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# *Huygens Professional*

**User Guide for version 15.05**



Scientific Volume Imaging B.V.

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# *Huygens Professional*

**User Guide for version 15.05**



**Scientific Volume Imaging B.V.**

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

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# Introduction

This guide is intended for the beginner and expert in deconvolution that start with the Huygens Professional toolkit.

In this introductory chapter we explain the structure of the guide and provide general information: installation instructions, license details, system requirements, contact addresses, conventions used throughout the manual and the status of the current release of Huygens Professional.

---

## *What is Huygens Professional?*

Huygens Professional is an image processing software package tailored for deconvolution of microscopic images. It enables you to deconvolve and analyze a wide variety of images ranging from 2D widefield (WF), to 4D multi-channel two-photon confocal, and Stimulated Emission Depletion (STED) microscope systems. Also people who use experimental set-ups like 4pi-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.
- Good's roughness Maximum Likelihood Estimation algorithm.
- PSF Distiller and 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 time series that enables you to correct for movement in the z (axial) direction that could have occurred for instance by thermal drift of the microscope table.

### **Basic Image Processing**

- 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, 2x32 bit complex.
- Per image undo/redo capabilities.
- An Image History that keeps track of all operations. This history can be saved along with the files or as a Huygens script to automate tasks.
- Scripting and batch processing environment based on Tcl (Tool Command Language).

### **Core Image Processing Functions**

- Create, delete, 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 two channels.
- Plots of energy flux as function of time and axial position.
- Plots of intensity profiles along lines in any orientation.

### **Image File I/O**

- Reads HDF5, ICS, Imaris classic, TIFF (file series with arbitrary numbering), Metamorph STK, Biorad PIC, Olympus TIFF (Fluoview and SIS), Delta Vision IMSubs (r3d), MRC files, IPLab IPL, Image-Pro SEQ, OME XML, and plain text (TXT and CSV).
- Optionally reads Zeiss ZVI, LSM and CZI, Leica LIF and LAS AF format version 2 and 3. Olympus OIF, Olympus VSI, BioVision IPM and IPM Tiff, Nikon ND2.
- Writes HDF5, ICS, Imaris classic, Biorad PIC, TIFF (Leica style and classic numbered TIFF), OME XML, Delta Vision IMSubs (r3d), plain text (TXT and CSV), and AVI (MJPEG). For Huygens Professional only: plain text (csv, txt). For Huygens Core only: JPEG (for web applications).

### **Analysis Functions**

- Threshold and label 3D image.
- Analyze labeled objects: compute center of mass, volume and integrated intensity.
- Estimate background.
- Measure distance.
- Plot line profiles.
- Compute image ratio.
- Compute colocalization coefficients.
- Compute the co-occurrence matrix of an image.

### **Visualization Capabilities**

- Thumbnail images.
- Multiple Slicers on one or more images. Each Slicer 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;
  - besides  $x$ - $y$ ,  $x$ - $z$  and  $y$ - $z$  slices, show slices in any arbitrary orientation;
  - display multi channel images in spectral or false color;
  - report individual pixel/voxel positions and values;
  - swing through planes or time;
  - dynamically link the position, orientation, time, and zoom.
- View your data from three sides simultaneously using the Orthogonal Slicer.
- Maximum intensity projections (MIP) of multi channel images in any orientation.
- Volume rendering using the SFP renderer. The Simulated Fluorescence Process (SFP) algorithm computes high quality 3D images based on the physics of light.
- Iso-surface rendering.
- Export of sophisticated 3D animations:
  - combine high quality MIP, SFP, and surface renderings;
  - use predefined animation presets;
  - animate the viewpoint, zoom, and any other visualization parameter;
  - use linear or smooth interpolation in transitions;
  - export to AVI and Tiff series.

---

## *Customizing Huygens Professional*

Huygens Professional has a button bar to quickly run commands or tools. This button bar can be personalized by pressing the grey arrow at the end of the button bar (▼). For each item in the menus there is a corresponding button/icon in the icon selector. In Figure 1.1 the icon selector window is shown with all its functionality highlighted.

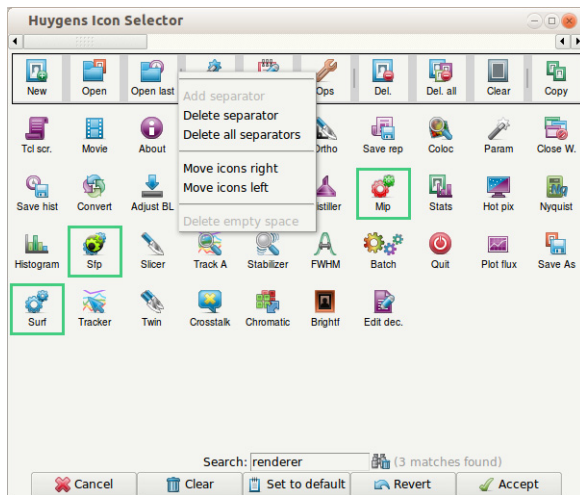
The top-bar in the icon selector represents the button bar of the main window of Huygens Professional. This top-bar can be scrolled from left to right. Below the top-bar is the icon field showing all icons not placed in the top-bar. Simply click and move icons to place them in or out the top-bar. If an icon already exists at the location where you release the mouse button, the icons will be switched.

In case you like to insert an icon between two adjacent icons in the top-bar, you first need to create an empty space. This is done by a right click in the space between the two icons and select e.g., “Move icons right”. This will create a space at the right of the mouse position and you can insert any icon that you want. More options are available under right click on the top-bar:

- *Add separator*: a separator may be inserted in between two icons to distinguish groups. A separator is a thin gray line between icons.
- *Delete separator*: added separators can also be deleted.
- *Delete all separators*: deletes all separators at once.
- *Move icons right*: moves all icons right from the cursor to the right. If icons would fall off at end of the top-bar, they are placed back in the icon field.
- *Move icons left*: moves all icons left from the cursor to the left. If icons would fall off at the beginning of the top-bar, they are put back in the icon field.
- *Delete empty space*: removes an empty space. This is not really necessary. Empty spaces will be ignored, so all icons will be placed side by side in the main window.

Below the icon field there is a search field. The entered key is matched with the icon names and tool tips. Matching icons are highlighted with a green border and the number of matches is printed beside the search entry. Since the top-bar can be scrolled you may have found an icon that is out of the view. Below the search entry, there are the following buttons:

- *Cancel*: cancels all changes made and closes the window.
- *Clear*: clears the top-bar and places all icons in the icon field.
- *Set to default*: sets the default button bar back in the top-bar.
- *Revert*: reverts all changes made in this instance of the icon selector.
- *Accept*: updates the button bar in the main window of Huygens Professional and closes the window.



**FIGURE 1.1. The icon selector showing all its functionalities.**

Huygens Professional can be downloaded from the SVI website<sup>1</sup>.

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*Microsoft Windows*

Double click on the Huygens installer executable, e.g. `huygens-1505p1_i386.exe`. Double click its icon to start the installation. During installation the directory `C:\Program files\SVI\` will be created by default. After completion the four Huygens icons appear on the desktop. Double clicking on the Huygens Professional icon starts the program.

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*Mac OS X*

Double click the package file, for instance `huygens-15.05-p1-Lion-i386-x86_64.pkg`. The archive manager expands it to a `.pkg` file, which will be placed in the same directory. Double click this file, and follow the installation wizard.

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*Linux (Debian)*

Debian packages are natively used by Ubuntu and other Debian-based Linux distributions. Double click the package file, e.g. `huygens_15.05-p1_amd64_olderGLIBC.deb`, and follow the steps in the package manager. To install the package through the command line:

```
dpkg -i huygens_15.05-p1_amd64_olderGLIBC.deb
```

---

*Linux (RPM)*

RPM (RedHat Package Manager) packages are natively used by RedHat, Fedora, SUSE, and other RPM-based Linux distributions. Double click the package file, e.g. `huygens-15.05-p1.x86_64.rpm`, and follow the steps in the package manager, or install the package through the command line:

```
rpm -ivh --force huygens-15.05-p1.x86_64.rpm
```

---

1. <http://www.svi.nl/Download>

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## After the Installation

After a first-time installation there is not yet a license available. However, the software can still be started. Without a license it will run in *Freeware mode* (See Figure 2.1). The *System ID*, necessary for generating a license, is obtained by pressing the REQUEST TEST LICENSE button when opening Huygens Professional. It can also be found in the HELP→LICENSE OVERVIEW menu. The next section explains how to obtain and install a license string.

---

## The License String

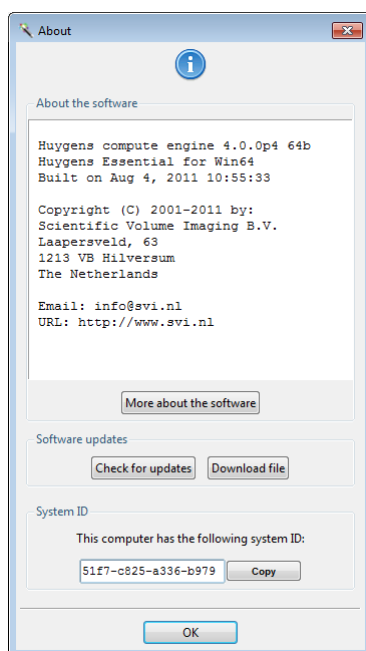
The license key used by all SVI software is a single string per licensed package. It may look as follows:

```
HuPro-15.05-wcnp-d-tvAC-emnps-eom2015Dec31-  
e7b7c623393d708e-{user@domain.com}-4fce0dbe86e8ca4344dd
```

At startup Huygens Professional searches for a license file `huygensLicense`, which may contain a license. A license string can be provided by SVI via e-mail. Installing a license string is the same for all platforms.

### Obtaining a License String

If upgrading is not handled from a previous installation it is likely that a license is not yet available. To enable us to generate a license string, we need the *fingerprint* of the computer used, the so-called *system ID* number.



**FIGURE 2.1.** The HELP→ABOUT window. The system ID is shown at the bottom.

If Huygens Professional is not already running, please start it. The system ID pops up as long as no valid license is available and is displayed in the HELP→ABOUT dialog (Figure 2.1). Send it to `sales@svi.nl`, and a license string will be provided.

To prevent any typing error use the COPY button to save the ID to the clipboard. It can be printed into the license mail message with the EDIT→PASTE menu item of the mail program.

This dialog box also contains a button to *Check for updates* on the SVI company server.

### Installing the License String

Select the license string in the e-mail message and copy it to the clipboard using EDIT→COPY in the mailing program. Start Huygens Professional and go to HELP→LICENSE: a dialog box pops up. Then press the ADD NEW LICENSE button and paste the string into the text field (Figure 2.2). Complete the procedure by pressing ADD LICENSE; this will add the string to the `huygensLicense` file. Please try to avoid typing the license string by hand: any typing error will invalidate the license. With an invalid license, the software will remain in Freeware mode. When the license is correct the message “*Added license successfully*” will appear.



Restart Huygens Professional to activate the new license!

### Location of the License File

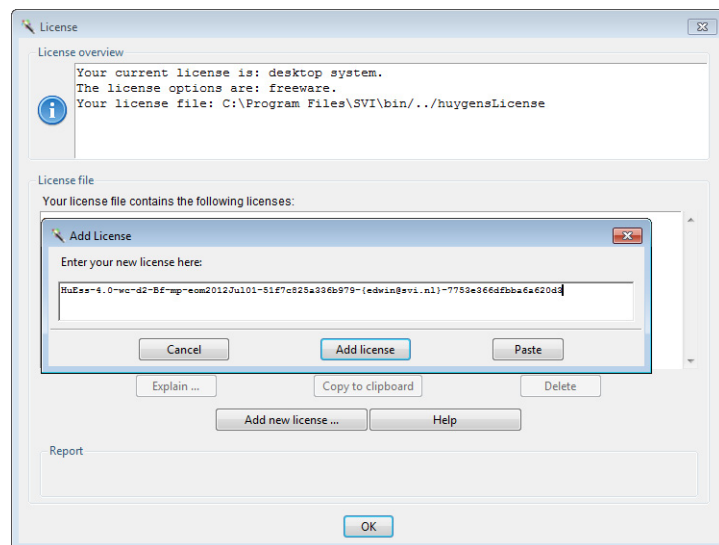
The license string is added to the file *huygensLicense* in the SVI directory (Table 2.1 on page 7).

**TABLE 2.1. The default installation paths per platform.**

Platform	Installation path
Windows	C:\Program files\SVI\
Windows 64 bit Edition	C:\Program files (x86)\SVI\
Mac OS X	/Applications/SVI/ <sup>a</sup>
Linux	/usr/local/svi/

- a. The path name on Mac OS X depends on where the software is installed. This is a typical example.

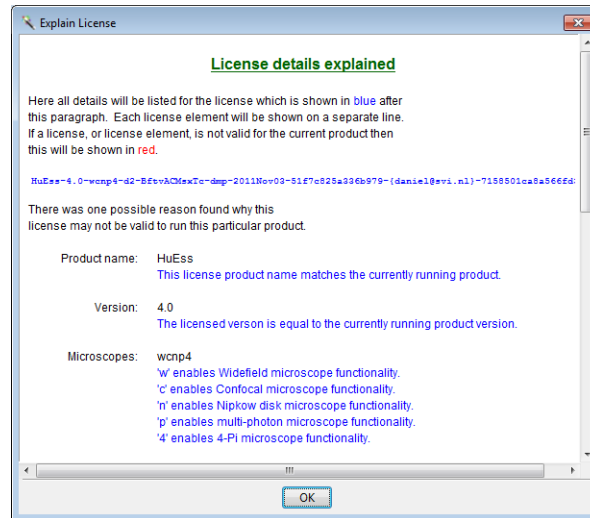
On Irix and Linux and Mac OS X an alternative location is the user's home directory. On OS X this is especially convenient when updating frequently.



**FIGURE 2.2. The license window allows to add, delete and troubleshoot licenses.**

---

## Troubleshooting License Strings



**FIGURE 2.3.** The *Explain License* window lists all license details.

The license string as used by SVI has the same appearance on all supported platforms. For each product it is required to have a license string installed. Select a license string in the license window (HELP→LICENSE) and press the EXPLAIN LICENSE button. All details for the current license will be listed (Figure 2.3). If running into licensing problems this information can be used to analyze the problem.

---

## Updating the Software

When the system is attached to the internet a pop-up window will appear when a newer version is available. The website can also be consulted for updates. Twice a year around April and October new releases will become available. Download the new version from the SVI website<sup>2</sup>. Proceed with the installation as explained above.

Do not uninstall the old version as this will delete the license string. The new version will (by default) automatically replace the old Huygens version. However, for the MAC OS X, we advise to first uninstall the software and then reinstall a newer version. Please read the next subsection “Removing the Software” carefully for more information.

---

## Removing the Software

Removing the software will also cause the license string to be removed. If it is preferred to uninstall the current version prior to installing a newer one, take care to store the license string in a safe place. See “Location of the License File” on page 7 to read where to

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2. <http://www.svi.nl/Download>

license string is stored. Table 2.2 on page 9 shows the uninstall procedure for each platform.

**TABLE 2.2. The uninstallation procedure per platform.**

Platform	Procedure
Windows	Open the start menu and select: PROGRAMS→HUYGENS SUITE→UNINSTALL→REMOVE THE HUYGENS SUITE.
Linux	Open the package manager, search for <i>huygens</i> and uninstall it. This could also be handled with the command line; type <code>dpkg -r huygens</code> to install a Debian package or <code>rpm -e huygens</code> to install an RPM package.
Mac OS X	Drag the installed version to the waste basket.

*System Requirements for Huygens Professional*

Tables Table 2.3, Table 2.4, and Table 2.5 list the requirements for Windows, Mac OS X, and Linux.

**TABLE 2.3. System requirements for Microsoft Windows.**

Operating system	Huygens runs on Microsoft Windows Vista, Windows 7, and Windows 8 (64 bit)
Processor	AMD Athlon 64 or Intel Pentium 4 and higher.
Memory	2 Gb or more.
Graphics card	Any fairly modern card will do.

**TABLE 2.4. System requirements for Mac OS X**

Operating system	Huygens runs on Mac OS X Mountain Lion, Mavericks, Yosemite (X11, XQuartz) <sup>a</sup> .
Processor	G5 PowerPC or Intel.
Memory	2 Gb or more.
Graphics card	Any fairly modern card will do.

a. OS X 10.7 or higher with X11 is required for full 64 bit capabilities.

**TABLE 2.5. System requirements for Linux**

Operating system	Most popular distributions like Ubuntu, RedHat, Fedora, and SuSE are supported. Linux (64 bit)
Processor	AMD Athlon 64 or Intel Pentium 4 and higher.
Memory	2 Gb or more.
Graphics card	Any fairly modern card will do.

*Support on Installation*

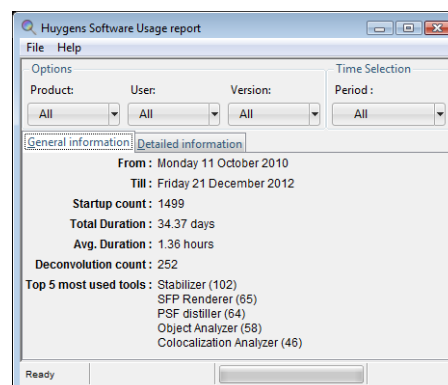
If any problem is encountered in installing the program or the licenses which could not be solved with the guidelines here included, please search the SVI Wiki<sup>3</sup> or contact SVI (See “Contact Information” on page 201).

---

## Measuring the software usage

The usage of the Huygens basics (e.g. Essential, Professional) software is logged everytime the software is launched. In addition, the usage of those tools that require a license-flag in your license string is saved. This usage can be viewed with the Usage Report window, see Figure 2.4.

Go to HELP → USAGE REPORT and wait for the software to analyze the data. Once the analysis is done the *General information* tab shows the total usage of the software. The *Detailed information* tab shows the usage per tool.



**FIGURE 2.4.** The usage report window.

The usage can be filtered by product, user, version and time. Simply select one of the options in the menu-buttons and the data is re-analyzed. This usage report can be saved through FILE → SAVE USAGE as a .CSV file.

The usage is written to a log file located in the installation directory in the directory UsageLogs and all users are able to write to it. Each year a new log-file will be created and the Usage Report tool analyzes all usage logs in this directory. When the software is updated and/or uninstalled, the usage logs are kept.

---

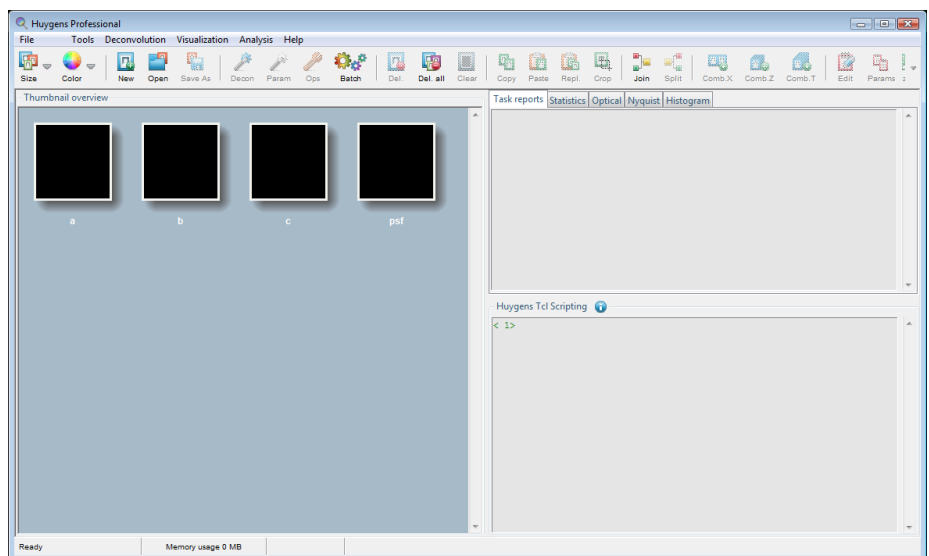
3. [http://www.svi.nl/FAQ#Installation\\_questions](http://www.svi.nl/FAQ#Installation_questions)

# Getting Started

This chapter will help you to get through the basic procedures in deconvolving an image and to become familiar with the user interface components of Huygens Professional. Note that several steps described below are automated in the deconvolution run (step 7).

## Step 1: Start Huygens Professional

You can start the software by clicking on its desktop icon or, in case you are using a Unix shell in Linux, by typing `huygenspro`. If Huygens is used remotely, you need to use a client software<sup>1</sup>. If Huygens Professional is started from a Unix shell, it is a good idea to navigate to your image directory first. After starting up the software, a graphical user interface (GUI) appears with four empty thumbnail images (Figure 3.1).



**FIGURE 3.1.** The main window on Microsoft Windows. Four empty thumbnails are shown by default.

1. <http://ww.svi.nl/RemoteDisplay>

---

Huygens Professional initially loads four empty images, named `a`, `b`, `c`, and `psf`. These images are tagged with default microscopy parameters, but the image data consists of just zero-es. The fourth image, `psf`, will be used in the following steps as a container for the point spread function.

From the Leica LAS AF/X software you can directly start Huygens and open an image in Professional. To do this, right-click with your cursor in the left column within LAS AF/X on the complete LIF project or a sub-image and select `Export→HUYGENS PROFESSIONAL`.

Huygens Professional can also be directly opened with selected images from Huygens Titan. For more information see chapter “Titan” on page 23.

---

## Step 2: Load an Image

If you like to open a new or another image in Professional, select `FILE→OPEN` from the menu in the main window and find the image. As mentioned above, you can also use Huygens Titan (again) for this purpose.

In the distribution you will find the demo image ‘`faba128`’. The image consists of the file pair `faba128.ids` and `faba128.ics`. The IDS file contains the image data and the ICS file the meta data, including the microscope parameters. You may click on one of these files to open the image.

In a Unix shell, steps 1 and 2 can be combined in a single command that loads the images automatically after starting Huygens Professional:

```
huygenspro faba128.ics dama.ids&
```

This command loads the files ‘`faba128`’ and ‘`dama`’ and shows their thumbnail images in the main window. The ampersand symbol (&) makes sure that the command prompt will return.

If you would like to open a series of Tiff slices, please read “Numbered Tiff Series” on page 194 for the naming convention in order to be able to read a multi-dimensional image as a whole.

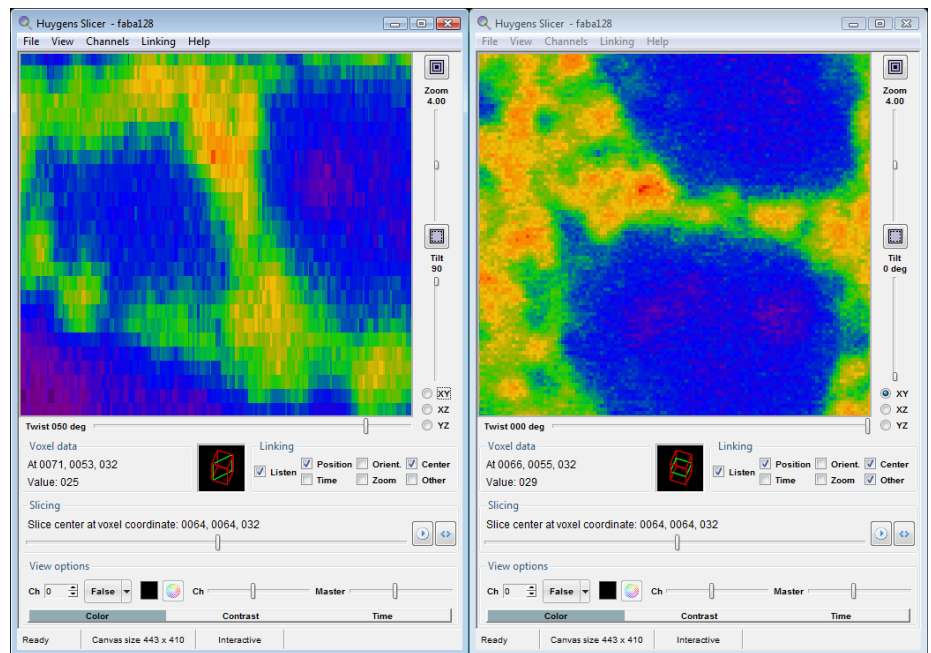
---

## Step 3: Inspect your Image

### The slicer

Select the ‘`faba128`’ image and open the Slicer via the menu `VISUALIZATION→SLICER` in the main window, or by right-clicking the thumbnail and choosing `VIEW SLICER`. The Slicer enables the user to show a single 2D plane extracted from a 3D volume. There are controls to select any plane orientation in space, zoom, and scroll through the available planes. For time series, a separate control is available to scroll through the available time frames. Furthermore, controls are available to set the contrast, brightness and color scheme for the different channels. Color schemes include ‘`spectral`’ colors, i.e. the displayed color of a channel matches the true color of the emission wavelength in so far this is possible.

You can open as many Slicers as you like, on the same image or on different images (Figure 3.2). Multiple Slicers can be linked and ‘listen’ to other Slicer windows. When linking is active, right clicking on the main Slicer will change the other view’s center to the clicked coordinate, regardless of its orientation. Note that the option ‘other’ from the ‘linking’ panel has to be selected in order listen to Slicer windows of other images.

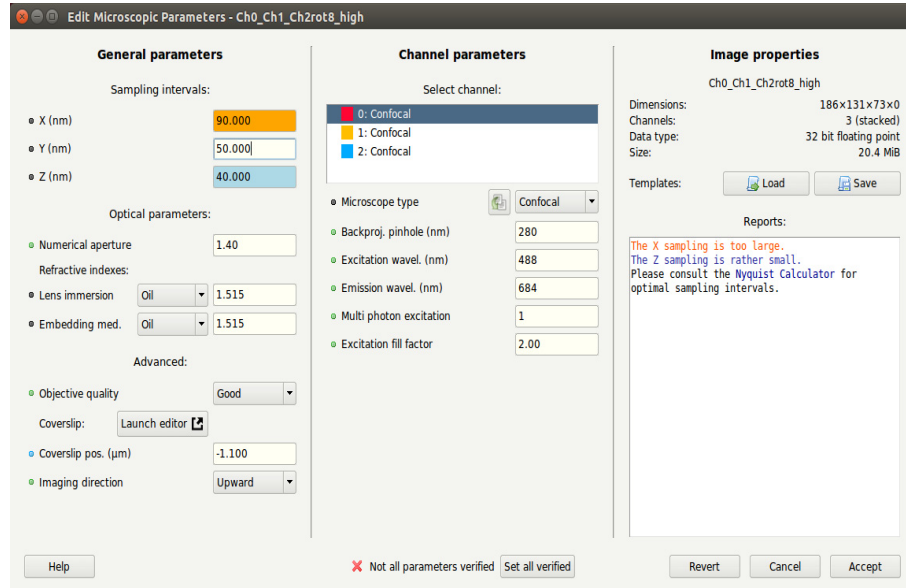


**FIGURE 3.2.** Two slicers opened with different views of the same image.

More information on the Slicer and other Huygens visualization tools can be read starting from “The Slicer” on page 61 onwards.

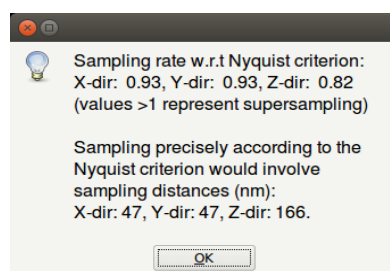
## Microscopy Parameters

Verify the microscopic parameters (needed for the generation of a PSF) like NA, microscope type, sample size, etc. by selecting EDIT→EDIT MICROSCOPIC PARAMETERS from the main menu (Figure 3.3).



**FIGURE 3.3.** The parameter editor. The values in this window should be checked carefully before deconvolution.

Did you use the correct sampling size during the image acquisition? To find out select ANALYSIS→NYQUIST RATE from the main menu, which displays an information window as shown in Figure 3.4. You can also look under the tab NYQUIST in the main window. Read more about this important topic at the SVI-wiki<sup>2</sup>. It is also important to make sure that the sample sizes reported by the microscope during acquisition are correct<sup>3</sup>.



**FIGURE 3.4.** The Nyquist information window. Values smaller than one indicate undersampling

The 'faba128' image is fairly good sampled in the  $x$  and  $y$  directions with a Nyquist rate of 0.93, which is close to the value of one. However, the  $z$  direction has a Nyquist rate of 0.82, which indicates undersampling (the sampling was too coarse). It is advised to sample in the  $z$  direction using a sample distance of 166 nm or less. If the Nyquist tool leaves you with values smaller than one, the image is undersampled. Your future recordings may benefit from this information. Note that supersampling is not a problem, although your dataset becomes unnecessarily large without providing extra information. For now we continue with the  $z$  undersampled demo image.

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

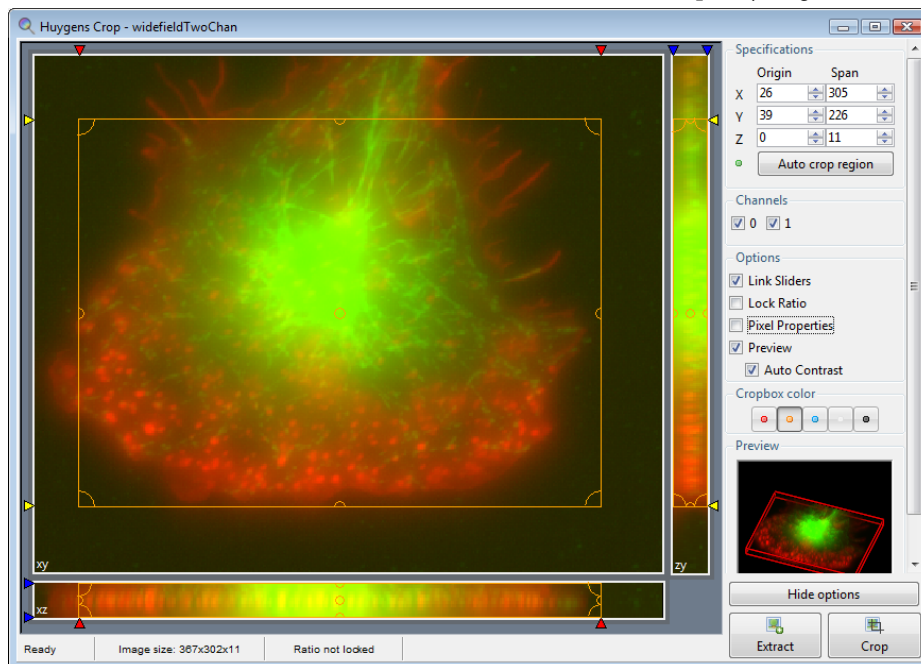
3. <http://support.svi.nl/wiki/CalibrateAxialDistances>



### 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 Professional is equipped with an intelligent cropper that automatically surveys the image to find a reasonable proposal for the crop region. In computing this initial proposal the microscopic parameters are taken into account, making sure that cropping will not have a negative impact on the deconvolution result.

To start the cropper, select **TOOLS** → **CROP** from the menu in the main window. Initially, the cropper suggests that the bottom planes from 'faba128' can be removed without significant loss of information that will decrease the deconvolution quality (Figure 3.5).



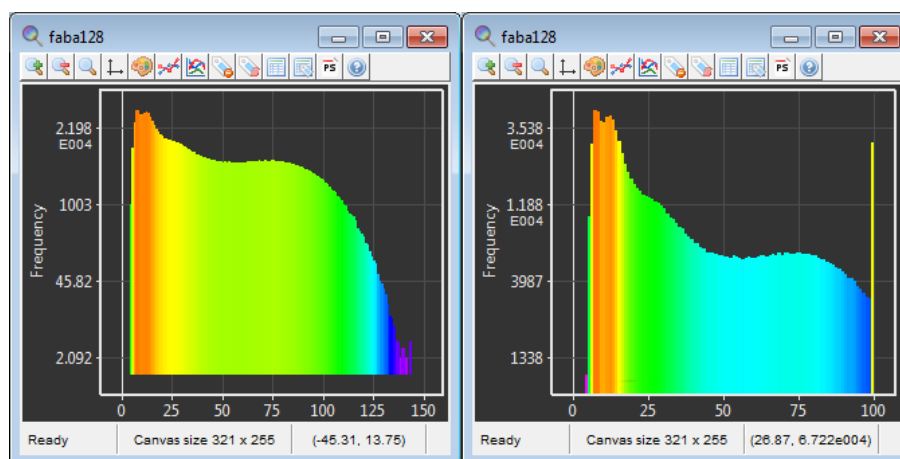
**FIGURE 3.5.** The cropper window.

To adjust this crop region manually, drag the corners or sides of the cropbox to the desired position or use the entries in the Specifications panel. Accept the new borders by pressing the **CROP** or **EXTRACT** button. The latter one will create a new image instead of adjusting the original. Do not crop the object too tightly, because you would remove blur information relevant for deconvolution. See “The Intelligent Cropper” on page 34. for more details about its options.

### The Image Histogram

Select image 'faba128' in the main window and display its histogram via **ANALYSIS** → **IMAGE HISTOGRAM** in the main menu. The histogram enables you to visually inspect the intensity distribution of the image. The distribution of 'faba128', as seen in the left window in Figure 3.6, is of reasonable quality, i.e. there are no sharp peaks at both sides of the histogram that suggest clipping. The right window in Figure 3.6 shows an example of the histogram of a clipped image.

Clipping occurs when input signals that are too high, are mapped to the highest value available, or when 'negative' input signals are mapped to zeroes by the CCD camera.



**FIGURE 3.6. Two image histogram windows. In the right image, the spike at the right hand side indicates clipping (saturation).**

Clipping at the right hand side of the histogram indicates saturation, as is the case in the right histogram in Figure 3.6. Saturation is caused by overexposure, e.g. by raising the laser intensity above the maximum pixel value the image file can accept.

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 widefield images. See “Clipping” on page 190. for more information.

A histogram may also show an offset at the left hand side, which indicates a positive blacklevel. A large positive blacklevel value reduces the effective dynamic range of your microscope, but will do no harm to the deconvolution with Huygens Professional. For more information, see “Black Level” on page 183 or the SVI wiki page<sup>4</sup>.

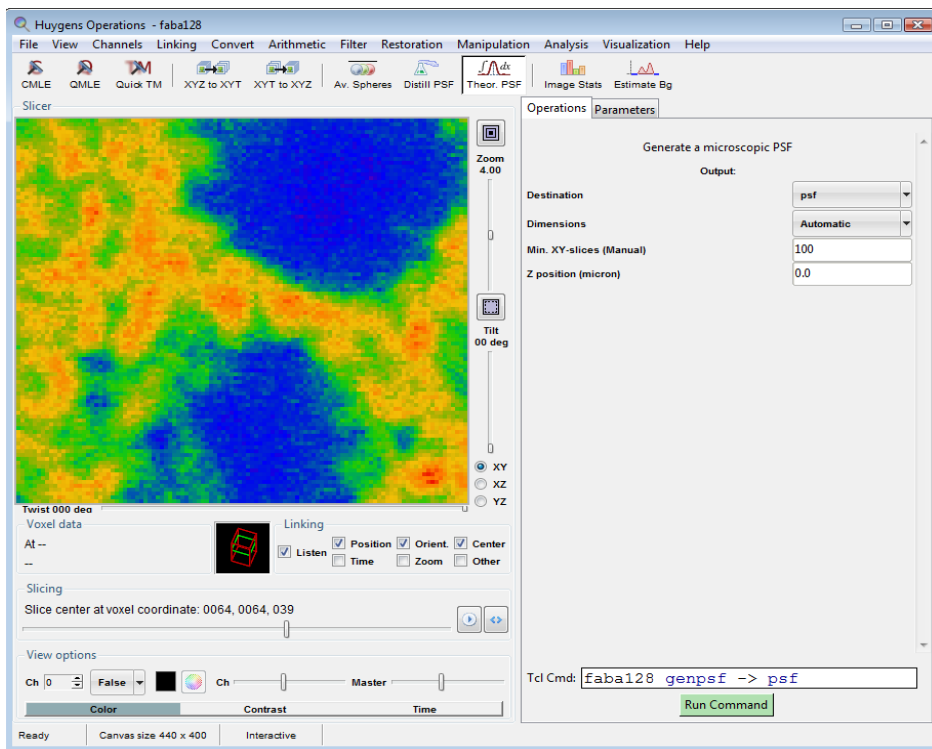
### The Operations Window

In the former steps all operations were performed without any ‘operation parameters’, i.e. variables or options applicable to a particular function. 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. However, most operations, like adding some constant value to an image, need extra input data. This can be done in the ‘operations window’, which can be accessed via **TOOLS → OPERATIONS WINDOW** in the main menu (Figure 3.7).

In the operations window, an operation can be selected from the menu bar or from the quick access buttons. Note that the left side of the operation window is similar to the Slicer. Each function can be controlled by editing the parameters in the ‘operations’ panel. To execute the operation, press the **RUN** button. Note that the corresponding Tcl command is displayed at the bottom of the panel and, after execution, also in the Tcl shell of the main window.

Using the operations window, it is also possible to change or convert the dimensions of an image. By selecting a menu item under **CONVERT**, you can e.g. change xyz into xyt or convert a 3D stack into a 4D time series.

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



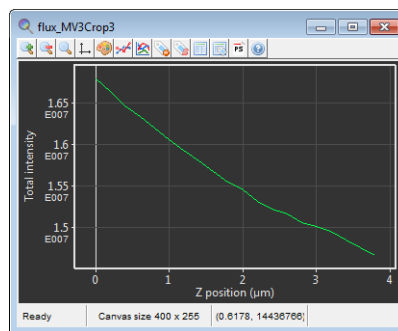
**FIGURE 3.7.** The operations window where a Theoretical PSF is selected. Various operations can be selected from the menu bar or the quick access buttons.

Most used conversion tools are also available using the Convert tool in the main window through TOOLS → CONVERT...

**Intermezzo: Bleaching Correction.**

This extra check is given here for the sake of completeness for those using this schematic approach for widefield images or confocal time series.

From the menu in the operations window, select ANALYSIS→PLOT FLUX. A typical example is shown in Figure 3.8. Note that the demo image 'faba128' is a regular confocal image; a bleaching correction test makes no sense here.

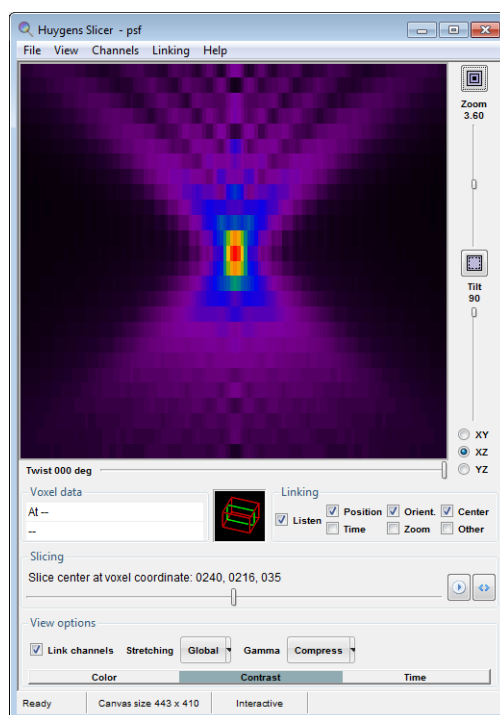


**FIGURE 3.8.** A flux plot generated by the 'Plot flux' tool. The plot shows a typical example of the intensity distribution along the z axis of a bleached widefield image.

---

### Step 4: Generate a Point Spread Function (PSF)

The point spread function (PSF) is the way in which an imaginary, infinitesimal small light source would be sampled by your microscope. Often this object is no longer a point but blurred and spread out. The aim of the PSF generation is to estimate the amount of blurring along the  $x$ ,  $y$ , and  $z$  axes. In the final step of deconvolution the PSF is used to come to a measured deconvolution, in contradiction to the so-called 'blind deconvolution'.



**FIGURE 3.9.** The PSF in x-z view. False coloring and a compressed contrast highlight the low intensity values showing the typical 'diabolo shape' in lateral view.

A PSF can be obtained either by *recording small beads* with known bead diameter --180 nm beads work fine for e.g. confocal and widefield images-- and reconstructing a measured PSF from the bead image, or by *calculating a theoretical PSF* from the information about your microscope settings. Huygens Professional has many tools to handle experimental PSF's, but these are beyond the scope of this user guide.

A theoretical PSF can be generated by clicking the button Theor. PSF from the 'operations' panel in the operations window. This function computes a PSF from the microscopic parameters. By default, the result will be displayed in the image named 'psf', but any available image can be selected as destination. Pressing the RUN button will execute the function. The Slicer can be used to examine the generated PSF in more detail (Figure 3.9).

Note that although this step gives you insight in the PSF, it is actually better letting the deconvolution run generate the PSF on the fly. Especially while using large images that are processed brick-wise (see "Brick-Wise Processing" on page 181), 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 a time instead for all at once.

All images can be cleared using the command EDIT→CLEAR IMAGE in the main window. You can do this for the image 'psf' as it will not be used in the next steps.

---

### Step 5: Estimate the Average Background Value

Huygens estimates the average background in a volume image. The average background is thought to correspond with the noise free equivalent of the measured (noisy) image. It is important for the search strategy that the microscopic parameters of the image are correct, especially the sampling distance and the microscope type are of importance.

The average background estimation is invoked by selecting ANALYSIS→ESTIMATE BACKGROUND from the menu in the operations window. The following choices are possible:

- *Lowest value (Default)*

The image is searched for a 3D region with the lowest average value. The axial size of the region is about 0.3  $\mu\text{m}$ , the lateral size is controlled by the 'radius' parameter which is set to 0.7  $\mu\text{m}$  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 a planar region with the lowest average value. Again, the size of the region is controlled by the 'radius' parameter.

In case of the image 'faba128', the best choice is 'Lowest value', because we are looking for the background outside the object in a confocal image. After pressing the RUN button the calculated value is displayed in the 'Task reports' panel of the main window. It is one of the values that should be known in the next steps.

Note that like PSF generation, this step is automated in the deconvolution run (step 7) too.

---

### *Step 6: Estimate the Signal to Noise Ratio (SNR)*

The signal to noise ratio (SNR, or S/N) is used as a parameter that controls the sharpness of the deconvolution result. It is advised to estimate these values manually, because automatic SNR estimation is tricky. The easiest way to obtain some SNR is to look at the textures of bright areas in your object image. Figure 28.1 on page 182 shows examples of an image having different noise levels. Inspect your image and decide if your image is noisy (SNR wiki, which is accessible in a fast way by typing the command `help SNR` in the Tcl shell).

---

### *Step 7: Perform a Deconvolution Run*

In the 'operations' panel, there are different deconvolution methods to choose from. These choices are 'Classic Maximum Likelihood Estimation' (CMLE), 'Quick Maximum Likelihood Estimation' (QMLE), Good's roughness Maximum Likelihood Estimation (GMLE), and 'Quick Tikhonov-Miller restoration' (Quick-TM). Quick-TM is intended for very special circumstances only. CMLE is the most general restoration method available and should be used in most circumstances. For more information on these restoration methods, visit the SVI wiki<sup>5</sup>.

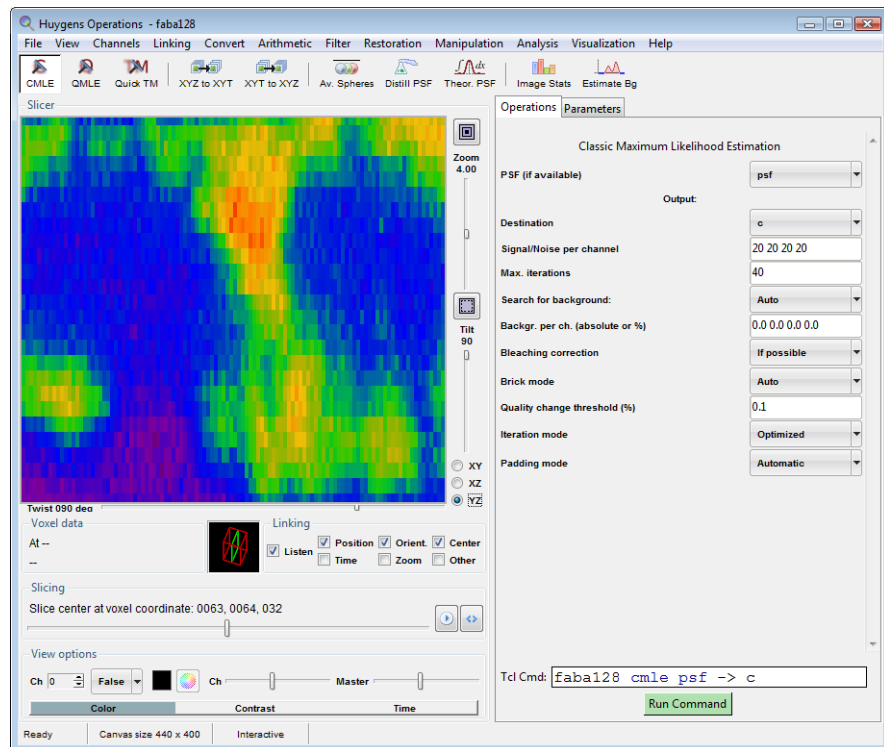
After clicking the CMLE button, the following parameter values can be adapted (Figure 3.10).

- *PSF (if available)*

From the list of all opened images, an image can be selected that is used as the PSF. This can be either a measured PSF or an earlier generated PSF (using the PSF button from the operations window). If you do not use a measured PSF, it is recommended to select an empty image. In this case, a PSF is 'not available' and the software uses the microscopic parameters to generate PSF's on the fly.

---

5. <http://www.svi.nl/RestorationMethod>

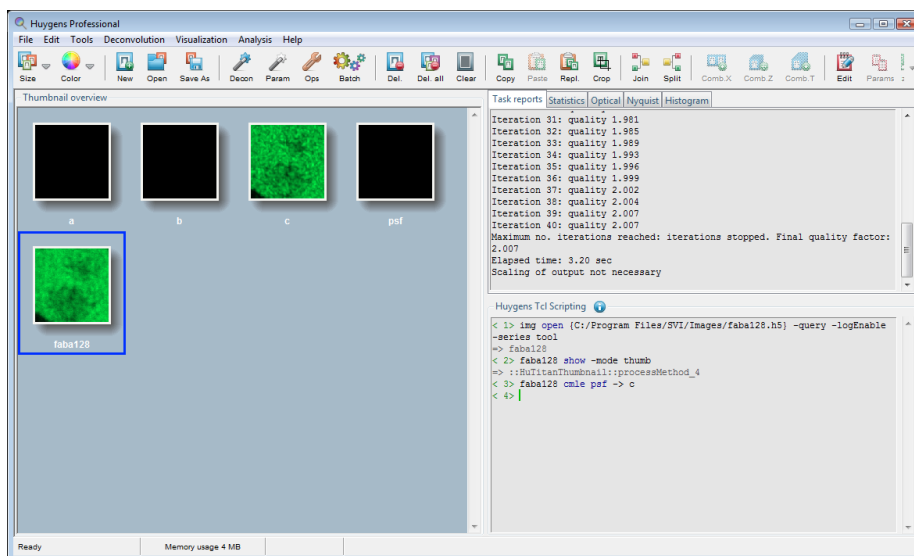


**FIGURE 3.10.** The operations window where CMLE is selected. The right panel shows the parameters for the CMLE deconvolution run.

- *Destination*  
The destination image in which the result will be stored.
- *Signal/Noise per channel*  
This is the value that was estimated in step 6.
- *Max. iterations*  
The deconvolution process will stop when the maximum number of iterations is reached or when the increase of quality drops below the 'Quality change threshold'. Depending on initial quality of your image, a higher number of iterations will result in a better restoration result.
- *Search for background*  
The option 'Auto' will choose the best estimation mode for you and calculates the background value. In 'Manual' mode the background value should be specified in the field below. This value was calculated in step 5.
- *Backgr. per ch. (absolute or %)*  
Multi channel images may have different background values per channel. If the previous parameter was set to 'Manual', this list of values holds the background for each of the channels. 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.
- *Bleaching correction*  
If this parameter is set to 'If possible', bleaching correction will be performed for widefield images or confocal time series showing exponential behavior.

- *Quality change threshold*  
This number gives the minimum quality increase between subsequent iterations. If the increase of quality drops below the 'Quality change threshold', the deconvolution process will stop. The lower the threshold value and the larger the number of iterations, the higher is the quality of deconvolution.
- Other parameters like 'Brick mode'<sup>6</sup>, 'Iteration mode'<sup>7</sup>, and 'Padding mode'<sup>8</sup> are best kept in their default modes. More information on these modes can be found in the SVI wiki pages.

Pressing the RUN COMMAND button will end up with a restored result in the destination image (Figure 3.11).



**FIGURE 3.11.** The main window with a deconvolved image 'c'. Note that the 'Task reports' panel shows information on the deconvolution process.

You may compare this result with the original image using two linked Slicers (see step 3 on page 12).

## Step 8: Saving your Image

If you are done and wish to save the result, select the thumbnail of the restored image and save the file by selecting FILE→SAVE AS from the menu in the main window. There are different formats to choose from.

### Saving your deconvolution template

In the *Operations Window*, you can find next to the RUN COMMAND button the button EXPORT TO TEMPLATE. This button opens an *Edit Deconvolution Template* window. Here, you can change the deconvolution settings. More information on these settings can be

6. <http://support.svi.nl/wiki/BrickSplitting>
7. <http://support.svi.nl/wiki/IterationMode>
8. <http://support.svi.nl/wiki/PaddingMode>

---

found under “Deconvolution Templates: Configuring the Restoration Process” on page 52. Upon clicking on ACCEPT the settings are saved to a template file. Template files are saved in the directory as defined under the directories tab in the *Preferences* window, which can be found under EDIT→PREFERENCES.

### **Why automatic deconvolution?**

If the procedure is not automated, what should you do to deconvolve an n-channel image? You will have to 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 Huygens Professional, it is a time consuming task. Not to speak about multi channel time series!



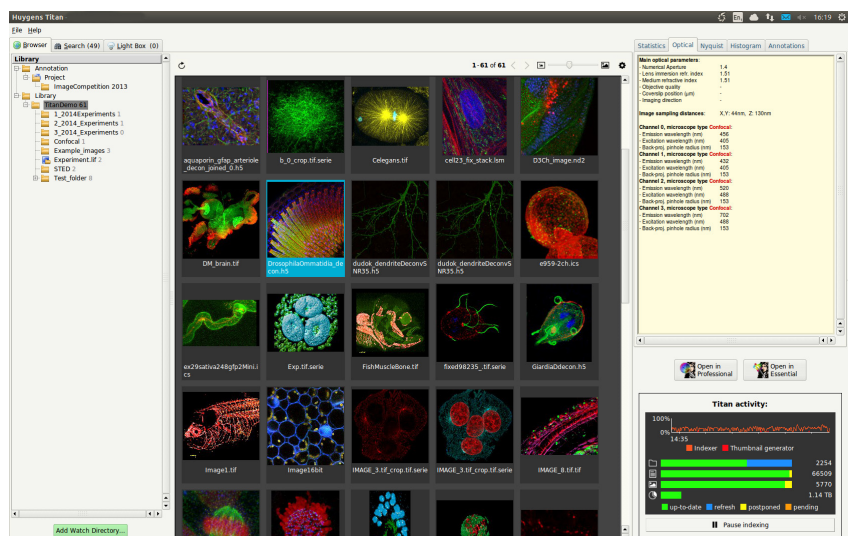
How to deal with my growing amount of image data and how to find my images fast without too much effort. These are questions that most microscopists will face at some point.

