HUYGENS PROFESSIONAL APLICATION GUIDE

[S.HÄRTEL 01/01/04]
[HUYGENS PROFESSIONAL IS VALID 24/09/03 + 30 DAYS]

Microscope Type :	Widefield / Confocal / Nipkov Disc
Objective Lens Numerical Aperture :	10 X: 0.3 20 X: 0.5 40 X: 1.3 [Oil] 63 X: 1.4 [Oil] 63 X: 1.2 [Water]
Lens Medium Refractive Index : The Numerical Aperture of an objective is the refractive index of the immersion medium times the sine of the half-aperture angle. Because the sine <= 1.0, the NA is always lower than the medium refractive index. If a 1.4 NA oil immersion lens is used to image into a watery object total internal reflection will truncate the NA. As a rule of thumb the effective NA is .1 lower than the manufacturer's specification.	1.518 [Zeiss Oil N518] 1.33 [Water] 1.0008 [Air]
Medium Refractive Index : This is the refractive index of the medium in which the specimen is embedded. For a frequently used medium such as glycerol (75%) this is 1.44. For low values of Rm such as 1.32 for water, the computed PSF for an oil immersion lens is only valid near the coverslip-medium interface due to spherical aberration effects in deeper layers. In such cases we recommend lenses optimized for the refractive index of the medium.	1.32 [Water] 1.44 [Glycerol 75%] 1.0008 [Air] 1 [Air]
Excitation & Emission Wavelength[nm]:	See experimental conditions
Pinhole radius [nm]: Huygens works with the **backprojected pinhole radius (r_b):** **r_{phys} :** physicel pinhole diameter (LSM image information) **m_{obj} :** objective magnification factor (10, 20, 40 or 63X) **m_{sys} :** fixed internal magnification (2 for Pascal in CECS) **NA :** numerical aperture	$r_b = r_{phys} / (m_{obj} * m_{sys})$ $r_b = 0.61 * \lambda * N_{AiryDisc} / NA$
XYZ-Sample Size [µm]:	See experimental conditions

Signal to Noise Ratio (**SNR**):

The SNR can be estimated with the following rules of thumb: Good quality Wide Field images recorded with a cooled CCD camera microscope can easily have S/N ratios above 30. Moderate WF images have an SNR of around 20. Good quality confocal fluorescence images have a S/N ratio between 20 to 40.

In noisy confocal images the SNR can be computed from the pixel value of a single photon event in the background: SNR = (maximumVoxelValue / single Hit-Intensity)$^{0.5}$.

Alternative Method for SNR-estimation:

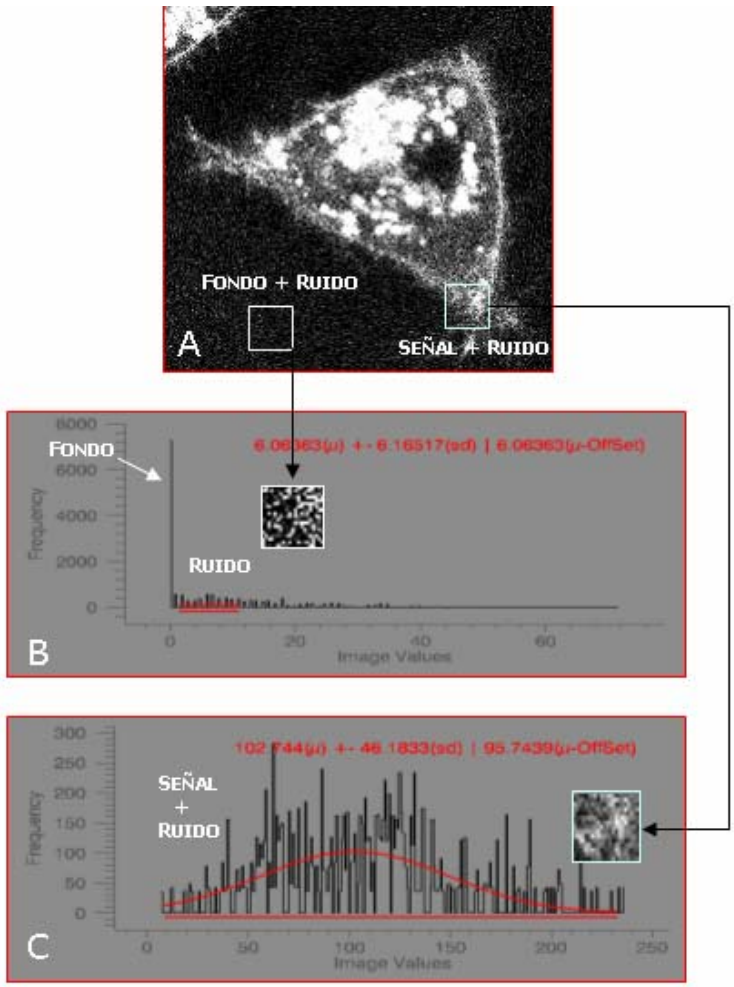
El cuociente entre Señal y Ruido (**SNR**) es un parámetro variable, que requiere el sistema de deconvolución, para poder estimar para cada píxel dentro de la imagen si su contenido corresponde a un evento aleatorio (ruido que obedece a una estadística de Poisson) o una señal real.

SNR se obtiene de la siguiente manera:

1. Obtener una imagen representativa (ver Fig. A).
2. Generar un histograma de frecuencias f en una zona de la imagen que no contiene señal (**f**(**FR**) es una función que depende del fondo (**F**) y del ruido (**R**), ver Fig. A/B).
3. La intensidad del fondo **I**(**F**) (background) de la imagen se define por el valor mínimo del Histograma **f**(**FR**): **I**(**F**) = min(**f**(**FR**)) = 0 en el ejemplo Fig. B).
4. La intensidad del ruido **I**(**R**) obedece a una estadística de Poisson. En una distribución de Poisson, el valor del promedio **μ**(**I**(**R**)) es igual a su varianza σ^2 (**I**(**R**)).
5. El promedio de la intensidad de una señal **μ**(**I**(**S**)) se aproxima con una estadística de Gauss (**μ**(**I**(**S**) = 102, ver Fig. C).
6. Con estos parámetros, la **SNR** se calcula de la siguiente manera:

$$SNR \cong \sqrt{\frac{\{\mu(I(S)) - I(F)\}}{\{\mu(I(R)) - I(F)\}}} \cong 4$$

See pages 13/20 in User Guide and below.



