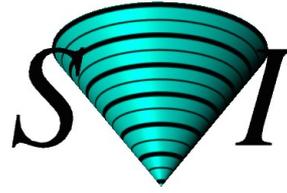


Huygens

Deconvolution Workshop guide



Scientific Volume Imaging



Huygens[®] *Workshop Guide*

Version 3.7

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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[®]. Macrophage fluorescently stained for tubulin (yellow/green), actin (red) and the nucleus (DAPI, blue). Left part: original data; right part: as deconvolved with the classical Maximum Likelihood Estimation method (MLE).

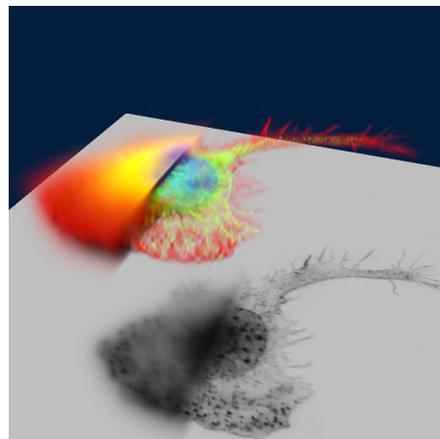


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1. The image restoration process

The processing stages in the guided part

Huygens Essential guides you through the process of microscopic image deconvolution (also referred to as 'restoration') in several stages. Each stage is composed of one or more tasks. While proceeding, each stage is briefly described in the bottom-left *Task Info* window pane. The stage progress is indicated at the right side of the status bar. Additional information can be found in the **HELP > ONLINE HELP** as well as by clicking on the highlighted help questions.

The following steps and stages are to be followed:

- Opening an image. A demo image (the `faba64.ics/ids` file pair) is placed in the `images` subdirectory of `svi`.
- stage P: The Preprocessing stage: loading an image, if necessary converting data sets, parameter check and cropping.
- stage 1: Tune the parameters. This stage will be skipped if you went through the preprocessing stage first. In that stage you have already checked the parameter settings for the intelligent cropper - intelligent since it uses a-priori knowledge for setting the optimal cropping boundaries automatically. If you wish to fine-tune your parameters for the best deconvolution result, -in particular when you like to set your parameters slightly different when using multi-channel images (see page 17)- you will enter this stage again from the last stage if clicking the <<RESTART button.
- stage 2: Inspecting the image histogram.
- stage 3: Background estimation.
- stage 4: The deconvolution run.
- saving the result.

The different stages will be explained below.

Loading an image

Select **OPEN** from the **FILE** menu to enter the file browser and move to the directory where your images are stored. Select the image to be deconvolved, e.g. the `faba64.ics/ids` file pair in the `images` subdirectory of `svi`.

Most file formats from microscope vendors are supported, but some of them require a special option in the license to be read. For updated information see <http://support.svi.nl/wiki/FileFormats>

When the file is read successfully you can either press **START DECONVOLUTION** to begin processing your image or you can convert your data set with the **TOOLS** button.

If you have loaded a bead image you also can proceed selecting **START PSF DISTILLER** and proceed with generating a Point Spread Function (PSF, see page 14) from measured beads (see page 27).

You can **OPEN ADDITIONAL** images for reference purposes, but only the one named 'original' will be deconvolved during the guided restoration.

Preprocessing

Converting a data set (optional)

Before you press the `START DECONVOLUTION` button you can convert a 3D stack into time series images (Convert XYZ to XYZT) or vice versa, or you can convert a 3D stack into a time series of 2D images (XYZ to XYT) or vice versa. These functions can be found in the `TOOLS`.

Time series

A time series is a sequence of images recorded along time at uniform time intervals. Every recorded image is a time frame. The Huygens Essential is capable of automatic deconvolution of 2D-time or 3D-time data. There are some tools that are intended only for time series, as the confocal bleaching corrector or the z-drift corrector (page 16).

Adapting the image

In the `TOOLS` menu you can find a contrast inverter helpful for the processing of brightfield images, see below.

A `CROP` tool is also available, but its use is recommended only after properly tuning the image parameters and will be explained in a later stage.

In the `TOOLS` menu you can also find a `MIRROR ALONG Z` tool, to flip the image when the *coverslip is on top*. This is specially important in case of a refractive index mismatch.

Processing 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 (PSF, see page 14), 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 say 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, 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. Still, 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 you may reverse to the original contrast setting.

Setting the image channel colors

The Color picker tool (TOOLS > PREFERENCES > EDIT GLOBAL COLORS) allows you to alter the colors for the different image channels (see *Multi channel images* on page 17). The color of a particular channel can be edited by clicking the corresponding button.

Image statistics

Right-mouse-click on a thumbnail image and select SHOW PARAMETERS and you will find, 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.

Verifying the microscopic parameters

Next to the basic voxel data the Huygens Essential also tries to read as much information as possible about the microscopic recording conditions. However, depending on the file type this information may be incomplete or incorrect. In this first stage all parameters relevant for deconvolution are displayed and can be modified:

Optical parameters (first page):

- Microscope type
- Lens and medium refractive index
- Numerical Aperture (NA)

Optical parameters (second page):

- Backprojected pinhole radius in nm. 'Backprojected' means the size of the pinhole as it appears in the specimen plane.
- Backprojected distance between the pinholes in microns (only visible if the microscope type is 'Nipkow').
- Excitation and emission wavelengths
- Photon count (number of excitation photons involved in the fluorescence)
- Voxel sizes in the three directions x , y , z (third page)
- Summary of all parameters now in effect

If values are displayed in a **red background**, they are highly suspicious. An **orange background** indicates a non-optimal situation. Oversampling is also indicated with a **cyan background**, that becomes **violet** when it is very severe. Figure 1 shows settings which do not fulfill the criterion for the critical sampling distance versus numerical aperture.