Related questions like: How can I add keywords and experimental information to images without modifying them; How to get a quick overview of the acquisition parameters; Can I group images into projects without actually editing or moving the raw data; I am not looking forward to re-organize my data and use a database approach, what alternative do I have.

If you recognize yourself in these questions, you may find *Huygens Titan* useful.

### *Titan as Freeware*

Titan is not modifying or moving your valuable microscopy data. It indexes your files and gives you a quick overview of all your images with thumbnails, metadata, histograms, statistics, and annotations (Figure 4.1). Titan reads the common microscope file



**FIGURE 4.1.** Huygens Titan shows microscopy data in *Freeware mode*.

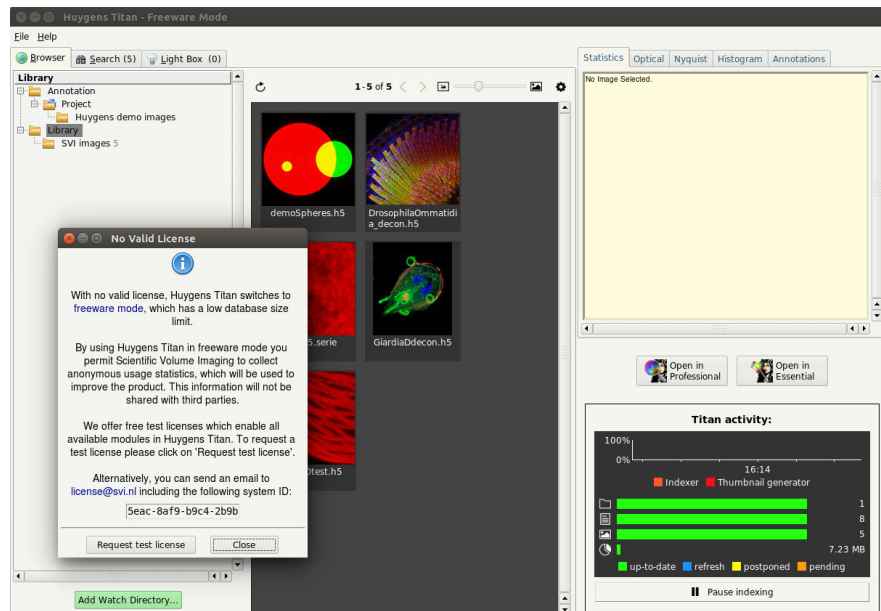
formats, and shows embedded images from container formats such as Leica LIF and Zeiss CZI. Titan has no problem with the location of the images. Microscopy data may have been saved centrally, located on external hard disks or USB drives, and spread over multiple file storage locations. The *Freeware version* of Titan has a capacity of 200 Gigabyte image data. If you need more than that, you can contact us for a test license (see “Contact Information” on page 201).

### *Titan does not edit or move your valuable image data*

There is no need to go through all your existing data if you want to organize your images with Titan. Titan simply does that for you! Just start by specifying in Titan the location of your images. This can be even multiple locations. Titan just visits the images and indexes them automatically, smartly focusing on images that you want to see first. You can quickly search for specific images across all indexed storage locations with search terms including file or folder names, Nyquist values, and/or metadata properties such as channel number, excitation/emission wavelength values, and microscope type (like for example Confocal, STED, Widefield). Keywords and comments can be added to the images, and it is possible to group images into projects. Again, this can all be done without editing the raw images and their location. Selected images can be opened in the *Light Box*, which is a viewer that allows you to inspect multi-dimensional image data (2D-5D). Exporting images to Essential and Professional for further processing is easily done with their corresponding launch buttons.

### *Starting Titan*

Click on the Huygens Titan desktop icon to get started. Titan can run in *FreeWare* mode up to 200 Gigabyte of data, and will immediately start indexing the demo images included with the Huygens Suite. You will see a window appearing for requesting free test license, with such a license you can index more files (*Terabytes* of data). See Figure 4.2. We will be happy to reply to your request and send you a test license.



**FIGURE 4.2.** Titan starts up in *Freeware mode* and can already index up to 200 Gigabyte of data. You will receive a test license with terabytes of index capacity if you send the systemID to us.

### **Define the location of your images**

Use within the main window the green ADD WATCH DIRECTORY button at the bottom-left, or look under FILE, to open the *Preferences* window for adding image storage locations (directories) to be indexed. Library name and indexing priority can be adjusted by clicking on these fields. You can also define here at what times Titan should leave enough computer resources available for other activities, and what should be the maximum file size to be indexed. The number of CPU's available for Titan can be set, as well as the storage location of where Titan should store the image thumbnails it generates.

Upon hitting the Apply button, Titan will immediately start indexing, as can be seen in the *Titan Activity* window (bottom-right). The *Library* name with the number of images is displayed in the left main window. If a location, such as for example a USB stick or external hard disk, is no longer available, a red cross will appear on a library name. Re-connecting the file storage location will remove this cross again.

Display options for colors and how the thumbnails should be sorted can be set with the settings (wheel) button located on the right above the thumbnail window. The slider next to it enables you to adjust the size of the thumbnails. If the number of images exceed one hundred they will be listed over multiple pages. Scrolling through these pages can be done with the arrow buttons.

At the right side within the main Titan window there are several tabs located with more image information of the selected thumbnail, and for annotations concerning the selected image (see "Annotations and Projects" on page 26).

### **Thumbnail messages**

There are several messages that can appear on the thumbnails. They show the indexing status of this specific image. An overview of these messages can be seen on this wiki page<sup>1</sup>.

### **Scheduling index activity**

Indexing can be a computational and time-consuming process, so if you have many data to be indexed you may need to leave Titan active for some time. However, once your images are indexed you may appreciate how fast Titan shows their thumbnails and metadata. By default, Titan runs in background mode between 7:00 and 19:00 hours on working days. Under the INDEXER tab within the *Preferences* window, you can manage these settings to save computer resources.

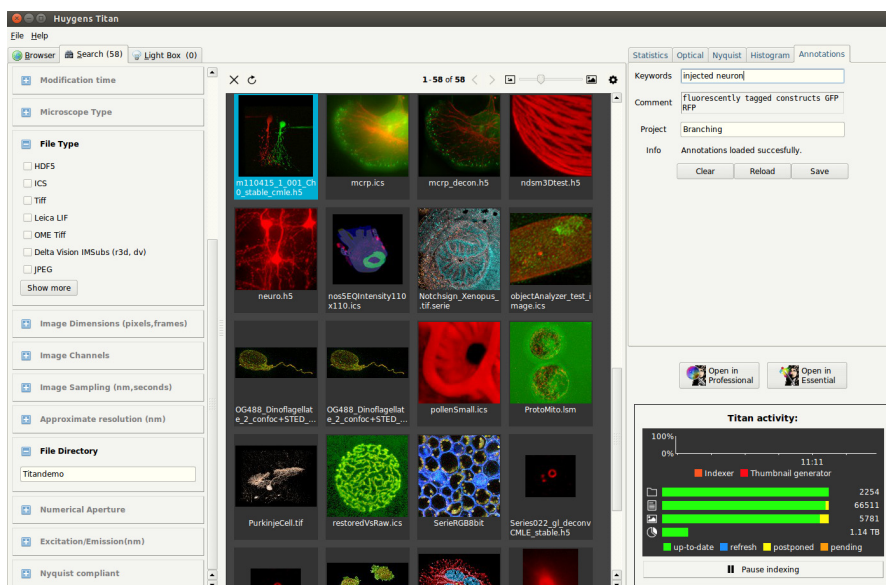
---

## *Searching images*

Titan allows you to easily search through your images using keywords and multiple metadata properties. Quick searches are available to select for images that have been acquired with correct Nyquist sampling, and for those that are multichannel (suitable for colocalization analysis), and have multiple frames (stabilization). Images can also be searched on file type, and with what microscope type they were acquired. Combinations of search terms can also be applied, and search parameters can be cleared with cross button above the thumbnail window (Figure 4.3 on page 26)

---

1. <http://www.svi.nl/TitanHelp>



**FIGURE 4.3.** Titan offers several parameters that can be set to narrow down your search for images.

## Annotations and Projects

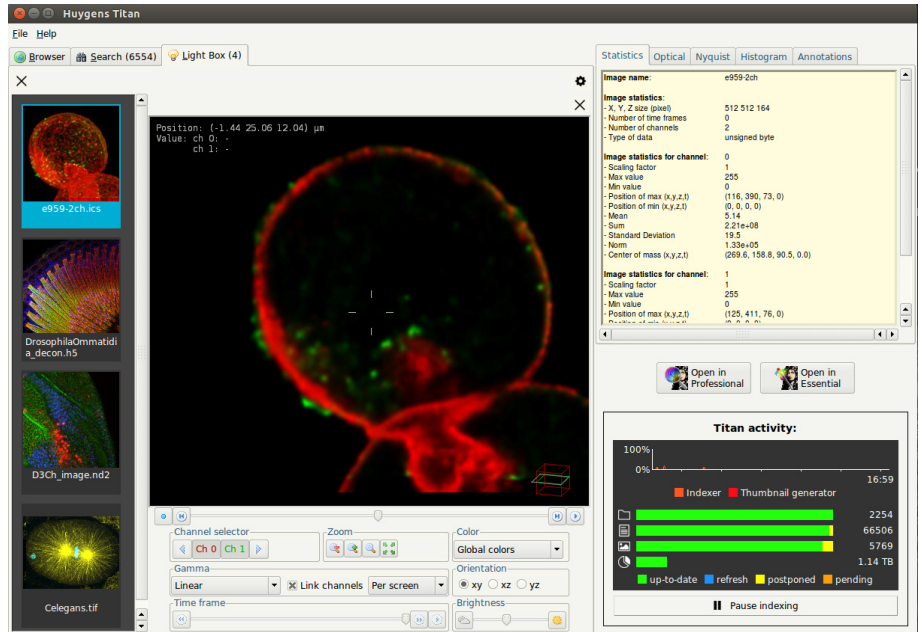
You can tag images with text, and group images into projects using the ANNOTATIONS tab (top-right). Upon selecting a thumbnail and writing a project name into the Project field under this tab, a new project will be made. This project will not be shown immediately. For that, you first need to manage the projects, which can be done in the left column "Library" by right-clicking on *Project* or on one of the existing project names. A new window *Manage Projects* will open where your new project name is shown. Other new projects can also be added or removed here, and project names can be selected for display under *Library*. You can assign multiple images to one project by specifying their project name.

## Viewing and forwarding images

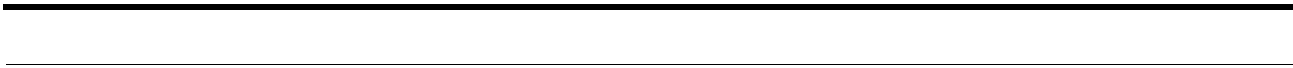
At any stage, you can double-click on a thumbnail to add the image to the *Light-box* in which another double-click allows detailed multi-dimensional viewing (Figure 4.4 on page 27). Thumbnails can be selected with the left mouse button (hold the Shift or CTRL-key to select more) and send with the right-mouse button for further processing to the File Manager, or Huygens Essential or Professional. You can also use the HUYGENS ESSENTIAL and HUYGENS PROFESSIONAL icons in the Titan for this purpose. Please note that the display of channel colors may change if the image is opened in Essential or Professional. This is due to a different color setting in the software which can be changed easily (see "Adjusting the Global Color Scheme" on page 195).

For more information and a tutorial video we like to refer you to the wiki on Huygens Titan<sup>2</sup>.

2. <http://www.svi.nl/HuygensTitan>



**FIGURE 4.4.** Slicing through 2D-3D time series and multichannel images is images in Titan *Light box*.



## *Deconvolution Wizard*

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### *The Processing Stages in the Wizard*

The deconvolution wizard guides you through the process of microscopic image deconvolution in several stages.

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

The following steps and stages are to be followed:

- Loading an image.
- Verifying microscopy parameters.
- Start Stage: here, an overview is given of the most important parameters for ensuring optimal deconvolution. You can continue with using the wizard or applying a deconvolution template.
- Loading a measured PSF that has been distilled from bead images.
- Preprocessing Stage: this stage goes through all preprocessing steps.
  - Cropping stage
  - Stabilization (STED data)
  - Select channel
  - Inspecting the image histogram.
  - Background Estimation
- Deconvolution Stage:
  - Setting the final deconvolution parameters.
  - Run the deconvolution.
  - Select final result
- Post-processing Stage: correct for Z-drift if any and accept the result.
- End Stage: Option to Restart, Save deconvolution template and Finish.

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

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## Loading an Image

Select FILE→OPEN or the OPEN icon in the Huygens main window to open the file dialog, browse to the directory where the images are stored, and select the image, for example faba128.h5. A demo image (faba128.h5) is placed in the Images subdirectory of the installation path (see Table 2.1 on page 7). For searching your image, you may find Huygens Titan a very useful option as it shows your images as thumbnails and offers advanced search tools (see “Huygens Titan” on page 12).

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

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

### Converting a Dataset

Before applying deconvolution, it is important that the image dimensions are correctly defined. Dimensions can be changed with the conversion tools listed under the TOOL menu. For example, a 3D stack can be converted here into a 3D time series () or vice versa, or a 3D stack can be converted into a time series of 2D images () or vice versa. In case of a refractive index mismatch, it is important to set the orientation of the image correctly with respect to the imaging direction and also to the coverslip position (). The MIRROR tab in the converter tool can also be used for that purpose. Read more at “Setting the Coverslip Position” on page 196.

### Time Series

A time series is a sequence of images recorded at uniform time intervals. Each recorded image is a time frame. Huygens Professional is capable of deconvolving 2D-time or 3D-time data automatically with the *Time option* (see Table 31.2 on page 203). There are some tools that are intended only for time series, such as the widefield *bleaching corrector* or the *z-drift corrector*.

### Adapting the image

In the menu, you can find under a contrast converter () which is required for the processing of brightfield images (See “Brightfield Images” on page 198.). A Crop tool is also available, but its use is recommended only after properly tuning the image parameters. This tool will be explained at a later stage in this manual.

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

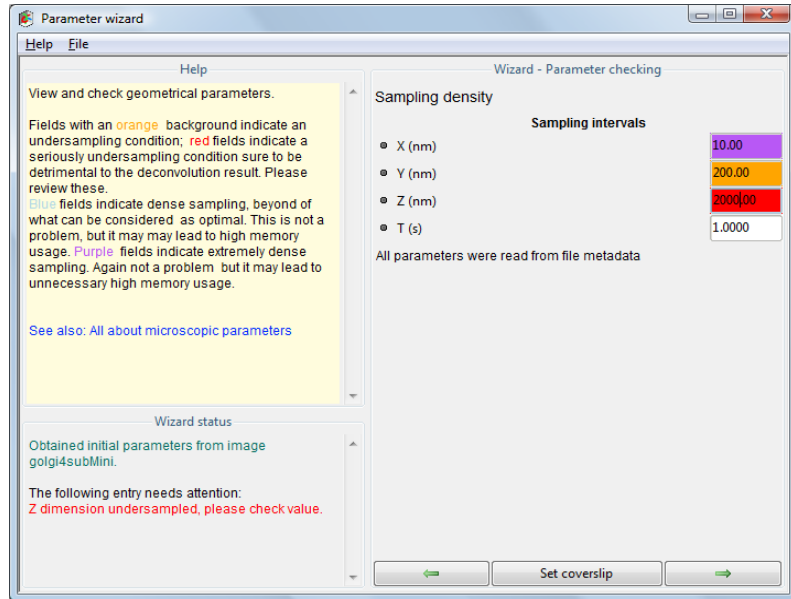


## Verifying Microscopy Parameters

**TABLE 5.1. Optical parameters explained.**

Parameter	Explanation
Microscope type	Select from <i>widefield</i> , <i>confocal</i> , <i>spinning disk</i> , <i>4Pi</i> or <i>STED</i> , <i>SPIM</i> , or <i>unspecified</i> . For a multiphoton microscope insert an <i>excitation photon count</i> higher than 1. Select <i>widefield</i> if a nondescanned detector was used, and <i>confocal</i> in case a pinhole of less than a few Airy disc units was used.
Numerical aperture	The NA of the objective lens.
Objective quality	Select from perfect, poor, or something in between.
Coverslip position	The <i>position of the glass interface</i> relative to the nearest slice of the stack. See also page 196
Imaging direction	Select from <i>upward</i> or <i>downward</i> . Upward means that the objective lens is closest to the bottom slice in the stack. Upward stands for an inverted microscope where the objective points upward.
Backprojected pinhole radius	The radius (in nm) of the pinholes in the spinning disk as it appears in the <i>specimen plane</i> . This is the physical pinhole radius divided by the total magnification of the detection system.
Backprojected pinhole spacing	The distance (in $\mu\text{m}$ ) between the pinholes in the spinning disk as it appears in the <i>specimen plane</i> . This is the physical pinhole distance divided by the total magnification of the detection system.
Lens refractive index	The RI of the immersion medium for the objective lens.
Medium refractive index	The RI of the specimen embedding medium.
Excitation wavelength	The wavelength (in nm) of the excitation light (usually a laser line).
Emission wavelength	The wavelength (in nm) of the emitted light.
Excitation photon count	Number of photons used in <i>multi-photon</i> microscopy.
Excitation fill factor	The width of the beam relative to the aperture. The default value is 2, meaning that the aperture has a diameter of $2\sigma$ , where $\sigma$ is the standard deviation of the Gaussian distribution in the beam.
STED depletion mode	This mode determines the type of STED depletion that is being used. You can set this to Pulsed, CW non-gated detection, or CW gated detection.
STED saturation factor	Factor describing how much the fluorescence is suppressed by the STED beam. <sup>a</sup>
STED wavelength	Wavelength of STED depletion laser beam (nm)
STED immunity fraction	Fraction of fluorophore immune (photoresistant) to the depletion beam.
STED 3X	Percentage of laser power used for the Z depletion beam, The remaining part is used for the vortex beam
Deviating microscope type	The deviating microscope type for the current channel.

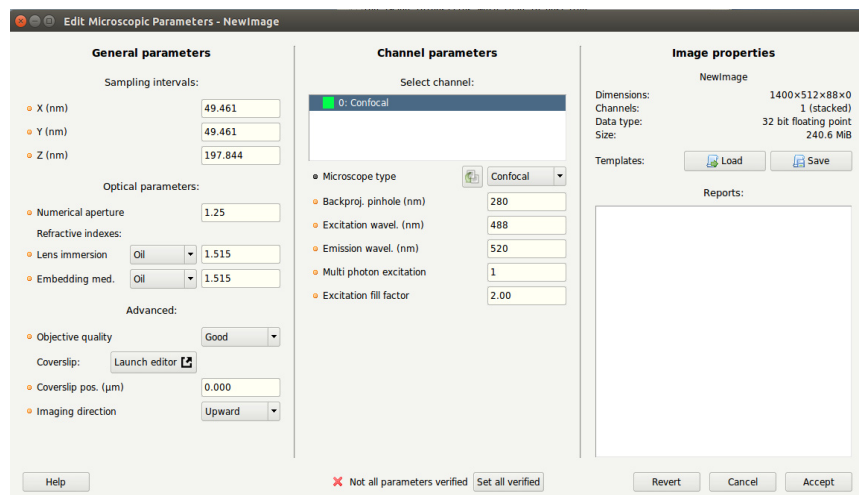
a. Chapter 8 on page 55



**FIGURE 5.1. Parameter wizard: check sampling density. Red coloring indicates a suspicious value, and orange a non-optimal value.**

By using the parameter wizard or the parameter editor, one can modify the parameters that are explained in Table 5.1. If values are displayed in a *red* background, they are highly suspicious. An *orange* background indicates a non-optimal situation (See Figure 5.1). Oversampling is also indicated with a *cyan* background, that becomes *violet* when it is significant.

The editor and wizard can be found by right-clicking on the image thumbnail and selecting or PARAMETER WIZARD. The wizard is also in the DECONVOLUTION menu. Figure 5.1 shows the *Parameter Wizard*. The *Parameter Editor* is shown in Figure 5.2.).



**FIGURE 5.2. The microscopy parameter editor. This window can be opened by right-clicking on the thumbnail and selecting .**

## Microscopic Template Files

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

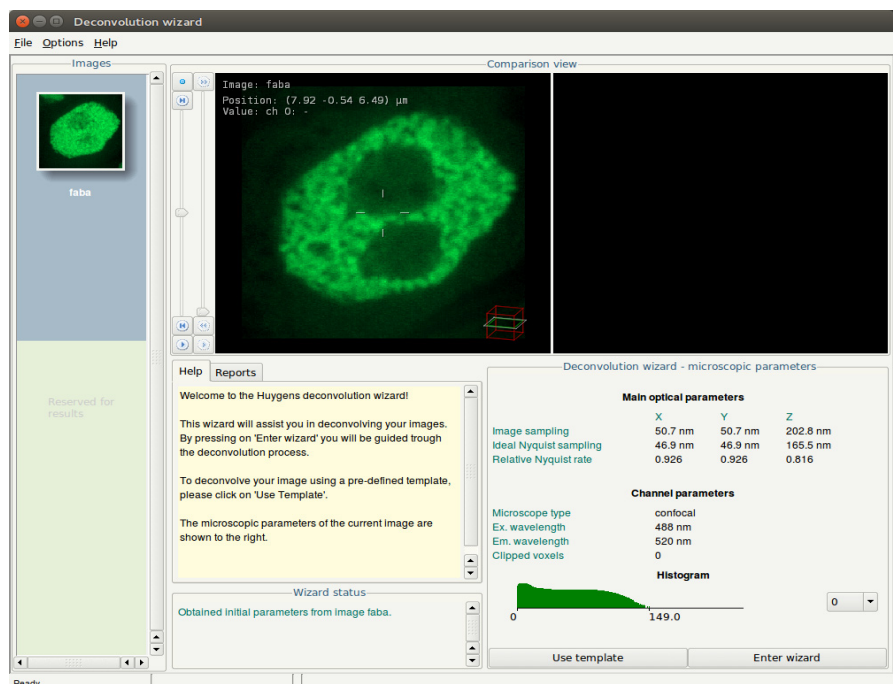
The LOAD button allows the selection of a template from a list of template files. The Huygens common templates directory is named Templates, and resides in the Huygens installation directory (See Table 2.1 on page 7). The user's personal templates directory is called SVI/Templates and can be found in the user's home directory<sup>2</sup>.

## Starting the Deconvolution Wizard

Once all microscopy parameters have been verified, you can start the *Deconvolution Wizard* by pressing the deconvolution wizard icon in the main task bar, by selecting the wizard from the DECONVOLUTION menu, or by using the right mouse button on a thumbnail and selecting the DECONVOLUTION WIZARD option from the menu that appears.

If you prefer to generate a *point spread function* from measured beads first, select PSF DISTILLER in the DECONVOLUTION menu (See Chapter 6 “The PSF Distiller” on page 43). A special license is needed for activating the PSF Distiller option.

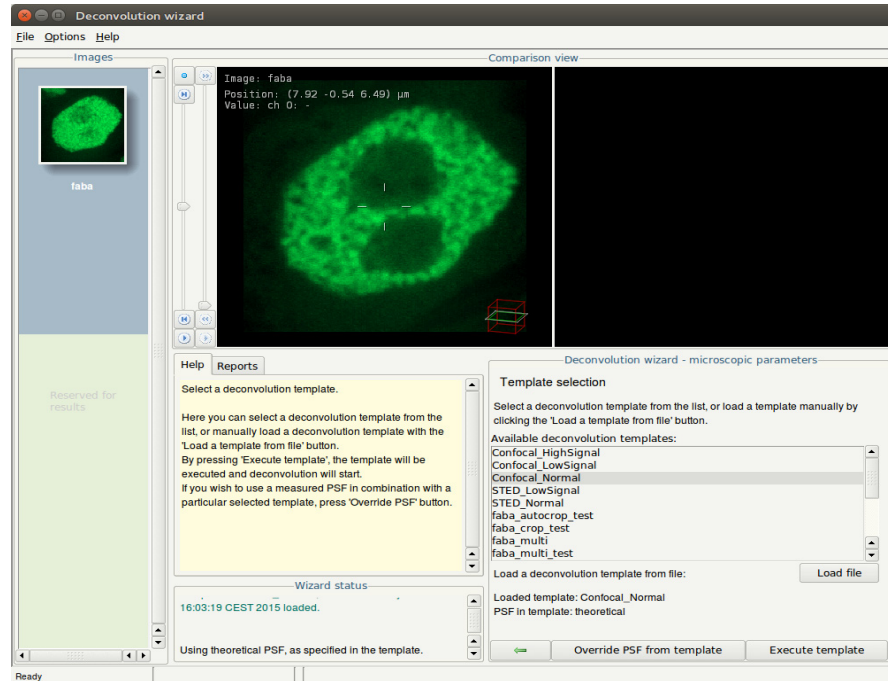
The first window of the *Deconvolution Wizard* shows a summary of the most important parameters for ensuring optimal deconvolution. Review them carefully before proceeding if you have not done so already (see Figure 5.3). You can continue with using the deconvolution wizard or you can execute a deconvolution template..



**FIGURE 5.3.** Starting window of the *Deconvolution Wizard* with a summary of the most essential parameters.

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

The latter option will show a list of available templates and a button to load a template file. Below the list of files is shown whether the template considers an experimental or a theoretical PSF (Figure 5.4). You can overrule this by using the button **OVERRIDE PSF FROM TEMPLATE**. A next screen, will give you the option to select an experimental PSF.



**FIGURE 5.4.** Deconvolution templates can be selected from the list or can be loaded. A template specifies if an experimental or theoretical PSF is used, but this can be overruled with the button **OVERRIDE PSF FROM TEMPLATE**.

### Using a Measured PSF

Measured PSF's improve the deconvolution results and may also serve as a quality test for the microscope. If the measured PSF contains less channels than the image, a theoretical PSF will be generated for the channels that have no available PSF. See Chapter 6 “The PSF Distiller” on page 43 and “The Point Spread Function” on page 193 for more information.

A previously created measured Point Spread Function (PSF) can be loaded from a file or directly loaded from the main Professional window. A measured PSF should only be used for deconvolution if the image and the bead(s) were recorded with the same microscope at the same parameter settings. The remaining steps in the wizard will be skipped when selecting the button **EXECUTE TEMPLATE**.

### The Intelligent Cropper

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

Huygens Essential and Professional are equipped with an intelligent cropper which automatically surveys the image to find a reasonable proposal for the crop region (See Figure 5.5). 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 parameters it is recommended to use the cropper as final step in the preprocessing stage (press YES when the wizard asks to launch the cropper), but it can also be launched from outside the wizard through the menu .

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

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

The three views shown are *maximum intensity projections* (MIPs) along the main axes. For more information about these projections read Chapter 12 “The MIP Renderer” on page 75. By default the entire volume (including all time frames) is projected. The red, yellow, and blue triangles can be dragged to restrict the projected volume.

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

### Cropping in Time and removing channels

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

All available channels are listed under CHANNELS in the *Operation Window* and can be removed individually. By using the right-click on a channel check box, all channels can

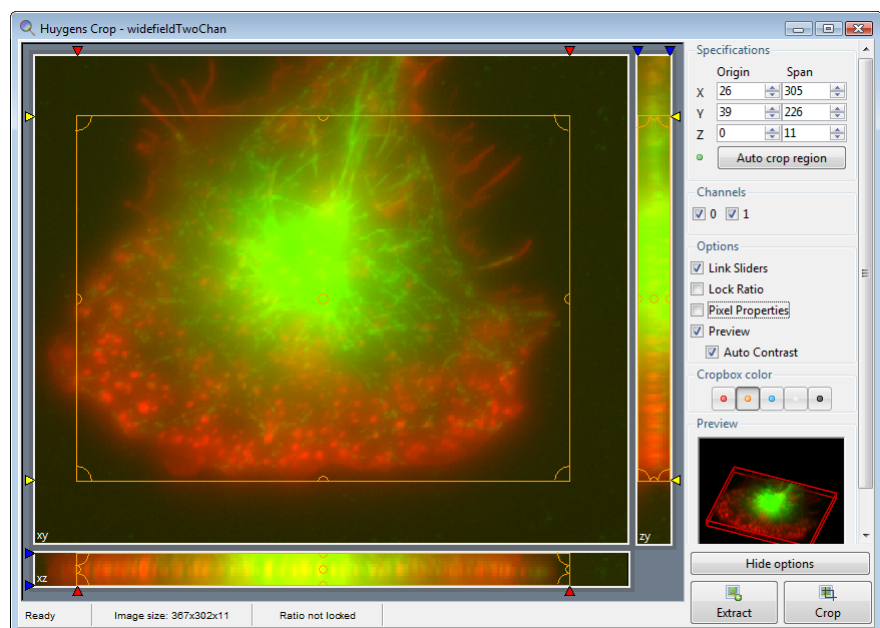


FIGURE 5.5. The crop tool.

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## Stabilizing STED data

be turned on or off, except for the selected channel. In the main window you can separate the image into individual channels using SPLIT, which can be found under the TOOLS menu or as a taskbar icon.

### Cropper Customizations and Options

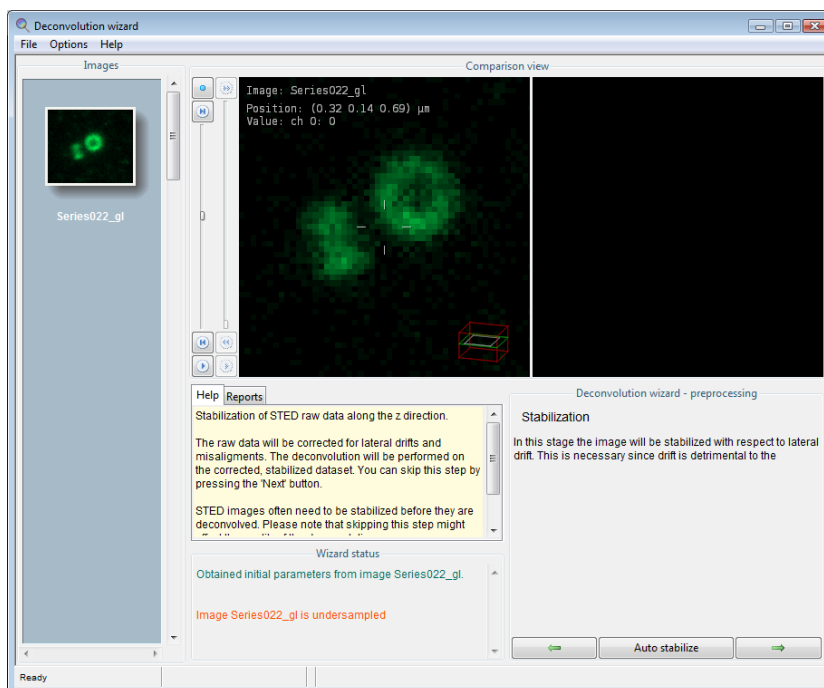
The cropper is extended with extra visualization options available under OPTIONS. LOCK RATIO fixes the aspect ratio of the cropping box for each projection separately. PIXEL PROPERTIES shows the pixel intensities and position of the mouse if you hover over the image. The intensities, however, are only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

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

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

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

To stabilize the image in the Deconvolution Wizard just click on the “AUTO STABILIZE” button at the “STABILIZATION” stage (See Figure 5.6).



**FIGURE 5.6.** Stabilization stage for STED data in the Deconvolution Wizard.

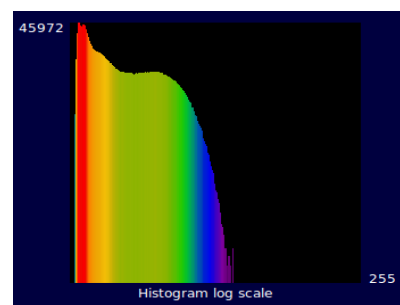
## The Image Histogram

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

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

The histogram in Figure 5.7 shows that the intensity distribution in the demo image is of reasonable quality. The narrow peak shown at the left represents the background pixels, all with similar values. The height of the peak represents the amount of background pixels (note that the vertical axes uses *logarithmic* scaling).

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



**FIGURE 5.7.** The image histogram. The vertical mapping mode can be selected from linear, logarithmic or sigmoid.

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 allowed digital value. Usually, all values above the maximum value are replaced by the maximum value. On rare occasions they are replaced by zeros. Clipping will have a negative effect on the results of deconvolution, especially with widefield images.

The histogram stage is included in the deconvolution wizard for examining purposes only. It does not affect the deconvolution process that follows<sup>3</sup>.

## Estimating the Average Background

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

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

- **Lowest value** (default): The image is searched for a 3D region with the *lowest average value*. The axial size of the region is about 0.3  $\mu\text{m}$ ; the lateral size is controlled by the radius parameter which is by default set to 0.7  $\mu\text{m}$ .

3. Learn more about histograms at <http://www.svi.nl/ImageHistogram>

- **In/near object:** The *neighborhood around the voxel with the highest value* is searched for a planar region with the lowest average value. The size of the region is controlled by the radius parameter.
- **Widefield:** First the image is searched for a 3D region with the lowest values to ensure that the region with the least amount of blur contributions is found. Subsequently the background is determined by searching this region for the planar region with radius  $r$  that has the lowest value.

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

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

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

The last option determines the deconvolution algorithm. The default is *Classical Maximum Likelihood Estimation* (CMLE). In case of widefield images, it can also be set to *Quick Maximum Likelihood Estimation* (QMLE), as the QMLE can typically be applied to images with a high signal-to-noise level. For STED images you have the option to use either the CMLE or the *Good's roughness Maximum Likelihood Estimation* (GMLE) algorithm. So, the wizard offers a different set of algorithms depending on what microscope type is used. Within the Operation window in Huygens Professional, you can choose either one of these algorithms or the *Quick-MLE-time*, *Quick-Tikhonov-Miller*, and *Iterative Constrained Tikhonov-Miller* algorithms.<sup>4</sup>

If done press ACCEPT to proceed to the deconvolution stage.

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## The Deconvolution Stage

Figure 5.8 shows the deconvolution stage where the deconvolution parameters can be set. The following parameters are available for the CMLE algorithm. The options for QMLE are almost the same, except that the Quality threshold and the Iteration mode are not available.

1. **Number of iterations.** MLE is an iterative process that never stops if no *stopping criterion* is given. This stopping criterion can simply be the maximum number of iterations. This value can be adjusted, depending on the desired final quality of the image. For an initial run the value can be left at its default. To achieve the best result this value can be increased to e.g. 100. Another stopping criterion is the *Quality threshold* of the process (See Item 3).
2. **Signal to noise ratio.** The SNR is a parameter that controls the sharpness of the restoration result. Using a too large SNR value might be risky when restoring noisy originals, because the noise could just be enhanced. A noise-free widefield image usually has SNR values higher than 50. A noisy confocal image can have values lower than 20. See also "Signal to Noise Ratio" on page 181

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4. <http://www.svi.nl/RestorationMethod>



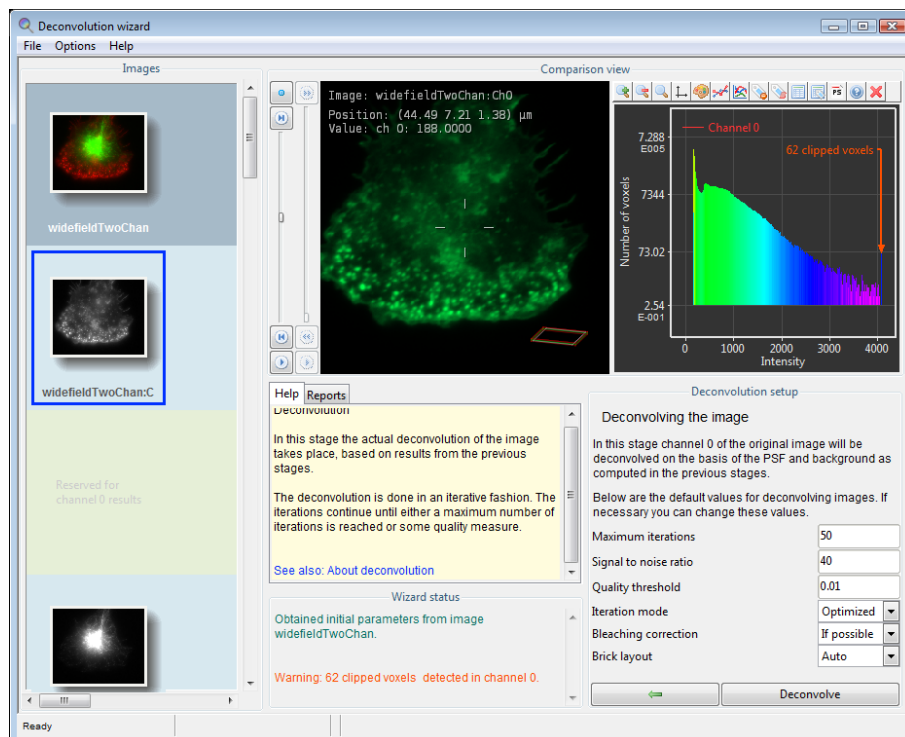


FIGURE 5.8. The Deconvolution Stage in the wizard.

3. **Quality threshold.** Beyond a certain amount of iterations, typically below 100, the difference between successive iterations becomes insignificant and the progress grinds to a halt. Therefore it is a good idea to monitor the progress with a quality measure, and to stop the iterations when the change in quality drops below a threshold. At a high setting of this quality threshold, e.g. 0.01, the quality difference between subsequent iterations may drop below the threshold before the indicated maximum number of iterations has been reached. The smaller the threshold the larger the number of iterations that will be reached; the higher the quality of the restoration. Still, the extra quality gain becomes very small at higher iteration counts. The absolute value of the final quality factor much depends on the data, the microscope type, and the background. It is a global value computed over the entire image, so the contribution of a local resolution increase can be small, as such it can only be used in a relative way to compare iterations and should not be seen as a value to compare the quality of different images.
4. **Iteration mode.** In *optimized* mode (highly recommended) the iteration steps are bigger than in *classical* mode. The advantage of classical mode is that the direction of its smaller steps is sure to be in the right direction; this is not always the case in optimized mode. Fortunately, the algorithm detects if the optimized mode hits upon a sub optimal result. If so, it switches back to the classical mode to search for the optimum.
5. **Bleaching correction.** If this option is set to *if possible*, then the data is inspected for bleaching. 3D stacks and time series of widefield images will always be corrected. Confocal images can only be corrected if they are part of a time series, and when the bleaching over time shows exponential behavior.
6. **Brick layout.** When this option is set to *auto*, then Huygens Professional splits the image into bricks in two situations:
  - a. The system's *memory* is not sufficiently large to allow an image to be deconvolved as a whole.

- 
- 
- b. *Spherical aberration* is present, for which the point spread function needs to be adapted to the depth.

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

---

### *Finishing or Restarting a Deconvolution Run*

When a deconvolution run is finished, the result is shown in the right viewer, and you can choose to RESTART ALL, RESTART CHANNEL, RESUME or ACCEPT/ALL DONE the restoration:

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

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### *The Final Stage*

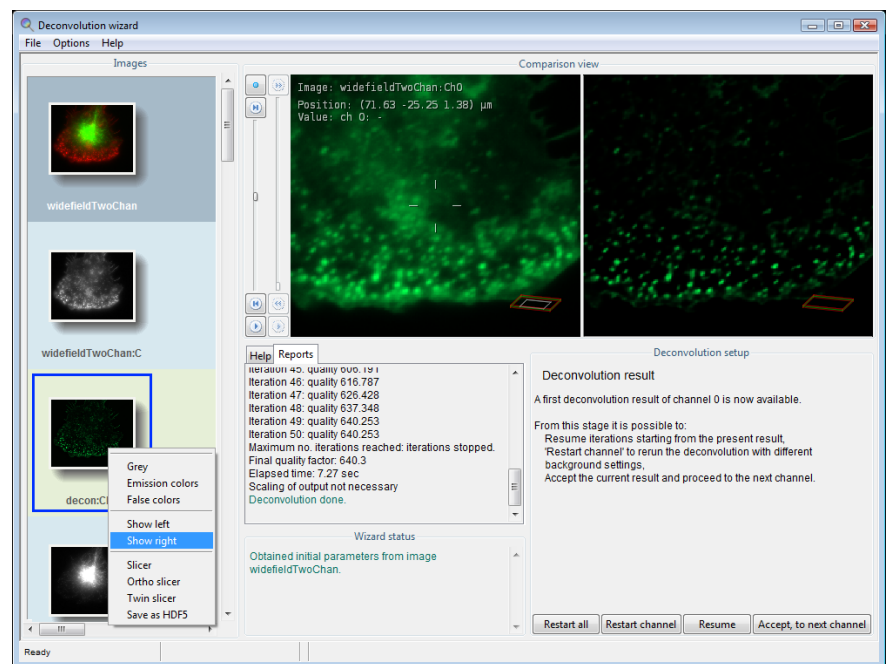
The last deconvolution result of each channel is automatically selected for the final image. By moving image names from the *Available* to the *Selected* list, you can combine channels as desired. You can use the mouse-scroll to scroll the lists. Also, when clicking on a thumbnail in the left *Images*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

What now follows is the possibility to RESTART all again (which discards the results), SAVE TEMPLATE for deconvolution, or press DONE to export the result to the main window of Huygens Professional.

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### *The Comparison View*

Detailed image comparisons of all the thumbnails in the left panel of the Wizard can be done using the *Comparison View* displays with loading images via a right-click on their corresponding thumbnails and select “Show left” or “Show right” (See Figure 5.9).



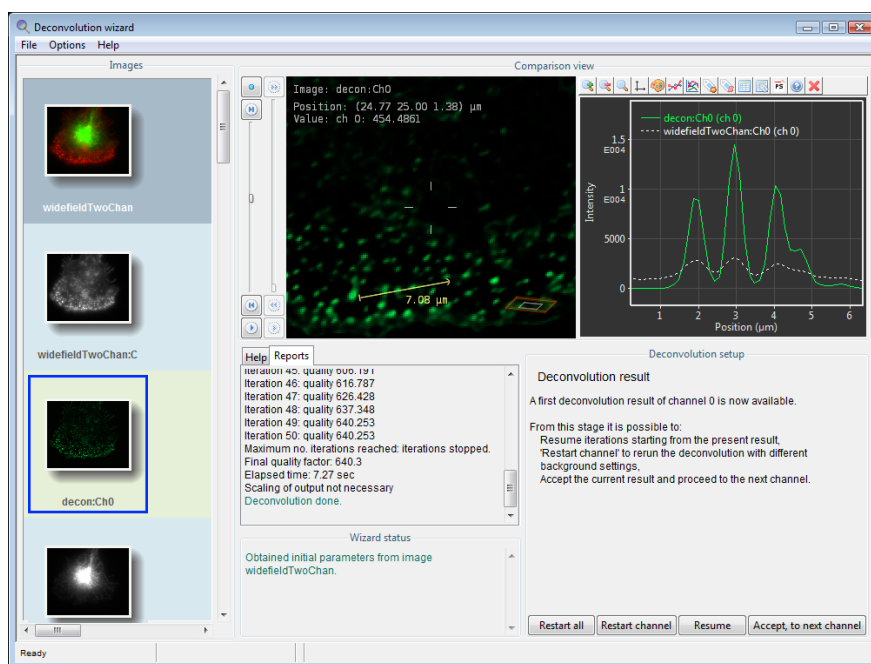
**FIGURE 5.9.** Showing an image on the Comparison View: right-click on the thumbnail and select “Show left” or “Show right”.

In order to plot the image intensities along a specific path click on the image and draw a line without releasing the mouse. A plot will be shown on the Comparison View display (left or right) opposite to where the line is drawn. To hide the plot, click a point of the Comparison View off the drawn line. To show the same plot again, click on the drawn line. This operation can be performed on both sides of the Comparison View. The plot will show the intensities of the two images loaded on the displays, for comparing purposes, see Figure 5.10 on page 42. If one of the displays is empty the plot will only show the intensities the image shown.

## Multi-channel Images

Multi channel images can be deconvolved in a semi-automatic fashion. Still, the results can be fine-tuned for each individual channel. After the preprocessing stage the multi-channel image is split into single channel images named *<imagename>:Ch0*, *<imagename>:Ch1*, etc. The first is automatically selected for deconvolution.

The procedure to deconvolve a multi channel data set is exactly the same as for a single channel image. Therefore multiple reruns on the channel can be done manually, just as with single channel data. Press ACCEPT, TO NEXT CHANNEL in the last stage, to select the following channels for deconvolution. There is the possibility to skip one or more channels.



**FIGURE 5.10.** Comparing intensity profiles of two images in the Deconvolution Wizard. Notice that the plot prompts at the display opposite to where the line is drawn.

When the last channel has been processed, the wizard offers the option to specify what channels and in what order they should be combined for the final deconvolved image..

## Z-drift Correcting for Time Series

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

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

## Saving the Result

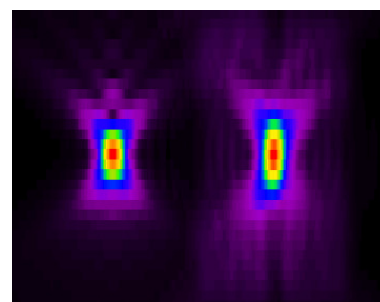
After exporting the deconvolution result to the main window, the result can be saved. Select the image to be saved and select FILE→SAVE AS... in the menu bar. The *HDF5* file format preserves all microscopic parameters and applies a lossless compression. To see which other file formats the Huygens software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

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

## The PSF Distiller

Huygens Professional is optionally equipped with the *PSF Distiller*. This wizard driven tool guides the user through the process of distilling a *Point Spread Function* (PSF) from 3D bead images. It helps in selecting bead images, and in creating and saving the PSF for further deconvolution runs. The wizard is able to measure a PSF from one or multiple images, each containing one or more fluorescent beads. It is also possible to distill multi-channel PSF's from multi-color bead images, or to assemble a multi-channel PSF from separate single channel bead images PSF's.

Measured PSF's improve deconvolution results and may also serve as a quality test for the microscope<sup>1</sup>. The measured PSF acts as a calibration of the microscope in the sense of relating a physical known object (bead) with what the microscope actually measures (bead image). Figure 6.1 shows an example of a theoretical, and a measured PSF.



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

### *Beads suited for PSF Distillation*

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

1. We advise to measure the PSF after a change in the recording setup and certainly after each maintenance job in which the optics or scanning device was serviced.
2. <http://www.svi.nl/HalfIntensityWidth>

insufficient signal for accurate averaging, resulting also in reduced accuracy. Beads ranging from 120 to 250 nm can be used. Typically beads with a diameter of 160 nm perform very well for many types of microscopy.

Confocal and two photon bead images of 60 nm beads should look like smooth fuzzy blobs with a relatively low level of noise. Use the default SNR settings. If possible, it is recommended to average 2 to 5 beads. Two photon bead images may look slightly noisy. If so, set the SNR to 20 and average 4 to 10 beads.

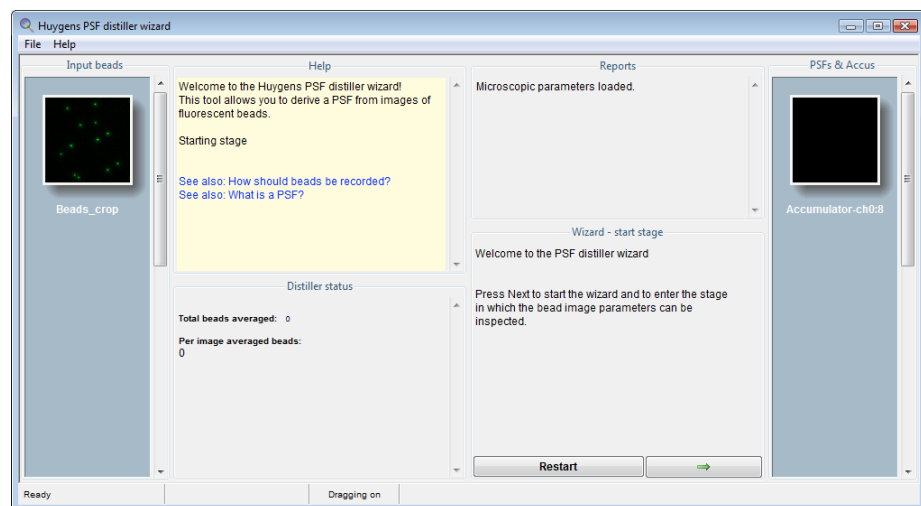
Widefield images taken from 160 nm beads should look like smooth fuzzy blobs with no visible noise. The default SNR settings can be used. It is not necessary to average any more beads.

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

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## *The PSF Distiller Window*

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



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

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

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

- *The Distiller status pane*  
Keeps track of all the steps during the distiller run and reports the progress.
- *The Reports pane*  
Displays the progress report of the distiller process and the status of the distilling process.
- *The PSFs & Accus pane*  
Displays the intermediate results of the distilling process. The thumbnails can be inspected with the Sliders. The slider on the right side can be used to scroll down if many files are displayed.
- *The Wizard pane*  
Shows the steps that will take you from checking image parameters to distilling and averaging the beads from your images.

---

### *The Processing Stages in the Wizard*

The following steps and stages are to be followed:

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

The next sections of this chapter will explain the wizard stages in detail. See “Loading an Image” on page 30 and “Saving the Result” on page 42 for more information on handling image files.

### **Starting the Distiller**

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

### **Verifying Microscopic Parameters**

See “Verifying Microscopy Parameters” on page 31 for more information on the microscopic parameters. Next to the optical parameters listed in Table 5.1 on page 31, it is in particular important to check the *sampling densities*.

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

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

## Averaging Stage

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

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

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

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

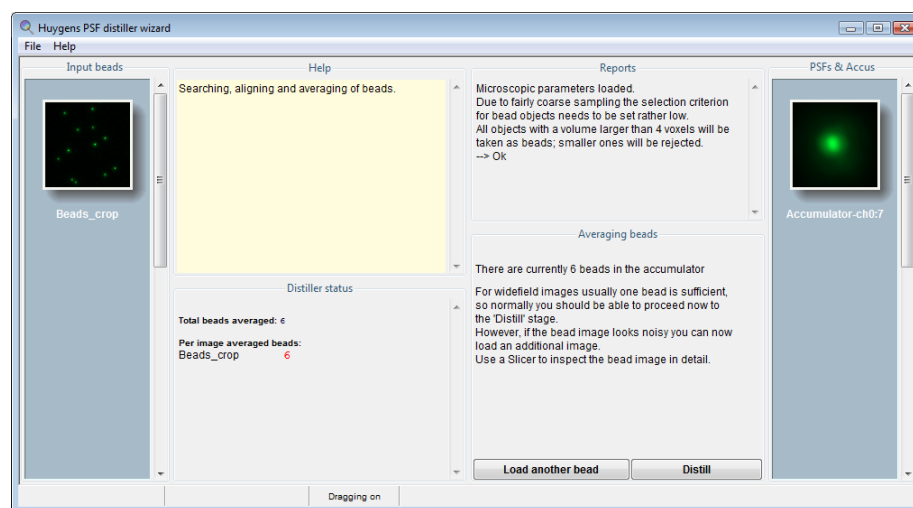


FIGURE 6.3. The averaging stage in the PSF Distiller wizard.

