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A Fatigue-Resistant Photoswitchable Fluorescent Protein for Optical Nanoscopy**

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data storage · fluorescent probes · live-cell imaging · photochemistry · STED/RESOLFT

The function of biological macromolecules can be fully understood only within the context of their complex natural environment. Thus, recent years have witnessed an upsurge of studies aimed at exploring functional processes within living cells, tissues, and organisms. Fluorescence-based optical microscopy is a key method in this pursuit because it is minimally invasive and allows imaging in two or three dimensions over extended periods of time. The need to specifically label structures of interest often requires substantial efforts but affords enormous selectivity, so a particular functional process can be observed with minimal interference from the many other processes happening at the same time. Moreover, fluorescence microscopy is exquisitely sensitive: even individual fluorescence emitters can be detected. A drawback of conventional far-field optical microscopy, however, is its moderate spatial resolution, limited by wave diffraction to about half the wavelength of visible light (200–400 nm). Because typical biomolecules have dimensions of only a few nanometers, a resolution gap of about two orders of magnitude exists that precludes molecular-scale imaging.

In recent years, a variety of optical microscopy techniques have emerged that truly overcome the so-called Abbe diffraction barrier and significantly narrow the resolution gap.[1] All these super-resolution, or nanoscopy, concepts have in common that they utilize fluorescent probes the emission of which can be controlled by light irradiation. In a recent paper in *Nature*, Hell, Jakobs and co-workers have introduced a new fluorescent protein (FP) variant, reversibly switching enhanced green fluorescent protein (rsEGFP), which has superb properties for optical nanoscopy.[2] It promises to become an important tool for live-cell super-resolution imaging.

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Stimulated emission depletion (STED) microscopy was the first practical implementation of far-field super-resolution fluorescence microscopy, introduced by Hell and co-workers in 1994.[3] The image is acquired pixel by pixel by scanning in the focal plane with a tightly focused, diffraction-limited spot (Airy disk) a few hundred nanometers in diameter. The spot size limits the resolution of conventional laser scanning confocal microscopy: all fluorophores within the spot are excited simultaneously, and their emission signals cannot be separated (Figure 1a). In STED microscopy, the fluorophores, while electronically excited, are additionally exposed to a so-called depletion beam, which spatially overlays the excitation beam. It has a donut-shaped intensity profile and zero intensity in the center. All fluorophores in the spot periphery are deexcited by stimulated emission; those near the center remain excited and subsequently emit fluorescence, which is detected and stored in the image pixel at the associated scanner position. This excitation/depletion sequence is repeated while the image is raster scanned with nanometer precision. The spatial confinement of the fluorescence emitters to the donut center and, thereby, the achievable image resolution is governed by the strength of the depletion beam and is theoretically unlimited.[1]

STED microscopy has the great advantage that it exploits an intrinsic physical property of fluorescent dyes, that is, their ability to undergo stimulated deexcitation. However, the high spontaneous deexcitation rates corresponding to short (typically 1–5 ns) excited-state lifetimes require a high intensity of the STED depletion beam to efficiently deexcite the fluorophores before they decay spontaneously. It was quickly realized that the STED concept of activating and deactivating fluorophores can be generalized;[4] all these approaches have been subsumed under the acronym RESOLFT (reversible saturable optical fluorescence transition). Any fluorophore featuring highly efficient light-induced and low spontaneous transition rates between an emitting and a non-emitting state is well suited for RESOLFT. With slow spontaneous transitions, for example in the millisecond range, the depletion beam can be orders of magnitude less intense for RESOLFT than for STED. The principle is depicted in Figure 1b. A group of fluorophores, initially in their non-emitting state, are switched to their fluorescent state. Then, a donut pulse switches off the fluorophores in the periphery, so only molecules close to the donut center can still fluoresce.

