Imaging Chromophores With Undetectable Fluorescence by Stimulated Emission Microscopy

Supplementary Information

Detailed apparatus

10W solid-state 532nm green laser (Millennia, Spectral-Physics) is used to pump a Ti-sapphire oscillator (Mira, Coherent) to produce a 2W mode-locked 100fs 76MHz pulse train at 830nm. This pulse train is then split and used to synchronously pump two fs optical parametric oscillators (OPOs) simultaneously: the first OPO's (Mira-OPO basic, Coherent) output wavelength is tuned by tuning the color of the pump Ti-sapphire output (under 830nm Ti-sapphire pumping, this OPO signal wave is at 1180nm), and the second OPO (Mira PP-OPO, Coherent) is tuned by adjusting the cavity length of the OPO (the range of the signal wave is between 1100nm and 1400nm).

For imaging of chromoproteins, X-gal hydrolysis product and TBO drug distributions, the first OPO output is frequency doubled outside the cavity by a BBO crystal to generate a fs pulse train around 590nm as the excitation beam, and the second OPO is intra-cavity doubled to generate its second harmonic signal between 550nm and 700nm as the stimulation beam.

For imaging blood vessels, a home-made mode locked Yb pump laser and two home-made synchronously pumped OPO are used. A high power Yb laser was built to operate at 75MHz, ~200fs, at power levels of 10W at a wavelength of 1040nm. More than 60% conversion to the second harmonic generation at 520nm is routinely achieved with an angel tuned LBO crystal at room temperature (theta=90 degrees, phi=13 degrees, 4mm

long, Casix). The doubled 520nm is then used to synchronously pump two homemade OPOs, which both use temperature tuned 6mm long LBO crystal. By changing the temperature of the crystal, the signal wave is tunable from 680nm to 1000nm, and the idler wave is available at wavelengths from 1080nm to more than 2000nm. The signal wave from the first OPO provides 830nm beam for two photon excitation of the heme, and the idler wave (~1200nm) from the second OPO is frequency doubled outside the cavity by a BBO crystal to generate a 200 fs pulse train ~600nm as the stimulation beam.

For experiments with both one photon and two photon excitation, the excitation beam and the stimulation beam are spatially overlapped with a dichroic beam splitter. Temporal delay between two excitation and stimulation pulse trains is set with a translation delay-stage and measured with an autocorrelator (APE GmbH). The exact time zero is adjusted by optimizing the coherent anti-Stokes Raman scattering signal around 534nm generated by the pump beam at 590nm and Stokes beam at 660nm.

The excitation beam is modulated by an acousto-optical modulator (AOM) (model 3080-122, Crystal technology) at 5 MHz which is driven by a square-wave function generator. We note that the AOM crystal adds significant chirp to the pulses. To compensate for this, a pulse compressor consisting of a pair of SF11 prisms (Thorlabs) is built into the excitation beam path to control its pulse width.

Excitation and stimulation beams are coupled into a modified laser scanning inverted microscope (IX71, FV300, Olympus). The beam size is matched to fill the back-aperture of objective. A 60X 1.2 N.A. water objective (UPlanSApo, Olympus) is used for excitation, and a 20X 0.95 N.A. long-working distance objective (XLUMPlanFI, water,

Olympus) is used as a condenser. Another lens is used to image the scanning mirrors onto a silicon amplified photo-diode (PDA36A, Thorlabs) to avoid beam movement during laser scanning. Two high OD bandpass filters (HG650/45X, Chroma Technology; Brightline fluorescence filter 655/40, Semrock) are used together to block the excitation beam completely and only transmit the stimulation beam. For imaging blood vessels, high OD filters (3^{RD}800SP and 3^{RD} 760SP, Omega Optical) are used together to block the two photon excitation beam completely.

