case you choose the manual setting the absolute value is automatically stored in the template.

The last option determines the deconvolution algorithm. The default is *Classical Maximum Likelihood Estimation* (CMLE), but it can also be set to *Quick Maximum Likelihood Estimation* (QMLE).

If done press ACCEPT to proceed to the deconvolution stage.

1. Learn more about histograms at `http://www.svi.nl/ImageHistogram`

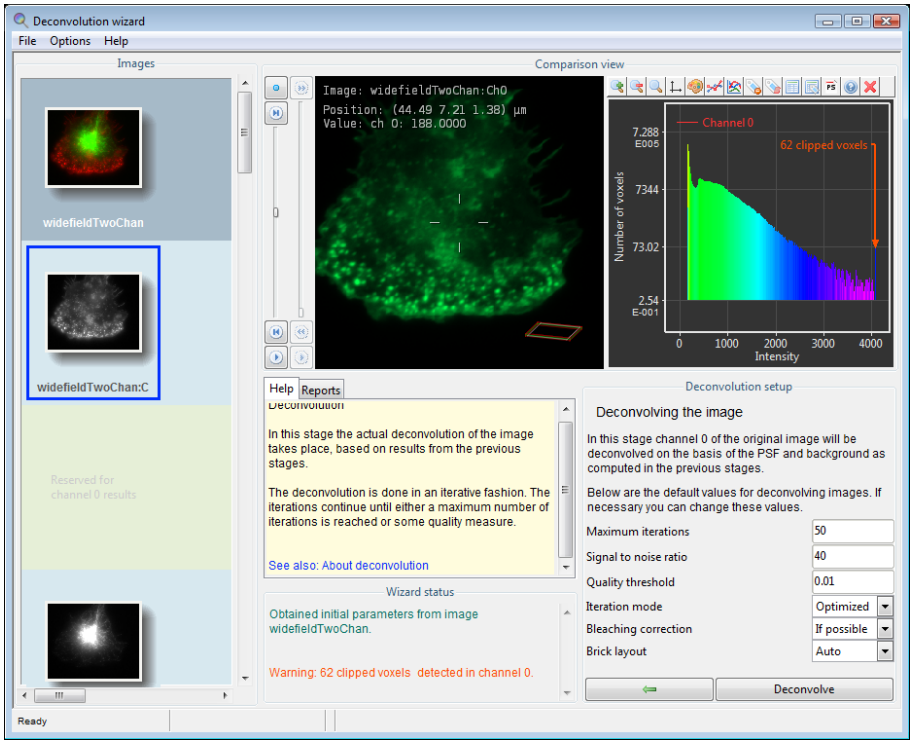


FIGURE 2.6. The Deconvolution Stage in the wizard.

The Deconvolution Stage

Figure 2.6 shows the deconvolution stage where the deconvolution parameters can be set. The following parameters are available for the CMLE algorithm. The options for QMLE are almost the same, except that the Quality threshold and the Iteration mode are not available.

1. **Number of iterations**. MLE is an iterative process that never stops if no *stopping criterion* is given. This stopping criterion can simply be the maximum number of iterations. This value can be adjusted, depending on the desired final quality of the image. For an initial run the value can be left at its default. To achieve the best result this value can be increased to e.g. 100. Another stopping criterion is the *Quality threshold* of the process (See Item 3).
2. **Signal to noise ratio**. The SNR is a parameter than controls the sharpness of the restoration result. Using a too large SNR value might be risky when restoring noisy originals, because the noise could just be enhanced. A noise-free widefield image usually has SNR values higher than 50. A noisy confocal image can have values lower than 20.
3. **Quality threshold**. Beyond a certain amount of iterations, typically below 100, the difference between successive iterations becomes insignificant and the progress grinds to a halt. Therefore it is a good idea to monitor the progress with a quality measure, and to stop the iterations when the change in quality drops below a threshold. At a high setting of this quality threshold, e.g. 0.01, the quality difference between subsequent iterations may drop below the threshold before the indicated maximum number of iterations has been reached. The smaller the threshold the larger the number of iterations that will be reached; the higher the quality of the restoration. Still, the extra quality gain becomes very small at higher iteration counts. The absolute value of the final quality factor much depends on the data, the microscope type, and the background. It is a global value computed over the entire image, so the contribution of a local resolution increase can be small, as such it can only be

used in a relative way to compare iterations and should not be seen as a value to compare the quality of different images.

4. **Iteration mode**. In *optimized* mode (highly recommended) the iteration steps are bigger than in *classical* mode. The advantage of classical mode is that the direction of its smaller steps is sure to be in the right direction; this is not always the case in optimized mode. Fortunately, the algorithm detects if the optimized mode hits upon a sub optimal result. If so, it switches back to the classical mode to search for the optimum.
5. **Bleaching correction**. If this option is set to *if possible*, then the data is inspected for bleaching. 3D stacks and time series of widefield images will always be corrected. Confocal images can only be corrected if they are part of a time series, and when the bleaching over time shows exponential behavior.
6. **Brick layout**. When this option is set to *auto*, then Huygens Essential splits the image into bricks in two situations:
    a. The system's *memory* is not sufficiently large to allow an image to be deconvolved as a whole.
    b. *Spherical aberration* is present, for which the point spread function needs to be adapted to the depth.

Press DECONVOLVE to start the restoration process. Pressing STOP DECONVOLUTION halts the iterations and retrieves the result from the previous iteration. If the first iteration is not yet complete an empty image will be shown.

Finishing or Restarting a Deconvolution Run

When a deconvolution run is finished, the result is shown in the right viewer where you can inspect the result in detail. Depending on the outcome one can choose to RESTART ALL, RESTART CHANNEL, RESUME or ACCEPT/ALL DONE the restoration:

- **Restart all** will return to the start stage and all results of all channels are discarded.
- **Restart channel** keeps the result so far and will resturn to the background stage where you can change the background setting and rerun the process with different deconvolution parameters. A new result will be generated to compare with previous results. This can be repeated several times.
- **Resume** keeps the result and returns to the stage where the deconvolution parameters can be entered. The software will ask to continue where it left off, or to start from the raw image again. A new result will be generated to compare with previous results. This can be repeated several times.
- **Accept, to next channel** proceeds to the first channel that has not yet been deconvolved. In case of **All done**, all channels have at least one deconvolution result or it was not a multi-channel image and it will proceed to the final stage.

The Final Stage

In the final stage the final image can be selected. The last deconvolution results of each channel is automatically selected. By moving imagenames from the *Available* to the *Selected* list, you can combine a multichannel image as desired. You can use the mouse-scroll to scroll the lists. Also, when clicking on a thumbnail in the left Images pane, the corresponding imagename is highlighted in the *Available* list, if it was not already moved to the *Selected* list. Not only the deconvolved results are available, also the original can be included in the final result. Once satisfied with the selection you can continue to the next stage. Please note, pressing **Restart** here will discard all the results and will return to the start stage.

What now follows is the possibility to restart all again (which discards the results), save the deconvolution template, quit the wizard (which discards the results) or press DONE to export the result to the main window of Huygens Essential.

The Comparison View

The Deconvolution Wizard allows for detailed image comparisons of all the thumbnails shown on the left panel. The Comparison View consists of two adjacent displays where images can be loaded on demand, and where plots of those images can be shown.

To show two images on the “Comparison View” right-click on the corresponding thumbnails and select “Show left” or “Show right” (See Figure 2.7).

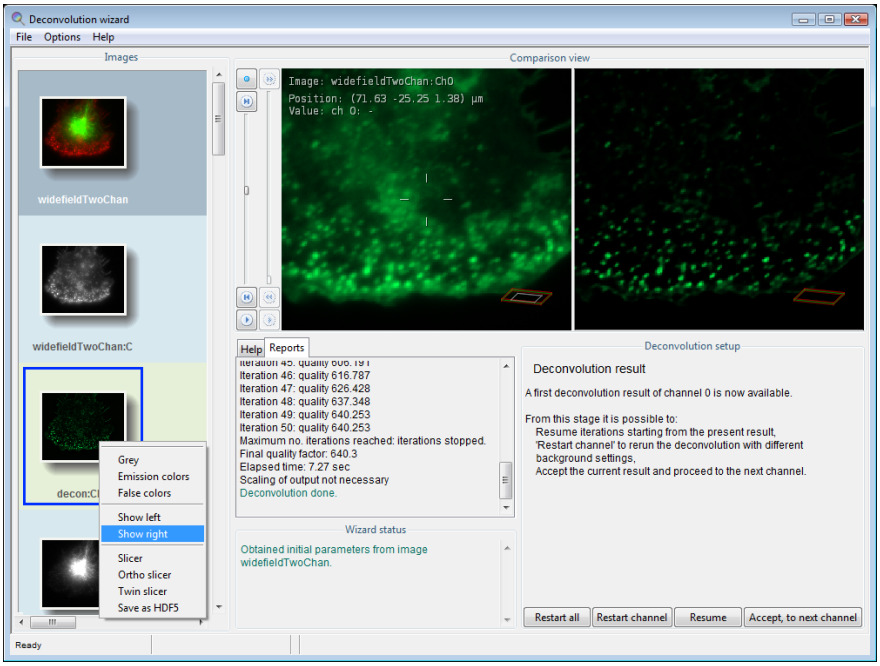


FIGURE 2.7. Showing an image on the Comparison View: right-click on the thumbnail and select “Show left” or “Show right”.

In order to plot the image intensities along a specific path click on the image and draw a line without releasing the mouse. A plot will be shown on the Comparison View display (left or right) opposite to where the line is drawn. To hide the plot, click a point of the Comparison View off the drawn line. To show the same plot again, click on the drawn line. This operation can be performed on both sides of the Comparison View. The plot will show the intensities of the two images loaded on the displays, for comparing purposes, see Figure 2.8 on page 15. If one of the displays is empty the plot will show the intensities of the existing image. Figure 2.9 on page 16 shows the result of plotting across the intensities of one image

Multi-channel Images

Multi channel images can be deconvolved in a semi automatic fashion, to give the opportunity to fine-tune the results obtained with each individual channel. After the preprocessing stage the multi channel image is split into single channel images named

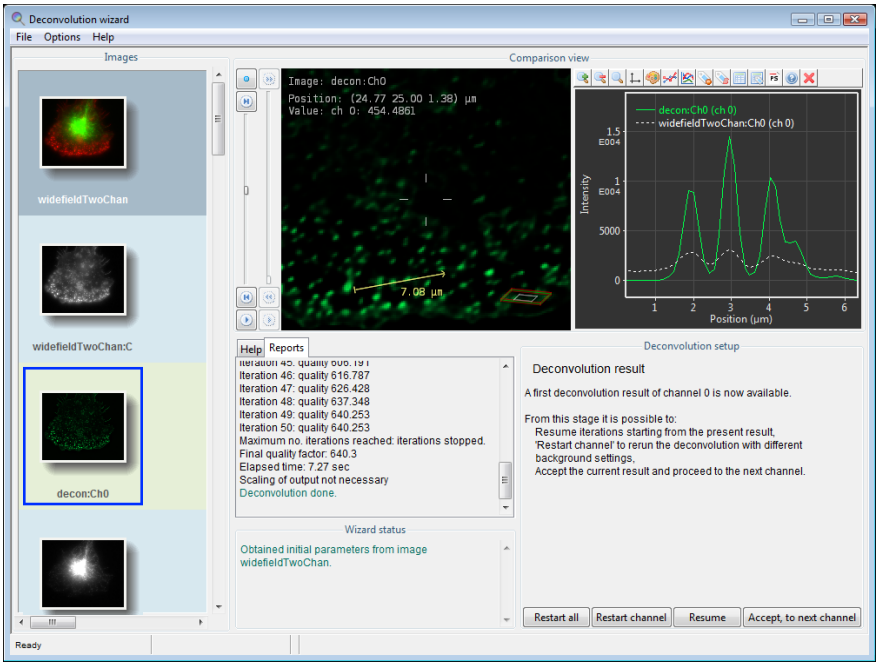


FIGURE 2.8. Comparing intensity profiles of two images in the Deconvolution Wizard. Notice that the plot prompts at the display opposite to where the line is drawn.