## Confocal and Two Photon Bead Images

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



### Widefield Bead Images

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

### Distillation Stage

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

### Finalizing the result

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

### Full-width at half-maximum estimator

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

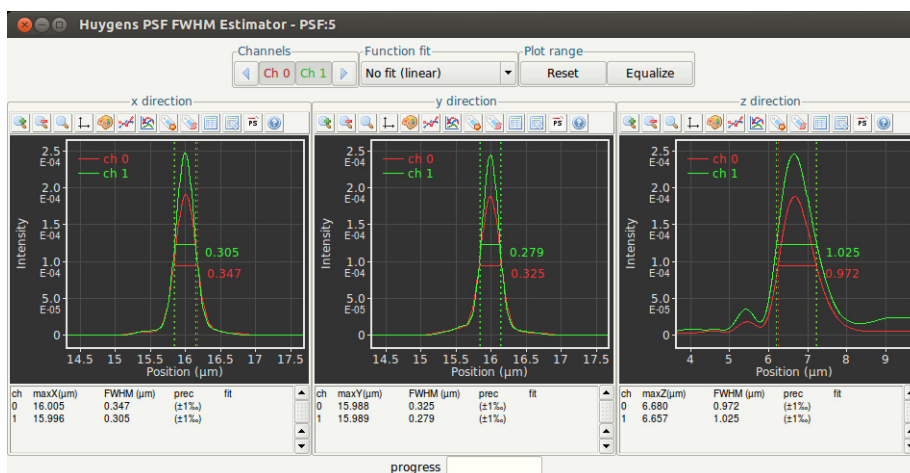


FIGURE 6.4. The PSF-FWHM overview. No curve fitting was selected.

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

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

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

## The Batch Processor

Once you are familiar with a particular kind of dataset and the restoration parameters are determined, more similar datasets can be restored automatically. This is called *batch processing*.

A batch process consists of a number of image restoration tasks (one per image), which are executed one by one until all are finished. Depending on the multi-threading capabilities of the computer, multiple tasks can be executed *in parallel*.

For example, batch scripts can be programmed with Huygens Scripting, which makes it possible to run scripts written in Tcl, using the extensive set of Tcl-Huygens image processing commands.

Batch processes can also be configured easily with the interactive Huygens Batch Processor. The Batch Processor is the tool to do large scale deconvolution of multiple images within Huygens Professional.

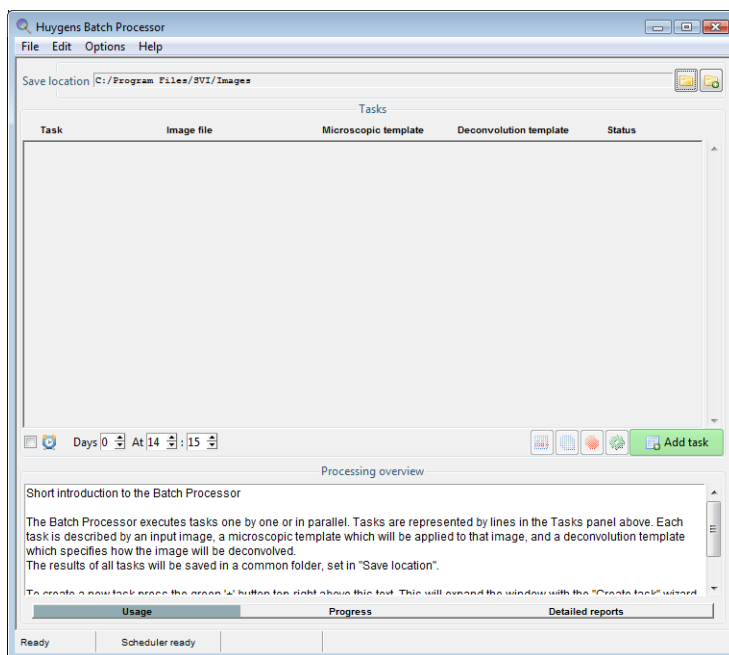
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### The Batch Processor Window

To launch the Batch Processor first open Huygens Professional, then click on the menu DECONVOLUTION→BATCH PROCESSOR.

The main Batch Processor window can be separated into five different elements from the top to the bottom. It shows initially no tasks (see Figure 7.1 on page 50). The elements are:

- **Save Location.** This is the directory where the resulting images will be placed during the batch run. With the two folder buttons a location can be respectively selected or a new location can be created in the currently selected folder.
- **The Tasks area** shows a list of tasks (empty at start). Tasks are jobs that will be processed by the Batch Processor one by one. Each task line consists of an image, a microscopic template and a deconvolution template. These templates can be updated after a task line is added to the list to tune the values in each particular case. In the *Usage* section this is explained in more detail.
- **The button bar** has a clock at the left side to delay the beginning of the processing, and buttons at the right side to delete, duplicate, stop, start, and add tasks to the list (one by one or many at the same time).



**FIGURE 7.1. The Batch Processor main window.**

- **The *Processing overview*** in the detailed information is given about the whole process in its different stages.
- **The *status bar*** at the bottom-end of the batch processor window supplies some status information about Huygens Professional. The leftmost tab shows the state of Huygens Professional, and the tab to the right of it shows information of the batch scheduler. The rightmost of these tabs gives information about the button the mouse is currently pointing at (now empty).

## Usage

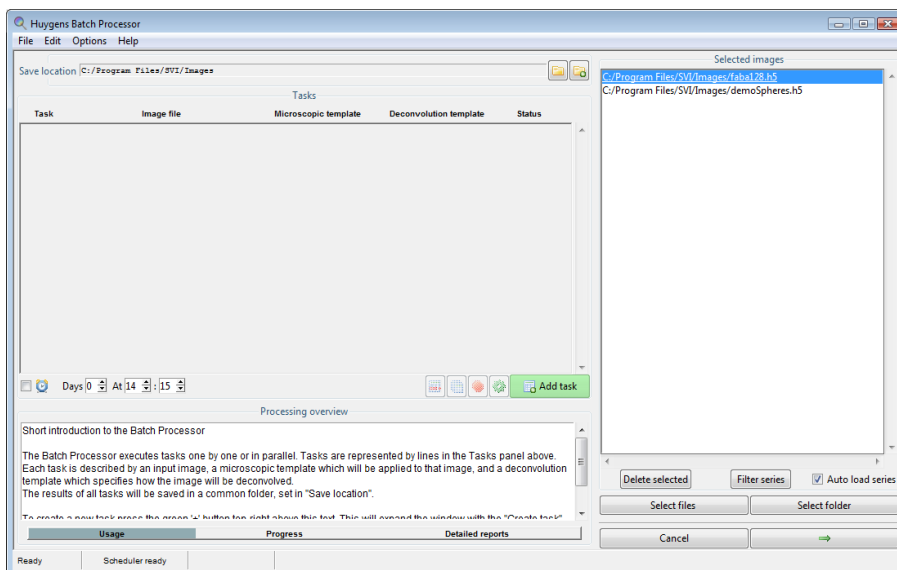
Before starting a batch processing job, the save location of the results should be defined (*Save location* field) and a file format should be selected in which the results should be stored (OPTIONS→OUTPUT FORMAT).

### Selecting Input Files

The Batch Processor has a wizard to guide in creating new tasks with only a few clicks. By clicking the green ADD TASK button below the task field a new menu entitled *Selected images* is expanded at the right (see Figure 7.2).

Either a complete folder containing images can be selected or folders can be browsed to select a single file. If a file is selected containing multiple sub-images (for example a Leica LIF file), a secondary menu will pop up to select which sub-image to deconvolve. Each selected sub-image will be added as a new task in the queue. Upon checking the *Auto load series* box (on), the Batch processor will try to interpret each listed file as a start of a file series.

If the box is unchecked (off), only the listed files will be deconvolved. If a complete file series is selected and you'd like to deconvolve that series as one image, you can click Filter series to prune all members of a file series except the first from the list.

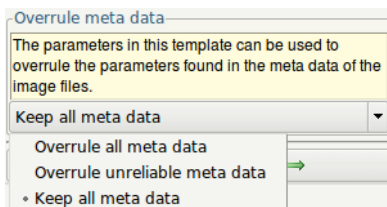


**FIGURE 7.2.** The Batch Processor with the *Selected images* field expanded.

If you select an entire folder containing one or more file series and the Auto load series box is checked (on), only the first file in a series will be listed. Leave Auto load series to unchecked (off) if you do not want this; you can always prune the list with Filter series.

After selecting the images to restore, the NEXT button (→) can be clicked to select or create a microscopic template.

### Microscopic Templates: Describing the Images



**FIGURE 7.3.** The Batch Processor metadata selector.

To guarantee the quality of the deconvolution, it is very important that the image acquisition conditions are properly described. Luckily, the parameters that the Huygens algorithms need are just a few.

The microscopic parameters can be edited and saved to a template so that they can be reused in the future. In addition to editing the microscopic parameters, Huygens also offers the possibility to deconvolve the image with the microscopic parameters stored in the image

metadata. Because different manufacturers save the microscopic data with different confidence levels<sup>1</sup> the Batch Processor allows you to choose whether only reliable metadata should be taken into account for the deconvolution (see Figure 7.3).

It is therefore important to understand what the different microscopic parameters refer to and know how to establish them. A typically conflictive one is the *backprojected pinhole radius*. This parameter is not difficult to calculate<sup>2</sup>, especially with the assistance of the online *backprojected pinhole calculator*<sup>3</sup>.

1. <http://www.svi.nl/ConfidenceLevels>

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As mentioned above, the entered parameters can be stored in *templates* for convenience's sake, so the same template can be applied to a series of images acquired with the same settings.

Click the NEXT button (⇒) again when finished with the microscopic template.

### Deconvolution Templates: Configuring the Restoration Process

Like the microscopic parameters, the deconvolution parameters can be saved in a template. Again, it is important that it is understood what the role of these parameters is (see “The Deconvolution Stage” on page 38 and the *Restoration Parameters* article<sup>4</sup>).

At this stage, you can load one of the existing deconvolution templates from the list or create a NEW TEMPLATE. If you select NEW TEMPLATE, the first tab allows you to LOAD a previously made template or SAVE a new template.

You can adjust the settings for your deconvolution procedure and select the deconvolution ALGORITHM you like to apply. Here, you can also choose to use a Theoretical PSF or to load and use an Experimental PSF.

With the Pre and Post taps, you will be able to select Autocrop, perform Stabilize along z for 3D images, Pre-adjust baseline and correct the z-drift with time series images. Furthermore, Chromatic Aberration correction is possible by loading a pre-made template (see “Working with templates” on page 117).

Once all settings are done, click the Done button at the bottom of the screen. You can also have a look at our detailed Batch Processor Tutorial on our support wiki<sup>5</sup>.

A typically conflictive point is setting the *signal-to-noise ratio*. Mind that this is not a number describing the image, but something that can be tuned to achieve different deconvolution results. Please see “The Deconvolution Stage” on page 38 and the *Set the Signal to Noise Ratio* article in the SVI Wiki<sup>6</sup>.

### Location of the Saved Templates

The microscopic and deconvolution templates are by default saved in the SVI/Templates folder in the user's home directory<sup>7</sup>. The next time the Batch Processor is used, the saved templates will be found in the wizard to set up the batch task.

There are some sample templates in a global location, where the system administrator can also store templates for everybody to use. This global location is a subdirectory Templates of the Huygens installation directory (See Table 2.1 on page 7).

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2. <http://www.svi.nl/BackProjectedPinholeRadius>

3. <http://www.svi.nl/BackprojectedPinholeCalculator>

4. <http://www.svi.nl/RestorationParameters>

5. <http://www.svi.nl/BatchProcessor>

6. <http://www.svi.nl/SetTheSignalToNoiseRatio>

7. The user home directories are usually located in C:\Users on Microsoft Windows Vista, Windows 7, and Windows 8 (64 bit). On Mac OS X Mountain Lion, Mavericks, Yosemite (X11, XQuartz) they are usually in /Users, and on Linux in /home.

### Adding the Task

After selecting the deconvolution template the DONE button appears. By clicking it the task is created and shown in the *Tasks* field. The task can be deactivated by clicking the READY TO RUN button in the status column, setting the status to DEACTIVATED. Marked tasks can be deleted by clicking the DELETE button (🗑️) or selecting EDIT→DELETE TASK from the menu.

New tasks can still be added to the queue before starting the computations, or start computing right away and add new tasks afterward.

### Duplicating Tasks

The DUPLICATE TASKS button (📄) is very convenient to prepare series of tasks for the same image. Just push the button and the copy of the selected line will be ready for modification. For example, it might be required to vary one deconvolution parameter to find the optimal value.

### Running the Batch Job

When the batch process is configured, its configuration can be saved for future reference by selecting FILE→SAVE in the menu.

Add as many tasks as required, single files of complete folders, the Huygens Batch Processor will run them all. By pushing the *start* button (🌀) the Batch Processor will start and go over the task list.

The progress of the Batch Processor and the report for each individual task are shown on the tabs in the *Processing overview* area. The status of each task in the task list changes accordingly to the progress of the process.

The restored and deconvolved images are saved in the selected destination directory as soon as they are ready, along with the image history and an independent task description that can be loaded later in the Batch Processor to re-execute it.

If the computations are very demanding for the system and should not block other activities, the beginning of the queue processing can be delayed by using the timer (🕒). Just adjust the delay in days (zero for today) and set the time of the day when the processing should start. The timer checkbox is then selected automatically; deselect it to disable the timer.

### Exiting the Batch Processor

If the Batch Processor is closed while running tasks, it will stop all running tasks. The Batch Processor window can be scrolled down while running tasks. Just exit the Batch Processor after all the jobs have been finished.

---

## Menus

The FILE menu can be used to save the tasks list for future reference. In this menu also the information reported during the batch processing can be saved.

The EDIT menu can be used to duplicate or delete tasks in the list.

The OPTIONS menu has three sub menus:

- 
- **OUTPUT FORMAT:** this sub-menu shows several options for the file format to select for saving the restoration result.

- **THREADS PER TASK:** this sub-menu allows to set the number of processors per job. Typically, in a run where tasks are processed sequentially, the computational work will still be distributed over the available processors, depending on license limitations.

The number of threads Huygens can use in parallel is by default set to *AUTO*, but in cases where it is required to restrict the computing resources, set a different value as threads.

- **CONCURRENT TASKS:** if the system has multiple processors it can be chosen to run multiple jobs at the same time. However, it is not necessarily true that concurrent execution of tasks is faster than sequential execution, because in the former case multiple tasks will compete for the available memory (*deconvolution demands a lot of memory*). If the available memory is insufficient, a slowdown will occur.



# Huygens STED Deconvolution

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## Introduction

Stimulated-Emission Depletion (STED) microscopy is a fluorescence microscopy technique which overcomes and improves the diffraction-limited resolution of regular confocal microscopy techniques up to 4-5 times<sup>1</sup>, leading to the so-called sub-diffraction resolution or super resolution in the lateral and, in case of STED 3D, also the axial direction .

The Huygens STED optical option offers support for deconvolving STED images, yielding stunning results in XY and in Z. Several other Huygens Professional tools offer an additional STED mode for STED-specific image processing. For example, the PSF distiller (see Chapter 6 on page 43) can generate Point Spread Functions and can estimate the STED microscopic parameters out of STED bead images, and the Object Stabilizer (see Chapter 21 on page 119) is equipped with a special STED stabilization mode. The Deconvolution Wizard (see Chapter 5 on page 29) and Batch Processor (see Chapter 7 on page 49) can automatically stabilize STED raw images, which often contain drifts along the z direction.

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

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## STED principle

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

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

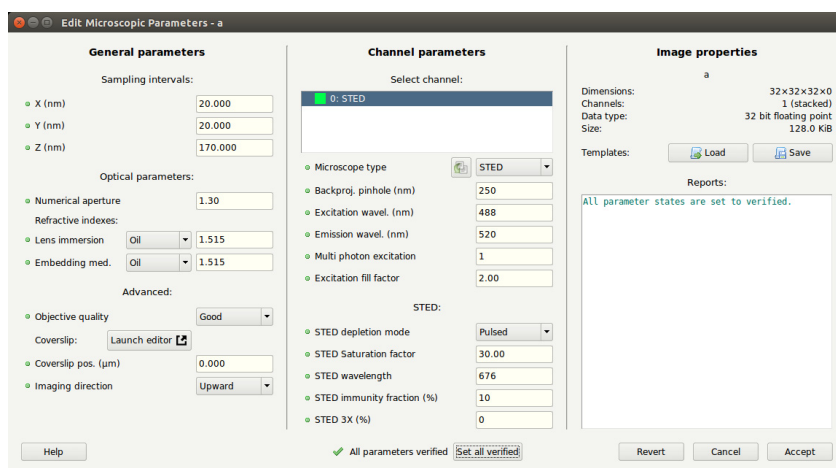
This results in a narrow region around the optical axis being hardly depleted, whereas beyond this region depletion increases steeply. The diameter of the depletion-free region can be as small as 25-50nm, easily resulting in a four fold increase in resolution over good quality confocal resolution.

## STED parameters

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

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

To edit the microscopic parameters of an image right-click on the image and select “PARAMETER EDITOR” or “PARAMETER WIZARD”, as explained in Chapter 5 on page 29. If “PARAMETER EDITOR” has been selected, the specific STED parameters will be presented as in Figure 8.1:



**FIGURE 8.1.** The specific STED parameters can be entered in the Parameter Editor.

- **STED depletion mode:** this mode determines the type of STED depletion that is being used. You can set this to Pulsed, CW (continuous wave) non-gated detection, or CW gated detection.
- **STED saturation factor:** this parameter describes how much the fluorescence is suppressed by the STED beam. The higher this factor the more fluorescence suppression off the optical axis, the more resolution. At values below 1, hardly any resolution is gained. Typical values are in the range of 10 to 50<sup>1</sup>. A very large value of the saturation factor usually also implies a large resolution improvement. Because this parameter can be difficult to quantify it is recommended to use the Huygens PSF distiller (see below) to get an automatic estimation. Also, please notice that in practice the fluorescence is never completely suppressed in the whole depletion region as the fluorophore molecules can be partly ‘immune’ to depletion.
- **STED wavelength:** the wavelength of the STED depletion laser beam (nm). The STED wavelength must be a value within the range of the fluorophore emission spectrum.

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

- **STED immunity fraction:** the fraction of fluorophore molecules not susceptible, 'immune', to the depletion beam. This parameter is specified as a percentage; 100% meaning that all the fluorophore molecules are immune to the depletion beam, 0% meaning that no fluorophore molecules are immune to the depletion beam. The value that should be entered here is usually between 0% and 10%. Because this parameter can be difficult to quantify it is advised to use the Huygens PSF distiller (see below) to get an automatic estimation.
- **STED 3X:** the percentage of power used in the Z depletion beam. The remaining power is used for the vortex beam path. This value is read from the image metadata
- **Deviating microscope type:** this parameter can only be edited when working with multichannel images. The parameter entry is hidden for single-channel images. If not all channels are STED, this parameter should be set to the other microscope type used for that channel.

The STED saturation factor can in principle be determined experimentally, see the footnote on page 56. An alternative is to use the Huygens PSF distiller which can estimate these parameters automatically from a suitable bead image.

*Example settings*

In Table 8.1 you find the (ranges of) normal values for the STED microscopic parameters for some STED systems.

**TABLE 8.1.**

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

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

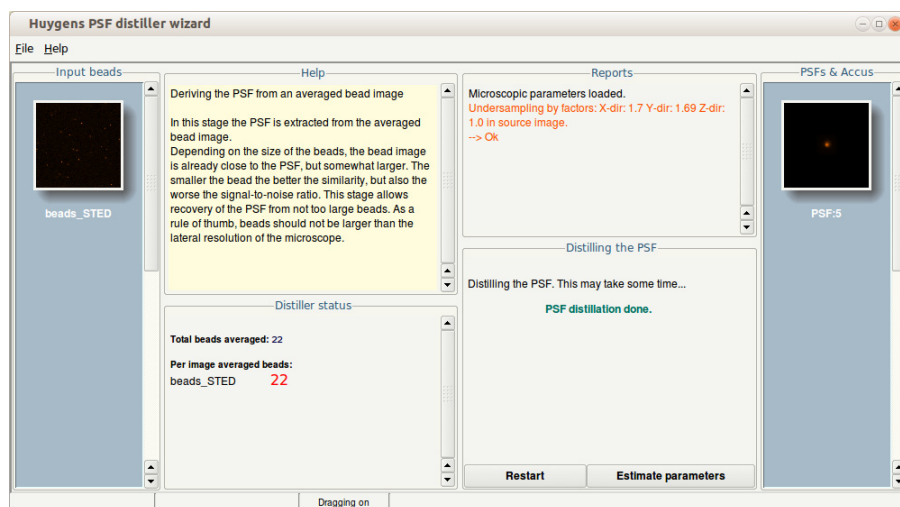
*Estimating STED parameters*

Estimating the STED parameters is an automated process guided by the PSF distiller. Open an image of STED beads in the PSF distiller and proceed to distill a PSF as explained in Chapter 6 “The PSF Distiller” on page 43.

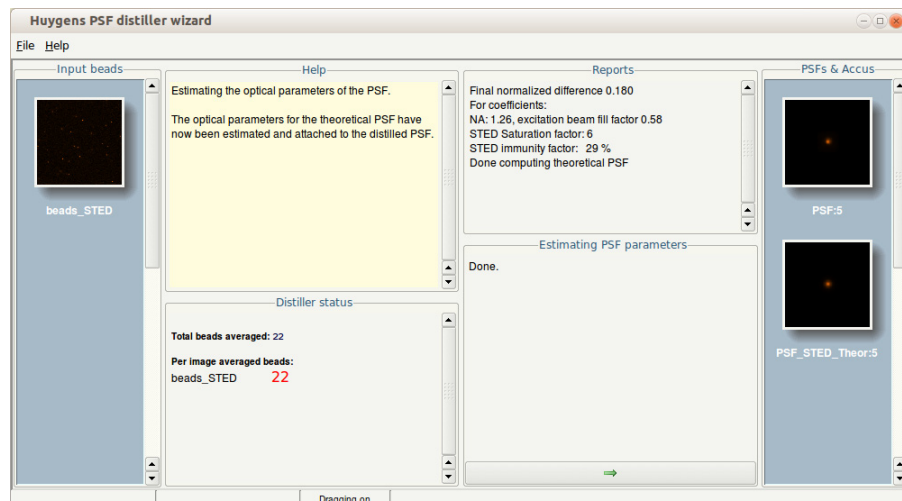
The PSF distiller will recognize that the image has been recorded with a STED microscope and will offer the possibility to estimate the STED parameters after distilling the PSF (see Figure 8.2).

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

Figure 8.3 shows the PSF distiller after estimating the STED parameters of a beads image. In this example, the estimated STED saturation factor is 6 and the (very high) immunity factor of 29%.



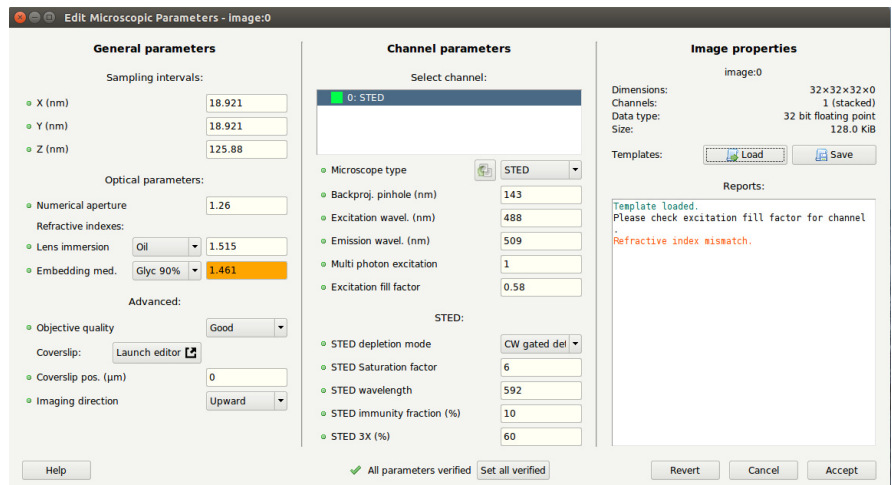
**FIGURE 8.2.** Estimating the STED microscopic parameters of a beads image automatically with the Huygens PSF distiller.



**FIGURE 8.3.** The PSF distiller showing the STED microscopic parameters automatically estimated from a beads image.

Estimated STED parameters will be attached to the new distilled PSF, which will be shown as a thumbnail in the main window of Huygens Professional. The parameters are reported by the PSF distiller (see Figure 8.3) and can be reviewed by, for example, opening the Parameter Editor by right-clicking on the PSF thumbnail (see Figure 8.4).

The microscopic parameters of the PSF can be exported as a template file within the Parameter Wizard or the Parameter Editor (see Figure 8.4). The microscopic parameter template file can, later-onwards, be applied to other STED images that have been recorded under the exact same conditions. This makes future parameter editing much easier and reliable.



**FIGURE 8.4.** The Microscopic Parameters of the distilled PSF now contain the values automatically estimated by Huygens Professional.

## STED Deconvolution

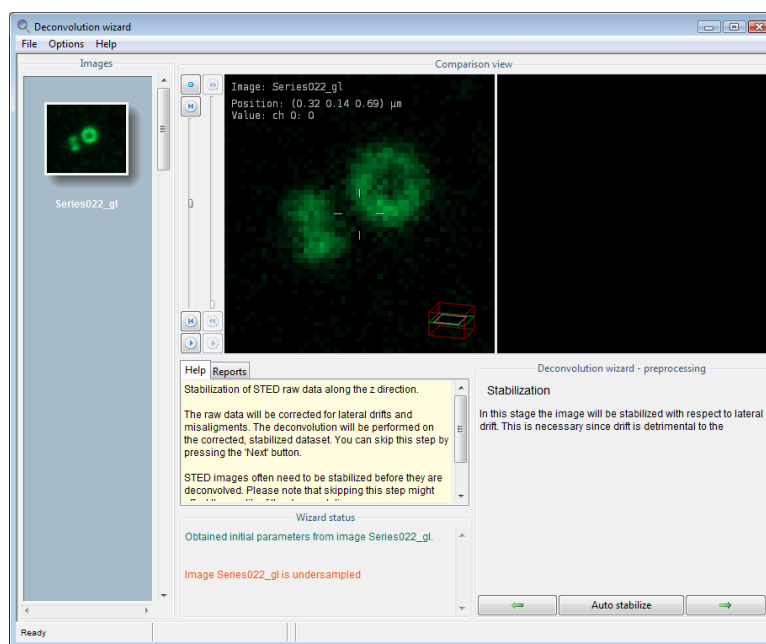
To deconvolve a STED image, open first the Parameter Editor or Parameter Wizard. Verify the microscopic parameters or load the microscopic parameter template file that was saved earlier. The latter option will import the PSF microscopic parameters to the STED raw dataset. Both the PSF and the raw image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

Continue to the next Wizard stage and proceed step by step as explained in Chapter 5 “Deconvolution Wizard” on page 29. The Deconvolution Wizard will recognize a STED dataset and can offer a stabilization option because STED image acquisition is often subjective to drift. The severity of drift in STED image data depends on how much of the STED power is used for vortex and for Z depletion. Stabilization is recommended for pure vortex-based STED images. With increased Z depletion in STED 3X data, drift will be more difficult to estimate but also less of an issue with respect to image quality. Huygens automatically decides whether the option to stabilize the image is needed, by taking the aspect ratio of the STED PSF into account.

To stabilize the image in the Deconvolution Wizard just click on the “AUTO STABILIZE” button at the “STABILIZATION” stage (See Figure 8.5). If Huygens applies stabilization a remark will be shown in the REPORT window. Note that the same level of stabilization will also be applied to the confocal channel, if present.

The stabilization stage can be skipped at the Deconvolution Wizard if the image has already been stabilized by the Huygens Object Stabilizer (See **Chapter 21 on page 119.**)

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



**FIGURE 8.5.** Stabilization stage for STED data in the Deconvolution Wizard.

*Summary:  
deconvolution  
procedure*

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

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

## The Slicer

The Slicer (see Figure 3.2 on page 13) allows you to quickly inspect an image. Even multiple Slicer windows can be opened simultaneously for comparing several images (e.g. a deconvolution result with the original) and/or multiple orientations of the same image.

To start the Slicer, select VISUALIZATION→SLICER from the menu in the main window, or double click the image's thumbnail. You can open as many Slicers as you like, on the same image or on different images, and multiple Slicers can be linked and 'listen' to other Slicer windows.

The Slicer enables the user to show a single 2D plane extracted from a 3D volume. There are controls to select any plane orientation in space, zoom, and scroll through the available planes. For time series, a separate control is available to scroll through the available time frames. Pixel intensity values for the cursor position on the image are displayed in the 'Voxel data' panel. You can move the image by clicking the middle mouse button and keeping it pressed while moving the image to the desired position.

---

### Color

For each channel, one out of five color schemes can be chosen:

- *Grey*  
Pixel values are assigned different shades of grey ranging from black for the lowest values to white for the highest values.
- *Emission*  
The displayed color of a channel matches the true color of the emission wavelength in so far this is possible.
- *False*  
Pixels values are assigned different colors ranging from black/dark purple for the lowest values to bright red for the highest value.
- *Global*  
In a multi-channel image, colors can be assigned to the different channels by using the 'global color palette'.
- *Custom*

---

A custom color can be chosen for each of the channels. As with RGB, pixel values are assigned different shades of a particular color ranging from black for the lowest values to the brightest possible shade for the highest values.

---

## *Contrast*

For the complete view, three basic gamma contrast settings can be chosen:

- *Linear*  
In this mode the pixel values are mapped to screen buffer color intensities in a linear fashion. Note that the actual translation of the screen buffer values to the actual brightness of a screen pixel is usually quite non-linear, for which the following gamma contrast settings are offered.
- *Compress*  
When an image contains a few very bright spots and some larger darker structures, using the Linear mode will result in poor visibility of the darker structures. Restoration of such images is likely to further increase the dynamic range resulting in the large structures becoming even dimmer. In such cases use, the compress display mode to increase the contrast of the low valued regions and reduce the contrast of the high-valued regions. Another way to improve the visibility of dark structures is the usage of false colors, as mentioned before.
- *Strong compression*  
Same as above, with stronger effect.
- *Widefield*  
In restoring widefield images it sometimes happens that blur removal is not perfect, for instance when one is forced to use a theoretical point spread function in sub optimal optical conditions. In such cases the visibility of blur remnants can be effectively suppressed.
- *Strong Widefield*  
Same as above, with stronger effect.

---

## *Time*

Slices of a time series can be selected by entering a frame number or using the slider.

---

## *Linking*

When you open two or more Slicers on the same image, then the Slicers are automatically linked. This means the position and orientation of both views are linked by default. A right-click on one of the images will change the center in the other Slicers.

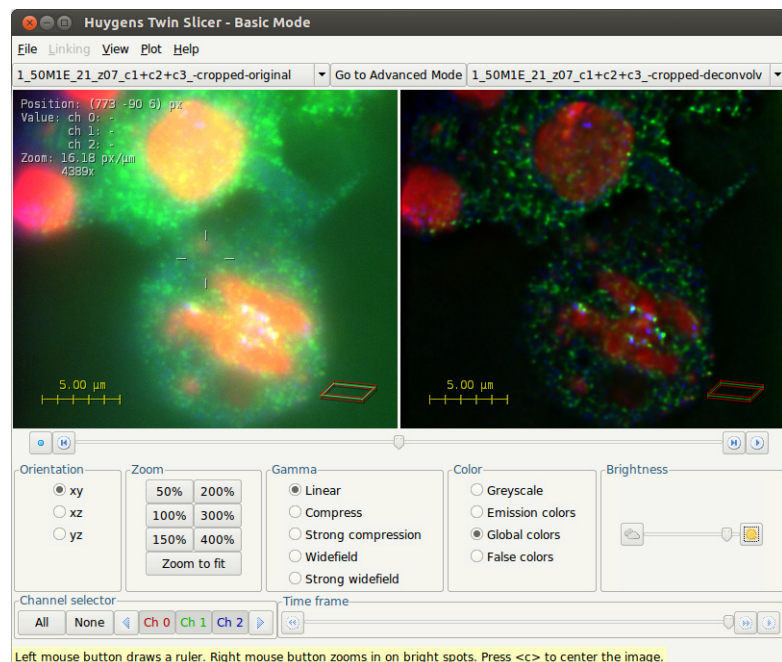
Besides centering and orientation, the 'Time', 'Zoom', and 'Twist' and 'Tilt' sliders can be linked by setting the appropriate option in the 'Linking' panel.

If you would like to link the Slicers of two different images, e.g. a deconvolved image and its original, select 'Other' in order to let the current Slicer listen to other Slicers.



The Twin Slicer allows to synchronize views of two images, measure distances, plot line profiles, etc. In *basic mode*, which is also available without a license, image comparison is intuitive and easy, while the *advanced mode* gives the user the freedom to rotate the cutting plane to any arbitrary orientation, link (synchronize) or unlink viewing parameters between the two images, and more.

To launch the Huygens Twin Slicer, select an image and select VISUALIZATION→TWIN SLICER from the main menu. To view another image in an existing slicer, click the image name in the drop-down menu above the left or right view port (See Figure 10.1).



**FIGURE 10.1.** The twin slicer in basic mode, showing an original and deconvolved image side-by-side.

---


## The View Menu

Use the VIEW menu to show or hide image properties and guides. These are listed in Table 10.1:





**TABLE 10.1. The options in the Twin Slicer's VIEW menu.**

Option	Description
POINTER COORDINATES	Display the position of the mouse pointer in $\mu\text{m}$ or in voxel coordinates.
TIME	Display the time for the current slice in seconds or frame numbers.
INTENSITY VALUES	Display the intensity values for all channels on the current pointer location.
ZOOM	Display the zoom value in screen-pixels per micron. A magnification factor is displayed as well; using the pixel density for the monitor, this value gives an estimation for the absolute magnification.
ROTATION ANGLES	Display the tilt and twist angles in degrees.
DROP SHADOWS	Enhance the contrast for the overlaid lines and text by showing drop shadows.
SLICE BOUNDARIES	Draws the slice boundaries for the left image in the right one and vice versa. This is helpful when both slicers are used.
WIREFRAME BOX	Show or hide the wireframe box, which gives visual feedback on the position and orientation of the cutting plane (green), and the displayed slice (gray) in the data volume (red).
SCALE BAR	show or hide the scale bar.
SVI LOGO	Show or hide the SVI logo in the lower right of the view port.
RESET TO DEFAULT	Sets all viewing settings to default.

## Panning

Click and hold the *middle mouse button* on the slice to move it around. If the middle mouse button is missing, click and hold the *left mouse button* while holding the `Ctrl` button. Clicking the *center* button (  ) or pressing the 'c' key centers the slice.

## Slicing

Drag the *slider below the view ports* to move the cutting plane back and forth. This can also be achieved using the buttons adjacent to the slider (  and  ), the up/down arrow keys on the keyboard, or by placing the mouse pointer over the slider and using the scroll wheel. The play button (  ) moves the cutting plane through the data volume. The pointer coordinates can be displayed through the VIEW menu. Note that it is possible to move the cutting plane out of the volume. Pressing the *center* button (  ) or pressing the 'c' key centers the plane again.

---

## Using the Slicer in Basic Mode

The button centered at the top of the window enables switching between *basic* and *advanced* mode. In basic mode, all controls are visible in the panels below the view ports (See Figure 10.1). In contrast to the advanced mode, which allows independent control of the left and right slicer (See "Using the Slicer in Advanced Mode" on page 66), the basic mode shows a single set of controls that apply to both slicers.

### Changing Time Frames

Drag the slider in the lower *Time frame* panel to change the time frame or press the play button (▶) to animate the time series. The time frame can be displayed through the VIEW menu.

### Orientation

Make a selection in the most left *Orientation* panel to change the plane used to display the image.

### Zooming

Click the buttons in the *Zoom* panel or use the scroll wheel to zoom in or out on the location of the mouse pointer.

### Changing Display Colors

Click an option in the *Color* panel to select a color scheme:

- *Greyscale*: the image is displayed in gray tints. For single-channel images, this gives a higher contrast than the emission or global colors.
- *Emission colors*: if the emission wavelengths are set correctly, this gives the most intuitive view.
- *Global colors*: the colors as defined in the *global color scheme*. The global color scheme applies to all visualization tools and can be modified via the Huygens Professional main menu: .
- *False colors*: a false color is given to each intensity value. This view gives a high contrast and makes it easy to spot areas of homogeneous intensity.

### Tuning the Brightness and Contrast

The brightness can be adjusted in the most right *Brightness* panel using the buttons (☰ and ☼), dragging the slider, or putting the mouse pointer over the slider and using the scroll wheel. The *Gamma* panel provides a linear and some nonlinear ways of mapping data values to pixel intensities. These are:

- *Linear* (default): pixel values are mapped to screen buffer color intensities in a linear fashion. Note that the actual translation of the screen buffer values to the actual brightness of a screen pixel is usually quite nonlinear.
- *Compress or Strong compression*: where an image contains a few very bright spots and some larger darker structures using linear mode will result in poor visibility of the darker structures. Restoration of such images is likely to further increase the dynamic range resulting in the large structures becoming even dimmer. In such cases use the compress display mode to increase the contrast of the low valued regions and reduce the contrast of the high-valued regions. Another way to improve the visibility of dark structures is the usage of *false colors* (See “Changing Display Colors” on page 65).
- *Widefield or Strong widefield*: in restoring widefield images it sometimes happens that blur removal is not perfect, for instance when one is forced to use a theoretical point spread function in sub optimal optical conditions. In such cases the visibility of blur remnants can be effectively suppressed.

### Automatic Panning, Slicing and Zooming

When the *right mouse button is clicked*, the Twin Slicer shows a context menu. The option “ZOOM IN ON BRIGHTEST SPOT” will automatically center and zoom in on the brightest spot in a 3D neighborhood around the cursor.

The button centered at the top of the window offers switching between *basic* and *advanced* mode. The advanced mode allows independent control of the left and right slicer. All controls are available in twofold and accessible through the tabs at the bottom of the window.

### Changing Time Frames

Drag the slider in the *Time frame* tab to change the time frame or press the play button (▶) to animate the time series. The time frame can be displayed through the VIEW menu.

### Zooming

Use the scroll wheel to zoom in or out on the location of the mouse pointer, or access the *Zoom* tab. The four buttons in this tab respectively *zoom out* (🔍), *zoom in* (🔍), *zoom 1:1* (🔍), and *view all* (🌐).

### Setting the animation speed.

Both the time slider and the slice sliders below each view port have a play mode to animate the slicing. With the slider between the slicers one can control the speed of this animation. For slow animation, move the slider towards the snail (🐌). This will add delays between the rendering of the scenes. When the slider is set all the way to the hummingbird icon (🐝) there is no delay between the renderings and the limitation of the visualization is simply how fast your computer can render the scenes. When decreasing the sizes of the view port, the rendering can go even faster, since small scenes are rendered more quickly than large scenes.

### Rotation

The three radio buttons in the *Orientation* tab can be used to switch between axial ( $xy$ ), frontal ( $xz$ ), and transverse ( $yz$ ) orientations. The *Twist* slider rotates the cutting plane around a  $z$ -axis, while the *Tilt* button rotates the cutting plane around an axis in the  $xy$  plane. The tilt and twist angles can be displayed through the VIEW menu. Note that the wireframe box in the bottom left of each view port gives visual feedback about the position and orientation of the slice.

### Changing Display Colors

Click the *Channels & Colors* tab key to view the color settings panel. The *Active channels* buttons (or drop down menu, if the image contains more than ten channels) can be used to enable or disable channels. The buttons colors correspond to the channel colors.

In addition to the *color schemes* that are available in basic mode (“Changing Display Colors” on page 65), the advanced mode allows the use of *custom colors*. Use the color picker (🎨) to manually select a color for each channel.

## Tuning the Brightness and Contrast

The brightness and contrast controls are accessible in the *Contrast* panel. The brightness can be changed per channel, or for all channels at once (master). The *Gamma* drop down menu provides a linear and some non-linear ways of mapping data values to pixel intensities (See “Tuning the Brightness and Contrast” on page 65 for an overview).

If the *Link* box is checked, the way of mapping data values to pixel intensities is the same for all channels; if not, the range is automatically adjusted according to the minimum and maximum intensity in each channel.

The contrast stretch can be either set globally (the complete dataset will be taken into account), or for each screen view.

## Linking, View & Plot

In the *Linking, View & Plot* panel are the most used options which you can also find in the menus. In one view you can easily see and set the links, view - and plot properties. More options are available in the menus. For more information about the linking options, see the Linking Controls below.

## Shortcut Information

In the *Shortcut Info* panel all the available shortcuts are listed which are explained in the previous section. If your mouse or laptop lacks a middle mouse button, one can use the left mouse button while holding the `Ctrl` key to simulate the middle mouse button press.

## Animate

The *Animate* tab allows you to set the frame rate in frames per second making it possible to create animations through time or through the z-stack.

## Linking Controls

The LINKING menu can be used to change the way in which both slicers communicate. The options in this menu are listed in Table 10.2. Note that settings get synchronized once the controls are being used.

**TABLE 10.2. The options in the Twin Slicer’s LINKING menu (accessible in advanced mode).**

Option	Description
POINTER LOCATION	Shows the position of the mouse pointer in the other slicer.
SLICE POSITION	Makes sure that the cutting plane for the right slicer crosses the center of the left slice, and vice versa.
TIME FRAME	Synchronize the time.
ZOOM LEVEL	Synchronize the level of magnification.
PANNING	This does not affect position of the cutting plane, but it shifts the right slice such that the projection of the center of the left slice is in the center of the right slice, and vice versa.
ROTATION	Makes sure that the rotation angles for both cutting planes are the same.

---


**TABLE 10.2. The options in the Twin Slicer's LINKING menu (accessible in advanced mode).**

Option	Description
ACTIVE CHANNELS	The left and right slicer will have the same channels enabled and disabled.
COLOR SCHEME	Makes sure that the left and right slicer use the same colors scheme.
CUSTOM COLORS	Use the same custom color scheme for both slicers.
BRIGHTNESS	Synchronize the brightness.
GAMMA	Synchronize the gamma setting.

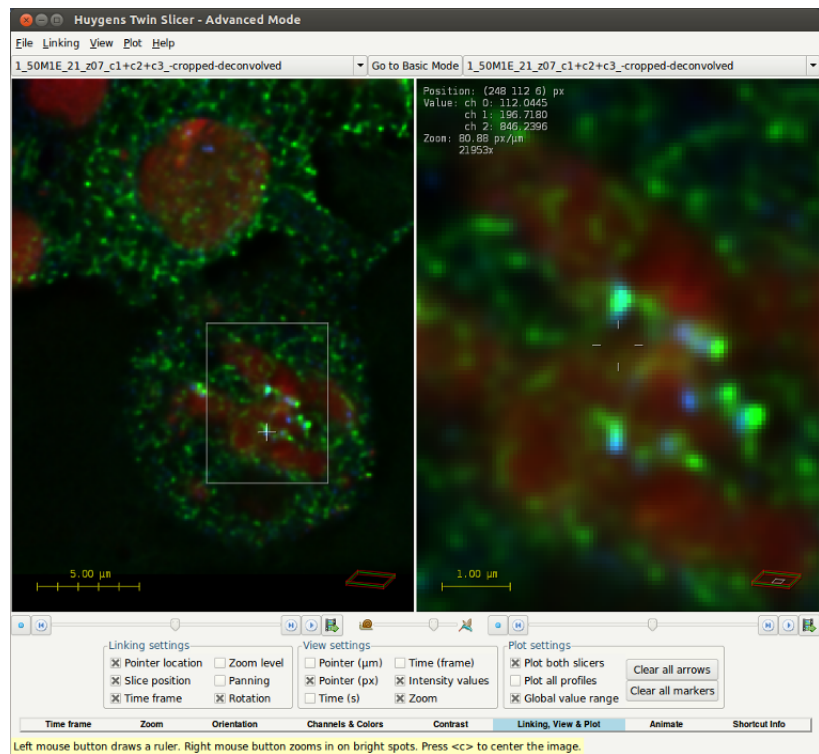
Some useful ways of linking the controls are:

- *Comparison mode*: to configure the Huygens Twin Slicer to compare two images, e.g. original and deconvolved, it is best to link all orientation parameters, i.e., slice position, time frame, zoom level, panning and rotation. This ensures that the same piece of data is displayed for comparison.
- *Orthogonal mode*: to view a part of an image in two orthogonal directions, for instance axial ( $xy$ ) and frontal ( $xz$ ), do the following:
  - Select the same image for both the left and right slicer.
  - Go to LINKING and link the slice position, time frame, zoom level, and panning. Unlink the rotation.
  - Select the *Orientation* tab at the bottom of the window and select the  $xz$  and  $xy$  orientation.

Now it is possible to zoom, pan, and slice while the centers of the left and right slice are always aligned. Note that when the cutting planes are not the same, the projected mouse pointer will show a distance (in  $\mu\text{m}$ ) beside it. If this number is positive, it means that real pointer is more towards the observer (in front of the screen).

- *Overview mode*: An easy overview mode can be configured as follows:
  - Select the same image for both the left and right slicer.
  - Go to LINKING (either the menu, or the panel *Linking, View & Plot*) and link the slice position, time frame, and rotation. Unlink the zoom level and panning.
  - Drag the sash to the left to make the left slicer a bit smaller.
  - Select the *Zoom* tab at the bottom and click the *view all* button (.

Now the right slicer can be used to zoom in on the data, while the left slicer shows the position in the image (See Figure 10.2).



**FIGURE 10.2.** The Twin Slicer in *advanced mode*, with all controls but zoom and panning linked.

## Measurement

### Markers

*Double click* in one of the images or right click and select SET MARKER to place a marker at the position of the mouse pointer. As configured in the VIEW menu, the marker shows the coordinates and intensity values beside it. To remove the marker, click it and press the Delete key.

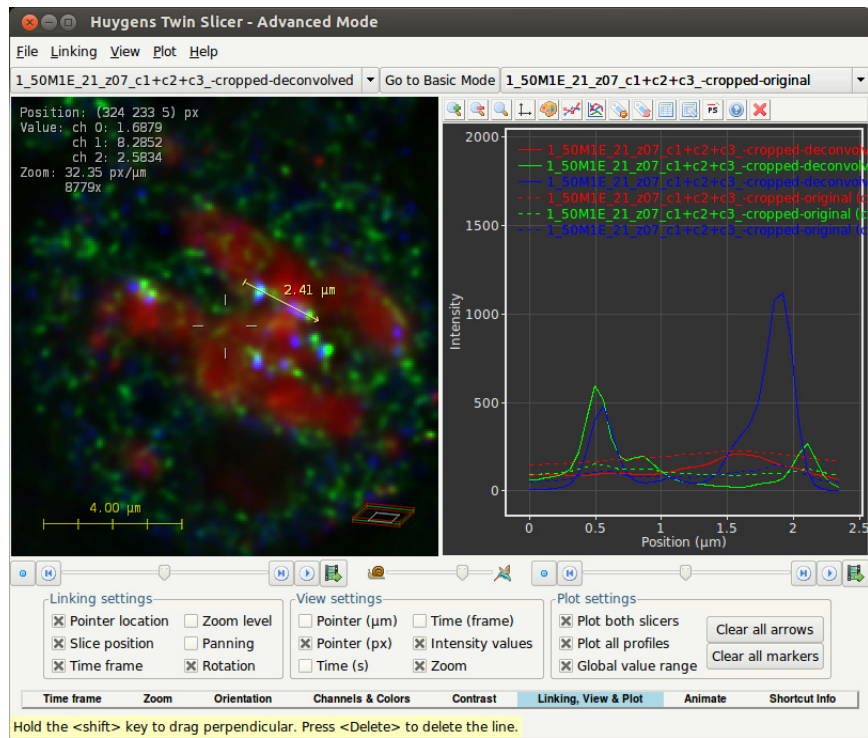
### Rulers

To overlay a ruler on the image, *hold the left mouse button and drag*. The length of the line in  $\mu\text{m}$  is displayed beside it. Press and hold the Shift key while creating a new ruler and the ruler will be parallel to one of the axis, depending on your mouse motion.

Click and drag the end points of the ruler to make adjustments. Press and hold the Shift key while dragging an end point to change length without changing direction. Click and drag the middle of the ruler to move it in its entirety, without changing length or direction. Press and hold the Shift key while dragging the ruler to move it perpendicular to its direction. To remove the ruler, click it and press the Delete key.

### Intensity Profiles

When a ruler in the left slicer is selected, the right slicer will be replaced by a plot window and vice versa. The intensity profiles for both the left and right image in the same plot are shown. Unselect PLOT→PLOT BOTH SLICERS from the menu to only show the plot for one image. The graphs for the left slicer will have solid lines, while the graphs for the



**FIGURE 10.3.** Measuring the intensity profile along a line. The plot can be configured such that it shows the profile of both images (left solid, right dashed).

right one are dashed (See Figure 10.3). See the online SVI Wiki<sup>1</sup> for more information on the data plotter.

1. <http://www.svi.nl/DataPlotter>



# The Orthogonal Slicer

The Huygens Orthogonal Slicer tool is shown in Figure 11.1. It displays the image that is selected from the main window. The Ortho Slicer is designed to show the same point in 3D space from three orthogonal directions;

- axial or  $xy$  (top left);
- frontal or  $xz$  (bottom left);
- transverse or  $yz$  (bottom right).

Each view is enclosed by a colored frame whose color indicates to which slices in the other views it corresponds.

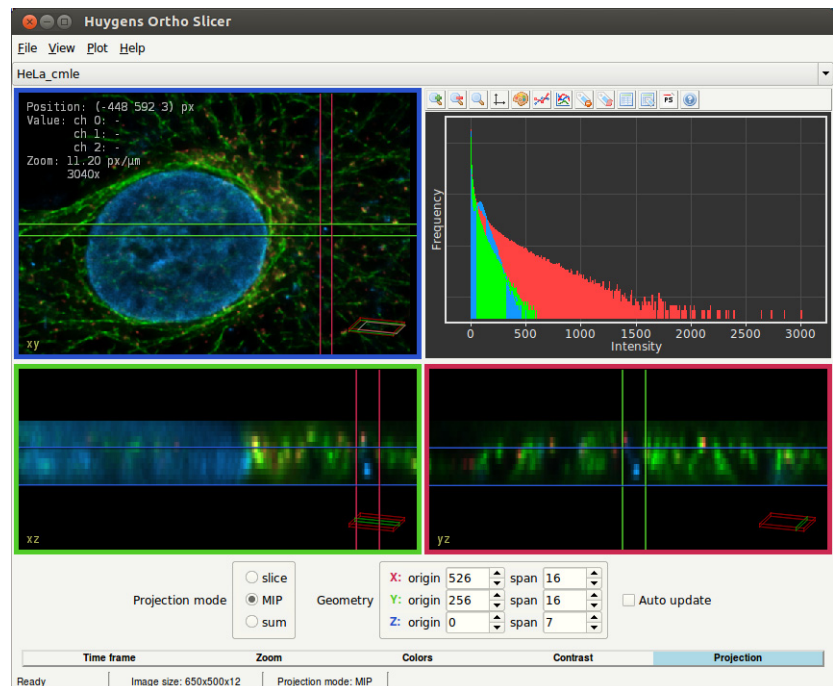


FIGURE 11.1. The Huygens Orthogonal Slicer.

