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The VisIR-765 “STED”– A Versatile Picosecond Pulsed Laser Module for Spectroscopy and Microscopy

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Introduction

Studying luminescence lifetime data is a very powerful analytical tool for spectroscopists and microscopists alike, as it provides insights into the excited state dynamics of molecules, complexes, nano particles, or semi-conductors. The fluorescence or phosphorescence lifetime is an intrinsic characteristic of a luminescent species. It indicates how long the species under consideration will remain in an electronically excited state before returning to the ground state. This lifetime is not dependent on instrumental settings such as excitation intensity or detector gain. Each emissive species has a characteristic luminescence lifetime that can be influenced by its environment.

A series of spectroscopy and microscopy methods based on luminescence lifetime have been developed which allow obtaining information that would be otherwise not accessible through steady-state experiments. For example, fluorescence lifetime imaging (FLIM) is a very well established imaging method in life science where the lifetime information is combined with spatial localization in the sample, allowing investigating biochemical or physical processes.[1] This combination of data can help to detect changes in the local environment such as pH, temperature, ion concentration, identify molecular interactions, or conformational changes via Förster Resonance Energy Transfer (FRET). FLIM is commonly used not only in biological imaging, but also in materials science for the characterization of important parameters such as in charge carrier mobility in semi-conductors.

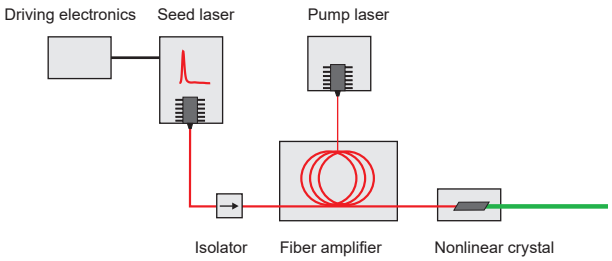
Time-resolved data acquisition is commonly performed through Time-Correlated Single Photon Counting (TCPSC); a versatile technique that allows for excellent time resolution and can cover lifetime ranges from typically ps to ms.[2] TCSPC requires periodic excitation e.g., from a pulsed laser along with single photon sensitive detectors (such as Photomultiplier Tubes (PMT), Micro Channel Plates (MCP), Single Photon Avalanche Diodes (SPAD), or Hybrid PMTs) as well as corresponding counting electronics and optical elements. In order to be useful as excitation sources for time-resolved applications, pulsed lasers need to fulfill certain requirements and ideally also provide a degree of flexibility.

Since fluorescence lifetimes are in the range of ns, the pulsed laser modules should be able to provide temporal pulse widths of a few tens to hundreds ps in order to measure them properly. A large choice with easy selection of pulse repetition rates for each laser is desirable as it permits optimal use of the TCSPC histograming channels with regard to the observed luminescence decay.

This Technical Note highlights the characteristics of pulsed modules from PicoQuant’s VisIR/VisUV laser platform, that make them ideal excitation sources for time-resolved spectroscopy and microscopy, with a particular focus on the VisIR-765 “STED”.

The VisIR-765 “STED” in a nutshell

PicoQuant’s VisIR/VisUV laser platform is designed to provide compact, high powered, stand-alone picosecond pulsed laser modules, that are ideally suited for all types of spectroscopy and microscopy applica-



Scheme 1: Scheme of the VisIR-765 "STED". A picosecond pulsed diode laser with integrated driving electronics operating at 1530 nm is used as a seed for a fiber amplifier in a MOFA arrangement. The infra-red emission is then converted to 766 nm using single pass second harmonic generation.

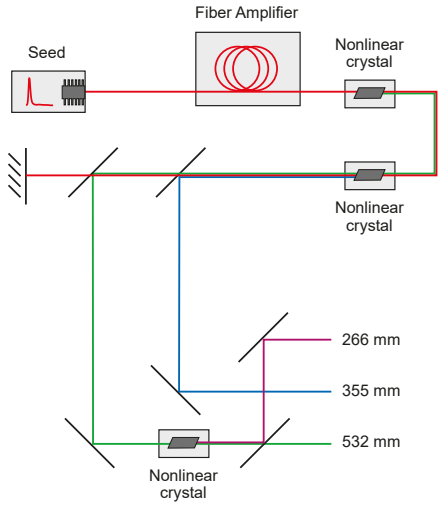
tions. Another key feature of these modules is their optimized heat dissipation, which enables stable and reliable long term operation at high output power levels. They are based on a versatile and flexible Master Oscillator Fiber Amplifier (MOFA) concept with optional frequency conversion, as shown in Scheme 1.

