Light that is incident on a molecule or ion that is in an excited electronic state may trigger radiative relaxation of the molecule and thereby cause the molecule to emit a coherent photon. This process, known as a stimulated emission, partially depletes the excited state of the molecule. In many cases, this result is very desirable. For example, Stefan Hell[1] and co-workers have developed a type of super-resolution optical microscopy called stimulated emission depletion (STED) microscopy that can be used to greatly enhance the normal resolution for imaging fluorescent probes. Stephan Hell was one of three people awarded the 2014 Nobel Prize in Chemistry for their major achievements in developing super-resolution microscopy.

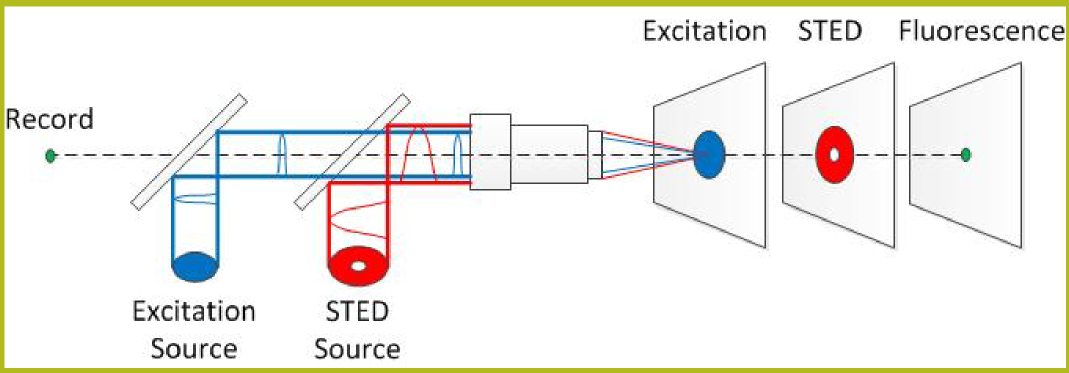
This application note demonstrates how our modeling software SimphoSOFT® allows a user to model and optimize the performance of STED using the SimphoSOFT features of multiple beams and stimulated emission. SimphoSOFT can be used

- to help design STED microscopes, and
- to optimize the performance of fluorescent probes used in STED microscopy.

Stimulated emission can reduce fluorescence emission and enhance resolution

In STED microscopy, organic molecules or nanoparticles are excited from their ground-state energy levels to higher energy levels by the diffraction-limited focused spot of a pump laser pulse as shown by the blue beam in the figure below. A second STED laser pulse (red in the figure below) that is donut-shaped and red-shifted is directed at the focus of the first pulse after a short delay. The second STED beam de-excites and depletes the fluorescence emission from the outer regions of the first pulse and allows emission only in the center of the donut where the STED pulse has zero or low intensity. The resulting fluorescence emission that occurs only at the center of the focal spot of the exciting light pulse results in an effective resolution for fluorescence emission that can be much smaller than the diffraction-limited spot size of the exciting pulse. Although these effects all occur in the same imaging plane, in the figure below the effects are shown in separate planes for easier visualization. STED microscopy can be done using either pulsed or continuous wave (CW) lasers. The highest resolution is done using pulsed laser sources and will be illustrated in the example below.

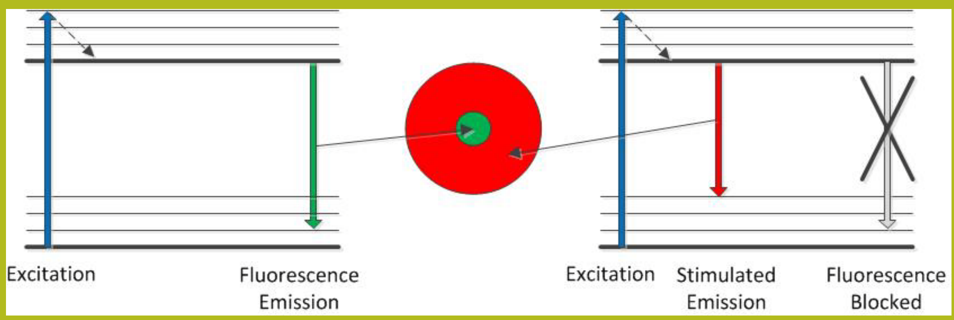
STED apparatus showing reduced spot size for fluorescence emission



