Supporting Material

Nanoscopy in a Living Multicellular Organism Expressing GFP

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ADDITIONAL DATA

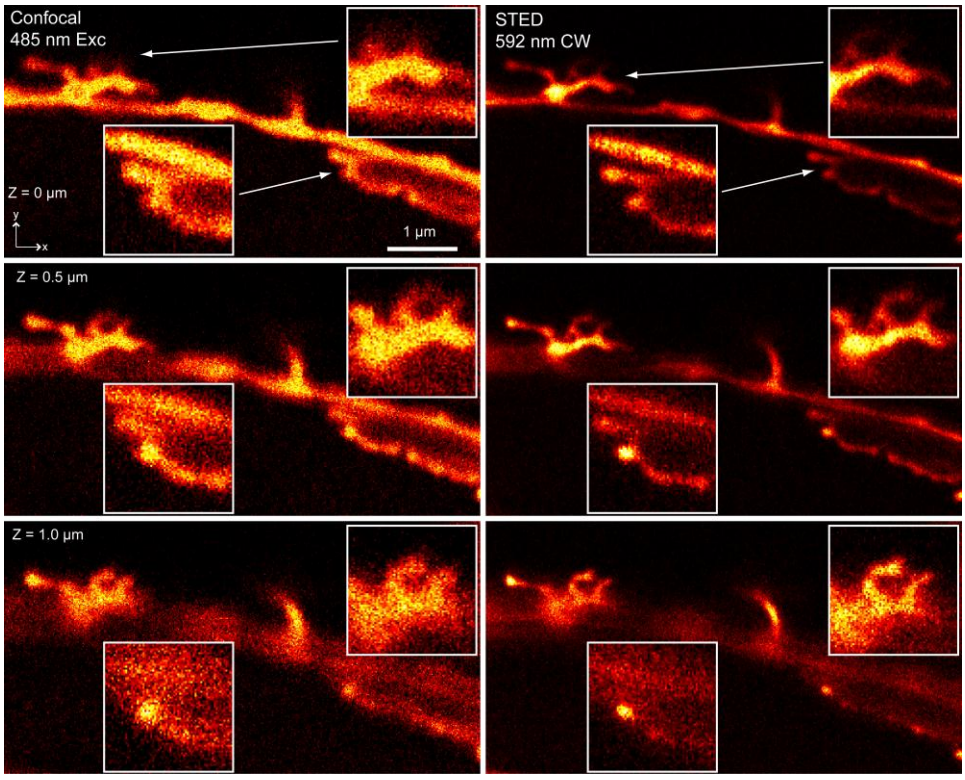

FIGURE S1 STED image stacks of NSM neuron structures. For reference, confocal images are shown at left, with corresponding STED images at right. The spacing between images in z (along the optical axis) is 0.5 µm. Images were taken with continuous wave laser sources; excitation was performed at 485 nm, and the STED wavelength was chosen to be 592 nm. The excitation and STED powers were 14 µW and 250 mW, respectively. A pixel size of 30 nm was used for both confocal and STED imaging, and with a pixel dwell time of 8 µs, acquired image frames of 12×12 µm were obtained in less than 2 seconds. The whole stack of eight images (of which only three are shown here, chosen for visual interest) was obtained in about 20 seconds. Neuron features too small to be distinguished in confocal but clearly revealed with STED are indicated with arrows.

MOVIE S1 Endoplasmic reticulum dynamics in cultured PtK2 cells. Following the confocal image, 20 STED recordings were taken continuously over 13 minutes. Imaging was performed in pulsed mode at 20 MHz, using 488 nm for excitation and 560 nm for STED, with employed average powers at the back aperture of the microscope objective lens ~7 µW and 4 mW, respectively.