In the case of the VisIR-765 "STED" module (Fig. 1), a gain-switched infrared laser diode provides pulses at 1530 nm with variable repetition rates of up to 80 MHz. The seed laser output is coupled into a multi stage fiber amplifier, which boosts the output from the seed laser by several dB, while maintaining its other characteristics such as emission wavelength, polarization, and pulse width. The high pulse energies of the amplified 1530 nm infrared laser

Figure 1: The VisIR-765 "STED" laser module, ideally suited for microscopy applications such as FLIM imaging with stimulated emission depletion (STED) based super-resolution.

permit an efficient wavelength conversion using single pass second harmonic generation (SHG). In this way it is possible to generate picosecond pulses at 766 nm with an average output power of more than 1.5 W and an excellent temporal pulse shape featuring a single peak with a width of typically 0.5 ns. The VisIR-765 "STED" and other modules from this Series can also be configured to generate pulses with a width of about 70 ps.

The VisIR/VisUV platform can be expanded by switching the wavelength of the infrared seed laser and using more than one nonlinear frequency conversion crystal. Changing the seed laser wavelength will of course also affect the result of the frequency conversion. Scheme 2 depicts a multi beam VisUV model where the seed laser generates pulses at 1064 nm, that are passed through a multi stage fiber amplifier. The amplified near infrared beam is routed towards up to three different nonlinear crystals, where second (SHG), third (THG), and fourth (FHG) harmonic generation occur. A fully equipped VisUV module can thus generate output beams with wavelengths of 532, 355, and 266 nm (simultaneously as well as individually).



Scheme 2: Scheme of the multi beam fiber amplified laser module from the VisUV platform series including second, third, and fouth harmonic generation from a 1064 nm oscillator. A near infra red diode laser is used as a seed for a multi-stage fiber amplifier in a MOFA arrangement. A series of nonlinear conversion crystals (SHG, THG, FHG) are used to generate emission wavelengths in the visible and UV spectral range.

Two modules from the VisUV platform line-up with dual output in the UV/green (280/560 nm) and UV/orange (295/590 nm) spectral regions are especially interesting for life science applications, including STED super-resolution microscopy. The emission wavelengths provided by them are optimally suited for exciting commonly used molecular probes or even naturally occurring tryptophan in time-resolved microscopy applications.

All modules from the VisIR/VisUV Series can be operated at 12 different internally selectable repetition rates from 31.25 kHz to 80 MHz or be triggered externally by TTL or NIM signals at any repetition rate between single shot and 80 MHz with, for example, PicoQuant's high-end, ultra flexible laser driver PDL 828 "Sepia II".[3] Repetition rates can be quickly and easily changed through either a graphical user interface on a PC or from the device's front panel,

without having to restart the laser system. Using the PDL 828 “sepia II” driver to operate a VisIR/VisUV laser module allow realizing complex pulse sequences in combination with other lasers as well as synchronizing them. Such a set-up is extremely useful for realizing a perfect synchronization between excitation and depletion laser in a stimulated emission depletion (STED) set-up.

Due to their capability for generating high powered near infrared laser pulses, the lasers from VisIR/VisUV Series are not only well suited for all kinds of time-resolved microscopy and spectroscopy, but also for ranging applications such as Light Detection And Ranging (LIDAR).

Application examples

Fluorescence lifetime imaging with STED super-resolution

In the recent years, super-resolution microscopy based on stimulated emission depletion (STED) has become a well established method for reaching optical resolutions beyond the diffraction limit. STED microscopy uses the principle of stimulated emission based depletion: After exciting the fluorophores in the focus of the excitation laser, a second laser beam of longer wavelength with a donut-shaped beam is used to actively quench the molecules in the periphery via stimulated emission.

