Spectroscopic Rationale for Efficient Stimulated-Emission Depletion Microscopy Fluorophores

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For decades, the power of far-field fluorescence microscopy has been hampered by the diffraction resolution limit,1,2 $d = \frac{\lambda}{2NA}$ > 200 nm,3,4 where $\lambda$ denotes the light wavelength and NA the numerical aperture of the lens. Identical fluorophores closer than $d$ cannot be discerned because their signals cannot be separated by the detector. However, in the recent past, it has been demonstrated that the diffraction limit can be fundamentally overcome5 by modulating or switching the ability of the dye to emit fluorescence, causing adjacent objects to be registered sequentially. To this end, stimulated-emission depletion (STED) microscopy5,6 and its derivatives modulate the fluorescence capability of dye ensembles using defined spatial patterns of light, whereas the techniques known as (delta)STORM9,10 and related concepts11 involve switching individual fluorophores stochastically in space followed by mathematical localization of their coordinates. Hence, all of these concepts harness a molecular mechanism that keeps the dye nonresponsive when it is exposed to excitation light.

In STED microscopy (Figure 1),12 the dyes are kept nonresponsive (dark) by subjecting them to red-shifted light, called the STED beam, which induces stimulated emission from their fluorescent state, $S_1$, to their ground state, $S_0$. The maximum occupation probability of the $S_1$ state decreases nearly exponentially with the intensity $I$ of the STED beam. Applying the condition $I \gg I_s$, where $I_s$ is a dye constant, virtually confines the dye to the dark $S_0$ state, even if it is subject to excitation light. Usually formed as a donut, the STED beam is superimposed upon the regularly spaced excitation spot, formed by the focused excitation beam to keep dark all of the dye molecules that cannot be discerned because their signals cannot be separated by the detector. However, in the recent past, it has been demonstrated that the diffraction limit can be fundamentally overcome by modulating or switching the ability of the dye to emit fluorescence, causing adjacent objects to be registered sequentially. To this end, stimulated-emission depletion (STED) microscopy and its derivatives modulate the fluorescence capability of dye ensembles using defined spatial patterns of light, whereas the techniques known as (delta)STORM and related concepts involve switching individual fluorophores stochastically in space followed by mathematical localization of their coordinates. Hence, all of these concepts harness a molecular mechanism that keeps the dye nonresponsive when it is exposed to excitation light.

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Figure 1. Jablonski diagram indicating transitions relevant for STED. Scale bars under the excitation and focal spots represent 500 nm.

darkening is minimized, STED should provide nanoscale resolution on the ensemble level with nearly any fluorophore.

Therefore, avoiding any process that competes with stimulated emission is prudent. Tuning the STED beam wavelength to the red edge of the fluorescence spectrum largely avoids excitation from the $S_0$ state, but transitions originating from excited states may still occur, such as crossing to the triplet states, $T_n$, as well as absorption from $S_0$ and $T_n$ states with $n > 1$ (Figure 1). In fact, many bleaching pathways involve triplet states,14,15 which are effectively populated through the $S_0$ states. The higher $S_n$ states can also lead to bleaching directly.16 Both processes compete with favorable ultrafast nonradiative nonradiative $S_n \rightarrow S_1$ (or $T_n \rightarrow T_1$) relaxation followed by spontaneous or stimulated emission.13

With these conditions in mind, we explored the use of rylene dyes for STED microscopy. In view of their large extinction coefficient, brightness, and photostability (especially when applied in materials sciences), these dyes should, at face value, be ideal for STED. Specifically, we investigated tetraphenoxysubstituted perylenediimide (pPDI) and tetraphenoxysubstituted terylenediimide (pTDI)18 dyes, which have revealed remarkable photostability and low intersystem crossing in single-molecule experiments.17 Single pPDI and pTDI molecules can be registered for over 1 h without photobleaching and with few excursions to dark states.17

STED microscopy has been carried out with excitation light pulses followed by pulses for STED or with continuous wave (CW) beams for excitation and STED. The pulsed-mode setup has been reported elsewhere,6,19 while the CW system used here is detailed in the Supporting Information. First attempts with pPDI in the CW mode using a STED wavelength of 700 nm exhibited pronounced bleaching, as only $\sim 40\%$ of the molecules seen in the confocal image were also found in the STED image (Figure 2a,b). Using 76 MHz pulse pairs with a STED wavelength of 740 nm slightly reduced the bleaching; a resolution gain by a factor of $\sim 3$ was observed ($\sim 65$ nm; see Figure S2 in the Supporting Information) when a STED power of 33 mW was applied in the focal plane.