<imagename>:Ch0, *<imagename>:Ch1*, etc. The first of these is automatically selected for deconvolution.

The procedure to deconvolve a channel in a multi channel data set is exactly the same as for a single channel image. Therefore multiple reruns on the channel can be done manually, just as with single channel data. When everything is done press ACCEPT, TO NEXT CHANNEL in the last stage. This will cause the next channel to be selected for deconvolution. Proceed as usual with the remaining channels. If it is not needed to process all the channels in an image one or more channels may be skipped.

When the last channel has been processed, the wizard allows to select the results which should be combined into the final deconvolved multi channel image. This means that up to this point it is still possible to decide which of the results to combine, even in what order..

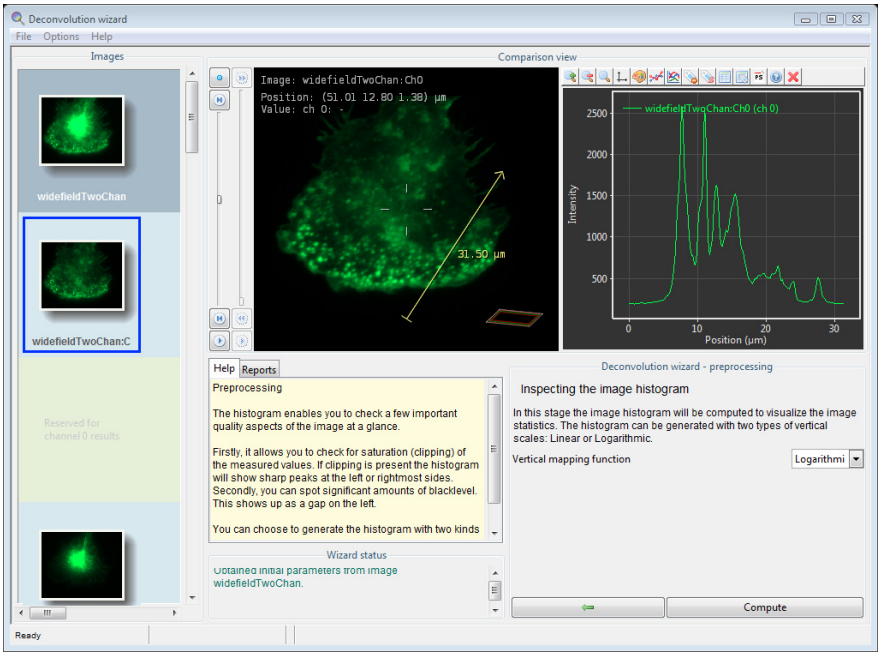


FIGURE 2.9. Getting an image intensity profile in the Deconvolution Wizard. Notice the image names on both displays (left and right).

Z-drift Correcting for Time Series

For 3D time series the wizard shows an additional stage to enable correction for movement in the *z* direction (axial) that could have been occurred, for instance, by thermal drift of the microscope table. In case of a multi channel image, the corrector can survey *All channels* and determine the mean *z* position of the channels, or it can take *One channel* as set by the *Reference channel* parameter.

After determining the *z* positions per frame, the *z* positions (not the image) can be filtered using a *median*, *Gaussian* or *Kuwahara* filter of variable width. If the drift is gradual, a Gaussian filter is probably best. In case of a drift with sudden reversals or outliers a median filter is best. In case the *z* positions show sudden jumps, we recommend the Kuwahara filter.

Saving the Result

After deconvolution when the final result is selected and exported to the main window, the result can be saved. Select the image to be saved and select FILE→SAVE AS... in the menu bar. The *HDF5* file format preserves all microscopic parameters and applies a lossless compression. To see which other file formats the Huygens software supports, see `http://www.svi.nl/FileFormats`.

Select FILE→SAVE STAGE REPORT to store the information as displayed in the Report tab.

CHAPTER 3

Huygens Deconvolution

The job of image restoration is to figure out what the instrument is actually trying to tell you. -- Prof. E.R. Pike, King's College London.

Introduction

Deconvolution is a mathematical operation used in Image Restoration to recover an image that is degraded by a physical process which can be described by the opposite operation, a convolution. This is the case in image formation by optical systems as used in microscopy and astronomy, but also in seismic measurements.

In microscopy this convolution process mathematically explains the formation of an image that is degraded by blurring and noise. The blurring is largely due to diffraction limited imaging by the instrument. The noise is usually photon noise, a term that refers to the inherent natural variation of the incident photon flux.

The degree of spreading (blurring) of a single pointlike (Sub Resolution) object is a measure for the quality of an optical system. The 3D blurry image of such a single point light source is usually called the Point Spread Function (PSF).

Image Formation

PSFs play an important role in the image formation theory of the fluorescent microscope. The reason for this is that in incoherent imaging systems such as fluorescent microscopes the image formation process is linear and described by Linear System theory. This means that when two objects A and B are imaged simultaneously, the result is equal to the sum of the independently imaged objects. In other words: the imaging of A is unaffected by the imaging of B and vice versa.

As a result of the linearity property the image of any object can be computed by chopping up the object in parts, image each of these, and subsequently sum the results. When one chops up the object in extremely small parts, i.e. point objects of varying height, the image is computed as a sum of PSFs, each shifted to the location and scaled according to the intensity of the corresponding point. In conclusion: the imaging in the fluorescent microscope is completely described by its PSF.

Convolution

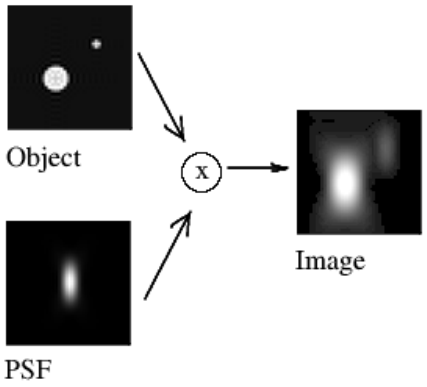


FIGURE 3.1. Diagram representation of

You can imagine that the image is formed in your microscope by replacing every original Sub Resolution light source by its 3D PSF (multiplied by the correspondent intensity). Looking only at one XZ slice of the 3D image, the result is formed like in Figure 3.1:

This process is mathematically described by a convolution equation of the form:

$$g = f * h \qquad \textbf{(EQ 1)}$$

where the image g arises from the convolution of the real light sources f (the object) and the PSF h. The convolution operator * implies an integral all over the space:

$$g = f * h = \iiint_{-\infty}^{\infty} f(\vec{x})h(\vec{x} - x')d^3\vec{x}' \qquad \textbf{(EQ 2)}$$

Interpretation

You can interpret Equation 2 as follows: the recorded intensity in a voxel located at $\vec{x} = (x, y, z)$ of the image $g(\vec{x})$ arises from the contrubutions of all points of the object f, their real intensitiesweighted buy the PSF h depending on the distance to the considered point.

Calculation

That means that for each voxel located at $\vec{x} = (x, y, z)$ the overlap between the object function f and the (shifted) PSF h must be calculated. Computing this overlap involves the computing and summing the value of $f(\vec{x})h(\vec{x} - \vec{x}')$ in the entire image. Having N voxels in the whole image, the computational costs is of the order N^2.

But this can be improved. An important theorem of Fourier theory, the convolution theorem, states that the Fourier Transforms G, F, H of g, f, h respectively are related by simply a multiplication.

$$G = F \cdot H \qquad \textbf{(EQ 3)}$$

This means that a convolution can be computed by the following steps:

1. Compute the Fourier transforms F and H of f and h
2. Multiply F times H to obtain G
3. Transform G back to g, the convolved image.

Because Fourier transforms require a number of operations in the order of $N\ log(N)$, this is more efficient than the previous integral.

To see how the application of different PSF's affect the imaging of an object, read the Cookie Cutter[1].

Deconvolution

If convolution implies replacing every original (sub-resolution) light source by its correspondent PSF to produce a blurry image, the restoration procedure would go the opposite way, collecting all this spread light and putting it back to its original location. That would produce a better representation of the real object, clearer to our eyes. (This increases the Dynamic Range of the image, and causes the background regions to look darker!).

Mathematically speaking, deconvolution is just solving the above mentioned Equation 1, where you know the convolved image *g* and the PSF *h*, to obtain the original light distribution *f*: an representation of the "real" object.

The relation in Equation 3 would seem to imply that it is possible to obtain the object function *F* by Inverse Filtering, just by dividing $F = G/H$. But due to the bandlimited character of *H* it has zeros outside a certain region (see Cookie Cutter), resulting in a division by zero for many spatial frequencies. Also, in a real general case the photon noise must be taken into account, so the equation that we actually have to solve is not Equation 1, but the equation shown in Equation 4.

$$k = f * h + \varepsilon \qquad \textbf{(EQ 4)}$$

where the acquired image *k* arises from the convolution of the real light source *f* and the PSF *h*, plus the photon noise ε. The division of ε by *H* would lead to extreme noise amplification and therefore tremendous artifacts due to the large areas of small values of *H* within the passband. (Also, we can not simply substract ε from *k*, as we can not know what the exact noise distribution is).

Thus, inverse filtering will never allow us to recover the true object function *f*. Instead, we must try to find an estimate *f'* which satisfies a sensible criterion, and is stable in the presence of noise.

