Two-Photon Excitation for Optical Nanoscopy and Light-Sheet Illumination Microscopy

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ABSTRACT

We report about coupling two-photon excitation (2PE) with two, comparatively young and successful optical methods specially realized for the improvement of resolution and 3D imaging of large samples, i.e. STED (stimulated emission depletion) and SPI (selective plane illumination) microscopy. In the former case we aimed to get a better resolution for 2PE microscopy while for the latter we aimed to obtain better penetration in scattering samples by shifting the light sheet wavelengths to the red in the non-linear domain. We show results obtained by adapting a commercial STED-CW microscope to 2PE and by implementing a 2PE-SPIM homemade system. The implementation of these systems is demonstrated by the imaging of living Saccharomyces cerevisiae cells.

KEYWORDS
light microscopy, two-photon excitation, fluorescence, STED, SPIM, superresolution, nanoscopy, deep penetration

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INTRODUCTION
Two-photon excitation (2PE) fluorescence microscopy [1] is a well-established far-field fluorescence optical microscopy technique for the study of the three-dimensional (3D) and dynamic properties of biological systems from cellular compartments, such as membranes, to thick specimens, such as brain slices or large cellular aggregates [2, 3].

Since the excitation process is restricted by the diffraction-limited spatial extension of the explored volume using near-infrared radiation, 2PE has some advantages and some drawbacks. The main advantages lie in the augmented penetration in scattering samples and in the reduced overall phototoxicity and/or photobleaching coupled to intrinsic 3D optical sectioning properties. Thus a confocal-like effect is obtained without the necessity of a confocal pinhole. As a matter of fact, the implementation of 2PE microscopy has been proven to be effective to reduce image degradation due to scattering and to increase the imaging penetration depth. Moreover, 2PE allows the excitation volume to be reduced and utilizes low-energy wavelengths, facilitating long-term measurements. In fact, due to the physics of the process, priming the fluorescence needs a high instantaneous photon flux density that can be obtained by a tight focusing of a laser beam. Thus, those regions away from the focal volume of the objective lens will therefore not suffer photobleaching or phototoxicity effects when 2PE is used: they are simply not involved in the excitation process. Unfortunately, also due to the doubling of the excitation wavelength, many features and organelles of biological specimens are still too small to be resolved, in agreement with Abbe’s law.

At the very same time, the main – but superable – drawbacks result from the loss of resolution and the poor signal efficiency with respect to the 1PE case. In the last few years there has been an increase of optical methods for improving resolution [4, 5]. Among these methods the breaking of the diffraction resolution barrier with stimulated-emission depletion (STED) [6] nanoscopy has been extensively demonstrated [7]. In a typical STED micro-

Figure 1:
Schematic of the setup realized to perform two-photon excitation stimulated emission depletion (STED) continuous wave (CW). It is based on a Leica TCS STED-CW microscope coupled to a pulsed infrared beam. The resulting system maintains the full functionalities of a confocal and STED microscope.

ND: neutral density. LPC: laser power controller [10].
scope, the diffraction limited excitation focus is overlaid with a red shifted STED beam featuring a zero intensity point. The STED beam transiently inhibits the fluorescence ability of dye molecules located in the outer part of the excitation area, confining the spatial extent of fluorescence, to the central part of the spot by effective molecular excitation, and hence of producing a direct improvement of resolution, theoretically without limitations [8].

Spot beyond the diffraction limit allows one to define it as a superresolution optical method or better as an optical nanoscope. The resolution of the STED microscope is thus a function of the spatial distribution and magnitude of the intensity of the depleting light, with no theoretical limit to the ultimate achievable value, currently set at 7 nm [9].

In this work we report about the coupling of 2PE and STED-CW in a commercial STED microscope [10]. Moreover, selective plane illumination microscopy (SPIM) has become an important approach for biological investigations of thick samples [11]. It has been found particularly useful in developmental biology applications since it provides the capability to perform fast imaging of living samples, reducing photobleaching and phototoxic effects [12]. The high signal-to-noise ratio and the intrinsic optical sectioning capability provided by SPIM suggest this technique as the best choice for imaging of thick, scattering samples. Unfortunately, imaging in depth of large samples suffers from a decrease in the image quality due to scattering effects. In order to contribute to the improvement of this method we decided to realize 2PE within the light sheet illumination scheme [13].

**TWO PHOTON EXCITATION STIMULATED EMISSION DEPLETION MICROSCOPY**

Supersolved two-photon excitation fluorescence imaging was achieved by using an ultrafast mode-locked titanium:sapphire laser (Chameleon Ultra II, Coherent, Santa Barbara, USA; 140 fs pulsewidth, 80 MHz repetition rate and 750 nm wavelength) coupled to a TCS SP5 STED-CW microscope (Leica Microsystems, Mannheim, Germany) [10].