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If you move one of the slices, the others will follow to make sure that the center of each of the slices intersects in the same point in space. This behavior makes the Ortho Slicer a useful tool to study small objects in 3D.

The histogram (top right) shows the number of pixels in the complete image plotted against the intensity value. More information on the orthoslicer can be found on the wiki<sup>1</sup>.

---

### *The Crosshair Cursor*

The position of your mouse is projected as a cross-hairs pointer on all views. The value besides the center of the cross-hairs gives the distance of the mouse position to this projection. If this number is positive, it means that the real pointer is more towards you (in front of your screen). If you click with your left mouse button on a position in one of the views, the slide-borders (indicated with colored lines) will be centered to that specific position.

---

### *Visualization parameters*

Changing the visualization parameters in the Orthogonal Slicer is similar to the Huygens Twin Slicer (on page 63). There are tools to;

- change time frames
- zoom in, out, fit or zoom 1:1
- change display colors
- tune the brightness and contrast
- change the projection mode.

Panning can be achieved by clicking and dragging the middle mouse button. To center the slice, use the menu that can be activated by placing the cursor on the view and clicking the right mouse buttons or by pressing 'c'.

---

### *Measurements*

To overlay a ruler on the image, hold the left mouse button and drag. The length of the line in microns is displayed beside it. Left-click and drag the end points of the ruler to make adjustments. Note that the other orthogonal directions show a projection of this ruler. Press and hold `Ctrl` while dragging an end point to change length without changing direction. Left-click and drag the middle of the ruler to move it in its entirety, without changing length or direction. Press and hold the `Ctrl` key while dragging the ruler to move it perpendicular to its direction. To remove the ruler, left-click somewhere else on the image.

If a ruler is drawn, the histogram panel will be replaced by a plot that shows the intensity profile along it. See the SVI wiki for more information about the data plotter's capabilities.<sup>2</sup>

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1. <http://www.svi.nl/OrthoSlicer>

2. <http://support.svi.nl/DataPlotter>

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*Auto-Zoom*

When you click the right mouse button, several options appear. The first can be used to automatically center and zoom in on the brightest spot in a 3D neighborhood around the mouse pointer.

---

*Display Options*

The VIEW menu allows you to show or hide information and guides within the image overlay, including pointer coordinates, time, intensity, zoom, rotation, graphics and the wireframe.

The “Global value range” option in the PLOT menu uses the maximum and minimum value of the image(s) to determine the visible range of the plot, otherwise it uses the maximum and minimum values of the plot data.

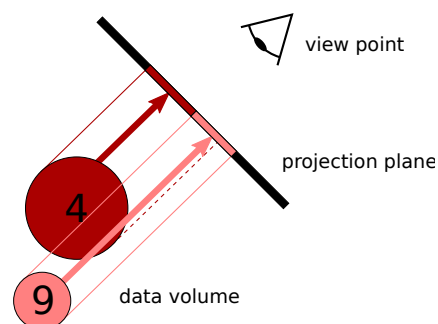


## The MIP Renderer

The *Maximum Intensity Projection* (MIP) Renderer enables the possibility to obtain an orthogonal projection of 3D data from any given viewpoint.

The MIP renderer projects the image voxels on the screen by tracing near-parallel rays from a viewpoint far away through the data volume. Along each ray the maximum intensity encountered is taken for the rendered image (See Figure 12.1). Notice that this implies that two MIP renderings from opposite viewpoints show symmetrical images.

To start the MIP Renderer, right-click on a thumbnail and select VIEW→MIP RENDERER from the pop-up menu, or choose VISUALIZATION→MIP RENDERER from the main menu.



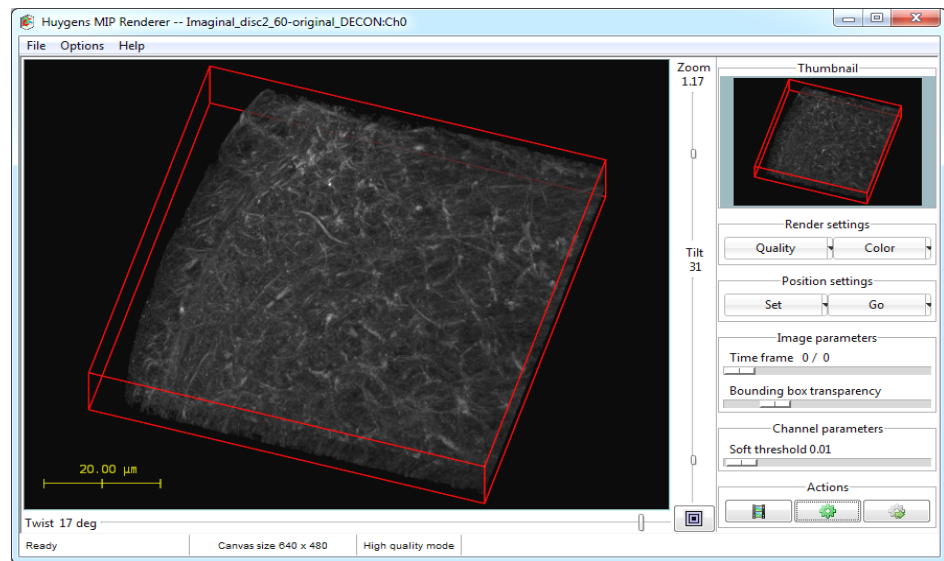
**FIGURE 12.1.** A schematic overview of MIP rendering. The maximum intensities on rays parallel to the screen are projected.

### Basic Usage

#### Orientation and Zoom

Adjust the viewpoint by moving the *Tilt* and *Twist* sliders (See Figure 12.2), or by clicking and dragging the mouse pointer across the scene. The magnification can be adjusted by using the *Zoom* slider or the scroll wheel. Use the center mouse button to *pan* the center of the projection.

Notice that the scene is rendered with low quality while being edited, which allows for fast scene edition. The scene is automatically rendered with good quality when the edition is finished and the scene released. This automatic rendering can be deactivated by pressing the SWITCH OFF rendering button (🛑). However, when either the FAST MODE render button (🚀) or the HIGH QUALITY render button (🌿) are pressed the automatic rendering occurs again upon releasing the MIP scene.



**FIGURE 12.2.** The MIP Renderer window.

## Threshold

The *Soft threshold* slider in the *Channel parameters* panel at the right affects the threshold level. The application of a threshold is a preprocessing step that *reduces the background* in the image, i.e. voxels with intensity values below the threshold value become transparent. Contrary to a standard threshold, which is ‘all or nothing’ (values above the threshold are kept, values below it are deleted), the soft threshold function handles images in a different way. It makes a *smooth transition* between the original and the deleted value.

## Saving Scenes

Choose FILE→SAVE SCENE... to save the rendered scene as a Tiff file.

*Advanced Usage***Render Parameters**

Table 12.1 gives an overview of the render parameters available in the MIP renderer.

**TABLE 12.1. MIP render parameters.**

Option	Description
ANIMATION FRAME COUNT	Set the number of frames that will be rendered in a movie. 180 frames with a frame rate of 24 fps result in a movie with a duration of 7.5 seconds.
ANIMATION FRAME RATE	Adjust the frame rate; a rate of 24 frames per second is fine for smooth movies.
RENDER SIZE	Adjust the size of the rendered image. When the render size exceeds the display area, then use the <i>right mouse button</i> to pick up and move the rendered image.
RENDER QUALITY	Set the default quality (FAST or HIGH QUALITY). This setting will be used for rendering animations.
COLOR MODE	Choose between GREY, EMISSION COLORS, GLOBAL PALETTE (See “Adjusting the Global Color Scheme” on page 195), FALSE COLOR, or CUSTOM COLOR.
BOUNDING BOX	Enable or disable the bounding box, or adjust the line color.
SCALE BAR	Enable or disable the scale bar, adjust the scale bar length and the line color.
SOFT THRESHOLD MODE	Adjust the smoothness of the soft threshold (See “Threshold” on page 76).
TIME FRAME	A selection of the time frame for time series.
CENTER SCENE	Undo both the panning of the rendered image (middle mouse button) and of the scene canvas (right mouse button).

**Render Options**

Table 12.2 gives an overview of the render options available through the OPTIONS menu.

**TABLE 12.2. Render options for the MIP Renderer.**

Option	Description
RENDER SIZE	Adjust the size of the rendered image. When the render size exceeds the display area, then use the <i>right mouse button</i> to pick up and move the scene canvas.
COLOR MODE	Choose between GREY, EMISSION COLORS, GLOBAL PALETTE (See “Adjusting the Global Color Scheme” on page 195) or FALSE COLOR.
SHOW SVI LOGO	Hide or show the SVI logo at the bottom right.

**Templates**


All scene settings, i.e. both the render options and all parameters, can be exported to a template file via FILE→SAVE SCENE TEMPLATE. The template files have the extension `.hgsv` and can be applied to any image that is loaded in the MIP Renderer.

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## *Simple Animations*

The Huygens *Movie Maker* (See “The Movie Maker” on page 91.) allows you to create sophisticated animations using the MIP, SFP, and Surface Renderer.

Without the Movie Maker the MIP Renderer has the option to make simple animations of the image, changing the view point in different movie frames. Set the render parameters for the first frame and click MOVIE tab, SET →FIRST SCENE. Now adjust the view-point for the final frame, and click SET→LAST SCENE. Set also the *frame count*, *frame rate*, and other general options such as colors. Finally press the *animate* button (  ), and select a directory to save the AVI movie or the TIFF frames to.

The exported AVI files use the MJPEG<sup>1</sup> codec and can be loaded in most movie players, including Windows Movie Player and Apple Quicktime. The TIFF frames are useful to combine multiple animations or edit the movie in e.g. Windows Movie Maker.

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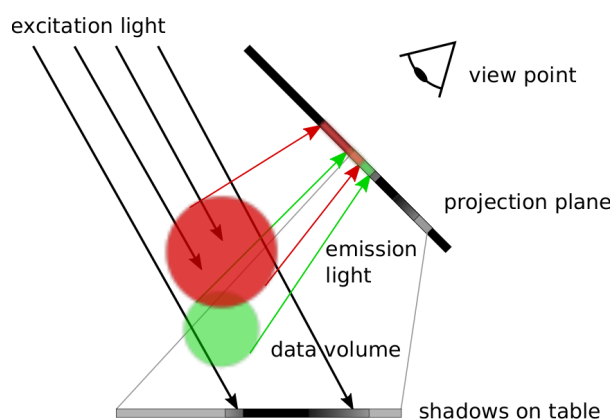
1. <http://en.wikipedia.org/wiki/Mjpeg>



The SFP Renderer generates *realistic 3D scenes*, based on the 3D microscopy image, which is taken as a distribution of fluorescent material. The computational work is done by the *Simulated Fluorescence Process* (SFP) algorithm<sup>1</sup>, simulating what happens if the material is excited and how the subsequently emitted light travels to the observer (See Figure 13.1).

The properties of this algorithm allow to render the object at different depths, unveiling layers under the object's surface. The SFP algorithm is not limited by boundaries or sharp gradients and is exclusively suited to render 3D microscopic data sets. Since the SFP algorithm is based on *ray-tracing* techniques it does not require a special graphic card.

To start the SFP Renderer, right-click on a thumbnail and select VIEW→SFP RENDERER from the pop-up menu, or choose VISUALIZATION→SFP RENDERER from the main menu.

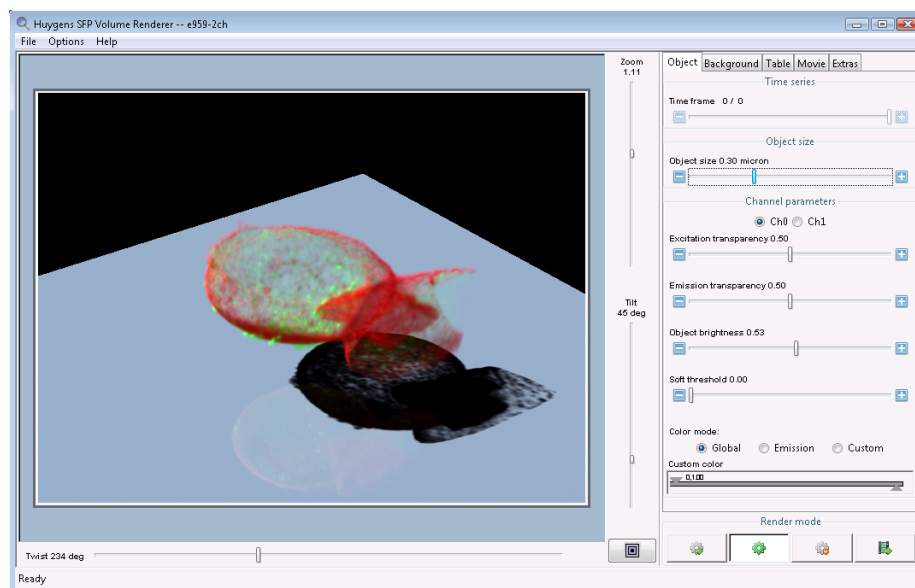


**FIGURE 13.1.** In the SFP renderer excitation and subsequent emission of light of fluorescent materials is simulated. Each subsequent voxel in the light beam is affected by shadowing from its predecessors. The transparency of the object for the emission light controls to what extent the viewer can peer inside the object.

1. <http://www.svi.nl/SFP>

## Orientation and Zoom

Adjust the viewpoint by moving the *Tilt* and *Twist* sliders (See Figure 13.2) or by clicking



**FIGURE 13.2.** The SFP Renderer window.

and dragging the mouse pointer across the scene. The magnification can be adjusted by using the *Zoom* slider or the scroll wheel.

## Render quality

The SFP renderer can render the image with three different levels of quality:

- **Low Quality.** This level is used when the scene is being moved or edited in any other way (zoomed in and out, adjusted with new parameter values, etc). This quality level is also used after editing the image if the Fast Mode and High Quality levels are switched off.
- **Fast Mode.** This quality level improves the scene greatly with respect to the Low Quality level. The Fast Mode level is used automatically when the renderer detects that the user stopped editing the scene (rotating, zooming, adjusting parameter values, etc) provided that the FAST MODE render button (🌀) is activated.
- **High Quality.** This mode can deliver even better quality than the Fast Mode although it can take more time to render the scene. The High Quality level is used automatically when the renderer detects that the user stopped editing the scene (moving, zooming, adapting parameters) provided that the HIGH QUALITY render button (⚙️) is activated.

Notice that the scene is rendered with low quality while being edited to allow for fast scene edition. The scene is automatically rendered with good quality (Fast Mode or High Quality) when the edition is finished and the scene released. This automatic rendering can be deactivated by pressing the SWITCH OFF rendering button (🛑). When either the FAST MODE render button (🌀) or the HIGH QUALITY render button (⚙️) are pressed the automatic rendering occurs again upon releasing the SFP scene.

When using poorly sampled images the FAST MODE and HIGH QUALITY modes allow for more limited zooming than the low quality mode. In that case a subtle zoom jump might be noticeable when switching between the low quality and the better quality modes.

### Threshold

The *Soft threshold* slider in the *Channel parameters* panel at the right affects the threshold level. The application of a threshold is a preprocessing step that *reduces the background* in the image, i.e. voxels with intensity values below the threshold value become transparent. Contrary to a standard threshold, which is 'all or nothing' (values above the threshold are kept, values below it are deleted), the soft threshold function handles images in a different way. It makes a *smooth transition* between the original and the deleted value.

### Penetration depth

The characteristic object size can be set by the *Penetration depth* slider in the *Image parameters* panel at the right side. This parameter affects both the excitation and the emission *transparency*. While traveling through the object, the light intensity is attenuated to some degree. This enables us to define some definition for penetration depth at which the light intensity is decreased to some extent, for instance 10 % of its initial value. This penetration depth should be in line with the object size. A transparent object is small with respect to the penetration depth. Thus for the same physical properties of the light one object can be transparent while the other is opaque due to its size. To find a reasonable range in transparencies the *Penetration depth* may be altered. The initial object size is computed from the microscopic sampling sizes and number of pixels the image is composed of. If the microscopic sampling sizes of the image are incorrect, then the *Penetration depth* is set according to some default parameters.

### Saving Scenes

Choose FILE→SAVE SCENE... to save the rendered scene as a Tiff file.

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## Advanced Usage

### SFP Fundamentals

The voxel values in the image are taken as the *density of a fluorescent material*. In case of a multi channel image, each channel is handled as a different fluorescent dye. Each dye has its specific excitation and emission wavelength with corresponding distinct absorption properties. The absorption properties can be controlled by the user (See the *transparencies* in Table 13.1 on page 82). The different emission wavelengths give each dye its specific color.

To excite the fluorescent matter light must traverse other matter. The resulting attenuation of the excitation light will cause objects, which are hidden from the light source by other objects, to be weakly illuminated, if at all. The attenuation of the excitation light will be visible as shadows on other objects. To optimally use the depth perception cues generated by these shadows, a flat *table* below the data volume is placed on which the cast shadows become clearly visible. In Figure 13.2 the table is rendered as a mirror.

After excitation the fluorescent matter will emit light at a longer wavelength. Since this emitted light has a different wavelength it is not capable to re-excite the same fluorescent matter: multiple scattering does not occur. Thus only the light emitted in the direction of the viewer, either directly or by way of the semi reflecting table is of importance. By sim-

ulating the propagation of the emitted light through the matter, the algorithm computes the final intensities of all wavelengths (the spectrum) of the light reaching the viewpoint.

The properties of the interaction between object and light (transparency), both for excitation and emission, can be adapted interactively by the user to produce different scenarios.

## Render Parameters

Table 13.1 gives an overview of all render parameters in the SFP Renderer.

**TABLE 13.1. SFP render parameters**

Parameter	Description
Time frame	A selection of the time frame for time series.
Penetration depth	Adjust the total transparency of the rendered object. See “Penetration depth” on page 81.
Excitation	Adjust the excitation transparency for the matter in the selected channel.
Emission	Adjust the emission transparency for the matter in the selected channel.
Object brightness	Set the intensity level for the excitation light source for the selected channel.
Soft threshold	Adjust the threshold level for the selected channel. See “Threshold” on page 81.
Color mode	Adjust the color of the selected channel.
Background color	Adjust the color of the scene background.
Compute shadow	Choose whether or not the object’s shadow should be included in the scene.
Table	Choose whether or not the table underneath the object should be included in the scene.
Table distance	Adjust the distance between the object and the table.
Table reflection	Adjust the degree of reflection of the table.
Table size	Adjust the size of the underlying table.
Table color	Adjust the color of the table
First scene	Set the viewpoint of the first scene of the movie.
Last scene	Set the viewpoint of the last scene of the movie.
Set number of movie frames	Set the number of frames that will be included in the movie.
Set movie frame rate	Set the number of frames per second of the movie.
Light direction	Set the position of the light source.
Light intensity	Set the intensity of the light source.
Render size	Set the size (in pixels) of the SFP scene.
Render mode	Set whether the scene must be rendered in fast mode, in high quality mode, rendered in a movie or not rendered.

## Render Options

Table 13.2 gives an overview of the different render options that are available through the OPTIONS menu.

**TABLE 13.2. Render options for the SFP Renderer.**


Option	Description
SHOW SVI LOGO	Show or hide the SVI logo at the bottom right of the scene.
VIRTUAL RENDER SIZE	Adjust the size of the rendered image. When the render size exceeds the display area, then use the <i>middle mouse button</i> to drag and drop the rendered image.
COLOR MODE	Choose between EMISSION COLORS or GLOBAL PALETTE (See “Adjusting the Global Color Scheme” on page 195.).

## Templates

All scene settings, i.e. both the render options and all parameters, can be exported to a template file via FILE→SAVE SCENE TEMPLATE. The template files have the extension `.hgsv` and can be applied to any image that is loaded in the SFP Renderer. Keep in mind that the sampling sizes of the data affect the transparency.

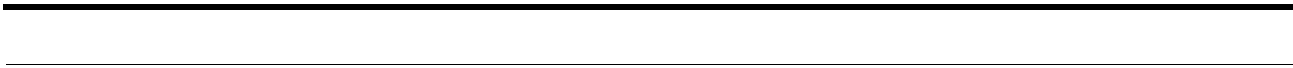
## Simple Animations

The *Huygens Movie Maker* (See “The Movie Maker” on page 91.) allows to easily create sophisticated animations using the MIP, SFP, and Surface Renderer.

Without the Movie Maker the SFP Renderer has the option to make simple animations of the image, changing the viewpoint in different frames. Set the render parameters for the first frame by clicking the MOVIE tab, SET →FIRST SCENE. Now adjust the viewpoint for the final frame, and click SET→LAST SCENE. Set also the *frame count*, *frame rate*, and other general options such as colors and light properties. Finally press the *animate* button (  ), and select a directory to save the AVI movie to.

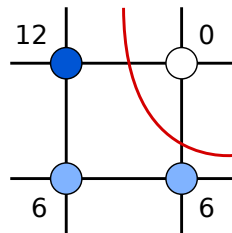
The exported AVI files use the MJPEG<sup>2</sup> codec and can be loaded in most movie players, including Windows Movie Player and Apple Quicktime.

2. <http://en.wikipedia.org/wiki/Mjpeg>



Huygens Surface Renderer is a powerful 3D visualization tool that enables the visualization of iso-surfaces of volumes.

An iso-surface is a 3D surface representation of points with *equal intensities* in a 3D stack; it is the 3D equivalent of a contour line. The iso-surface envelops voxels with intensities above a certain threshold. If those voxels are distributed in groups that are not spatially connected, they will be considered and labeled as independent objects.



**FIGURE 14.1.** A contour line for an interpolated value of 5. Because 5 is much closer to 6 than to 12, the distance of the contour to the voxel with value 12 is larger than the distance to the bottom-right voxel with value 6.

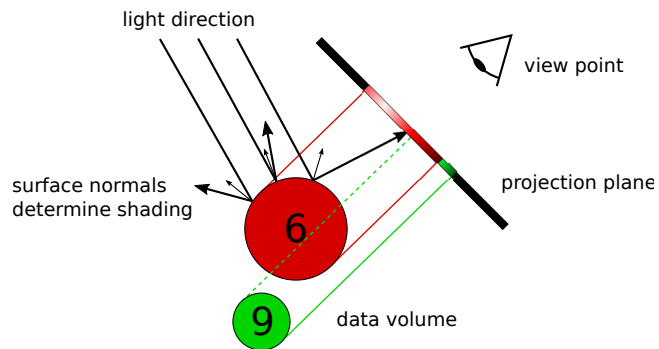
The iso-surface is a smooth surface through all points between the voxel locations which have an interpolated value equal to the threshold value (see Figure 14.1).

Shading enhances the perception of 3D shapes and texture (See Figure 14.2). Besides iso-intensity surfaces, this renderer is able to generate MIP projections which are blended with the surfaces to be used as a reference to the original microscopic data (See Chapter 12 on page 75).

Because the Surface Renderer is based on *fast ray tracing* that runs efficiently on multi-core computers, there is no need for any special graphics card as would be necessary for conventional polygon based techniques.

To start the Surface Renderer, right-click on a thumbnail and select VIEW→SURFACE RENDERER from the pop-up

menu. Alternatively, choose the corresponding icon from the taskbar or select the VISUALIZATION→SURFACE RENDERER from the main menu.

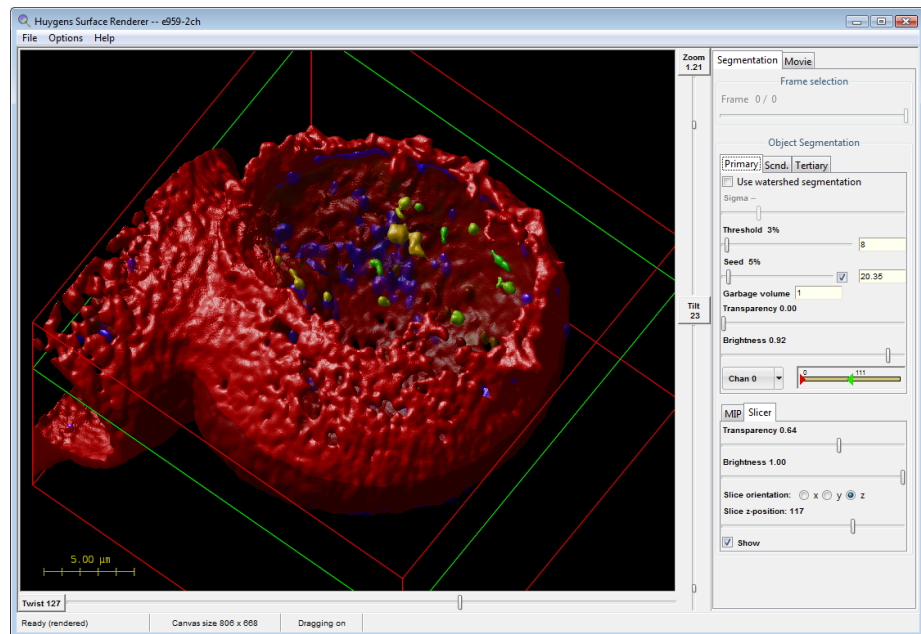


**FIGURE 14.2.** A schematic overview of surface rendering. The orientation of the surface determines the shading.

## Basic Usage

### Orientation and Zoom

Within the Surface Renderer window, you can adjust the viewpoint by moving the *Tilt* and *Twist* sliders or by dragging the mouse pointer on the large view (See Figure 14.3). The magnification can be adjusted using the *Zoom* slider or by using the scroll wheel. Use the middle mouse button to *pan* the center of the projection.



**FIGURE 14.3.** The Surface Renderer window.



## Threshold

Use the *Threshold* slider in the *Render pipes* panel to apply different thresholds to the data channels. Voxels that are spatially connected and have intensities above this threshold define *closed volumes*. These volumes are represented by the 3D (iso-intensity) surfaces containing them, each object having a different surface color.

The three *render pipes*, in the *Object Segmentation* frame, referred to as *primary*, *secondary*, and *tertiary*, allow us to define three threshold levels that can be applied to the same or to different data channels. The data channel can be selected using the menu button in the *Object Segmentation* panel. The color range in which the different objects inside a render pipe will be displayed can be adjusted with the hue selector next to it. To facilitate the segmentation of the objects, a watershedding can be applied. For more information see “Using the watershed segmentation” on page 139.

## Saving Scenes

Press the HIGH QUALITY button in *Actions* panel to apply full scene *anti aliasing* to the rendering and choose FILE→SAVE SCENE... to save the rendered scene as a Tiff file.

## Advanced Usage

### Adding a Maximum Intensity Projection

Besides the surface pipes there are additional rendering pipes to redirect data to the scene. The *MIP* pipe works by projecting the voxels with maximum intensity laying in the path of the rays traced along the viewing direction (See Chapter 12 on page 75). When the MIP pipe is used in combination with the surface pipes, a clearer representation can be obtained of the different objects in the image. In other words, the MIP of a channel can be used as a spatial reference for the objects present in other channels.

### Adding a Slice

The *Slicer* pipe is available to represent a single slice of the 3D dataset in its corresponding location.

### Render Parameters

Table 14.1 gives an overview of all render parameters in the Surface Renderer.

**TABLE 14.1. Surface render parameters**

Parameter	Description
Frame	Set the time frame (in case of a time series).
Threshold (surface)	Adjust the threshold level for the selected pipe, i.e. the intensity for which the iso-intensity surfaces are defined. See “Threshold” on page 87
Threshold (MIP)	Set the soft threshold level for the MIP pipe. See “Threshold” on page 76
Garbage volume	Volumes that contain less voxels than defined by the <i>garbage volume</i> parameter will not be rendered. This is useful for rendering only significant objects in noisy images.
Transparency	Set the level of transparency to other pipes.
Brightness	Adjust the brightness for the selected pipe.
Slice Z-position	Set the position of the slice in the <i>Slicer</i> pipe.

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## Render Options

Table 14.2 gives an overview of the different render options that are available through the OPTIONS menu. The ANIMATION FRAME COUNT, ANIMATION FRAME RATE and RENDER QUALITY apply to the rendering of simple movies as explained in the next section.

**TABLE 14.2. Render options for the Surface Renderer.**

Option	Description
ANIMATION FRAME COUNT	Set the number of frames that will be rendered in a movie. 180 frames with a frame rate of 24 fps result in a movie with a duration of 7.5 seconds.
ANIMATION FRAME RATE	Adjust the frame rate; a rate of 24 frames per second is fine for smooth movies.
VIRTUAL RENDER SIZE	Adjust the size of the rendered image. When the render size exceeds the display area, then use the <i>middle mouse button</i> to pick up and move the rendered image.
TRANSPARENCY DEPTH	This option defines how different surfaces are seen through the others:  <i>Simple</i> : see through one surface, the surface closest to the viewer. Quite often this is sufficient.  <i>Normal</i> : see through two surfaces.  <i>Deep</i> : consider many more screening levels, making the final rendering computationally more complex.
BOUNDING BOX	Enable or disable the bounding box, or adjust the line color.
SCALE BAR	Enable or disable the scale bar.
SHOW SVI LOGO	Hide or show the SVI logo at the bottom right.
High quality MIP	Render the MIP pipe in high quality mode.
CENTER SCENE	Undo both the panning of the projection center (right mouse button) and the rendered image itself (middle mouse button).

## Templates

All scene settings, i.e. both the render options and the parameters, can be exported to a template file via FILE→SAVE SCENE TEMPLATE. The template files have the extension `.hgsv` and they can be applied to any image that is loaded in the Surface Renderer.

---

## Simple Animations

The Surface Renderer has the option to make simple animations of the image. When selecting the movie-tab, one can set the render parameters for the first scene and click the FIRST SCENE button in the *Position settings* panel of the *Set view* settings. Now adjust the viewpoint for the last scene, and click the LAST SCENE button of the *Set view* settings. Also the *frame count*, *frame rate*, or other render options in the OPTIONS menu can be adjusted.

If you want to view or adjust the first or last scene, press the corresponding button next to *Show*. This will show you the stored scenes. You can adjust these settings and again store the scenes via the *Set view* buttons.

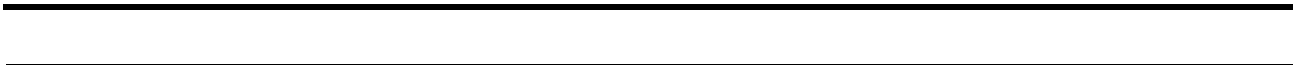
Finally press the *Animate* button in the *Actions* pane, and select a directory for storing the AVI movie or the Tiff frames.

The exported AVI files use the MJPEG<sup>1</sup> codec and can be played in most movie players, including Windows Movie Player and Apple Quicktime. Tiff frames are useful to combine multiple animations or edit the movie in e.g. Windows Movie Maker.

For more sophisticated movie making options, and for creating animations using combinations of MIP, SFP, and Surface rendered images, we like to refer you to the Huygens *Movie Maker* (See “The Movie Maker” on page 91.)

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1. <http://en.wikipedia.org/wiki/Mjpeg>



## *The Movie Maker*

The Movie Maker is a tool that allows the user to easily create sophisticated animations of multi-channel 3D images using the powerful Huygens visualization tools. Animations from the *MIP Renderer* (See Chapter 12 on page 75), the *SFP Renderer* (See Chapter 13 on page 79), and the *Surface Renderer* (See Chapter 14 on page 85) can be combined in a single movie.

The Movie Maker assists the user in creating the *key frames* that define the main scenes, and *transitions* between them. Interactive manipulation of the scenes is possible in the *e Timeline* or by using the interfaces of the renderers.

The movies can be exported to AVI files or to TIFF series that can be combined with other software. Movie projects can be saved for later editing or for usage with other 3D datasets.

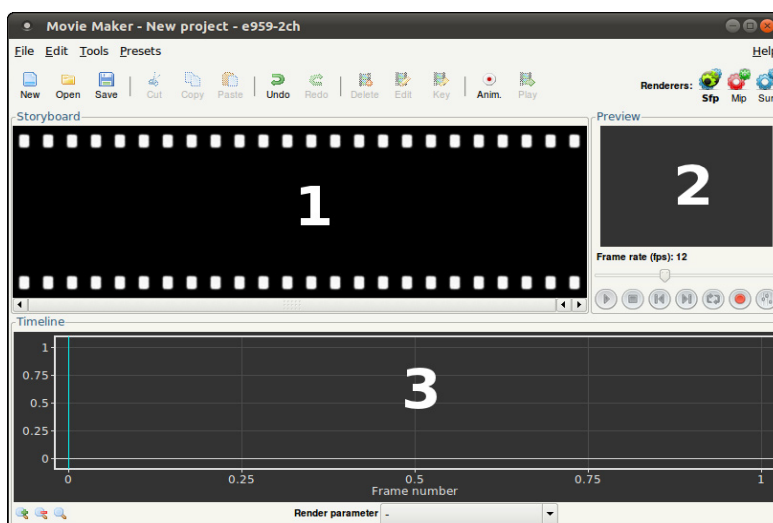
An introductory tutorial can be found in the HELP menu at the top right. This interactive tutorial guides the user step by step through the process of creating a simple movie.

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### *An Overview*

Figure 15.1 shows the Movie Maker's user interface. The numbered areas are:

1. The *Storyboard*: this filmstrip shows the main elements of the movie, which are the keyframes and the transitions between them.
2. The *Preview area*: this mini movie player quickly creates a low-resolution version of the movie.
3. The *Timeline*: this interactive plot shows how render parameters change over time. Use the menu-button below this timeline to the render parameter to be visualized. Green nodes, representing render parameters at each keyframe, can be dragged vertically.



**FIGURE 15.1.** An overview of the Movie Maker user interface, showing the *Storyboard* (1), the *Preview* (2), and the *Timeline*. (3).

## Creating and Adjusting Keyframes

### About Keyframes

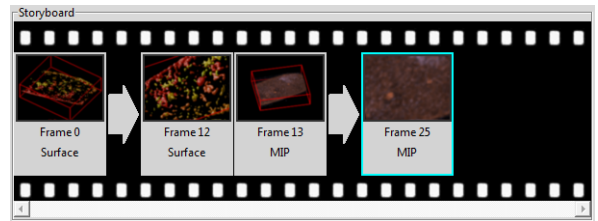
A keyframe defines a *control point* within a transition. This can be either a start point, end point, or an intermediate point in time. The appearance of the 3D rendered image is *fixed* in these frames. In between keyframes, the Huygens Movie Maker calculates a smooth or linear transition (a technique called *tweening*).

### Inserting New Keyframes

To add the first keyframe to the storyboard, one of the renderers should be launched from the Movie Maker window by pressing the corresponding button ( , , or ). Within the renderer, a scene can be defined using the available controls; see Chapter 12, Chapter 13, and Chapter 14 for more information. If the view of the first frame is satisfactory, press the *Add keyframe* button ( ) to capture this configuration and to add the keyframe to the storyboard. All render parameters are captured and most of them can be smoothly animated.

A movie needs at least two keyframes from the same renderer to set the start and end point of a *transition*. If the second frame view is satisfactory, press the *Add keyframe* button ( ) again. The storyboard will now show two keyframes with an arrow in between. This arrow indicates the transition from one keyframe to another.

The Huygens Movie Maker accepts a mix of keyframes from different renderers, but transitions can only be made between keyframes from the same renderer, as shown in Figure 15.2.



**FIGURE 15.2.** The Movie Maker's storyboard showing two surface-rendered keyframes, followed by two MIP renderer keyframes.

### Editing Keyframes

To edit an existing keyframe, double click it or select the frame and choose **EDIT**→**EDIT KEYFRAME** from the menu. This will load the keyframe's settings in the corresponding renderer. The renderer's controls can now be used to adjust the 3D scene. To submit the changes to the Movie Maker, press the *Add keyframe* button (📄) again. Because the original keyframe is still selected, the Movie Maker will ask if the original frame should be replaced.

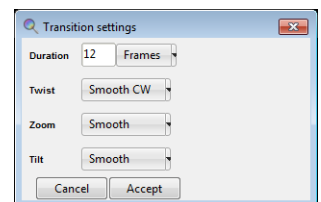
## Using the Storyboard

### Rearranging Keyframes

The storyboard allows the user to copy, delete, and rearrange keyframes. The *Cut* (✂), *Copy* (📄), *Delete* (🗑), and *Edit* (🔧) buttons are activated whenever a keyframe is selected. If there is no keyframe selected, the *Paste* button (📄) will append the copied or cut keyframe to the last frame in the storyboard. If one of the keyframes is selected, the Movie Maker will ask where the frame should be inserted. All these operations can be undone with the *Undo* button (↶).

### Changing Transitions

Transitions can be changed by double clicking the arrow that joins two keyframes. This will pop-up a window in which the duration of transition (in frames or seconds) can be changed, as well as the transition type of the parameters that will be animated (See Figure 15.3). For most parameters, the Huygens Movie Maker uses linear or smooth transitions. An exception to this is the twist, which also requires a direction of rotation (clockwise or counter clockwise) and a value for the number of rotations.

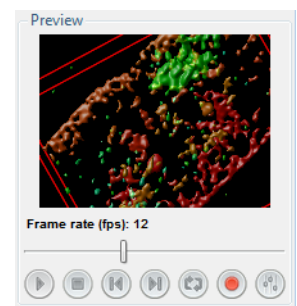


**FIGURE 15.3.** The transition settings dialog is shown by double clicking a transition arrow in the storyboard.

### Playing a Preview Movie

To preview the movie, just press the *play* button (▶) in the *Preview* area (See Figure 15.4). The Movie Maker quickly creates a low-resolution movie and displays it in the preview area. If *loop mode* (🔁) is on, the movie will be repeated until the *stop* button (⏹) is pressed.

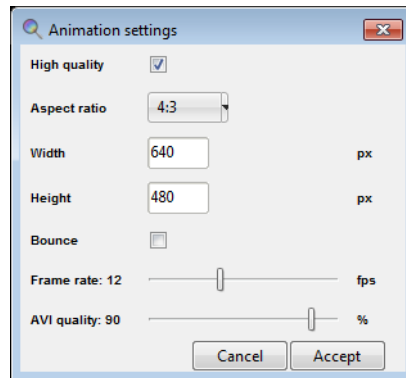
To change the animation settings (*aspect ratio*, *size*, *frame rate*, etc.), press the *render settings* button (⚙) in the *Preview* area. The *High quality* setting and the *AVI quality* are not applied to the movie preview, but only to the final result.



**FIGURE 15.4.** The Preview area.

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## Export to AVI or Tiff Series



**FIGURE 15.5.** The animation settings dialog.

Press the record button (●) (See Figure 15.4) to render the final movie and to export it to AVI or a Tiff series. Before the save dialog appears, the Huygens Movie Maker will show the animation settings dialog (See Figure 15.5), where the AVI quality can be set. Note that large movies will take several minutes to render.

Once the movie has been exported to AVI, it can be opened in a movie player like Apple QuickTime or Windows Movie Player. To quickly open the last saved movie in the operating system's default movie player, press the button labeled "Open AVI in external movie player" (🎬).

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## Working with Movie Projects

### Saving Projects

A collection of keyframes, transitions, and animation settings is called a *project*. The image itself does not belong to the project. To save the current project, press the *Save* button (💾) or choose FILE→SAVE PROJECT in the menu. Movie Maker project files have the extension `.hgsa` (Huygens animation template).

When closing the Movie Maker window, you are asked to save the project if unsaved changes have been made.

### Reloading and Appending Projects

If a saved project is reloaded in a Movie Maker that has the same image attached, then the final movie will be an identical copy. It is also possible to apply saved projects to other images, or to the current storyboard. To load or append a project from disk, select FILE→OPEN PROJECT... or FILE→APPEND PROJECT... from the menu, respectively.

The Movie Maker has some example projects available under the menu PRESETS that can be used.

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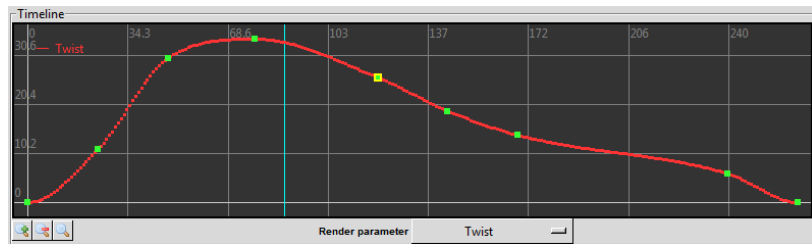
## Using the Timeline

### Visual Feedback

The *Timeline* is an interactive plot which shows the frame number on the horizontal axis and the value of a selected render parameter on the vertical axis (See Figure 15.6). It gives a more detailed visual feedback on values of each of the animated parameters. A mouse click in the *Timeline* area will select the nearest frame and displays a preview of this frame in the *Preview* area. The left and right arrow keys can be used to navigate through the frames.

To zoom in on the timeline, click near the frame of interest and use the scroll-wheel or the *magnifying glass* buttons below the timeline.





**FIGURE 15.6.** The *Timeline* area gives detailed visual feedback on the animated parameters.

### Changing Render Parameters

The keyframe nodes are displayed in green and can be dragged vertically to tune the value of the parameter. The (interpolated) transition frames are displayed as smaller red dots (See Figure 15.6). If a render parameter has been changed, the Movie Maker will recalculate the transitions, update the thumbnails in the storyboard, and update the still of this frame in the *Preview* area.

To select the render parameter to be shown in the graph, open the drop-down menu below the timeline. Because the number of parameters that can be animated is extensive, the menu only lists the ones that change during this movie. If “*Other render parameter...*” is selected, a dialog window will pop up that shows a list of all available parameters.

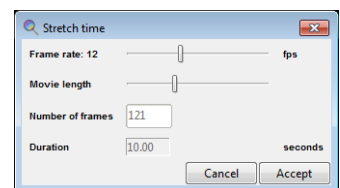
To change parameters in a frame which is not a keyframe, the frame first needs to be converted into a keyframe. To do this, select the frame in the timeline and choose EDIT→CONVERT TO KEYFRAME from the menu.

## Advanced Topics

### Stretching Movie Length

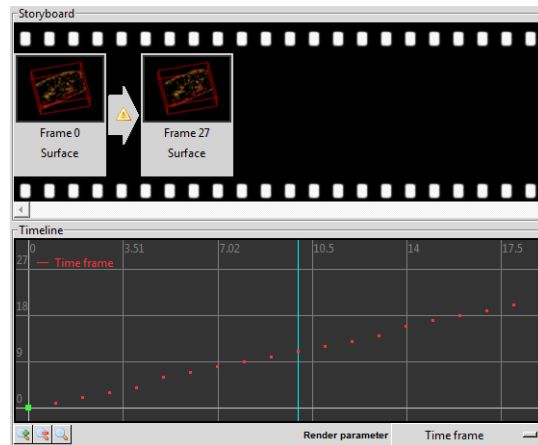
The number of frames in a transition can be changed by double clicking the transition arrow. However, if a movie gets complex it is easier to use the *stretch tool*. This tool can be found in the menu TOOLS→STRETCH MOVIE.

The stretch tool shows the *Frame rate*, the *Number of frames*, and the *Duration* in seconds (See Figure 15.7). The two sliders can be used to change the frame rate and the number of frames; changing these will affect the duration of the movie. The stretch tool tries to redistribute the total number of frames over the complete movie in such a way that the relative length of each transition does not change.



**FIGURE 15.7.** The stretch dialog helps increasing or decreasing the number of frames in a complex movie.

## Synchronizing Transitions in Time and Slice Plane Transitions



**FIGURE 15.8.** A transition in which the *time frame* parameter is out of sync with the number of frames. In this example, the transition counts 27 frames, while the *time frame* parameter increases linearly from 0 to 30. Note the irregular change of this parameter in the plot.

Most render parameters, like the *Tilt*, *Twist*, and *Zoom*, can be set to non-integer values. The *Time frame* (in case of a time series), and the *Slicer z-position*, however, are fixed to integer values. When the transition of such a parameter is not linear, or when the change in value does not match the number of frames, then this parameter is *out of sync*. In that case the Movie Maker will show a warning symbol (▲) on the transition arrow (See Figure 15.8). In the final result, these asynchronous transitions may show irregularities.

To restore the synchronization, right click on the transition arrow and choose SYNCHRONIZE TIME FRAMES... from the pop-up menu. The Movie

Maker will set the transition type to linear and add or remove some frames from the transition to achieve a 1:1, 2:1, 1:2, etc. synchronization.

## Creating Loopable and Bouncing Movies

In two simple steps, a movie can be made *loopable*, i.e. it can be played seamlessly in repeat mode:

1. Copy the first keyframe and paste it to the end.
2. Right-click on the final keyframe that has just been pasted, and click SKIP THIS FRAME in the pop-up menu.

The result is the best when all transitions are set to *smooth*.

To create a *bouncing* animation, i.e. an animation that is played in reverse when the last frame is reached, mark the *bounce* option in the animation settings dialog. Doing so will not insert extra keyframes on the storyboard, but the frames are appended in reverse to the final AVI file or Tiff series.

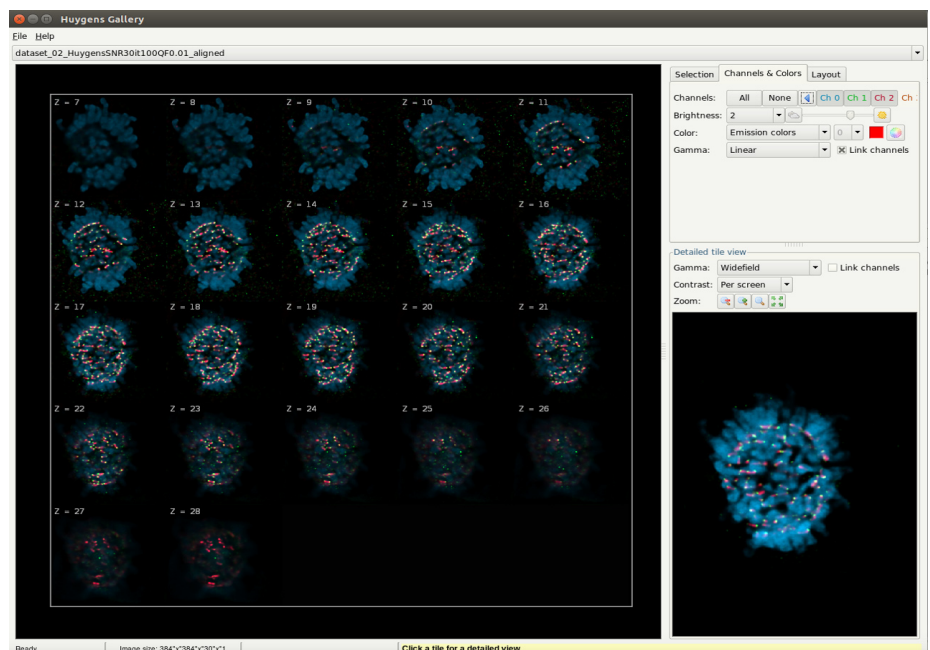
## About Movie Quality

In the Huygens Movie Maker, two types of quality can be set:

1. The *High quality* check box determines if the quality of the renderers should be set to the highest possible setting when rendering the final movie.
2. The *AVI quality* scale bar determines the compression level of the AVI file. Set to 100 %, the quality is the best, but then the file size will be large.

These quality settings are not applied to the movie preview, but only to the final result.

The *Gallery* can be found under the menu *VISUALIZATION* and gives you a quick overview of your microscopic image. Instead of slicing through your image using a slider, the *Gallery* tool displays the individual slices of your z-stack or time series next to each other (See Figure 16.1). As a user, you can control which z-slices or time frames are displayed



**FIGURE 16.1.** The *Gallery* tool showing the individual slices of a Z stack image.

in the gallery. Display settings for the overview can be adjusted using the options listed under the *CHANNELS & COLORS* tab. The *LAYOUT* tab offers the possibility to change the Layout and decoration of the overview. The overview can be exported as a tiff file via the *FILE* menu. Upon selecting a slice with the left mouse button, a detailed view appears in the window *Detailed tile view*. *GAMMA*, *CONTRAST* and *ZOOM* controls allow optimal and closer viewing of a selected slice in this window. For more details on the use of these controls see “Using the Slicer in Advanced Mode” on page 66.



## The Stitcher

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### *Stitching and Deconvolution*

When imaging large objects at high resolution, the microscope field of view can become too small. This problem is typically solved by subdividing the region to be imaged into multiple smaller images (tiles), which after acquisition are combined to a larger overview.

Improving such stitched images with deconvolution is a challenging task as extensive computer resources for allocating both raw and processed images are needed. The Huygens *Stitcher* wizard offers a unique solution for this by combining high-quality stitching with deconvolution. This integrated approach offers the advantage of minimizing the resources needed for obtaining restored contrast-rich and high-resolution images of large objects. Furthermore, the post-acquisition work-flow is considerably simplified for the user.

---

### *Vignetting and Shading correction*

Individual building blocks (tiles) of a stitched mosaic often suffer from vignetting and shading because of uneven field illumination. Such distortions can be seen in individual tiles, but are more apparent when tiles are positioned next to each other (stitched). To correct for these artifacts, the Huygens *Stitcher* is equipped with an automatic vignetting/shading correction. As an alternative, there is a “manual” vignetting and shading correction option for which flat-field and dark-frame image need to be loaded.

To summarize, the Huygens *Stitcher* is an easy-to-use wizard that allows for:

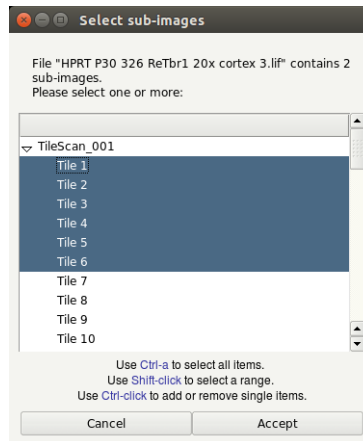
- optimal stitching of tiles.
- automatic vignetting and shading correction.
- integrated deconvolution.
- Stitching of 2D, 3D multichannel and time series images.
- Stitching of unlimited files sizes and numbers of tiles.

---

### *Starting the Stitcher*

The *Stitcher* wizard can be started in Huygens from the DECONVOLUTION menu. If Leica LIF or Zeiss CZI images with tiles, or a XML file with accompanying Tiff tiles are opened, the tile content will be automatically recognized and opened in the *Stitcher* wiz-

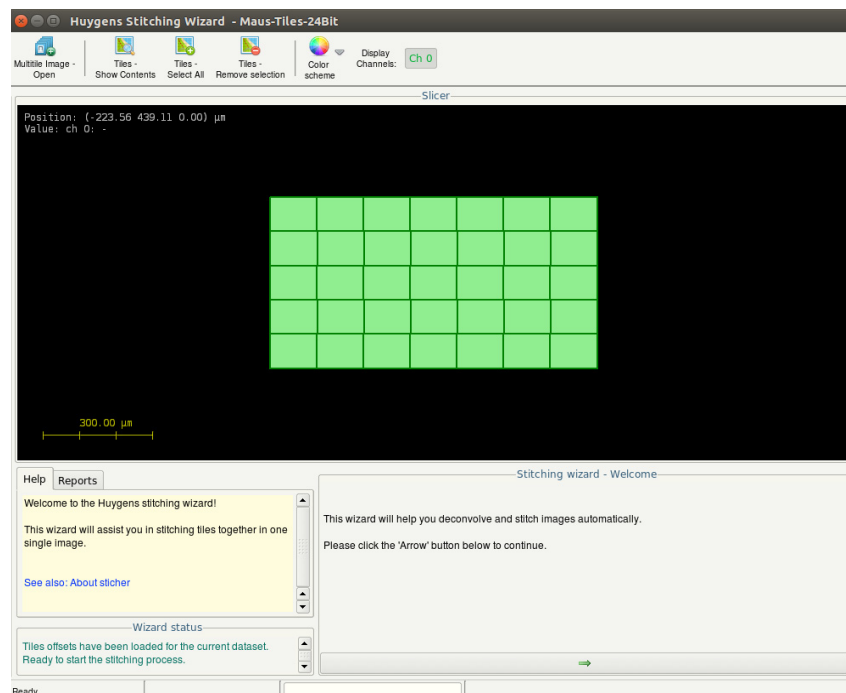
ard. Click the MULTITILE IMAGE – OPEN button located at the top bar, and select the tiles that should be loaded into the Stitcher. This opens the window Select sub-images (See Figure 17.1). Use CTRL-click to select or de-select individual tiles, SHIFT-CLICK to select a range, and CTRL+ A to selects all tiles.