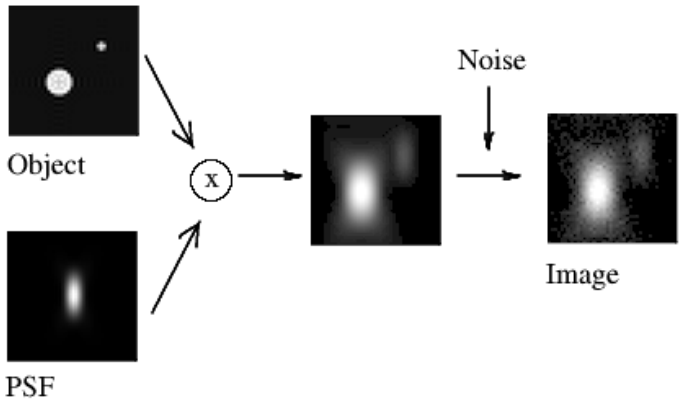


FIGURE 3.2. Diagram representation of convolution with photon noise.

1. Check the SVI Wiki `http://www.svi.nl/CookieCutter`

Some deconvolution methods (like Blind Deconvolution) try to solve Equation 4 without knowing the PSF term *h*. Although some constraints can be applied, this is always risky, as it introduces a lot of indetermination in the solution of the equation. (How many solutions *x, y* can you find for an algebraical equation of the form $x \times y = 5$?) These methods are also currently lacking of any scientific validation when applied to microscopy.

We must go for another solution.

The way Huygens works

The Huygens Software of Scientific Volume Imaging enables you to obtain a PSF in two ways:

- automatically computing a Theoretical Psf based on known Microscopic Parameters and a model of the microscope, or
- distilling an Experimental Psf from spherical bead images, after Recording Beads.

In the second case, given a model of the bead shape, the PSF is computed 'distilled' where its convolution with the bead model matches the measured bead image. That can be understood looking back at Equation 1 and Figure 3.1 on page 18. Now we know how the object *f* is (the exact size of the spherical bead must be known) and we have acquired its image *g*, thus we can distill the remaining unknown term *h* in the equation.

Once a PSF is provided Huygens can use different mathematical algorithms to effectively solve the convolution Equation 4 and do deconvolution:

- Classic Maximum Likelihood Estimation
- Quick Maximum Likelihood Estimation
- Iterative Constrained Tikhonov-Miller
- Quick Tikhonov-Miller

The Classic Maximum Likelihood Estimation (CMLE) is the most general Restoration Method available, valid for almost any kind of images. It is based on the idea of iteratively optimizing the likelihood of an estimate of the object given the measured image and the PSF. The object estimate is in the form of a regular 3D image. The likelihood in this procedure is computed by a Quality Criterion under the assumption that the Photon Noise is governed by Poisson statistics. (Photoelectrons collected by a detector exhibit a Poisson Distribution and have a square root relationship between signal and noise). For this reason it is optimally suited for low-signal images. In addition, it is well suited for restoring images of point- line- or plane like objects. See SVI Wiki[1] for more details.

There are however situations in which other algorithms come to front, for example when deconvolving 3D-time series, which is very compute-intensive. In this case you may consider to use Quick Maximum Likelihood Estimation-time (QMLE) which is much faster than the CMLE-time and will give excellent results as well.

An advantage of using measured PSF as in Huygens is that in essence it requires you to calibrate your microscope, and stimulates the use of standard protocols for imaging. Together, these will ensure correct functioning of the microscope and vastly increase the quality and reliability of the microscopic data itself, and with that of the deconvolution results.

1. `http://www.svi.nl/MaximumLikelihoodEstimation`

Lastly, an advantage of theoretical or measured PSFs is that they facilitate construction of very fast algorithms like the QMLE in Huygens Professional. Iterations in QMLE are about five times more effective than CMLE iterations and require less time per iteration.

Images affected by Spherical Aberration due to a Refractive Index Mismatch are better restored with Huygens Software through the use of depth-dependent PSF's (see SVI Wiki[1]).

Huygens algorithms generally do Intensity Preservation. Check the Huygens restoration applied to some accessible images in Convolving Trains on the SVI Wiki[2].

Examples

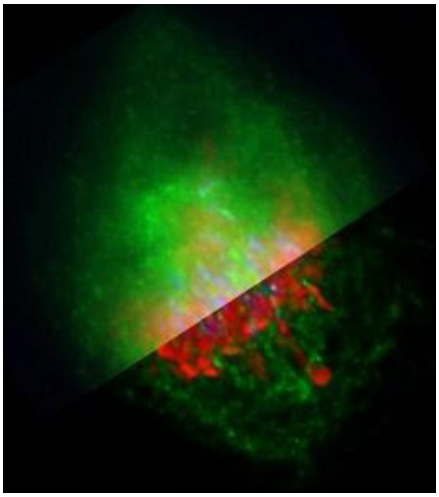


FIGURE 3.3. A metaphase human cell stained for DNA (red), centromeres (blue) and the anaphase promoting complex/ cyclosome (green). Upper part: original data. Lower part: deconvolved with Huygens Professional. Recorded by Dr. Claire Acquaviva, Dr. Pines Lab.

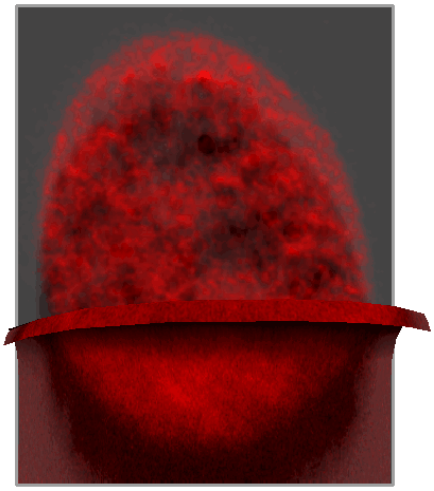


FIGURE 3.4. Nucleus of a human epithelium cell stained with an antibody against splicing factor. Bottom part: original image. Top part: image as restored by Huygens Professional. Both parts were visualized using the Sfp Renderer. Recorded by Dr. Marjolein A. Grande.

You can also find other images in Resolution Improvement and Evans Macrophage. A very simple and clarifying example of a deconvolution done with the Huygens Software is in Decon Example. Real microscopy images can be seen in the SVI image gallery[3].

Validation

The CMLE method used in Huygens is backed up by quite some scientific literature. We mention here just three relevant examples:

1. http://www.svi.nl/ParameterVariation
2. http://www.svi.nl/ConvolvingTrains
3. http://www.svi.nl/ImageGallery

- Verschure P.J., van der Kraan I., Manders E.M.M. and van Driel R. Spatial relationship between transcription sites and chromosome territories. J. Cell Biology (1999) 147, 1, pp 13-24.
- Visser A.E. and Aten J.A. Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei. J. Cell Science (1999) 112, pp 3353-3360.
- Hell S.W., Schrader M. and Van Der Voort H.T.M. Far-Field fluorescence microscopy with three-dimensional resolution in the 100-nm Range. J. of Microscopy (1997) 187 Pt1, pp 1-7 .

Further Reading

See `http://www.svi.nl/DoingDeconvolution` on the SVI Wiki for special topics and references. In "Support" on page 47 you will find many useful links to webpages, which will be a good starting point to learn more about the Huygens Software and image restoration.

CHAPTER 4

Huygens STED Deconvolution

Introduction

Stimulated-**E**mission **D**epletion (STED) microscopy is a fluorescence microscopy technique which overcomes and improves the diffraction-limited resolution of regular confocal microscopy techniques up to 4-5 times[1], leading to the so-called sub-diffraction resolution or super resolution in the lateral and, in case of STED 3D, also the axial direction .

The Huygens STED optical option offers support for deconvolving STED images yielding stunning results in XY, and in Z. Several existing Huygens Essential tools, offer an additional STED mode for STED-specific processing. For instance, the PSF distiller (see Chapter 6 on page 33) can generate Point Spread Functions and can estimate the STED microscopic parameters out of STED bead images. The Deconvolution Wizard (see Chapter 2 on page 3) can automatically stabilize STED raw images, which often present drifts along the z direction.

This chapter describes the STED parameters and the restoration procedure to achieve optimal deconvolution results with STED images. A short introduction to the STED principle is also included. A step-by-step summary of the restoration process for STED images is listed at the end of the chapter.

STED principle

The STED microscope overcomes the diffraction-limited resolution of the conventional fluorescence and confocal microscopes, by exploiting a strong non-linear effect in the depletion of excited fluorophores. In the STED microscope two laser beams are focussed on the same location. The first laser beam excites the fluorophore molecules located in the imaged volume, in the same way as the confocal microscope. The second laser, also referred to as depletion beam or STED beam, goes through a shape changing phase filter resulting in a doughnut-shaped focus. In the outer ring of this focus, where the intensity is high, excited fluorophore molecules are forced out of the excited state. This depletion effect is very non-linear, so that in effect above a certain intensity depletion rises quickly. This results in a narrow region around the optical axis being hardly depleted, whereas beyond this region depletion increases steeply. The diameter of the depletion-free region

1. "Two-photon excitation STED microscopy". Gael Moneron and Stefan W.Hell. Optics Express 17 ; 17 (2009).

can be as small as 25-50m, easily resulting in a four fold increase in resolution over good quality confocal resolution.

STED parameters

To achieve optimum results with the Huygens STED deconvolution it is recommended to verify the microscopic parameters of the STED image.

If the image metadata contains information about the STED parameters Huygens Essential will incorporate them into the image. Otherwise, parameter defaults will be loaded instead. Therefore, it is advisable to review the parameter values of the STED datasets.

To edit the microscopic parameters of an image right-click on the image and select or "PARAMETER WIZARD", as explained in Chapter 2 on page 3. If has been selected, the specific STED parameters will be presented as in Figure 4.1:

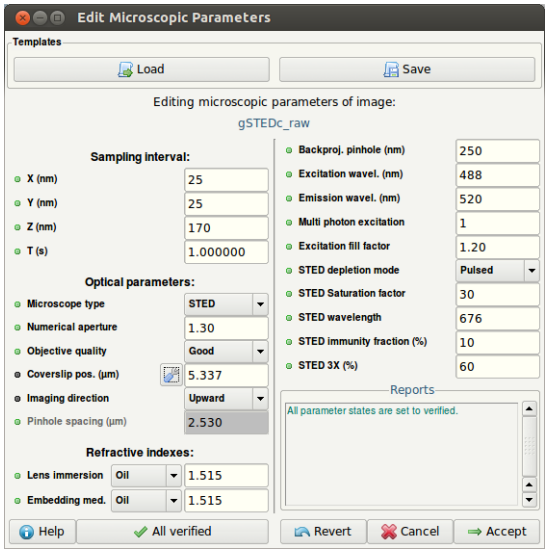


FIGURE 4.1. The specific STED parameters can be entered in the Parameter Editor.