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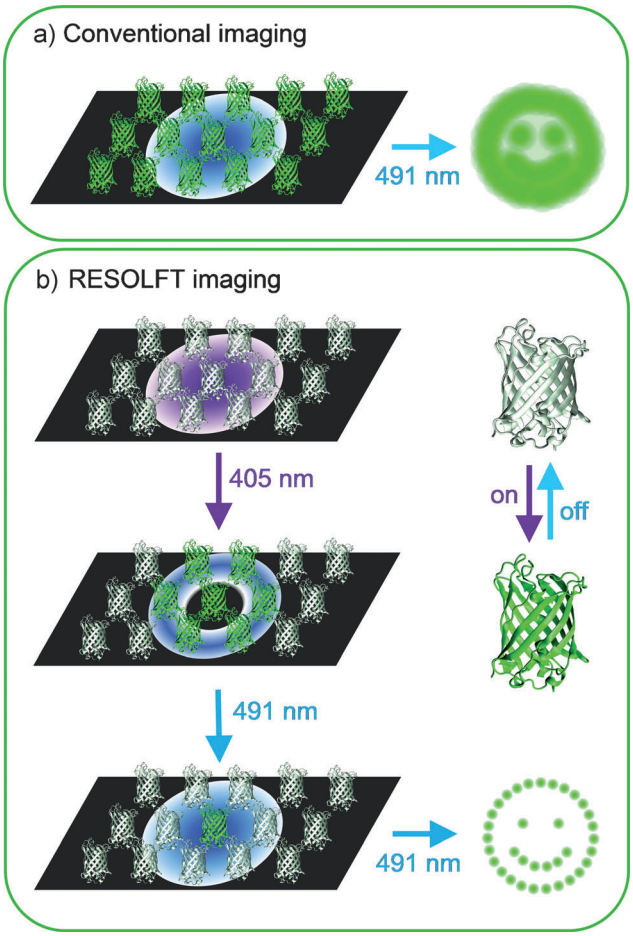

Figure 1. a) In conventional imaging, many fluorophores are excited simultaneously by a focused spot of light that is raster-scanned across the sample. b) In RESOLFT microscopy, chromophores are locally photoactivated by a spot of 405 nm light for roughly 100 µs. Subsequently, a depletion spot of 491 nm light is applied for roughly 10 ms featuring zero intensity in the center; this switches FPs in the periphery back to the nonfluorescent state. Only rsEGFP molecules near the center remain fluorescent and can be detected by their emission.

Instead of scanning a focused spot, periodic line-pattern illumination can also be used in combination with photoswitchable fluorophores in a widefield microscope to produce super-resolution optical images by saturated structured illumination microscopy (SSIM).[5] Yet another concept has been implemented in a family of super-resolution localization microscopy methods (PALM/STORM) [PALM (photo-activation localization microscopy) and STORM (stochastic optical reconstruction microscopy)].[6] In a widefield microscope, photoactivatable fluorophores are initially held in their nonfluorescent state. They are sparsely activated so that their individual fluorescence emission patterns do not overlap within a single camera frame. The centroid positions of the optical signals from single emitters are determined with great precision and, subsequently, a super-resolution image is reconstructed from a large number of probe locations in typically 10^2–10^4 camera frames.

Specific labeling in live-cell imaging has been greatly simplified by the development of a wide variety of fluorescent proteins (FPs) from the GFP family.[7] These marker proteins can be fused to a protein of interest on the DNA level, and the gene of the fusion construct can be introduced into the cell so that it synthesizes a fusion protein, which often functions normally despite its additional marker domain (although this needs to be confirmed for every instance). In recent years, a variety of photoactivatable FPs have been identified and further optimized to serve as fluorescent probes in super-resolution imaging. Two types of photoactivation are distinguished: irreversible photoconversion, either from a non-fluorescent to a fluorescent state or from one emission wavelength to another one, and reversible photoswitching between a fluorescent and a nonfluorescent state. For localization-based imaging, both types of photoactivatable FPs can be employed because only a single activating event is needed. For RESOLFT imaging, however, markers have to be cycled between on and off states once for each scanner position. Roughly speaking, a tenfold improvement in resolution requires ten switching cycles per spatial dimension and thus 10^2 (10^3) cycles for 2D (3D) imaging. Current FPs are less photostable than good synthetic dyes, and many photoswitching FPs can be cycled between on and off states only a few times until they photobleach. The key importance of the work of Grotjohann et al.[2] lies in the fact that their novel rsEGFP survives more than 10^3 on–off cycles, thereby enabling RESOLFT imaging in all three spatial dimensions.