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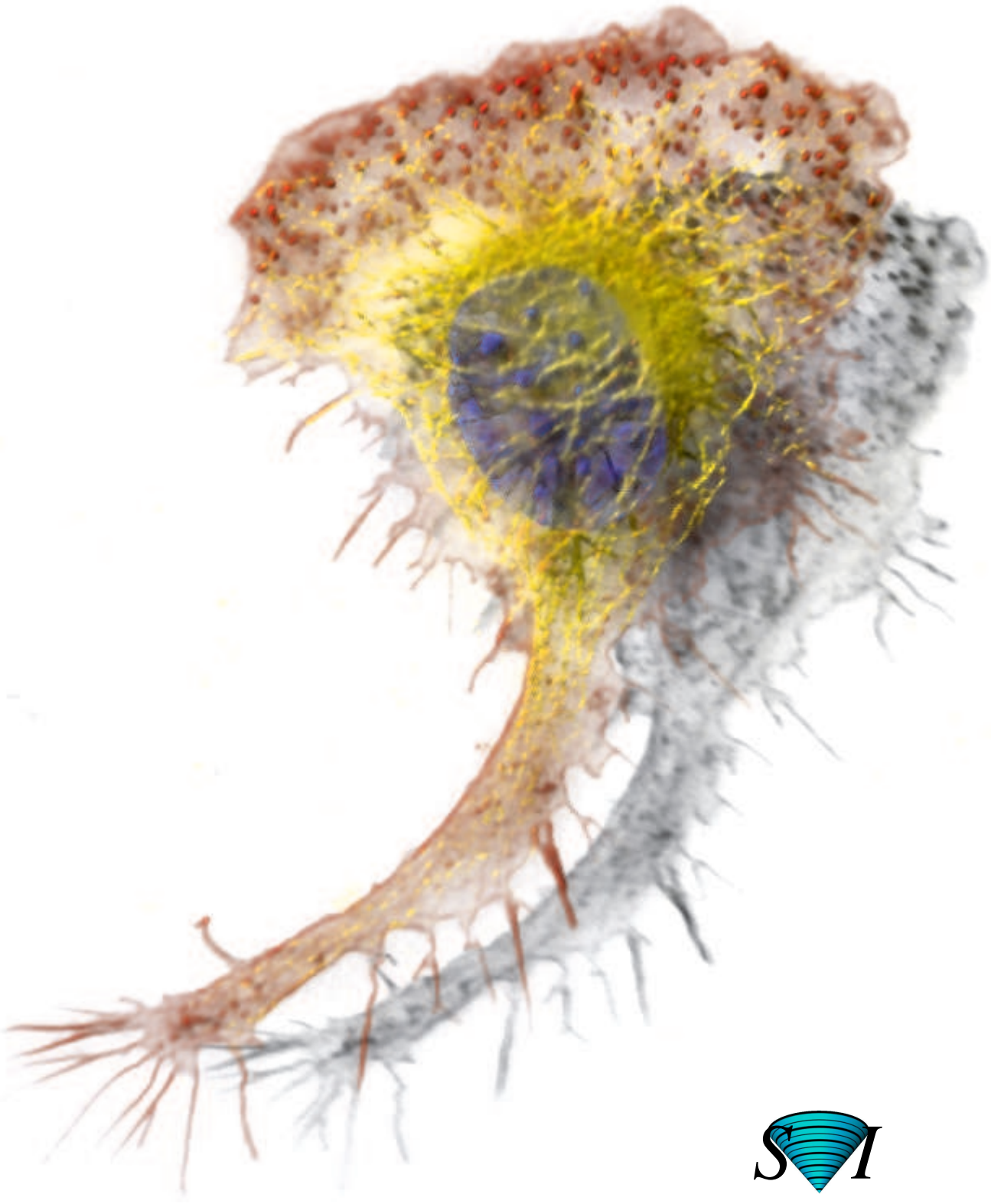


Scientific Volume Imaging

Huygens Professional User Guide

Scientific Volume Imaging b.v.
Alexanderlaan 14
1213 XS Hilversum

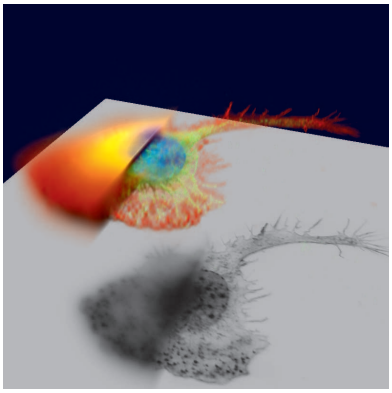
P.O. box 615
1200 AP Hilversum
The Netherlands

Cover illustration:

Macrophage recorded by Dr. James Evans, Whitehead Institute, MIT, Boston MA, USA, using wide field microscopy.

At the right the same dataset: 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 Liklihood Etimation method (MLE).

The image was visualized using FluVR. FluVR is the Simulated Fluorescence Process (SFP) volume rendering package from Scientific Volume Imaging.



CHAPTER 1

Introduction

This guide is useful to the beginner in deconvolution and to the expert that starts using the Huygens Professional toolkit.
For the expert we recommend also to study the Huygens Reference Manual.

contents

In chapter 1 we explain the installation
In chapter 2 you deconvolve your first image
In chapter 3 we help you determining some important parameters and give you hints for basic image data-acquisition

What is Huygens Professional?

Huygens professional is an image processing software package tailored for deconvolution of microscopic images. It enables you to deconvolve a wide variety of images ranging from 2D widefield (WF) images to 4D multi-channel two-photon confocal images or from scanning disk confocal microscopes. Also people who use experimental set-ups like 4p-microscopes may benefit from the software. The Huygens Professional toolbox contains the following features:

Image deconvolution functions

- Accelerated Maximum Likelihood Estimation restoration algorithm optimized for low light level images
- Iterative Constrained Tikhonov-Miller restoration algorithm
- Quick Tikhonov-Miller and Quick MLE restoration algorithms
- Point Spread Function (PSF) measurement tool box to derive a microscopic PSF from finite sized micro bead images, containing:
- Automatic alignment and averaging procedure to combine the signal from different micro beads in one or more images
- PSF reconstruction tool to correct for the finite size of micro beads
- Generates a theoretical Point Spread Function for widefield, confocal and two-photon microscopes based on electromagnetic diffraction theory
- Automatic bleaching correction of 3D and 4D widefield images and 4D confocal and multi-photon images
- Z-drift corrector tool for timeseries 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.

Basic image processing capabilities

- Capability to handle multiple images
- Time series support
- Multi parameter (multi channel) image elements (stacked or packed)
- Basic data types: unsigned byte, 16 bit signed integer, 32 bit float, 2 x 32 bit complex
- Per image undo/redo capabilities
- Scripting and batch processing environment based on Tcl (Tool Command Language)

Core image processing functions

- Create, destroy, copy, copy block, convert, split, join, zoom, rotate, iso-sample, shift, replicate image
- Add/remove border, shift to sub-pixel accuracy, mirror image, swap image octants
- Arithmetic operations on two image operands, one image operand and a scalar, mathematical functions on one image operand, soft clipping & thresholding
- 4D Gaussian filter of arbitrary widths, 4D Laplacian filter
- Generate solid and hollow bandlimited spheres, generate Poisson and Gaussian noise
- Real and complex 4D Fast Fourier transforms

Reporting & display operations

- Image statistics
- Report sampling density with respect to Nyquist rate
- Image histograms of images with up to three channels
- Plots of Energy Flux as function of time and axial position

Image file I/O

- Reads ICS, Imaris® classic, numbered series TIFF, Zeiss Lsm, Metamorph STK, Biorad pic, Olympus 'Fluoview', Leica files and Delta Vsion IMSubs (r3d), IPLab Tiff.
- Writes ICS, Imaris® classic, Biorad pic, TIFF (Leica style and classic numbered series TIFF).
- 4D read support: ICS, numbered 'stk', numbered Leica-TIFF, numbered TIFF
- Export to FluVR, the volume renderer based on spectral fluorescence
- Export to Imaris®

Analysis functions

- Threshold and label 3D image
- Analyze labelled objects: compute centre of mass, volume and integrated intensity
- Estimate background
- Measure distance
- Compute image ratio
- Compute colocalization coefficients
- Compute the co-occurrence matrix of an image

Visualization

- Thumbnail images
- Multiple Expand viewers on one or more images. Each Expand viewer is able to
  - Show x-y, x-z or y-z slices for selectable points in time while optimizing contrast on a global or per-plane basis
  - Show Sum or MIP projection, animate projections of time series
  - Display single channel images in False or True color; multi channel images in True color
  - Report individual pixel/voxel positions and values
- Swing through planes or time
- Slicing positions in expand viewers can be dynamically linked for easy image comparison

Installation and system requirements

Installing the Huygens Professional.

Installing the software

Linux

The Huygens professional Linux distribution is a so called 'rpm' file.

Example: Your rpm file is called:huygens-2.3.7-17.i386.rpm

To install this file you open a shell, go to the directory were this file is located, become super-ruser and type:

rpm -Uvh huygens-2.3.7-17.i386.rpm

note: capital 'U' and space between 'rpm' and '-'.

After installing the software type hugens2 in a shell to start the software. A directory /usr/local/svi will be created; the executables will be installed in /usr/local/bin

Irix

Currently the Irix distribution is a single 'tardist' file containing various components. By default all components are installed. Become superuser and type:

swmgr -f dist65-2.3.5-2.tardist

Press 'Start' in the Software Manager window.

A directory /usr/local/svi will be created; the executables will be installed in /usr/local/bin and /usr/sbin.

Obtaining a license key

To run the software beyond the free ware functionality you will need a license key. This temporary license key can be obtained by sending SVI a reply on the email that was sent to you when you downloaded the software.

Please answer the questions in the email and paste your computers system ID at the correct place. Your computers system ID can be read in the Menu item **Help>Product information**.

Once we have received the system ID together with the information from your reply and the original email, we will generate a personalized license for your system. This license will only work on that specific computer

Installing the license key

The installation created a file huygensLicense in the directory /usr/local/svi. Become root and add the license string on a separate line in the file.