- **STED depletion mode**: this mode determines the type of STED depletion that is being used. You can set this to Pulsed, CW (continuous wave) non-gated detection, or CW gated detection.
- **STED saturation factor**: this parameter describes how much the fluorescence is suppressed by the STED beam. The higher this factor the more fluorescence suppression off the optical axis, the more resolution. At values below 1, hardly any resolution is gained. Typical values are in the range of 10 to 50[1]. A very large value of the saturation factor usually also implies a large resolution improvement. Because this parameter can be difficult to quantify it is recommended to use the Huygens PSF distiller (see below) to get an automatic estimation. Also, please notice that in practice the fluorescence is never completely suppressed in the whole depletion region as the fluorophore molecules can be partly 'immune' to depletion.
- **STED wavelength**: the wavelength of the STED depletion laser beam (nm). The STED wavelength must be a value within the range of the fluorophore emission spectrum.
- **STED immunity fraction**: the fraction of fluorophore molecules not susceptible, 'immune', to the depletion beam. This parameter is specified as a percentage; 100% meaning that all the fluorophore molecules are immune to the depletion beam, 0% meaning that no fluorophore mol-

1. "Resolution scaling in STED microscopy". Harke B, Keller J, Ullal CK, Westphal V, Schönle A, Hell SW. Optics Express 16 ; 6 (2008).

ecules are immune to the depletion beam. The value that should be entered here is usually between 0% and 10%. Because this parameter can be difficult to quantify it is advised to use the Huygens PSF distiller (see below) to get an automatic estimation.

- **STED 3X**: the percentage of power used in the Z depletion beam. The remaining power is used for the vortex beam path. This value is read from the image metadata
- **Deviating microscope type**: this parameter can only be edited when working with multichannel images. The parameter entry is hidden for single-channel images. If not all channels are STED, this parameter should be set to the other microscope type used for that channel.

The STED saturation factor can in principle be determined experimentally, see the footnote on page 24. An alternative is to use the Huygens PSF distiller which can estimate these parameters automatically from a suitable bead image.

Example settings

In Table 4.1 you find the (ranges of) normal values for the STED microscopic parameters for some STED systems.

TABLE 4.1.

Parameter	Microscope types			
	TCS STED	TCS STED CW/TCS SP8 STED (3X)		
	pulsed STED	STED CW	gSTED	STED 3X
specified resolution (FWHM)*	70	80	50	n.d.**
typical resolution (FWHM)	60	70	40	n.d.**
excitation fill factor	0.5-1.0 (0.8)	1.2	1.2	1.2
STED saturation factor	< 60 (40)	< 40 (25)	< 40 (25)	< 40 (25)
STED wavelength (nm)	750-780	592	592	592/660/775
STED immunity fraction	<25% (10)	<25% (10)	<10% (5)	<10% (5)
Imaging direction	downwards	downwards	downwards	downward

* measured on 40nm beads; ** not determined yet

Estimating STED parameters

Estimating the STED parameters is an automated process guided by the PSF distiller. Open a STED beads image in the PSF distiller and proceed to distill a PSF as explained in Chapter 6 “The PSF Distiller” on page 33.

The PSF distiller will recognize that the image has been recorded with a STED microscope and will offer the possibility to estimate the STED parameters after distilling the PSF (see Figure 4.2).

The possibility to generate a theoretical PSF with the estimated STED parameters prompts in the PSF distiller. This can be of interest to compare the distilled PSF with the theoretical PSF.

Figure 4.3 shows the PSF distiller after estimating the STED parameters of a beads image. In this example, the estimated STED saturation factor is 3 and the (very high) immunity factor of 29%.

Estimated STED parameters will be attached to the new distilled PSF, which will be shown as a thumbnail in the main window of Huygens Essential. The parameters are

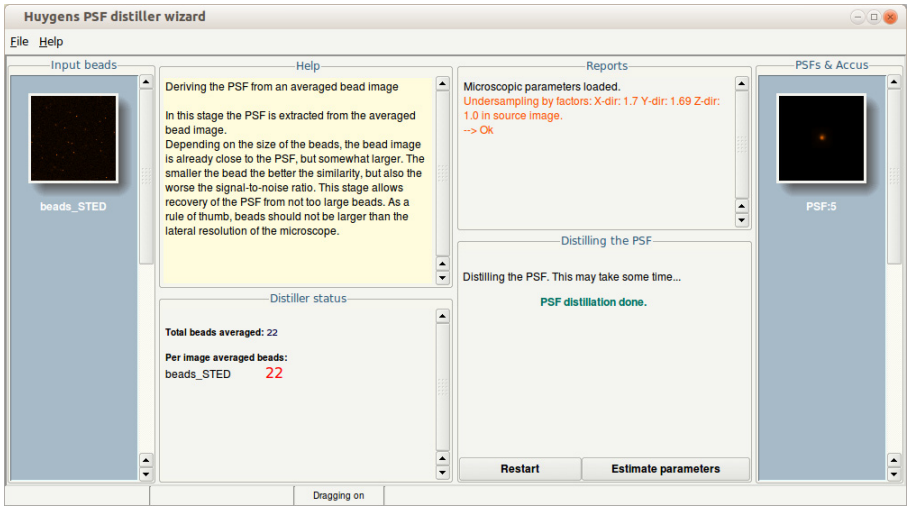


FIGURE 4.2. Estimating the STED microscopic parameters of a beads image automatically with the Huygens PSF distiller.

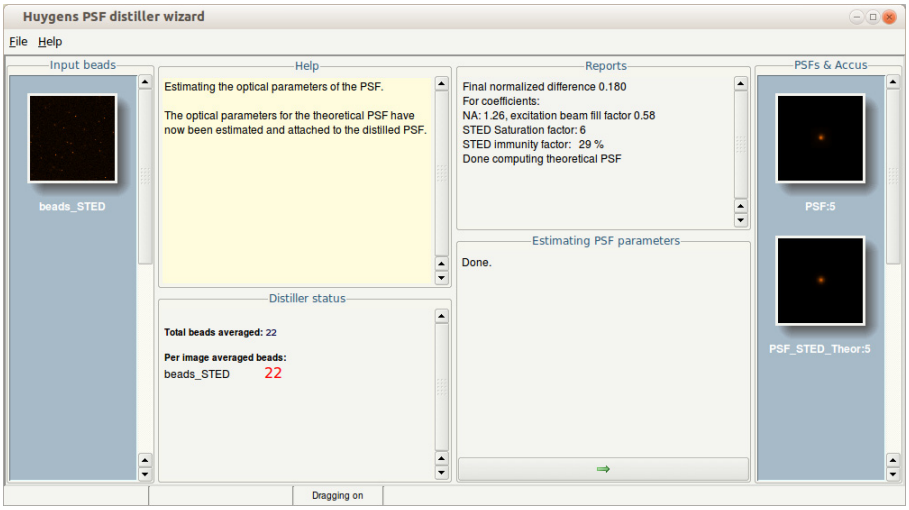


FIGURE 4.3. The PSF distiller showing the STED microscopic parameters automatically estimated from a beads image.

reported by the PSF distiller (see Figure 4.3) and can be reviewed by, for example, opening the Parameter Editor by right-clicking on the PSF thumbnail (see Figure 4.4).

The microscopic parameters of the PSF can exported as a template file within the Parameter Editor or the Parameter Wizard (see Figure 4.4). The microscopic parameter template file can, lateron, be applied to other STED images that have been recorded under the exact same conditions. This makes future parameter editing much more comfortable and reliable.

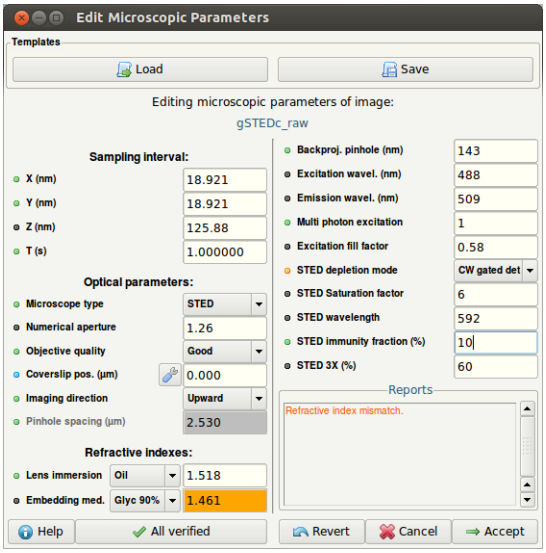


FIGURE 4.4. The Microscopic Parameters of the distilled PSF now contain the values automatically estimated by Huygens Essential.

STED Deconvolution

To deconvolve a STED dataset, open the image in the Parameter Editor or Parameter Wizard. Verify the microscopic parameters or load the microscopic parameter template file saved before. The latter will import the PSF microscopic parameters to the STED raw dataset. Both the PSF and the raw image will now have the same estimates of the relevant STED parameters. Next, open the data set in the Deconvolution Wizard (select the image → DECONVOLUTION → DECONVOLUTION WIZARD). Select the PSF at the first stage of the Deconvolution Wizard. If no PSF is provided, the Deconvolution Wizard will create a theoretical PSF from the microscopic parameters of the image.

Continue to the next Wizard stage and proceed step by step as explained in Chapter 2 “Deconvolution Wizard” on page 3. The Deconvolution Wizard will recognize a STED dataset and will offer a stabilization option because STED image acquisition is often subjective to drift. The severity of drift in STED image data depends on how much of the STED power is used for vortex and for Z depeletion. Stabilization is recommended for pure vortex-based STED images. With increased Z depletion in STED 3X data, stabilization becomes less of an issue as the PSF becomes less elongated in the axial direction. Huygens will take the

To stabilize the image in the Deconvolution Wizard just click on the “AUTO STABILIZE” button at the “STABILIZATION” stage (See Figure 4.5). Whether Huygens really applies stabilization depends on the percentage of STED 3X, will be shown in the REPORT window.

The stabilization stage can be skipped at the Deconvolution Wizard if the image has already been stabilized by the Huygens Object Stabilizer (See **Chapter 17 on page 91.)**

After this stage, complete the remaining Deconvolution Wizard stages as usual.

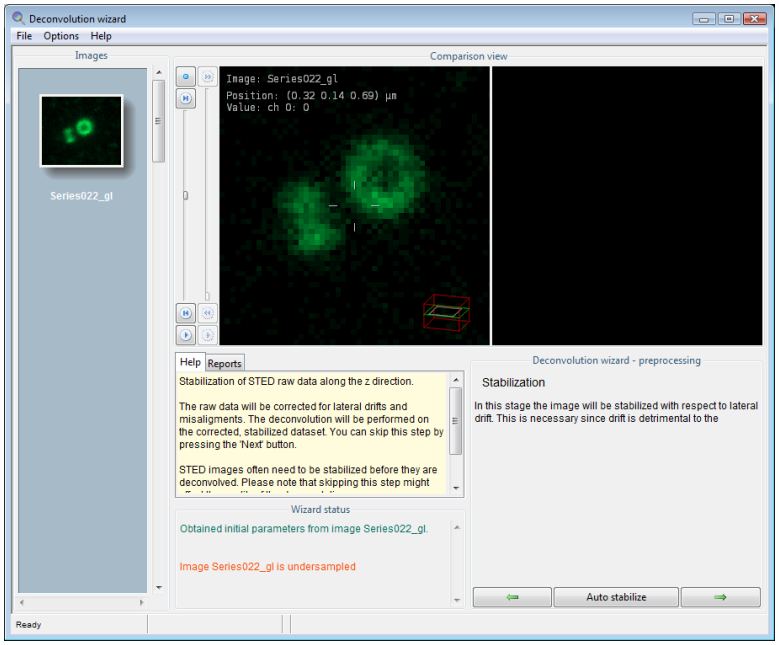


FIGURE 4.5. Stabilization stage for STED data in the Deconvolution Wizard.

