MICROSCOPY

STEDy progress

Continuing progress in the development of stimulated emission depletion microscopy (STED) is slowly but surely fulfilling the method's promise of ultrahigh-resolution fluorescence microscopy and may bring unexpected benefits to other imaging modalities as well.

The concept of STED microscopy was first described by Stefan Hell in 1994 and was demonstrated a couple of years later. Since then his research group has continued improving the technique, which recently has been instrumental in elucidating novel biological findings (Willig, *et al.*, 2006a; Kittel, *et al.*, 2006). Despite this progress, the method has not found much traction outside his laboratory. This may be due to the pulsed tunable lasers required for constructing a STED microscope and is reminiscent of the situation that two-photon microscopy faced in its early days when it was not always clear that its advantages outweighed its costs.

Two reports just published by Hell and colleagues, one in the *Proceedings of the National Academy of Sciences* (Donnert *et al.*, 2006) and one in this issue of *Nature Methods* (Willig *et al.*, 2006b), demonstrate the continuing progress being made in the development and application of STED microscopy. The report in this issue shows for the first time that STED microscopy can be used with fluorescent proteins—an important step toward live-cell imaging with STED.

The advance reported in the other paper is potentially even more significant. Donnert *et al.* report the surprising finding that extending the length of the gaps between the paired pulses of illumination used during STED results in lower photobleaching and higher fluorescence emission.

Photobleaching is always observed in STED microscopy because the technique uses an illumination intensity that is 10- to 100-fold larger than that typically used for single-photon fluorescence microscopy, albeit at a wavelength at which the dye is not expected to absorb. Although this intensity is 100- to 1,000-fold lower than the intensity of excitation in multiphoton imaging, STED uses the same visible region of the spectrum as single-photon imaging and a similar number of photons in the pulse.

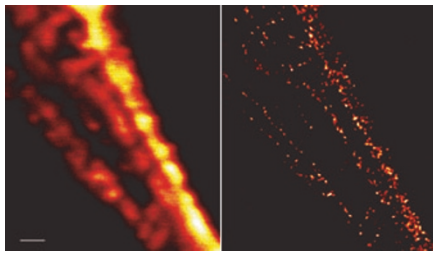

Figure 1 | Side-by-side comparison of confocal and STED microscopy images. Imunofluorescence imaging of the neurofilament–heavy subunit protein in neurofilaments of human neuroblastoma cells by confocal microscopy (left) and by STED microscopy with nonlinear deconvolution (right). Scale bar, 500 nm. Image courtesy of G. Donnert and S.W. Hell, Max Planck Institute for Biophysical Chemistry, Göttingen.

Bleaching commonly results from a transition of fluorophores to an excited energy state known as the triplet state. This is a long-lived state that can absorb photons over a wider wavelength range and is very conducive to bleaching. "It became clear that there must be a link between the triplet state and the intense STED pulse," says Hell. "We decided that we could change the setup in such a way that the repetition rate is much slower." The longer time between pulses should provide time for any molecules in the triplet state to transition back to the ground state. They call this illumination method triplet relaxation (T-Rex) STED.

Not only did reducing the repetition rate decrease the rate of bleaching, it also resulted in a substantial increase in the fluorescence signal. Hell says, "The signal increased so strongly that the effective elongation of the recording time only increased by a factor of four to ten" despite the 30- to 80-fold decrease in actual illumination time. This simple change allowed them to apply a tenfold stronger intensity of the STED beam. According to the square-root law governing STED microscopy resolution, the tenfold stronger intensity resulted in a further threefold resolution increase, down to 20 nm, that is, an order of magnitude below the diffraction barrier (**Fig. 1**).

Even though decreasing the repetition rate resulted in such a large improvement in emission intensity Hell is quick to add, "This is not the ultimate solution that I would recommend. The real solution is quick scanning. If you scan really quickly so that only one or two pulses hit the same molecule and then the beam is gone, you get the same effect but you maintain the flux of fluorescence coming from the sample." Unfortunately it has not yet been technically possible to implement fast scanning in STED microscopy.

Such developments are improving the prospects for STED microscopy becoming an important imaging modality capable of providing ultrahigh resolution in fluorescence imaging. The next big improvement is likely to be beam scanning. This should not only allow live-cell imaging but provide an ideal way of implementing T-Rex STED.

Intriguingly, there is no theoretical reason why the benefits of T-Rex should not be applicable to any imaging modality that uses a pulsed laser, such as two-photon microscopy. It may turn out that the use of a pulsed laser with T-Rex for conventional single-photon imaging could have some advantages as well. It would be ironic if in the process of improving STED microscopy, one of the resulting innovations ended up having a larger impact on fluorescence microscopy in general.

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RESEARCH PAPERS

Donnert, G. *et al.* Macromolecular-scale resolution in biological fluorescence microscopy. *Proc. Natl. Acad. Sci. USA* **103**, 11440–11445 (2006).

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Willig, K.I. *et al.* Nanoscale resolution in GFP-based microscopy. *Nat. Methods* **3**, 721–723 (2006b).