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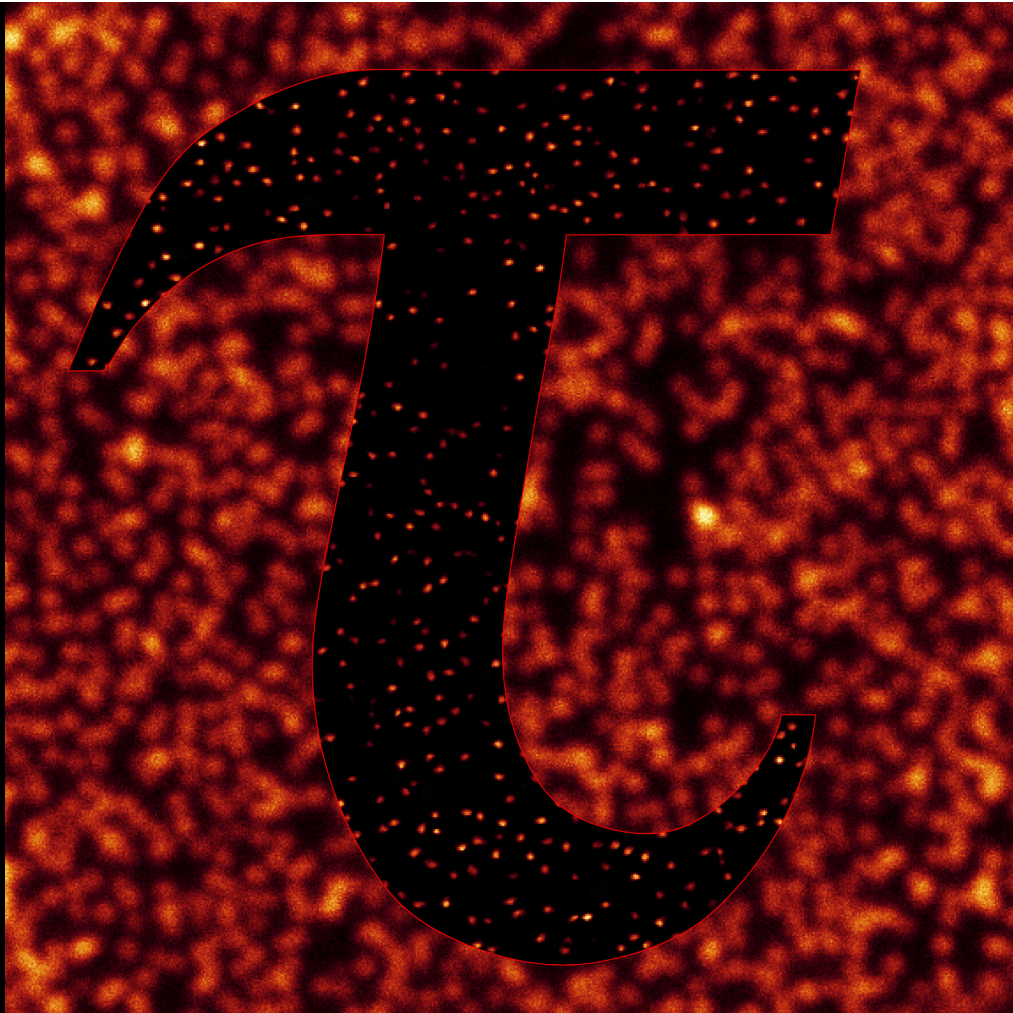
Application Note

NANOSCOPY MEETS LIFETIME: τ-STED

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τ-STED combines the optical signals from STED and the photophysical information from the fluorescence lifetime at unprecedented speeds.
This new approach to STED uses phasor analysis in a novel way. τ-STED enables an increased STED resolution and elimination of uncorrelated background noise, even at low excitation and STED powers.

Introduction

Since the advent of super-resolution fluorescence microscopies, the characterization of macromolecular complexes and cellular structures at the sub-diffraction level has become a reality. It also opened the door to the next challenge: make it work in conditions closer to the specimen's native state. Among the available approaches, STED* Nanoscopy (Hell and Wichmann, Opt. Letters 1994) is a widespread confocal-based technology delivering resolutions in the tens of nm and isotropic resolutions below one hundred nm, depending on factors such as fluorophore photophysics, labeling density, and labeling strategy.