Summary: deconvolution procedure

The STED deconvolution procedure can be summed up in the following steps:

- Load a STED beads image.
- Distill a PSF from the beads image with the PSF distiller. Allow it to estimate the STED parameters automatically.
- Export the microscopic parameters of the new PSF to a template file (via Parameter Editor or Parameter Wizard).
- Load the STED raw dataset.
- Import the PSF microscopic parameters template file to the STED raw data (via Parameter Editor or Parameter Wizard).
- Open the raw dataset in the Deconvolution Wizard.
- Specify the PSF image in the Deconvolution Wizard and continue to the subsequent Wizard steps.
- At the “Stabilization” stage select “Auto Stabilize” if the STED raw data has not yet been stabilized.
- Complete the remaining stages of the Deconvolution Wizard normally.

CHAPTER 5

Data acquisition pitfalls

This chapter summarizes some typical experimental difficulties when acquiring microcopic images. A mismatch between the Lens Refractive Index and the Medium Refractive Index will cause several serious problems.

Geometrical distortion: the fishtank effect

The objective moves along the optical axis a certain distance (a z-step that is recorded in the data file), but the focus shifts inside the sample an actual different step. Therefore objects will appear elongated or shortened in the microscope. Huygens Essential will automatically adapt the PSF to this situation, but it will not modify the image geometry.

Spherical aberration (SA)

Spherical Aberration will cause the oblique rays to be focused in a different location than the central rays. The distance in this focal shift is dependent on the depth of the focus in the specimen. If the mismatch is large, e.g. when going from oil immersion into a watery medium, the PSF will become asymmetric at depths of already a few micron. Especially harmful for WF deconvolution. Workaround: keep the Z-range of the data as small as possible. Solution: use a water immersion lens. The Huygens Software can correct the S.A. under certain conditions, see Spherical Aberration.

Total internal reflection

When the lens Numerical Aperture is larger than the Medium Refractive Index, Total Internal Reflection will occur, causing excitation light to be bounced back into the lens and limiting the effective Numerical Aperture.

Clipping

The light intensities from the microscopic object are converted to electrical signals that pass an adjustable amplifier. Also an electrical DC component can be added or subtracted by the microscope operator. The electrical signal may thus range from negative to highly positive. These electrical signals must be converted to numbers processed by the computer. This converting stage is done in the CCD camera and its electronics. Most CCD cameras have an 12-bit converter limiting the output numbers to a range of 0 to 4095. Negative input signals are usually converted to 0 while positive input values

exceeding some value are all converted to 4095 (clipping): information in the clipped samples is lost.

In practice: be suspicious if you find values at the extremes in your image, probably clipping occurred.

Undersampling

One of the rules of measurement that is often overlooked is that one takes too few XY slices from the microscopic object. In that case the Sampling Distance is too large (too few samples: UnderSampling) which leaves you with a 3-D stack with hardly any relation between the adjacent planes. It is important to know how the sampling conditions should be established in order to recover an image from the sampled values. How you should sample your object depends on your microscope type -Wide Field Microscope or Confocal Microscope- and on the microscope parameters used, like the Numerical Aperture and WaveLength of the light. The correct Sampling Distance can be calculated using the Nyquist Calculator, or the formulas as given in the Nyquist Rate.

Do not undersample to limit photodamage

Some times undersampling is done to limit photodamage (Bleaching Effects) to live cells. If photodamage plays a role it is better to distribute the available photons over more pixels, resulting in an apparently noisier image, than putting the photons in fewer pixels to get a low noise, but undersampled, image (see Under Sampling). Of course there are limits, but a fair trade-off can be often found.

It is better to record 10 separate noisy slices 100 nm apart than slices on 1000 nm each averaged 5 times on order to reduce noise.

Bleaching

Bleaching Effects are practically unavoidable phenomena in fluorescence microscopy. Because the image planes are acquired sequentially, bleaching will vary along the Z direction. Assuming it is not strong it will not affect deconvolution results on confocal or two photon images. But in Wide Field Microscopes (WF) deconvolution bleaching is more of a problem. Fortunately, usually the bleaching in WF images can be corrected quite easily. Huygens will do so automatically. However, if the bleaching is strong the correction might not be perfect, resulting in lower quality deconvolution results.

Illumination instability

Some Wide Field Microscopes are equipped with unstable arc lamps. Huygens will correct this instability, but when the instability is severe it cannot do so sufficiently.

Jitter: Irregular deviations in time from an average value which causes, for example, a flickering lamp.

Mechanical instability

Mechanical instability can take many shapes, for example:

- Vibrations sometimes seen in confocal images. They may seriously hamper deconvolution.
- Z-stage moves irregular or with sudden jumps. Near-fatal for confocal or WF deconvolution.

- Specimen moves. If in WF data the object can clearly be seen moving when slicing along over a few micron in Z this will cause problems for the deconvolution. Best cause of action, apart from speeding up acquisition, is limiting the Z-range of the data as much as possible. Confocal data of moving specimen causes less problems.

Thermal effects

Thermal effects are known to affect calibration of the Z-stage, especially if piezo actuators without feedback control are used. In particular harmful for WF data. In time series the effect can be seen as a drift of the Z-postion, or even a periodic movement induced by periodic switch on and off of an airconditioner system. The Z-drift corrector is able to correct this in most situations though.

Internal reflection

At high Numerical Aperture (NA) the angle of incidence of the most oblique rays can be close to 70 degrees. When a ray has to cross the cover-glass to medium interface at such an angle total reflection may occur. To be precise, Total Internal Reflection occurs when the NA of your lens is higher than the Medium Refractive Index. This will reduce the effective NA of the lens.

CHAPTER 6

The PSF Distiller

Huygens Essential is optionally equipped with the *PSF Distiller*. This wizard driven tool guides through the process of measuring a microscope's *Point Spread Function* (PSF). It guides you in selecting your bead images, creating and saving the PSF for further use in deconvolution runs. The wizard is able to measure a PSF from one or more images of fluorescent beads, each containing one or more beads. It is also able to distill multi-channel PSF's from information collected from multi wavelength beads, or assemble a multi-channel PSF from single channel PSF's.

Measured PSF's improve deconvolution results and may also serve as a quality test for the microscope[1]. The measured PSF acts as a calibration of the microscope in the sense of relating a physical known object with what the microscope actually measures. Figure 6.1 shows an example of a theoretical PSF next to a measured one.

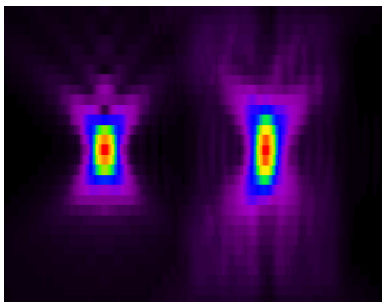


FIGURE 6.1. An *xz* cross section of a theoretical PSF (left) versus a measured PSF (right) for the same confocal setup.

The PSF Distiller is able to measure a PSF from one or more 3D stacks, each containing one or more fluorescent beads. It can distill *multi channel PSF's* from information collected from multi wavelength beads, or assemble a multi channel PSF from single channel PSF's.

Beads suited for PSF Distillation

The PSF distiller works by inspecting average images from small beads that are almost sub resolution in size, so they contain much of the PSF information. In order to measure a PSF from beads, the diameter of the beads should be in the order of the *half intensity width*[2] (HIW; also referred to as *full width at half maximum*, FWHM) of the expected PSF. Larger beads will reduce the accuracy of the Distiller, while smaller beads yield insufficient signal for accurate stacking in the averaging procedure, resulting also in

1. We advise to measure the PSF after a change in the recording setup and certainly after each maintenance job in which the optics or scanning device was serviced.

2. `http://www.svi.nl/HalfIntensityWidth`

reduced accuracy. Beads ranging from 120 to 250 nm can be used. Typically beads with a diameter of 160 nm perform very well for many types of microscopy.

Confocal and two photon bead images from 160 nm beads should look like smooth fuzzy blobs with hardly visible noise. Use the default SNR settings. If available, it is a good idea to average 2 to 5 beads. Two photon bead images may look slightly noisy. If so, set the SNR to 20 and average 4 to 10 beads.

Widefield images taken from 160 nm beads should look like smooth fuzzy blobs with no visible noise. The default SNR settings can be used. It is not necessary to average any more beads.

Beads should be recorded with the same microscopic parameters that you will use later to image your specimens. Please find more practical information about beads for PSF measurements on the SVI Wiki[3].

The PSF Distiller Window

If your license includes the PSF Distiller option, select DECONVOLUTION→PSF DISTILLER to start the Distiller. In the opening window (Figure 6.2) different panes show the input beads field, the Help field, the report field, the wizard fields and the PSFs & Accus field:

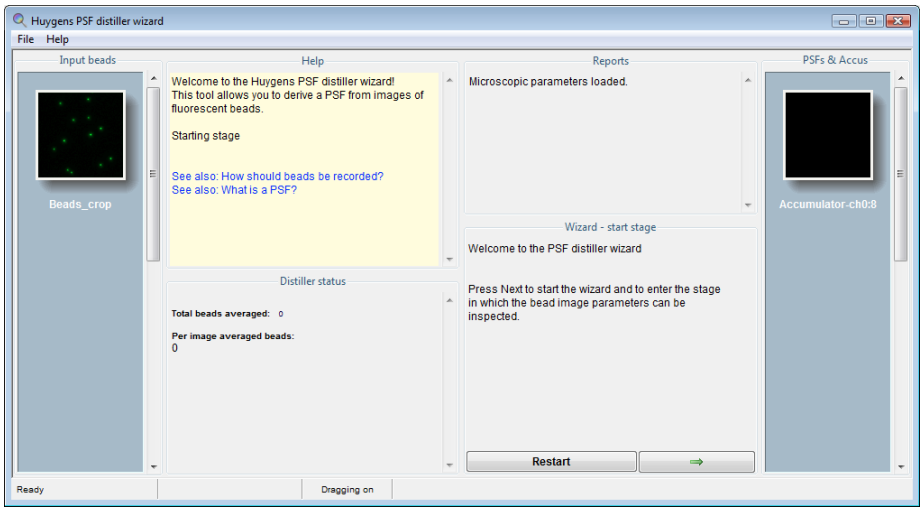


FIGURE 6.2. The PSF Distiller window. Different panes show the input beads field, the Help field, the report field, the wizard fields and the PSF and Accus field.

