APPLICATION NOTE

Leica MICROSYSTEMS

TauSTED: pushing STED beyond its limits with lifetime

The new TauSTED technology from Leica Microsystems sets the new standard for straightforward, gentle STED using lifetime-based information. TauSTED combines the optical signals from STED and the physical information from fluorescence lifetime at confocal speeds. This approach uses phasor analysis in a novel way, delivering outstanding STED resolution and image quality while removing background noise, even at low excitation and STED powers. This newfound flexibility opens new avenues for super-resolution applications in live specimens.

Stimulated emission depletion (STED) is one of the super-resolution techniques that have revolutionized fluorescence imaging, promoting a better understanding of the spatial distribution and relationships of cellular components and processes[1]. To realize STED in two dimensions, the diffraction-limited point spread function (PSF) is reduced to a subdiffraction-sized PSF by overlaying a donut-shaped laser beam ("STED beam") onto the excitation beam. STED provides a de-excitation path for the fluorophores to return to the ground state via stimulated emission (Fig. 1a), confining the volume from which fluorescence is observed up to a few tens of nanometers. This concept has also been extended to three dimensions.

Conventional STED approaches have been challenging to adapt to dynamic processes in live cells, mainly because of the light dose plus the photon budget and signal-to-noise ratio needed to attain such high resolutions[1]. These limitations are particularly relevant when the only available information is the fluorescence intensity signal versus the spatial coordinate. Pioneering work explored the use of fluorescence lifetime to gain information and enhance STED performance. This aspect of STED microscopy sparked the concepts behind gated STED, SPLIT and phasor-STED[2–5]. The STED process competes with the fluorescence emission, lowering the fluorescence