A schematic view of a STED super-resolution set-up as realized in the MicroTime 200 time-resolved microscope from PicoQuant is shown in Fig. 2.[4] The microscope has an illumination system featuring at least two pulsed diode lasers: one or more for exciting the fluorescent labels and a STED laser to

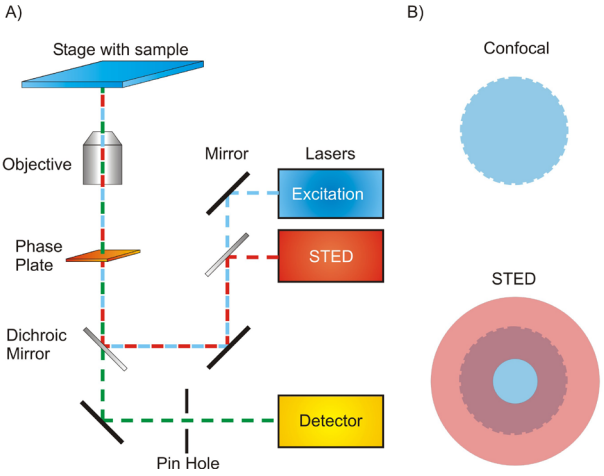


Figure 2: (A) Schematic overview of a confocal microscope equipped with STED add-on. (B) Cross sections of the focal plane with and without STED donut (red), altering the observation area (shown in blue).

deactivate their emissive state. All lasers are spatially overlaid before being coupled into a single mode fiber and pass through a so-called EASYDOnut phase plate. This segmented phase plate modulates the polarization profile of the STED laser, leading to a donut shape in the focal plane, while the beam profile of the excitation lasers is retained.

The STED donut deactivates the excited state of the fluorescent species present in its area by inducing stimulated emission at a longer wavelength than that of the STED laser. Light from the excitation and STED lasers as well as the stimulated emission are excluded from detection by means of appropriate bandpass filters. Therefore, only the desired emission from the center of the STED donut will be detected, leading to a smaller effective observation area with a diameter below the diffraction limit.

The VisIR-765 “STED” has been designed to be ideally suited as depletion laser for STED applications: The generated laser light has a wavelength of 766 nm, which is well suited for quenching many fluorophores commonly used in life science. The pulses have an extended duration of 0.5 ns (full width at half maximum). The extended pulse duration of 0.5 ns is

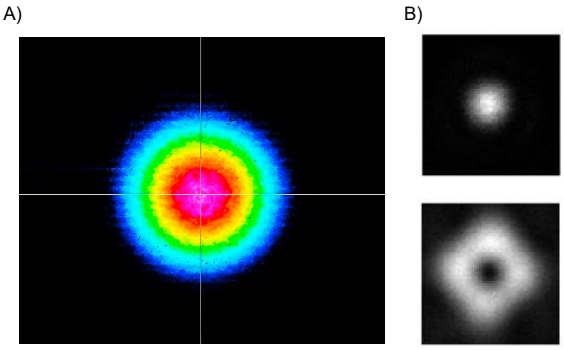


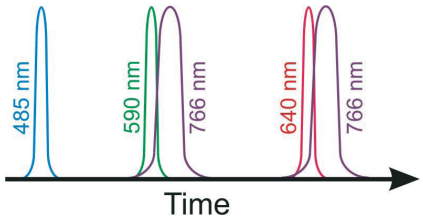
Figure 3: (A) Beam profile (TEM_{00}) of the VisIR-765 “STED” laser with a beam diameter of 2.2 mm and an M^2 value of 1.02. (B) Back reflections images from 80 nm gold beads for both the excitation (top) and the STED lasers (bottom). The excitation laser spot profile is circular, while the STED laser exhibits a donut shape in the focal plane.

an excellent compromise for STED microscopy since longer pulses or even continuous-wave excitation can expose the sample to an unnecessary amount of radiation, leading to increased photobleaching. Furthermore, the beam profiles of all VisIR/VisUV modules feature a nearly perfectly circular and Gaussian shaped beam profile (TEM_{00} mode with an M^2 value of typically 1.02; see Fig. 3A). This excellent beam quality makes it possible to accurately shape it into a donut with help of the segmented phase plate, as can be seen from the back reflection images in Fig. 3B.

The following example aims to demonstrate the power of the VisIR-765 “STED” as a depletion laser for super-resolution FLIM imaging. The axon terminals and dendrites of neuronal synapses were each

labeled with fluorophores having different absorption and emission spectra. FLIM images with both confocal and STED super-resolution were obtained by running the STED and all excitation lasers in Pulsed Interleaved Excitation (PIE) mode.

The pulse sequence used in this example is shown in Scheme 3 and consists of a first excitation pulse at 485 nm, which generates autofluorescence from the cell walls. This excitation pulse is not followed by a STED pulse; meaning that, in both the super-resolution and confocal image, the cell walls were imaged with the diffraction limited confocal



Scheme 3: Pulse sequence used to image a synaptic cleft with STED based super-resolution where the axon terminals and dendrites were labeled with different dyes.

resolution. The two fluorescent labels were excited with pulses at 590 and 640 nm respectively and were each followed by a slightly delayed STED laser pulse at 766 nm. The same area was also imaged under confocal conditions using a similar pulse sequence but without STED laser pulses.