[1] S. W. Hell and J. Wichmann, "Breaking the diffraction resolution limit by stimulated emission: stimulated emission depletion microscopy," Opt. Lett. 19, 780-782 (1994)

The corresponding Jablonski energy level diagram, representing excitation and relaxation transitions between ground and excited states, is shown below. For simplicity, the diagram has two main electronic energy levels: a ground state level and an excited state level. At the center of the donut-shaped STED beam, fluorescence can occur and is shown in green. Within the red STED beam, stimulated emission can occur that depletes the excited state and prevents fluorescence from occurring within that region. The stimulated emission is at longer wavelengths and shorter times than fluorescence and can be prevented from reaching the fluorescence detector by use of appropriate bandpass filters and/or detector timing gates.

Jablonski energy level diagram



The traditional maximum diffraction-limited resolution of a focused laser spot with no STED enhancement is given by:

$$\Delta r = \frac{\lambda}{2n\sin\alpha}$$

With STED enhancement, the effective resolution is improved[1] and is given by:

$$\Delta r = \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_{sat}}}}$$

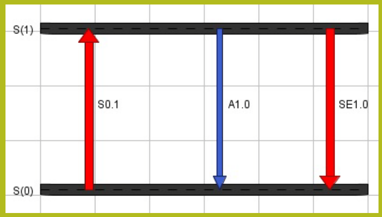
where λ is the wavelength, n is the refractive index of the material, the angle α is the half-angular aperture of the focusing lens and I_{sat} is the STED pulse peak intensity such that the total fluorescence emission is reduced by a factor of $1/e$.

In the example below, we assume that molecules with two singlet states are dispersed in a host material. The molecules are excited by one-photon absorption (1PA) from state S(0) to S(1) using 405 nm blue light. The molecules can emit fluorescence from the S(1) state to the ground state S(0). In our simulation example, we will assume a fluorescence radiative relaxation time of 1000 ps or 1 ns. One-photon stimulated emission (1PSE) from a second STED laser pulse at 640 nm can selectively deplete portions of the S(1) state to reduce the area of fluorescence emission.

SimphoSOFT energy level diagram

The SimphoSOFT M-CAD energy level diagram shown below includes one-photon absorption at 405 nm (S0.1 on the diagram), fluorescence (A1.0) and one-photon stimulated emission at 640 nm (SE1.0). Vibrational states are not shown since vibrational relaxations are very fast and usually do not affect the overall results. However, a user may add vibrational relaxations to the simulation if desired. The user may also add additional electronic energy levels for more complex molecules if needed.

Screenshot of energy level diagram shown in SimphoSOFT® M-CAD

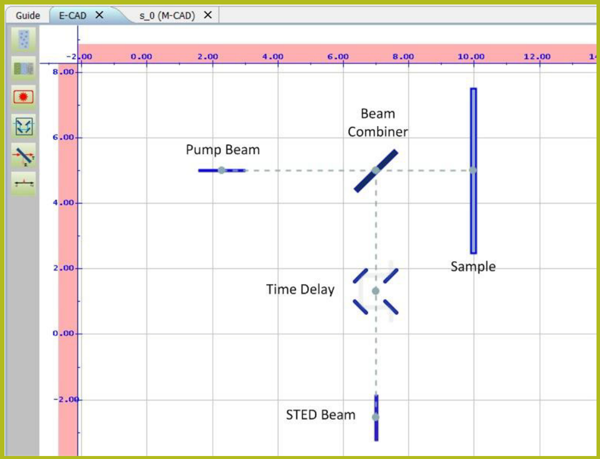


From level(s)	To level(s)	Cross-section	Relaxation time
S(0)	S(1)	1.0×10^{-17} cm^2 (1PA absorption)	
S(1)	S(0)	1.0×10^{-17} cm^2 (stimulated emission)	1000 ps (radiative)

SimphoSOFT E-CAD experimental setup

The experimental setup in SimphoSOFT E-CAD is shown below. The setup includes two laser beams components ("Pump Beam" and "STED Beam"), that are combined by a "Beam Combiner". A "Time Delay" element delays the center of the STED pulse by 30 ps with respect to the center of the pump pulse.