System requirements

Memory requirements

Recommended RAM memory: 512 MB or larger.

Irix

All SGI equipment running Irix 6.5 running Irix 6.5 on a MIPS R5000 processor or higher. On Octane or higher systems automatically a 64bit multiprocessing executable is installed. This allows the software to access more than 4GB of RAM and to distribute the computational work over many processors.

- All SVI packages on Irix require the SCSL mathematical library. This is distributed along with Irix; an up to date version can be downloaded from the SGI web site.

Linux

Linux: RedHat and SuSE distributions. Since Linux versions evolve rapidly best consult SVI's www.svi.nl web page to see which Linux distributions are currently supported.

A standard ethernet card is required to provide your computer with a system ID.

- Processor: Pentium III or IV (Intel) or Athlon (AMD).
- Graphics card: any fairly modern card will do.

Addresses and URLs

Where can we be reached?

Scientific Volume Imaging b.v.

Alexanderlaan 14
1213XS Hilversum
The Netherlands

You can call us directly by phone:

++31 35 6859405 or ++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/distributors

Support and FAQ

Please visit the following web site for support issues and to consult the FAQ:

http://support.svi.nl

On this web site you'll also find a form to submit questions to SVI's support team.

Where can I find more about Silicon Graphics?

Please visit the SGI web site:

http://www.sgi.com

There you can find addresses of sales and support offices which exist in many countries.

IBM Life Sciences

http://www-1.ibm.com/industries/lifesciences/

CHAPTER 2

Getting Started

This chapter will help you to get through the basic procedures in deconvolving an image.

Deconvolving my first image

Searching the SVI Knowledge Base

It's 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. FAQ's are available for many items such as deconvolution and general microscopy, installation, memory management, visualization, file formats, platforms and reported bugs.:
http://support.svi.nl/

Step 1: Start Huygens Pro

Starting Huygens Pro

- IRIX: You may start the Huygens program by clicking the 'Icon Catalog' on your Irix desktop.
- LINUX: Via the KDE application menu.
- LINUX / IRIX: By opening a shell and typing 'huygens2'. It is a good idea first to go to the directory where your images are stored, using the command 'cd'.

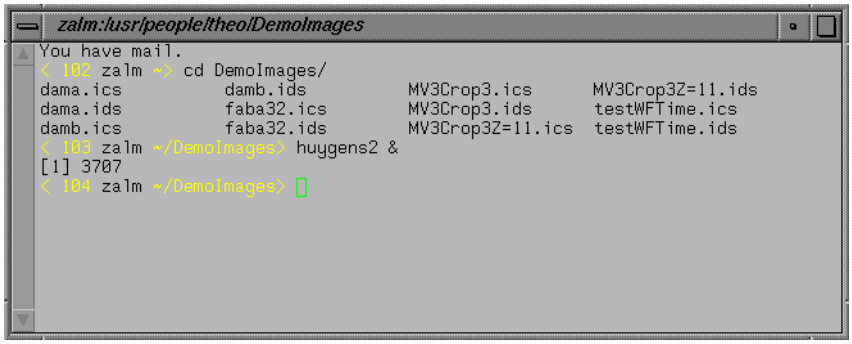


Figure 1. Starting Huygens Pro. If you type an ampersand (&) after the command the prompt (>) will return.

The `huygens2` command starts the program that comes up with the Main Window with 4 black thumbnail images (see Figure 2).

Figure 2. The Main Window with empty thumbnail images loaded by default.

Thumbnail images

The Main Window is initially loaded with 4 black thumbnail images, images that are already prepared for you, but where all image data are zero's. One of the images is called 'psf' which will be used in the deconvolution steps as you will learn soon.

Step 2: Load an image

Loading your image

First you have to load an image. Select 'Open' from the File menu in the Main Window and find the file to be processed.

In the distribution you will find the demo image faba32 (the file pair faba32.ids and faba32.ics, where faba32.ids is the file containing the raw data and faba32.ics is a header file, containing all microscope parameters). Click on one of these, no matter which one.

You may combine steps 1 and 2: starting Huygens and loading images using a single command line:

```
huygens2 faba32.ics dama.ids&
```

This command appends the images faba32 and dama to the Main window.

If you have Tiff slices to be processed please read "Tiff file series naming convention" on page 27 for the naming convention in order to be able to read a multi-dimensional image as a whole.

Step 3: Inspect your image.

Inspect your image.

- Use the slicer

Expand viewer

Open the Expand viewer by pressing the Arrow-up button  from the thumbnail of faba32. The Expand viewer is intended as a general purpose image inspection tool. It is composed of a menu bar and a tabbed deck consisting of a Projection page, a Slicer Page

and a Parameters page. You can open as many Expand viewers as you like, on the same image or on different images.

image is not a time series.
‘t’-slicing is not available.

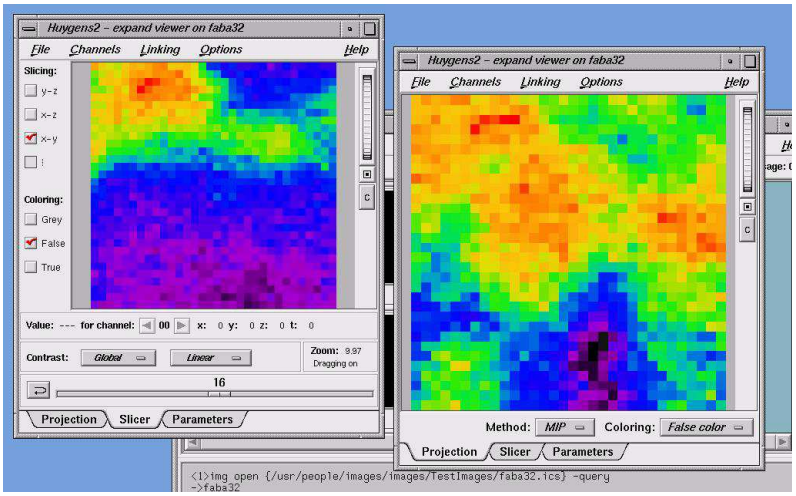
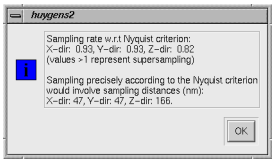


Figure 3. Two Expand Viewers opened from the same image. Left the Slicer Tab is selected, right the Projection Tab.

With the Viewer’s Slicer you can scan through the slices (in different directions). Since this example image is not a time series, the time (t) select box is greyed.

Microscopic parameters

- Verify the microscopic parameters (needed for the generation of a PSF) like Numerical Aperture, Microscope type, sample size, etc. by selecting the Parameters Tab from the Expand viewer (see Figure 3).



Did you use the correct Sampling size? To fi nd out, select faba32 in the Main window, by clicking on the image (the name is displayed in blue, when selected!). Now select ‘Nyquist rate’ from the Edit menu and you will be informed.
We see that the image is fairly good sampled in the x and y directions since the nyquist rate is near to the value of one. However, the z (the x-y slices) is undersampled (the sampling was too course). Advised is to sample in the z-direction with a distance of 166 nm or less.
Conclusion: if the Nyquist tool leaves you with values smaller then one you have under-sampled your image. Tough luck for you now, but hopefully future recordings may benefi t from this information.
(Super sampling of an image is not a problem, although your dataset becomes unnecessarily large without providing extra information).
For now we continue with the z-undersampled demo image.

Histogram

- The image histogram
  Select the image in the Main Window and select Image histogram from the Edit menu. An image histogram is created as a thumbnail image named faba32:histo. You may use the Expand viewer for a better view. The histogram enables you to inspect the quality of the image visually. The one in Figure 4 is of reasonable quality: No sharp peaks at the extreme sites of the histogram image indicating clipping. An example of a clipped image is shown below.
  Not also that the black space at the left hand of the histogram. It signifi esan electronic mismatch in the recording of the image. See “Blacklevel” on page 20.