* STimulated Emission Depletion

The STED principle relies on the ability to control the states of a fluorophore, i.e. emitting vs. dark states. In STED, the emission of fluorophores residing within a diffraction-limited spot is confined to a sub-diffraction region by overlaying a donut-shaped laser beam of appropriate wavelength (STED beam) onto the excitation beam. This forces the fluorophores affected by the STED beam to return to the ground state via an alternative path (stimulated emission), and the effective focal volume is reduced below the diffraction limit (Fig. 1).

The competition between fluorescence emission and STED to send molecules to the ground state can be explored using fluorescence lifetime (the average time that a fluorophore spends in the excited state). Essentially, any additional process competing with the spontaneous fluorescence emission (stimulated emission in this case) will shorten the fluorescence lifetime of the fluorophore. In STED imaging, the fluorescence lifetime of the fluorophore has a maximum at the center of the STED donut-shaped beam, and decreases across the donut profile, as the STED competing process generates an alternative route for de-excitation (Fig. 2). Pioneer work utilizing fluorescence lifetime to enhance STED performance originated the concepts of gated STED (Vicidomini et al., Nat. Methods 2011, Vicidomini et al., PLoS One 2013, Vicidomini webinar) and more recently SPLIT (Separation of Photons by LIfetime Tuning), based on phasor analysis (Lanzanò et al., Nat Comm 2015), and phasor-STED (Wang et al., Nanoscale 2018). These concepts overcome issues associated with intensity-based approaches to improve STED resolution, such as significant image quality loss due to high light dose (excitation and STED) and artifacts derived from adaptive illumination.
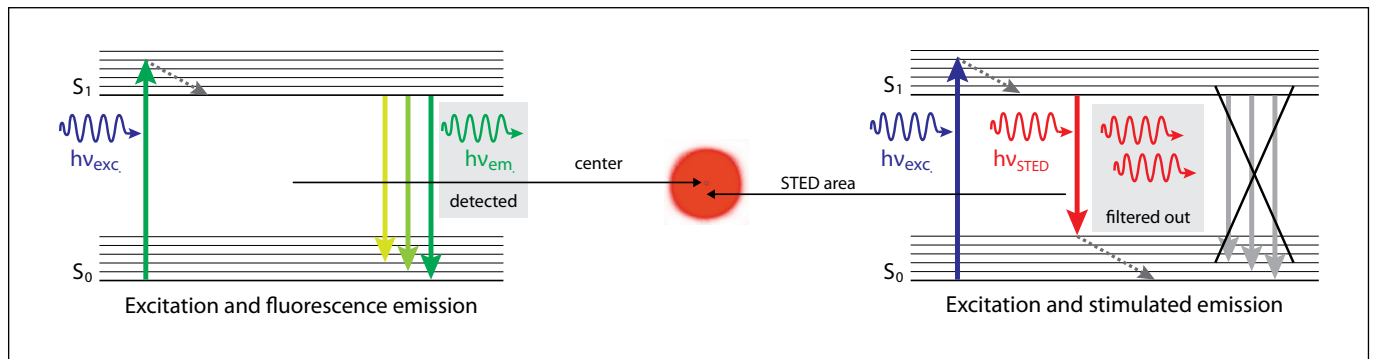

Figure 1: The involved photophysical processes are confined to different areas of the STED scanning spot. The conventional excitation of the fluorophores that is followed by spontaneous emission of photons dominates inside the ring, where the STED intensity is close to zero. The STED laser depopulates the excited electronic state S1 by inducing stimulated emission in the periphery. The released photons are indistinguishable from the STED laser photons and spectrally filtered out. The process is not related to bleaching and can be repeated many thousand times.
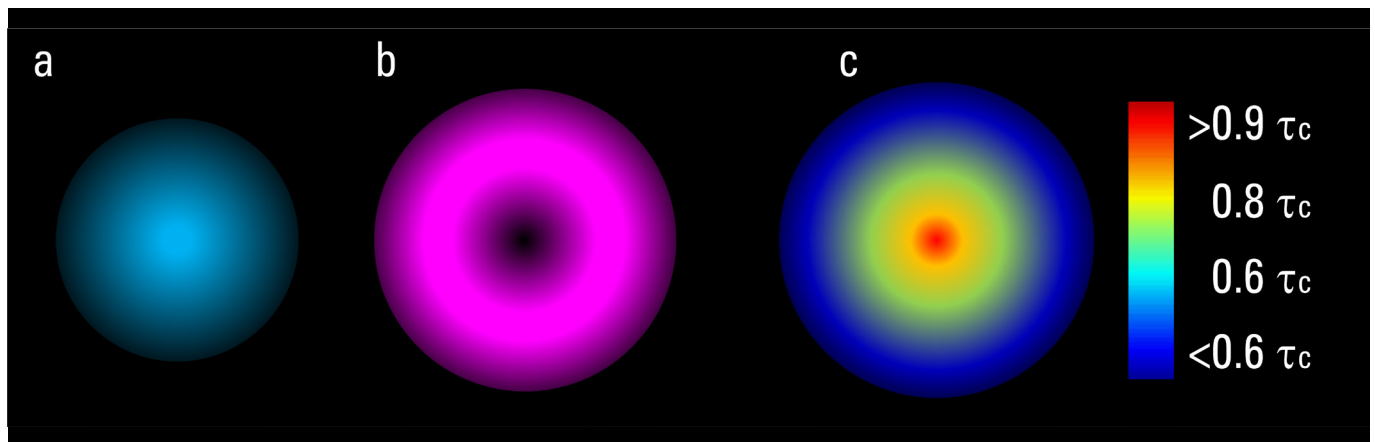