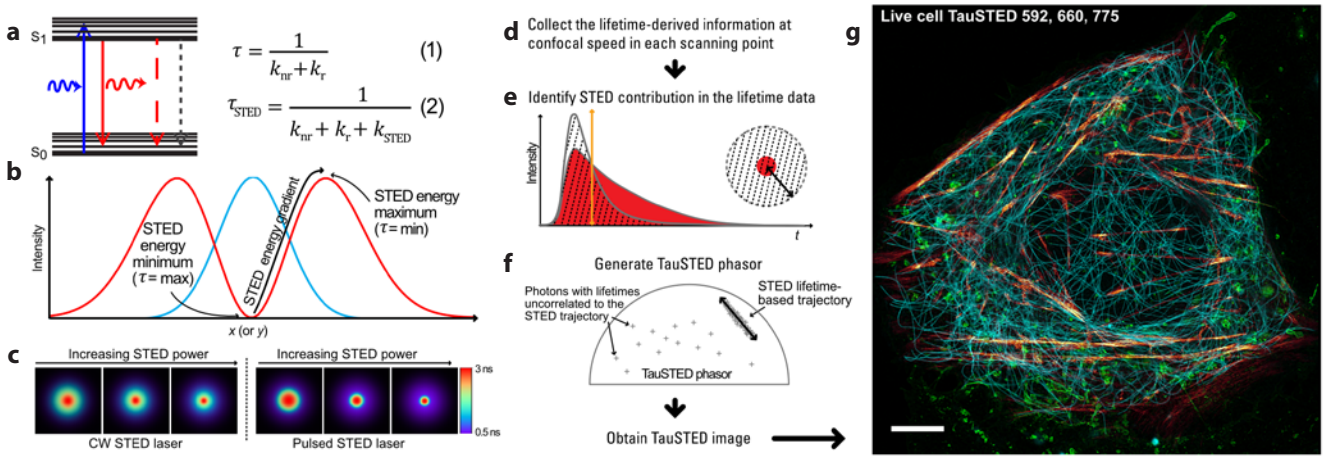

Figure 1 | Basic concepts of TauSTED. **a**, Jablonski–Perrin diagram: fluorescence process, fluorophore excitation (blue), photon emission (red), and competing processes (STED, red dashed arrow; non-radiative processes, dotted arrow). (1, 2): observed rates for fluorescence process with and without STED. τ, lifetime; k_r, k_{nr} and k_{STED}, rates of radiative, non-radiative and STED de-excitation, respectively. **b**, Radial line profile of excitation and STED energies, and related gradient. **c**, Simulated PSFs with intensity and lifetime-derived information, for CW STED and pulsed STED lasers (650 ps pulse). **d**, TauSTED acquisition pipeline. **e**, Temporal dynamics of fluorescence and STED process as lifetime decay curves and relationship to scanning spot geometry. Orange arrow, temporal position for a single gate-out approach; photons to the left are lost. **f**, TauSTED phasor. STED trajectory and additional signals from the image. **g**, Live-cell TauSTED in U2OS cells. Stains for actin (SiR-actin, glowscale), microtubules (SPY555-tubulin, cyan) and membranes (WGA-488, green). Scale bar, 2 µm. SiR and SPY probes available from Spirochrome.

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lifetime-derived readouts as the applied STED energy increases (Fig. 1, equations (1) and (2)). As a result, a lifetime gradient is generated on the effective PSF that follows the energy gradient of the STED beam geometry (Fig. 1b). Simulated data for lifetime-coded PSFs illustrate

this point for any scheme of STED depletion (continuous wave or pulsed) and at increasing STED powers (Fig. 1c). This information is in principle available at every scanning point in a STED experiment (Fig. 1d). The simplest approach to exploit this extra layer of information, gated STED, relies on a single time gate that excludes photons with early arrival times (Fig. 1e, cross-hatched region) that contribute low-resolution signal[2,3]. The overall resolution increases, but the signal from the photons that are excluded is lost. This temporal window (Fig. 1e, left of the orange arrow) still contains significant signal from the center of the donut, and omitting this information leads to lower signal-to-noise ratios than those observed for less-resolved, non-gated images. Therefore, it would be advantageous to preserve these photons. This can be achieved with the more advanced lifetime-based approaches mentioned above, but they require a deep knowledge of both nanoscopy and fluorescence lifetime imaging microscopy (FLIM), making their widespread adoption difficult[4,5]. Leveraging our fast FLIM and lifetime-based technologies[6,7], we have developed TauSTED (Fig. 1d–h), a new approach to STED super-resolution to deliver high quality, multicolor STED in two and three dimensions, compatible at the same time with live-cell imaging of highly dynamic processes. TauSTED combines the optical signals from STED with the physical information from the fluorescence lifetime acquired at typical confocal speeds. Our approach uses phasor analysis[8] in a novel way and works in an automated manner, enabling increased STED resolution and elimination of uncorrelated background noise even at low excitation and STED light dose.

For TauSTED, we first must describe the STED process in the lifetime dimension. We determine the two characteristic limits of the operative range of STED: the maximum energy delivered by STED sets the shortest lifetime in the sample while the minimum STED energy (at the center of the donut) marks the longest lifetime, where fluorophores undergo the least fluorescence lifetime change (Fig. 1b). These two values, mapped into a phasor plot, define a STED trajectory that will contain all the photons emanating from the STED process (Fig. 1f). Building the STED trajectory is far from trivial, especially because of the limited photon budget associated with STED measurements. We have automated this step in TauSTED by leveraging a newly published approach with a complex wavelet filter. This approach improves the determination of the fluorescence lifetime behavior on the phasor plot[8], preserving the fine structures in the image even at very low photon budget[9]. The result of this automation is a TauSTED phasor with two parameters: TauBackground Suppression, which removes background from the physical readout of lifetime contributions uncorrelated to the STED process, and TauStrength, which improves resolution from the lifetime-derived gradient of the STED trajectory mapped on the plot. A unique aspect of this approach is that the only information needed comes from the STED experiment itself; no additional confocal readout is required.

In TauBackground Suppression, the two-dimensional distribution of the lifetime fingerprints on the phasor space makes it straightforward to assess any signal contributions that do not emanate from the STED process (Fig. 1f). The photon signals with fluorescence lifetime signatures that are not correlated to the STED trajectory are most likely generated by noise and background, and these are precisely the signals that are removed (Fig. 2a). TauBackground Suppression can be activated and deactivated, providing a tool to evaluate its effect on the TauSTED image.