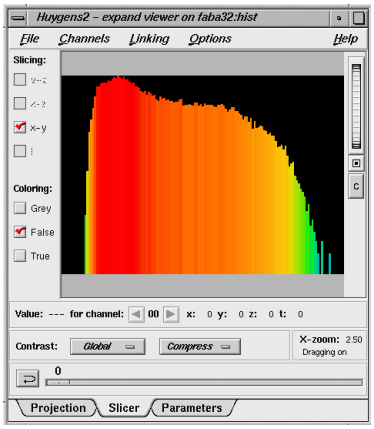


Figure 4. The image histogram from the example

A clipped image example, see Figure 5.
If you see a narrow peak at the left hand side of the histogram this signifi es ‘clipping’. It occurs when negative input signals are mapped to zero’s by the CCD camera. See also “Clipping” on page 22.
If a peak is visible at the extreme right hand side of the histogram it indicates saturation. Saturation is a type of clipping caused by raising the laser intensity above the maximum pixel value available on your microscope. Usually, all values above the maximum value are replaced by the maximum value. On rare occasions they are replaced by zeroes. Clipping will have a negative effect on the results of deconvolution, especially with WF images

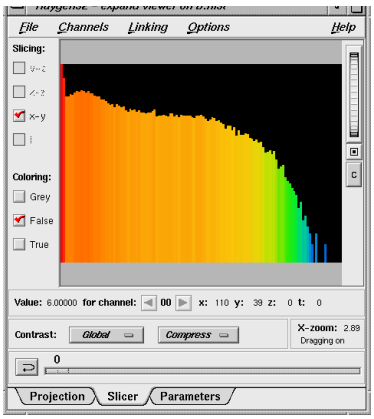


Figure 5. The image histogram from a clipped image. A spike is seen at the left hand site.

Operations window

In the former steps all operations were performed without any operation *parameters* (functions applicable on a particular image). For example if one wants to know the image statistics no extra information is needed but the name of the image. All these types of operations are accessible from the main Window. Most operations, however, need extra input data. For example adding some constant value to an image. This can be done in the Operations Window

Since in the next steps all image processing operations need one or more parameters you have to work with the Operations window. You open an Operations window on an image by clicking on the small button labelled **O** in the images’ Thumbnail component.

Subsequently you select an operation from the Menu bar or from the Quick access buttons. The

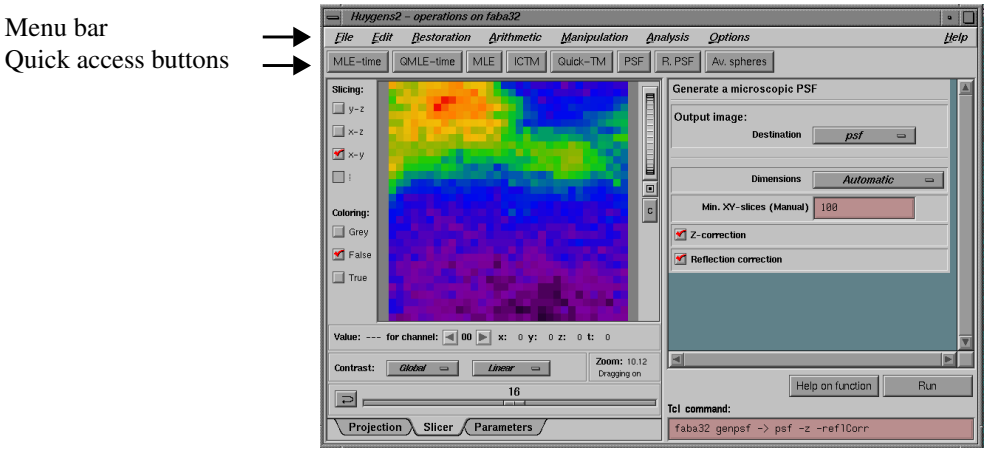


Figure 6. The Operation window with the PSF button pressed.

left side of the operation window is similar to the Expand viewer. At the right side you will find the parameter entry area. By editing the parameters you control the way the function operates. To execute the operation, press the **RUN** button.

bleaching correction

Intermezzo: check for bleaching correction

Remember, we were inspecting our image. This extra check is given here for the sake of completeness for those using this schematic approach for widefield images or confocal time series. Select 'Plot Flux' from the 'Analysis' menu in the **Operation window**. A typical example is shown in Figure 7. The Demo image is a regular confocal image: bleaching correction test makes no sense here!

- Extra check to see if bleaching correction is needed in *widefield* images or confocal *time* Series. While recording widefield images or confocal time series strong bleaching may occur.

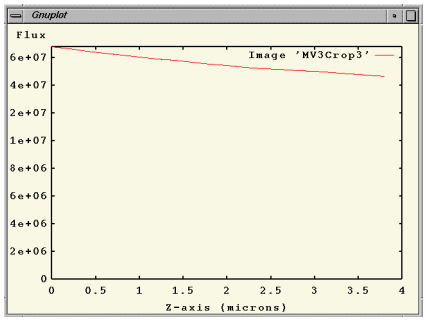


Figure 7. A typical example of a bleached widefield image. Use the 'Plot Flux' tool from the Analysis menu available in the Operation window.

Step 4: Generate a Point Spread Function (PSF)

The PSF is the way in which a point of your original object is visualized in the image you've loaded in step 1. Often this point is no longer a point but blurred and spread out. The aim of the PSF is to calculate the measure of blurring in the x,y,z axis. In the final step of deconvolution this PSF is used to come to a (in contradiction to the so-called 'blind deconvolution) measured deconvolution. A PSF can be obtained either by *recording small beads* with known bead diameter (180 nm beads work fine) and reconstructing a PSF from the bead image or by *calculating a theoretical PSF* from the information about your microscope settings, see " Microscopic parameters" on page 9. Huygens Pro has many tools to handle the first method but these are beyond the scope of this demo. For this first image demo we generate a theoretical one.

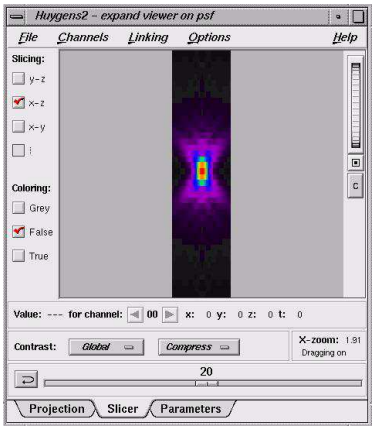


Figure 8. The PSF in x-z view, using 'compress' contrast mode. 'Compressed contrast' highlights the low intensity values showing the typical diabolo shape in lateral view.

A theoretical PSF can be generated by clicking the button **PSF from the Operation Window**. This tool computes a Point Spread function from the microscopic parameters. You need not change by now the parameters. By default the psf-image will be displayed in the destination 'psf', which is still empty, but any available image can be selected. Press the **Run button** located in the right down corner. In thumbnail 'psf' you may select the Expand viewer to see the Point Spread Function in detail. (Figure 8)

Step 5: 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 measured (noisy) image. It is determined by searching the image first for a region with low values. Subsequently the background is determined by searching this region for the planar region with radius *r* which has the lowest 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 average background estimation is invoked by **Estimate background** from the Analysis menu in the Operation window. 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 set to 0.5 micron by default.
- 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.

We are looking for the background outside the object, in a confocal image, therefore we choose for the default 'Lowest value' setting. Again press the **Run button**. The result is displayed in the Main window's Task reports. It is one of the values that should be known in the next step.

Step 6: Estimate the Signal to Noise ratio (SN ratio)

The Signal to Noise ratio (SN) is a number not always easy to estimate. The easiest way to obtain some figures is to look at the textures of bright areas in your object image. In the figure at left you see examples of such textures obtained from originally the same object image to which various levels of poisson noise were added.

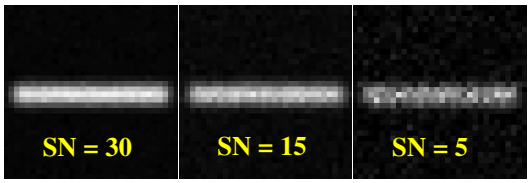


Figure 9. Images with different generated noise levels

Step 7: Perform a deconvolution run, applying the MLE (Maximum Likelihood Estimation) method.

