Ultrafast dynamics microscopy

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We report the three-dimensional imaging of the vibrational, solvent, orientational, and electronic relaxation in organic fluorescent samples at 200–500 nm spatial resolution. This is achieved in steady-state recordings by exciting the fluorophore with a femtosecond pulse and subsequent quenching with a time-delayed, redshifted femtosecond pulse through stimulated emission. Temporal resolution of 380 fs is solely determined by the pulse widths and is further reducible. Images of submicron structures revealing vibrational and solvent relaxation gradients are shown. Furthermore, we introduce contrast modes based on stimulated emission depletion and apply them to cellular imaging. © 2000 American Institute of Physics.

Imaging the excited state lifetime is of great interest in fluorescence microscopy because the relaxation of fluorescent molecules provide important information about their chemical environment. Therefore, a number of methods have been developed to quantify and map excited state lifetime in microscopy, relying both on time as well as on the frequency domain measurements, and using both continuous wave and pulsed lasers. For a review see Refs. 1–3, and the references therein. In all cases, the attained resolution was limited to a few tenths of nanoseconds, so that only slow transitions, such as fluorescence emission were quantified.

In this work, we report the improvement of resolution of lifetime microscopy by three orders of magnitude using the effect of stimulated emission. Our approach is particularly convenient as it only involves steady-state recordings. The attained temporal resolution allows us to establish ultrafast processes, such as vibrational, solvent, and orientational relaxation as contrast modes in microscopy with the potential for retrieving hitherto unavailable information about the variation of the molecular dynamics in the sample.

Stimulated emission has been introduced and developed in microscopy4–7 as a powerful tool to deplete the fluorescent excited state \( S_1 \) of molecular probes. At the same time, stimulated emission has been developed in cuvette based spectroscopy where it enabled the study of the dynamics of organic molecules with femtosecond resolution.8–11 Whereas in a cuvette experiment stimulated emission can be readily used for light amplification,10 in microscopy the limited number of molecules (< 10⁴) restricts the use of stimulated emission to inhibition of fluorescence.5

The underlying principles are sketched in Fig. 1. An ultrashort pump pulse (≈280 fs) excites the molecule to a Franck–Condon state \( S_1^o \), from which the molecule relaxes nonradiatively within \( \tau_{\text{nr}} \ll 1 \) ps to the fluorescent state \( S_1 \). After an arbitrary delay \( \Delta t \), the excitation pulse is followed by a more intense stimulated emission depletion (STED) pulse (≈240 fs) whose wavelength is in the (red edge of the) emission spectrum of the dye, coupling the \( S_1 \) to empty, higher vibrational substates \( S_0^h \) of the ground state. From \( S_0^h \) the molecule further relaxes to \( S_0 \) by vibrational relaxation. The respective populations are denoted with \( N_0 \), \( N_0^h \), \( N_1 \), and \( N_1^h \), the lifetime of \( S_1 \) with \( \tau_1 \), the stimulated transition cross section with \( \sigma \), and the STED pulse photon flux with \( h(t) \). It is predicted that if the rate of the stimulated transition \( h(t)\sigma(N_1^h - N_0^h) \) is of the order of that for spontaneous relaxation \( k_{\text{fl}} = \tau_1^{-1}N_1 \) or higher, an ultrafast STED pulse of duration \( \tau_{\text{STED}} \) effectively forces molecules to \( S_0^h \) by stimulated emission.4,8 The result is a reduction of \( N_1 \) within \( \tau_{\text{STED}} \) that is manifested as a decrease of total fluorescence emission.

An attempt of stimulated emission lifetime microscopy6 involving asynchronous pumping and phase demodulation detection attained temporal resolution of \( \tau_{\text{res}} \approx 1–5 \) ns. In contrast, the steady-state lifetime microscopy reported herein realizes a resolution \( \tau_{\text{res}} \approx 380 \) fs. \( \tau_{\text{res}} \) is determined by the pulse width alone and therefore further reducible. Through varying the time difference \( \Delta t \), STED monitors the full evolution of \( S_1 \) over four orders of magnitude in time,12 that is from the femtosecond scale of intramolecular vibrational relaxation up to the nanosecond scale of spontaneous emission.