Such complex pulse sequences can be very easily realized by operating both the excitation and STED lasers with a PDL 828 “Sepia II” driver. This high-end laser driver features an oscillator and burst generator module with up to 8 individually addressable

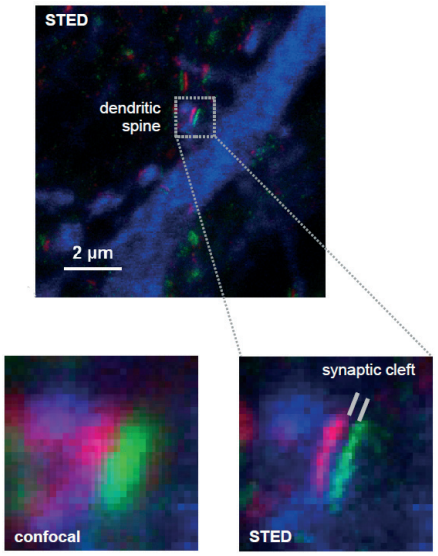


Figure 4: FLIM images of a dye-labeled dendritic spine imaged under both confocal and STED super-resolution. The synaptic cleft can be easily recognized in the STED FLIM image (bottom right).

channels.

These channels can be combined and delayed relatively to each other with picosecond accuracy. The oscillator module allows defining the output pulse pattern at each channel individually. The pattern is derived from a combination of burst generator, delayer, and combiner operation.

The delay function allows shifting the output of individual channels by ± 1 ms in 25 ps steps, which is extremely useful to fine tune the temporal spacing of different laser heads with a very high accuracy for, e.g., STED microscopy.

The resulting image (Fig. 4) shows the neuronal cell wall autofluorescence in blue, while the axon terminal and dendrite labels are shown in green and red, respectively. In the STED super-resolution image, the synaptic cleft can be easily seen as a colorless band between the red and green zones. In the confocal image, however, the cleft cannot be seen, as the resolution is too low.

Pulsed interleaved excitation STED based fluorescence correlation spectroscopy (PIE-STED-FCS)

Another application where the capabilities of the VisIR-765 “STED” laser module are invaluable is pulsed interleaved excitation based fluorescence correlation spectroscopy using STED (PIE-STED-FCS). Fluorescence Correlation Spectroscopy (FCS) is a well established method for studying the diffusion behavior of molecules in solution or membranes.

Using a fully Pulsed Interleaved Excitation (PIE) scheme allows detecting more than one type of fluorophore in a single experiment. The PIE scheme in a PIE-STED-FCS measurement involves generating a sequence of pulses where the excitation lasers fire

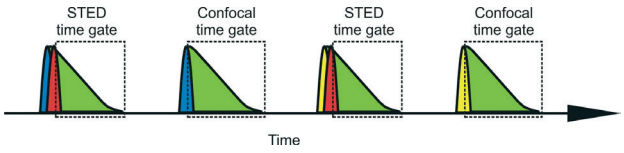


Figure 5. Schematic representation of the pulsed interleaved excitation pattern used for quasi-simultaneous, multi species FCS probing under STED and confocal conditions. Excitation laser pulse (blue and yellow), STED pulse (red), and resulting fluorescence (green). The dashed boxes indicate the time gates applied in the data analysis step for separating STED and confocal contributions.

in an alternating manner to individually excite and detect each fluorophore type (see Fig. 5). Each of these excitation pulses can be individually coupled to a STED pulse or not.

The VisIR-765 “STED” features both high flexibility in repetition rate selection and the ability to be externally triggered, which makes it an excellent choice for this type of application. By operating it and the excitation lasers with an advanced laser driver such

as the PDL 828 “Sepia II”, complex pulse sequences as shown in this example can be realized through an easy to use graphical user interface.