Applying the deconvolution process

You can choose between various deconvolution methods. Only in some special cases you should use the ICTM (Iterative Constraint Tikhonov-Miller) method as explained in the reference manual. While selecting the **MLE-button** you'll see the following parameter values which can be adapted:

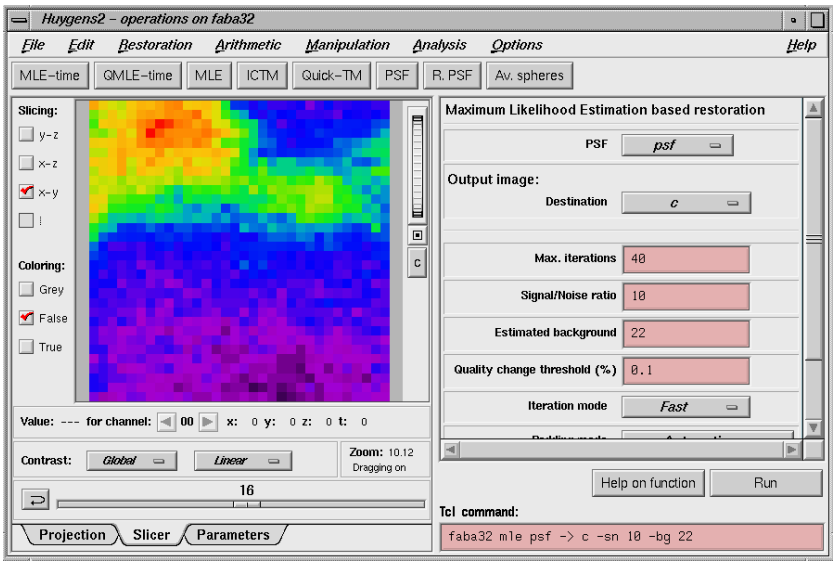


Figure 10. The Operations window with the MLE button pressed.

- Number of iterations: this depends on the initial quality of your image.(e.g. 20 to 40)
- Signal to noise ratio: You have to make an estimation of the Signal to noise (SN) value from your recorded image. Inspect your image and decide if your image is noisy (S/N < 10), has moderate noise (10-20) or is a low-noise image (S/N> 20). See examples of noisy images in Figure 9
- Background: This value was calculated in step 5. Copy the value from the Tasks reports (Main window) into the field.
- Threshold quality: This number gives the maximum difference between the subsequent iterations. If one chooses a high number (e.g. 0.1) the deconvolution may stop before the indicated number of iterations has been completed as the threshold value has been reached beforehand. The smaller the threshold and the larger the number of iterations the higher the quality of deconvolution.
- Other parameters like bleaching correction and padding mode are best kept in their default modes.

- Press the **Run** button! You will end up with a restored image ‘c’ in the Main window.

After inspecting the result in the Expand viewer you may want to perform another deconvolution run. Maybe you also wish to change one or more parameters. After pressing the **Run** button again you will be asked, in the case the output image destination is not changed, if you like to do a completely new run (the destination image is zero-ed before) or one on top of the first run.

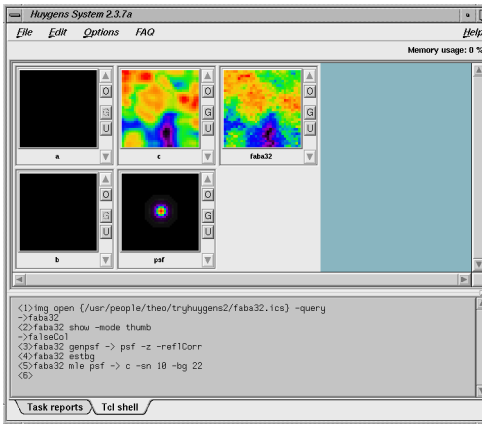


Figure 11. The Main Window with a deconvolved image ’c’.

Step 8: Saving your image

If you are done and wish to save the result go to the Main window and select the restored image (c) by clicking its thumbnail (the name is in blue when selected!). From the File menu you select the Save as ICS (International Cytometry Standard file format) or the Save as ... for some other file formats.

The easy way

In the former part you learned some basic procedures for the deconvolution process and familiarized you with some User interface components. This approach is in two minds about education and demoing functionality. Deconvolution can be done easier.

Quick and easy deconvolution

Following the steps from the former example you have learned that a PSF is essential to deconvolution. You restored your data using a PSF-image located in ’psf ’, which is either an existing measured PSF obtained from bead images or a theoretical PSF generated from the parameters of the parent image. You have generated a theoretical PSF and you may have noticed that you need not inspect nor manipulate this image. And also that the background estimation tool generated a number that need to be copied by hand in the MLE deconvolution process. Since there is no basic user interaction these steps can be automated. This is done in the MLE-time or the QMLE tools available (Buttons) from the Operation window.

MLE-time

The MLE-time tool is the most versatile deconvolution tool accessible from the Huygens Pro. It can be used for any type of data, no matter whether this is a 2D widefield image or a confocal time series. If a theoretical PSF is good enough to get by, you can skip generating one yourself as it is done on the fly.

- There is just one thing to wait for, no extra button presses.
- Especially while using large images that are processed brick-wise the PSF is prevented from being unnecessary large, it adapts its size to the brick size.
- In case of a multi channel image the PSF is generated for one channel at the time instead for all at once

- The PSF image is thrown away as early as possible, at least before the iterations begin.

Downside is that an automatically generated PSF is never re-used.

Note: The MLE-time is basically the same deconvolution tool as used in the Huygens Essential. Therefore there is no difference between the theoretical PSF's as generated by Huygens Pro and the Huygens Essential.

We recommend to use the MLE-time tool for all your deconvolution work. Only in special occasions you have recourse to other tools as is explained in 'Which deconvolution method should you use?' from the Huygens Recipes.

From step 4 we repeat the deconvolution process using the 'MLE-time'.

Proceed as follows: either Exit Huygens Pro by 'Exit' from the File menu in the Main window and start a Huygens Pro from scratch and open faba32 again, or continue the session after having cleaned images 'psf' and 'c'. (click on the thumbnail image, and select 'Zero image' from the edit menu, or use the shortcut 'ALT+Z').

Open an Operation Window from the original image (faba32). Now press Button MLE-time and you see the display as shown in Figure 12.

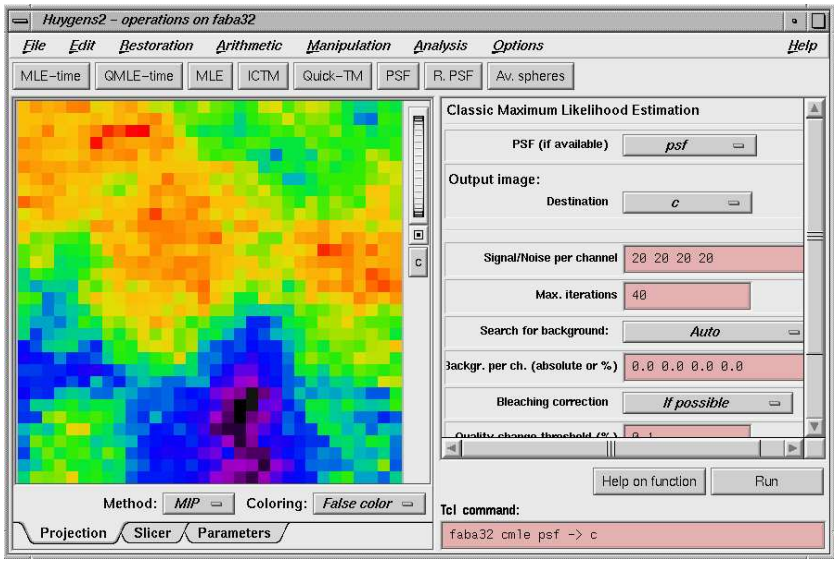


Figure 12. The Operations window with the MLE-time button pressed.

Use all default values except the 'Signal/Noise per channel'. We will use the value '10' here instead of the default value '20'. In the input field you see 4 numbers as prepared for a 4 channel images. We are dealing with a single channel image here therefore only the first number should be changed to '10'. If you do so and press 'Run', your restored image will be made as 'c'.

MLE-time Buttons and input fields

Since the MLE-time is the key deconvolution tool we will explain the MLE-time related parameters one by one.

- PSF (if available)
  Here you may select from the list of all opened images that particular image that is used as the PSF image. This can be either a measured PSF or an earlier generated PSF (using the 'PSF' button from the Operations window). If you don't use a measured PSF we recommend to select the empty image 'psf'. In this case a PSF is 'not available' and the software generates PSF's on the fly.

