

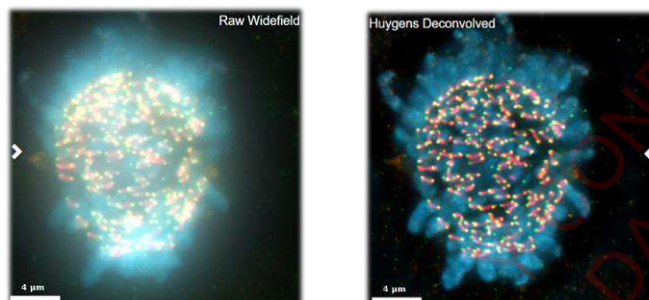


## Huygens Professional Deconvolution Guide

### - What is Deconvolution?

Deconvolution is a computational technique applied to images to compensate for the optical limitations of the system used in their acquisition by reducing the out-of-focus blurring. The use of different Deconvolution algorithms is particularly effective when processing data obtained in Widefield Microscopy, Laser Scanning Confocal Microscopy (LSM), Spinning Disk Confocal Microscopy, and Super-resolution Microscopy...

*"A Practical Guide to Deconvolution of Fluorescence Microscope Imagery". David S.C. Biggs*



### - Key Aspects when acquiring images to deconvolve

1. The size of each pixel must satisfy the Nyquist criterion
2. Avoid saturated pixels
3. Optimize the Signal to Noise (S/N) ratio
4. Acquire a minimum of 3 Z planes

System	Image saving format
Zeiss LSM510 y LSM710 Confocal	<b>.lsm format</b>
Zeiss LSM800 y LSM900 Confocal	<b>.czi format</b>
NikonA1R in vivo Confocal	<b>.nd2 format</b> ; and the <b>.xml file</b> containing the acquisition information
Olympus SpinSR10 Confocal	<b>.vsi format</b> ; and save the <b>folder containing the ".ets" file</b> (metadata)
Leica Stellaris 8 STED+FLIM Confocal	<b>.lif format</b>
Zeiss Widefield Microscopes (sCMOS y F.R.E.T.)	<b>.stk format</b>

### - For more information:

You can find detailed information about the deconvolution program "Huygens" on the following website:

<https://svi.nl/Huygens-Deconvolution>

- More information about the possibilities of this software could be found through the following link:

<https://svi.nl/FAQ>

## How to Calculate pixel size of images to deconvolve

When acquiring images using a microscope and saving them, the ideal is to achieve a sampling density that meets the Nyquist criterion.

Huygens (*Scientific Volume Imaging*) has an online calculator where you will have to select:

<https://svi.nl/NyquistCalculator>

### Nyquist rate and PSF calculator

Microscope type: Confocal (Microscope type (Widefield, Confocal, STED...))

Numerical aperture: 1,3 (Objective Numerical Aperture (N.A.))

Excitation wavelength: 488 nm (Excitation and Emission wavelengths (nm))

Emission wavelength: 520 nm (Excitation and Emission wavelengths (nm))

Number of excitation photons: 1 (Number of excitation photons:  
1 For Widefield Microscopes and Laser Scanning (LSM) and spinning disk (SDCM) Confocals  
2 2 photons microscopes)

Lens immersion refractive index: Oil 1,515 (Immersion refractive index)

Calculate a Point Spread Function

Calculate

#### Results

This is the parameter list used in this calculation:

Parameter	Value
Microscope type	Confocal
Numerical aperture	1.3
Excitation wavelength	402
Emission wavelength	450
Number of excitation photons	1
Lens immersion refractive index	1.515

The optical axis lays along z. Your Nyquist sampling is:

x: 38 nm Recommended pixel size (XY)  
y: 38 nm Recommended step size for Z-Stack  
z: 136 nm

- Set your zooms and scanning steps so that each pixel covers a x-y area of 38 nm × 38 nm (or smaller)
- Calibrate and set your z-stopper so that it takes steps of 136 nm when acquiring a 3D stack (or smaller)

Depending on the values obtained (X, Y, Z); the different parameters (zoom, z-stack step size...) must be adjusted to satisfy these requirements.

#### IMPORTANT

When images are acquired in confocal microscopes using a **pinhole value of 1 Airy Unit (AU)**, the lateral sizes (XY) obtained in the calculator, and those listed in the following tables, can be **increased up to 1.6 times** without detriment to the quality of the Deconvolution.

In cases where **small pinhole values (< 0.5 AU)** are used, these sizes can be **increased up to 1.3 times**; and in the case of using **large pinholes (> 4 AU)**, these sizes can be **increased up to 2 times**.

## STED IMAGE DECONVOLUTION

When acquiring Super-resolution images (STED), 86X (1.2) and/or 100X (1.4) objectives will be used.

To perform a correct Deconvolution of the acquired images, we will use the SVI Huygens online calculator to get the recommended pixel size (XY) and Z-step needed in our image acquisition.

<https://svi.nl/NyquistCalculator>

### Nyquist rate and PSF calculator

Microscope type	<input type="text" value="STED"/>	
Numerical aperture	<input type="text" value="1,3"/>	Objective numerical aperture: <b>1.2</b> (86X obj.) or <b>1.4</b> (100X obj.)
Excitation wavelength	<input type="text" value="488"/> nm	Excitation and emission peak wavelengths of the fluorophore
Emission wavelength	<input type="text" value="520"/> nm	
Number of excitation photons	<input type="text" value="1"/>	
Lens immersion refractive index	<input type="text" value="Oil"/> <input type="text" value="1,515"/>	Immersion medium <b>Water</b> (86X) or <b>Oil</b> (100X)
Backprojected pinhole radius	<input type="text" value="250"/> nm	Backprojected pinhole value of the selected objective. See below.
STED Depletion wavelength	<input type="text" value="775"/> nm	Depletion laser wavelength ( <b>775 nm</b> )
STED 3x percentage	<input type="text" value="0"/>	STED Depletion Laser Power Percentage
STED Saturation factor	<input type="text" value="30,0"/>	Percentage used in the z (axial) depletion beam ( <b>STED 3D</b> )
<input type="checkbox"/> Calculate a Point Spread Function		

A true Nyquist rate does not exist for STED. Instead, we calculate a sampling rate that is both practical and high enough to capture all information realistically available.