The output of the photodiode is bandpass filtered (15542, DC-48MHz low-pass filter, Mini-Circuits) to suppress the strong signal at the pulsing repetition rate (76 MHz), and then terminated with 50Ω. A high-frequency lock-in amplifier (SR844, Stanford Research) is used to demodulate the stimulated emission signal. The analog on phase component x-output of the lock-in amplifier is fed into the A/D converter of the laser scanning microscope (FV300, Olympus) input. The time constant is set for 1 sec and 100 μs under spectroscopy and microscopy experiments, respectively. For imaging, 512 by 512 pixels are acquired. ImageJ is used to process the data.

When the stimulation beam is physically blocked, stimulated emission images vanish. We have double checked this on all of our samples (it is simple to check experimentally).

Sample preparations

High purity crystal violet powder is used as purchased (Sigma Aldrich). Aqueous solutions are prepared with deionized water. For spectroscopy, we built a flow-cell from

two No.1 coverslips and a spacer (~130μm) to allow quick concentration exchange without moving the sample position or focusing depth inside the sample. The absolute concentration is checked by a UV-vis spectrophotometer around 590nm.

Chromoprotein gtCP was expressed in the DH10B *E. coli* using pQE30 expression vector without induction. After cell growth in LB medium at 37 °C to A_{600} of 0.6, the culture was moved to 22°C shaker for 24 -36 hrs to ensure complete maturation of the chromophore. cjBLUE was overexpressed from a pRSETB vector in BL21(DE3) *E. coli* cells. After growth in LB at 37 °C, expression was induced with 1mM IPTG at A_{600} of 0.6 and moved to 22 °C shaker for 36 -48 hrs.

Wild-type *E. coli* cells are incubated with 50 μM X-gal solution in 37ºC for 30 min, and then concentrated and sandwiched between two No. 1 coverslips. No inducer for lacZ gene is added.

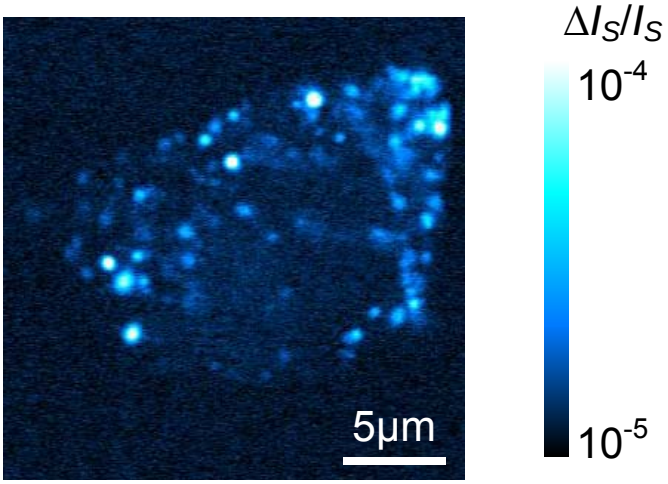
Toluidine blue O (TBO) is used as purchased (Sigma Aldrich). Human embryonic kidney (HEK) 293 cell line was obtained from American Type Culture Collection (ATCC, Rockville), HEK 293 cells are maintained in DMEM (ATCC) supplemented with 10% fetal bovine serum (ATCC) at 37 ºC in a humidified 5% CO_2 air incubator. Cells are cultured on uncoated glass bottom dishes (P35G-1.0-14-C, MatTek Cooperation). The image is taken one hour after incubating the cells with 10 μM TBO/PBS solution.

Mouse skin tissue from wild-type white mice is obtained from Harvard Mouse Facility. Thin ear is harvested for drug incubation immediately after sacrificing the mouse. Approximately 25 μl of a 10 μM TBO/PBS solution is pipetted onto a 5X5 mm piece of

skin surface, and the tissue is then incubated at 37 ºC and saturating humidity for one hour. The whole ear tissue is then placed between two No. 1 coverslips for imaging.

Absorption spectrum is taken under a spectrophotometer (DU 800, Beckman Coulter).