Screenshot of experimental setup in SimphoSOFT® E-CAD



Sample properties (typical values)	
Fluorescent molecule dopant density (concentration) in the host material	3.1×10^{16} molecules/cm^3
Host material linear refractive index	$n_0 = 1.5$ (405 nm and 640 nm)
Host material linear absorption	0 cm^{-1}
Sample length	0.1 mm

Gaussian shaped pump laser beam

The pump (excitation) beam has a Gaussian radial dependence and Gaussian time dependence. The formula for the intensity of the incident Gaussian pump beam is

$$I(r,t) = I_0 e^{-\left(\frac{t}{T_0}\right)^2} e^{-\left(\frac{r}{R_0}\right)^2}$$

, where

$$T_0 = t_{FWHM} / 2\sqrt{\ln 2}$$

$$R_0 = w_{HW1/e^2M} / \sqrt{2}$$

$$I_0 = \frac{E_{in}}{\pi \sqrt{\pi} R_0^2 T_0}$$

For the Gaussian pump beam, the peak intensity is at *r=0*. FWHM is the abbreviation for Full Width at Half Maximum.

Pump laser parameter	SimphoSOFT E-CAD Values
Radial shape	Gaussian
Pulse energy	2.5 nJ
Pulse radius (HW1/e^{2}M)	1 µm
Pulse radius (R_0)	0.707 um
Pulse FWHM	5 ps
Laser wavelength	405 nm

Donut-shaped STED laser beam

The STED beam has a Donut-shaped radial dependence and Gaussian time dependence. The formula for the Donut shape is

$$I(r,t) = I_0 e^{-\left(\frac{t}{T_0}\right)^2} \left(r/R_0 \right)^2 e^{-\left(\frac{r}{R_0}\right)^2}$$

, where

$$T_0 = t_{FWHM} / 2\sqrt{\ln 2}$$

$$R_0 = w_{HW1/e^2M} / \sqrt{2}$$

$$I_0 = \frac{E_{in}}{\pi\sqrt{\pi} R_0^2 T_0}$$

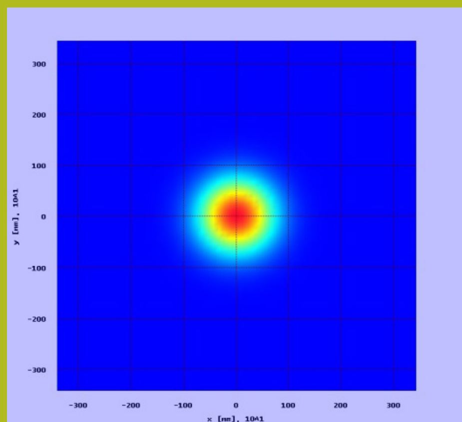
Note that the Gaussian time dependence is the same for both the Gaussian pump beam and the Donut STED beam. However, the radius input to SimphoSOFT E-CAD beam properties for the Donut has a different meaning than the radius input for the Gaussian pump. The SimphoSOFT Donut input parameter for radius, 1 μm, is R_0 and is the radius for the peak intensity of the Donut.

STED laser parameter	SimphoSOFT E-CAD Values
Radial shape	Donut
Pulse energy	0 – 16 nJ (various)
Pulse intensity at peak	0 – 8.8 GW/cm^2 (various)
Radius (R_0) of peak intensity	1 μm
Pulse FWHM	20 ps
Laser wavelength	640 nm
Delay (center-to-center)	30 ps

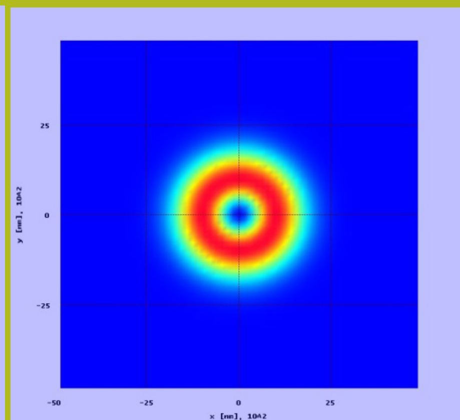
Laser beam plots

SimphoSOFT can generate transverse plots of pump and STED laser beams intensities as shown on the next page. The Gaussian pump beam (left) has highest intensity (the red color) at $r=0$. The Donut-shaped STED beam has the highest intensity at $r=R_0$.