- *The Input beads pane*

  Shows the selected file with the bead images for the PSF distiller process. You can import and use multiple files with beads for the distillation of a PSF. With the cursor you can hover over the thumbnail and with a right mouse click you can roll down a menu to open the image in one of the slicers. The edit parameters function is greyed out here, you can only modify the microscopic parameters in the main microscopic parameter window or the PSF wizard.

- *The Help pane*

  Explains the different steps and displays links to relevant wiki pages.

3. http://www.svi.nl/RecordingBeads

- *The Distiller status pane*

  Keeps track of all the steps during the distiller run and reports the progress.
- *The Reports pane*

  Displays the progress report of the distiller process and the status of the distilling process.
- *The PSFs & Accus pane*

  Displays the intermediate results of the distilling process. The thumbnails can be inspected with the Sliders. The slider on the right side can be used to scroll down if many files are displayed.
- *The Wizard pane*

  Shows the steps that will take you from checking image parameters to distilling and averaging the beads from your images.

The Processing Stages in the Wizard

The following steps and stages are to be followed:

- Loading an image.
- Start Stage: here the possibility exists to load a microscopic parameter template and check the microscopic parameters.
- Averaging Stage: in this stage (all channels of) the image are searched for beads that meet the selection criteria. After each successful or unsuccessful search there is the possibility to load in additional bead images, or go to the next stage.
- Distillation Stage: in this stage the PSF is measured from the averaged beads, for all available channels.
- Finalizing the result: in case it is desired to combine results from earlier distillations with the current result to obtain a multi channel PSF, an earlier result can be added here. It is also possible to add single or multi channel previous results to a current multi channel result.
- Save the result.

The next sections of this chapter will explain the wizard stages in detail. See "Loading an Image" on page 4 and "Saving the Result" on page 16 for more information on handling image files.

Starting the Distiller

After launching Huygens Essential, open the first bead image via FILE→OPEN. If the license includes the PSF Distiller option, start the PSF Distiller via the menu DECONVOLUTION -> PSF DISTILLER. When the window is opened, one or more *accumulator images* will be created into which later on the averaged beads will be kept. Now the *start* stage will be entered.

Verifying Microscopic Parameters

See "Verifying Microscopic Parameters" on page 5 for more information on the microscopic parameters. Next to the optical parameters listed in Table 2.1 on page 5, it is in particular important to check the *sampling densities*.

Do not use undersampled bead images. If any of the entry fields for the sampling density turns orange or red, the data is *unusable* for distilling PSF's.

If there are multiple bead images, then the parameters of bead images loaded at a later stage should match the ones to establish in this stage; a warning of any mismatch will be given.

Averaging Stage

After setting the *bead diameter* and estimating the *Signal to Noise Ratio* (SNR), the image is searched for beads that meet the following selection criteria:

- A bead should not be *too close to another bead*. If a bead is too close to another bead, their signals will interfere. In widefield bead images this is quite problematic due to the large size of the blur cone. Fortunately, widefield PSF's can be derived from a single bead within an image.
- A bead should not be *too close to an image edge*. After all, another bead might be located just over the image edge.
- The *intensity* of a bead should not deviate too much from the median intensity of all beads. If it is brighter then it may be a cluster of two or more beads. If it is dimmer then it is not likely to be a bead. In both cases the object geometry is unknown so they are unusable.

If for some reason no usable beads are found, an explanation and some advice will be displayed in a pop up window. First, the software will try to find beads with ideal selection criteria. If this does not yield a single bead, it will automatically retry with reduced inter-bead distance criteria.

After having added the last bead recording press DISTILL to enter the *distillation* stage (See Figure 6.3).

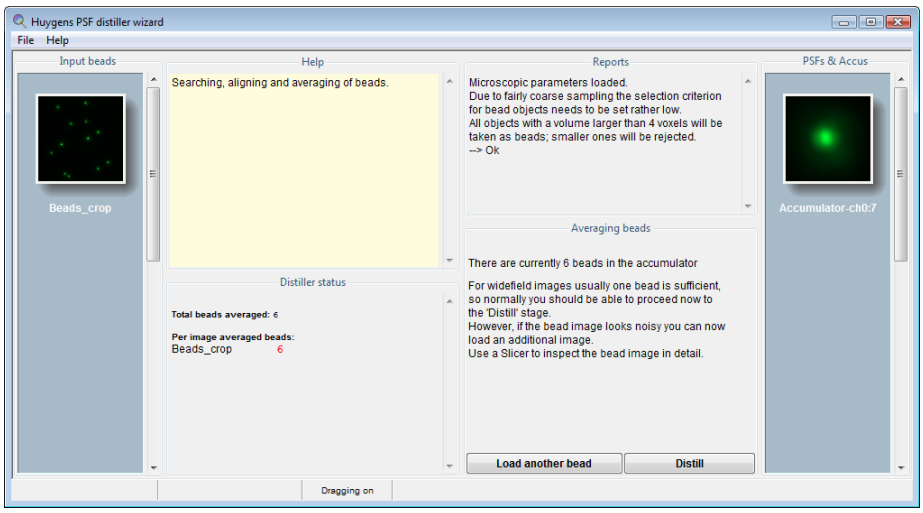


FIGURE 6.3. The averaging stage in the PSF Distiller wizard.

Confocal and Two Photon Bead Images

Images from 160 nm beads should look like smooth fuzzy blobs with hardly visible noise. Use the default SNR settings. If available, it is a good idea to average 2 to 5 beads. Two photon bead images may look slightly noisy. If so, set the SNR to 20 and average 4 to 10 beads. To load more bead images press LOAD ANOTHER BEAD in the averaging stage and either select an image from the main window or open a new one.

Widefield Bead Images

Images from 160 nm beads should look like smooth fuzzy blobs with no visible noise. Use the default SNR settings. Averaging beads is not necessary for widefield images.

Distillation Stage

The distillation stage usually requires no user intervention, though in some cases a pop up window will be displayed with a question or a warning. All channels will be processed automatically.

Finalizing the result

At this stage, a previously obtained PSF image can be added as a channel before or after the current result. Press ADD CHANNELS if this is desirable. The Distiller will compare the microscopic image parameters of the selected PSF image and check its content. In case there are differences, the software will ask to decide between ignoring these and discarding the selected file. Press DONE, if you are finished adding channels to the distilled PSF.

Full-width at half-maximum estimator

This stage also offers the possibilty to measure the quality of the PSF using the PSF full-width at half-maximum (FWHM) Estimator tool. If you have the PSF distiller option in your license, you can also find this tool in the task bar menu of the Professional under ANALYSIS.

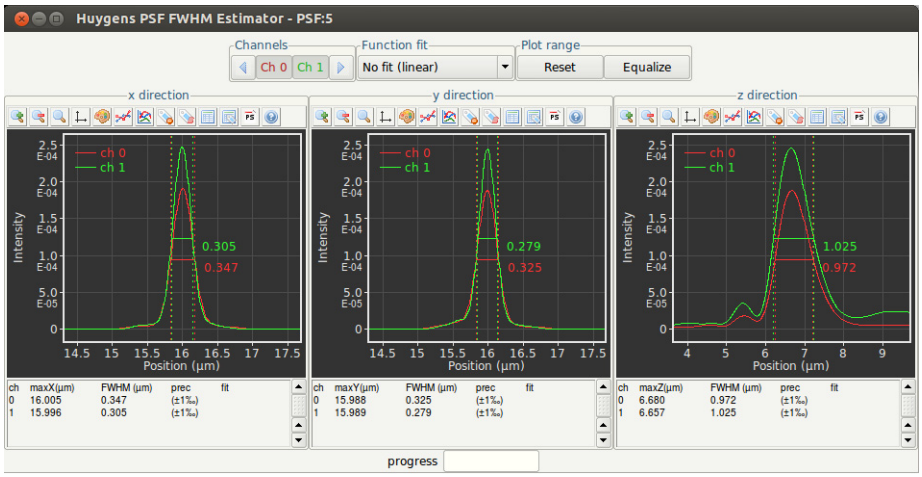


FIGURE 6.4. The PSF-FWHM overview. No curve fitting was selected.

The PSF FWHM Estimator creates a line intensity profile through the center of the PSF in all dimensions, and measures the X value of the peak maximum and the width of each peak at approximately half of the maximum value using a curve fitting procedure. Curve fitters such as Gaussian, Lorentzian, Voight and Pearson, are available for this. This is done for all channels. For further background information on the FWHM[4], PSFs and the PSF Distiller[5], please refer to the SVI Wiki.

4. http://www.svi.nl/HalfIntensityWidth
5. http://www.svi.nl/PsfDistiller

Finally, press EXPORT & CLOSE. This will export the distilled PSF to the main window, where the result can be saved or used immediately in a subsequent deconvolution run.

CHAPTER 7

Appendix

The Point Spread Function

One of the basic concepts in image deconvolution is the *point spread function* (PSF). The PSF of the microscope is the image which results from imaging a point object in the microscope. Because of wave diffraction[1] a point object is imaged (spread out) into a fuzzy spot: the point spread function. In fluorescence imaging the PSF completely determines the image formation. In other words: *all microscopic imaging properties are packed into this 3D function*. In Huygens Essential, a PSF can be obtained in two different ways:

1. *Generating a theoretical PSF*: When a measured PSF is not available, Huygens Essential automatically uses a theoretical PSF. The PSF is computed from the microscopic parameters attached to the data. Because a theoretical PSF can be generated without any user intervention Huygens Essential does the calculation in the background without any notice.
   Images affected by spherical aberration are better restored using a theoretical depth-dependent PSF.
2. *Measuring a PSF*: By using the PSF Distiller a measured PSF can be derived from images of small fluorescent beads (See "The PSF Distiller" on page 33.). Measured PSF's improve deconvolution results and may also serve as a quality test for the microscope

Quality Factor

Deconvolution as it is done in Huygens Essential is based on the idea of finding the best estimate of the object that is imaged by the microscope. To assess the quality of an estimate, Huygens Essential simulates the microscopic imaging of each estimate (the estimated is convolved with the PSF) and compares the simulation with the measured image. From the difference a quality factor is computed. The difference is also used to compute a correction factor to modify the estimate in such a way that the corrected estimate will yield a better quality factor. The quality factor as reported by the software is a measure relative to the first estimate and therefore a number greater than or equal to 1. If the increase in quality drops below the *quality threshold* the iterations are stopped.

1. http://www.svi.nl/ImageFormation

File Series

There are many ways in which Tiff files or other file series are named. These files can have multiple counters (referring to *slices*, *time frames*, or *channels*), and these counters can have arbitrary prefixes and ordering.