**FIGURE 17.1. Selection of tiles contained in a dataset.**

## Selection of Tiles

The tiles' initial positions are read from the metadata and shown in the *Stitcher* window (See Figure 17.2 ). To view the actual content of the tiles, click the TILES – SHOW CONTENTS button in the top menu of the *Stitcher* window. By default, all tiles of the mosaic are selected. Use the *mouse left click* plus the CTRL/SHIFT keys to select a specific region of tiles.



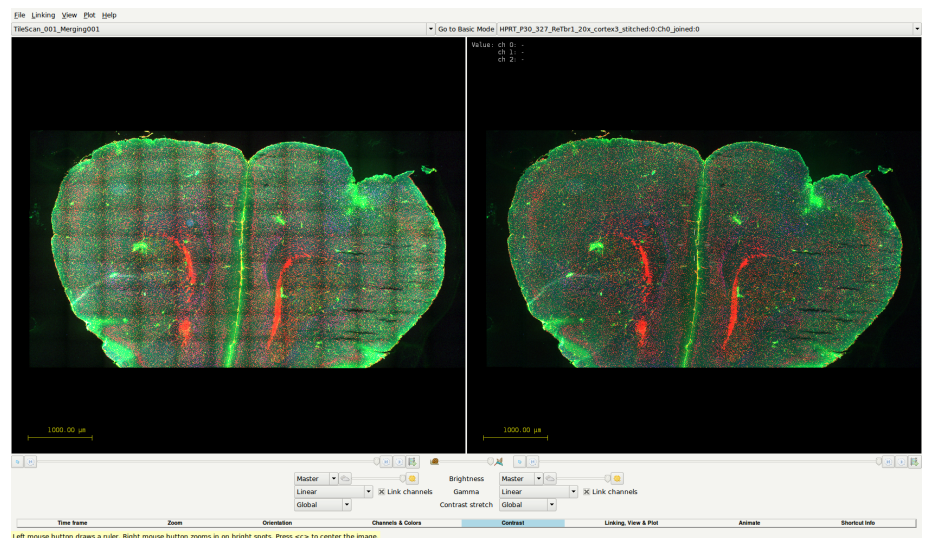
**FIGURE 17.2. The Stitcher window showing the tiles' initial positions.**

## Vignetting and shading correction

Continue and follow the instructions of the wizard to select whether automatic vignetting correction must be applied, or if this should be manually done by loading a flat-field and a dark frame image. The user can choose between a circular and an ellipsoidal model for the vignetting. The steepness of the vignetting model at the tile edges can also be adjusted. An example of automatic vignetting/shading correction is shown in Figure 17.3.

## Deconvolution of tiles

The *Stitcher* wizard offers the user the unique possibility to deconvolve each tile automatically before stitching. Every single tile is first deconvolved before being stitched into the mosaic. By doing so, Huygens not only simplifies the work-flow but also minimizes the processing workload that would be needed for deconvolving a stitched mosaic image, or for stitching tiles that have increased in size because they have already been deconvolved. To deconvolve the tiles before stitching, you just need to select a microscopic template and deconvolution template (See “Microscopic Template Files” on page 33., and See “The Final Stage” on page 40.). These templates should be created first before starting the *Stitcher* wizard by using the *Deconvolution Wizard* (Chapter 5 on page 29) or the *Batch Processor* (Chapter 7 on page 49).



**FIGURE 17.3.** Huygens stitched tiles of widefield fluorescent Leica LIF data. The same data is shown in the Twin Slicer without (left panel) and with (right panel) Huygens automatic vignetting correction. Note that the overlapping regions are less apparent after vignetting correction. Image represents a developing mouse cortex (P30) stained for Tbr1, reelin and an unspecified molecule .





## *The Crosstalk Corrector*

The Crosstalk Corrector detects and corrects for (linear) crosstalk between all channels in multi-channel images.

---

### *What is crosstalk?*

In fluorescence microscopes, crosstalk (or bleedthrough) can occur when acquiring a multi-channel image. In that case, the emission radiation of a given emission wavelength is detected by the wrong detector because part of the photons go through the wrong optical path inside the microscope (e.g. because the filters efficiency is not 100%). Therefore, some signal is actually recorded as coming from a certain dye when it really comes from a different one.

To avoid this situation, microscopes usually excite each dye alternatively, making sure that all the detected radiation comes from a single dye type. But some experiments (like e.g. Fluorescence Resonance Energy Transfer - FRET) require simultaneous acquisition of signal from all the present dyes, with the possible risk of crosstalk.

Crosstalk within multi-channel images dramatically affects almost any type of data analysis, including colocalization analysis.

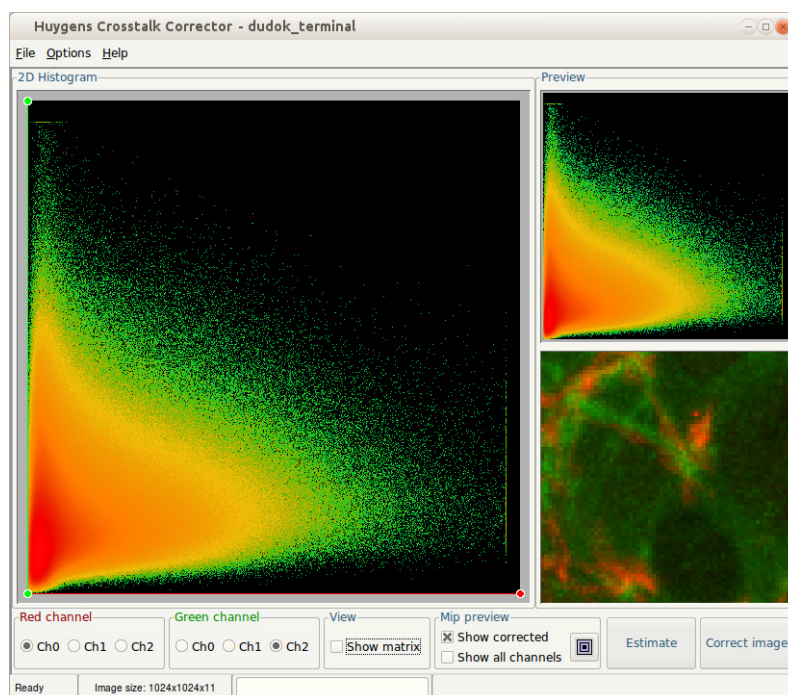
In line with our aim to improve microscopic image quality and measurements, we have implemented a Crosstalk Corrector tool in Huygens Professional to correct for this imaging artifact.

---

### *Starting the Crosstalk Corrector*

To start the Crosstalk Corrector in Huygens Professional, select an image and go to DECONVOLUTION > CROSSTALK CORRECTOR. This tool can handle multi-channel time serie images up to 32 channels.

The tool does not automatically estimate the crosstalk at startup. This is to avoid slow loading time, especially if the image contains many channels. The more channels there are in the image, the longer the estimation will take.



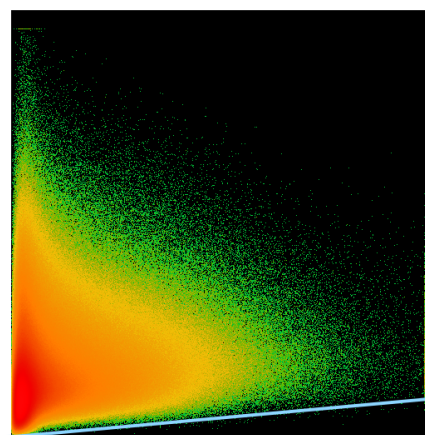
**FIGURE 18.1.** The startup window of the Crosstalk Corrector with a large 2D histogram of the original data (left) and a preview of the 2D histogram and MIP of the corrected data (right).

The Crosstalk corrector shows a large 2D histogram in the left panel of the startup window, see Figure 18.1. This histogram is from the original raw data set and is useful for the detection and visualization of the crosstalk coefficients.

Assume, for example, that in a 2-channel image the first channel bleeds into the second channel. Then at least a fraction of every signal (or intensity) in the first channel exists in the second channel. And so, for every signal in the first channel no intensity combination exists with low signal (smaller than a fraction of the first channel) in the second channel. This is visualized by an empty (except for some noise) triangular shape, from the origin along the axis of the first channel, see Figure 18.2.

Note that the colors within the 2D histogram have nothing to do with the colors used to represent the channels. The colors show the density or count of intensity combinations within the two channels.<sup>1</sup>

The slope of the diagonal of the triangle is the crosstalk coefficient of, in this case,



**FIGURE 18.2.** A 2D histogram, showing the intensity range of the first channel on the x-axis and the intensity of the second channel on the y-axis. The triangle shows that for every intensity in the first channel there is always a fraction of it in the second channel which is typical for crosstalk.

1. Read more about 2D histograms at <http://www.svi.nl/2Dhistogram>

channel 0 in channel 1. For each crosstalk or bleedthrough in each channel there is a crosstalk coefficient. For a 2-channel image there are two crosstalk coefficients, for a 3-channel images there are six, for a 4-channel image there are twelve.

In general, for an  $n$ -channel image there are  $n^2 - n$  crosstalk coefficients. These coefficients can be displayed in a matrix with element  $(i,j)$  the crosstalk coefficient of channel  $i$  in channel  $j$ . The elements  $(i,i)$  can be ignored, since comparing channels with itself will result in a crosstalk of 1.0. Note that the crosstalk coefficient matrix is not symmetric.

The crosstalk corrector can handle multi-channel images up to 32 channels, but the histogram can only show two channels at a time. That is why you can select two channels to visualize the 2D histogram, but the correction will take place on all the channels simultaneously.

Next to the large 2D histogram, within the preview frame, the 2D histogram and MIP view of the corrected data are shown. This way you can quickly validate the crosstalk correction.

With the options at the bottom of the window you can adjust all of the available display settings: set the two channels for the histogram views, toggle between histogram view and matrix view, toggle between corrected and original data, change views between all channels (shown in grey) or only the selected channels (shown in their corresponding channel selector color) and to center the MIP preview. Use the mouse scroll to zoom the MIP preview.

With the ESTIMATE button you can estimate all the crosstalk coefficients. With the CORRECT button you apply the final crosstalk coefficient matrix to the image and the result will be shown in the main window of Huygens Professional.

---

### *Adjust the crosstalk coefficients*

There are three ways to set the crosstalk between channels. The simplest is to press the ESTIMATE button and let the tool determine the crosstalk coefficients. If the estimation is not satisfactory the crosstalk coefficients can be adjusted manually.

The crosstalk coefficients can be manually adjusted by moving the handles within the 2D histogram, which are the small circles at the end of the crosstalk lines. When the handles are moved, the crosstalk line changes, and thus the crosstalk coefficient.

Click and drag on the middle of a crosstalk line, to move this line completely. This does not change the crosstalk coefficient, because the slope does not change. It is intended for verification only.

The crosstalk coefficient can also be adjusted directly by switching to matrix view and changing the element in the matrix which corresponds to the correct crosstalk coefficient. For example, if the crosstalk coefficient of channel 2 in channel 3 needs to be adjusted, edit element (2,3) (row, column) in the crosstalk coefficient matrix.



## *The Hot Pixel Remover*

The *Hot Pixel Remover* can detect and correct for hot pixels automatically.

---

### *Why correct for hot pixels?*

Hot pixels appear as bright pixels within your image. They do not originate from the specimen or noise, but are caused by individual sensors of the CCD camera with higher than normal rates of charge leakage. In fact, all pixels on a CCD detector have some charge leakage. This is why the amount of hot pixels will increase when you increase the exposure time. The charge leakage will even get worse at higher temperatures. Hot pixels are unwanted intensities which you can remove from the image, using the Hot Pixel Remover.

Removal of hot pixels is useful for three reasons:

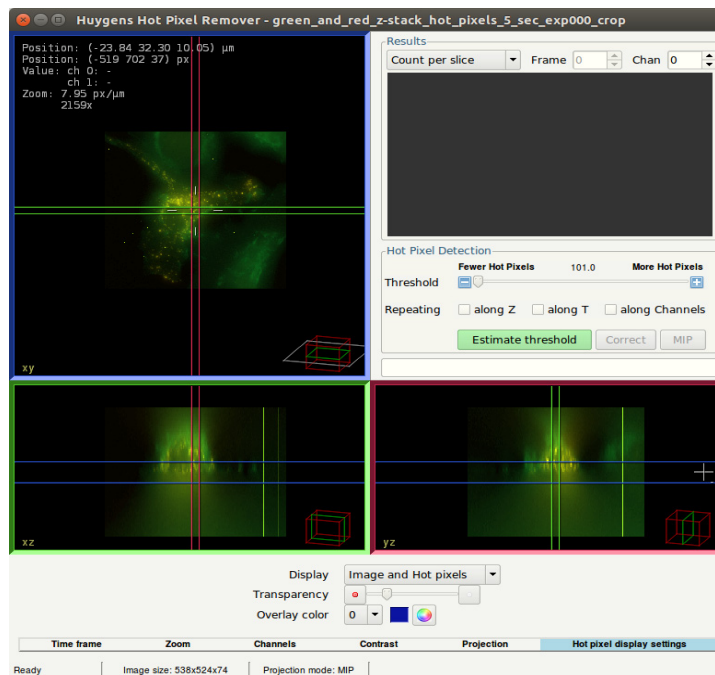
- For deconvolution; hot pixels affect the deconvolution result, because they disturb the normal signal distribution in your image. Especially when those hot pixels are not due to strong noise, but caused by deficiencies of the camera.
- To avoid false colocalization; when hot pixels occur at the same position in each channel, this will affect the colocalization analysis.
- For visualization; hot pixels disturb the contrast range and could force the contrast of the image to be very low. This may result in that objects of interest with lower intensity values become almost invisible.

Hot pixels may also be a product of deconvolution. If a very tiny object in your original image fits the PSF perfectly, deconvolution may result in one very bright spot in your image. This can disturb the contrast range and as such the visualization of the image. We have come across this situation a few times. So, if the image seems black after deconvolution, try to remove hot pixels first and see if this brings back the original contrast of the image.

---

### *Starting the Hot Pixel Remover*

The *Hot Pixel Remover* can be started from the DECONVOLUTION menu in Huygens Professional. .



**FIGURE 19.1.** The start-up window of the Hot Pixel Remover.

The window of the Hot Pixel Remover shows orthogonal views of the image. At the bottom of the window there are several tabs with which you can change the visualization of the image and the hot pixels (see Figure 19.1):

- *Time frame*: opens a slider for changing the time frame
- *Zoom*: simply zoom in or out by using the buttons or the mouse scroll.
- *Channels*: if you are only interested in specific channels, channels can be selected or deselected and the colors can be adjusted. By default, all channels are shown.
- *Contrast*: When the hot pixels show a large variety of intensities, it can be useful to increase the contrast.
- *Projection*: here you can switch between a single slice, Maximum Intensity Projection (MIP), or Sum projection.
- *Hot pixel display settings*: the hot pixels are visualized as an overlay over the original image. The image and overlay can be switched on or off to verify whether the hot pixels are correct. Transparency and color of the hot pixels can be adjusted here.

## Options

### Threshold

Threshold is used to filter hot pixels on a value calculated with an algorithm that takes the intensity and location of the neighboring pixels into account. A high threshold results in a lower count of hot pixels, and a low threshold in a higher count. At start-up the strength is always set to 50.

### Repeating

If the hot pixels originate from an abnormal charge leakage or defect in the CCD, then these defects are most likely visible at the exact same xy-locations for each z-slice, time frame, and very likely also for each channel. If you know that the hot pixels are con-

served in a particular dimension, you can set these dimensions here. This extra information is then used to detect hot pixels even better. False positives will most likely be eliminated if the information of other z-slices is taken into account. By default the `in Z` is turned on, because hot pixels are likely to be conserved in every slice of a 3D stack.

If you want to remove hot pixels which are a product of deconvolution, all the repeating options should be turned off. Because in that case, you are searching for a single hot pixel which is not conserved in any dimension.

### **Results**

The results of the hot pixels are displayed in a plot as the hot pixel count per slice for a specific channel. You can switch between the channels. For time series images, the pixel count can be plotted per frame.

### **Viewing the result**

You can zoom in the scene and change contrast and color settings to optimize the visualization. Still, it may be difficult to find hot pixels. Then, it may be useful to right click on the image and use the option `FIND CLOSEST HOT PIXEL`, which will move the mouse to the closest detected hot pixel.

---

## *Estimating and finalizing*

### **Estimating Threshold**

The strength is set as default to 50. It is difficult to determine what the correct strength settings is that separates the real hot pixels from the false positives. To minimize false positives, you can press the `ESTIMATE THRESHOLD` button which will set the strength to an estimated position. To prevent centers of small bright objects to be included as hot pixels, fine tuning with the plus and minus sign may be necessary.

If the estimated threshold is higher than 100, the hot pixel remover makes a safe decision and indicates that no hot pixels are detected.

The reason that the estimation is not done at startup is because it may be a time-consuming process. Note that every time you change the repeating settings you need to re-run the estimation.

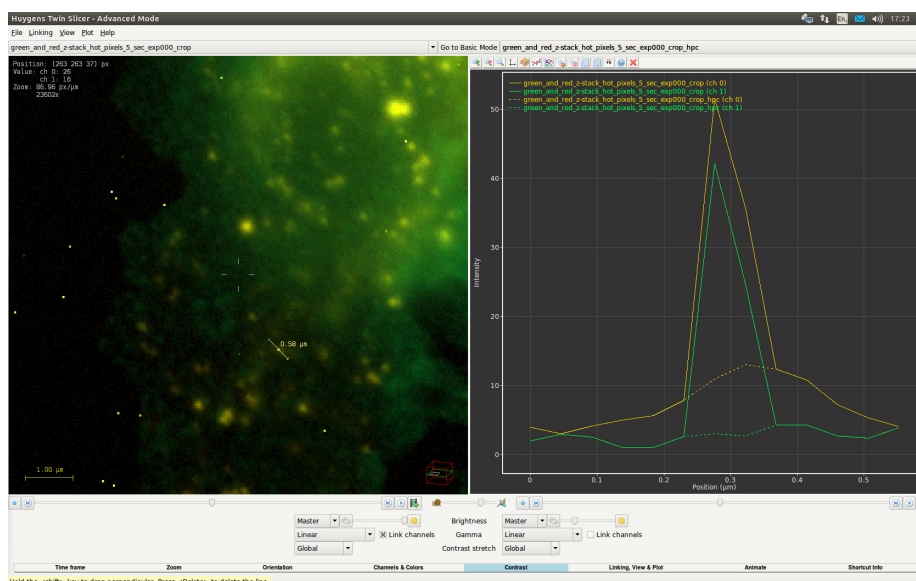
### **Remove hot pixels**

Once you are satisfied with the hot pixel detection, you can correct your image. Simply press `CORRECT` and a new image will be created with the hot pixels corrected. This image will be shown in the main window of Huygens Professional. The hot pixels are replaced by the median of their neighborhood.

---

## *Comparing the results*

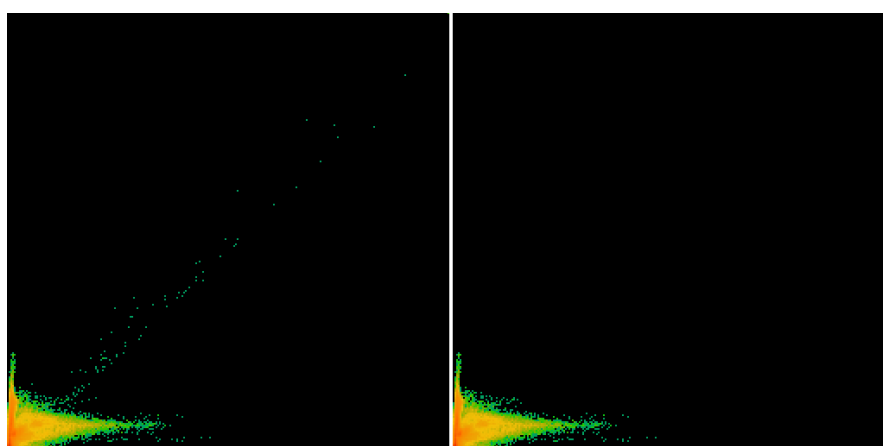
After the removal of the hot pixels, the correction can be visualized using the `Twin-slicer`. Figure 19.2 shows the line profiles of an example image and its corrected version. The solid lines are the intensity profiles of the original image (2 channels) and the dashed lines show those of the corrected image. The high peaks are clearly replaced by a value that match the continuity of the line profile. This example shows also nicely that the Hot Pixel Remover detects and corrects for *local* hot pixels, even in regions with a high mean intensity or gradient.



**FIGURE 19.2.** Comparing the results of hot pixel correction using the line plot feature within the Twin Slice. The dashed lines show the corrected values in this two channel example image.

The results of hot pixel correction can also be verified with histograms. Since the example is a two-channel image, 2D histograms can be compared. Histograms can be viewed by a right-click on the image in the main window of Huygens Professional and selecting ANALYZE > HISTOGRAM, or by using the `hist2Ch` command in the tcl-shell. Type `a hist2Ch ?` within the tcl-shell to see how to use the command.

Figure 19.3 shows 2D the histograms before and after hot pixel removal. The hot pixels are located at the same positions in both channels, as indicated by the diagonal scatter within the histogram of the original image (shown left). The diagonal scatter is clearly absent in the histogram of the corrected image (shown right). Note that the histogram does not show all hot pixels, since hot pixels may have a high intensity locally, but can have a relative low intensity if compared to the intensity range of the complete image



**FIGURE 19.3.** The 2D histogram of the original (left) image shows hotpixels as a diagonal scatter that are successfully removed in the corrected image (right).







## *The Chromatic Aberration Corrector*

Since Huygens version 15.05, the Chromatic Shift Corrector option has been renamed Chromatic Aberration Corrector as, besides removing misalignments between channels, it is now also capable of automatically estimating and correcting differences in scaling and rotation between channels.

The support for templates in this option allows chromatic correction to be applied from one image to other images. This is particularly interesting when the estimation is carried out on a multi-channel bead image. The use of templates for chromatic aberration correction is also available in the batch processor.

---

### *Causes of chromatic aberration*

The chromatic shift, usually being the largest component of the chromatic aberration, is a misalignment across the channels of a multichannel image which can be corrected by simply shifting the channels.

There are several circumstances that can lead to chromatic shifts in the microscopic images:

- Chromatic aberrations in the microscope optics.
- Misaligned beam splitters (confocal microscopes).
- Misaligned excitation lasers (confocal microscopes).
- Misaligned colour filters (widefield microscopes).
- Faulty colour interpolation in colour cameras.
- Any other internal misalignments in the microscope.

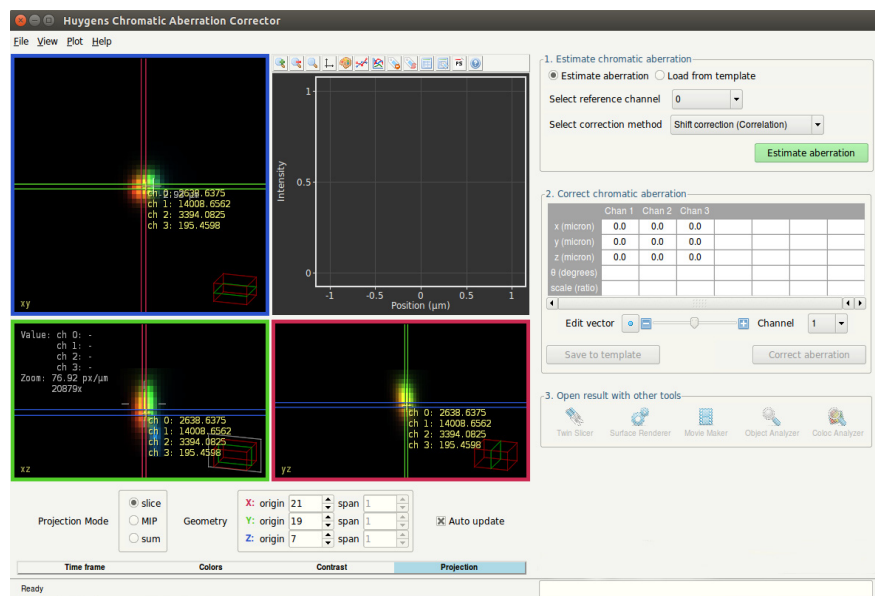
Therefore, chromatic aberration can be a rather frequent - though correctable - imperfection in multichannel images.

## Starting the Chromatic Aberration Corrector

- Launch Huygens Essential Professional.
- Load a multichannel image to be corrected for chromatic aberration.
- Select the image thumbnail and in the top menu go to DECONVOLUTION->CHROMATIC ABERRATION CORRECTOR.

The Chromatic Aberration Corrector will open and show the image on an orthogonal slicer where the existing chromatic shifts can be seen in a 3D view (For operating the slicer See “The Orthogonal Slicer” on page 71.). Below the orthogonal slicer a Z slicer, a time slicer, and other visualization tools such as contrast, color scheme, channel selection, and zoom tools can be found. These tools are useful to enhance the view of the image for a better visualization of the chromatic shifts.

A view of the Chromatic Aberration Corrector at start-up with a loaded two-channel bead image is shown in Figure 20.1.



**FIGURE 20.1.** The Chromatic Aberration Corrector at start-up, prior to estimating the chromatic aberration.

## Estimation of the chromatic Aberration

The following three methods can be chosen for the automatic estimation of chromatic aberration:

- Shift Correction (Cross correlation). This can be considered an 'all-round' method. The software searches for the best alignment across channels by maximizing the overlap.
- Shift Correction (Center of mass alignment). This method works best if the image contains a single object. The object should not touch the image borders, and the contrast between object and background should be high.
- Full Correction (Cross Correlation). This approach corrects for shifts, scaling, and rotation between channels. The software searches for the best alignment across channels by maximizing the overlap. Both scaling and rotation correction are performed with respect to the image center

Chromatic shifts will be quantified by a three dimensional vector, indicating how much a channel is shifted with respect to the given reference channel.

The channel to act as reference can be selected by the user via the Reference Channel selection box. Because this channel will have no chromatic aberration with itself, it will not be reported.

If an estimation method and a reference channel have been selected, the chromatic aberration will be estimated and reported upon clicking on the ESTIMATE ABERRATION button.

---

### *Visualization of the chromatic aberration*

The estimated chromatic aberration will be reported within the table. The aberration of each channel but the reference will be listed. The length unit of the three dimensional shift vectors is set to micrometers. Rotation will be presented in degrees, and a difference in scaling will be represented as a ratio value.

The user can select any channel for editing except for the one used as a reference. This can be done by using the EDIT CHANNEL selection box under the vector table.

The shift vector selected for editing will be drawn on the orthogonal slicer, so that the estimated shift can be easily assessed. Notice that the vector components are projected onto each orthogonal view correspondingly. The total length of the vector is displayed next to each vector projection. This length is a measure of the estimated chromatic shift.

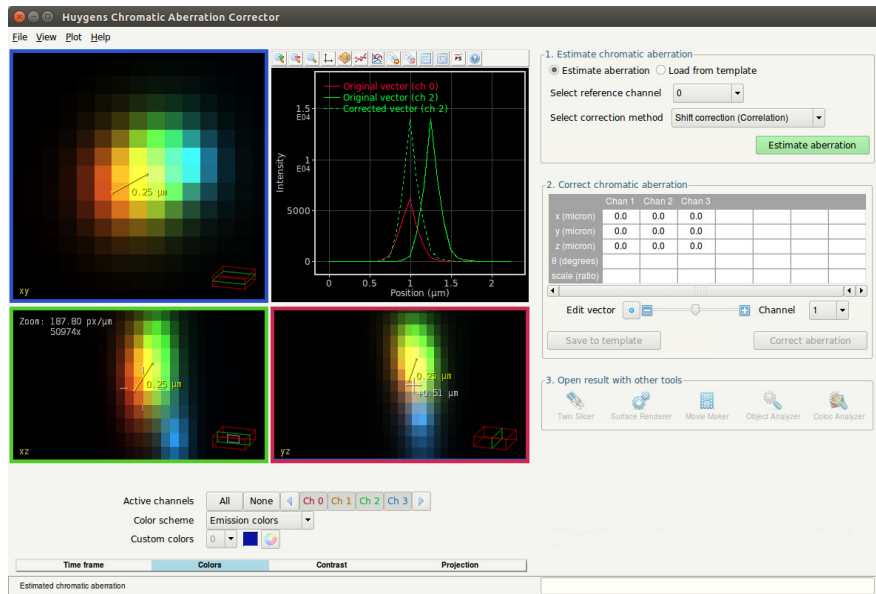
At the same time, a plot shows the intensity profiles along the direction of the shift vector estimated for the reference channel and the channel selected for editing. The plot also shows the intensity profile of the edited channel as if it were corrected with the existing estimated shift (dashed line).

Therefore, the plot serves as a comparison between the reference channel, the edited channel and the corrected edited channel, and allows us to see to what extent the intensity of the edited channel is shifted with respect to the reference channel. At the same time, it shows in advance whether the estimated shift vectors will correct for the existing chromatic shifts properly.

The result of a chromatic aberration estimation is shown in Figure 20.2. The shift between the intensity profiles of the reference channel and the edited channel is visible in the embedded plot. Additionally, the dashed line in the plot shows the intensity profile of the edited channel as if it were corrected with the estimated shift.

Ideally, the intensities of the corrected channel (dashed line) and the reference channel should show no remaining shift, having similar shapes and peaks roughly located at the same x positions.

The Chromatic Aberration Corrector will return accurate and reliable estimations of the existing chromatic aberration. Still, a possibility to edit and customize the estimated shifts is offered so that the user can reach more precision if necessary.



**FIGURE 20.2. The Chromatic Aberration Corrector** after estimating the shifts. A plot shows the gap between channel 2 and reference channel 0. The estimated shift is drawn as a vector in the orthogonal slicer scenes.

### *Editing the chromatic shift vectors*

The user can also edit the shifts that have been estimated automatically by the Chromatic Aberration Corrector. The components of the selected shift vector can be modified by using the EDIT VECTOR tool, which allows to shorten and lengthen the estimated shift.

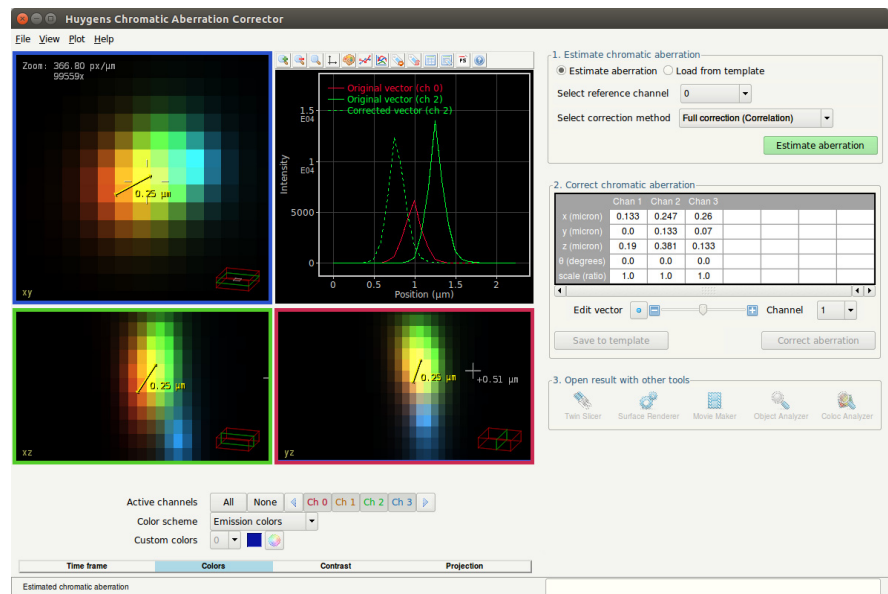
While the shift vector of the edited channel is shortened or lengthened the plot updates itself to show how the gap between the reference channel and the edited channel is increased or decreased.

In this way, the contents of the estimated shifts can be modified while checking in advance whether the image will be corrected appropriately.

The chromatic shift estimation of a four-channel image is shown in Figure 20.3. Channels can be edited one by one and their shifts modified with again the EDIT VECTOR tool.

Alternatively, any customized shifts can be typed in the vector table for each channel.

Upon clicking on the CORRECT ABERRATION button the image will be corrected with the chromatic shifts listed in the vector table. A new corrected image will be created.



**FIGURE 20.3. Using the Edit Channel tool of the Chromatic Aberration Corrector.** The estimated vector has been manually lengthened, and as a result the dashed line plot is slightly moved to the left.

## Working with templates

The estimated aberration correction can be saved to a template by using the template tool of the Chromatic Aberration Corrector.

Saved templates can be imported and applied to other images. The template values will be loaded and listed in the table. Furthermore, the plot will show the corresponding shift in the intensity profiles as if the ESTIMATE ABERRATION button had been pressed.

Subsequently, the image can be corrected by clicking on the CORRECT ABERRATION button, which will create a new corrected image in the main window. This will also activate buttons with which the corrected image can be immediately opened in Huygens tools for visualization and analysis. Some of these tools are available as options.

It is recommended to apply templates to images that have the same emission and excitation wavelengths as the image with which the template was created.





## The Object Stabilizer

The Huygens Object Stabilizer measures and corrects for cell motion, thermal drift, shaking, and other types of movement (i.e. translation in  $x$ ,  $y$ , and  $z$ , and axial rotation). Both the measurement and subsequent stabilization are done in 3D and at sub-pixel level. The Stabilizer not only stabilizes 2D or 3D time series, but it also allows the alignment of slices within a 3D stack.

To launch the stabilizer, select an image and select DECONVOLUTION→OBJECT STABILIZER from the main menu.

---

### Stabilization of 3D Time Series

Stabilization over time works best if the 3D time series has been deconvolved first. Deconvolution enhances resolution and reduces noise, which helps to analyze motion.

For time series there are four stabilization methods available, which are explained in the next paragraphs:

- *Cross correlation*
- *Model-based correlation*
- *Multi object tracking*
- *Center of mass alignment*

If a 2D time series was read in as a 3D stack, then the Huygens Tcl command `convertZ2T` can be used to convert  $z$  dimension to the time dimension.

#### Pre-processing

The first step in the wizard is to select which stabilization method to use. After selecting a method a pre-processing step is shown, that offers the possibility to launch the cropping tool (`()`), and disable channels that are not relevant. The channel selector is hidden for single-channel images.

## The Cross Correlation Method

This can be considered an *all-round method*. It can correct for both *x-y-z* translation and axial rotation. Adjacent time frames are compared and the program tries to find the best alignment by maximizing structural overlap. A spherical region of interest can be defined to stabilize a particular part of the image.

After the pre-processing step, the *correlation settings* screen appears in which rotation detection can be enabled or disabled (rotation detection takes more time), and where it is possible to select a region of interest in the image, as shown in Figure 21.1. The NEXT button will start the alignment process.

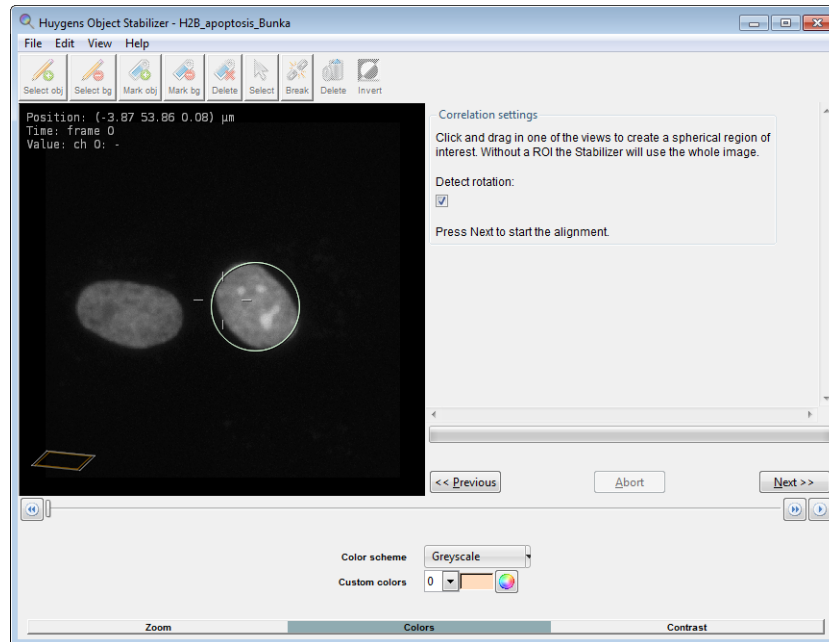


FIGURE 21.1. The *Correlation settings* screen in the Object Stabilizer.

When the Object Stabilizer has finished measuring the displacements, the *Stabilization* screen is shown. Continue reading “Stabilization Settings” on page 123.

## The Model-Based Correlation Method

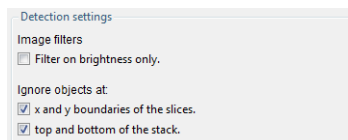
When the geometry of the imaged object did not change much during the acquisition, then the time series can be stabilized using a model of the object. The stabilizer creates the model automatically.

Pushing the NEXT button on the pre-processing screen immediately starts the alignment process, because this method does not require any additional user input. When the Object Stabilizer has finished measuring the displacements, the *Stabilization* screen is shown. Continue reading “Stabilization Settings” on page 123.

## The Multi Object Tracking Method

When your image contains well-defined objects, i.e. nuclei or small particles, then the image may be stabilized using object tracking. Objects are tracked over time and their average movement is used to stabilize the time series.

After the pre-processing step, the *detection settings* step is shown (Figure 21.2). In this screen the detection algorithm can be configured to *filter on brightness only*. This is a little faster and usually sufficient for tracking bright (or dark) spots in images with a high contrast between the spots and the background.



**FIGURE 21.2.** The *Detection settings* screen.

When the detection algorithm finds many false objects at the image borders (this may happen for very small object sizes), then the Object Stabilizer can be configured to *ignore objects* at  $x$  and  $y$  boundaries, and at the top and bottom of the stack.

Next, the user is asked to use the *Select object* tool (🔪) and *Select background* tool (🔪) to mark a few objects and background areas in the first time frame. Figure 21.3 shows an example of selected objects (green selection) and background (red selection). It is important to make sure that the size of the object selections roughly matches the real size of the objects, because this is a parameter that is used to tune the detection filters.



**FIGURE 21.3.** An example of object and background selections.

Note that the selections are three-dimensional, i.e. spheres instead of circles. The  $x$ - $z$  and  $y$ - $z$  slicers can be used to view and modify the position of the selections along the optical ( $z$ ) axis.

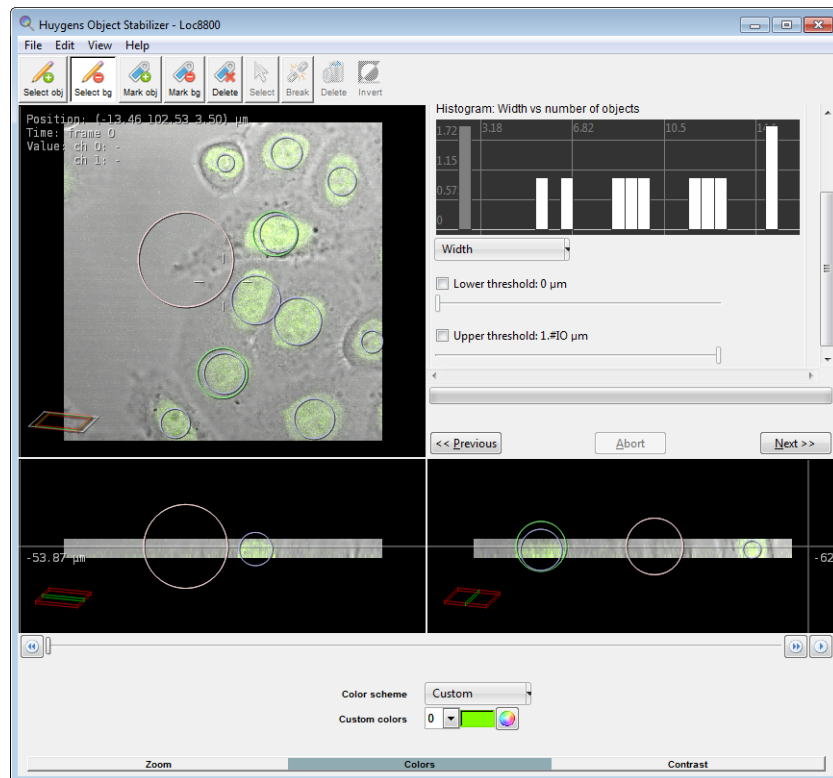
When the NEXT button is pressed, the Object Stabilizer analyzes the selections and uses that information to detect objects in the first time frame. The detected objects are marked by blue spheres as shown in Figure 21.4. When the detection fails, then the selection tools can be used to select new objects and background, mark detected objects as object (🔪) or as background (🔪), and delete selections. When the selections are modified, pressing NEXT will recompute the detection instead of proceeding to the next screen.

The histogram and sliders in the *optimizing object detection* screen (See Figure 21.4) can be used to apply thresholds on the *number of objects*, *score*, *width*, and *brightness*. Objects that fall outside of one of these thresholds will be ignored. The *score* is a statistic that reflects the certainty of the detection algorithm, i.e. objects with a low score are probably noise. When the thresholds are modified, pressing NEXT will recompute the detection instead of proceeding to the next screen.

In the next screen the tracking parameters can be modified. A limit can be set to restrict the maximum distance over which an object is allowed to move in between two time frames. This means that the tracker will not connect two objects in subsequent time frames if their mutual distance is larger than this value.

The detection algorithm can be configured to automatically adapt its settings per time frame (to correct for bleaching, for example). The tracking algorithm can be configured to use the geometry and orientation of the detected objects –besides the position, brightness, and filter values– for connecting the tracks (default).

The NEXT button will start the tracking process. When this is finished the *edit tracks* screen is shown (See Figure 21.5 on page 123). In this stage the user can select (🖱),



**FIGURE 21.4.** The *Optimizing object detection* screen in the Object Stabilizer.

break (🔪), and delete (🗑️) the detected tracks. The Object Stabilizer averages the remaining tracks to correct for the average displacement of the objects.

When the NEXT button is pressed again, the *Stabilization* screen is shown. Continue reading “Stabilization Settings” on page 123.

### The Center of Mass Alignment Method

This method works best if the image contains a single large object. No objects should cross the image borders, and the contrast between object and background should be high.

Pushing the NEXT button on the pre-processing screen immediately starts the alignment process, because this method does not require any additional user input. When the Object Stabilizer has finished measuring the displacements, the *Stabilization* screen is shown. Continue reading “Stabilization Settings” on page 123.

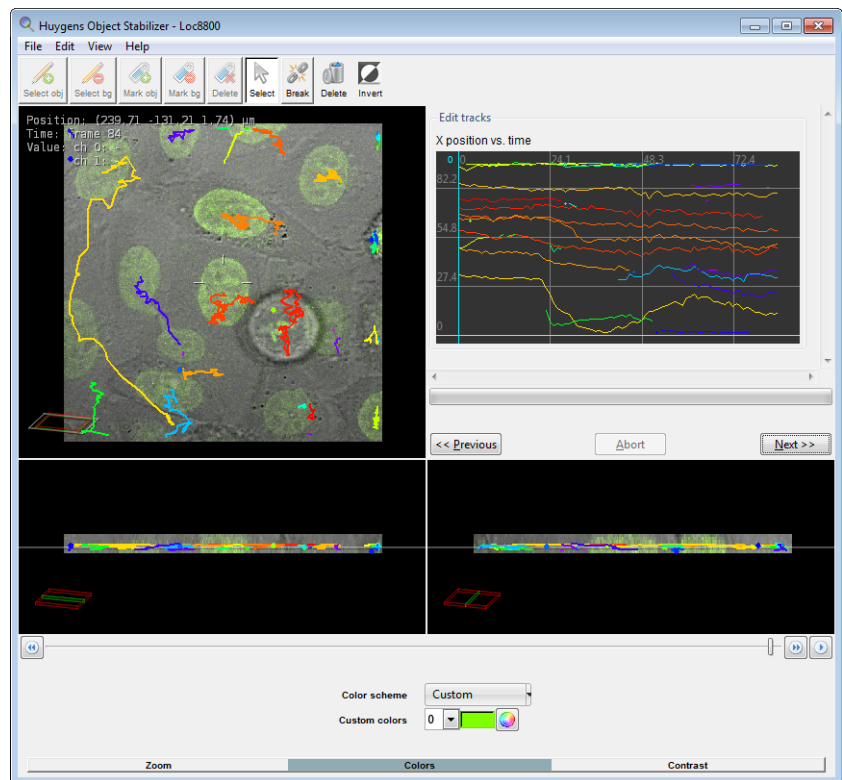


FIGURE 21.5. The *Edit tracks* screen in the Object Stabilizer.

### Stabilization Settings

The *Stabilization settings* screen shows the measured displacements in  $x$ -,  $y$ -, and  $z$ -direction, and the rotation in four plots. If the axial displacement is small no correlation will be applied, unless the checkbox is ticked. The three sliders above the plots can be used to filter outliers, drift, and noise from the displacement curves. When the displacement curves shows sudden steps, then the outlier filter can be used to remove those steps. The drift and noise filters can be used to respectively remove the drift and keep the random motion, or keep the drift and remove the random motion. The effect of those filters is shown in Figure 21.6.

At the bottom of the *Stabilization settings* screen there is the option to select the quality of the interpolation filter that is used to resample the images. The *high quality* mode is not available when correcting for rotation, because it uses Fourier transforms to shift the image.

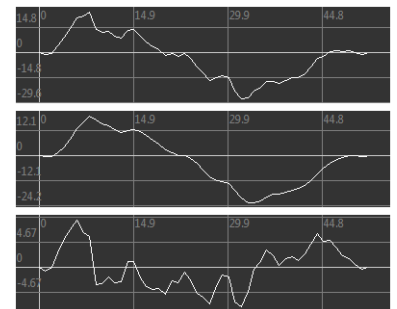
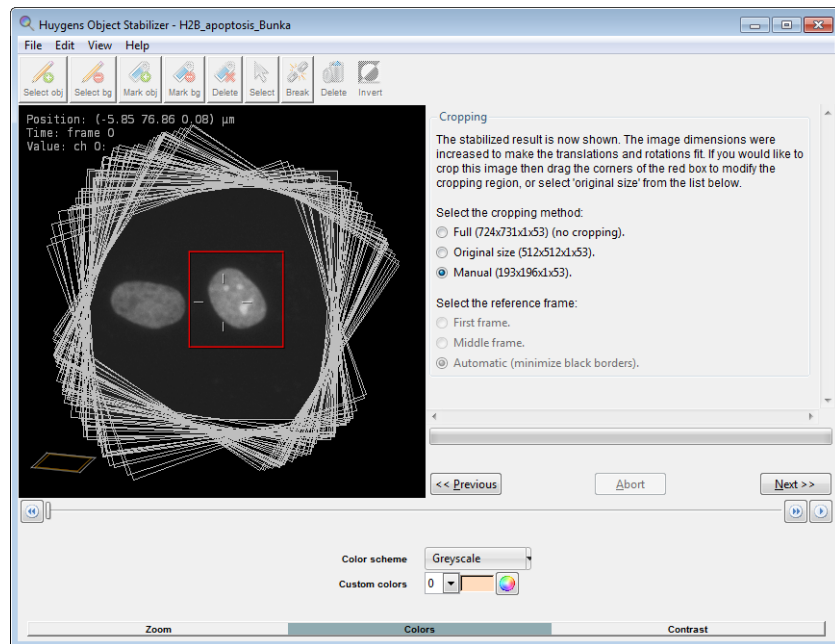


FIGURE 21.6. Effect of filters in the *Stabilization settings* screen. The top shows the measured displacement, the curve in the middle is noise filtered, and the bottom is drift filtered.

Push the NEXT button to apply the stabilization and proceed to the cropping screen.



**FIGURE 21.7.** The stabilization result can be cropped manually to cut out the objects of interest. The original image borders are shown as grey rectangles.

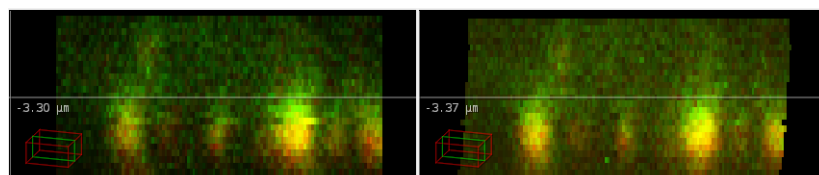
### Cropping the Result

Because there is some extra space needed for the translated and rotated frames, the stabilized image will be larger than the original one. The *Cropping* screen (See Figure 21.7) allows the user to manually cut off the black borders, crop the image to the original size, or keep the full size. When selecting *Original size*, the reference frame needs to be selected.

### Alignment of Slices in 3D Stacks

3D alignment can be applied prior to deconvolution if the stack suffers from *severe* misalignment between adjacent slices. In those cases the Object Stabilizer may improve the deconvolution of 3D stacks and can be applied beforehand. In all other cases applying deconvolution first is preferable.

The 3D alignment method always uses the cross correlation method for comparing adjacent frames, and it can correct for *x* and *y* translation and rotation. The steps in the wizard are the same as for the stabilization of time series, as explained in “The Cross Correlation Method” on page 120. A comparison between a misaligned and aligned *z*-stack is shown in Figure 21.8.



**FIGURE 21.8.** An *x-z* slice of a misaligned *z*-stack (left), and the alignment result (right). The chromatic shift in stack like this one can be correcting using the Chromatic Shift Corrector.

Note that 3D alignment deforms the volume. It is advisable to check the alignment result carefully in using the  $x$ - $z$  and  $y$ - $z$  views of the Twin Slicer (See “Using the Slicer in Basic Mode” on page 64.).





# The Object Tracker

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## Introduction

The Huygens Object Tracker wizard and the Track Analyzer can be used to study the 3D motion of cells and smaller particles.

By manually selecting a few objects and background regions, the Object Tracker is trained to differentiate between object and background volumes, and thus to detect new objects. In this way not only can bright spot tracking be done, but also tracking of more complex scenes. Because the Object Tracker is designed to detect and track objects with a well-defined center, it works best with spherical, disk-like (flat) or slightly elongated objects.