- Destination of the Output image
  Select an image into which the result will be stored.
- Signal/Noise per channel
  Here you may give the S/N for the separate channels.
- Max. iterations
  Absolute stopping criterion. See also 'Quality change threshold' below.
  Deconvolution as it is done in the Huygens compute engine hinges around the idea of finding the best possible estimate of the object that is imaged by the microscope. To assess the quality of an estimate, the deconvolution algorithm computes the image of each estimate as it would appear in the microscope and compares it 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. This is an iterative process that should have a stopping criterion. If the process has not yet been stopped by the 'Quality change threshold' it will be explicitly stopped by the number of Maximum iterations.
- Search for background
  Several choices can be made: AUTO, MANUAL, LOWEST VALUE, IN/NEAR OBJECT OR WIDEFIELD MODE.
  In manual mode the background value should be specified in the Estimated background input widget.
- Backgr. per ch. (absolute or %)
  This parameter should be set to the estimated mean background in the measured image. When restoring time series the background is likely to vary from frame to frame. The time-enabled deconvolution tools can therefore make a survey of the background in all frames and channels. The way this survey is carried out is determined by the 'Search for background' option.
  Since the backgrounds established by the survey are conservative values (unless In/near object is selected) they can be increased by a percentage. For instance 10% means all background estimates are increased by 10%. Negative percentages are also valid.
  If the 'search for background' is in Manual-mode the number is an absolute value and will be applied 'as is' to all frames. This value will be subtracted from all images in all frames.
- Bleaching correction
  The bleaching correction can be 'If possible' or 'Off'. In the If possible mode the software corrects for bleaching automatically. The correction is possible for 3D and 4D widefield images or 4D confocal images. For 3D confocal images automatic bleaching correction is not possible.
- Threshold for quality factor increase (%)
  This is the primary stopping criterion. After each iteration a quality factor is computed from the new estimate. This factor is based on the so-called I-divergence or cross-entropy measure of the original image and the imaged estimate. You can set a stop criterion in the form of a threshold on the quality increase. When the quality drops below the threshold for a few iterations in succession, iterations are stopped.
  When the quality is decreasing, iterations are stopped immediately. This occurs with poor quality PSFs.
  When set to 0 the stop criterion is inactive and even in the case of decreasing quality, iterations will continue.
- Iteration mode
  This allows the selection of a Fast mode or a High Quality mode. In Fast mode the iterations are more effective at the cost of a marginal increase of computing time per iteration and the addition of one extra (hidden) temporarily image.
- Padding mode
  Padding means that a border will be added around an image which is not only needed for trivial things like creating extra space to be able to rotate an image without having its corners cutted off, but also to prevent 'wrap around' effects caused by the use of discrete Fourier Transforms (DFT) which interprets the image as periodic, i.e. the image is continued on all sides by exact copies of itself. Also one or more dimensions of the image hamper effi -

cient computations of its DFT. Effi cient DFT computation can be done on images with dimensions composed of powers of 2 or small primes like 2,3,5 and 7.
AUTOMATIC: The padding depends on the image size and microscope type.
OFF (PARENT): Takes the size of the parent, so in fact no padding is made. If the sizes can be factored into small primes, like powers of 2, and the object in the image is surrounded by empty space off can very well be used.
PADDED PARENT: In this mode extra volume is added to the image. The border size computed by the software is a trade-off between FFT compute effi ciency and the size of the original image. The size of the border therefore depends on the size of the original image. As an example: consider a image with 31 layers. The next size, 32, would allow effi cient Fourier transforms because it is a power of two. However, to avoid wrap around effects a single extra layer is not enough. In this case the software will select a total of 40 slices as compromise between compute effi ciency, image size and wrap around effects.
FULLY PADDED PARENT: This mode is especially relevant for widefi eld images; for other microscope types it is equivalent to Padded Parent.

Padding and widefi eld images

In restoring widefi eld images the software must remove the contributions (blur!) of all XY slices of the object to each slice of the object. This means that to compute the top slice of the object everything below must be taken into account, using the bottom half of the PSF. For the bottom part of the object the top part of the PSF is needed. Thus the full PSF must be twice as high as the object. Worst case the object is just contained in the image, so in that case the PSF must be twice as high as the image. Since the use of Fourier transforms requires the PSF and the image to be of the same size the image must be padded to double its size. In practice however, objects are usually generously contained in the image. In that case the automatic padding mode will be suffi cient.

Why is automatic deconvolution essential?

If the procedure is not automated what should you do to deconvolve an n-channel image? You must split your parent image into n single-channel images and then generate a PSF, estimate the background and the Signal to Noise Ratio for each of them. Next you have to run the deconvolution algorithm channel by channel which ends you up with n restored one-channel images. Finally you have to ‘join’ the separate images to a restored n-channel image. Although all tools are available in the Huygens Pro it is a time consuming task. Not to speak about multi channel Time series!

CHAPTER 3

Establishing image parameters

Image size

Computing time. The amount of computing time involved in deconvolving images is more than proportional to the image size. It is therefore sensible to limit the data size as much as possible. With WF images we recommend to not record planes below and above the object which only contain blur. Huygens Pro does not need these planes to restore your object. Since the blur in these planes might be affected by hard to correct bleaching they might even reduce the quality of the deconvolution result.

Brick wise processing

Computer memory. Deconvolving images requires much computer memory because all computations are done in 32-bit floating point format, and because several extra (hidden) images are needed to store intermediate results. To reduce the memory requirements Huygens Pro will split your images into bricks, deconvolve the bricks sequentially, and fit the bricks together in a seamless fashion. Brick wise processing is an automatic feature of Huygens Pro.

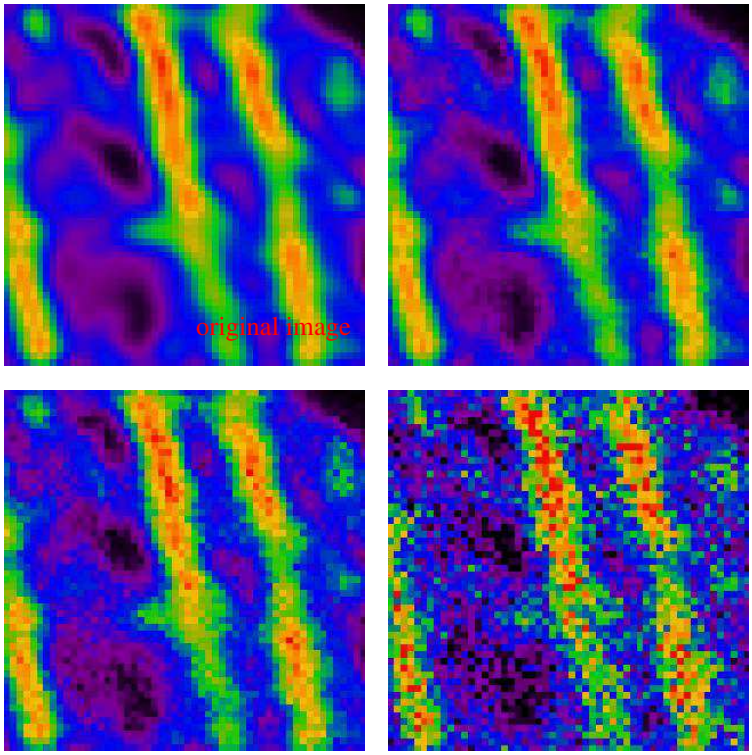


Figure 13. Example of different SNR values. Same image, different SNR ratios Top left: original image. Top right: image with SNR=30. Bottom left image with SNR=15, Bottom: right: image with SNR=5

Signal to Noise Ratio (SNR)

The SNR should be estimated from the quality of the image. In Figure 13 you find some examples of recordings where different noise levels were added to an original (restored) image.

Blacklevel

Figure 14 shows the histograms of three synthetic images. At left an image homogeneously filled with the value 5. At the middle we applied Poisson noise as if the image was build by a CCD camera. At the image on the right a value of 20 was added to simulate electronic shift. This shift is called 'blacklevel'. A large blacklevel value will reduce the effective dynamic range of your microscope, but will do no harm to the deconvolution since it is automatically accounted for in the background estimation stage. However, it is also possible that the blacklevel is negative. An image histogram will show a spike on the left (Clipped at zero values).