Figure 2: Schematic of Gaussian excitation and STED profile (a, b). Schematic representation of the lifetime distribution in the STED PSF (c). The fluorescence lifetime is shorter where the depletion is stronger, τc indicates fluorescence lifetime in the absence of STED.

Inspired by these approaches combining STED and fluorescence lifetime, we developed a new concept for STED Nanoscopy: τ-STED. τ-STED exploits the fluorescence lifetime gradient induced by the STED beam in a novel way to identify and remove uncorrelated background, improve image quality and increase image resolution in an automated way.

τ-STED is only possible thanks to the unique combination of STED, ultra-fast photon-counting detectors, and FLIM as provided by the FALCON approach (Alvarez et al., Nat Methods 2019). The main benefits of τ-STED are at two levels: first, τ-STED identifies the signal in the sample coming from the STED process and singles it out from background and spurious contributions, using fluorescence lifetime information available from the FALCON approach; second, it decodes the fluorescence lifetime gradient to deliver the desired resolution at dramatically lower light dose, (Fig. 3) or to push the resolution (Fig. 4) beyond what conventional STED provides. The results are crisp, high quality images at cutting-edge resolution better than 30 nm (lateral) and 100 nm (axial).*
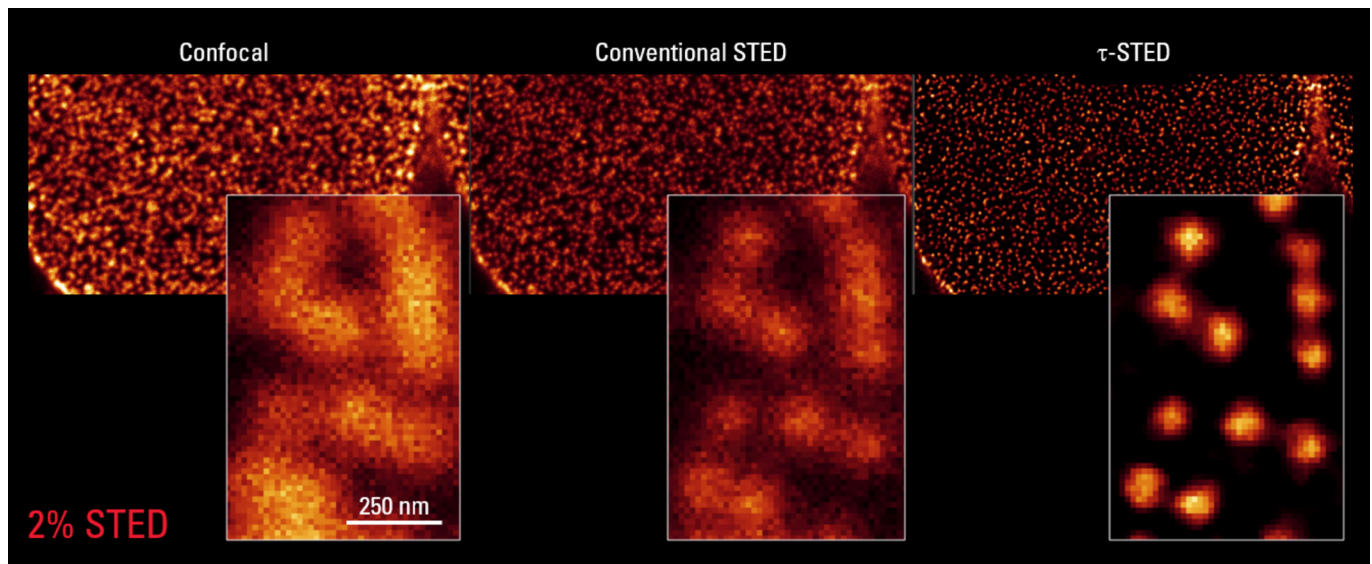