You can see and correct the image parameters not only at this deconvolution stage, but also at any time by right-clicking on the image

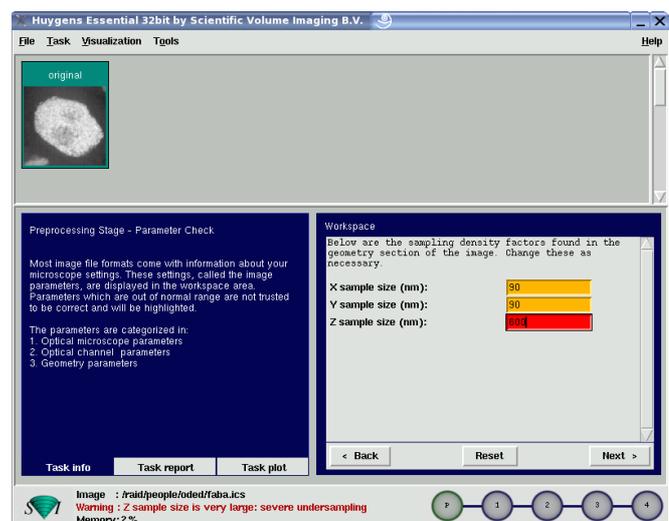


Figure 1. Parameters check stage: Sampling. Red coloring indicates a suspicious value, and orange a non-optimal value.

thumbnail and selecting **SHOW PARAMETERS** or **CORRECT PARAMETERS**. Correcting will pop up the window shown on Figure 3.

Parameters Templates

Once the proper parameters have been set and verified, they can be saved to a Huygens template file (suffix `.hgst`). Those template files are loadable in the very start of the wizard, hence the user can skip the parameters verification stage, provided that an image is to be restored with the same optical properties as the ones which were recorded on the template.

The **IMPORT MICROSCOPIC TEMPLATE** button will allow you to choose a template from a list of pre-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, namely `/usr/local/svi/Templates` on Unix systems, `C:\Program Files\SVI\Templates` on Windows

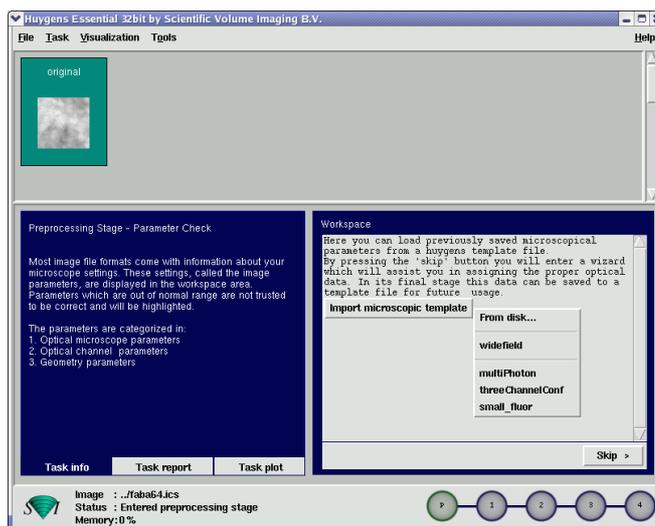


Figure 2. Importing a microscopical parameters template

and `/Applications/SVI/Templates` on the Mac OS X. The user's personal templates directory is called `.svi` on the Unix platforms and `SVI` on Windows, and it can be found in the user's home directory on Unix, and in `C:\Documents and Settings\user_name` on Windows. You can also choose to load a template file from a different location by pressing the **FROM DISK...** option.

The **EXPORT TEMPLATE** button will allow you to either save the template to the personal template directory by choosing the **TO DISK...** option, or overwrite one of the existing templates by selecting them from the list.

The Huygens template is a simple `.xml` file which can be edited 'by hand' as well (see examples in the common **Templates** directory).

The intelligent cropper

The time needed to deconvolve an image increases more than proportional with its volume. Therefore, 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. In computing this initial proposal the microscopical parameters are taken into account, making sure that cropping will not have a negative impact on the deconvolution result. Because the survey depends on accurate microscopical parame-

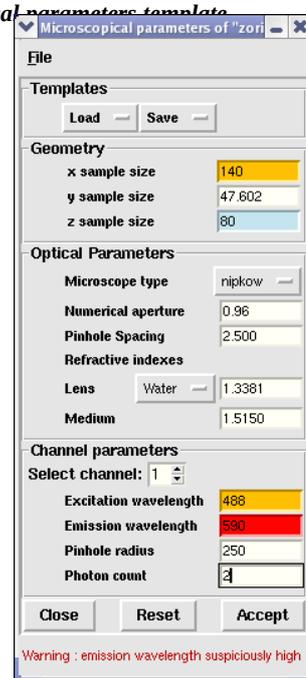


Figure 3. Microscopical parameters corrector.

ters it is recommended to use the intelligent cropper as final step in the preprocessing stage, but you can launch it before the restoration process from the **TOOLS > CROP ORIGINAL IMAGE** menu. Once you have cropped your image during the guided restoration process you can not crop it again except after closing the image and reloading it again.

Cropping an image in x y z

After you have verified your image parameters the Crop tool is launched if you press the **YES** button to the question 'Launch the cropper?'. The cropper will look as in Figure 5 (the image will be in gray scale mode if it is a single channel). Red lines indicate the borders of the proposed cropping region. This is computed from the image content and the microscopic parameters at launch time of the cropper.