Color-coded plots of the intensities of
the Gaussian pump beam (left) and the Donut STED beam (right)



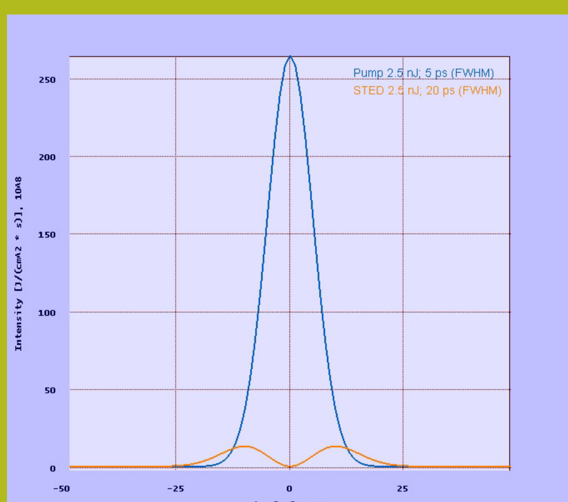
range: [-300, 300] × 10 nm



range: [-30, 30] × 10^2 nm

The plot shown below shows the cross-section intensities of a 2.5 nJ, 5 ps FWHM, Gaussian pump beam (blue curve) and a 2.5 nJ, 20 ps FWHM, Donut STED beam (orange curve) at the temporal centers of each beam. For the pump excitation beam, the curve is for $t=0$ ps (the temporal center of pump). For the STED beam, the curve is for $t=30$ ps (the temporal center of the STED pulse that is delayed 30 ps with respect to the pump). The maximum intensity of the Donut STED beam is at $r=R_0$. The plot was generated using SimphoSOFT 'Plot Creator' by superimposing 2D intensity plots for the Gaussian pump beam and the Donut STED beam. The horizontal axis label *rho* is equivalent to the parameter *r* in the equations above. Although both pulses have the same energy, 2.5 nJ, the maximum intensity of the STED beam is significantly lower than the pump beam partly because the STED beam has a temporal FWHM of 20 ps and the pump beam has a FWHM of only 5 ps.

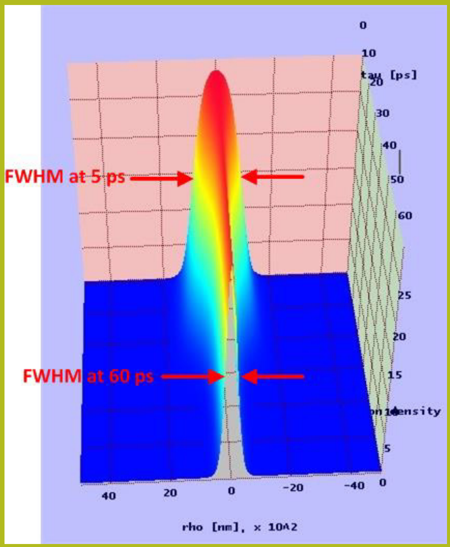
Cross-section intensities of
the Gaussian pump beam (blue) and the Donut STED beam (orange)



SimphoSOFT simulations with Donut STED to determine resolution enhancement factor