Starting from the bright, monomeric EGFP, five mutations (Q69L/V150A/V163S/S205N/A206K) were introduced by combining site-directed and error-prone mutagenesis to obtain rsEGFP. Its fluorescence can be switched on with purple light (405 nm) and switched off with cyan light (491 nm) (Figure 1b). A fluorescence reduction to 1–2% of the initial intensity was reported upon exposure to 491 nm wavelength light in vitro (at pH 7.5).

Through the use of fusion proteins, excellent performance was shown with live-cell RESOLFT imaging of cultured *E. coli* and PtK2 cells as well as slice preparations of mouse hippocampus, yielding image resolutions of approximately 40 nm. Because the depletion beam intensity is roughly six orders of magnitude lower than with STED, even higher spatial resolution may be possible by using a more intense depletion beam. Note that with STED, increasing the depletion beam intensity has its limitations owing to photodamage by multiphoton absorption. RESOLFT image acquisition times were comparable to other those of super-resolution techniques. They depend on the switching kinetics of the rsEGFP probes and possibly can be further accelerated by mutations that affect the free energy barriers separating the on and off states.

Besides cellular imaging, high-density 2D and 3D data storage are further exciting applications for photoactivatable FPs.[8] Taking advantage of the switching stamina of rsEGFP, Grotjohann et al.[2] demonstrated 2D data storage, with both WMRM (write many, read many) and WORM (write once, read many) storage schemes.

Many laboratories all over the world are striving to advance FP technology. On account of their importance in

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super-resolution optical microscopy, novel photoactivatable FPs are met with particular excitement.[9] Photostability and photoswitching stamina are parameters that are crucial for marker performance. With their new rsEGFP, Hell, Jakobs, and co-workers have made a huge leap forward in the development of versatile and robust FP markers.

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[1] a) S. W. Hell, *Science* **2007**, *316*, 1153 – 1158; b) P. N. Hedde, G. U. Nienhaus, *Biophys. Rev.* **2010**, *2*, 147 – 158.
[2] T. Grotjohann, I. Testa, M. Leutenegger, H. Bock, N. T. Urban, F. Lavoie-Cardinal, K. I. Willig, C. Eggeling, S. Jakobs, S. W. Hell, *Nature* **2011**, *478*, 204 – 208.
[3] S. W. Hell, J. Wichmann, *Opt. Lett.* **1994**, *19*, 780 – 782.
[4] S. W. Hell, M. Dyba, S. Jakobs, *Curr. Opin. Neurobiol.* **2004**, *14*, 599 – 609.
[5] R. Heintzmann, M. G. Gustafsson, *Nat. Photonics* **2009**, *3*, 362 – 364.
[6] a) S. T. Hess, T. P. Girirajan, M. D. Mason, *Biophys. J.* **2006**, *91*, 4258 – 4272; b) E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, H. F. Hess, *Science* **2006**, *313*, 1642 – 1645; c) M. J. Rust, M. Bates, X. Zhuang, *Nat. Methods* **2006**, *3*, 793 – 795.
[7] G. U. Nienhaus, *Angew. Chem.* **2008**, *120*, 9130 – 9132; *Angew. Chem. Int. Ed.* **2008**, *47*, 8992 – 8994.
[8] V. Adam, H. Mizuno, A. Grichine, J. Hotta, Y. Yamagata, B. Moeyaert, G. U. Nienhaus, A. Miyawaki, D. Bourgeois, J. Hofkens, *J. Biotechnol.* **2010**, *149*, 289 – 298.
[9] a) T. Brakemann, A. C. Stiel, G. Weber, M. Andresen, I. Testa, T. Grotjohann, M. Leutenegger, U. Plessmann, H. Urlaub, C. Eggeling, M. C. Wahl, S. W. Hell, S. Jakobs, *Nat. Biotechnol.* **2011**, *29*, 942 – 947; b) J. Fuchs, S. Böhme, F. Oswald, P. N. Hedde, M. Krause, J. Wiedenmann, G. U. Nienhaus, *Nat. Methods* **2010**, *7*, 627 – 630.

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