Evaluation of the excited state absorption spectrum

In Fig. 1 the ground state absorption and fluorescence emission spectra are shown with the excited state absorption (ESA) spectrum determined by pump-probe measurement 100 ps after excitation. Fig. S2 shows the data used to determine the ESA spectrum. The ground state absorption and fluorescence emission spectra are shown for reference (upper panel). The transient absorption (TA) spectrum (middle panel) of eGFP was measured at a pump-probe delay of 100 ps as described as below in Materials and Methods. The TA is the sum of ESA, ground state absorption, and stimulated emission (SE). The middle panel shows that for wavelengths >520 nm the SE line shape as derived from the fluorescence emission spectrum is almost identical with the TA spectrum. Hence the properly scaled SE spectrum was subtracted from the TA spectrum, shown by the pink curve. Above 500 nm the curve TA - SE is markedly similar to the spectral shape of the ground state absorption spectrum. This is clarified in the lower panel of Fig. S2 where the amplitude of the inverted ground state absorption spectrum was adjusted to match the edge of the curve representing TA-SE above 500 nm. After subtraction of the contribution of ground state absorption the ESA spectrum is obtained, shown in red.

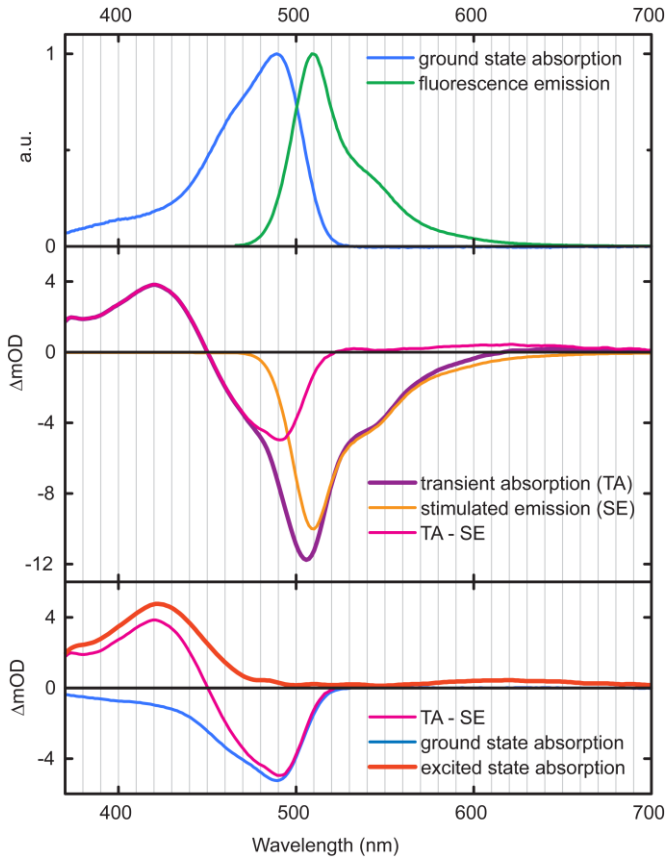

FIGURE S2 The ground state absorption and fluorescence emission spectra are shown for reference (upper panel). The transient absorption (TA) spectrum, measured by pump-probe spectroscopy, is used to calculate the ESA spectrum by subtracting the scaled contribution of stimulated emission (SE) (middle panel) to obtain the curve showing TA-SE, and then subtracting the scaled contribution of the depletion of the ground state absorption to obtain the ESA spectrum.

MATERIALS AND METHODS

Protein purification of eGFP

A pQE31 (Qiagen, Hilden, Germany) expression vector containing the coding sequence for eGFP was transformed into the *Escherichia coli* strain SURE (Stratagene, La Jolla, Ca, USA). For protein expression, cells were grown to an OD600 of 0.6 in LB medium containing 100 μg/ml ampicillin and were induced with 1 mM isopropyl beta-D-1-thiogalactopyranoside. Following 6 h incubation at 25°C, the resultant biomass was pelleted by centrifugation, resuspended in PBS with protease inhibitors and sonicated. The bacterial lysate was cleared by centrifugation, and the fluorescent proteins were affinity purified by Ni-NTA (Ni^{2+}-nitrilotriacetate) affinity chromatography and subsequent size-exclusion chromatography according to standard procedures. The purified proteins were concentrated by ultrafiltration and taken up in 20 mM Tris/HCl and 120 mM NaCl (pH 7.5).