After the Object Tracker wizard has finished processing the time-lapse data the Track Analyzer is launched. The Track Analyzer can also be launched directly if tracks were stored from a previous session of the Object Tracker. The Track Analyzer helps to filter, edit, and analyze the results. It presents the properties of the measured tracks in informative graphs and allows to export track data to a file.

---

## The Object Tracker Wizard

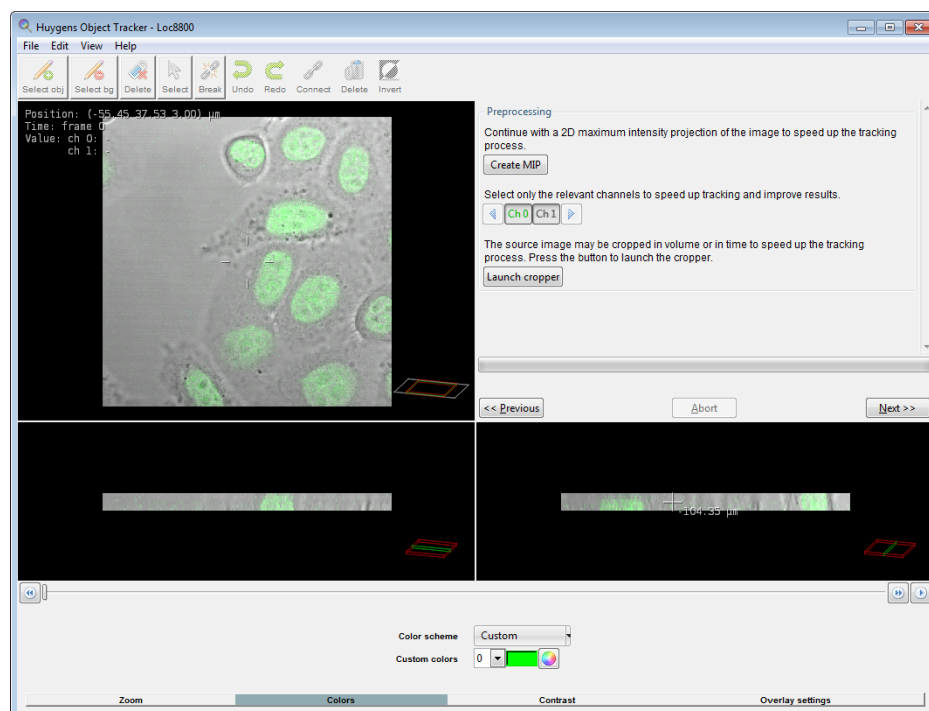
### The Preprocessing Stage

When the Object Tracker is launched –through the main menu ANALYSIS→OBJECT TRACKER or the thumbnail pop-up menu– it will start the *preprocessing* stage (Figure 22.1). The wizard checks if the sampling distances and time interval are reliable (reported from file or verified) and will pop-up a parameter editor (Figure 3.3 on page 14) if those parameters need attention.

Depending on the image dimensions (2D/3D, single/multi channel) the preprocessing stage will give the option to create an axial maximum intensity projection (MIP), select relevant channels, and/or crop the image (See “The Intelligent Cropper” on page 15.). These preprocessing operations help to speed up and enhance the tracking process.

### Detection Settings

In this stage the parameters that control the object detection can be adjusted. The first screen of this stage shows the choice to use a wizard or to load a template of detection settings saved in a previous session.



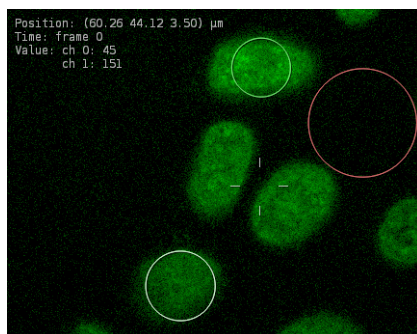
**FIGURE 22.1.** The preprocessing stage in the Huygens Object Tracker.

The next screen gives the option to *filter on brightness only*. This disables other image filters, and is faster and usually good enough for bright (or dark) spot tracking.

The *ignore artifacts* choices give the option to discard objects that are detected close to the image borders, as image borders can be sensitive to false positives.

### Object Detection Stage

In the *object detection stage* the user is requested to select a few objects and background areas. In this stage one can also create a region of interest (ROI) such that only objects within this ROI are analyzed. By pressing the DEFINE A ROI, a new window will be opened in which an ROI can be created. When done, press the CLOSE AND EXPORT ROI button. The ROI will be visualized in the tracker as a new overlay image. The search for objects is then limited to object within the ROI.



**FIGURE 22.2.** Two object selections (green) and a background selection (red).

To start the training, use the *object selection tool* (👉) to draw green outlined spheres to select objects. Use the *background selection tool* (👉) to draw red outlined spheres to select regions containing mostly background voxels (Figure 22.2).

Like the Orthogonal Slicer (page 71), the Object Tracker displays the same point in space (the image center) from three orthogonal directions ( $xy$ ,  $xz$ , and  $yz$ ). The object and background selections are visible and can be drawn and modified in either of these projections.

It is important to make sure that the diameter of the object selections is about the same as the diameter of the real objects, because the Object Tracker uses that information to tune the detection filters. The size of the background selection does not affect the detection.

When at least one object selection and one background selection is created, pressing the NEXT button will initialize the object detection and advance to the next step in the wizard.

Note that you can choose any slice for training, but the objects and background selection is only possible in one slice. During training, Linear Discriminant Analysis is used to find the best features to distinguish between object and background. In combination with the watershed segmentation method (See “Using the watershed segmentation” on page 139.), objects are defined. This process is done for each slice.

### Optimizing Object Detection

After pressing the NEXT button in the object detection stage, the wizard will highlight the detected objects with a light blue overlay. A ‘plus’ symbol marks the center of each detected object (Figure 22.3).

In this screen it is still possible to add, delete, or modify the selections. In case the selections are altered, the NEXT button will reload this screen instead of advancing to the next stage.

The right side of the wizard shows a histogram of the number of objects versus their *score*. The score is a qualitative measure for the probability that the detected object should indeed be classified as ‘object’. By default all objects with a positive score are taken into account, but this threshold can be changed in order to make the Object Tracker less or more sensitive. To change the threshold, tick the *lower threshold* box and drag the slider (Figure 22.4).

Besides the score, thresholds can be applied to the *number of objects*, and the *width* and *brightness* of the objects. The width and brightness can be limited by both a lower and upper threshold. There is by default a threshold of 100 objects on the number of objects. An important thing to note is that *all* thresholds are applied, not only the currently selected one.

When one of the thresholds is modified, the NEXT button will reload this screen instead of advancing to the next. When the detection is satisfactory, press NEXT again to advance to the next step.

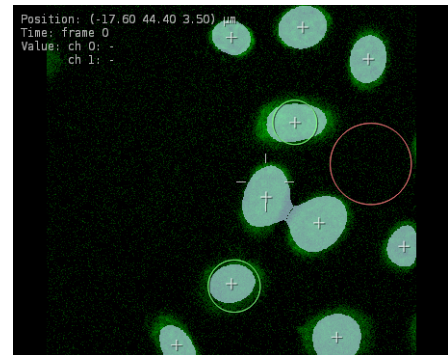


FIGURE 22.3. Detected objects.

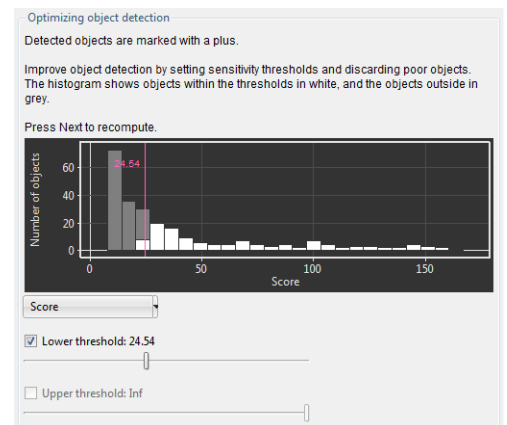


FIGURE 22.4. Increase the threshold on the score to reject more objects.

## Tracking Parameters

This is the final stage before the automated tracking starts. The parameters that can be modified in this screen affect the linking of the detected objects between subsequent time frames.

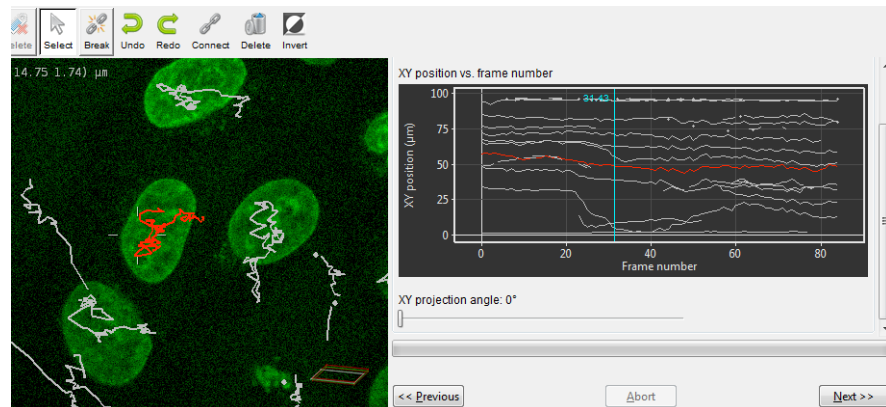
The first parameter limits the distance over which an object is allowed to move between time frames. This means that the tracker will not connect two objects in subsequent time frames if their mutual distance is larger than this value.

The second option enables or disables the use of object geometry and orientation for linking objects. Enabling this option makes sense if the objects of interest have distinct sizes.

## Quick Edit Tracks



When all time frames are processed and the detected objects have been linked into tracks, the wizard advances to the *quick edit tracks* stage. In this stage broken tracks can be connected, and wrong tracks can be deleted or broken down into smaller pieces that can be re-connected.

The graph shows the position of the objects in the  $xy$  plane versus the time frame number (Figure 22.5). The projection angle can be changed using the slider below the graph.



**FIGURE 22.5.** The *quick edit tracks* stage allows to connect, break, and delete tracks.

An angle of  $0^\circ$  projects on the  $x$ -axis while  $90^\circ$  projects on the  $y$ -axis.

To break tracks down into smaller pieces, simply select the *break* tool () and click between the nodes that should be disconnected. Multiple tracks can be selected using the CTRL key. If the nodes of the selected tracks do not overlap in time, then those tracks can be joined by pressing the *connect* button ()

Pressing Next will show the last stage where you can save the tracks to a file, and/or save the tracker settings in a template. The tracks will be saved in a `.tracks` file and this file can be imported within the Track Analyzer. The tracker template can be used to quickly track similar images. The tracks can also be saved in the Track Analyzer, but the tracker template can only be saved at this stage.

Pressing NEXT again will close the wizard and load the tracks in the Track Analyzer.

## The Track Analyzer

The Track Analyzer is automatically launched after the Object Tracker has finished processing the data, but can be started individually as well. Select the image which corresponds to the `.tracks` file to be analyzed and go to the main menu

ANALYSIS → TRACK ANALYZER. The `.tracks` file can be imported through FILE → OPEN TRACKS....

### The Filter Tab

The Track Analyzer can be used to analyze the tracks that have been generated by the Object Tracker. The scene on the top left displays these tracks, which can be saved via FILE > SAVE TRACKS. Viewing options can be selected under VIEW in the menubar. Tracks can still be edited using the break tool (✂) and connect tool (🔗), or the delete button. The window at the bottom left shows the collection of tracks and the objects for each track. Clicking on the track or object number highlights the concerned track in the image. Vice versa, the track number is highlighted when clicking on a specific track in the image.

The filter tool (Figure 22.6) can select a group of tracks based on common properties.

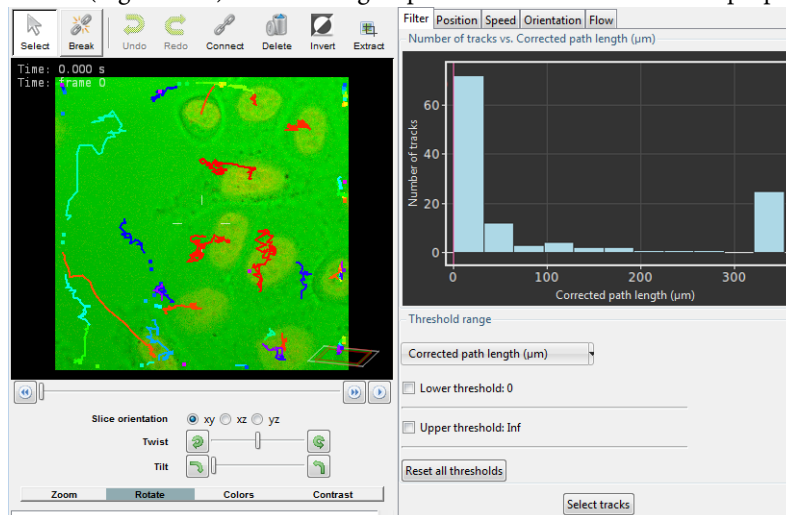


FIGURE 22.6. The filter tab in the Track Analyzer.

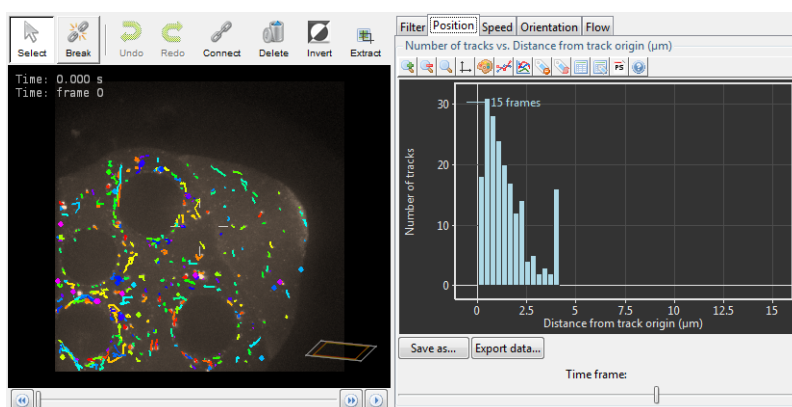
These properties can be the number of *detected objects* (nodes) per track, *start time*, *duration*, the *path length*, *average speed* and the *average angular speed*. The upper and lower threshold sliders can be used to define a range within those properties. When the SELECT TRACKS button is pressed, all objects within the threshold ranges are selected. The INVERT button in the taskbar inverts the selection to the other tracks.

An important thing to note is that *all* thresholds are applied when SELECT TRACKS is pressed. Press the RESET ALL THRESHOLDS button to disable all thresholds.

To remove tracks with less than 4 objects, for example, select the *detected object per track* property, tick the *upper threshold* box, drag the upper threshold slider to a value just above 4, and click SELECT TRACKS. Now those tracks are selected. These tracks can be removed by pressing the DELETE button.

### The Position Tab

This tab provides information on the position and displacement of the tracked objects. The histogram at the top, as shown in Figure 22.7, gives the number of objects versus the distance to their track origins for a specific duration. Example; if the time frame slider is



**FIGURE 22.7.** In case of a large number of objects that show Brownian motion, the shape of the histogram in the *position* tab will be a half-normal distribution.

set on 3 frames, and the height of the bar at a distance of 1.2 µm is 9, then there are 9 objects that travelled a net distance of 1.2 µm in 3 time frames.

The second and third plot in this tab are closely related. This second plot shows the average displacement for a certain duration, as given in Equation 1. In this equation  $t$  is the duration in frames and  $N$  is the number of objects.  $\bar{x}_{i,t}$  is the position vector of object  $i$  at time frame  $t$ .

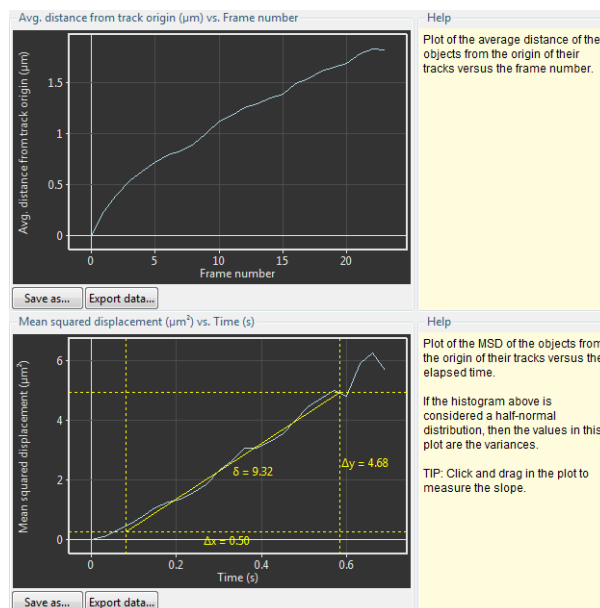
$$MD(t) = \frac{1}{N} \sum_{i=1}^N |\bar{x}_{i,t} - \bar{x}_{i,0}| \quad (\text{EQ 1})$$

The third plot shows the mean squared displacement, or *MSD*, as given in Equation 2. Note that the  $x$ -axis of this plot shows the duration  $t$  in seconds instead of frames.

$$MSD(t) = \frac{1}{N} \sum_{i=1}^N |\bar{x}_{i,t} - \bar{x}_{i,0}|^2 \quad (\text{EQ 2})$$

There is also a close relation between the *MSD* and the histogram in the first plot (Figure 22.7). In case of pure Brownian motion and a large number of objects, the histogram will show a half-normal distribution. In this special case, the variance of that distribution is given by  $MSD(t)$ . The rate of change of this variance is known as the diffusion coefficient  $D$ , which thus can be estimated by measuring the slope of the *MSD* plot (Figure 22.8).

Just click and drag in any of the plots to measure distances and slopes.

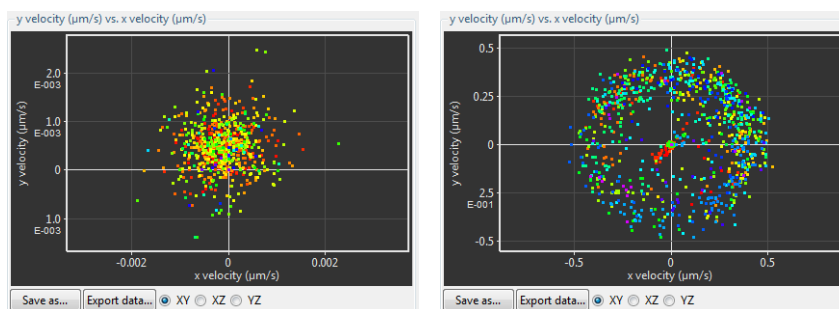


**FIGURE 22.8.** The slope of the mean squared displacement plot indicates a diffusion coefficient of  $9.32 \mu\text{m}^2/\text{s}$ .

### The Speed Tab

This tab displays information on the velocity of the tracked objects. The first plot is a histogram that shows the distribution of the speed of the objects, while the second plot shows the average speed over time.

The third plot informs about the velocity distribution of the objects. This not only gives information on the speed (a scalar value), but also on the direction of the velocity, as shown in Figure 22.9.



**FIGURE 22.9.** Left: An *xy* velocity scatter plot showing a drift in the positive *y* direction. Right: An *xy* velocity scatter plot of objects moving with a constant speed of about  $0.35 \mu\text{m}/\text{s}$  in random directions.

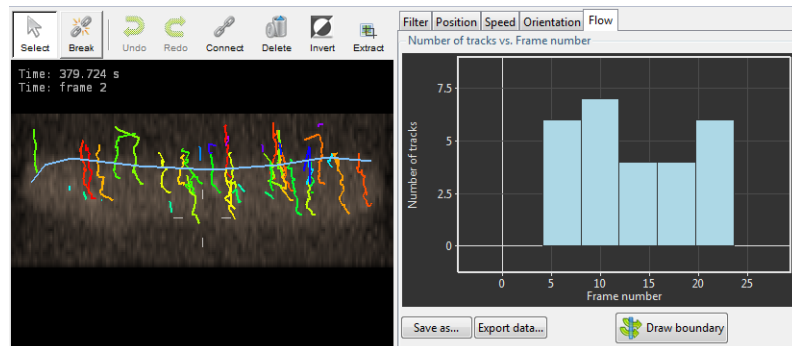
### The Orientation Tab

The first graph under this tab shows the axial rotation of objects in time, while the second graph show the 3D angular speed versus time (indicated as frame numbers).

## The Flow Tab

This tab can be used to gain insight into the amount of objects that cross a certain boundary over time. Boundary surfaces can be defined as a 2D poly line in the  $xy$  slice image. These lines are extruded to planes in the axial direction. To start drawing one, click the DRAW BOUNDARY button (🌿), and then click on the first point of the boundary. A single click adds a new line segment and a double click finishes the poly line.

When a boundary has been defined, then the plot in the Flow tab shows the number of objects that cross this boundary at a certain time frame (Figure 22.10).



**FIGURE 22.10.** The flow histogram shows that objects are crossing the boundary between time frame 4 and 24.

## Exporting Data

The plot graphics, as well data in the plots and the track data can be exported to a file. The buttons below each of the plots, SAVE AS... and EXPORT DATA..., save the plot as an Encapsulated PostScript file (EPS) and export the data as comma separated text (CSV), respectively. The track data of the selected tracks can be exported to a CSV file using the menu FILE→EXPORT→SELECTED TRACKS AS CSV.

The EPS format can be imported by most office programs, including Microsoft Office and OpenOffice. The CSV format is plain text and can be parsed by most spreadsheet programs and other software like Matlab and Mathematica.



## Introduction to the Object Analyzer

The interactive Object Analyzer (OA) tool allows you to obtain *statistics of individual objects* by clicking on them, or analyzing all objects with a single button press.

In this context, an object is a distinct group of interesting voxels that are spatially connected one to another. Interesting voxels are distinguished from the background by using a seed and threshold criterion. Therefore, defining objects in an image implies:

1. *Segmentation*: Separating interesting voxels from the background according to a given criterion;
2. *Labeling*: Grouping them together and assigning them a distinct name or label.

This is done interactively by the Object Analyzer. To exclude objects that are too small before starting the analysis, a garbage level can be used. Any object smaller than this garbage volume will be discarded. After removing these small objects, all remaining objects are automatically labeled and sent to a continuous iso-surface renderer (See Chapter 14 on page 85).

The Object Analyzer is an extended option, and is enabled by a special flag in the Huygens license string.

This chapter is written in the form of a step-by-step, introductory tutorial to the basic functions of the Object Analyzer. A reference guide that describes all the user interface components of this option that can be found on page 151.

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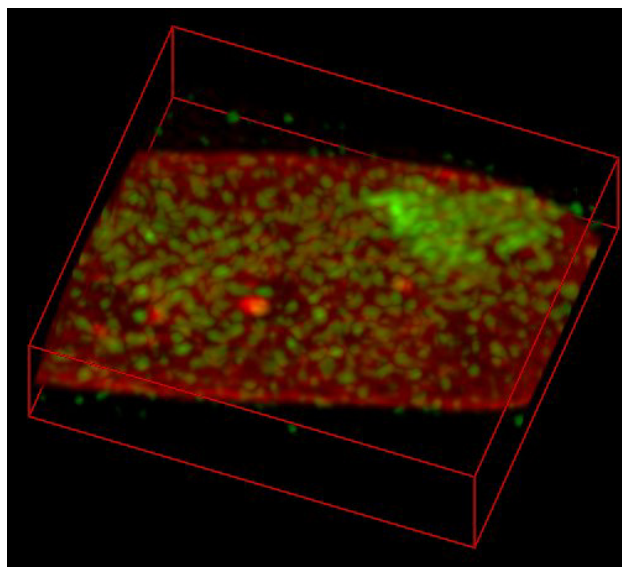
### *Starting the Object Analyzer*

- Launch Huygens Professional .
- Load an image you want to analyze. To explore all the OA possibilities, better use a multi channel image.
- Select the image thumbnail, and in the top menu go to ANALYSIS→OBJECT ANALYZER ADVANCED.

You can find this introductory tutorial on-line on the SVI Wiki<sup>1</sup>, from where you can also download the test image we will use in the following steps.

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You can explore the image with the Twin Slicer (See Chapter 10 on page 63) for an initial impression of the data. The demo image is a deconvolved image. It is always recommended to perform deconvolution before analysis. Deconvolution will reduce noise and blurring significantly and will thereby improve the analysis quality.



**FIGURE 23.1. Maximum intensity projection of the test image.** Cell nucleus FISH-stained, recorded at the Nuclear organization Group, SILS, University of Amsterdam (head: Prof. Roel van Driel), under the 3D-Genome research project.

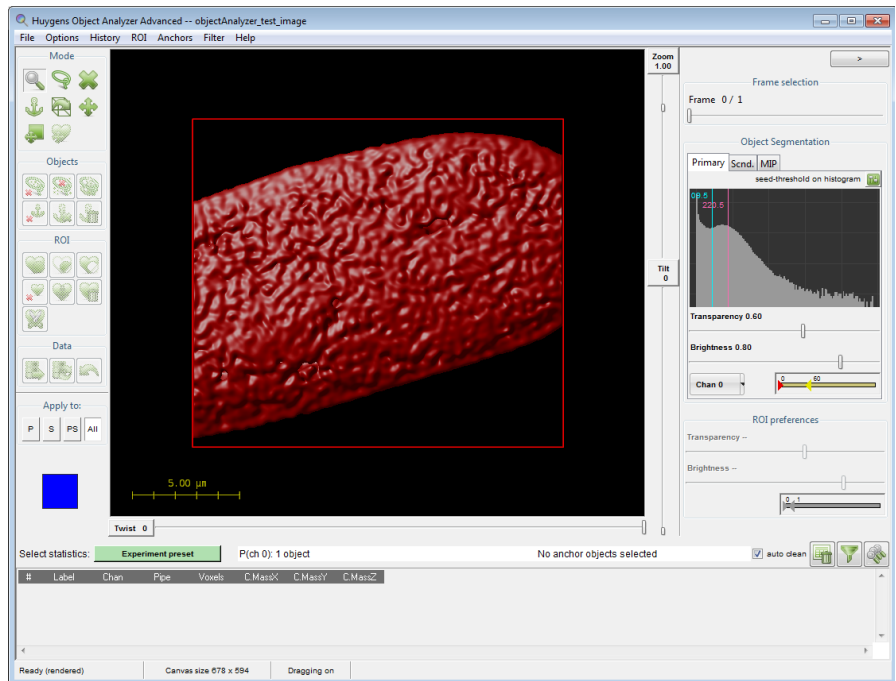
The image in Figure 23.1 is a MIP projection of the example 3D dataset. Notice that there are a few bright objects in the red channel against a more or less homogeneous background (it is actually a quite flat cell nucleus), and lots of scattered objects of different sizes and intensities in the green channel. The red channel is in general dim with the exception of the inner bright objects and some increase in intensity in the peripheral area, making something like a border.

When the image is opened in the OA, a first view and segmentation is presented (Figure 23.2). The initial segmentation is based on Otsu's segmentation.

A large bright object is visible in red and viewed from the top. The image was automatically rendered with default settings. If more than one object is present, different colors will be assigned to each object. The specific color range can be adjusted with the hue and saturation sliders.

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1. <http://www.svi.nl/ObjectAnalyzerTutorial>

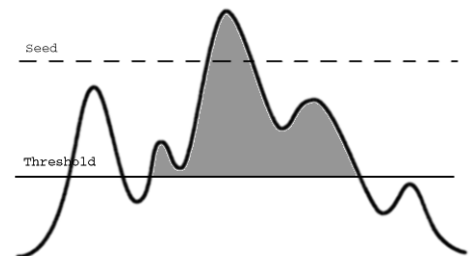


**FIGURE 23.2.** Start up screen of the Object Analyzer. The object in the image's first channel is automatically segmented with initial parameters calculated based on the intensity distribution (Otsu's segmentation).

### Segmenting the Objects: Setting the Threshold

The segmentation method in the Object Analyzer uses a combined seed-and-threshold method, see Figure 23.3. The seed acts as a secondary threshold level, so that objects that do not reach this intensity (in at least one voxel) are discarded. More details on how the threshold-seed segmentation works can be found in the expert online tutorial<sup>2</sup>.


Let's try different segmentation parameters and see what happens. First we increase the threshold value from the automatically calculated value to something around 360 (the mentioned numerical values refer to this particular test image, of course). You can drag the blue line in the channel histogram and shift it to higher values, or click on the blue-font label showing the threshold value to enter any number. The threshold lines on the histogram are found at the right of the window, in a pane labeled *Object Segmentation*.



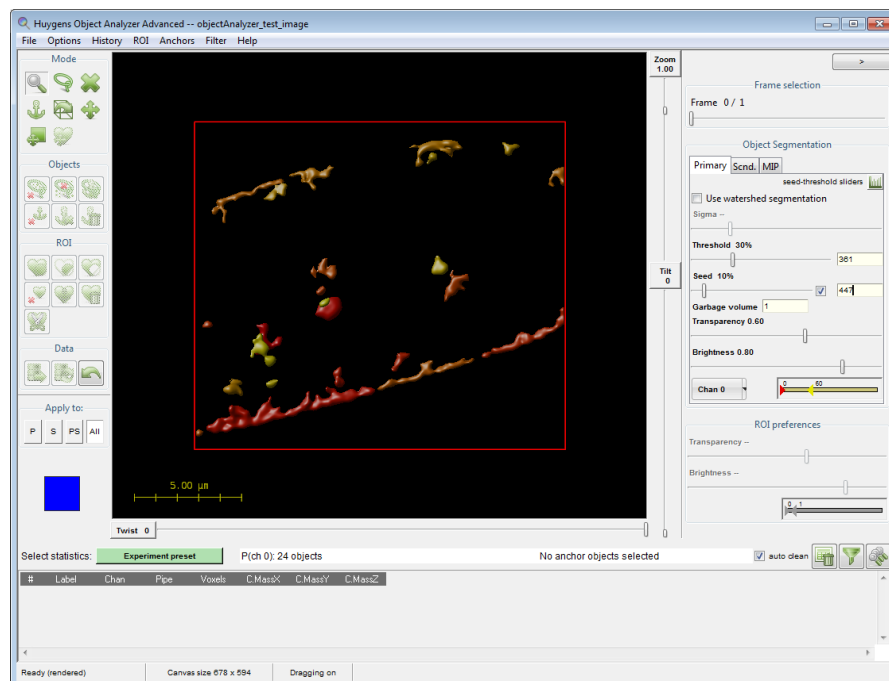
**FIGURE 23.3.** Visual representation of the seed-and-threshold segmentation method.

2. <http://www.svi.nl/ObjectAnalyzerExpertTutorial>

The magenta-colored line is the seed level. When you shift the blue line, the magenta line also moves, as by default the two are linked.

You can also switch to a percentage representation of threshold and seed by clicking the small button  at the top-right of the histogram. In this alternative slider view, you can also control whether the seed is linked to the threshold value or not, by enabling or disabling the check box next to the *Seed* slider.


When we shift the threshold to higher values the rendering scene will change. First, the object we had defined will shrink in size and we see many smaller objects starting to appear. Some objects that were connected earlier shrink so much that they now get separated, and they define new objects. To visualize the object details better, you can increase the zoom factor up to 1.30 to see what is shown in Figure 23.4.



**FIGURE 23.4. Different segmentation conditions.** Increasing the threshold decreases the objects size, and adds more objects to the scene.

The increased threshold breaks the single large object into multiple smaller objects. The very small objects can be easily removed by setting a garbage volume.

### Setting a Garbage Volume Level

A quick way of removing small objects, is the *Garbage Volume* option. You can find this entry in the alternative slider view of the threshold, to which you can switch by clicking the small button  at the top-right of the histogram. The garbage entry is shown below the seed slider. Objects with a voxel volume below the garbage level are discarded. This means that when you set it to 1, no object is discarded, but if you set it to for example 100, any object with a volume smaller than 100 voxels will be removed from the scene and from further analysis.

Additionally, you can apply some post-segmentation filtering. Details on this are explained in the expert tutorial. In the top menu you can also find some predefined filters for discarding objects. These filters are based on the geometrical properties of individual objects or on how objects are related to other objects.

### Using the watershed segmentation

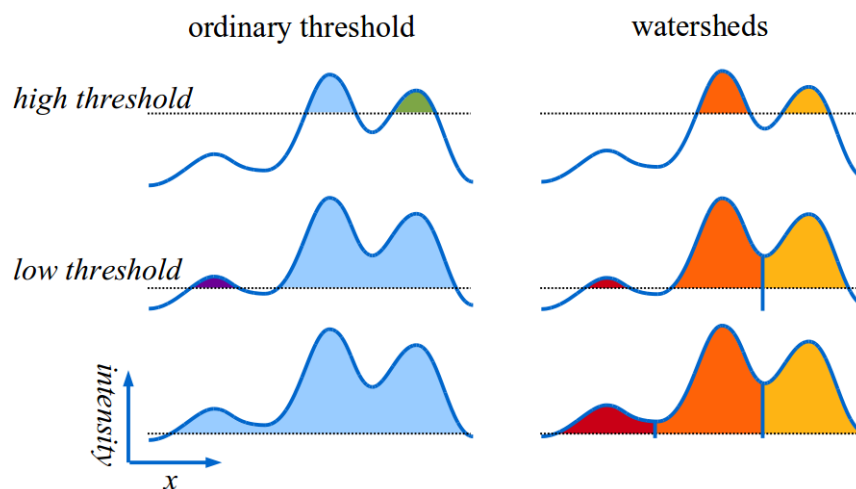
When using the seed and threshold method, it is possible that objects merge at a certain threshold level. To further tune segmentation, you can apply a watershed segmentation.

The watershed segmentation builds “watersheds” at local minimum values to separate merged objects. This can also be explained as a flooding algorithm where the inverse intensity profile is flooded with water. A watershed is build in order to separate the different water-reservoirs. The difference between threshold and watershed segmentation is illustrated in Figure 23.5.

The *Seed* setting has the same meaning for the watershed as it has for the threshold segmentation method, however, the garbage volume filtering uses a slightly different approach. The garbage filter removes objects with a volume below the given value, but if this concerns an object that was separated from an other object by the watershed algorithm (a neighbor) it will first be merged with this neighbor, after which the volume of the new merged object is again reviewed. This merging process will be repeated with new neighbors until it has no direct neighbors. If the volume is it still below garbage level and it has no neighbors anymore, it will be removed, otherwise the complete object is kept.

The watershed segmentation method uses the sigma setting as an extra input. This setting is used to avoid over-segmentation of the data by smoothening intensities with a gaussian filter. Especially with very noise images, local minimum value are frequently present, resulting in many small objects. The Object Analyzer can handle at most 32768 ( $= 2^{15}$ ) objects.

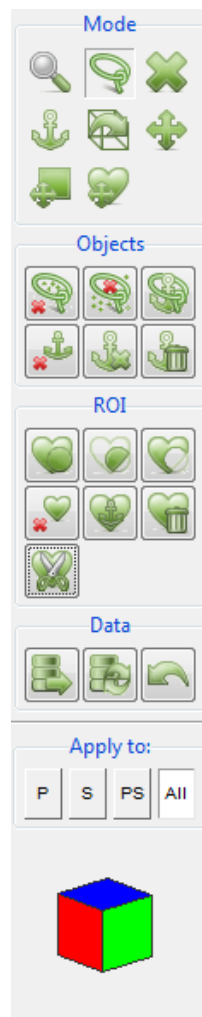
If you want to use the watershed segmentation, we recommend to first use the simple seed-and-threshold segmentation. After setting the threshold correctly, the watershed can be activated. Due to its complexity, the watershed segmentation may take some time, especially in comparison with the more simple seed-and-threshold segmentation.



**FIGURE 23.5.** Difference between the seed-and-threshold and watershed segmentation methods.

---


## Interaction with the Objects



On the left side of the Object Analyzer window you can find a column of buttons. Many of the buttons are deactivated at this moment, they will be activated when it makes sense later. But almost all icons in the first group are always available. The first group of icons control the *Mouse mode*, i.e. the action of the mouse when you left-click in the scene. A more detailed description of these icons are described in “Mouse Modes (2)” on page 154 .

The buttons in the other groups (Objects, ROI and Data) execute operations on previously defined conditions, for example deleting the objects under a selected area, or discarding every object that has not been selected as anchor. We will not consider them in this basic tutorial, as you don't need them to perform basic measurements. Please see the “Object Analyzer Component Reference” on page 151 for a detailed description of these toolbox buttons.

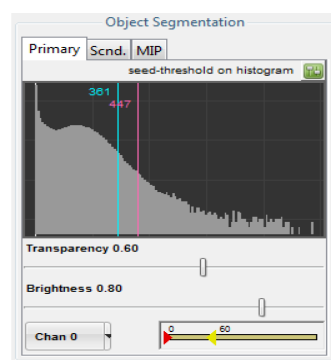
At the end of the buttons column there is a colored reference cube that will help you to orientate in space when you rotate the dataset, especially with large zoom factors that do not let you see the surrounding box frame in the rendered image. The initial view of this cube is the blue top face corresponding to  $z = 1$  . Hovering the mouse over the cube faces brings a tooltip with the face label ( $x$ ,  $y$  and  $z$  with values 0 or 1).

The *Analyze object* mouse mode  is the default mode. Click on an object and see what happens. Notice that some parameters are reported on the table at the bottom of the window. Later in this tutorial we will learn how to analyze more parameters and more objects at once.

First, we need to briefly explain what a pipe is, because you need to know how to select which channels in the image you want to analyze.

---

## Render Pipes



**FIGURE 23.6. Rendering pipes control panel.**

When we opened the analyzer, only the first channel of the image is shown on the screen. However, we can change this by simply selecting another channel from the drop-down menu in PRIMARY PIPE: where it reads *Chan 0* you just select *Chan 1* (See Figure 23.6).

With this approach, you can also explore two channels at the same time?

The term *pipe* suggests that data flows from your original image to the final rendering in the Object Analyzer through a ‘computational duct’ in which some processing occurs. You have two of these pipes in the Object Analyzer to direct data through.

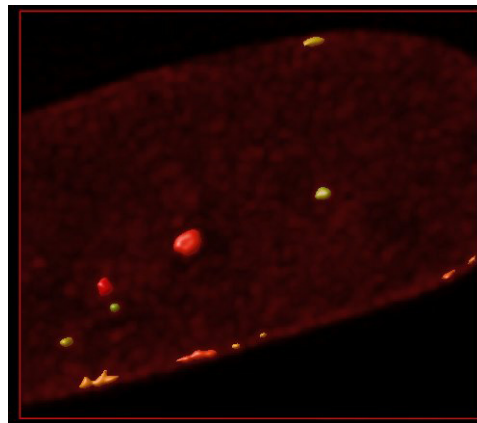
You can activate the control pane of the secondary pipe by clicking on the tab that reads SCND. ‘Secondary’ does not mean here ‘less import-

ant, it is just that we have the first pipe (*Primary*) and the second pipe (*Secondary*), also abbreviated as P and S. The primary and secondary pipe should not be confused with the numbering of the image channels. For example; you can even segment the same channel twice, but with different conditions in each of the two pipes.

All the different objects in a pipe are colored differently to be able to distinguish them. The range of colors assigned to each pipe can be controlled with a HUE SELECTOR (See “Hue Selector” on page 196.). If you want all objects in a pipe have the same color, you can collapse this range completely by moving the two triangular sliders together.

There is also a maximum intensity projection (MIP) pipe. This pipe does not interfere with the analysis, but can be used to set a visual reference of the (unsegmented) data.


Before continuing our exploration, let's enable channel 0 again in the primary pipe with a threshold of 40% (488 in absolute terms), nothing (Off) in the secondary pipe, and channel 0 again in the MIP, so we see something like Figure 23.7.



**FIGURE 23.7. Objects and MIP.** Objects in a surface pipe rendered together with a MIP pipe for spatial reference. The data channel is the same one in both pipes.

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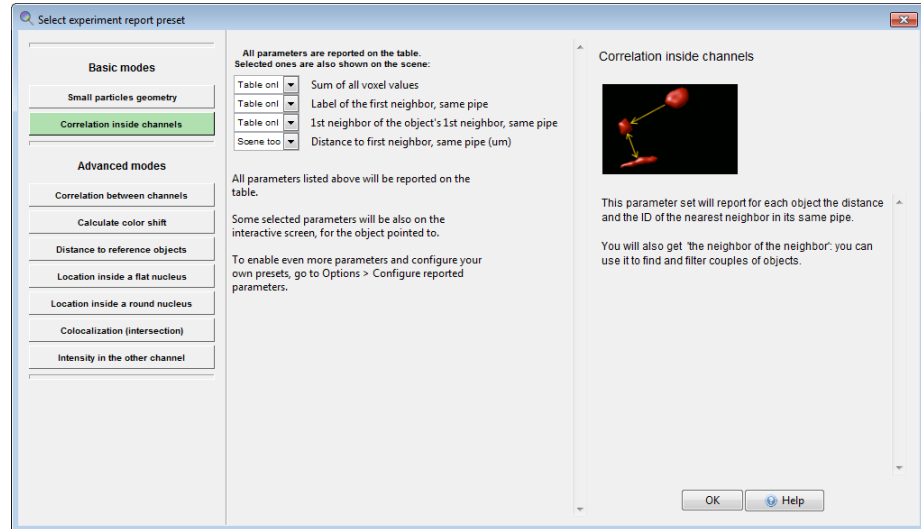
## Object Statistics

The Object Analyzer is able to measure many different parameters, but only a few are computed and reported under the default settings. To obtain information about a specific object, click on this object while you are in the *Analyze object* mouse mode. To automatically process all the segmented objects in all pipes, simply press the ANALYZE ALL button  on the top-right of the table. (If you have a region of interest (ROI) selected on the screen which encloses a few objects, this button analyzes only the objects inside this ROI).

There are many additional useful parameters that you can choose from. Let's have a look at how to retrieve all this information.

## Configuring the Reported Parameters

To simplify the usability of the Object Analyzer, there is a big button next to *Select Statistics* on the top left of the table that reads EXPERIMENT PRESET at start-up. Click on it and you will get a selection dialog as shown in Figure 23.8.



**FIGURE 23.8.** The Experiment Presets dialog. Collections of parameters can be selected to be reported on the table and the scene, depending on your specific experimental needs.

The left column shows a series of different experiments. If you click on them, a new list of parameters is listed in the middle column, and a description is shown on the right column. When you hover with your mouse over the listed parameters, a tooltip explains each parameter. Please read the descriptions briefly to get an impression of them. Let's select the preset called CORRELATION INSIDE CHANNELS and inspect it in more detail. Please read the descriptive text.


*"This parameter set will report for each object the distance and the ID of the nearest neighbor in its pipe."*

For each object we are interested in, we are going to find also the closest object.

Notice that among the listed parameters (center column) of this preset, only one of the parameters is selected to be reported in the scene. All parameters will be calculated and reported in the results table, but only the selected ones will be shown on the rendering canvas as well when you interact with the objects. In this parameter set, the distance is a useful parameter to report in the scene, as it provides a visual reference of the distance measurement between neighboring objects.

Once you have selected this CORRELATION INSIDE CHANNELS preset, press OK to continue.

## Measuring the Objects

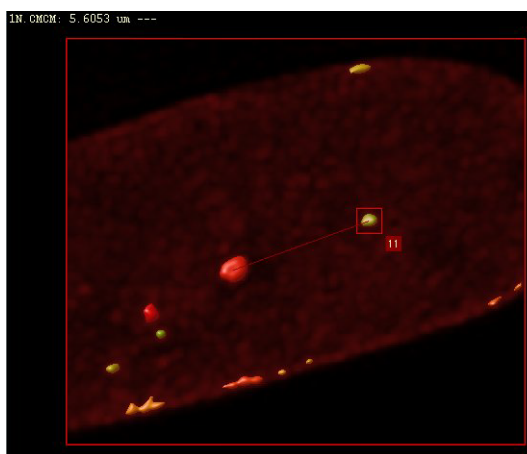
Make sure you have the *Analyze object*  mouse mode selected, click on an object in the scene and notice the new columns that appear in the statistics table.



Before showing the actual object details, the first row in the table informs you about the segmentation conditions for this pipe. If you move your mouse over the column header, a description of the corresponding parameter will be reported at the very bottom of the window. You can always find out what each parameter abbreviation stands for by looking at its tooltip. The description of each parameter will be also stored in your file when you export the results table later.

The tooltip of the column header also shows the sum and the average value of all the cells in the column. It is possible to include data of two pipes, or a single given pipe only.

Click on another object to apply a measurement and add the results to the table. You will see that the segmentation conditions are not reported again, because they have not changed. A checkbox option at the top-right of the table can be used to automatically clear the table when the segmentation or report conditions change, so you always have an organized table. When you deselect this option, all the results (despite different segmentation conditions) will accumulate in the table. The button located next to the checkbox allows you to clear the table at any time.



**FIGURE 23.9. Interaction with the objects.** When the distance to the nearest neighbor is computed, the distance line is also displayed on the screen for the object aimed at.

You may have noticed that something else happens when you interact with the rendered objects. In the example of Figure 23.9, the user clicked on object 11. A line joins the center of mass (CM) of this object with the center of mass of the nearest neighbor, and this distance is reported at the top of the window. This is the only parameter reported on the canvas because it is the only that was selected from the preset selection dialog to be shown in the scene.

### Other Measuring Parameters


The presets are organized in **basic** and **advanced** modes.

We can distinguish two basic parameter presets: the one we have used here to explore the nearest neighbors, and another one to retrieve morphological parameters about objects, called SMALL PARTICLES GEOMETRY. This parameter preset can report object information like *length*, *width*, *aspect ratio*, and *sphericity*. Details on how these parameters are defined can be found on “Object Analyzer Geometry Measurements” on page 147.

The advanced modes require that you define some conditions first. For example, that you first define a ROI. The advanced modes are not intrinsically more complex, but they demand from the user some knowledge on how to set these reference conditions.

For more details about any parameter preset just click the HELP button at the lower-right part of the preset selection dialog, and follow the on-screen tooltips during the interaction with the module.

### Exploring the Table

Let's try another thing: click on the ANALYZE ALL button  at the top-right of the table so the data of all objects are gathered. When the calculations are done (it should be quite fast in this example) move your mouse over the table rows and see that the cursor highlights the table rows (see Figure 23.10). Furthermore, the object corresponding to the current row will be highlighted on the canvas, and the distance to its nearest neighbor will be shown.

47	36	1	S	14	102.79	92.715	13.935	0	2636.93	2	1.7649	-8.1495	6.2257	-8.1499
48	37	1	S	7	144.58	163.71	14	0	1316.35	8	3.0245	27.968	-35.824	-2.0866
49	38	1	S	6	217.81	172.84	14	0	1130.53	8	4.2358	-45.366	-44.955	-2.0866
50	39	1	S	11	32.902	78.999	15	0	1983.05	9	1.273	15.083	-10.844	-1.7127
51	40	1	S	40	77.396	94.902	15.224	0	8238.29	2	2.2204	17.244	4.0366	-9.4394

Ready (rendered) Canvas size 678 x 577 Dragging on 38/S 1NP.CMCM: 4.2358 um

FIGURE 23.10. Exploring the table.

You can find an object in the results table by clicking on the object in the rendering canvas while the *Analyze object* mode is active: the table will be shifted to show its corresponding row, and it will be highlighted.

You can right-click on a column and select STATS for more detailed statistics.

To plot a histogram of the distribution of values in a column, select the column (or a subset), click with the right mouse button, and select HISTOGRAM from the pop-up menu. Figure 23.11, for example, shows a plot of the particle sphericity distribution.

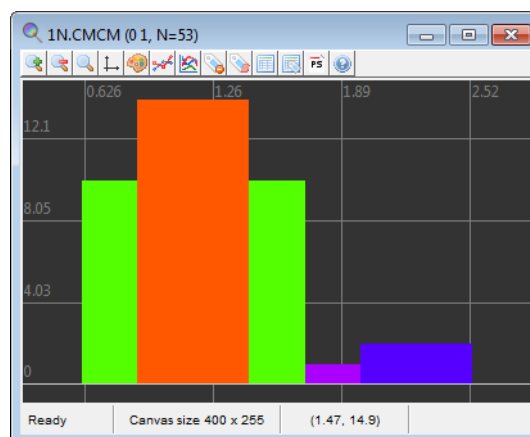


FIGURE 23.11. Histogram representation of one column values.

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### *Storing your Results*

In the top menu you can select FILE→SAVE OBJECT STATISTICS to export the table to a file for further analysis and/or calculations. The table can be stored as a plain text file, a csv-file, that can be imported in e.g., Microsoft Excel, LibreOffice Calc, GNUplot, and as a m-file, that can be imported in Matlab.

The current scene as it is currently visible on the rendering canvas can be saved to a TIFF file using FILE→SAVE SCENE. In the OPTIONS menu you can find different options that affect how the scene is rendered. You can set the MIP pipe to high quality for example, or render each analyzed object together with its numeric ID label, so that you can link them with the exported data visually.

In the HISTORY menu, you can save your analysis history as a template. Such a template can be used on the same dataset to reproduce the analysis, or on other datasets.

---

### *Further Reading*

This tutorial has covered the very basic features to the Huygens Object Analyzer. If you want to learn much more, consider following the expert on-line tutorial<sup>3</sup> and the accompanying wiki pages, where you will learn many other powerful things you can do with this versatile analyzer.

A reference description of the Object Analyzer components can be found in Chapter 25 on page 151.

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3. <http://www.svi.nl/ObjectAnalyzerExpertTutorial>



## Object Analyzer Geometry Measurements

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### *Iso-surface*

In the Object Analyzer, the threshold for the segmentation is used to define an iso-surface around the object.

An iso-surface is a 3D surface representation of points with equal values in a 3D data distribution. Is the 3D equivalent of a 2D contour line (See Figure 14.1 on page 85).

Based on the iso-surface, one can measure volume and surface in *high resolution*, by fine polygonization at a sub-pixel level.

---

### *Principal Axis*

Segmented objects are geometrically analyzed in terms of their principal moments of inertia. (In this sense, the recorded light intensity registered in the image is used as density: the pixel 'values' are interpreted as local mass, so brighter regions weight more).

The principal axes of an object establish a natural system of reference based on its mass distribution. When you rotate an object around one of its principal axes, the angular momentum is parallel to it. This does not happen in general, and is what makes these axes so special.

Around these axes the principal moments of inertia of the object are defined. For one of these axes, the rotation inertia of the object is minimal (around this axis the object would rotate with the least effort). This axis usually lies along the length of the object. The other two axes are orthogonal to it, and orthogonal to each other.

---

### *Length and width*

One can easily define a box, with dimensions  $L$ ,  $pBoxW0$ ,  $pBoxW1$  in the system of reference of the principal axes, that encloses the object completely. The sides of this box are in general not parallel to the main planes of the image, because the principal axes do not coincide with the image  $x$ ,  $y$ , or  $z$ -axes in general. It is as if the principal box is rotated with respect to the image in order to properly enclose the object, which may not be aligned with any of the image axes.

---

The length of the object is the largest distance measured along the three principal axes, it coincides with the largest dimension of the principal box  $L$ .

One could use the other two dimensions of the principal box as width and thickness of the object, but for some practical uses this may be too simplistic.