Our microscope [Fig. 1(a)] employs a mode-locked Ti:sapphire laser (Coherent, Santa Clara, CA) emitting a \( f_r = 76 \) MHz train of 160 fs pulses at \( \lambda_{\text{STED}} = (735–750) \) nm. At this pulse width the spectral width of the pulse is 100 cm⁻¹ meaning that possibly more than one vibrational substate may be involved in the transition. The Ti:sapphire light is partly up-converted into \( \lambda_{\text{EXC}} = (550–555) \) nm by a synchronously pumped intracavity second harmonic generation optic parametric oscillator (OPO, APE GmbH Berlin, Germany). A delay path ensures that the STED pulse follows its up-converted counterpart by \( \Delta t \). The temporal resolution of the microscope is given by the width of the intensity cross-correlation function of the two pulses, which we established as \( \tau_{\text{res}} \approx 380 \) fs by two-photon excitation of an ultraviolet-absorbing dye.9 Imaging is accomplished by piezoscanning the sample. Using a 1.4 numerical aperture oil objective we obtained a focal volume of 20–30 attoliter, as defined by the full width half maxima of the main diffraction maximum. Due to the setting of the wavelength, we investigated dyes
with emission spectra in the 640–750 nm range, such as Pyridin 2 and Nile Red (Lambda Physik, Göttingen, Germany).

Figure 1(b) depicts the evolution of the fluorescent population $N_1(t)$ initiated by the excitation pulse, as predicted by a four-level model, with and without STED. The assumed pulses of 200 fs duration feature a Gaussian temporal shape. The maximum of the STED pulses arrive with a delay of $\Delta t_1 = 150$ fs and alternatively with $\Delta t_2 = 400$ fs. We further assumed $\tau_{\text{ vib}} = 100$ fs, $\tau_0 = 0.5$ ns, and a time-averaged value of $h\sigma = 1.5 \times 10^8$ s$^{-1}$. The onset of $N_1(t)$ without STED is determined by the vibrational relaxation from $S_1^\ast$, $\tau_{\text{ vib}}$, and the excitation pulse width. The timing of the STED pulse, $\Delta t$, is crucial for the efficiency of STED, which is defined as

$$\varepsilon(\Delta t) = 1 - \int \frac{N_1^{\text{with STED}}(t)dt}{\int N_1(t)dt}.$$  \hspace{1cm} (1)

The integrands denote the population of $S_1$ with and without STED pulse. $\varepsilon$ is unity for complete suppression of fluorescence. The residual fluorescence is larger for $\Delta t_1 = 150$ fs than for $\Delta t_2 = 400$ fs, because at $\Delta t_1$ the vibrational decay of $S_1^\ast$ is not completed [Fig. 1(b)]. In fact, $\varepsilon(\Delta t)$ probes the population $N_1(t)$. In Fig. 1(c), we displayed the function $1 - \varepsilon(\Delta t)$ over four orders of magnitude temporal range, revealing that STED monitors the full evolution of $N_1(t)$.12

Ultrafast lifetime images are readily obtained by recording a series of fluorescence images with different $\Delta t$ and subsequent comparison of their signal. The results of such an experiment is described in Fig. 2, where we imaged Pyridin 2 stained latex beads immersed in a Pyridin 2/1-pentanol solution. Whereas Fig. 2(b) displays a confocal intensity image sectioning the center of the bead, Fig. 2(c) shows the STED-efficiency image for $\Delta t = 26$ ps, representing spatial variations of $\varepsilon(\Delta t = 26$ ps). To disclose the temporal evolution of the excited state Fig. 2(a) displays $1 - \varepsilon(\Delta t)$ for the first 25 ps at fixed points in typical sample regions. The fitted time constant $t_i \approx \tau_{\text{res}}$ indicates the presence of a fast vibrational decay which is not fully resolved with the pulse widths available in this experiment. The surrounding solution reveals a somewhat slower component of $t_1 = 9.4$ ps which can be attributed to solvent relaxation.10 In the alcohols ethanol, propanol, pentanol, and butanol, we obtained $t_2 = 4.2, 6.4, 9.4$, and 16.2 ps, respectively. In these measurements data points are taken at intervals of 100 fs.