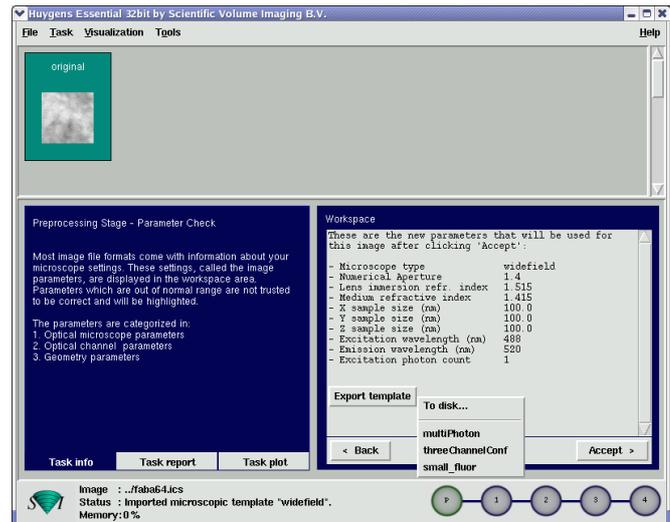


Figure 4. Exporting a microscopical parameters template.

The cropper allows manual adjustment of the proposed crop region. To adjust the crop region put the cursor *inside* the red boundary, press the left mouse button and keep it pressed to sweep out a volume. Accept the new borders by pressing the **CROP** button. Do not crop the object too tightly, because you would remove blur information relevant for deconvolution. Do not crop the image to make it too large along the optical axis Z, an aspect ratio close to 1:1:1 (or less than 1 for Z) is much better.

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.

By default the projections are over the whole dataset (including all the frames in time series), but this might be confusing sometimes. The small colored triangles can be used to restrict the projections within a specific range of slices.

Cropping an image in time

You also can reduce the number of time frames by selecting **TIME > SELECT FRAMES** from the **CROP** menu, as shown in Figure 6. This applies to time series (see *Time series* on page 8).

Removing channels

You can remove channels from a multi-channel image (see p. 17) using the crop tool's channels selecting tool CHANNELS > SELECT CHANNELS.

Stage 1: Parameter tuning

Stage 1 enables you to tune your parameter settings as set in the pre-processing stage. After you have finished cropping, stage 1 is skipped and you will directly jump into the image histogram (stage 2). Still you may wish to tune your parameter settings afterwards. You can enter stage 1 by pressing the RESTART button in the latest stage.

Stage 2: The image histogram

The next stage shows you the image histogram.

The histogram image is an important statistical tool for *inspecting* your image. It is included to let you spot problems that might have occurred during the recording. It has no image manipulation options as such, it just may prevent you from future recording problems.

The histogram shows the number of pixels as a function of the intensity (gray value) or groups of intensities. If your image is an 8-bit image (gray values from 0–255) 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-bits gray values are collected to form a 'bin' (for example gray values from 0–9 form bin 0, values from 10–19 form bin 1, etc.) The histogram is now the number of pixels in every bin.

The histogram from the demo image as shown in Figure 7 is of reasonable quality. The narrow peak you see at the left represents the background pixels, all with similar values. The height of the peak represents the amount of background pixels. Because in this particular image there are many voxels with a value in a narrow range around the background the peak is higher than the other.

In this case there is also a small black gap at the left of the histogram. This indicates an electronic offset (blacklevel) in the signal recording chain of the microscope.

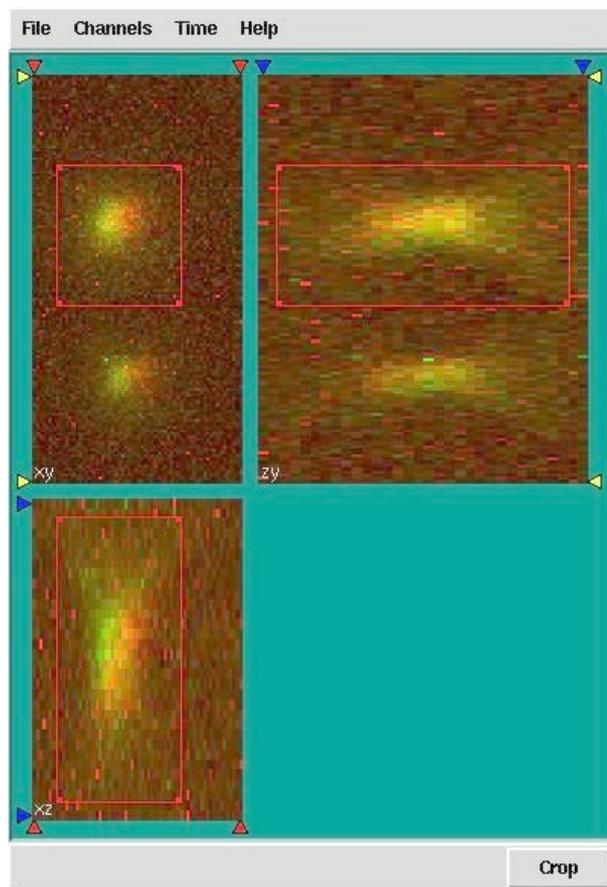


Figure 5. The Crop tool.

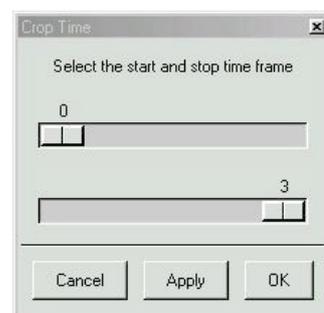


Figure 6. Reducing the number of time frames.

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 your 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 WF images.

The histogram stage is included for examining purpose only. It has no meaning for the deconvolution process that follows¹.