The width of the objects is actually computed with a search algorithm that acts as a virtual caliper held perpendicular to the length axis. To find the largest width of an object one would rotate the caliper around the object and repeat this procedure while sliding the caliper along the length axis. However, because microscopic data, even when it is deconvolved, often shows orientation dependent imaging due to the lower axial resolution, structures are often elongated in the axial direction. Moreover, in most cases, the voxels themselves are much higher than they are wide, causing all small objects to be elongated.

Clearly, without correction, the anisotropy in resolution would result in an overestimation of the width. To avoid this problem the rotation angles at which the caliper is held are divided in axial directions and lateral directions.

To measure in the axial directions several slices are taken out of the object and analyzed one by one. Each of these slices is parallel to the 'caliper plane', perpendicular to the length axis of the object. In any slice there are directions more oriented towards the optical axis (axial directions) than others. The largest axial width of each slice is obtained by holding the caliper in these directions and searching for the largest among them.

After all slices along the length axis have been examined, the largest axial width of the object is reported as  $WiAx$ .

The lateral directions in the caliper plane are the directions closer to the  $xy$ -plane. The caliper measures now the width of each slice in directions near-perpendicular to the optical axis. Taking the largest figure among these lateral directions might again introduce a bias due to elongation, so now two values are computed per slice: the largest and the smallest width along the lateral directions.

After exploring all slices some global relevant figures are reported. The largest lateral width obtained while sliding the caliper plane along the length axis is reported as  $WiLat$ . The largest of the smallest widths is reported as  $WiLatC$ . In case of small objects and moderate to high ratios between the axial and lateral resolution this last value is likely to suffer least from the orientation dependent imaging.

The waist (the smallest of the smallest widths) in the lateral directions is reported as  $WaistLat$ .

What about objects with a vertical length axis? In that case the 'caliper plane', perpendicular to the length axis, will be horizontal, parallel to the  $xy$ -plane. In a horizontal caliper plane all directions in it are perpendicular to the vertical  $z$ -axis. As a result there is no 'most axial direction' in that plane. The software then orients the lateral width towards the  $x$ -axis and the axial direction towards the  $y$ -axis.

---

## Sphericity

The sphericity is reported in two ways in the Object Analyzer:

1. The axial sphericity is defined in general as the ratio of the volume of an ellipsoid with axes length  $L$ , width  $W$  and thickness  $T$  to the volume of a sphere circumscribed around the segmented object, defined by its length.

Because it is based on three axes, it gives an idea of the 3D aspect ratio of the objects. Depending on which of the previously reported parameters we choose for  $W$  and  $T$  we have different practical definitions of axial sphericity.

Probably the most intuitive one is the axial sphericity of the principal box  $axSphPB$ : the ratio of the volume of an ellipsoid with axes  $L$ ,  $pBoxW0$ ,  $pBoxW1$  to the volume of a sphere circumscribed around the principal box (see above) using the largest side (the length of the object  $L$ ) as diameter (Equation 1).

$$AxSphPB = \left( \frac{pBoxW0 \cdot pBoxW1 \cdot L}{L^3} \right)^{1/3} \quad (\text{EQ 1})$$

- Another definition (reported as  $AxSph$ ) involves the lateral and axial widths discussed above, obtained with the virtual caliper algorithm (Equation 2).

$$AxSph = \left( \frac{L \cdot WiAx \cdot WiLatC}{L^3} \right)^{1/3} \quad (\text{EQ 2})$$

The roughness sphericity characterizes the roughness of the iso-surface, it measures how close the volume-to-surface ratio is to the one of an ideal sphere. This is conventionally defined as:

$$SurfSph = \frac{\pi^{1/3} \cdot (6V_i)^{2/3}}{A_i} \quad (\text{EQ 3})$$

where  $V_i$  is the iso-volume and  $A_i$  is the iso-surface of the segmented object. Both sphericity values become 1 for an ideal sphere. The  $SurfSph$  is the inverse of the 'surface factor'  $fs$  used in Goetze et al.<sup>1</sup>

---

## Aspect Ratio

Again, the aspect ratio of an object can be defined in terms of different dimensions:

- The axial aspect ratio  $AxRatio$  is the ratio of the object length to its axial width  $WiAx$ .
- Similarly, the lateral aspect ratio  $LatRatio$  is the length divided by  $WiLatC$ .

---

## More Parameters and Filtering

Many additional parameters, geometrical and of other kinds, can be calculated, reported, and used to filter the data by the Object Analyzer. Please refer to the on-line tutorials to learn how to use the different parameters and Experiment presets.

Parameters are available that report:

- Correlation* inside and between channels, by analyzing neighbor objects
- Location of objects with respect to *reference objects* (anchors).
- Location of objects inside *regions of interest* (for example bodies inside a cell nucleus)
- Colocalization* of objects (by computing the volume and the intensity overlap of segmented objects in different pipes).

Any of the calculated parameters can be used to filter out objects and further segment your image in elaborated ways.

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1. Goetze et al., *Molecular Cellular Biology* 27, p. 4475-4487 (2007)

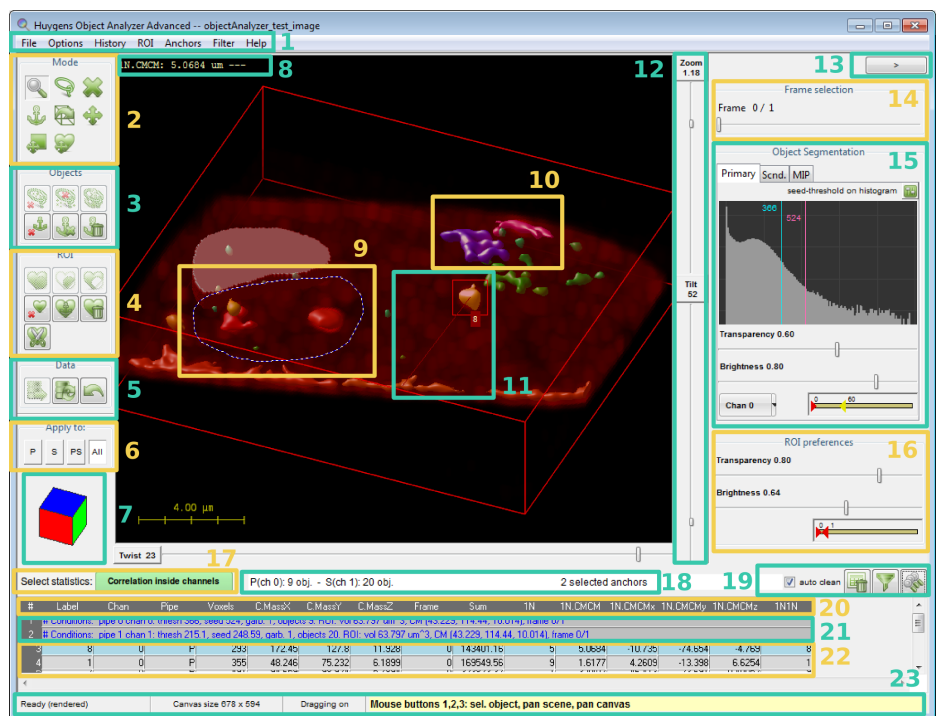




# Object Analyzer Component Reference

This section describes the components of the Object Analyzer (OA) interface.

This section is intended as a quick reference. See “Introduction to the Object Analyzer” on page 135 to learn how to use the basic components in context.



**FIGURE 25.1. The Object Analyzer interface.** Different regions are enumerated to describe them in sections of this chapter.

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## Main window components

The OA main window is shown in Figure 25.1 with most of its components in an active state. Different regions of the interface are enumerated to describe them conveniently along this chapter.

When the OA is launched, not all the buttons in the toolbox are enabled. Most buttons are automatically enabled when they are usable, depending on conditions set by the user.

### Main Menu (1)

**FILE:** Entries to save the rendered image, export or clear the object statistics, and analyze all time frames in a series.

**OPTIONS:** Rendering options and advanced statistics configuration:

- Virtual render size: sets the size of the canvas on which the Ray Tracing algorithm renders the image. This canvas can be larger than the OA window or even the screen (that's why you can pan the canvas to inspect other regions). This way you can render and save high-resolution TIFF images.
- Transparency depth: controls the number of surfaces considered by the renderer in order to show inner cavities and objects inside objects.
- Bounding box: shows or hides the reference 3D bounding box
- Scale bar: shows a scale bar on the scene. The distances are calculated based on the voxel sizes in the original image microscopic parameters.
- Show ID labels: render the scene showing the numerical ID label of each of the analyzed objects.
- Show SVI logo.
- High quality MIP: enables or disables the high quality rendering mode for the MIP pipe
- Show reference cube (See "Reference Cube (7)" on page 157).
- Show on-screen tooltips for interactive actions.
- Relaxed selection: when active, objects partially outside the selected 2D area are also considered.
- Center scene: moves the point of view to show the center of the dataset.
- Configure statistics report: shows all available parameters to let you select which ones are calculated and reported on the table. This is intended for advanced users, beginners should better use the *Experiment presets* (See "Experiment Presets (17)" on page 160).

### HISTORY

- Undo the last operation, or Redo it again.
- Reload original data after cropping the image or discarding objects.
- View the whole operations history up to the current point.
- Load and save analysis templates, so that the current view and parameters can be stored and retrieved, or a whole operations history re-executed with other data. This is also useful to store your analysis steps and reproduce them.
- Set analyzer as in any other open instance of the tool.

**ROI:** operations to define a region of interest in complex ways, and to modify and save the currently defined ROI.

- Set:
  - Set to extruded selected area: uses the interactively defined 2D area to set the ROI to the volume below it. The 2D area is projected along the line of view.
  - Set using MIP threshold: use the threshold and data channel of the MIP pipe to set a 3D ROI
  - Make coincide with objects: use the currently segmented objects to define a 3D ROI. Objects from the Primary, Secondary or both pipes can be used depending on the active pipes and the pipe mode (See “Active Pipe Mode (6)” on page 156).
  - Make coincide with anchors: use the currently selected anchors to define a 3D ROI. This may leave holes in the interior of the ROI if the anchor is not a solid object.
  - Make coincide with intersection: this is interesting to do object analysis with colocalizing volumes only. When you have two pipes active and some objects in one channel intersect with the other. A ROI can defined, and applied to discard objects and parts of objects outside the region, so that only the colocalizing intersecting regions remain.
  - Envelop anchors: use the currently selected anchors to define a 3D ROI, so that also holes inside the objects are 'filled in' and in the ROI.
  - Enclose anchor in a box: define a prism that is an envelop to the selected anchors.
  - Make a spherical ROI of a given diameter, centered in the image. You can shift it later with the shift ROI mouse mode.
  - Set to all volume: sets the ROI to the complete dataset volume.
- Modify:
  - Fill inner cavities: a ROI defined by using a threshold may contain inner cavities (visible when selecting the deep Transparency Depth). This operation fills them in automatically.
  - Fill inner and cutoff cavities: A cutoff cavity is a hole in the surface of a ROI that touches the image limits.
  - Grow/shrink: the currently defined ROI can be enlarged or reduced in a certain number of VoXels, independently in the *xy*-plane or in the *z*-direction. A 3D (*xyz*) reduction is also possible: here the number of voxels in the *xy*-plane will be partially adapted to the entered voxels along *z* to, considering the voxel anisotropy, grow/shrink the volume proportionally.
  - Outer shell: re-define the ROI considering only an outer shell of given thickness.
  - Invert the ROI volume.
- Storage
  - Export current ROI to the main window, to have the ROI made available for other tools in Huygens.
  - Save current ROI to file
  - Load ROI from file
  - Add ROI from file
  - Intersect with ROI from file
  - Subtract ROI from file
- Center ROI on the anchor CM: align the Center Of Mass (CM) of the currently defined ROI with the CM of the selected anchors.
- Clear ROI

- Keep only objects inside the ROI, discarding anything else. The relaxed selection mode in the options also affects how objects partially inside the ROI are handled.
- Analyze ROI volume computes and reports information on the table about the ROI itself.
- Analyze all objects inside the ROI reports in the table information about objects inside the ROI, or partially outside it, depending on the relaxed selection option.
- Help on Regions of Interest.

#### ANCHORS

- Anchor objects under selection
- Select all objects as anchors.
- Set anchors by filtering. This opens a filter dialog as explained in “Table and Analysis Shortcuts (19)” on page 161, but allowing you to select or deselect anchors instead of discarding objects.
- Invert current anchor set.
- Deselect all anchors.
- Keep anchor objects, discarding anything else
- Discard all anchor objects.
- Analyze only objects select as anchors

**FILTER:** some useful predefined filters to remove objects based on their features, and access to a full control filter tool and to reload the original data.



- Quickly remove objects that are touching the borders of the image, as they are surely incomplete.
- Quickly remove objects based on size or sphericity
- Quickly remove non-colocalizing objects
- Quickly find pairs of objects, inside the same pipe or by combining the two pipes.
- Advanced filtering shows the same filter dialog explained in “Table and Analysis Shortcuts (19)” on page 161.







**HELP:** shows on-line help and tutorials

#### Mouse Modes (2)

These buttons control the Mouse mode, this defines the mouse functionality when you left-click on the image. Hoovering your mouse over these buttons allows you to get a tip on what they are for. If they have a keyboard shortcut, this is also shown in the tooltip. For example, the first mouse mode is intended to *analyze objects*, and you can always activate this mode from within the rendering view by pressing the keyboard key ‘1’.

From left to right, and from top to bottom, the mouse modes are:

-  **Analyze object** lets you click on different defined objects and obtain the local statistics.
-  **Select area** lets you define a 2D region on the current view of the image. This allows you to analyze or discard objects below it, anchor them as references, or interactively define regions of interest (ROI). We will see what all this is useful for.




-  **Discard object** lets you discard irrelevant objects one by one. Just select this mode and click on the uninteresting objects.
-  **Select object** (as anchor) lets you select and deselect objects to be 'anchors', for example to act as references to measure distances from other objects with respect to the anchor object. When you set an object as a reference anchor it will 'light up' and change color on the screen to indicate the anchor status. It is possible to select a group of anchor objects, and you can operate with them through the Anchors menu.
-  **Rotate scene** interacts with the full image to rotate it in the space, by dragging the mouse pointer on the rendering view. That can also be achieved by moving the Tilt and Twist sliders along the rendering.
-  **Pan scene** interacts with the full image to move it in space laterally. This means that you can pan the scene in the 2D plane of your screen,
-  **Pan canvas** This mode allows you to explore the canvas while not re-rendering the scene. This only makes sense when you have a canvas larger than your rendering window, See OPTIONS→VIRTUAL RENDER SIZE in the top menu.
-  **Shift the ROI**. This is the only mouse mode that is not always enabled: you need to have defined a region of interest (ROI) before moving it around.

For the advanced users: some of these mouse modes have 'shortcuts'. Most of the times you can 'pan scene' independently of the selected mouse mode if you use your mouse right button instead of the left one. Similarly, you can 'pan canvas' using the middle button at any time. When a mouse mode is active and the cursor is inside the canvas, the tooltip in the status bar (See Figure 25.1, item 23) shows you what each mouse button can do.

### Selection Interactive Operations (3)

The rest of the buttons in the toolbar are not mouse modes, but they execute operations on previously defined conditions. For example, deleting the objects inside a defined ROI. They are all disabled until these conditions have been set (in the example, until you define a ROI to operate with).



After having drawn a 2D selection (See “The Selected Area (9)” on page 157) in the *Select area* mouse mode, you can click on:

-  Keep all objects under the selected area, discarding anything else.
-  Discard all objects under the selected area
-  Set all objects under the selected area as anchors

Some objects may partially cross the border of the defined ROI. These can be considered to be either in- or outside the ROI. You can change this definition in the top Options menu: the area can consider only objects fully under the selection, or also objects partially outside it (relaxed selection mode). In any case, the objects not affected by the selection are rendered with a lower intensity to clearly indicate which objects are selected.







When you have selected certain objects as anchors, you can apply further operations:

-  Deselect all anchors.





-  Keep the selected anchors, discarding anything else.
-  Discard the selected anchors.

More operations are available at the top ANCHORS menu. The main purpose of an anchor is to serve as analysis reference in some experiment presets, for example to measure distances relative to these anchor objects. But you can also use the selected anchors to delete some other objects, or define a 3D ROI based on the anchor object shape. When you have defined a ROI, you can add/subtract new ROI shapes, or perform additional operations with the ROI Interactive Operations buttons:

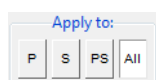
#### ROI Interactive Operations (4)

-  Reduce the current ROI to the volume under the selected 2D area (intersects the ROI with the selection)
-  Add the volume under the selection to the ROI volume (union of the ROI with the selection).
-  Remove the volume under the selection from the defined ROI (difference of the ROI with the selection).
-  Set all objects inside the ROI as anchors.
-  Keep objects inside the ROI, discarding anything else.
-  Discard the defined ROI, reverting to the whole image.

#### Data Cropping and Restore Operations (5)

-  Once a ROI is defined, you can use this button to crop the dataset and delete (set to zero) all voxel values outside the ROI.
-  Export the current data (as cropped by the ROI, or after deleting objects interactively) to the original image.
-  If deleted data was not yet exported to overwrite the original, you can always reload the original dataset with this button.
-  Undo last operation.

#### Active Pipe Mode (6)



These radio buttons control, on which of the active data pipes (*Primary*, *Secondary* or both) and channels (*Selected pipe*, or *All*) they operate. Pipes are explained in “Render Pipes (15-16)” on page 159.

Examples of operations controlled by these buttons are:

- Interactively clicking on objects. When you click on the scene, only objects in the active pipe are considered.
- Analyze all / Analyze selection
- Set ROI to the visible objects
- Sum all column values (when hovering over a column header in the table)
- Apply the ROI to crop the dataset

There are only a couple of practical differences between the PS and All modes:

When cropping data by applying a ROI, you can crop the channel in the Primary pipe (P), the channel on the Secondary pipe (S), on both (PS), or all channels in the image (All), even if they are not shown in any active pipe.

When summing cell values in a column of the table, you can include in the summation cells for the channel in the Primary pipe (P), the channel on the Secondary pipe (S), both (PS), or all cells (All), including those reporting about the ROI.

For all the other operations, the PS and All modes are equivalent.

### Reference Cube (7)



At the end of the buttons column you can notice a colored reference cube that will help you to orientate in space when you rotate the dataset, specially with large zoom factors that do not let you see the surrounding box frame in the rendered image. The initial view of this cube is the blue top face corresponding to  $z = 1$ . Hovering the mouse over the cube faces brings a tooltip with the face label ( $x$ ,  $y$  and  $z$  with values 0 or 1).

### The Interactive Rendering Canvas (8-11)


The canvas shows the scene, the result of the ray tracing<sup>1</sup> algorithm rendering the segmented objects.

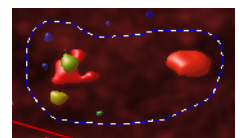
The scene is determined by the objects orientation (*tilt* and *twist*), the *zoom*, the *brightness* of the pipes, what point is centered on the view and so on. All these parameters are taken into account by the renderer, which generates the scene and displays it in the canvas on the screen. Notice that depending on the render size (OPTIONS→VIRTUAL RENDER SIZE) the canvas can be made larger than your screen.

### The On-Screen Reported Parameters and Tooltips (8)

The currently selected Experiment preset (See “Experiment Presets (17)” on page 160) selects the statistics to report many parameters to the table (See Figure 25.1, item 20, 21, and 22) A few of these parameters can be also reported on the screen for the current object for easy reading. The magnitude of the distance that is plotted on the screen is indicated by a triple dash ‘---’

### The Selected Area (9)

The *Select area*  mouse mode allows you to define a region in the image. So for example you can: analyze objects ‘inside’ the ROI, anchor them as references, or use the selected volume to define a region of interest (ROI).



The *selection* is not yet a 3D ROI, but a 2D area. That is why ‘inside’ is quoted in the previous paragraph: objects are inside the selection only from the current point of view, so it would more appropriate to say ‘below’ the current selection. It allows quick and simple interaction with the objects, specially on flat images here the objects remain more or less in a plane.

1. <http://www.svi.nl/RayTracing>

---

In the Select area mouse mode you can use the right mouse button to shift the defined selection around and reuse it multiple times in different locations.

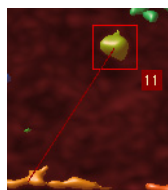
### Anchor Objects (10)



Objects that act as references (anchors) are shown in the rendering with magenta or violet colors, depending on the pipe they belong to, so that they are clearly distinct from the other objects.

There are different ways to set objects as anchors, interactively (See “Selection Interactive Operations (3)” on page 155) or by using filtering operations (top ANCHORS menu). You can also visit the on-line article about anchor objects for more details on their utility.

### Aim (11)



Objects under your cursor are highlighted. Once a certain object is analyzed, the object will also show a red box surrounding the object. To analyze an object just click on the object with the *Analyze object* mouse mode, or press the ANALYZE ALL button (See Figure 25.1, item 19).

Objects in the Primary pipe are framed with a red box, and objects in the Secondary pipe with a green one.

A small label showing the object's number ID is also shown when pointing at it. The background color also indicates if it belongs to the primary (red) or the secondary (green) pipe. Yellow labels are shown whenever the pointer has two objects below it, from different pipes.

If a distance is configured to be reported on-screen (See Figure 25.1, item 8), it is also plotted when pointing to an object.

If you hover over an object's row in the table and point to a cell containing a distance parameter, it will also be plotted on the rendering canvas. Like this you can interactively explore many reported distances.

Notice that, depending on the active pipe mode (See “Active Pipe Mode (6)” on page 156), the interaction with the scene may highlight and affect objects in one pipe only.

### Scene Control Sliders (12)



Three sliders run along the canvas (vertically, on its right, and horizontally, on its bottom) to control the point of view of the scene:

- **Zoom:** the number is just indicative, 1 meaning that the whole dataset is shown in the canvas.
- **Tilt:** the angle of rotation (in degrees) around the canvas  $x$ -axis.
- **Twist:** the angle of rotation (in degrees) around the image  $z$ -axis.

By clicking on the labels you can enter numerical values manually to quickly switch to the desired scene.

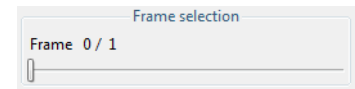


### Hide Pane Button (13)

This button at the top right of the window collapses the control pane to make more room for the rendered scene. Once you have defined the segmentation conditions for all pipes, you don't need to interact with those controls anymore, but with the objects, so you can hide the pane to focus on the scene.

### Time frame selector (14)

When time series are loaded in the OA, this slider controls which time frame is taken for visualization and analysis.



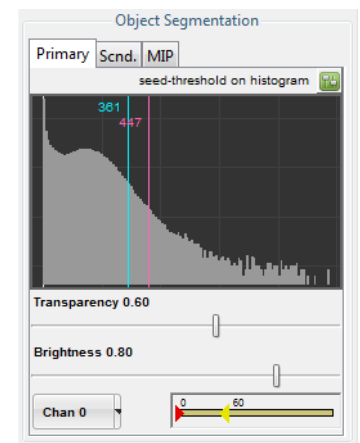
Changes to the current frame (like discarding objects or selecting anchors) are remembered when you change the time frame. But voxel editions (using the ROI to crop the dataset) are lost unless you export them first with *Export the applied ROI to original* (📁).

You can analyze all frames in a time series, accumulating the data in the table, through the FILE→ANALYZE TIME SERIES menu entry.

### Render Pipes (15-16)

The Huygens Object Analyzer has two surface pipes (named *Primary* and *Secondary* pipes) for you to put image data through for object analysis and another pipe to simultaneously visualize a MIP of one of the channels (See Figure 25.1, item 16).

'Secondary' does not mean here 'less important', it is just that you can use the first pipe (*Primary*) and the second pipe (*Secondary*), abbreviated *P* and *S*. We keep numbers to refer to image channels, which is something different, because in each of these pipes one can put any image channel, in any order. One can even segment the same channel twice with different conditions in each of the pipes if necessary!

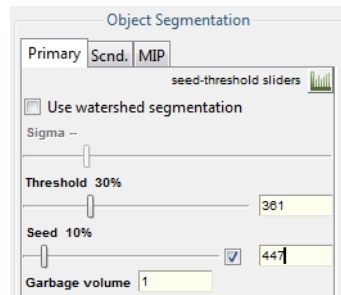



This pane controls which data channel goes through which pipe, how its objects are segmented (with a seed and a threshold<sup>2</sup>), and how the data is rendered (transparency and brightness). A garbage volume can also be set to remove little spurious objects: objects with a volume smaller than this garbage volume will be discarded and not rendered.

You can see the seed as a secondary threshold. The first threshold segments the data and makes independent objects, but then only objects with intensity that reaches above the seed level will remain, while the rest are discarded.

At start-up, the threshold and seed levels are represented by vertical blue and magenta lines, respectively, on top of a histogram of the channel in the pipe. An alternative representation of these levels is also available. This alternative representation also shows their values as a percentage of the channel maximum and allows you to link the seed with the threshold.

2. <http://www.svi.nl/SeedAndThreshold>



The garbage volume control is also in the slider pane, not in the histogram. To switch between the histogram and the sliders control panes you have to click on the small button (  ) at the right of the pane title.

The threshold ranges between the minimum and the maximum values in the channel intensities, considering all the time frames. Its percentage representation refers to the maximum value.

By default, the seed is linked to the threshold value, so when you vary the latter, the seed absolute value also changes in such a way that its relative value remains constant. In its linked mode, the seed is set referred to the span between the actual threshold and the maximum, and ranges from the threshold value itself (0%) to the image maximum (100%). This is because the seed, being an 'upper threshold', can never be lower than the threshold. It is also useful to be able to set the seed to 0% so that it is not used at all and you retrieve the classical threshold-only segmentation. Still you may find convenient to express the seed relative value in the same terms you use for the threshold (relative to the image intensity range), or to be able to set the seed to a fixed value independent of the threshold. For that, deselect the seed checkbox so that 0% also represents the same value as the 0% threshold (the image minimum, or zero), and the two sliders are unlinked. The linking mode of the seed does not really affect the segmentation, it is just a matter of convenient representation of relative values: what is applied to the image as threshold and seed are always the absolute numeric values shown on the entry widgets and next to the histogram lines, that you can edit directly by clicking on them.

All the different objects in a pipe are colored differently to distinguish them. The range of colors assigned to each pipe can be controlled with a *Hue Selector* (See “Hue Selector” on page 196.). You can collapse this range completely if you want so all objects in a pipe get the same color.

### Experiment Presets (17)



This button opens a preset selection dialog that allows to select an experiment preset, a collection of parameters that make sense to

be reported together in the context of certain experimental needs (See Figure 23.8 on page 142).

Users are very much welcome to send their own suggestions to implement new presets. We will gather all the feedback in different wiki articles that will explain what parameters are best for certain experiments and how to interpret them. Please feel free to tell us about your experiences!

In this dialog you can notice three columns. On its left column a series of different experimental needs are listed. When you click on each of them, a different list of parameters is listed in the middle column, and a description is shown on the right column. Even more, when you hover with your mouse over the listed parameters, a tooltip will appear, explaining each parameter with more detail.

By selecting a particular preset all the listed parameters (apart from the basic ones) will be reported and calculated. Moreover, all the listed parameters will also be available for filtering the objects (See “Filtering Objects” on page 161). The check boxes allow a few of these parameters to be also reported on the screen (See Figure 25.1, item 8) for the current object. The magnitude of the distance that is plotted on the screen is followed by a

triple dash '---'. Only one distance can be plotted at a time by clicking on an object, but many can be reported on the table and explored interactively there by simply moving the cursor over the cells.

Only one preset can be selected at a time, and all its parameters will be reported. Advanced and more flexible configuration of the parameters is always possible through OPTIONS→CONFIGURE STATISTICS REPORT in the menu. In this configuration dialog users can also store any set of reported parameters as a new preset.

### General Object Information (18)

This little bar reports:

Pipe P - ch 0: 9 obj., Pipe S - ch 1: 17 obj.    2 selected anchors

- left: the number of objects currently segmented in each surface pipe.
- right: the total number of objects selected as anchors.


### Table and Analysis Shortcuts (19)

These widgets provide quick access to some table operations.



- Auto clean checkbox: this option makes the table to be cleaned whenever the segmentation or analysis conditions change, to always have a fresh start. Deselect this option to keep all data and keep accumulating rows in the table.
- Clear statistics table: manually delete the table contents
- Filter: opens a dialog that allows you to filter objects out based on the reported parameters (see below).
- Analyze all / Analyze sel.: This button runs the analysis procedure on all the currently segmented objects, for objects on the pipes selected by the Pipe mode radio-buttons (See Figure 25.1, item 6). When a 2D area has been selected (See Figure 25.1, item 9) this buttons analyzes only the objects under the selection.

### Filtering Objects

The Filter button  opens a pop up dialog that allows you to discard objects:

- Based on any of the reported parameters...
- Using a certain arithmetic operator...
- To compare the parameter with either a fixed value or with another reported parameter

The pipes that are filtered can be controlled with the active pipe radio-buttons.

You have to select one of the two options (fixed value or another parameter) and enter the value or select the parameter you want to compare with.

Because the filter is based on the reported parameters, you may need to configure the reported parameters or select other statistics first in order to filter based on the desired property.

For interesting usages of this filter, see for example the neighbors article in the SVI Wiki<sup>3</sup>.

For time series, an additional button allows filtering all time frames.

### Statistics Table (20-22)

The statistics table is the place where all the objects parameters are reported after the analysis. You can explore the table values in interesting ways directly on the Object Analyzer (see below), or export the contents for further analysis in other program.

When you move your mouse over the table rows, the objects they refer to are highlighted on the rendering canvas. When you point to a cell reporting a distance, this distance is also plotted on the screen.

#	Label	Chan	Pipe	Voxels	C.MassX	C.MassY	C.MassZ	Frame	Sum	1N	1N.CMCM	1N.CMCMx	1N.CMCMy
0	# Image: objectAnalyzer_test_image_analyzed with HuPro 3.6.0p0 on 2010-06-03												
1	# Conditions: pipe 0 chan 0 thresh 366 seed 524.07 gaib. 1 objects 9 ROI: vol 0.34587 um <sup>3</sup> , CM (170.13, 213.42, 9.1209), frame 0/1												
2	# Conditions: pipe 1 chan 1 thresh 203 seed 254. gaib. 1 objects 17 ROI: vol 0.34587 um <sup>3</sup> , CM (170.13, 213.42, 9.1209), frame 0/1												
3	1	0	P	355	48.246	75.232	6.1899	0	169549.56	9	1.6177	4.2609	-13.398
4	2	0	P	491	94.659	98.924	5.7894	0	323823.32	1	3.4402	-46.413	-23.691
5	3	0	P	1677	60.758	26.59	6.7792	0	730828.35	7	2.6129	-34.511	16.137
6	4	0	P	514	238.13	79.466	6.5037	0	220270.08	5	5.3358	-76.415	-26.321
7	5	0	P	396	161.71	53.145	7.1591	0	164795.01	8	5.0684	10.735	74.654

The contents of the table can be copied to your clipboard, or stored to a file on your disk by using the FILE menu.

### The Table Columns and Their Headers (20)

**Distance to first neighbor, same pipe (um)** When you move your mouse over the column titles at the top of the table a description of each parameter will be shown at the very bottom of the window (See Figure 25.1, item 20). You can always find out what each parameter is by looking at its tooltip. The description of each parameter will be also stored in your file when you export the table later.

This brief parameter description, plus longer explanations are also given in the selection and configuration dialogs that selects or configures the reported parameters.

For columns with numeric values, basic descriptive statistics of all values in the column are also reported along with the parameter description. You can select whether the sum runs for both pipes (ALL or PS) or only for one of the pipes (P or S) depending on the selected pipe mode (See Figure 25.1, item 6). This provides a quick way of finding the total number of voxels in the object list, or those that are colocalizing, for example. Many interesting questions can be answered by the summations and ratios between them.

You can right click on a column to pop-up a contextual menu, from which you can retrieve more detailed statistics (STATS) for that column. The descriptive statistics will analyze pipes separately and together, and also include ROI information if present. The reported values are the maximum (MAX), the median (MED), the minimum (MIN), the number of items considered (N), the summation of the values (SUM), the average value

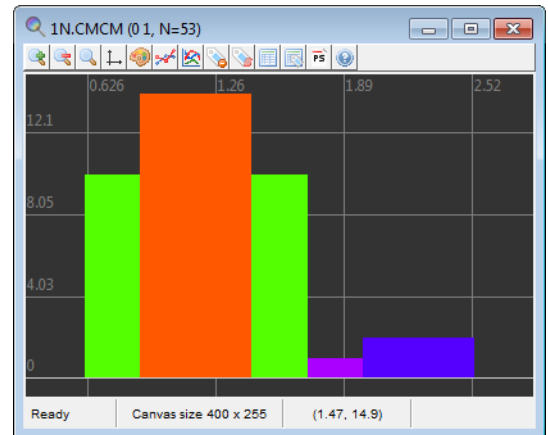
3. <http://www.svi.nl/ObjectAnalyzerNeighbors>

(AVG) and the standard deviation for the  $N$  items (SD- $N$ ), reported also as a percentage relative to the average value.

Clicking on a column title selects the whole column for you to copy. Multiple columns can be selected by holding the `Ctrl` key. To plot a histogram of the distribution of values in a column, select the column (or a subset) and select Histogram from the pop-up menu.

Selected columns also act as 'special parameter selector'. The value of the parameter for selected columns will be reported on the status bar (See Figure 25.1, item 23) when you explore cell values for given objects.

Like this, you can quickly compare different parameter values for the same object, by looking at the report in the status bar while you move your mouse over the table cells.



A similar thing can be also done by selecting rows, see below.

When multiple columns are selected, the STATS popup dialog will report descriptive statistics for all them.

### Conditions Report (21)

When new segmentation or analysis conditions are set, they are reported when you analyze one or all objects. One row is added to describe the image and the time of the analysis, and another row per active pipe is added that reports the image channel in that pipe, the segmentation conditions, the number of objects in that pipe, and the volume and geometrical center of mass of the ROI:

```
# Conditions: pipe 0 chan 0: thresh 504.8, seed 540.52,
garb. 1, objects 12. ROI: vol 1103 um^3, CM (130, 115,
10)
```

The parameters about the ROI (volume and CM) are the same for both pipes, in case two are active. This is because there is only one ROI for all pipes and channels, and the Center Of Mass (CM) here reported is calculated considering the ROI as a uniform, solid object.

There is another way to calculate the CM of the ROI, not considering it homogeneous but taking into account the real image intensities in envelopes. These intensities, being different per pipe, yield to different ROI's CM per pipe. This is not reported in the conditions rows, but as object rows in the table when you select Analyze all. In this case, the ROI itself is treated as a new object in each pipe and more detailed information is reported in separate rows in the table. See the wiki article about ROI<sup>4</sup>.

4. <http://www.svi.nl/ObjectAnalyzerROI>

## The Table Rows, How to Explore Them (22)

Each row in the table is either a *Conditions report* (See “Conditions Report (21)” on page 163) or information about one of the segmented objects.

In the second case, the object the row refers to is identified by some mandatory parameters (parameters that are always reported):

- *Label*: an integer index that is unique, per object, inside its pipe.
- *Chan*: the image channel this object belongs to
- *Surf*: the surface pipe in the current analysis the object belongs to (*P* is *Primary*, *S* is *Secondary*).
- *Voxels*: the number of voxels in that object
- *C.Mass*: three columns (*x*, *y*, *z*) for the center of mass location of the object, in the image coordinate system, with voxels as units.

The second letter in the *Surf.* column informs whether the object you clicked was in the front (*F*) or the back (*B*) position. This is only relevant in the following situation: it may happen that two objects in different pipes overlap in space (or they apparently do from the current point of view) and when you click somewhere on the screen you are actually selecting both of them, if the pipe interaction mode is PS or ALL (See “Active Pipe Mode (6)” on page 156). In that case, two rows are added to the table, and this second letter lets you know which of the two was in front of the other, from the current point of view.

By moving the mouse over the table rows the corresponding object is highlighted on the canvas (See Figure 25.1, item 11). Objects in the Primary pipe are highlighted in red, and objects in the Secondary pipe in green.

A quick way to find the parameters of an object in a very long results table is by clicking on it on the rendering canvas while the *Analyze object* mode is active: the table will be shifted to show its corresponding row, which will be highlighted.

When you move over the table cells the current value is shown on the tooltip bar at the bottom (See Figure 25.1, item 23). This, combined with the selection of rows (see below) or columns (See Figure 25.1, item 20), allows a quick exploration of the table and the comparison of different parameter values.

In the following example, when the cursor is moved over one cell that contains the distance to the first neighbor, the following is reported in the tooltip: 38 / S 1NP.CMCM : 4 . 2358  $\mu\text{m}$ . The first part is the label of the object in the current row: label 38 on the Secondary pipe. Then the parameter tag 1NP.CMCM refers to the CM-to-CM distance between this object and the nearest object in the other pipe. The value of the distance itself is also shown: 4.23 microns.

47	36	1	S	14	102.79	92.715	13.935	0	2636.93	2	1.7649	-8.1495	6.2257	-8.1499
48	37	1	S	7	144.58	163.71	14	0	1316.35	8	3.0245	27.868	-35.824	-2.0866
49	38	1	S	6	217.81	172.84	14	0	1130.53	8	4.2358	-45.366	-44.955	-2.0866
50	39	1	S	11	32.902	78.999	15	0	1983.05	9	1.273	15.083	-10.844	-1.7127
51	40	1	S	40	77.396	94.902	15.224	0	8238.29	2	2.2204	17.244	4.0386	-9.4394

Ready (rendered) | Canvas size 678 x 577 | Dragging on | 38/S 1NP.CMCM: 4.2358  $\mu\text{m}$

That tooltip region can also show other values that can be used as reference. Try this: while keeping the `Ctrl` key pressed on the keyboard, select a couple of rows by clicking on the row number at the very left of the table. The selected rows will turn green. If you now hover the mouse over a cell on any other row, you will get the value not only of that

cell, but also those in the corresponding cells of the selected objects (rows). This is a quick way to compare results for different objects that can be well separated in the table.

44	33	1	S	9	139.23	130.45	12.681	0	1610.24	8	2.2042	33.217	-2.5615	-0.76713
45	34	1	S	26	59.195	72.512	14.466	0	5065.94	9	0.82804	-11.211	-4.3561	-1.1784
46	35	1	S	40	60.92	87.086	14.049	0	7706.94	9	1.5209	-12.936	-18.93	-0.76123
47	36	1	S	14	102.79	92.715	13.935	0	2636.93	2	1.7649	-8.1495	6.2257	-8.1499
48	37	1	S	7	144.58	163.71	14	0	1316.35	8	3.0245	27.868	-35.824	-2.0866
49	38	1	S	6	217.81	172.84	14	0	1130.53	8	4.2358	-45.366	-44.955	-2.0866
50	39	1	S	11	32.902	78.999	15	0	1983.05	9	1.273	15.083	-10.844	-1.7127
51	40	1	S	40	77.396	94.902	15.224	0	8238.29	2	2.2204	17.244	4.0386	-9.4394

If you select columns instead of rows in the table the tooltip will display the corresponding parameter values for the same object. It is possible to highlight columns and rows at the same time but this is confusing to interpret, and thus not recommended.

### The Status Bar and Tooltip (23)

The bottom part of the window is a status bar that also shows an contextual tooltip.

Ready (rendered)	Canvas size 678 x 577	Dragging on	38/S 1NP.CMCM: 4.2358 um - (34 S): 0.82804 - (36 S): 1.7649
------------------	-----------------------	-------------	---

The left side reports the current status of the renderer and analyzer. You can see whether a long computation is running or if the analyzer is ready for further interaction.

Then the current size of the canvas is reported. When you first start the OA, the canvas size is adapted to the exact room left for the scene rendering, but it can be larger or smaller at wish (OPTIONS→VIRTUAL RENDER SIZE).

The 'Dragging' status refers to the automatic rendering of the scene while the user interacts with the scene. In slower systems, the dragging is turned off automatically and the rendering only happens after the user released the mouse buttons or finished moving the segmentation sliders.

The tooltip (the region with light yellow background) shows contextual information:

- A more detailed description of the reported parameters when you hover over the table headers (See Figure 25.1, item 20) and a sum of the cell below them.
- The value in the cell under the cursor when you point at table rows, plus selected reference values.
- The different actions bound to the mouse buttons, when the cursor is inside the rendering canvas.





## *The Colocalization Analyzer*

The *Colocalization Analyzer* provides information about the amount of spatial overlap between structures in different data channels, for 3D images and time series.

This overlap can be defined in various ways; Huygens supports the *colocalization coefficients* most commonly used in literature: *(Object) Pearson*, *(Object) Spearman*, *Overlap*, *Intersection*, and *Manders M and K*. The SVI Wiki provides extensive information on these coefficients<sup>1,2</sup>.

The Colocalization Analyzer works in two steps: First, it searches for colocalizing regions in the complete image and calculates the coefficients values. Then in the second step it segments these regions and treats them as objects required for further visualization, and for the analysis of the object-based coefficients.

Note that the Object Analyzer module (See page 135) also provides a form of colocalization measurement which complements the functionality of the Colocalization Analyzer. Whereas the Colocalization Analyzer takes the whole image into account, the Object Analyzer works at the object level; it first segments the image into objects and then determines if these objects colocalize.

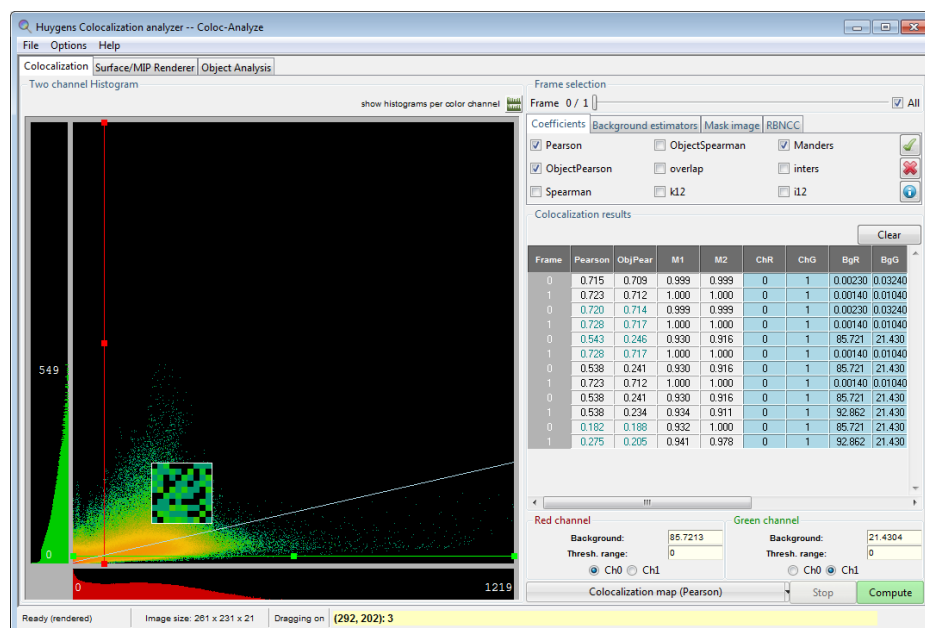
The *Colocalization Analyzer* is an optional module, and is enabled by the C flag in the license string (See “License String Details” on page 202.).

---

### *How to use the Colocalization Analyzer*

To *start* the Colocalization Analyzer in Huygens Professional, right-click an image thumbnail to open the contextual menu, then select ANALYZE → COLOCALIZATION ANALYZER. Alternatively, select a thumbnail and select in the menu bar ANALYSIS → COLOCALIZATION ANALYZER. The image must be a multi-channel image (See “Multi-channel Images” on page 41.) as colocalization is based on the overlap of different channel intensities. Wait for the analyzer to initialize and to compute the 2D histogram with the default settings.

- 
1. <http://www.svi.nl/ColocalizationTheory>
  2. <http://www.svi.nl/ColocalizationCoefficients>



**FIGURE 26.1.** The first tab of the Colocalization Analyzer showing the colocalization coefficients and histograms.

When the colocalization analyzer is opened you will see three main tabs (See Figure 26.1), of which the first is visible. In this tab the colocalization coefficients can be calculated. You can choose the time frame, select colocalization coefficients, set the background settings (See “Background Estimators” on page 172.), select a mask image (See “Selecting a mask image” on page 174.), select a reference image for RBNCC (See “RBNCC” on page 174.) and choose a colocalization map (See “The colocalization map” on page 169.). On startup, all these settings are set to the default values, so you can immediately press COMPUTE.

First we select the data to analyze. For time series, the *Frame* slider selects the time coordinate. In the lower part of the window we can select which data channels from our image we want to use. For the channels we follow the usual naming convention in colocalization theory: *Red* (R) for the first channel, *Green* (G) for the second channel.

Whenever we change the Red or the Green channel selection a two-dimensional *histogram* is calculated. This histogram provides an rough indication of the degree of overlap between the selected channels; for two channels with a high degree of overlap, the histogram pixels concentrates along the diagonal  $y = x$  line. In contrast, total absence of overlap would produce a 2D histogram with values only on the coordinate axes. The histogram can give an indication of some image defects such as clipping, hot pixels, and dynamic range differences. For more information on the 2D histogram see the SVI Wiki<sup>3</sup>.

Along the x and y line of the 2D histogram, there is a separate 1D histogram for each color channel. Clicking the SHOW HISTOGRAM PER COLOR CHANNEL button near the top-right corner of the histogram shows enlarged versions of these histograms.

3. <http://www.svi.nl/TwoChannelHistogram>

By moving the colored *background* lines in either the 2D-histogram, the enlarged 1D-histograms, or by changing the numeric values in the input fields, These values are subtracted from the voxels intensities when calculating the coefficients (if the result is negative, it is understood as a zero). Generally the colocalization coefficients depend much on correct estimation of the image background and resolution. For these reasons we strongly recommend to compute colocalization coefficients only on deconvolved images.

One sets a background value prior to the calculation to remove signal that would otherwise lead to spurious colocalization. This is intended for minor tuning, or just in case it is really needed to calculate colocalization in raw images that still have all the measured background. See “Backgrounds vs. thresholds in colocalization” on page 171..

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### *The colocalization map*

Next we select what *colocalization map* we want to calculate: *(Object) Pearson, (Object) Spearman, Overlap, Manders M or K or Intersection*. You can find the selector at the bottom of tab 1 next to the COMPUTE button.

Notice the difference between *maps* and *coefficients*: the *colocalization coefficients* parametrize the degree of colocalization of the full image, while a *colocalization map* parametrizes the colocalization locally. In a map, a single colocalization value is calculated per voxel creating a 3D distribution that is represented in a 3D image by iso-surfaces. In other words, the colocalization map shows the contribution of each voxel to the colocalization coefficient.

The Colocalization Analyzer computes only the map selected by the user, but it always computes the selected coefficients. When the COMPUTE button is pressed, the pane *colocalization coefficients* will show the selected colocalization coefficients for the selected time frame. If you have checked the COMPUTE ALL option next to the time frame slider, the coefficients will be calculated for all time frames sequentially.

Your choice of the colocalization map is needed for the functionality in the second tab (See Figure 26.2). In this tab an iso-colocalization surface is generated based on the colocalization map. This iso-surface rendering is only possible if a colocalization map exists. So, at startup the iso-colocalization surface sliders are deactivated, as we have to calculate a colocalization map first, by pressing COMPUTE.

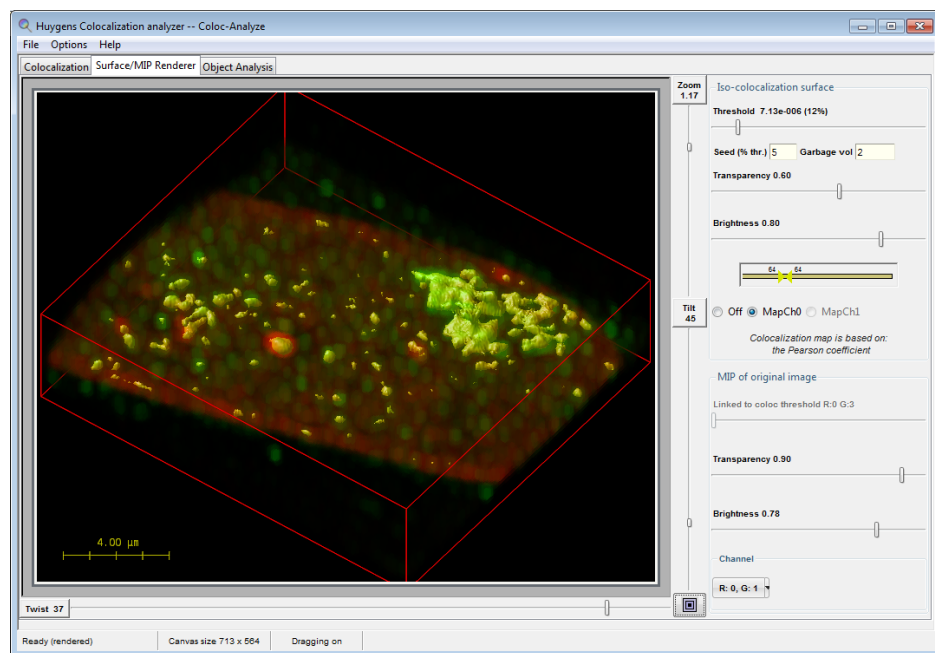
Together with a surface renderer a MIP renderer is available. The viewpoint of the MIP and surface can be selected by moving the Tilt and Twist slide. Also try changing the zoom.

The obtained colocalization map is represented in the renderer window by iso-colocalization surfaces. These iso-surfaces represents points which all have the same colocalization value, thus regions in which the degree of colocalization exceeds a certain value become objects. This “certain value” can be controlled by the *threshold* slider in the iso-colocalization surface parameters. The transparency and the brightness of this surface pipe can be controlled with the correspondent sliders. The color range in which the objects are displayed can be modified using a hue selector (page 196). One can also switch the surface pipe off.

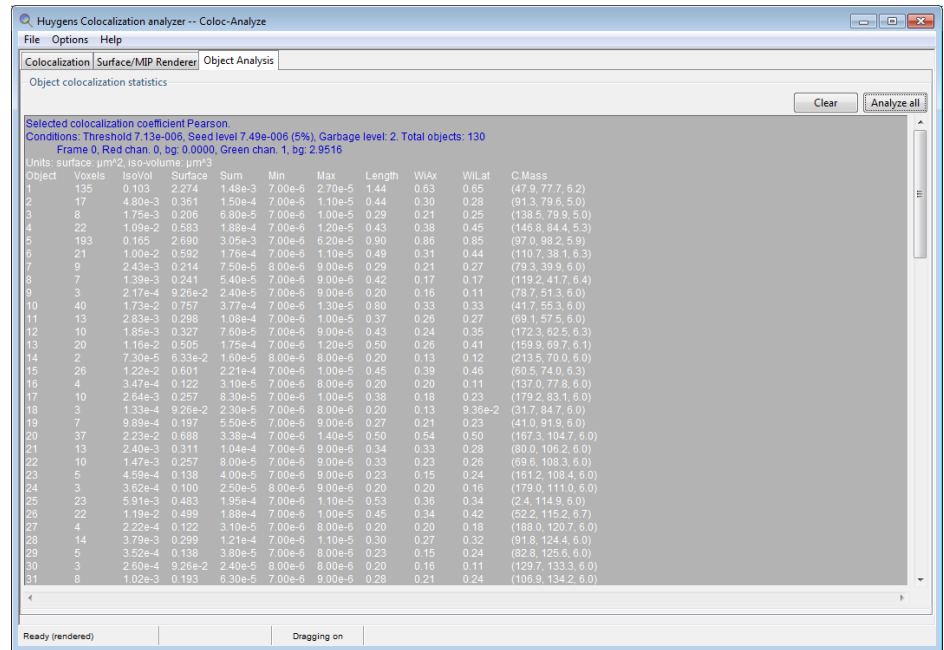
Some modes generate two-channel *colocalization maps*: colocalization of red with respect to green, and vice versa, e.g. in case of the *Manders M1* and *M2* coefficients. In these cases, the iso-colocalization surface parameters will offer the possibility of rendering any of the two channels, and thus the threshold is referred to the active one.