Under confocal conditions, the minimal size of the FCS observation volume is restricted by the diffraction limit. However, this limit can be circumvented by employing Stimulated Emission Depletion (STED) and the observation volume can be shrunk

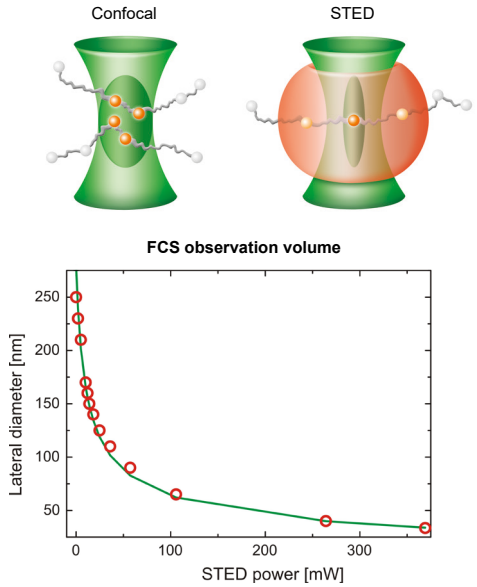


Figure 6: Top: Depiction of confocal and STED observation volumes in a PIE-STED-FCS experiment. Due to the STED donut in the focal spot (right, in red), the observation volume is smaller allowing achieving a better spatial resolution. Bottom: Plot of the observation spot size as a function of the STED laser power, as determined from images of immobilized crimson beads with 20 nm diameter.

in a gradual manner by increasing the STED laser intensity.[5]

At low STED laser powers, a small increase in intensity leads to a rapid shrinking of the lateral observation area diameter. Going from 0 to 50 mW reduces the spot diameter from 250 to less than 100 nm, depending on the fluorophore used. At high STED powers, however, increases in intensity will result in slower shrinking until a diameter below 50 nm can be reached (see plot in the bottom part of Fig. 6).

The temporal pulse shape and width of the VisIR-765 “STED” remains independent of the optical output power over a large range, which, along with the excellent beam quality, make it an ideal STED laser for this type of application.

Shrinking the detection volume helps in overcoming averaging issues in long transit paths and also enables determining the type of hindered diffusion behavior of fluorophores in lipid membranes. Furthermore, using a fully Pulsed Interleaved Excitation (PIE) illumination scheme for excitation and STED lasers allows collecting FCS data under both confocal and STED conditions quasi-simultaneously. PIE-STED-FCS allows also for a straightforward check whether the STED laser has an influence on the investigated diffusion dynamics.

Conclusions

The picosecond pulsed laser modules from PicoQuant’s VisIR/VisUV platform have a series of features that make them ideally suited for time-resolved spectroscopy or microscopy applications. These features include supporting a range of repetition rates going from 1 Hz to 80 MHz with the ability to change them on the fly, temporal pulse shapes and widths that are independent of output power over a broad range, and the ability to be easily interfaced with advanced laser drivers such as the PDL 828 “Sepia II” to generate complex pulse patterns.

As we have shown here, the VisIR-765 “STED” is ideally suited for microscopy applications involving STED super-resolution due to its pulse width of typically 0.5 ns, excellent beam shape, and high optical output powers of up to 1.5 W. The pulse width ensures good depletion efficiency while minimizing photo-bleaching, while the nearly perfect circular and Gaussian beam profile allows for good beam shaping via segmented phase plate.

In combination with other pulsed diode lasers and a high-end laser driver such as the PDL 828 “Sepia II”, the VisIR-765 “STED” is an excellent choice for demanding applications in time-resolved spectroscopy and microscopy.

Further reading

[1] S. Trautmann, V. Buschmann, S. Orthaus, F. Koberling, U. Ortmann, R. Erdmann, Fluorescence Lifetime Imaging (FLIM) in Confocal Microscopy Applications: An Overview; Technical note, Picoquant GmbH, 2012

[2] M. Wahl, S. Orthaus-Müller, Time Tagged Time-Resolved Fluorescence Data Collection in Life Sciences, Technical Note, PicoQuant GmbH, 2014

[3] Website containing information regarding the PDL 828 “Sepia II” laser driver: http://www.picoquant.com/products/category/picosecond-pulsed-driver/pdl-828-sepia-ii-computer-controlled-multichannel-picosecond-diode-laser-driver

[4] Website containing information regarding the MicroTime 200 STED: http://www.picoquant.com/products/category/fluorescence-microscopes/microtime-200-sted-time-resolved-confocal-fluorescence-microscope-with-super-resolution-capability#description

[5] M. Koenig, P. Reisch, R. Dowler, B. Kraemer, S. Tannert, M. Patting, P. Clausen, S. Galiani, C. Eggeling, F. Koberling, R. Erdmann, ns-time resolution for multispecies STED-FLIM and artifact free STED-FCS, Proceedings of SPIE, Vol. 9712, 97120T, 2016

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