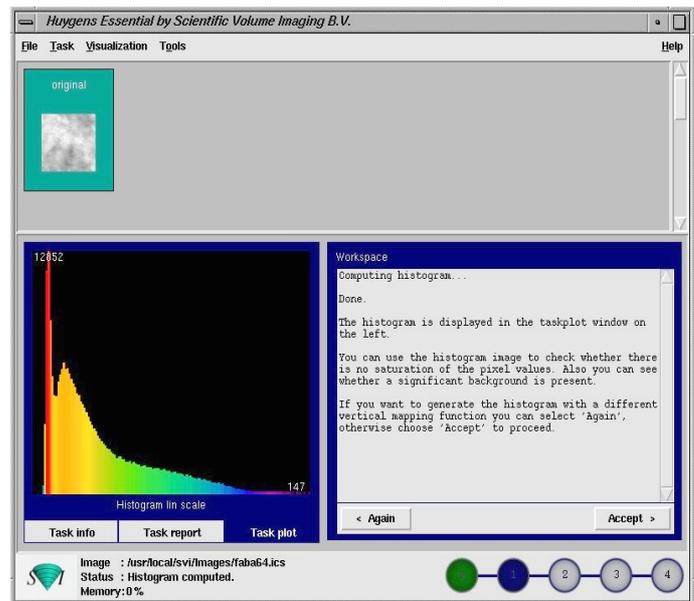


Figure 7. **The image histogram.** The vertical mapping mode can be selected from linear, logarithmic or sigmoid.

Stage 3: Estimate the average background in the image

In this stage the *average* background in a volume image is estimated. The average background is thought to correspond with the noise-free equivalent of the background in the measured (noisy) image. It is determined by searching the image first for a region with low values. Subsequently the value for the background is determined by searching in this region for the area with radius r which has the lowest average value. It is important for the search strategy that the microscopic parameters of the image are correct, in especially the sampling distance and the microscope type.

The following choices are possible here:

- **Lowest value (Default):** The image is searched for a 3D region with the lowest average value. The axial size of the region is around 0.3 micron; the lateral size is controlled by the *radius* parameter which is default set to 0.5 micron.
- **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.

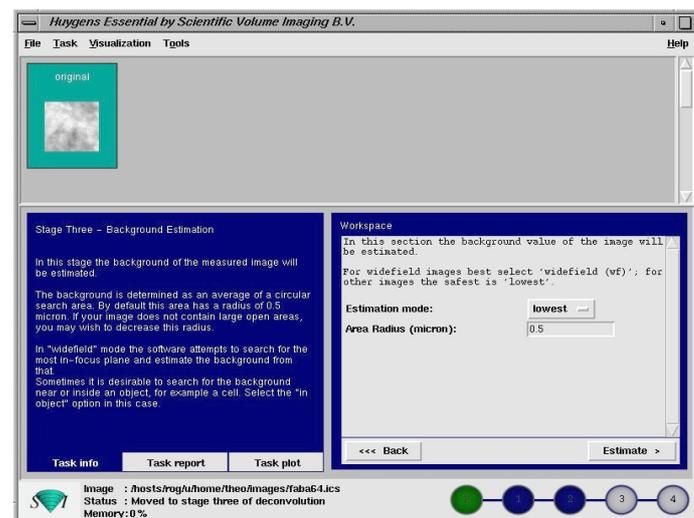


Figure 8. **Estimating the average background.**

¹ Learn more about histograms in <http://support.svi.nl/wiki/ImageHistogram>

- *WF*: 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 **ESTIMATE** button to continue. You may now adapt the value if you like to, either by altering the value in the **ESTIMATE BACKGROUND** field or in the **RELATIVE BACKGROUND** field. Setting this last to -10, for example, lowers the estimated background with 10%. If you are done press **ACCEPT** to proceed to the last stage, the true deconvolution process.

Stage 4: Deconvolution

Before starting the actual iterative deconvolution run, stage 4 first carries out several preprocessing steps:

1. **Background**: This value is calculated in stage 3. You can verify whether this value represents areas in the image which you consider background by opening the **Twin Slicer** (see Error: Reference source not found) and moving the mouse pointer over the areas of interest. The voxel values are displayed below the image. Modify the value as you see fit.
2. A **Point Spread Function (PSF)** (see below) was generated from the established microscopic parameters. This took place off the screen and is fully transparent to the user.
3. If the size of the computer's RAM is too small to deconvolve the image as a whole, it is split up in parts called 'bricks'. SVI's **Fast Classic Maximum Likelihood Estimation (MLE)** algorithm runs on the image or on all the bricks and fits the deconvolved bricks seamlessly together.

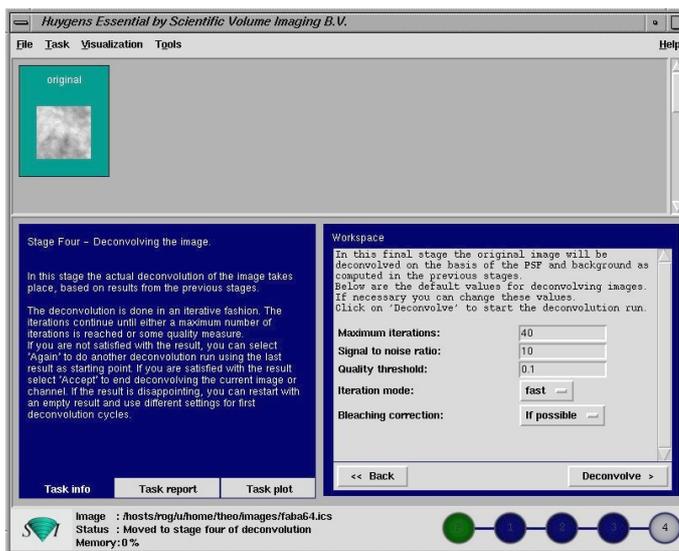


Figure 9. Stage 4: starting the deconvolution.

The Point Spread Function (PSF)

One of the basic concepts in image deconvolution is the Point Spread Function. The PSF of your microscope is the image which results from imaging a point object in the microscope. Due to wave diffraction a point object is imaged, 'spread out', into a fuzzy spot: the PSF. In fluorescence imaging the PSF completely determines the image formation. In other words: all microscopic imaging properties are packed into this 3D function. The 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 that come with your image and which you have double-checked in the preprocessing 'P' stage or in stage one. 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 (S.A.) due to a refractive index mismatch are better restored through the use of theoretical depth-dependent PSF's..