By clicking on the rendered objects local colocalization parameters are computed and reported, which will be discussed in more detail in “Iso-colocalization object analysis” below.

There is one *Maximum Intensity Projection* (MIP) pipe available to redirect the data channels to. The *MIP rendering* of one channel (maybe one different from those used for colocalization) or the two channels under analysis can be a good *spatial reference* for the objects from the colocalization map. When an original channel is selected, the *threshold*



**FIGURE 26.2.** Tab two of the Colocalization Analyzer shows surfaces for regions of overlapping channels.



**FIGURE 26.3.** Tab three of the Colocalization Analyzer if for object analysis for the localized regions of the colocalization map.

*slider* can be used to select what voxels are considered for the MIP rendering, depending on their intensities. Notice that here the threshold is simply used for *representation*. If both R and G channels are selected to be rendered, their correspondent backgrounds as selected in the histogram will be used as projection thresholds. As with the surface pipes, the *transparency* and the *brightness* of this MIP can also be controlled.

All the obtained information can be saved to external data (text or image) files through the FILE menu.

## Iso-colocalization object analysis

One of the features of the colocalization analyzer is iso-colocalization *object analysis* (Figure 26.3 on page 171). It allows to determine quickly the properties of the different colocalization regions in the data. This is realized by visualizing the colocalization map as iso-colocalization surfaces. In this way regions in which the degree of colocalization exceeds a certain value become objects. By clicking on the objects local colocalization parameters are computed and reported. To relate the iso-colocalization objects to the original data the surface objects can be blended with a MIP projection of the data (See “The MIP Renderer” on page 75.). The color range in which these objects are displayed can be modified using a hue selector (page 196).

## Backgrounds vs. thresholds in colocalization

*Backgrounds* are for removing signal prior to the calculation. In an ideally restored image that would not be necessary, because all the signal present in the image gives a valid representation of it. The background would have been removed during the image restoration.

*Thresholds* are used to split the colocalization maps in two regions: what are interesting regions, and what are not. Local colocalization values are calculated for every image

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voxel, but the zero value would be very rarely achieved. In most of the cases there are some non-zero colocalization everywhere, the interest probably is in regions where colocalization exceeds typically a value of 20 % of the maximum.

With the background settings, voxels are in- or excluded based on their voxel intensities: intensities higher than the background are kept, while lower intensities are disregarded for the calculation. Note, the intensity value of voxels do not relate to the level of colocalization. In fact, it may well be that high colocalization levels occur in regions with low voxel intensities.

Therefore, background settings should not be used to remove signal that can still have some colocalization level. Ideally work with deconvolved images and consider all the signal, then study the colocalization levels using thresholds to split regions of high colocalization from regions with low colocalization, something that can not be known beforehand! Setting the background is used for removing really constant background signals.

Thresholds do not affect colocalization, but only the way colocalization maps are represented on the screen and regions of interests (visualized as surfaces) are defined. Depending on the locally calculated colocalization surfaces may merge or split. What affects colocalization, as explained in the colocalization theory at the SVI Wiki, are the backgrounds. In the computation of *Manders* coefficients the background values act like in this example: to the computation of *MI* only pixels in *R* contribute when their corresponding pixel in *G* is above the background.

More information can be found at the SVI Wiki<sup>4</sup>.

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## Background Estimators

The Colocalization Analyzer consists of a number of background estimators. They are meant to help to determine the correct background setting to compute the colocalization coefficient. As is explained in the previous section “Backgrounds vs. thresholds in colocalization” the background setting affects the colocalization analysis.

The background estimators are:

- Gaussian minimum
- Costes method
- Optimized search

### Gaussian minimum

The gaussian minimum is the simplest background estimation method. The background setting is the minimum intensity value after applying a gaussian filter on the image. Before the extension of the Colocalization Analyzer with the background estimations, this method was used as an initial setting. This method is fast, but maybe too simple. In case of image with not too much noise, this method is good enough.

### Costes Method

The Costes method is based on the estimation explained in article “*Automatic and Quantitative Measurement of Protein-Protein Colocalization in Live Cells*” by Sylvain V. Costes,

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4. <http://www.svi.nl/ColocalizationAnalyzer>

Dirk Daelemans, Edward H. Cho, Zachary Dobbin, George Pavlakis and Stephan Lockett, *Biophysical Journal*, volume 86, June 2004 (3993-4003).

They explain an automatic background searching algorithm within a 2D histogram over the linear regression line. This regression line is calculated with the least squares line fit algorithm. Each point on the regression line is a combination of background values for the red and green channel. Starting with the highest intensity value for red, the backgrounds are decreased, in such a way that the points lie on the regression line, i.e. points  $(r, ar + b)$  for a decreasing  $r$  with  $a$  the slope and  $b$  the intercept of the regression line. This process stops when a point is reached for which the Pearson's coefficient of the area *below* the background settings is zero (or closest to zero).

Because this method searches the regression line from high to low, it may take some time to reach the desired point.

### **Optimized search**

This method is an extension of the Costes method, but where the assumption that the ideal background combination lies on the regression line is dropped. This method uses an iterative process to search the whole 2D histogram to find the point where the Pearson coefficient of the area *below* the background settings is zero.

This method is more precise, but could also take more time to reach the desired point. This iterative process however uses a smart searching algorithm, for which is known to quickly convert to a solution.

For both the Costes and the Optimized search it is assumed that the noise in the image is poisson distributed, because the Pearson coefficient of two poisson distributions is zero.

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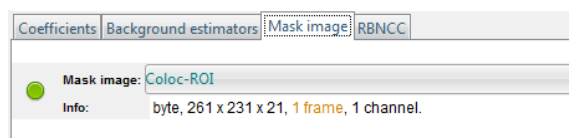
## *Background Options*

Besides the background estimators, there are some additional options to set the background settings, especially for different time frames.

- *Stop*: This button is disabled by default, but when an estimation method is started, the costes or the optimized, the process can be stopped by pressing this button. This will *not cancel* the process and it will return the background settings, which is found before you pressed Stop.
- *Delete*: This will delete all the background settings for all time frames at once and sets them at the minimum values of the time frame.
- *Clone*: In case the background is correctly set for a time frame, this will copy the *exact* background settings to the other time frames.
- *Reset*: This will reset any manually changed background of the current time frame, back to the estimated background setting of the selected method.
- *Recalc*: To redo the estimation process, which could be useful for the costes and the optimized method.

## Selecting a mask image

A mask image can be selected to calculate the colocalization in a region of interest (ROI). The mask option can be enabled by selecting the *Mask* tab and pressing on the red button on the right. It will change to green to denote it is enabled. When the mask option is enabled, it is required to select an image that meets the dimensions of the original source image. When you hover over and click on the image name, a different image can be selected. The mask image should already be opened in the main window of Huygens Professional.



**FIGURE 26.4.** The mask option enabled with information about the mask image parameters.

The mask image may only contain 1 channel. Whether the mask image meets the criteria is reported at *Info*. You can hover over the highlighted image parameters to read the tooltip in the statusbar to see what is wrong.

Once all the criteria are met, simply press the *COMPUTE* button to only calculate the colocalization in the area denoted in the mask image. The mask image used for the analysis is also reported in the table.

## RBNCC

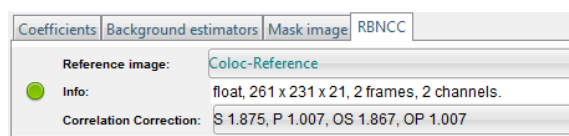
The colocalization analyzer is extended with Replicate-Based Noise Corrected Correlation (RBNCC). Noise causes difficulties around colocalization analysis, since noise also introduces colocalization. This can be handled by using a background threshold, which is explained in the previous subsections, but when the noise is quite severe a threshold may not be sufficient.

With the RBNCC algorithm one can correct for noise that contributes to the underestimation of the colocalization coefficients, Pearson and Spearman coefficients. The RBNCC method has been developed by J. Adler, S.N. Pagakis and I. Parmryd from Uppsala University; *Journal of Microscopy* 230, 121-133; 2008. More information can be found at the SVI Wiki<sup>5</sup>.

Colocalization coefficients are calculated and directly corrected with RBNCC when this option is activated. To use the RBNCC method a license with the RBNCC option is required.

When RBNCC is activated in the license, then within the *RBNCC* tab a button shows red when the RBNCC is disabled and green when it is enabled. If the button shows grey, the license is insufficient to work with RBNCC.

With RBNCC enabled, a reference image can be selected by hovering over and clicking on the visible image name, see Figure 26.5. The reference image should already be opened in the main window of Huygens Professional and should have the same dimensions as the original. Whether the reference image meets the



**FIGURE 26.5.** The RBNCC option is enabled and a reference image can be selected.

5. <http://www.svi.nl/RBNCC>



criteria is shown at Info and hovering over any highlighted parameters shows a tooltip in the statusbar what is wrong. When the reference image is selected, simply press COMPUTE and the coefficients will be corrected automatically.

The correction factors applied to the coefficient are shown in the *RBNCC* frame, where the abbreviations stand for a specific coefficient:

- S: Spearman coefficient
- P: Pearson coefficient
- OS: Object Spearman coefficient
- OP: Object Pearson coefficient



# *The Performance Tune Wizard*

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## *Why performance tuning*

Huygens uses multi-threading to speedup the image processing algorithms. Normally, Huygens uses the maximum amount of cores as specified by the license (type of server option), and available in a system as the default number of threads. This means that, under normal circumstances, all the allowed number of cores will be used simultaneously. During processing, Huygens competes for CPU time with all other programs in the system. Since the threads of Huygens perform the image processing in close collaboration, there will be a severe penalty if one or more of the threads can't run on the CPU at a given time because other software (e.g., an office suite, web browser, virus scanner) is already running on CPU. In such a case, it is more beneficial to let Huygens not use all cores so that interference with other software is reduced. This is just one of the reasons why it might be more beneficial for Huygens performance not use all the cores. There are several others arguments for this, but they lie beyond the scope of this manual.

The Performance tune wizard will execute several deconvolution runs in order to determine the optimal number of threads of your system that should be used by Huygens.

### **Workflow**

The performance tune wizard consists of several stages:

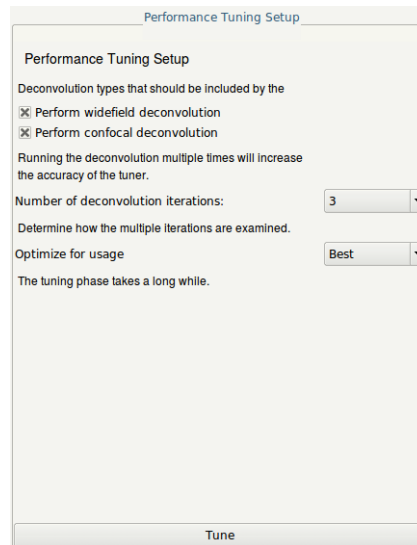
- Welcome screen
- Setup and tune
- Results

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## *Welcome screen*

A brief overview of the *Performance Tune Wizard* is given. Note that the results of the *Performance Tune Wizard* will be send to SVI, unless you disable this on this screen. We use this information to better understand how our software runs on different kinds of machines, which helps us to determine where we might need to optimize the software even more. Moreover, it will help us to provide better information to our customers on the optimal system requirements for Huygens.

In this stage, the configuration for the tuning can be given (See Figure 27.1).



**FIGURE 27.1. Configuration window for adjusting the tuning settings.**

### **Deconvolution types**

Depending on your license it is possible to run a widefield and or confocal deconvolution. The results of the two types of deconvolution runs with regard to the optimal number of threads are equal most of the times, so it is safe to select both the types even if you typically run only one the types. In order to speedup the performance tune wizard you could deselect the widefield deconvolution since it is significantly slower then the confocal deconvolution.

### **Number of deconvolution iterations**

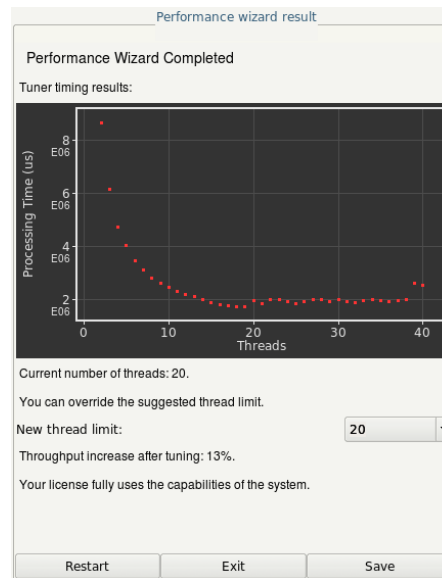
The deconvolution runs are executed several times in order to increase the accuracy of the result. This is not the same as the iteration count for the deconvolution itself.

### **Optimize for usage**

If the deconvolution is run several times this option determines how the results of those different runs are used. When used with the 'best' option the fastest run is used, and when used with the 'typical' option the average of the runs is used. Use 'best' when you would normally not use the system during a normal deconvolution run, and use 'typical' when the system remains in use when performing a deconvolution run. The result of using 'best' makes it more likely that a larger number of threads is used, which increases the chance of performance interference if other software is running simultaneously.

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## Performance Results



**FIGURE 27.2.** Graph in which the processing time is displayed against the thread count of a hyper-threaded system with twenty physical cores.

In the results stage a graph of the processing time versus thread count is shown (See Figure 27.2). This specific graph indicates that the processing time decreases significantly when more cores are used. You can also see that the processing time decreases until 18 threads are used. After that the processing time slightly increases and remains roughly the same. In this example, the system is hyper-threaded and has 20 physical cores. We can see that the hyper-threads actually decrease the performance and that, if we only use 18 of the 20 physical cores the interference with other software is reduced. Hence, the wizard suggests that we should use 18 threads. You can override this setting if you think that the wizard made a measurement error, and if you prefer to configure -for example - 20 cores.

The wizard also estimates the expected performance increase (measured in throughput), when compared with the currently configured amount of threads. In this case the system was already configured for 20 threads and we only see a performance increase of 13%. But when compared with the default setting of 40 threads, we can see a significant performance increase of more than 50%.

Furthermore, the wizard determines if your license is able to use all the cores in the system and will detect if you can increase the performance by using a larger server option in the license. You can contact [sales@svi.nl](mailto:sales@svi.nl) for more information.

Lastly, the results of the wizard need to be saved or discarded with the SAVE or EXIT button, respectively. It is also possible to restart the *Performance Tune Wizard* if you suspect that other software interfered too much during the tuning phase.



## *Establishing Image Parameters*

The deconvolution algorithm needs to know some of the parameters describing the image acquisition. These are not too many, but careful determination may significantly enhance the deconvolution results.

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### *Image Size*

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. Regarding widefield images we recommend to not record planes below and above the object which only contain blur. Huygens Professional does not need these planes to restore the 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. In any case, never crop the objects of interest. As a rule of thumb, leave about *one extra*  $\mu\text{m}$  above and below the objects.

### **Brick-Wise Processing**

Deconvolving images requires much computer memory than the image size, 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 Professional will *split the 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 Professional. To find out the best number of bricks, let the software run in automatic mode for splitting. It will consider many options and go for the most optimal one.

More information can be found on the SVI Wiki<sup>1</sup>.

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### *Signal to Noise Ratio*

The Signal to Noise Ratio (referred to as SNR or S/N) is in the Huygens Software used as a regularization parameter, i.e. as a parameter that controls the *sharpness* of the restoration result. The higher this value, the sharper the restored image will be. Therefore it

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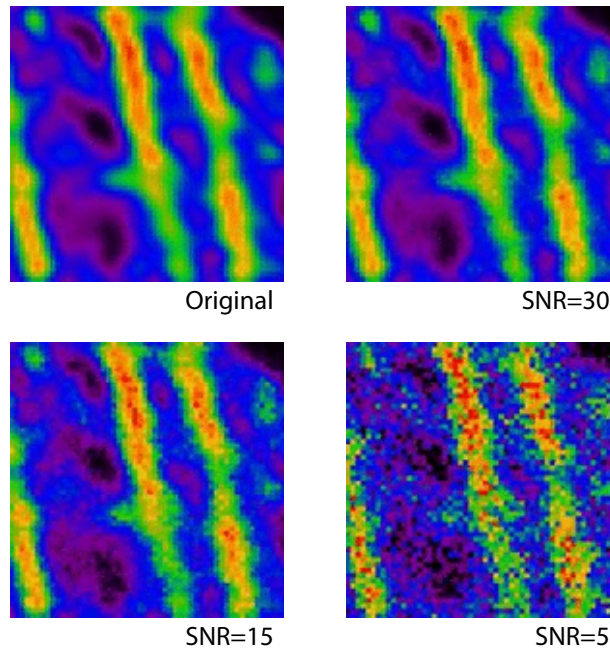
1. <http://www.svi.nl/BrickSplitting>

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should not be considered as a parameter describing the original image, but more as a tunable parameter that controls the deconvolved result.

Using a too large SNR value might be risky when restoring noisy originals, because the noise could just be enhanced. A noise-free widefield image usually has SNR values higher than 50. A noisy confocal image can have values lower than 20.

A good starting SNR value can be estimated simply by visual inspection from the quality of the image. Figure 28.1 shows some examples of recordings where different noise levels



**FIGURE 28.1.** Examples of different SNR values. Same image, different noise levels.

were added to an original (restored) image.

Estimating the SNR in noisy images is fairly easy. It is based on the idea of establishing the voxel intensity value  $s$  corresponding to a single photon hit by looking for such an event in a dark areas of the image. Knowing the intensity value  $M$  of the brightest voxel in the image, one can now calculate how many photons are involved in it. The SNR is now defined as:

$$SNR = \sqrt{\frac{M}{s}} \quad (\text{EQ 1})$$

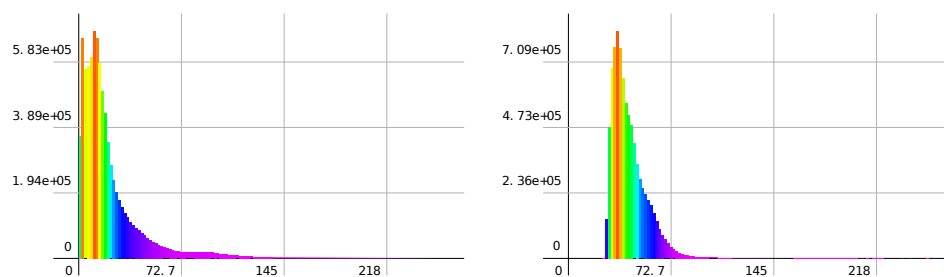
If a significant blacklevel is present, it should be subtracted from  $M$  and  $s$ . See “Black Level” on page 183 and the SVI Wiki<sup>2</sup> for more details.

In low noise images this is much more difficult, as single photon events are no longer easily observed. Fortunately, in such cases the establishment of the precise SNR is not very important for the restoration method, and a rough estimation based on the appearance of the image is usually enough (See Figure 28.1).

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2. <http://www.svi.nl/SNR>





**FIGURE 28.2.** Left: The histogram of an image without black level. Right: The histogram of a similar image with a significant black level.

## Black Level

The blacklevel (also called *base line* or *electronic shift*) is the output of the photo-multiplier if no light is coming through.

A positive black level (See Figure 28.2) will do no harm to the deconvolution since it is automatically accounted for in the background estimation stage. A large black level value, however, will reduce the effective dynamic range of the microscope. Besides that, a large black level will prevent the bleaching decay analyzer to do its job correctly, and the bleaching correction may turn out to be poor or even impossible. To prevent this Huygens Professional has a tool to adjust the base line of an image, and the Batch Processor templates show the option to remove the black level as a preprocessing step.

It is also possible that the black level is *negative*. In the image histogram this will show as a spike on the left. This causes clipping (See “Clipping” on page 190.) in the lower intensity limit, and it is impossible to correct: clipped images should be reacquired.

More information can be found on the SVI support Wiki<sup>3</sup>.

## Sampling Density

The sampling density is the number of recorded *samples per unit area volume (3D) or area (2D)*. It is a microscopic parameter that describes the conditions of the image acquisition, established by the way the microscope is configured (usually by the zoom factor). The ideal sampling density depends on the system optics and is determined by the *Nyquist rate*. It is recommended to sample as close to the Nyquist rate as possible. Note that the *actual* sampling distances from the acquisition must be used in the deconvolution.

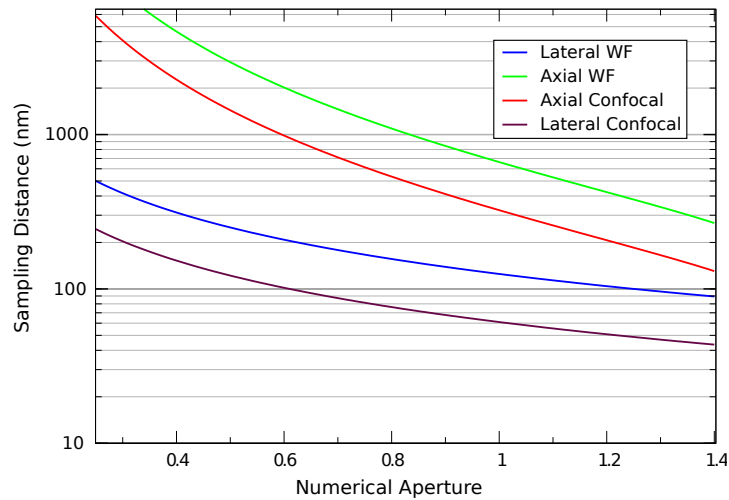
The SVI Wiki has an on-line tool<sup>4</sup> that computes the Nyquist rate for any widefield, confocal, spinning disc, and 4-Pi microscope.

Sampling according to the Nyquist rate makes sure that *all* information generated by the optics of the microscope is captured in digital form. It can be shown that if the sampling distance is smaller than the so-called *critical sampling distance*, no new information about the object is captured. 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, on the contrary.

3. <http://www.svi.nl/BlackLevel>

4. <http://www.svi.nl/NyquistCalculator>

Figure 28.3 shows the dependency of this critical sampling distance on the numerical



**FIGURE 28.3. Critical sampling distance vs. NA. The curves above show the critical sampling distance in axial and lateral directions for wide-field and confocal microscopes. The emission wavelength in both cases is 500 nm.**

aperture for a wavelength of 500 nm. To apply this plot of to another wavelength, simply scale the vertical axis by that wavelength. For example, if a widefield microscope with NA 1.3 is used, there can be noticed from the plot that the critical lateral Nyquist sampling distance at 500 nm emission is 95 nm. For an emission wavelength of 570 nm, this becomes  $(500/570) \times 95 = 83$  nm .

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 widefield system and the sampling distance can be set accordingly. We do not recommend to increase the axial sampling distance appreciably beyond the critical distance.

In a multi photon excitation microscope, it is the excitation wavelength divided by the photon count which determines the sampling.

More information can be found on the SVI Wiki<sup>5</sup>.

### Computing the Backprojected Pinhole Radius and Distance

Throughout Huygens Professional pinhole sizes of confocal systems are specified as the *backprojected radius* in nm. *Backprojected* means the size of the pinhole *as it appears in the specimen plane*, i.e. the physical pinhole radius  $r_b$  divided by the total magnification of the detection system. This total magnification is the product of the (variable) objective magnification  $m_{obj}$  multiplied by a fixed internal magnification  $m_{sys}$  :

5. <http://www.svi.nl/NyquistRate>

$$r_b = \frac{r_{phy}}{m_{obj}m_{sys}} \tag{EQ 2}$$

The SVI Wiki has a calculator<sup>6</sup> to automatically compute the backprojected pinhole radius for specific microscope models. Note that the Bio-Rad MRC500, 600, and 1024 microscopes have a very high magnification in the detection system (See “Checking the Bio-Rad System Magnification” on page 186.).

The equations that can be found in the next pages are intended to orientate the user in finding out the backprojected value for different types of microscopes, but the idea is always the same: given a diameter  $d$  of the real pinhole, we might need to multiply it by a factor for unit conversion (to obtain the radius in nm), and divide the result by some other factor that takes account of the magnification of the microscope. These include both the objective and the intrinsic system magnification. In some microscopes with pinholes that are not circular, a geometrical correction will also be needed.

### The Airy Disk as Unit for The Backprojected Pinhole

Some confocal microscopes report their pinhole size with the *diameter of the Airy disk*<sup>7</sup> as unit. shows how to compute the backprojected pinhole radius:

$$r_b = \frac{0.61\lambda_{ex}N_{Ad}}{NA} \tag{EQ 3}$$

with  $NA$  the numerical aperture of the lens,  $N_{Ad}$  the number of Airy disks, and  $\lambda_{ex}$  the excitation wavelength. In principle using  $\lambda_{ex}$  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.

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

### Square Pinholes and the Shape Factor

The shape correction from a square to a circular pinhole is based on equalizing the area for both pinholes.

Because the area  $A = d^2 = \pi r^2$  (See Figure 28.4), the shape factor  $c$  for a square pinhole becomes

$1/(\sqrt{\pi}) = 0.564$ . For circular pinholes  $c = 0.5$  to just convert the diameter to a radius.

If the size of a square pinhole is given in Airy disk units, then the backprojected radius is a combination of Equation 3 and the shape factor  $c$  for a square pinhole. Taking into account that Equation 3 already converts a diameter into a radius, the relation becomes:

$$r_b = \frac{0.69\lambda_{ex}N_{Ad}}{NA} \tag{EQ 4}$$

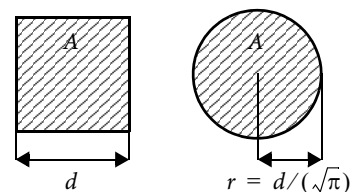


FIGURE 28.4. A square and a disk with an equal area  $A$ .

6. <http://www.svi.nl/BackprojectedPinholeCalculator>  
 7. <http://www.svi.nl/AiryDisk>

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## Converting from an Integer Parameter

Unfortunately, quite a few microscopes do not report the physical pinhole size or the Airy disk size. Instead, often an integer size parameter  $p_8$  in the range [0,255] (8 bit) is specified. If  $p_8$  maps to a physical size in linear fashion, then Equation 5 can be used to translate that parameter into a backprojected pinhole radius.

$$r_b = 10^3 c \frac{(p_8/255)(s_{max} - s_{min}) + s_{min}}{m_{obj}m_{sys}} \quad \text{(EQ 5)}$$

Here,  $c$  is the shape factor and  $s_{min}$  and  $s_{max}$  are the minimal and maximal pinhole size in  $\mu\text{m}$ . The factor  $10^3$  converts from  $\mu\text{m}$  to nm.

## The backprojected Pinhole Spacing for 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 frequently used Yokogawa disks<sup>8</sup>, for example, the physical spacing is about 253  $\mu\text{m}$ . This can be checked by imaging a stopped disk. Using a 100 $\times$  lens for example, the backprojected distance is about 2.53  $\mu\text{m}$ . If an extra zoom lens is placed between the disk and the sample, its magnification must be also considered.

## A Supplied Calibration Curve

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

## An Example: the Olympus FV1000

As reported by Olympus engineers, the FV1000 confocal microscope has a square pinhole and an internal magnification of 3.82 $\times$ . The pinhole size reported in the Olympus software is the side length of this square pinhole. Therefore,  $c = 1/(\sqrt{\pi}) = 0.564$ , and  $m_{obj} = 3.82$ . Using a 100 $\times$  objective lens and a reported pinhole size  $d$  of 150  $\mu\text{m}$  the backprojected pinhole radius  $r_b$  in nm is:

$$r_b = c \frac{d}{m_{obj}m_{sys}} = 0.564 \frac{150 \cdot 10^3}{100 \cdot 3.82} = 221 \quad \text{(EQ 6)}$$

The on-line calculator<sup>9</sup> knows the system magnifications and pinhole shapes for most popular microscopes.

## Checking the Bio-Rad System Magnification

The Bio-Rad MRC 500, 600, and 1024 microscopes have a very high magnification in the detection system. The fixed system magnification is, according to Pawley<sup>10</sup>,

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8. <http://www.svi.nl/YokogawaDisk>

9. <http://www.svi.nl/BackprojectedPinholeCalculator>

10. Pawley, J. B., *Handbook of Biological Confocal Microscopy*, 2<sup>nd</sup> edition, 1995. Plenum Press, New York and London. ISBN 0-306-448262. Page 30.

$53 \times m_{tube}$ , with  $m_{tube}$  between 1.0 and 1.56 (factor 1.25 for the fluorescence attachment multiplied by factor 1.25 for the DIC attachment). The factor of 53 includes the 8× eyepiece just below the scan head, but does not 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 the viewing of the diffraction pattern (Airy disk) at the pinhole plane directly by eye. To enable the correctness verification of the values for the system magnification used in the on-line calculator, the way the system magnification was derived is now explained.

In a Bio-Rad MRC600 with a NA 1.3 60× objective, the Airy disk has a diameter of around 2 to 2.5 mm at the pinhole plane. The diameter of the first Airy zero ring is 7.6 lateral optical units<sup>11</sup> (o.u.), using Equation 7 to express a distance  $r$  in dimensionless o.u.:

$$v = r \frac{2\pi}{\lambda} NA \quad \text{(EQ 7)}$$

In the system described here, an o.u. is  $0.3 \pm 0.033$  mm. At the specimen plane (backprojected) a lateral o.u. is in this case around 61 nm. The total magnification is in that case 4918×, the system magnification  $4918/60 = 82 \pm 9 \times$ . This value corresponds well with the largest possible system magnification for the MRC600 ( $53 \times 1.25 \times 1.25 = 83$ ).

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11. <http://www.svi.nl/OpticalUnits>



# Improving Image Quality

In this chapter discusses basic suggestions on how to acquire better microscope images. These are based on common problems that we find frequently in data provided by users. The recommendations go on the direction of obtaining the highest quality images from the microscope, from the point of view of acquiring as much information as clean as possible. This alone is worth the effort, but it will also be very valuable for the deconvolution afterwards.

Some basic guidelines to improve the deconvolution results are also listed. More information can be found in the SVI Wiki<sup>1</sup>.

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## Data Acquisition Pitfalls

### Refractive Index Mismatch

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

- **Geometrical distortion:** Frequently referred to as *the fish tank effect*<sup>2</sup>. The axial sampling distance that is recorded in the image file is the step size by which the objective lens moves along the  $z$ -axis. The focal point inside the sample, however, shifts due to the fish tank effect by a different step size. Therefore objects will appear elongated or shortened in the image data.

Huygens Professional will automatically adapt the PSF to this situation, but it will *not* modify the image geometry. *After deconvolution* the geometric distortion can be corrected by multiplying the  $z$ -sampling distance by the medium refractive index divided by the immersion refractive index.

- **Spherical aberration:** this phenomenon causes the oblique rays to be focused in a different location than the central rays<sup>3</sup>. 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

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1. <http://www.svi.nl/>

2. <http://www.svi.nl/FishtankEffect>

3. <http://www.svi.nl/SphericalAbberation>

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of already a few microns. This is especially harmful for the deconvolution of wide-field images.

A *workaround* for this problem is to keep the *z*-range of the data (the number of slices) as small as possible. The *solution* is to use a water immersion lens instead.

- **Total internal reflection:** When the *numerical aperture of the objective lens is larger than the medium refractive index*, total internal reflection<sup>4</sup> will occur. This is causing excitation light at high angles to be bounced back into the lens and therefore limiting the effective NA.

If spherical aberration is unavoidable, the image can still be improved during restoration using an adaptive point spread function (See “Refractive Index Mismatch” on page 189.).

### Clipping

The intensity of the light emitted by the microscopic sample is converted to electrical signals that pass an adjustable amplifier. These electrical signals must be converted to numbers processed by the computer. This conversion is done by the CCD camera. Most scientific CCD cameras have a 12 bit converter limiting the output numbers to the range [0,4095] . Negative input signals are usually converted to 0 while positive input values exceeding the *dynamic range*<sup>5</sup> are all converted to 4095. This phenomenon is called *clipping*: information in the clipped samples is lost.

In practice: be suspicious if the data contains intensity values at the extremes of the numerical range. These ranges are [0,255] for 8 bit data, [0,4095] for 12 bit data, and [0,65535] for 16 bit data.

Read more on the SVI Wiki<sup>6</sup>.

### Undersampling

One of the rules of measurement that is often overlooked is sampling according to the *Nyquist rate* (See “Sampling Density” on page 183). Especially the sampling distance along the optical axis is frequently too large; too few *xy* slices are imaged. This leaves as result a 3D stack in which there is hardly any relation between the adjacent slices.

It is important to know how the sampling conditions should be established in order to recover an image from the sampled values. How the objects should be sampled depends on the microscope type (widefield, confocal, etc.) and on the microscopic parameters used, like the numerical aperture and wavelength.

The SVI Wiki has an on-line tool<sup>7</sup> that computes the ideal (Nyquist) sampling distances for any widefield, confocal, spinning disc, and 4-Pi microscope.

### Do Not Undersample to Limit Photodamage

Sometimes undersampling is done to limit photodamage to live cells. However, if photodamage plays a role it is actually better to *limit the number of photons per sample* than

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4. <http://www.svi.nl/TotalInternalReflection>

5. <http://www.svi.nl/DynamicRange>

6. <http://www.svi.nl/ClippedImages>

7. <http://www.svi.nl/NyquistCalculator>



to limit the number of samples. Having less photons per sample means that the overall photon dose can remain largely constant; as a result bleaching does not need to get worse. Although this results in an apparently noisier image, there is actually *more information* in the data because sampling density is higher. Deconvolution with Huygens Professional removes the noise and may enhance the image quality dramatically. Of course there are limits, but a fair trade-off can be often found.

Concluding, it is better to record 10 separate noisy slices 100 nm apart than two slices 1000 nm apart and averaged 5 times in order to reduce noise.

### **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 widefield deconvolution bleaching is more of a problem. Fortunately, the bleaching in widefield images can usually be corrected quite easily. Huygens Professional 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 widefield systems are equipped with unstable arc lamps which can cause amongst others jitter. These are irregular deviations from the average intensity in time. Huygens Professional 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.
- The *z*-stage moves irregular or with sudden jumps. This deforms the data along the *z*-axis and is fatal for widefield and confocal deconvolution.
- The specimen moves. If in widefield data the object can clearly be seen moving when slicing along over a few  $\mu\text{m}$  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 widefield data. In time series the effect can be seen as a drift of the *z*-position, or even a periodic movement induced by e.g. an air-conditioning system switching on and off. The *z*-drift corrector is able to correct this in most situations though.

### **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 the lens is higher than the refractive index of the embedding medium. This will reduce the effective NA of the lens.

### **Acquire an Experimental PSF**

A *point spread function* or *PSF* is the image of a single point object<sup>8</sup>. The degree of spreading (blurring) in the image of this point object is a measure for the quality of an optical system. The imaging in a fluorescent microscope is completely described by its PSF. Although in many cases a theoretically calculated PSF very well matches the real one, ideal theoretical calculations can not predict actual misalignments or other problems inside the optical path. Therefore it is always recommendable to measure an experimental PSF and, if it is very different from the ideal one, use it for deconvolution instead of the theoretical one.

The experimental PSF can be measured by acquiring the image of a small bead. When the size of the bead is known, then the PSF Distiller can distill the real shape of the PSF from the recording (See Chapter 6 “The PSF Distiller” on page 43).

### **Spherical Aberration Correction**

When there is a refractive index mismatch, then the Huygens software automatically correct for spherical aberration<sup>9</sup> by adapting the theoretical PSF to the sample depth.

In case the image suffers from severe spherical aberration, it might be better to use a theoretical PSF with this depth-dependent correction than an experimental one.

### **Improve the Deconvolution Parameters**

Some deconvolution parameters, for example the SNR and the background level, can be fine-tuned to get the best out of the restoration process. See the SVI Wiki<sup>10</sup> for detailed steps in configuring the restoration process.

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8. <http://www.svi.nl/PointSpreadFunction>

9. <http://www.svi.nl/SphericalAberration>

10. <http://www.svi.nl/DeconvolutionProcedure>

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### *The Point Spread Function*

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

1. *Generating a theoretical PSF*: When a measured PSF is not available, Huygens Professional automatically uses a theoretical PSF. The PSF is computed from the microscopic parameters attached to the data. Because a theoretical PSF can be generated without any user intervention Huygens Professional does the calculation in the background without any notice.  
Images affected by spherical aberration (See “Refractive Index Mismatch” on page 189) are better restored using a theoretical depth-dependent PSF.
2. *Measuring a PSF*: By using the PSF Distiller a measured PSF can be derived from images of small fluorescent beads (See “The PSF Distiller” on page 43.). Measured PSF's improve deconvolution results and may also serve as a quality test for the microscope

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### *Quality Factor*

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

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

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## File Series

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

### Numbered Tiff Series

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

### Leica Numbering

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

```
im_tNN.tif
```

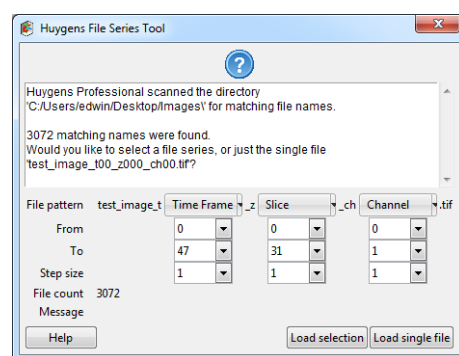
Here, NN is replaced by the time index for each frame. A more complex, multi-channel 3D time series has this pattern:

```
im_tNN_zNNN_cNN.tif
```

In this series, the second channel of the fourth slice of the third time frame has the file-name:

```
im_t02_z003_c01.tif
```

### The File Series Tool



**FIGURE 30.1.** The Huygens File Series Tool automatically scans a directory for a file series.

Although Huygens Professional uses Leica style numbering for writing files, the software attempts to detect *any type* of file series for reading. Whenever a file is opened that appears to be part of a file series, Huygens Professional shows the *File Series Tool* dialog (Figure 30.1). This tool enables the user to select a subset of a file series, and select a dimension for each the indices in the file name, so that each image is assigned to the correct *z*-plane, time frame, and channel. Only select the first file of a series. If you select more, Huygens will attempt to see every selected file as a start of a new series. Consequently, many windows of the File Series Tool will be opened.

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

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

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

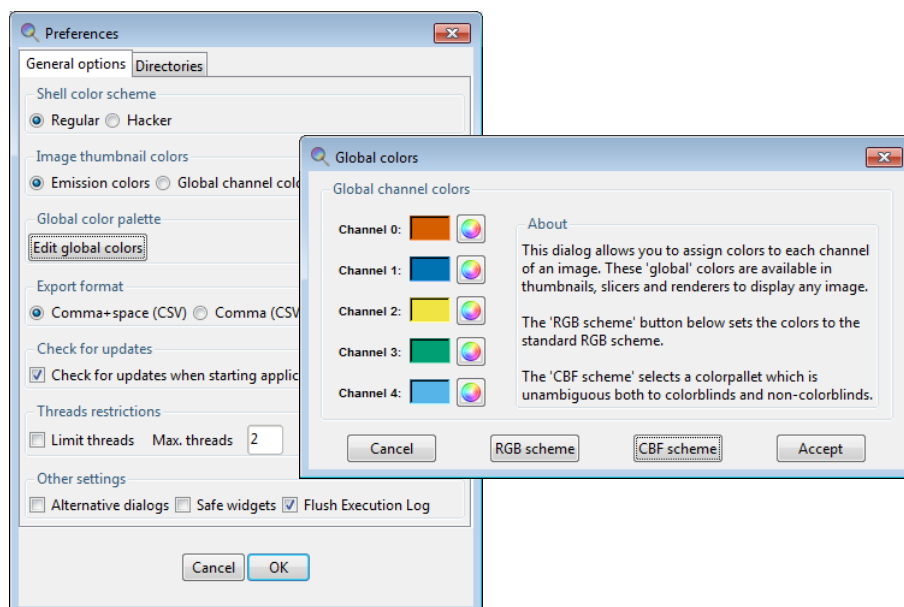
Note that the selection has to be unique, i.e. it is impossible to have ignored variables without having a *Slice*, *Time Frame*, or *Channel* counter.

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

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

## Adjusting the Global Color Scheme

Huygens Professional uses a global scheme for coloring the different channels in multi-channel images. These colors can be adjusted through the *Preferences* window via (See Figure 30.2).



**FIGURE 30.2.** The global color scheme can be modified through the *Preferences* window.

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

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

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## Hue Selector

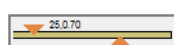
The *hue selector* is a component that allows adjustment of the color range in which objects are displayed (See “The Surface Renderer” on page 85 and “The Colocalization Analyzer” on page 167). Objects belonging to different channels can be represented in different hue ranges to make them clearly distinct. The gradual differences inside the selected range make independent objects distinguishable. Also a range can be collapsed to have all objects in a channel displayed with exactly the same color. In Huygens the *hue selector* does appear in two flavors.

### Hue Range



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

### Hue Range and Saturation



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

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## Image Statistics

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

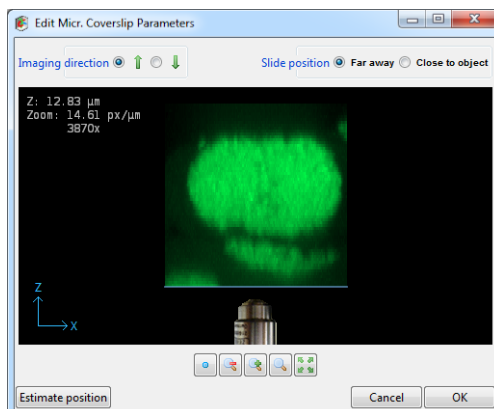
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## Setting the Coverslip Position

If there is a mismatch between the refractive index for which the microscope's objective is designed and the actual refractive index of the embedding medium, the shape of the point spread function (PSF) will be distorted due to spherical aberration (See “Refractive Index Mismatch” on page 189). As deeper layers in the specimen are imaged, moving away from the coverslip, this distortion will progressively worsen. To compute the level of spherical aberration at a specific depth within the specimen, it is necessary to know the distance from the coverslip. Because in many cases the coverslip position does not coincide with the first plane in the data, this position can be set in the microscopic parameter editor. To our knowledge none of the existing microscopic image files record the coverslip position in the meta data. Next to direct numerical input, the coverslip position and imaging direction can be set using a visual editor that can be started using the LAUNCH EDITOR button located in the parameter editor window (Figure 30.3).

### Inverted Microscope

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



**FIGURE 30.3.** The coverslip position editor showing an  $xy$  MIP of the data along the  $y$ -direction. The coverslip position can be adjusted by dragging the blue line. The imaging direction, here *upwards*, is indicated by the position of the objective relative to the data as shown. The  $z$ -position shown top-left in the image indicates the distance in  $\mu\text{m}$  of the coverslip to the first data plane.

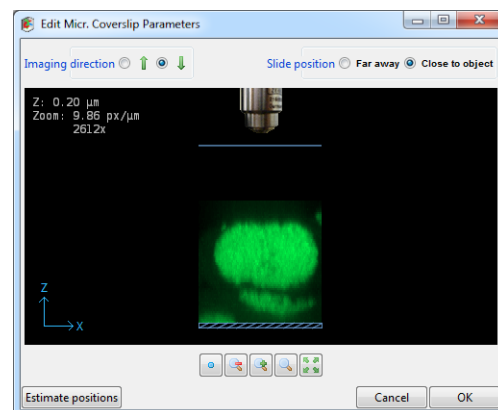
### Upright Microscope

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

### Slide Position

When the specimen is mounted on the coverslip, the distance from the object to the slide is probably in the range from 50 to 100  $\mu\text{m}$ , outside of the image. In this case, or in the case there is no slide, select *Far away* in the top-right selector.

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

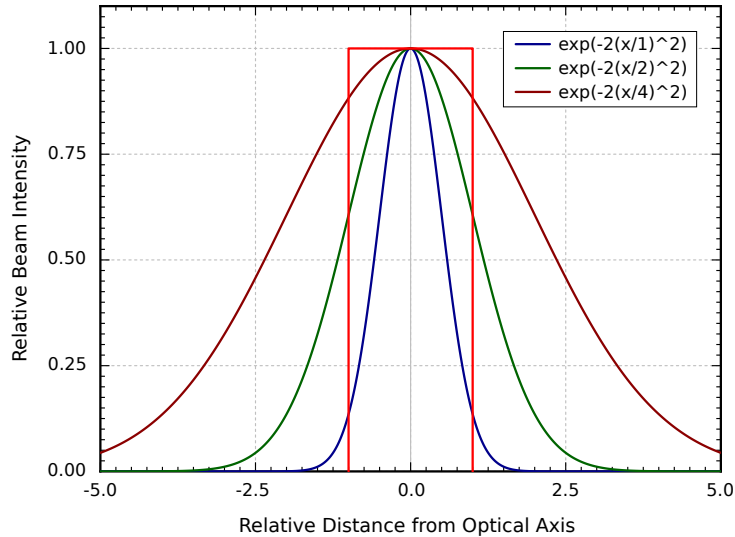


**FIGURE 30.4.** The Coverslip editor with the slide position set to *Close to object*.

### Excitation Beam Overfill Factor

In confocal microscopes, the entry pupil of the microscope objective is illuminated by a laser beam. Usually, laser beams have a Gaussian intensity profile<sup>3</sup>. As a result, the illumination intensity is not constant over the pupil but will decrease towards the edges.

Lower edge intensities will lower the effective NA and therefore negatively affect resolution. In most confocal microscopes this is remedied by using a beam width which is significantly larger than the entry pupil, at the cost of loss of excitation power. The ratio between the beam width and the pupil diameter is the excitation beam overfill factor (See Figure 30.5) and is typically in the range from 2 to 4.



**FIGURE 30.5.** Lens entry pupil (red), beam profile with overfill factor 1 (blue), 2 (green), and 4 (dark red). At overfill factor 1, the beam intensity is 14 % of the maximum, at overfill factor 2 the edge intensity is 61 % of the maximum.

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

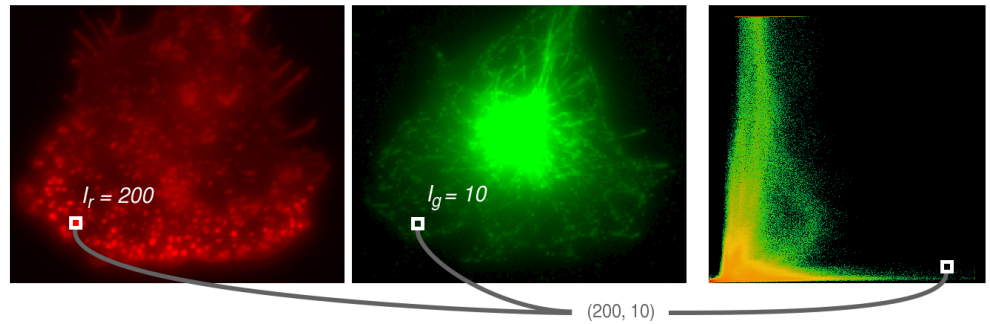
## Brightfield Images

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

In practice one goes about deconvolving brightfield images by inverting them (using ) and processing them further as incoherent fluorescence widefield images. The Tikhonov Miller algorithm was proven to work excellently for brightfield data. This algorithm is available in the Huygens Professional only. With the MLE algorithm one should watch out sharply for interference like patterns (periodic rings and fringes around objects) in

3. [http://en.wikipedia.org/wiki/Gaussian\\_beam](http://en.wikipedia.org/wiki/Gaussian_beam)





**FIGURE 30.6.** Example how a 2D histogram is computed, showing the first and second channel and their corresponding 2D histogram. At each position within the channels, the corresponding intensities of both channels are combined to form a coordinate within the 2D histogram. The count at this coordinate of the 2D histogram is then increased by one.

the measured image. As a rule these become pronounced in low contrast images. After the deconvolution run a reverse to the original contrast setting is possible.

## 2D Histogram

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

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

The 2D histogram is the same as the 1D histogram with the difference that it counts the occurrence of *combinations* of intensities. To compute a 2D histogram the images need to be equal in size. See the example in Figure 30.6; at position (30,20) the first channel has an intensity of 200 and the second image has an intensity of 10. Then this will add one to the count in the 2D histogram at position (200,10).

The difficulty with a 2D histogram is how to show the actual count per intensity combination. For a 1D histogram the height of the bars represent the count, but this height for a 2D histogram requires a third dimension which is difficult to visualize. Instead, to visualize the count of the combinations, colors are used. In the histogram in Figure 30.6 colors range from green to red. Note that these colors have nothing to do with the colors used to represent the channels. Within the 2D histogram, green represents low counts while red represent high counts.

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

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

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More information and examples are in the SVI wiki<sup>4</sup>.

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

# *Support and Contact Information*

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## *Contact Information*

### **Addresses and Phone Numbers**

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URL	<a href="http://www.svi.nl/">http://www.svi.nl/</a>

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

### **Distributors**

An up-to-date list of distributors can be found on our web site<sup>1</sup>.

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## *Support*

### **SVI Support Wiki**

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

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

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

- 
1. <http://www.svi.nl/distributors/>
  2. <http://www.svi.nl/FrontPage>

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

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## License String Details

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

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

```
HuPro-15.05-wcnp-d-tvAC-emnps-eom2015Dec31-
e7b7c623393d708e-{user@domain.com}-4fce0dbe86e8ca4344dd
```

Table 31.1 lists the building blocks from which this string is composed.

**TABLE 31.1. The building blocks of the Huygens license string.**

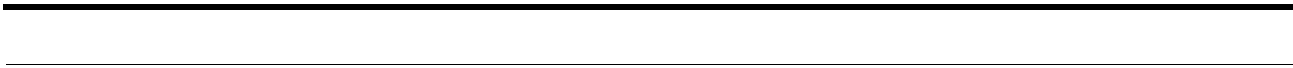
Substring	Description
Product	The product to which the license string applies. This can be HuEss, HuPro, HuScript, HuCore, and HuTitan.
Version	The version number of the product.
Microscope types	This substring consists of one or more characters representing the microscope types for which the deconvolution is enabled. These are 'w' (widefield), 'c' (confocal), 'n' (Spinning disk), 'p' (multi-photon), 's' (STED), 'S' (STED 3X) and '4' (4-Pi experimental microscopes).
Server flag	Determines the number of cores that are enabled for multi-threading. A hyper-threaded core is counted as a single core. It can be 'd' (desktop; 2 cores), 's' (small server; 4 cores), 'm' (medium server; 8 cores), 'l' (larger server; 16 cores), and 'x' (extreme server; 512 cores).
Option flags	This is a set of characters that list the enabled optional modules. An overview of these modules is given in Table 31.2.

**TABLE 31.1. The building blocks of the Huygens license string.**

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

**TABLE 31.2. Identifiers for the optional modules.**

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



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