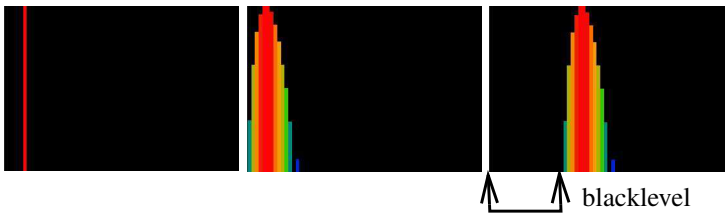


Figure 14. Histogram of images with various blacklevel values.

Sampling densities

It is very important for the quality of a deconvolution result that all information generated by the optics of the microscope is captured in digital form. It can be shown that if the sampling density is higher than a certain value all information about the object is captured. We'll call this value the critical sampling distance, corresponding to the Nyquist rate. Apart from practical problems like bleaching, acquisition time and data size there is no objection at all against using a smaller sampling distance than the critical distance, to the contrary.

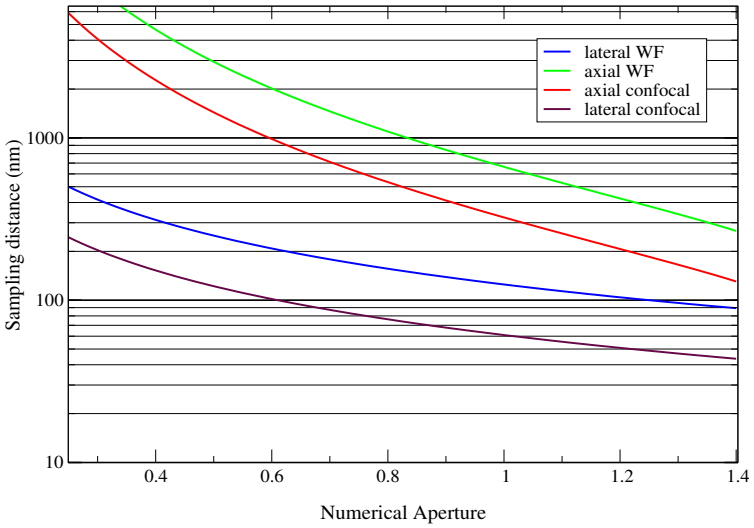


Figure 15. Critical sampling distance vs. NA
The curves above show the critical sampling distance in axial and lateral directions for WF and confocal microscopes. The emission wavelength in the WF case was 500nm; the excitation wavelength in the confocal case is

Figure 15 shows the dependency of this critical sampling distance on the NA for specific wavelengths. To apply the plot of Figure 15 to different wavelengths you can simply scale the vertical axis with the wavelength. Example: you are working with a WF microscope with NA 1.3 at emission wavelength 570nm. From the plot you read that the critical lateral Nyquist sampling distance at 500nm emission is 95nm, so in your case this becomes 570/500 * 95nm = 108nm.

In the confocal case it is the excitation wavelength which determines the Nyquist sample distance. In theory the pinhole plays no role, but larger pinholes strongly attenuate fine structures at the resolution limit. Therefore, as a rule of thumb, with a common pinhole diameter of 1 Airy disk the lateral critical sampling distance may be increased by 50% with negligible loss of information. In cases were the pinhole is much larger, the lateral imaging properties much resemble those of a WF system and the sampling distance can be set accordingly. We do not recommend to increase the axial sampling distance appreciably beyond the critical distance.

Data acquisition pitfalls

Refractive index mismatch

A mismatch between the refractive index of the lens immersion medium and specimen embedding medium will cause several serious problems:

- Geometrical distortion: the fishtank effect
  Objects will appear elongated in the microscope. Huygens Essential will automatically adapt the PSF to this situation, but assumes the image geometry is *not* corrected.
- Spherical aberration (SA)
  SA will cause the oblique rays to be focussed 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.

- Total internal reflection
  When the lens NA 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 NA.

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: undersampled) 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 -WF or confocal- and on the microscope parameters used, like the numerical aperture and wavelength of the light. How to calculate the correct sampling distance can be calculated using the formulas as given in "The Nyquist rate" from **The Huygens Recipes**.

Do not undersample to limit photodamage

Some times undersampling is done to limit photodamage to live cells. If photodamage plays a role it's 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. 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 2 slices on 1000 nm each averaged 5 times on order to reduce noise. See also "A typical example" from the **Huygens Recipes**

Bleaching

Bleaching is a practically unavoidable phenomenon 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 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 WF systems are equipped with unstable arc lamps. Huygens will correct this instability, but when the instability is severe it cannot do so sufficiently

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*. 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.

Internal reflection

At high 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 reflection occurs when the NA of your lens is higher than the refractive index of the embedding medium. This will reduce the effective NA of the lens.

Computing the backprojected pinhole radius

Throughout the Huygens Pro and Huygens Essential pinhole sizes of confocal systems are specified as the **backprojected radius in nanometer** (r_b) 'Backprojected' means the size of the pinhole as it appears in the specimen plane: the physical pinhole size (r_{phys}) divided by the total magnification of the detection system. This total magnification is the product of the (variable) objective magnification times a fixed internal magnification:

$$r_b = \frac{r_{phys}}{m_{obj} \cdot m_{system}} \quad \textbf{(EQ 1)}$$

with m_{obj} the magnification factor of the objective and m_{system} is the fixed magnification of system.

Airy disk as unit for the backprojected pinhole

Some confocal microscopes report the pinhole size (diameter) with the Airy disk (diameter) as unit. The backprojected pinhole radius can then be computed with:

$$r_b = \frac{0.61 \lambda_{ex} N_{Airydisks}}{NA} \quad \textbf{(EQ 2)}$$

with $N_{Airydisks}$ the number of Airydisks and λ_{ex} the excitation wavelength. In principle using λ_{ex} is not correct because the Airy diffraction pattern is formed by the emitted light. However, we suspect microscope manufacturers prefer to use the excitation wavelength because it is better defined and does not depend on settings of devices like adjustable band filters. For this reason in the formula above we too use λ_{ex}.

Note that this relation bypasses the need to know internal system and lens magnifications.

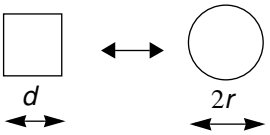
Converting from integer parameter

Unfortunately, quite a few commercial microscopes do not report the physical pinhole size or the Airy disk size. Instead, often an integer size parameter is specified with a range 0...255 (8-bit). Matters are further complicated by the use of non-circular pinholes. To compensate this we introduce a shape factor c_{shape} which takes care of the conversion from *size* (diameter or edge size) to *radius*. The following formula can be used to translate the 8-bit machine number into the backprojected pinhole radius:

$$r_b = \frac{(P_8/255) \cdot (s_{max} - s_{min}) + s_{min}}{m_{obj} \cdot m_{system}} \cdot c_{shape} \cdot 1000 \quad \textbf{(EQ 3)}$$

where p_8 is the 8-bit machine number for the pinhole, s_{max} is the maximal pinhole size in *micron*, s_{min} is the minimal pinhole size in micron. The factor 1000 is to convert from microns to nanometer.

The shape correction from a square to a circular pinhole is based on setting the area of the square pinhole equal to the area of the replacing circular pinhole.



$$d^2 = \pi r^2 \text{ and thus } r = \frac{d}{\sqrt{\pi}} \quad \textbf{(EQ 4)}$$

For a square pinhole $c_{shape} = 1/\sqrt{\pi} = 0.564$. For circular pinholes $c_{shape} = 0.5$ to just convert from diameter to radius.

Airy disk as unit for the backprojected radius of a square pinhole

The relation between the edge size of square pinhole in Airy disk units and the backprojected radius is a combination of (EQ 2) and (EQ 4). Taking into account that (EQ 2) already converts a diameter into a radius we get:

$$r_b = \frac{0.69\lambda_{ex}N_{Airydisks}}{NA} \quad \textbf{(EQ 5)}$$

with $N_{Airydisks}$ the number of Airydisks.

Computing the backprojected pinhole distance in Nipkow spinning disks

As is the case for the backprojected pinhole diameter, the distances between the pinholes in spinning disks must be divided by the system magnification. For the most used Yokogawa, for example, the pinhole distance is 2.5 µ. You can check this by stopping the disk. So with an 100x lens the backprojected distance is about 2.5 µ.