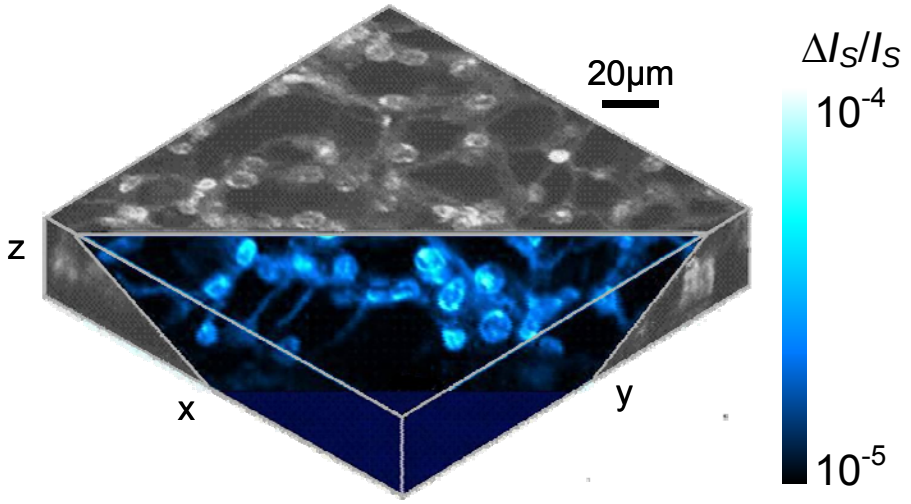
Sup. Figure 1. TBO distribution in a human embryonic kidney (HEK) 293 cell, one hour after incubation of 10 µM TBO/PBS solution. Local accumulation of the dye is observed inside cytoplasm instead of in the membrane or the nucleus. Color table (also for Fig. 4 a and b): cyan hot. The brightest and darkest colors correspond to ~ 10^{-4} and ~ 10^{-5} of the relative modulation depth, respectively.



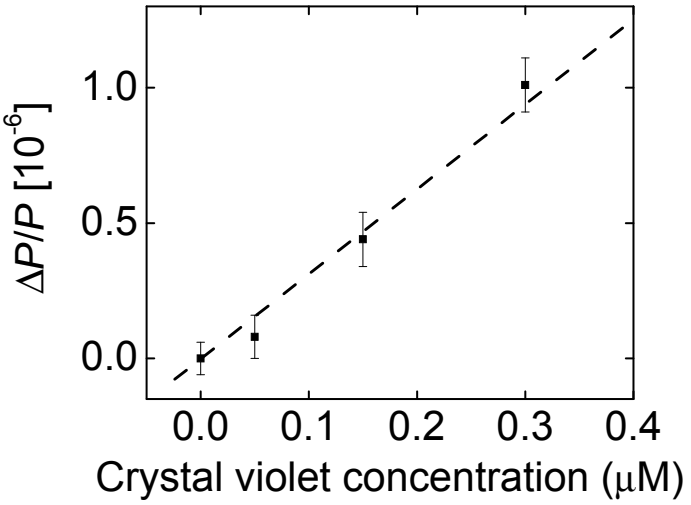
Sup. Figure 1.

3D optical sectioning of hematoxylin stained kidney tissue

Imaging chormophore stained tissues with intrinsic 3D optical sectioning is another application. Various types of chromophore staining are widely used in histology for medical diagnosis. For example, hematoxylin, as in H&E staining, is widely used to stain basophilic structures such as nuclei. In the conventional approach, thin sections have to be physically cut piece-by-piece, because the traditional wide-field transmission microscopy does not have optical sectioning ability. As a result of the nonlinear intensity dependence, stimulated emission microscopy can selectively record images at different depths (Sup. Fig. 2) without being affected by the out-of-focus contribution, because the signal is only generated at the laser focus, where the laser intensity is the strongest. 590nm and 660nm for excitation and stimulation, respectively, are used for imaging hematoxylin. Hematoxylin stained tissue sample is used as purchased (Science Stuff). The kidney IS. is from Model B17106.



Sup. Figure 2.



Sup. Figure 3. An enlargement of the lowest-concentration data points of Fig. 2d.