2. *Measuring a PSF*: By using the PSF Distiller or the tools in Huygens Professional you can derive a measured PSF from images of small (< 200 nm) fluorescent beads. You can load a previously measured PSF with FILE > OPEN PSF... (Main menu). If you load a PSF, Huygens Essential will automatically use it. If the measured PSF contains less channels than the image, a theoretical PSF will be generated for the channels where there is no PSF available.

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 as the bead image(s).

The Classical Maximum Likelihood Estimation (CMLE) algorithm

Huygens Essential uses the Classical Maximum Likelihood Estimation for the deconvolution process. This method is an extremely versatile algorithm, applicable for all types of data sets².

The following option values may be set:

Number of iterations

MLE-based deconvolution uses an iterative process that never stops if no stopping criterion is given. This stopping criterion can simply be a maximum number at which the process will stop. This value depends on the desired final quality of your image. For an initial run you can leave the value at its default. To achieve the best result you can increase this value.

Another stopping criterion is one based on the Quality change of the process, see *Quality threshold* below.

Signal to noise ratio

You have to make an estimation of the SNR from your recorded image. Inspect your image and decide if your image is noisy (SNR < 10), has moderate noise (10 < SNR < 20) or is a low-noise image (SNR > 20). Panels for estimating the SNR can be found on <http://support.svi.nl/wiki/SNR>.

Quality threshold

Beyond a certain amount of iterations, typically below 100, the difference between successive iterations becomes insignificant and progress grinds to a halt. Therefore it is a good idea to monitor progress with a quality measure, and to stop iterations when the change in quality drops below a threshold. At a high setting of this quality threshold (e.g. 0.1) the quality difference between subsequent iterations may drop below the threshold before the indicated maximum number of iterations has been completed. The smaller the threshold the larger the number of iterations which are completed and the higher the quality of restoration. Still, the extra quality gain becomes very small at higher iteration counts.

² Huygens Professional also has Quick-MLE-time, Quick-Tikhonov-Miller, and Iterative Constrained Tikhonov-Miller algorithms available.

Iteration mode

In **FAST MODE** (highly recommended) the iteration steps are bigger than in **HIGH QUALITY** mode. More information can be read in the **DICTIONARY** from the **HELP** menu.

Bleaching correction

The data is inspected for bleaching. 3D images and time series of WF 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.

Stopping the MLE algorithm

Pressing **RESTORE** starts the iterative MLE algorithm; a **STOP** button appears. Pressing **STOP** halts the iterations and retrieves the result from the previous iteration. If the first iteration is not yet complete a empty image will result.

Finishing or restarting a deconvolution run

When a deconvolution run is finished use the **Twin Slicer** to inspect the result in detail. Depending on the outcome of that you can choose **AGAIN**, **RESUME** or **ACCEPT**:

AGAIN discards the present result, and re-runs the deconvolution, possibly with different parameters.

RESUME re-runs the MLE procedure without discarding the result, and with the possibility to change the deconvolution parameters. The software will ask you to continue where you left off (keeping improving the image, quite recommendable) or to start from the raw image again. A new result will be generated to compare with the previous one, for instance using the **Twin Slicer**. You can repeat this several times.

ACCEPT proceeds to the final stage or, if the data was multi-channel, to the next channel (see page 17). If you generated several results by resuming the deconvolution you will be asked to select the best result as the final one, that will be renamed to 'deconvolved'. The other results will remain as well in case you want to save them.

z-drift corrector for time series

For 3D time series the program pops-up an additional tool that enables you to correct for movement in the z (axial) direction that could have been occurred for instance by thermal drift of the microscope table. In case of a multi channel image (see p. 17), 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 can be filtered with a **MEDIAN**, **GAUSSIAN** or **KUWAHARA** filter of variable width. When 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 edge preserving Kuwahara filter.

Saving the result

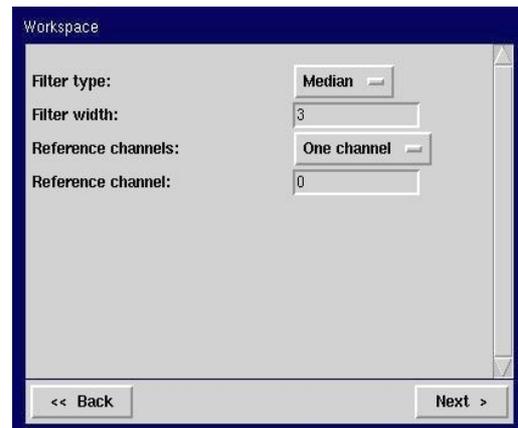


Figure 10. The z-drift corrector.

Saving the restored image

After each deconvolution run you can save the result. Select the image to be saved and do **FILE > SAVE 'IMAGE NAME' AS...** in the menu bar. You can save the image as an Image Cytometry Standard (ICS or ICS2) image file, a TIFF file series, an Open Microscopy Environment-XML (OME) file, an Imaris-Classic file or a Biorad .pic file. Only the ICS and OME file type save all the microscopic parameters. For information on how to proceed with multi-channel data see *Joining the results* on page 18.

The ICS file format actually uses two separate files: a header file with .ics extension and other, much bigger and with the actual image data, with .ids extension. On the other hand the newer ICS2 file format uses only one single .ics file with both the header and the data together.

Saving your Task report

Select from the Main menu bar **TASK > SAVE TASK REPORT** to store the information as displayed in your *Task report* tab deck.

Multi channel images

Fluorescent microscopes can usually register different emission wavelengths (almost) simultaneously, allowing you to image different dyes on the sample. In the terminology of the Huygens Software, one channel in an image refers to the intensity distribution recorded at a given fixed wavelength, independently of what device made the acquisition. Thus, it is a logical channel of stored data, and not necessarily a physical channel (as all the image channels could have been measured by a single photomultiplier, for instance).