Pump-probe spectroscopy

The transient excited state spectrum of eGFP was measured using femtosecond transient absorption spectroscopy with broad-band white-light continuum probing. The laser system is based on a 1 kHz amplified Ti:sapphire laser system (Clark MXR, CPA-2001) producing 900 μJ pulses 150 fs in duration at a wavelength of 773 nm. For exciting eGFP a portion of this laser light was frequency doubled and used to generate 490 nm pump pulses with a non-collinear optical parametric amplifier. The white-light continuum was produced by focusing a small part of the 773 nm light into a CaF_2 plate. The white light was split into a probe and a reference beam. The eGFP sample was excited with pump pulse intensities of ca. 90 nJ focused down to a spot size of ≈ 200 μm and overlaid with the white-light probe pulse. The relative plane of polarization of pump and probe light was set to 54.7°. Probe and reference spectra were dispersed in imaging spectrographs (Jobin Yvon Horiba, CP 140-2951) onto 256 element diode arrays covering a spectral range of 360-770 nm. For the experiments 15 μM solutions of GFP in water at pH 7.5 were prepared using a Tris buffer (100 mM Tris·HCl, 150 mM NaCl). The sample was loaded in a flow system consisting of a peristaltic pump (Gilson Miniplus 3) and a flow cell with an optical path length of 1 mm.

Laser sources

We employed both pulsed and CW lasers within the ~550–600 nm spectral range appropriate for inhibition of fluorescence of GFP with stimulated emission. A pulsed source based on stimulated Raman scattering (SRS) in a single-mode optical fiber (1, 2) provided a comb spectrum of Stokes lines conveniently spanning the tail of the emission spectrum of GFP, allowing the flexibility to easily select the STED wavelength within this range. The SRS light source employed had a repetition rate 20 times that of a similar source previously reported (2). A single-mode fiber of type S630-HP (Nufern, East Granby, CT, USA), with a pure silica core to avoid photodarkening due to high intensities at the pump wavelength of 530 nm, was pumped by a 20 MHz, 532 nm fiber laser offering pulse energies of ~100 nJ with pulses 700 ps in length (Mobius Photonics, Mountain View, CA, USA). The frequency shift between Stokes lines of 13.2 THz was determined by the peak of the Raman gain spectrum of silica glass (3). Individual Stokes lines were selected from the spectrum for use as STED light with a bandpass filter. The spectral lines at 556 nm, 570 nm, and 585 nm were tried; best results were obtained with 556 nm and 570 nm light. Additionally, a dedicated pulsed source providing light at 560 nm and operated at 20 MHz was employed (MBP Photonics, Pointe-Claire, Canada), which produced results comparable to the 556 nm Stokes line of the SRS light source. For pulsed STED configurations, excitation light was produced by a triggerable diode laser operating at 488 nm (Pico TA 490, Picoquant, Berlin, Germany).

GFP STED images using continuous wave fiber lasers were obtained using a laser operating at 592 nm (MPB Photonics, Pointe-Claire, Canada). A 485 nm laser diode (Picoquant, Germany) was used as an excitation light source, running in CW mode. In this case, CW excitation produces a higher fluorescence signal rate resulting in shorter dwell times for a given signal to noise ratio and consequently higher acquisition speeds.

STED microscope setups

Both STED microscopes employed beam scanning for fast image acquisition; a TILL Photonics Yanus III scanning unit (Munich, Germany) was used with the pulsed STED system and a 15 kHz resonant scanner (SC-30, EOPC, USA) in the CW STED system (4). 4F lens configurations were implemented in each setup to image the scanning mirror(s) in the back focal plane of the objective lens in order to keep the intensity and phase distributions of the beams stationary in that plane while scanning the excitation and STED beams through the sample. 1.4 numerical aperture, oil immersion objective lenses