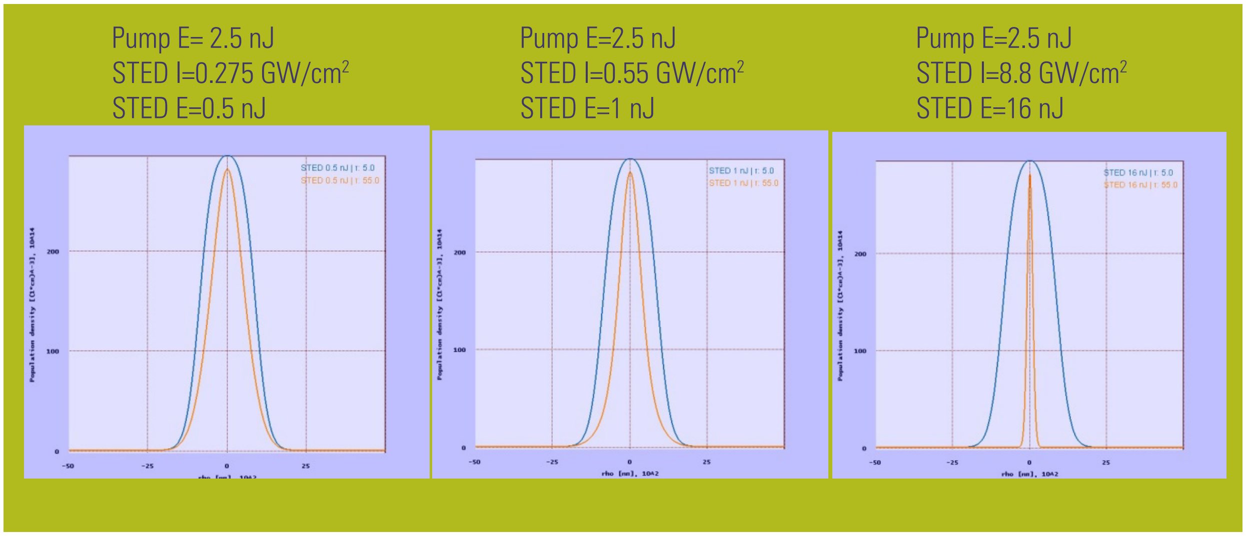
We now show some results for STED simulations using SimphoSOFT. We will show the FWHM value of the width of the singlet S1 population just before and just after a STED pulse. The singlet S1 state is the state from which fluorescence occurs. The width of the S1 population corresponds to the width of the fluorescence emission. During the time of the Donut STED pulse, the width of the S1 population can be reduced by stimulated emission. An example 3D plot of the S1 state for a Donut STED pulse of intensity 2.2 GW/cm^2 (E=4 nJ) is shown below. It is readily apparent that the FWHM of the population distribution is greatly reduced at a time of 60 ps (after the Donut pulse has passed through the sample) compared to a time of 5 ps (which is just before the Donut pulse). Note that the center of the STED pulse is at 30 ps since the STED pulse is delayed 30 ps with respect to the pump pulse that is centered at 0 ps.

3D plot of S1 population for Pump E=2.5 nJ and Donut STED I=2.2 GW/cm2 (E=4 nJ) for the time domain of the STED pulse from 5 ps (just before the STED pulse) to 60 ps (just after the STED pulse)



The 2D plots below show how the FWHM of the S1 population distribution decreases as the STED pulse peak intensity increases. Three STED pulse peak intensities, I=0.275 GW/cm^2 (E=0.5 nJ), I=0.55 GW/cm^2 (E=1 nJ) and I=8.8 GW/cm^2 (E=16 nJ), have been chosen. The cross-sections of the S1 population distributions are plotted for 5 ps (blue line; before the Donut STED pulse enters the sample and 55 ps (orange line; after the Donut pulse has passed through the sample). The substantial narrowing of the S1 population distribution with increased pulse intensity (and energy) is readily apparent.

S1 population cross-sections at 5 ps (blue; before Donut STED pulse) and 55 ps (orange; after Donut STED pulse)



The above representative figures were done by using SimphoSOFT 'Plot Creator' to plot 2D population cross-sections at 5 ps and 55 ps starting with 3D plots for the S1 population versus time and radius. We now compile FWHM data for a wide range of pulse intensities and show the results in the table below.