Numbered Tiff Series

If a series is simply numbered like: `slice001.tif`, `slice002.tif`, `...`, `slice0nn.tif`, then Huygens Essential will read the series into a single 3D image. Because Tiff files usually carry no additional microscopic information, check the parameters carefully.

Leica Numbering

Huygens Essential natively supports both reading and *writing* Tiff series with Leica style numbering, if there is more than one channel, slice, or time frame. A single channel 2D time series would be numbered according to the scheme:

```
im_tNN.tif
```

Here, `NN` is replaced by the time index for each frame. A more complex, multi-channel 3D time series has this pattern:

```
im_tNN_zNNN_cNN.tif
```

In this series, the second channel of the fourth slice of the third time frame has the filename:

```
im_t02_z003_c01.tif
```

The File Series Tool

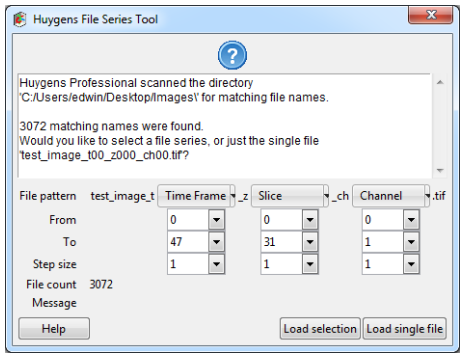


FIGURE 7.1. The Huygens File Series Tool automatically scans a directory for a file series.

Although Huygens Essential uses Leica style numbering for writing files, the software attempts to detect *any type* of file series for reading. Whenever a file is opened that appears to be part of a file series, Huygens Essential shows the *File Series Tool* dialog (Figure 7.1). This tool enables the user to select a subset of a file series, and select a dimension for each the indices in the file name, so that each image is assigned to the correct *z*-plane, time frame, and channel. Only select the first file of a series. If you select more, Huygens will attempt to see every selected file as a start of a new series. Consequently, many windows of the File Series Tool will be opened.

The file pattern is shown in the first row in the dialog. The counters in the file name are replaced by menu buttons for selecting the appropriate dimension for each counter. The options are:

- **Slice**: The range of this counter becomes the *z*-dimension.
- **Time Frame**: The range of this counter becomes the time dimension.
- **Channel**: The range of this counter becomes the channel dimension.
- **Ignore**: the variable is ignored. This is useful to omit e.g. the value of time stamps.

- **The value of the counter** in the selected file: the value of this counter has to match the value in the selected file.

Note that the selection has to be unique, i.e. it is impossible to have ignored variables without having a *Slice*, *Time Frame*, or *Channel* counter.

In the second, third, and fourth row, the range for each of the counters can be defined. A range from 0 to 9 with step size 2 will load the files 0, 2, 4, 6, and 8. Note that the time (in seconds) and *z*-sampling intervals (in nm) are not adapted to the step sizes.

Press the LOAD SELECTION button to load all files in the series into a single image. Before the dialog is closed, the tool will check if all files in the selection are really present in the directory.

Adjusting the Global Color Scheme

Huygens Essential uses a global scheme for coloring the different channels in multi-channel images. These colors can be adjusted through the *Preferences* window via EDIT→PREFERENCES...→EDIT GLOBAL COLORS (See Figure 7.2).

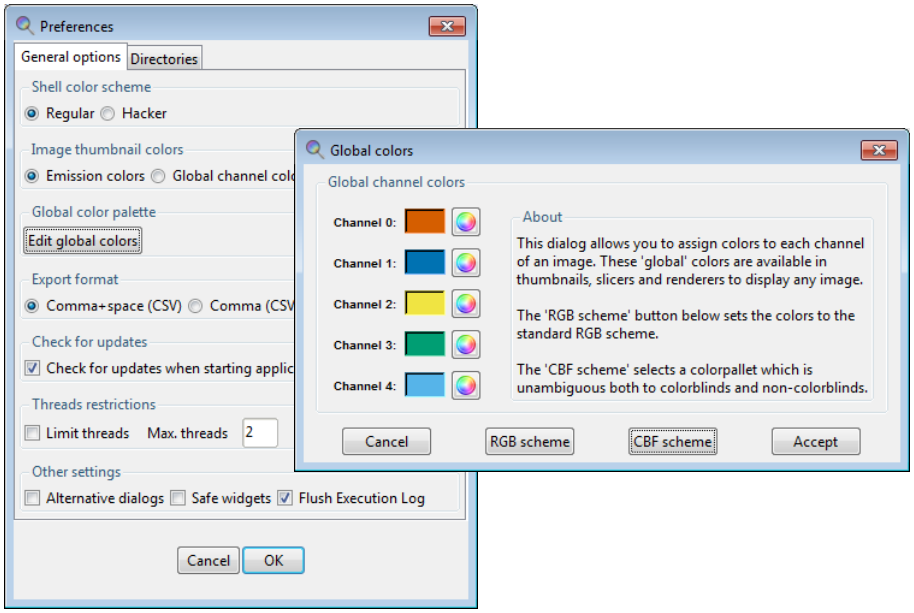


FIGURE 7.2. The global color scheme can be modified through the *Preferences* window.

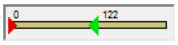
There are two color scheme available which are the RGB and CBF schemes. The RGB scheme (Red Green Blue scheme) is the color scheme that starts with the red, green and blue colors for the first 3 channels. The CBF scheme (Color Blind Friendly scheme) are contrasting colors that are unambiguous both to colorblinds and non-colorblinds.[2]

2. Published by Okabe and Ito, "*How to make figures and presentations that are friendly to Color-blind people*", J*FLY, 2002

Hue Selector

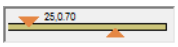
The *hue selector* is a component that allows adjustment of the color range in which objects are displayed. Objects belonging to different channels can be represented in different hue ranges to make them clearly distinct. The gradual differences inside the selected range make independent objects distinguishable. Also a range can be collapsed to have all objects in a channel displayed with exactly the same color. In Huygens the *hue selector* does appear in two flavors.

Hue Range



This selector allows the adjustment of a *hue* range. The objects on which this selector acts will get a color that lies within this range. The assignment of colors is based on the position of an object or on another parameter.

Hue Range and Saturation



This selector allows the adjustment of a single *hue* value and a *saturation*. The upper triangle defines the color, while the lower triangle sets the saturation for this color; left is white, right is fully saturated.

Image Statistics

Right-click on a thumbnail image and select SHOW PARAMETERS from the pop-up menu. This window shows, besides the parameter settings, statistical information of the particular image. Amongst them are the mean, sum, standard deviation, norm, and position of the center of mass.

Setting the Coverslip Position

When there is a mismatch between the refractive index for which the microscope's objective is designed and the actual refractive index of the embedding medium, the shape of the point spread function (PSF) will be distorted due to spherical aberration. As deeper layers in the specimen are imaged, moving away from the coverslip, this distortion will progressively worsen. To compute the spherical aberration it is necessary to know the distance from the coverslip. Because in many cases the coverslip position does not coincide with the first plane in the data, this position can be set in the microscopic parameter editor. To our knowledge none of the existing microscopic image files record the coverslip position in the meta data.

Next to direct numerical input, the coverslip position and imaging direction can be set using a visual editor (Figure 7.3), reachable from the parameter editor by clicking the wrench button ( ).

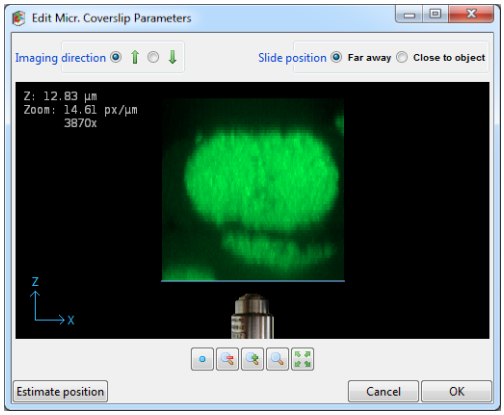


FIGURE 7.3. The coverslip position editor showing an *xy* MIP of the data along they *y*-direction. The coverslip position can be adjusted by dragging the blue line. The imaging direction, here *upwards*, is indicated by the position of the objective relative to the data as shown. The *z*-position shown top-left in the image indicates the distance in µm of the coverslip to the first data plane.

Inverted Microscope

The editor shows the coverslip position and imaging direction relative to the data as read from the microscopic file. In an inverted microscope, with the objective physically below the specimen it is likely that the first *xy*-plane in the data, corresponding with the lowest location in the *xz* maximum intensity projection (MIP) on the screen, corresponds with the *xy*-plane scanned closest to the objective. This situation is shown in REF TO FIG. However, since scan directions and data planes might have been reordered, this match is not guaranteed. Fortunately, it is often easy to spot the flat side of the object where it adheres to the glass, so the orientation can be verified.

Upright Microscope

In an upright microscope, and a *z*-scan starting away from the coverslip, the first plane is also likely to be physically the lowest plane. In that case, the imaging direction should be set to downwards and the coverslip position in the top part of the *xz* MIP projection. However, if the scan started close to the coverslip while storing these first planes first in the data set, the MIP projection will show the data upside down. Consequently, the coverslip position will be in the lower part of the MIP, and the imaging direction is upward.

Slide Position

When the specimen is mounted on the coverslip, the distance from the object to the slide is probably in the range from 50 to100 µm, outside of the image. In this case, or in the case there is no slide, select *Far away* in the top-right selector.

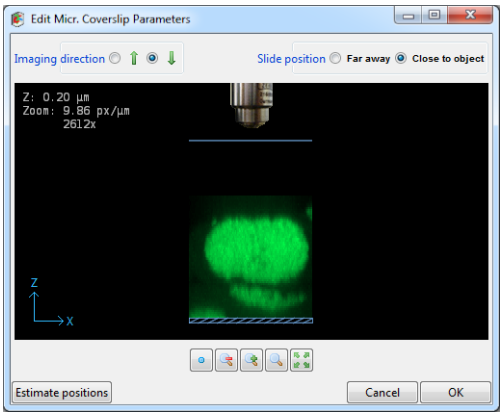


FIGURE 7.4. The Coverslip editor with the slide position set to *Close to object*.

When the specimen is close to or mounted on the slide, select *Close to object* (upper right corner). Drag the coverslip to its proper location. When this location is at some distance from the data it might be necessary to zoom out. The image can be dragged by holding down the right mouse button. In terms of imaging quality, when there is a refractive index mismatch between embedding medium and immersion medium, this is not an ideal situation since the light from and to the objective must travel hundreds of wavelengths through the embedding medium, possibly resulting in strong spherical aberration induced bloating of the PSF.