Multi channel images can be deconvolved in a semi automatic fashion, giving you 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 'channel-0', 'channel-1', and so on. The first of these is automatically selected for deconvolution. To deconvolve it, proceed as follows:

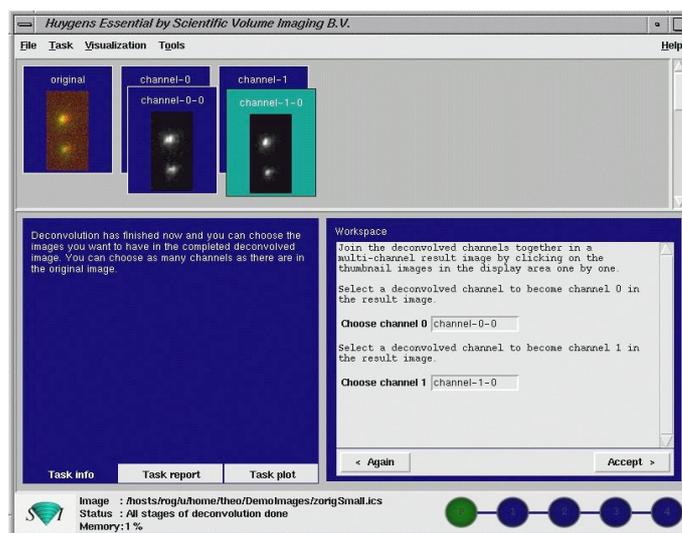


Figure 11. Deconvolving a two channel image.

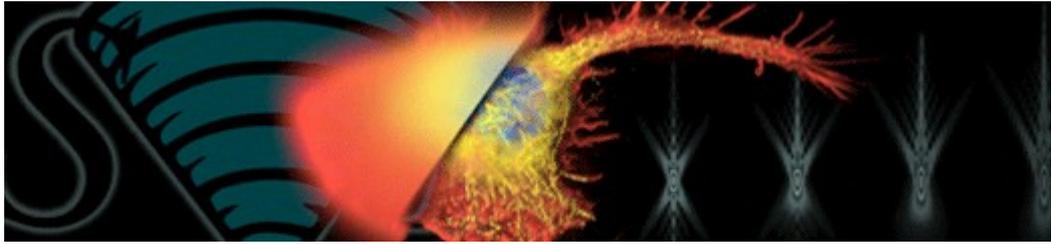
Deconvolving a channel in a multi channel image

The procedure to deconvolve a channel in a multi channel data set is exactly the same as for a single channel image. You can therefore do multiple reruns on the channel at hand, just as you can with single channel data. When you are done press **ACCEPT** in the last (stage 4) screen. This will cause the next channel to be selected for restoration. Proceed as usual with that channel and the remaining channels. If you do not want to process all the channels in an image you may skip one or more channels.

Joining the results

When you press **ACCEPT** for the last channel you enter a screen which allows you to select the results which you want to combine into the final deconvolved multi channel image. This means that up to this point you can still change your mind as to which of the results you want to combine, even in what order. Once you press **ACCEPT** a multi channel image named *Restored* is created. To save it go to the **FILE > SAVE 'RESTORED' AS** menu.

2. 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).

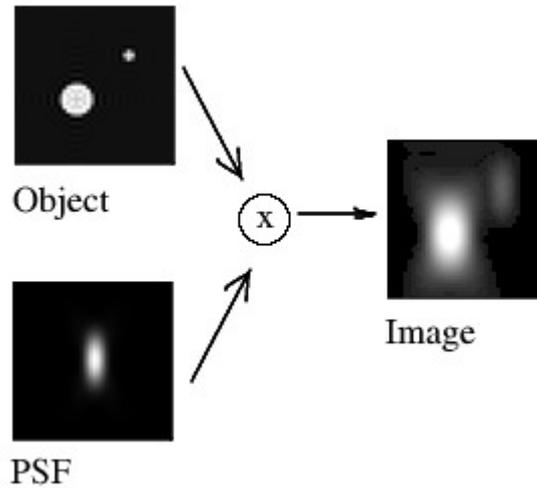
Image Formation

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

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 this:



(Fig. 1)

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

$$g = f * h \quad (\text{Eq. 1})$$

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:

$$g = f * h = \iiint_{-\infty}^{\infty} f(\vec{x}) h(\vec{x} - \vec{x}') d^3\vec{x}' \quad (\text{Eq. 2})$$

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 contributions of all points of the object f , their real intensities weighted by 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 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 cost is of the order of 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:

$$G = F \cdot H . \quad (\text{Eq. 3})$$

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.

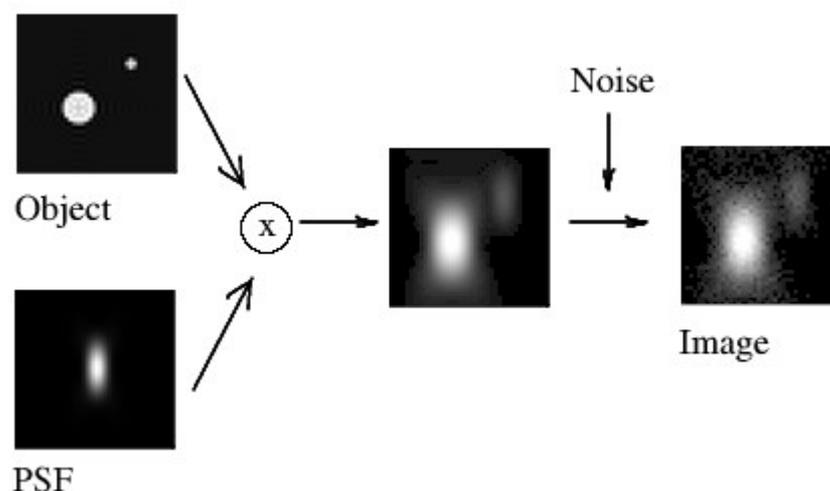
Deconvolution

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 abovementioned Eq. 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 Eq. 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 Eq. 1, but this:

$$k = f * h + \epsilon \quad (\text{Eq. 4})$$



(Fig. 2)

where the acquired image k arises from the convolution of the real light sources 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 subtract ϵ from k , as we can not know what is the exact noise distribution).