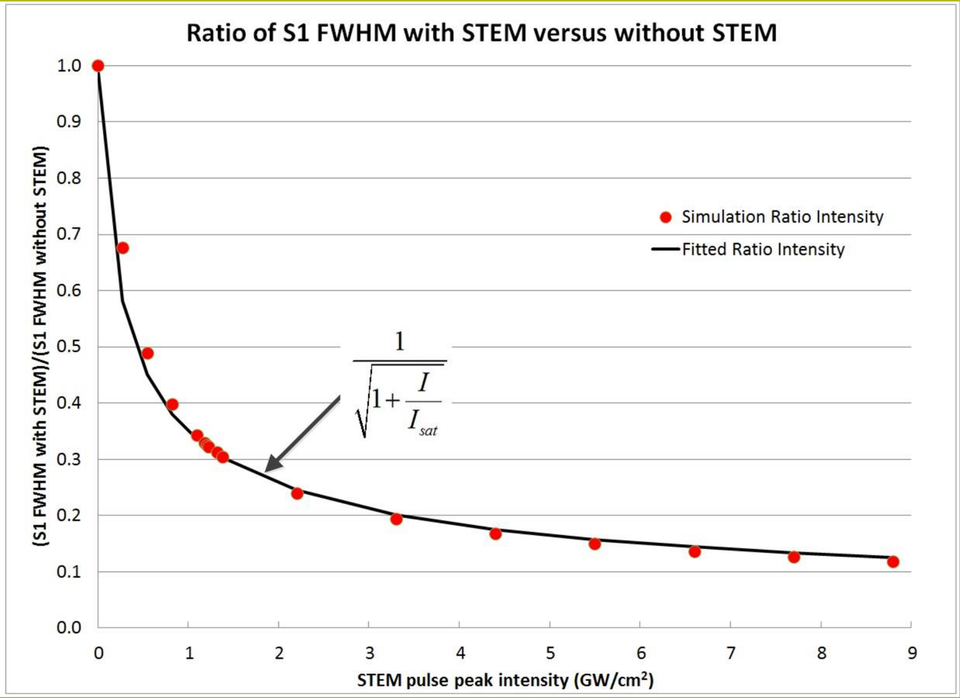
The following table lists the values for FWHM widths and ratios of FWHM widths for the S1 populations at 5 ps (before the STED pulse, which is centered at 30 ps) and 55 ps (after the STED pulse); the values were determined as a function of STED Donut peak intensity.

Peak Intensity (GW/cm^2)	S1 FWHM (nm) at 5 ps	S1 FWHM (nm) at 55 ps	Simulation Ratio	Ratio Using Best Fit for I_{sat}
0.00	1768	1768	1.000	1.000
0.28	1768	1196	0.676	0.581
0.55	1768	864	0.489	0.450
0.83	1768	704	0.398	0.381
1.10	1768	606	0.343	0.336
1.18	1768	582	0.329	0.325
1.22	1768	570	0.322	0.320
1.32	1768	552	0.312	0.310
1.38	1768	538	0.304	0.303
2.20	1768	424	0.240	0.245

3.30	1768	344	0.195	0.202
4.40	1768	298	0.169	0.176
5.50	1768	266	0.150	0.158
6.60	1768	240	0.136	0.144
7.70	1768	224	0.127	0.134
8.80	1768	210	0.119	0.125

The simulation FWHM ratios and the fitted enhancement ratios in the above table are plotted below. In the plot, the value of I_{sat}=0.14 GW/cm^2 is a best fit to the data and is not independently simulated.

Plot of the ratio of S1 FWHM before STED versus after STED as a function of intensity



The SimphoSOFT results clearly show resolution enhancement with increased STED pulse intensity and are consistent with the theory for STED. The biggest improvement in the resolution occurs for STED pulse energies, I_{STED}, less than about 1.5 GW/cm^2. Since the I_{sat} best fit is determined to be 0.14 GW/cm^2 for the given sample and laser conditions, the biggest improvement in resolution occurs for ratios of I_{STED}/I_{sat} less than about 11. Higher STED intensities do lead to better resolution, but the improvements in resolution are not as large as at lower intensities.

Helpful hints for STED

1. Both fluorescence and stimulated emission depend on having an adequate electronic population in the excited S1 state. Using a pump pulse energy sufficient for population inversion (population S1 > population S0) is advantageous. SimphoSOFT can easily determine if population inversion is occurring for any laser and sample conditions.
2. For a fixed Donut pulse energy, a longer Donut pulse length (e.g. 20 ps) can result in greater resolution enhancement than a shorter Donut pulse length (e.g. 5 ps).

Summary

These results show how it is possible to use SimphoSOFT simulations to design a STED microscope or to determine the best materials and experimental conditions for STED resolution enhancement. The results illustrate the importance of having a software program that includes both multiple beams and stimulated emission for doing simulations. 'Multi-Beam' and 'Stimulated Emission' are built-in features of SimphoSOFT Suite.

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