The pulsed infrared beam was coupled to the microscope through the multifunction port on the top of the scanning head (see Figure 1). A neutral density filter controlled the laser power so that it was on average 10-25 mW at the sample. The emitted fluorescence was collected in the 500-550 nm spectral range using a colour filter placed in front of an internal avalanche photodiode (APD) detector. The STED beam wavelength was currently set at 592 nm running in continuous wave (CW) mode. The depletion beam power, i.e. the attainable resolution, was controlled by an internal acusto-optical-tunable-filter (AOTF).

The spatial alignment of the two-photon excitation beam and the STED beam was crucial to obtain the resolution enhancement. Sub-resolved gold beads have been used in reflectance mode to check the correct overlay of the three delivered beams 488 nm, 750 nm and 592 nm laser lines used for confocal and 2PE-STE D imaging. In order to demonstrate the performance of the microscope, yellow green fluorescent 40 nm beads (FluoSpheres carboxylate-modified microspheres, Invitrogen, Carlsbad, CA, USA) dried on a coverslip were imaged [10] (Figure 2). Since they were sub-resolution objects for all the four imaging techniques used (confocal, STED, 2PE, 2PE-STE D) they allowed us to demonstrate the resolution improvement. The four images were acquired with the same settings except for the pinhole size.

The line plots in Figure 3 demonstrated the improved lateral resolution of the STED images with respect to the confocal and 2PE ones. The resolution achieved by 2PE-CW was close to the STED-CW, i.e. 80 nanometres. The application of the STED to 2PE allowed us to achieve a 2PE resolution that was equivalent to the 1PE one, despite the excitation wavelength.
TWO PHOTON EXCITATION
STIMULATED SELECTIVE PLANE
ILLUMINATION MICROSCOPY

A single-plane illumination setup suitable for two-photon excitation is shown in Figure 4 [13]. The setup was composed by an illumination unit, which created the light sheet from the side of the detection focal plane. The illumination unit was composed both by solid state diode lasers operating at different wavelengths in the visible range and by an ultrafast tunable IR laser. Lasers at three different wavelengths in the visible range could be used: a UV laser (Coherent CUBE 405-100mW), a 488 nm laser (Coherent Sapphire OPLS 488 nm 200 mW) and a 561 nm laser (Coherent Sapphire OPLS 561 nm 200 mW).

The 2PE light sheet was generated by means of a mode-locked titanium-sapphire laser operating with pulses of 140 fs and a repetition rate of 90 MHz having the maximum power of 4 W (Chameleon Ultra2, Coherent). A cylindrical lens (f = 200 mm) focused the light along one direction, forming a thin laser light sheet. A low-NA infinity-corrected objective (Nikon Plan 10/0.3), properly placed after the cylindrical lens, generated a diffraction-limited light sheet with reduced aberration effects.

The detection pathway was similar to the detection scheme used in conventional fluorescence microscopy. Water-dipping lenses were used to collect the emitted signal and a tube lens formed the image on an high-quality back-illuminated electron multiplying CCD camera (Andor Ixon DU-897E-C50-BV). For two photon excitation a special dichroic mirror (Chroma Technology Corp. T670LP IR) allowed us to separate the IR excitation light from fluorescence emitted photons and a dichroic filter selected the fluorescent signal (Chroma Technology Corp. T420LP).

To demonstrate two-photon excitation within the light sheet geometry the quadratic dependence of the emitted signal over the excitation power has been verified (data not shown).

The two-photon excitation process primed in single plane illumination architecture was used to observe nuclei of living yeast cells (Figure 5). Saccharomyces cerevisiae cells were embedded in agarose gel and included in a glass capillary in order to allow imaging in the SPIM architecture. The sample was placed in a customised water chamber made from plastic material. Saccharomyces cerevisiae were grown under standard conditions and stained with Hoechst 33342 (Invitrogen). The high density and the large amount of cells mounted in agar gel also represented a model of turbid and highly scattering media [14].

CONCLUSIONS

In this work we have shown a two-photon excitation fluorescence (2PEF) stimulated...
emission depletion (STED) microscope adapting a commercial confocal Leica TCS STED-CW to a two-photon excitation microscope. The relevance of this is also reported by other early work [15, 16] and the advent of a commercial STED-CW architecture can expand the applications. In fact, the infrared beam has been coupled without compromising the capability of the commercial microscope and taking advantage of its STED ‘standard’ functioning [10]. Therefore, we have shown the possibility of performing confocal, STED-CW, 2PE and 2PE-STED-CW on the very same device.

Moving to the increasing relevance of light sheet illumination methods [17-20], we have built a scanless two-photon excitation imaging system based on single-plane illumination [13]. The light sheet illumination geometry and the two-photon excitation volume induced by a femtosecond infrared laser source have been characterized [13, 14]. The implementation of 2PE-SPIM was demonstrated by the imaging of living Saccharomyces cerevisiae cells.

REFERENCES

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