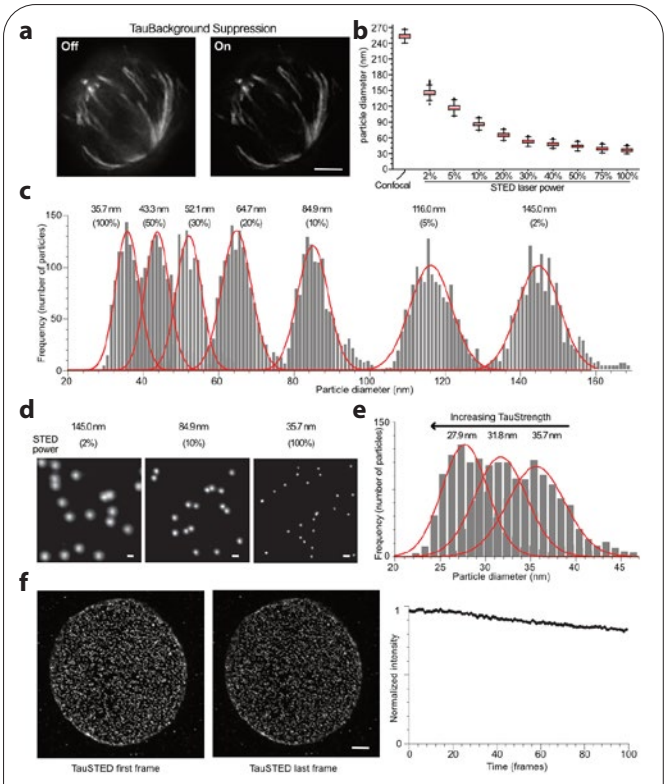

Figure 2 | TauSTED operating principles. **a**, TauBackground Suppression on a TauSTED image of tubulin (560nm-excitable fluorophore; see Fig. 3a). Scale bar, 2 µm. **b**–**e**, TauSTED 775 of GATTA-Beads R (nominal size 23 nm) at increasing STED dose (% of available STED power). **b**, Diameter estimation with a Lorentzian function fitting each particle. $n_{particles} > 500$ per condition. **c**, Distribution of nanoparticle diameters in the series. **d**, Representative images for data used in **c**. All images are adjusted to the same look-up table (0–100 photon counts). Scale bars, 200 nm. **e**, Effect of TauStrength on the diameter estimation at STED 100%. **f**, Time series on cells immunostained for basket proteins of nuclear pores (point-like structures) imaged at low excitation and STED light dose. 85% of the signal is preserved after 100 frames of continuous STED imaging, with ~80 nm FWHM for individual pores. Scale bar, 2 µm.

In TauStrength, the intensity values of the image are weighted on the basis of their positions with respect to the limits of the TauSTED trajectory. This weighting determines a range of values that correspond to a default scale and the resolution that can be achieved with it. The so-called TauStrength parameter can take any of those values. The scale can then be further tuned to push resolution along the resulting readout of the lifetime gradient, following the rules of phasors[8]. Here, as TauStrength values increase, the longer lifetime-containing signals are given more weight than the shorter ones. The limit to the achievable resolution is set by the photon budget and signal-to-noise ratio.

To illustrate how TauSTED works, we imaged a commercially available fluorescent bead sample (GATTA-Beads R, nominal diameter 23 nm), increasing the STED light dose and keeping the excitation

light dose constant. The sampling conditions (4k image format, 7 nm pixel size) and the beads density in the sample ensured a statistically significant assessment of the TauSTED performance ($n_{particles} > 500$ for each image; Fig. 2b–d). The results showed the expected decrease in the estimated diameter of the nanoparticles at increasing STED light dose, using the default settings for TauSTED (Fig. 2b). Once we have reached the optical limits, we can further push the resolution with TauStrength (Fig. 2e), where we estimate an average diameter for the GATTA-Beads R of (27.9 ± 2.1) nm.

The ability of TauSTED to increase the resolution at a given STED light dose enables gentle excitation and STED regimes while delivering high STED quality and resolution. We show this by performing continuous TauSTED imaging of a sample of Cos7 cells immunostained for proteins forming the nuclear pore basket (Fig. 2f) at extremely low STED light dose (5%). The labeling at the central region of the pores gives point-like structures as a result. The total intensity trace over time shows that 85% of the fluorescence intensity remains after 100 consecutive images, with an estimated nuclear pores central region size of 80 nm (from full width at half maximum (FWHM) measurements on individual structures). This means that we can protect the sample for extended periods to achieve longer time-lapse sessions and larger volumes.