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For this power setting, ∼50% of the molecules could be observed after acquisition of the STED image. Using a lower repetition rate of 1 MHz reduced the bleaching to some extent, since the associated time between the pulse pairs (1 µs) allowed relaxation of dark states before the arrival of the succeeding pulse pair\(^{15,20}\) and gave slightly better resolution (55 nm; see Figure S3). In contrast, as shown in Figure 3, pTDI exhibited excellent performance at 76 MHz, with ∼95% of the molecules in the confocal image also emerging in the STED counterpart. The resolution obtained with the employed STED beam power was 35 nm (without further data treatment). pTDI showed equally good performance in the CW mode too, with 90% recovery and 75 nm resolution. Longer interpulse breaks were not required.\(^{20}\)

Since in the reported single-molecule (excitation only) experiments the photostability of pPDI was comparable to that of pTDI, we found the differences observed with STED rather puzzling. We therefore carried out femtosecond transient absorption measurements to elucidate the photophysics and photochemistry of the excited states of the two fluorophores.\(^{21}\)

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In the case of pPDI, the situation is different. While stimulated emission undoubtedly occurs, the fluorophore can effectively absorb a photon and move on to a higher-energy \(S_n\) state. The fluorophore can rapidly (within <5 ps) convert back to the \(S_1\) state, from which...
it fluoresces, undergoes stimulated emission, or re-enters the same cycle, each with a probability corresponding to the transition probability of the respective process. Unfortunately, non-negligible excited-state absorption fosters repeated excitation to the $S_n$ state and hence photobleaching, either by facilitating crossing to the $T_n$ state or by starting a bleaching reaction straight from the $S_n$ state. Clearly, the average number of cycles into higher excited states per fluorophore increases with smaller $\rho$.

The deconvolution of the transient absorption spectrum (500–740 nm) into the corresponding ground-state absorption, stimulated-emission, and excited-state absorption spectra reveals that pPDI has no “good wavelength band” to achieve de-excitation without substantial excitation to higher states. Nevertheless, when the (photoinduced oxidation) bleaching pathways from the higher states or the triplet state are blocked by working under a nitrogen atmosphere, improved STED images of pPDI can be recorded, as shown in Figure S4. Like the 1 MHz repetition rate experiment, this finding indicates the participation of long-lived (triplet) states in bleaching while also underscoring the relevance of avoiding pumping to higher states in STED microscopy.

In conclusion, our work provides a rationale for the suitability of dyes for STED microscopy. We have demonstrated that the excited-state properties are essential for transiently keeping the fluorophore dark by stimulated emission. Dyes for which the $S_1 \rightarrow S_n$ absorption spectrum is shifted with respect to its stimulated-ethanol counterpart satisfy the prerequisite for excellent performance in STED microscopy. For a given fluorophore, the location of the excited-state absorption band is difficult to predict, but for many dyes, the excited-state properties have been published. For example, a dye with little $S_n \rightarrow S_1$ absorption is the red-emitting dye Atto647N, which to date has been the most popular dye for STED at $\sim$750 nm. Hence, our investigations suggest suitable fluorophores and guide the optimization of the experimental settings. Moreover, they pinpoint a chief criterion, namely, spectral decoupling of the stimulated-emission and $S_1 \rightarrow S_n$ bands, for designing and selecting dyes for a non-diffraction-limited far-field fluorescence microscopy that is based just on molecular state transitions.

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Supporting Information Available: Structures and complete chemical names of pPDI and pTDI, sample preparation for STED microscopy, details of the experimental setup for CW STED microscopy, STED images of pPDI with the 76 MHz system, CW STED images of pPDI under nitrogen, pulse-energy dependence of STED resolution, and a reference for triplet absorption. This material is available free of charge via the Internet at http://pubs.acs.org.

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