The recording of a set of images with this sampling density would involve unacceptably long recording times. However, the ultrafast temporal evolution of the excited state can also be represented by two $\varepsilon(\Delta t)$ values. Therefore, we introduce the $\Gamma$ contrast with $\Gamma(\Delta t_1: \Delta t_2) = [1 - \varepsilon(\Delta t_2)]/[1 - \varepsilon(\Delta t_1)]$. The corresponding $\Gamma$ image is shown in Fig. 2(d), disclosing variations of the solvent relaxation dynamics.

The STED-beam intensity is highest in the center of the diffraction maximum and falls off as dictated by diffraction. Therefore, $\varepsilon(\Delta t)$ is largest at the focal point and falls off accordingly. When imaging structures that are substantially smaller than the focal volume, the $\varepsilon$ and $\Gamma$ contrast mode also reflect the substructure of the focal intensity distribution of the STED beam. Clearly, the resolution of the STED-efficiency contrast modes are limited by diffraction, as in any standard far-field fluorescence microscope.

In the experiments shown in Fig. 2 the excitation and the STED pulse were linearly polarized in the same direction. Figure 3 displays two measurements of $1 - \varepsilon(\Delta t)$ of a Pyridin 2/ethanol solution, whereby the STED pulse was oriented parallel and then perpendicular to the excitation pulse and shifted over a time span of $0 \leq \Delta t \leq 1.5$ ns. Figure 3 reveals a strong dependence of the STED efficiency on the mutual orientation of the light field as anticipated from the selection rules for dipole transitions.8,10 For crossed polarization STED is less efficient, so that we define an anisotropy function $R(\Delta t) = (\varepsilon_\parallel - \varepsilon_\perp)/(\varepsilon_\parallel + 2 \varepsilon_\perp)$, the semilogarithmic plot of which is shown in the inset of Fig. 3, revealing a mono-exponential decay with $\tau_{\text{OR}} = (155 \pm 3)$ ps. Within about this time the excited molecules assume a random orientation in space. In pentanol we have measured a longer $\tau_{\text{OR}}$ of $(801 \pm 28)$ ps, which traces back to the ~4 fold higher viscosity of pentanol at room temperature. Hence, one can envisage imaging of the local viscosity in 20–30 attoliter volumes in three dimensions.
Figure 4 reveals the ultrafast fluorophore dynamics in the lipid structures of a yeast cell labeled with Nile Red (Phenoxazon 9, Lambda Physik, Göttingen, Germany). Figure 4(a) shows a confocal intensity image thereof, along with its \( \varepsilon(\Delta t = 26\) ps) image shown in (c), and the \( \Gamma(0.9\) ps; 26 ps) image in (d) map the ultrafast dynamics in the sample. The time-averaged power of the STED beam was 0.3 mW; \( \lambda_{\text{STED}} = 750\) nm and \( \lambda_{\text{EXC}} = 555\) nm.

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In conclusion, we have demonstrated that STED microscopy visualizes a range of ultrafast processes of the molecular excited state including vibrational, solvent, and orientational relaxation within a focal volume of 20 attoliter. Ultrafast dynamics microscopy by STED should allow the observation of specific dye couplings to other molecules or dye-environment interactions. Advances in pulse width reduction\textsuperscript{15} will enable ultrafast dynamics three-dimensional microscopy by STED with temporal resolution of 5–20 fs both in fixed and live cells.