Calculate 

#### How to calculate the Backprojected pinhole?

To calculate the Backprojected Pinhole we will use the online calculator available at the following link:

[https://svi.nl/LeicaConfocal\\_TCS\\_SP8](https://svi.nl/LeicaConfocal_TCS_SP8)

#### (1) In case of adjusting the Pinhole in terms of Airy Units (AU)

In this case, we have to take into account that Leica presents the Pinhole value in reference to the 580 nm excitation line; so that when we adjust the AU to our reference channel, we will have to see what this value is for the wavelength 580 nm; along with the numerical aperture of the objective used.

Number of Airy disks	<input type="text"/>
Lens numerical aperture	<input type="text" value="1.3"/>
Calculate	<input type="button" value="Calculate"/>

#### (2) In case of adjusting the Pinhole in terms of microns (µm)

In this case, we will set the Pinhole to be displayed as microns (µm) in the LAS X program, noting that value and the objective magnification to perform the calculations.

Pinhole side (microns)	<input type="text"/>
Objective magnification	<input type="text" value="100"/>
Calculate	<input type="button" value="Calculate"/>

## Objectives and Numerical Apertures (N.A.) available at the SMOC

### - ZEISS CONFOCAL

LSM510 vertical & LSM710 (Inverted and Vertical)		LSM800 Inverted and LSM900 Vertical	
Objective (Immersion Media)	Numerical Aperture (N.A)	Objective (Immersion Media)	Numerical Aperture (N.A)
25X (Oil)	0.8	20X (Air)	0.8
40X (Oil)	1.3	25X (Oil)	0.8
63X (Oil)	1.4	40X (Oil)	1.3
100X (Oil)	1.4	63X (Oil)	1.4
63X (Water) <i>LSM710 Inverted only</i>	1.2	100X (Oil)	1.4
100X (Oil) <i>LSM510 vertical only</i>	1.3	63X (Water) <i>LSM800 Inverted only</i>	1.2

### - OLYMPUS SPINSR10 CONFOCAL

Objective (Immersion Media)	Numerical Aperture (N.A)
10X (Air)	0.4
20X (Air)	0.8
30X (Silicone)	1.05
40X (Air)	0.95
40X (Silicone)	1.25
60X (Silicone)	1.3
100X (Oil)	1.45

Objective	50 µm Disk + Lens 1X		SoRa Disk + Lens 1X		SoRa Disk + Lens 3.2X	
	Backprojected Pinhole (nm)	Pinhole Spacing (µm)	Backprojected Pinhole (nm)	Pinhole Spacing (µm)	Backprojected Pinhole (nm)	Pinhole Spacing (µm)
10X	2500	25.3	1250	12.7	390.63	3.95
20X	1250	12.7	625	6.4	195.31	1.98
40X	625	6.33	313	3.17	97.66	0.99
60X	416.7	4.22	208.4	2.11	65.11	0.66
100X	250	2.53	125	1.27	39.06	0.40

#### IMPORTANT WHEN DECONVOLVING SPINNING DISK IMAGES

When deconvolving spinning disk images, we have to take into account which **Disk (50 µm or SoRa)** and **magnification lens (1X or 3.2X)** combination was used in the image acquisition, since the Backprojected pinhole and the pinhole spacing values vary depending on the combination selected.

Likewise, we must check if the excitation wavelengths coincide with the channels that we want to deconvolve (**405: BFP, DAPI, Hoechst**; **488: Alexa 488, FITC, GFP**; **561: Alexa 555, Alexa 594, Rhodamine, TexasRed, TRITC**; **640: Alexa647, Cy5, To-Pro3**).

In the Microscopic Parameters window, we will have to check if the values shown agrees with the combination that was used during the image acquisition.

- NIKON A1R IN VIVO CONFOCAL

Objective (Immersion Media)	Numerical Aperture (N.A)
20X (Aire / Air)	0.75
20X (Aceite / Oil)	0.75
40X (Aceite / Oil)	1.3
60X (Aceite / Oil)	1.4
60X (Agua / Water)	1.2

- LEICA STELLARIS 8 STED+FLIM CONFOCAL

Objective (Immersion Media)	Numerical Aperture (N.A)
20X (Aire / Air)	0.75
40X (Aceite / Oil)	1.3
63X (Aceite / Oil)	1.4
86X (Agua / Water)	1.2
100X (Aceite / Oil)	1.4

- WIDEFIELD MICROSCOPES: F.R.E.T. and sCMOS (ZEISS)

FRET		sCMOS	
Objective (Immersion Media)	Numerical Aperture (N.A)	Objective (Immersion Media)	Numerical Aperture (N.A)
10X (Air)	0.45	10X (Air)	0.3
20X (Air)	0.8	20X (Oil)	0.5
25X (Oil)	0.8	25X (Oil)	0.8
40X (Oil)	1.3	40X (Oil)	1.3
63X (Oil)	1.4	63X (Oil)	1.4
100X (Oil)	1.3	100X (Oil)	1.3
40X LD (Air)	0.6	40X (Air)	0.6