were used (Leica Microsystems, Mannheim, Germany and Olympus, Tokyo, Japan). In both configurations, the STED pattern featuring a zero-intensity-point in the focal plane on the optical axis was created by a vortex polymer phase mask inducing a radial phase ramp from 0 to 2π (RPC Photonics, Rochester, NY, USA) in the wavefront of the STED beam in the back focal plane of the objective lens. Dichroic mirrors (AHF Analysentechnik, Tübingen, Germany) were used to combine the excitation and STED light into a common beam path before the scanning units of each setup and allow the passage of fluorescence light to the detector. For pulsed STED an avalanche photo-diode (idQuantique, Geneva, Switzerland) was used for detection; for CW STED, depending on the brightness of the sample, either a fast photo-multiplier tube or an avalanche photo diode was used (4).

STED imaging parameters

In Table S1 the imaging parameters used to obtain the data shown in this letter are summerized for reference. The optimum parameters depend largely on the sample to be imaged. Bright samples are forgiving of sub-optimal imaging parameters, whereas weakly labeled samples require careful optimization to obtain satisfactory images. As a rough guideline, the STED power for initial measurements is chosen such that the STED image is half the brightness as the confocal image, provided that the pixel size and pixel dwell times are the same for both imaging modes. Appropriate pixel sizes must be employed to provide satisfactory spatial sampling (adhering to the Nyquist-Shannon sampling criterion) at the obtained resolution. It is advisable to use the shortest pixel dwell time (i.e. scan as quickly as possible) necessary to acquire the desired signal-to-noise ratio, both to increase the speed of image acquisition and minimize photodamage in the sample. STED pulse durations significantly shorter than ~1 ns were not employed for imaging in this work and may also work well.

Wavelength (nm)	Repetition Rate (MHz)	Pulse Length (ns)	STED Power (mW)	Excitation Power (μW)	Pixel Size (nm)	Pixel Dwell Time (μs)
556	20	0.7	4	6	50	50
560	20	0.9	4	6	50	50
570	20	0.7	8	6	25	20
592	Continuous Wave	—	250	14	30	8

TABLE S1 Summary of the relevant imaging parameters used to obtain the data presented in this Letter. Optical powers were measured at the back aperture of the microscope objective lens. The values here are given as guidelines, and are by no means the only which can be used to obtain acceptable images.

Yeast cells

Standard methods were used for growth and manipulation of yeast strains. The yeast strain expressing the epitope-tagged fusion protein Nce102-GFP was purchased from Invitrogen (Carlsbad, CA, USA). For imaging, yeast cells growing in agar YPD plates for 24 hours at 25˚C were scraped from the plate, washed, and mounted in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7) immediately before imaging. Alternatively, yeast cells were grown in liquid YPD growth medium to logarithmic growth phase, being harvested by centrifugation and mounted in PBS immediately before imaging. After placing a drop of yeast cells suspended in PBS on the microscope slide, the coverslip was gently pressed against the slide to immobilize the cells.

Mammalian cells

PtK2 *(Potorous tridactylus* kidney cells) and Vero (*Cercopithecus aethiops* epithelial kidney cells) cell lines were grown and transfected as previously described (5). To tag Keratin19 at the C-terminus with GFP, the expression plasmids pMDKrt19-GFP was constructed by Gateway vector conversion (Invitrogen, Carlsbad, CA, USA) from the donor vectors pDONR223-Krt19 and the empty destination vector pMD-GFP-N. The plasmid targeting the GFP to the ER was constructed using standard cloning techniques. Cells were transfected and cultured on coverslips at least 24 hours prior to imaging.

C. elegans

Worms were raised at 20˚C on NGM media seeded with *E. coli* OP50. The strain TV432 was used for visualizing NSM neurons. TV432 was created by integrating Ptph-1:gfp. Ptph-1 is a promoter region of the tryptophan hydroxylase gene that drives expression of GFP cell-specifically in NSMs (6). Strain LX837 was also used for visualization of NSM. Nematodes were mounted on agarose pads on microscope slides with a drop of M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$) containing 0.05% tricaine and 15 mM levamisole for anesthetization for the purpose of immobilization (7).

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