Figure 3: τ-STED 660 at extremely low STED power on COS7 cells immunostained for nuclear pores (NUPs). The primary antibody recognizes several proteins of the nuclear pore basket, so the NUPs are labeled in the central region and as a result they appear as point-like structures in the images.
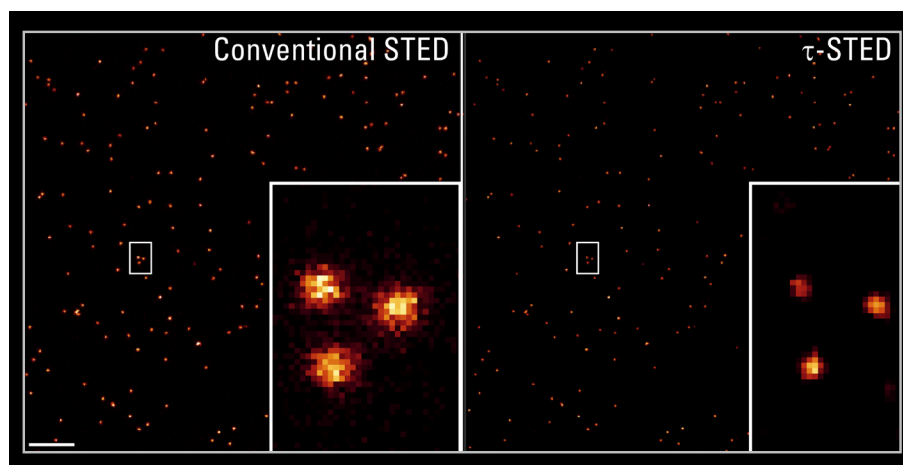

Figure 4: τ-STED imaging of GATTA-Beads 23 nm. Scale bar: 1um. Resolution (FWHM) better than 30 nm.

*depending on sample and fluorophore

τ-STED works for multi-color applications with 2D or 3D STED in live and fixed specimens (Fig. 5). The lower excitation and STED light dose translates into protection for the specimen, longer time-lapse experiments (more frames), or large volume imaging, without sacrificing spacial resolution. The raw data is always available for validation and quantification of results, avoiding setting artificial intensity thresholds and image masks that can miss key features and distort the outcome of the imaging.

Combined with FALCON, it is possible to perform multi-fluorophore STED with lifetime-based species separation, and take advantage of the use of the most suitable STED fluorophores that emit in the far-red spectral range. These dyes show a strong spectral overlap and cannot be combined in conventional STED with intensity-based only readout; instead, they can be distinguished by their distinct fluorescence lifetimes with a single detection window (Fig. 6).

With τ-STED it is now possible to perform:

> STED imaging at cutting-edge resolution with a dramatically lower light dose

> Gentle live-cell STED imaging for extended time-lapse experiments
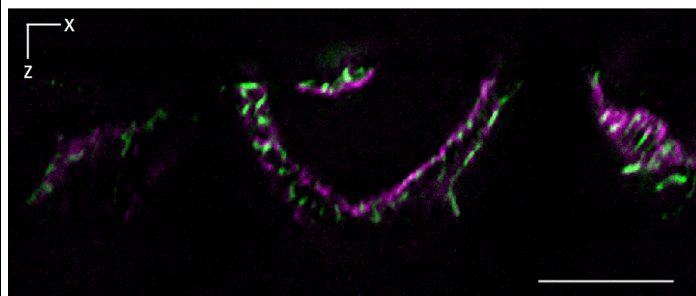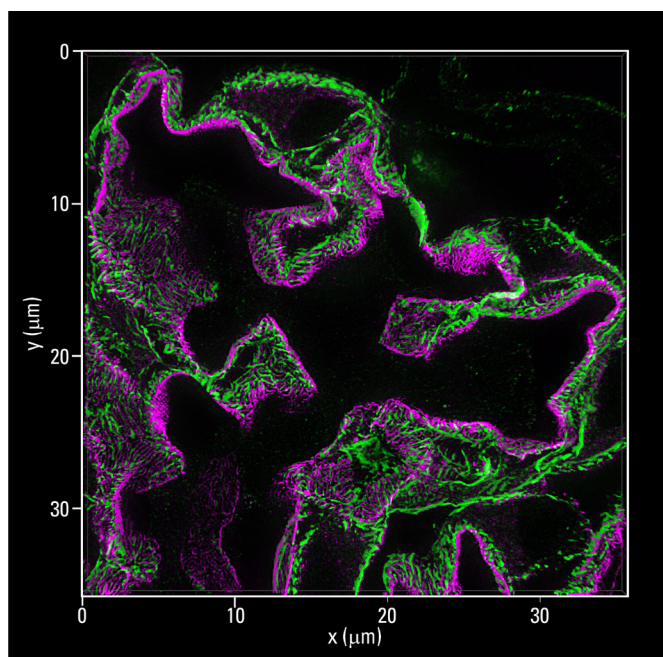
> Multicolor applications with the best STED probes