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.

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' which its convolution with the bead model matches the measured bead image. That can be understood looking back at figure 1 and equation 1. 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 Maximum Likelihood Estimation 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.

Lastly, an advantage of theoretical or measured PSFs is that they facilitate construction of very fast algorithms like the QMLE in Huygens Professional or the New Batch Processor Tutorial*. 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 Parameter Variation).

Huygens algorithms generally do Intensity Preservation.

See the Huygens restoration applied to some accessible images in Convolving Trains.

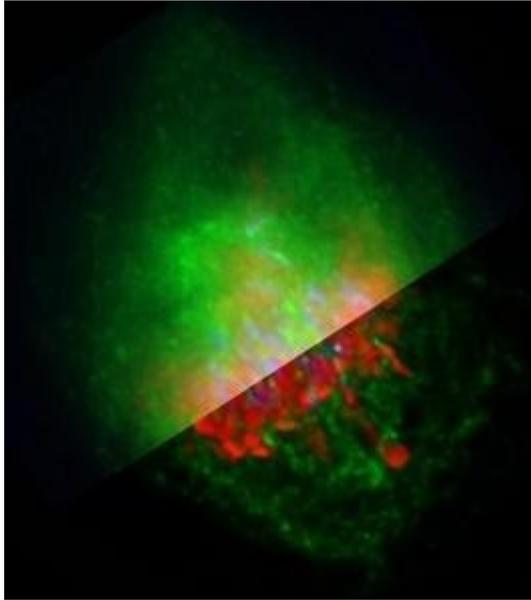
Validation

The CMLE method used in Huygens is backed up by quite some scientific literature. We mention here just three relevant examples (follow the previous link for a longer list):

- 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 (get pdf).
- 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 (get pdf).
- 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 (get pdf).

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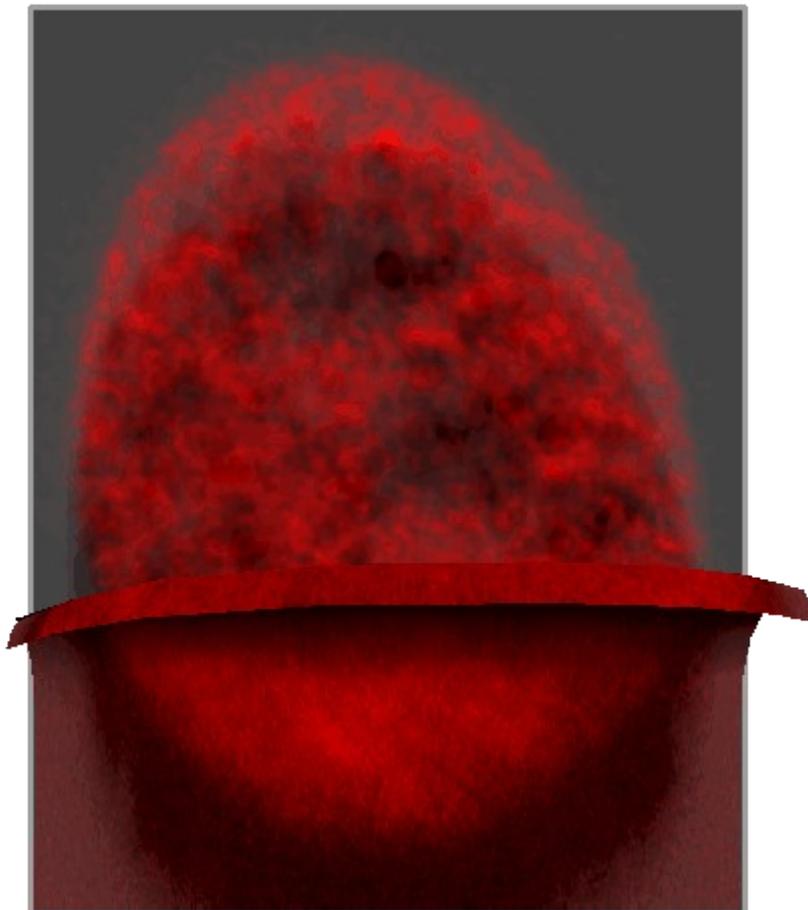
Examples



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.



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

You can also find other images in Resolution Improvement and in Evans Macrophage.

There is a very simple and clarifying example of a deconvolution done with the Huygens Software in Decon Example. For more accessible examples see Convolving Trains. Real microscopy images can be seen in the SVI web page, at <http://www.svi.nl/gallery/>

Further reading

See Doing Deconvolution for special topics and references.

Also see the list of Useful Links in the *Appendix*.

3. Data acquisition pitfalls

This article summarizes some typical experimental difficulties when acquiring microscopic images.

Refractive index mismatch

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: Under-Sampling) 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 UnderSampling). 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-position, 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.

4. Distilling a PSF from bead measurements

This post describes how to obtain a Point Spread Function (PSF) out of the recorded images of test beads (Recording Beads).

Procedure outline

Briefly, the procedure has the following sequence:

1. Prepare a specimen containing fluorescent latex beads.
2. Record a number of images at the microscope settings you also use when recording the images you want to restore.
3. Align and average the individual bead images from single or multiple files.
4. Reconstruct the PSF from the averaged bead image.
5. Store the resulting PSF in ICS image format for later usage.