Excitation Beam Overfill Factor

In confocal microscopes, the entry pupil of the microscope objective is illuminated by a laser beam. Usually, laser beams have a Gaussian intensity profile[3]. As a result, the illumination intensity is not constant over the pupil but will decrease towards the edges. Lower edge intensities will lower the effective NA and therefore negatively affect resolution. In most confocal microscopes this is remedied by using a beam width which is significantly larger than the entry pupil, at the cost of loss of excitation power. The ratio between the beam width and the pupil diameter is the excitation beam overfill factor (See Figure 7.5) and is typically in the range from 2 to 4.

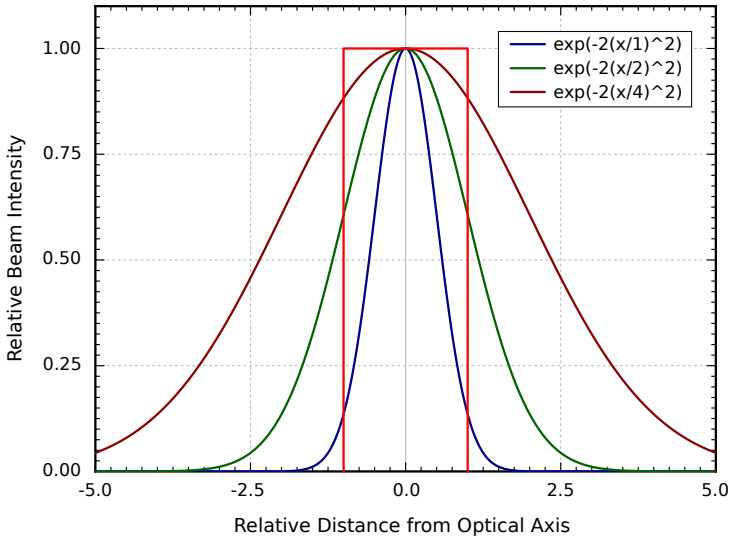


FIGURE 7.5. Lens entry pupil (*red*), beam profile with overfill factor 1 (*blue*), 2 (*green*), and 4(*dark red*). At overfill factor 1, the beam intensity is 14 % of the maximum, at overfill factor 2 the edge intensity is 61 % of the maximum.

3. http://en.wikipedia.org/wiki/Gaussian_beam

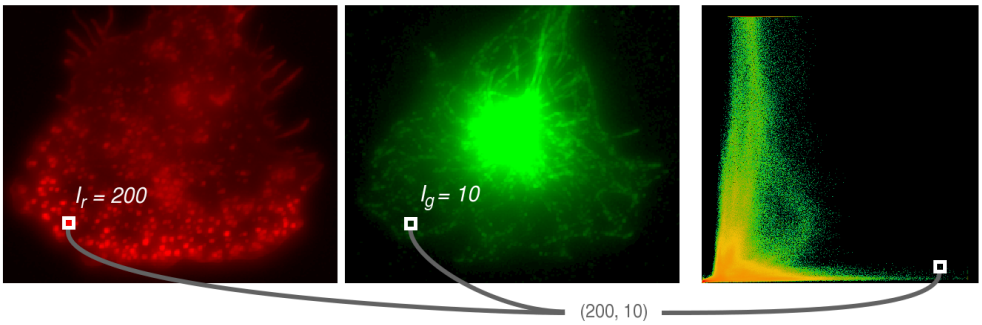


FIGURE 7.6. Example how a 2D histogram is computed, showing the first and second channel and their corresponding 2D histogram. At each position within the channels, the corresponding intensities of both channels are combined to form a coordinate within the 2D histogram. The count at this coordinate of the 2D histogram is then increased by one.

The overfill factor can be set as a microscopic parameter in Huygens Essential, and is taken into account when computing the point spread function.

Brightfield Images

Brightfield imaging is not a *linear imaging* process. In a linear imaging process the image formation can be described as the linear convolution of the object distribution and the point spread function, hence the name deconvolution for the reverse process. So in principle one cannot apply deconvolution based on linear imaging to non linear imaging modes like brightfield and reflection. One could state that the image formation in these cases *is* linear because it is governed by linear superposition of amplitudes. However, microscopes do not measure light amplitudes but rather intensities, i.e. the absolute squared values of the amplitudes. Taking the absolute square destroys all phase information one would need to effectively apply deconvolution. Fortunately, in the brightfield case the detected light is to a significant degree incoherent. Because in that case there are few phase relations the image formation is largely governed by the addition of intensities, especially if one is dealing with a high contrast image.

In practice one goes about deconvolving brightfield images by inverting them (using TOOLS→INVERT IMAGE) and processing them further as incoherent fluorescence widefield images. The Tikhonov Miller algorithm was proven to work excellently for brightfield data. This algorithm is available in the Huygens Professional only. With the MLE algorithm one should watch out sharply for interference like patterns (periodic rings and fringes around objects) in the measured image. As a rule these become pronounced in low contrast images. After the deconvolution run a reverse to the original contrast setting is possible.

2D Histogram

In image processing a 2D histogram shows the relationship of intensities between two images. The 2D histogram is mostly used to compare 2 channels, where the x-axis represent the intensities of the first channel and the y-axis the intensities of the second channel.

As a comparison, a 1D histogram is nothing more than counting how many voxels with a particular intensity occur in the image. The intensity range of the image is divided in bins. A voxel then belongs to the bin if its intensity is included within the range the bin represents.

The 2D histogram is the same as the 1D histogram with the difference that it counts the occurence of *combinations* of intensities. To compute a 2D histogram the images need to be equal in size. See the example in Figure 7.6; at position (30,20) the first channel has an intensity of 200 and the second image has an intensity of 10. Then this will add one to the count in the 2D histogram at position (200,10).

The difficulty with a 2D histogram is how to show the actual count per intensity combination. For a 1D histogram the height of the bars represent the count, but this height for a 2D histogram requires a third dimension which is difficult to visualize. Instead, to visualize the count of the combinations, colors are used. In the histogram in Figure 7.6 colors range from green to red. Note that these colors have nothing to do with the colors used to represent the channels. Within the 2D histogram, green represents low counts while red represent high counts.

2D histograms show interesting image properties and are therefore very useful:

- Offset
- Intensity factor
- Colocalization
- Clipping
- Crosstalk
- Hot pixels

More information and examples are in the SVI wiki[4].

4. http://www.svi.nl/TwoChannelHistogram.

CHAPTER 8

Support and Contact Information

Contact Information

Addresses and Phone Numbers

Mailing Address	Scientific Volume Imaging B.V. Laapersveld 63 1213 VB Hilversum The Netherlands
Phone	+31 35 6421626
Fax	+31 35 6837971
E-mail	`info@svi.nl`
URL	`http://www.svi.nl/`

We are directly reachable by phone during office hours (CET) or by e-mail 24/7.

Distributors

An up-to-date list of distributors can be found on our web site[1].

Support

SVI Support Wiki

The SVI Wiki[2] is a rapidly expanding public knowledge resource on 3D microscopy and deconvolution. Based on the WikiWikiWeb principle, it is open to contributions from every visitor. In addition it serves as a support medium for SVI customers and relations to discuss different aspects of the Huygens software.

This is a list of useful starting points in the SVI Wiki to learn more about the Huygens software and microscopical imaging in general:

- Information on the parameters describing the imaging conditions (sampling, numerical aperture, pinholes, etc.):
  `http://www.svi.nl/MicroscopicParameters`

1. `http://www.svi.nl/distributors/`
2. `http://www.svi.nl/`

- Information on the restoration parameters (signal to noise ratio, background, quality criteria, etc.) used by the deconvolution algorithms:
  `http://www.svi.nl/RestorationParameters`
- A step by step example on how to tune these parameters to achieve the desired restoration results:
  `http://www.svi.nl/DeconvolutionProcedure`
- Important issues regarding image acquisition and restoration (sampling, clipping, etc.):
  `http://www.svi.nl/ImportantFactors`
- Typical acquisition pitfalls (spherical aberration, undersampling, bleaching, etc.):
  `http://www.svi.nl/AcquisitionPitfalls`
- Information on recording beads to measure a PSF:
  `http://www.svi.nl/RecordingBeads`
- Tutorials and detailed information on using the different aspects of the Huygens software (restoration, visualization, analysis, programming, etc.):
  `http://www.svi.nl/Tutorials`
- Uploading images to SVI:
  `http://www.svi.nl/SendImagesToSvi`

License String Details

Detailed information about the installed license strings can be displayed via HELP→LICENSE. Select the license string of interest and click EXPLAIN LICENSE.

A Huygens license string consists of a set of substrings separated by dashes (-). These substrings describe e.g. the product, version number, options, etc. The checksum at the end of the string should match with all other substrings. A complete string looks like this:

```
HuEss-14.10-wcnp-d-tvAC-emnps-eom2014Dec31-
e7b7c623393d708e-{user@domain.com}-4fce0dbe86e8ca4344dd
```

Table 8.1 lists the building blocks from which this string is composed.

TABLE 8.1. The building blocks of the Huygens license string.

Substring	Description
Product	The product to which the license string applies. This can be `HuEss`, `HuPro`, `HuScript`, and `HuCore`.
Version	The version number of the product.
Microscope types	This substring consists of one or more characters representing the microscope types for which the deconvolution is enabled. These are '`w`' (widefield), '`c`' (confocal), '`n`' (Spinning disk), '`p`' (multi-photon), '`s`' (STED), '`S`' (STED 3X) and '`4`' (4-Pi experimental microscopes).
Server flag	Determines the number of cores that are enabled for multi-threading. A hyper-threaded core is counted as a single core. It can be '`d`' (desktop; 2 cores), '`s`' (small server; 4 cores), '`m`' (medium server; 8 cores), '`l` (larger server; 16 cores), and '`x`' (extreme server; 512 cores).
Option flags	This is a set of characters that list the enabled optional modules. An overview of these modules is given in Table 8.2.

TABLE 8.1. The building blocks of the Huygens license string.

Substring	Description
Locking policy	A set of characters that indicate to which properties the license is locked. These can be 'd' (expiry date), 'e' (e-mail address), 'm' (system ID), 'n' (number of cores), 'p' (processor type), and 's' (processor details).
Expiry date	The date on which the license or maintenance ends. When this substring starts with 'eom', then only the maintenance expires; the license remains valid.
System ID	A 16 character hexadecimal string containing hardware identification numbers.
E-mail address	The customer e-mail address.
Checksum	A 20 character hexadecimal checksum on the previous substrings.

TABLE 8.2. Identifiers for the optional modules.

Character	Module
a	Object Analyzer
A	Advanced Object Analyzer
b	Small file reader bundle
B	Complete file reader bundle
C	Colocalization Analyzer
f	PSF Distiller
L	Leica LAS AF - Huygens data exchange
M	Movie Maker
N	Enable new, pre-released features
s	Object Stabilizer
t	Time Series
v	Surface Renderer visualization
x	Chromatic Shift Corrector
c	RBNCC option for colocalization
T	Object Tracker
u	Crosstalk Corrector

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