TauSTED enables straightforward access to high fidelity, multicolor super-resolution imaging of live and fixed specimens (Figs. 1h and 3). We characterized the three-dimensional organization of the mitotic spindle (Fig. 3a) together with the distribution of CENP-C and BUB1, two key proteins required for kinetochore assembly[10], using TauSTED with multiple STED lines (592, 660 and 775 nm). We also achieved high resolution while examining delicate cristae in live mitochondria in HeLa cells[11] (Fig. 3b). The access to TauSTED with low photon budgets and the fast detection scheme in our confocal platform[12] enable the tracking of highly dynamic processes in live cells. As an example, we can follow the dynamics of the cytoskeleton and membrane vesicles (Fig. 3c).

TauSTED is the fusion of nanoscopy and lifetime, bringing excellent resolution, image quality and flexibility in a diversity of samples and experimental designs. STED benefits from gentle illumination for extended imaging of delicate specimens (more frames) or of larger volumes (more planes) without sacrificing resolution. The sample is protected by milder illumination regimes, particularly suited for sensitive fluorophores and for studies of cellular dynamics that require high imaging speeds. The outstanding performance of TauSTED at low light dose is key to rendering dynamic, live-cell super-resolution microscopy straightforward and accessible for researchers.

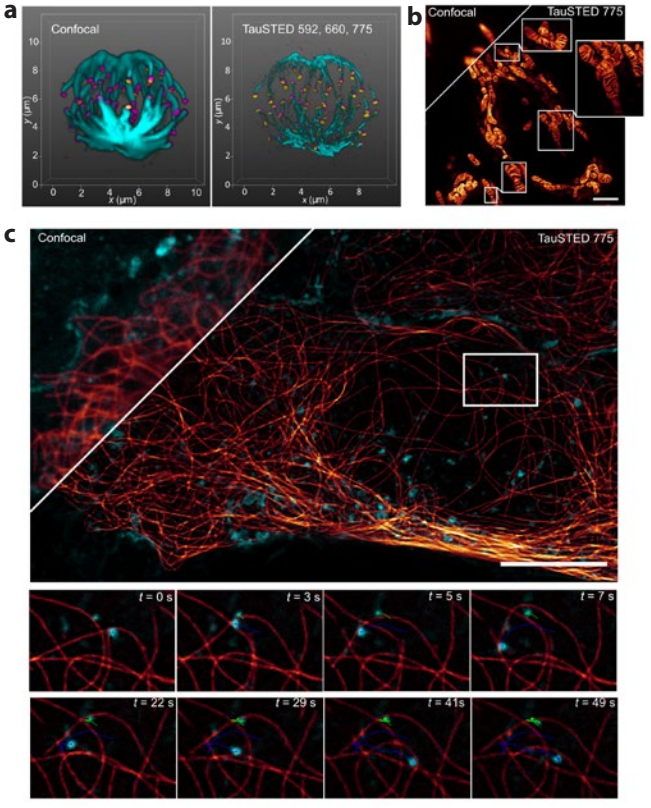

Figure 3 | TauSTED for biological applications. **a**, Three-color TauSTED 660, 592 and 775 on mitotic cells immunostained for tubulin (cyan; 560-nm excitation), CENP-C (yellow; 488-nm excitation) and BUB1 (magenta; 647-nm excitation). Sample courtesy Carlos Sacristan Lopez, Hubrecht Institute, Utrecht[10]. 3D movie at https://www.leica-microsystems.com/tausted-mitoticcell3d/. **b**, Live-cell TauSTED with STED at 775 nm: HeLa cells stably expressing COX8A-SNAP labeled with SiR-BG. Sample courtesy T. Dellmann and A. Garcia, CECAD, Köln. Cell line originally from S. Jakobs[11]. Scale bar, 2 µm. **c**, Two-color live-cell low power TauSTED; cytoskeleton (SPY-620 tubulin, glow) and vesicles (WGA-594, cyan). Scale bar, 5 µm. Fast dynamics captured at 1 frame/s. Montage shows a selection of time points from the inset; TrackMate Fiji plugin[13] traces three individual vesicles (red, green and blue). Movie at https://www.leica-microsystems.com/tausted-cell-dynamics/.

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