Figure 5: Two color, 2D STED (left) and 3D STED (right) imaging of mouse kidney section immunostained for synaptopodin (green) and nephrin (magenta). Sample courtesy: Dr. Victor Puelles, Dr. Milagros Wong and Dr. Jan Czogalla, Universitätsklinikum Hamburg-Eppendorf, Hamburg.
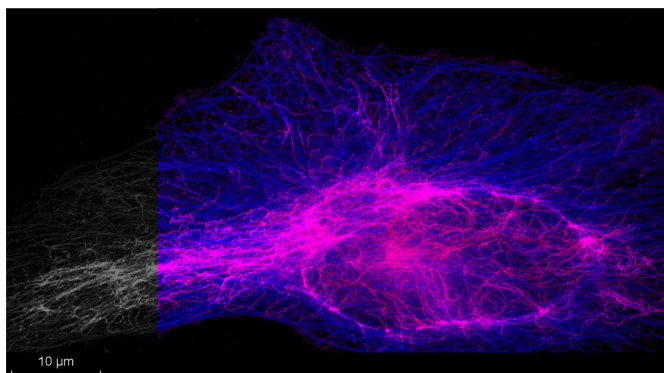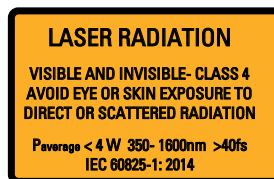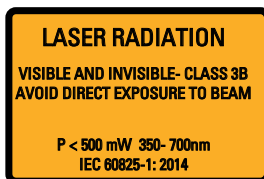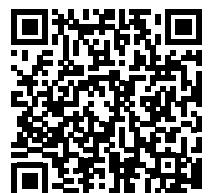

Figure 6: Multi-fluorophore STED-FALCON imaging of cell cytoskeleton. Vimentin immunolabeled with Atto647N (magenta), and tubulin immunolabeled with Alexa Fluor 647 (blue). Both fluorophores are spectrally highly overlapping but have distinct lifetimes, therefore can be separated based on their fluorescence lifetime information. On the left (gray), the STED intensity image is displayed.

References

[1] Hell SW and Wichman J: "Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion-microscopy", Opt. Lett. 19: 780–82 (1994).

[2] Vicidomini G, Moneron G, Han K, Westphal V, Ta H, Reuss M, Engelhardt J, Eggeling C, Hell SW: "Sharper low-power STED nanoscopy by time gating", Nat. Methods 8(7), 571-573 (2011).

[3] Vicidomini G, Schönle A, Ta H, Han KY, Moneron G, Eggeling C and Hell SW: "STED Nanoscopy with Time-Gated Detection: Theoretical and Experimental Aspects", PLoS One 8 (1): e54421 (2013).

[4] L. Lanzanò, I. Hernández, M. Castello, E. Gratton, A. Diaspro, G. Vicidomini: "Encoding and decoding spatio-temporal information for super-resolution microscopy", Nat. Comm. vol. 6, p. 6701 (2015).

[5] L. Wang, B. Chen, W. Yan, Z. Yang, X. Peng, D. Lin, X. Weng, T. Ye, J. Qu, "Resolution improvement in STED super-resolution microscopy at low power using a phasor plot approach". Nanoscale , vol. 10 iss. 34, pp. 16252-16260 (2018).

[6] Vicidomini G., Webinar Time-Resolved STED Microscopy https://www.labroots.com/webinar/time-resolved-sted-microscopy (2020).

[7] Alvarez L, Widzgowski B, Ossato G, van den Broek B, Jalink K, Kuschel L, Roberti MJ, and Hecht F: "SP8 FALCON: a novel concept in fluorescence lifetime imaging enabling video-rate confocal FLIM", Nat Methods (2019). https://www.nature.com/articles/d42473-019-00261-x

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