This post describes steps 3-5 of the procedure. To see steps 1 and 2, go to Recording Beads.

Averaging bead images

Huygens Essential

Huygens Essential has a Psf Distiller tool that enables you to generate a PSF from one or a series of bead recordings in one single procedure. You need to have the 'Distiller license'.

Huygens Pro

If you use the Huygens Professional, at this stage we assume that you have recorded a number of bead images.

1. Inspect and load all recorded bead images. In case of images containing few beads you can use the Copy part image or Add/Remove borders tool to isolate the beads in smaller images. Do not make such images smaller than 128x128x32, and keep the beads roughly centred.
2. Select the Av. spheres in an Operation window on a bead image. Change the parameters in the menu if necessary. The function will first do a survey of the image to locate suitable beads. It will report how many beads are found and how many are rejected. Beads which pass the first survey are aligned and accumulated in the 'Destination' image.
3. Repeat step 2 for all bead images, accumulating in the same Destination image.
4. Save the Destination image to disk, for example as 'beadAv'.

After accumulating 5-10 beads you should see a noise free, very smooth bead image in the Destination image.

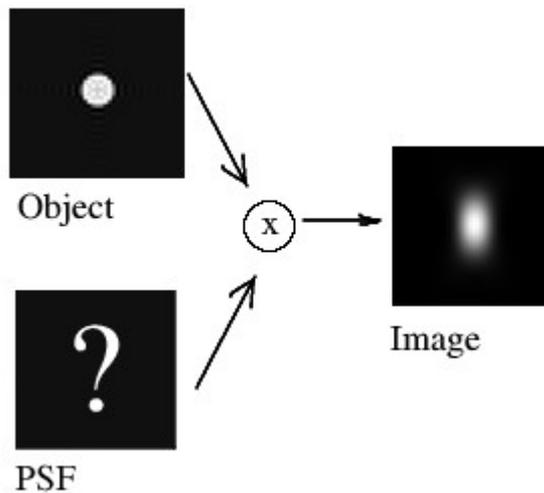
You can remove the Background in the averaged bead image by switching on the Final baseline adjust button in the last run of the Average spheres function.

This procedure applies only to single channel images. If you have a multicolor bead image with many channels, you will have to split it first into single channel images (with Edit > Split image, in the main menu). Then you can average spheres for each channel independently.

Reconstructing a PSF from an averaged bead image

If you use Huygens Essential you have already reconstructed the PSF in the previous step, but this is what is done:

The Reconstruct PSF function corrects for the broadening effect by the finite (non Sub Resolution) bead to derive the PSF. It is based on the following idea: The bead image is the result of a Convolution of the unknown PSF with the known object model. This problem is the same as a `normal' → DeConvolution problem, but with the roles of PSF and object reversed. Therefore, a `normal' deconvolution procedure can be used to recover the PSF from a bead image. Fortunately, we have in this case even some more a priori information since the Microscope Type (confocal, widefield) imposes constrictions on the shape of the PSF. The Huygens Software uses these constraints, and for this reason you have to specify some Microscopic Parameters in the Reconstruct PSF (R.PSF) function.



Storing the measured PSF

It is important to store the PSF in ICS file format. The reason is that the Huygens Software generates a normalized PSF in floating point format. Normalized means that the sum of all voxel is unity. The Save as ICS function automatically saves an image composed of float numbers in floating point format.

Appendix

Addresses and URLs

Where can we be reached?

Scientific Volume Imaging B.V.

Laapersveld 63
1213 VB Hilversum
The Netherlands

You can call us directly by phone:

+31 35 6421626 or +31 653 345445,
or fax us at: +31 35 6837971,
or email us at: info@svi.nl

<http://www.svi.nl>

Distributors

A list of distributors can be found on our web site:

<http://www.svi.nl/company/distributors.php>

On-line support

SVI[®] support server

It is good to know that an extensive support base is available where you may find answers to questions that come to front while reading this document. All our knowledge base can be found on-line, on our support server at

<http://support.svi.nl>

The recommended starting point for obtaining support is
<http://support.svi.nl/wiki/SviSupport>

The SVI-wiki

The SVI-wiki 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 addi-

tion it serves as a support medium for SVI® customers and relations to discuss different aspects of SVI's Huygens Software. The SVI-wiki can be found in

<http://support.svi.nl/wiki>

Starting points

Here you can find a selection of good starting points in the SVI-wiki to learn more about the Huygens Software and microscopical imaging:

- On the parameters describing your **imaging conditions** (sampling, numerical aperture, pinholes...) see <http://support.svi.nl/wiki/MicroscopicParameters>
- On the **restoration parameters** (signal to noise ratio, backgrounds, quality criteria...) used by the deconvolution algorithms, see <http://support.svi.nl/wiki/RestorationParameters>
- A step by step example on how to **tune these parameters** to achieve the desired restoration results: <http://support.svi.nl/wiki/DeconvolutionProcedure>
- Very **important factors** (sampling, clipping...) on image acquisition and restoration: <http://support.svi.nl/wiki/ImportantFactors>
- Typical **acquisition pitfalls** (spherical aberration, undersampling, bleaching...) explained: <http://support.svi.nl/wiki/AcquisitionPitfalls>
- On recording beads to **measure a PSF**: <http://support.svi.nl/wiki/RecordingBeads>
- **Tutorials** and detailed information on using the different software **tools** (restoration, visualization, analysis, programming...) can be found in <http://support.svi.nl/wiki/Tutorials>
- If you are **sending your images** to SVI® to get further assistance, please follow the instructions detailed in <http://support.svi.nl/wiki/SendImagesToSvi>.
- Do you want to **edit the wiki**, contributing to enhance its contents? Read <http://support.svi.nl/wiki/HowToUseWiki>
- With the **search tools** you can find much more information on the topic you want: <http://support.svi.nl/wiki/FindPage>