Pinhole radius tables

In the case of the Leica TCS4d, the Biorad MRC500/600, the Zeiss LSM310 and the Zeiss LSM410 type microscopes the pinhole geometry and system magnification is known resulting in the conversion formulas from the following tables:

Leica confocal microscopes

TCS 4D, SP1, NT

The size of the square pinhole is given as an 8-bit number which maps to the physical pinhole radius given in the following table:

TABLE 1. Leica TCS4d pinhole parameters

TCS4d	Range begin	Range end
Reported parameter (p_8)	0	255
Size (micron)	20	630 (earlier reported as 500)
Pinhole geometry	square	
System magnification	4.5	
Backprojected pinhole radius (nm)	$\frac{295\,p_8 + 2508}{m_{obj}}$ **(EQ 6)**	

If the pinhole is specified in Airy disk units, see "Airy disk as unit for the backprojected radius of a square pinhole" for information on how to convert to a backprojected radius.

TCS-SP2

The Leica TCS-SP2 has a system magnification of 3.6. However, the size of its square pinhole is usually specified in Airy disk units making it independent of the actual overall magnification. It is dependent though on wavelength and NA of the objective. See "Airy disk as unit for the backprojected radius of a square pinhole" for information on how to convert to a backprojected radius.

Zeiss confocal microscopes

TABLE 2. Zeiss LSM310 (upright) pinhole parameters

Zeiss LSM310 upright	Range begin	Range end
Reported parameter (p_8)	0	255
Size (micron)	0	500
Pinhole geometry	square	
System magnification	1.11	
Backprojected pinhole radius (nm)	$\frac{996 \cdot p_8}{m_{obj}}$ **(EQ 7)**	

TABLE 3. Zeiss LSM410 (inverted) pinhole parameters

Zeiss LSM410 inverted	Range begin	Range end
Reported parameter (p_8)	0	255
Size (micron)	0	1000
Pinhole geometry	square	
System magnification	2.23	
Backprojected pinhole radius (nm)	$\frac{992 \cdot p_8}{m_{obj}}$ **(EQ 8)**	

TABLE 4. Zeiss LSM510 pinhole parameters

Zeiss LSM510		
Size (p,diameter,micron)		
Backprojected pinhole radius (nm)	$\frac{p \cdot 1000}{6.66 \cdot m_{obj}}$ **(EQ 9)**	

Olympus confocal microscopes

TABLE 5. Olympus Fluoview

Olympus Fluoview					
Reported parameter	1	2	3	4	5
Size (p, diameter, micron)	0	100	150	200	300
Pinhole geometry	circular?				
System magnification	3.426				
Backprojected pinhole radius (nm)	$\frac{p}{6.85 m_{obj}}$ **(EQ 10)**				

Biorad confocal microscopes

TABLE 6. Biorad MRC600, 1024, Radiance

Biorad MRC600/ 1024/ Radiance	
Reported parameter (p_8)	n.a.
Size (diameter in mm)	0-8
Pinhole geometry	circular
System magnification [Pawley 1995, p 30]	53-83, reported 60 for the Radiance, 53 for the 1024
*Backprojected pinhole **radius** (nm) as function of the physical **diameter** d (mm)*	$\frac{d \cdot 10^6}{2 \cdot m_{system} \cdot m_{obj}}$ **(EQ 11)**

Checking the Biorad system magnification

The Biorad MRC500/600/1024 microscopes have a very high magnification in the detection system. The fixed system magnification is according to Pawley [Pawley 1995, p 30] $53 \times m_{tube}$, m_{tube} between 1.0 and 1.56 (a factor 1.25 for the 'fluorescence attachment and also a factor 1.25 for the 'DIC' attachment). The factor of 53 includes the 8× "eyepiece" just below the scan head, but doesn't include that variability in magnification due to the variations in tube-length that are result from the aligning the system.

The high system magnification allows you to view the diffraction pattern (Airy disk) at the pinhole plane directly by eye. To enable you to verify the correctness of (EQ 11) in the table above for your instrument we outline the way the system magnification was derived:

In a Biorad MRC600 with a 1.3 60x objective the Airy disk has a diameter of around 2-2.5mm at the pinhole plane. The diameter of the first Airy zero ring is 7.6 lateral optical units (o.u.), with a lateral optical unit v defined as:

$$v = x \frac{2\pi}{\lambda} n \sin\alpha \qquad \textbf{(EQ 12)}$$

with $n \sin\alpha$ the numerical aperture. In the system above an o.u. is 0.3 ± 0.033 mm. At the specimen plane (backprojected) a lateral o.u. is in this case around 61nm. The total magnification appears thus to be 4918, the system magnification 82 ± 9. This value corresponds well with the largest possible system magnification for the MRC600.

A supplied calibration curve

If a calibration curve was supplied with your microscope best use that curve to convert the displayed setting to a physical size and from there convert to the backprojected radius.

Questions

What does the quality factor mean while running Huygens?

Deconvolution as it is done in Huygens Pro hinges around the idea of finding an as good as possible estimate of the object that is imaged by the microscope. To assess the quality of an estimate, Huygens Pro computes the image of each estimate as it would appear in the microscope and compares it 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 Huygens Pro is a measure relative to the first estimate and therefore a number greater or equal to 1. If the increase in quality drops below a threshold the iterations are stopped.

Can I deconvolve a Tiff series?

Yes, if the series is a numbered series like: slice001.tif, slice002.tif.....slice0NN.tif, Huygens Pro will read the series into a single 3D image. Because Tiffs usually carry no additional microscopic information, check the parameters carefully.

Tiff file series naming convention

If you have Tiff images to be read into the Huygens Pro or the Huygens Essential you should know about the naming convention used.

If you select a file from a numbered series, the selected file and the following files will be interpreted as x-y planes of a 3D stack and read into a 3D image of suitable size and channel configuration.

A one-channel 3D images only go with numbers:
As an example a dataset called 'c' with 32 slices numbers as follows:

```
c000.tif
c001.tif
.......
.......
c031.tif
```

If you wish to work on the complete c stack you only have to select 'c000.tif' while opening. If you select file 'c020-tif the first 20 slices will be skipped.

Numbered series without the Tiff extension like

```
c04
c05
...
c18
```

are not read in as a series.

Huygens Essential and Professional read and write Tiff series with Leica style numbering if there are more channels (different wavelenghts), slices or frames(in time).

An image of four slices and two frames is named with Leica style numbering as follows:

```
c_t00_z000.tif
c_t00_z001.tif
c_t00_z002.tif
c_t00_z003.tif
c_t01_z000.tif
c_t01_z001.tif
c_t01_z002.tif
c_t01_z003.tif
```

And an image 'sTCh' of four slices, three frames and two channels:

```
sTCh_t00_z000_ch00.tif
sTCh_t00_z000_ch01.tif
sTCh_t00_z001_ch00.tif
sTCh_t00_z001_ch01.tif
sTCh_t00_z002_ch00.tif
sTCh_t00_z002_ch01.tif
sTCh_t00_z003_ch00.tif
sTCh_t00_z003_ch01.tif
sTCh_t01_z000_ch00.tif
sTCh_t01_z000_ch01.tif
sTCh_t01_z001_ch00.tif
sTCh_t01_z001_ch01.tif
sTCh_t01_z002_ch00.tif
```

```
sTCh_t01_z002_ch01.tif
sTCh_t01_z003_ch00.tif
sTCh_t01_z003_ch01.tif
sTCh_t02_z000_ch00.tif
sTCh_t02_z000_ch01.tif
sTCh_t02_z001_ch00.tif
sTCh_t02_z001_ch01.tif
sTCh_t02_z002_ch00.tif
sTCh_t02_z002_ch01.tif
sTCh_t02_z003_ch00.tif
sTCh_t02_z003_ch01.tif
```

Can I deconvolve a single plane widefield image?

Yes, single plane WF deconvolution works because the data are extrapolated into a region above and below the plane spanning typically between 10-20 planes of 100-300 nm sampling in Z. The software generates an appropriate PSF.

Can I deconvolve a single Tiff image?

Yes. Huygens Pro treats the image as the only known plane of a 3D stack and proceeds as usual. Set the z-sampling distance to the Nyquist value, see "Data acquisition pitfalls" on page 21.