Diffraction-unlimited all-optical imaging and writing with a photochromic GFP

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In a fluorescence microscope, diffraction prevents (excitation) light being focused more sharply than \(\lambda/(2NA)\), with \(\lambda\) being the wavelength of light and \(NA\) the numerical aperture of the lens. Thus, as they are illuminated together, features residing any closer together than this distance also fluoresce together and appear in the image as a single blur. The diffraction resolution barrier can be overcome by forcing such nearby features to fluoresce sequentially, but this strategy clearly requires a mechanism for keeping fluorophores that are exposed to excitation light non-fluorescent.1–3

In stimulated emission depletion (STED) microscopy, this is accomplished by the so-called STED beam, which turns the fluorescence capability of fluorophores off by a photon-induced de-excitation. Because at least a single de-exciting photon must be available within the lifetime of the fluorescent molecular state, the intensity of the focal STED beam must exceed the threshold \(I_0 = Cr^2\) with \(C\) accounting for the probability of a STED beam photon to interact with the fluorophore.4 The STED beam, usually formed as a doughnut overlaid with the excitation beam, features a central point of zero intensity at which the fluorophores can still assume the fluorescent state. As this point can be positioned with arbitrary precision in space, the coordinate of the emitting (on-state) fluorophores is known at any instant: it is the position of zero intensity and its immediate vicinity, where the STED beam is still weaker than \(I_0\). The diameter of this area is given by \(d = \lambda/(2NA \times (1 + I_0/I_s)\), with \(I_0\) (typically \(\gg I_s\)) denoting the intensity at the doughnut crest. Hence, features that are (just slightly) more apart than \(d < \lambda/(2NA)\) cannot fluoresce at the same time even when simultaneously illuminated by excitation light.5 Scanning the beams across the sample and recording the fluorescence yields images of subdiffraction resolution \(d\) automatically and irrespective of the fluorophore concentration in the sample.

De-excitation by stimulated emission is the most basic and general mechanism for modulating the fluorescence ability of a molecule. However, by requiring light intensities \(> I_0 \approx 1–10 \text{ MW cm}^{-2}\), attaining high resolutions by this mechanism necessitates large \(I_0\) values. For example, \(d < 40 \text{ nm}\) typically entails \(I_0 = 100–500 \text{ MW cm}^{-2}\) (ref. 6). Although intensities of this order have been demonstrated to be live-cell compatible, all-optical nanoscopy methods operating at fundamentally lower light levels are highly in demand, because they allow larger fields of view and can avoid photodamage.

A route to low light level operation is to replace STED with a fluorescence switching mechanism having a lower threshold \(I_s\) (refs 2, 5, 11–13). Following the equation for \(I_s\), this can be realized by exploiting transitions between fluorophore states of longer lifetime \(\tau < 1 \mu\text{s}\) (refs 2, 5, 11). Hence, it has been suggested that fluorescence can be switched by transferring the fluorophores transiently to a generic metastable dark (triplet) state of \(\tau \approx 10^{-3}–100\text{ ms}\) (refs 2, 15). A more attractive option is to use fluorophores that can be explicitly ‘photoswitched’, for example, by photoisomerization. Hence, in 2003 it was proposed to implement a STED-like microscope with STED being replaced by a reversible on–off switch as encountered in organic photochromic fluorophores and reversibly photoswitchable fluorescent proteins (RSFPs).5,11

In fact, this strategy is even more general because any reversible transition between a signalling and a non-signalling state can be used for breaking the diffraction barrier. Therefore, all concepts that switch the fluorescence capability of molecules at sample coordinates predefined by patterns of light have been generalized under the name RESOLFT, which stands for reversible saturable optical (fluorescence) transition exchange. Of these, the most powerful was the reversible switching of enhanced green fluorescent protein (rsEGFP), a fluorescent protein that can be reversibly photoswitched more than a thousand times. Distributions of functional rsEGFP–fusion proteins in living bacteria and mammalian cells are imaged at \(<40\text{-nanometre resolution. Dendritic spines in living brain slices are super–resolved with about a million times lower light intensities than before. The reversible switching also enables all-optical writing of features with subdiffraction size and spacings, which can be used for data storage.}

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read out. Biological imaging therefore remained unviable. Other studies using a variant of the RSFP called dronpa faced the same challenge. As a rule of thumb, an m-fold resolution improvement along a certain direction requires m switching cycles, meaning that m = 10 along the x- and y-axes entails m² = 100 cycles, whereas ~1,000 cycles are required for x, y and z (ref. 6). Thus, for RESOLFT super-resolution, the number of switching cycles afforded by the fluorophore assumes a vital role. Because they are able to generate an image with a single on–off cycle, the super-resolution concepts called (F)PALM and STORM, which have emerged in the interim, have successfully harnessed the switching between metastable states for gaining sub-diffraction resolution. However, these methods rely on the imaging and computation-aided localization of individual fluorophores amidst the scattering and autofluorescence background common in (living) cells and tissues. Moreover, rapid localization of a sufficiently large number of fluorophores requires the excitation light to be intense. In contrast, a RESOLFT approach is able to instantly record the emission from all fluorophores attached to the nanosized particle, remaining a monomer. 

Similarly, although STED/RESOLFT-inspired optical writing with photochromic compounds has been shown to yield structures ~<λ/2 (2NA), writing such structures with spacings ~<λ/(2NA) remained challenging again the impediment being the requirement of many on–off cycles before the structure is made permanent. Here we introduce a RSFP enabling both low-light-level all-optical nanoscopy of living cells and tissues, and far-field optical writing and reading of patterns of subdiffraction size and density.

Generating a reversibly switchable GFP

All fluorescent proteins have a similar fold, namely an 11-stranded β-barrel with a central helix containing the chromophore, which is typically in a cis-configuration. Light-driven switching of RSFPs generally involves an isomerization of the chromophore, frequently coupled with a change of its protonation state. We started from EGFP and identified, using its X-ray structure, amino acid residues the exchange of which was expected to facilitate isomerization. We expressed numerous EGFP variants in Escherichia coli and screened for colonies expressing an RSFP with an automated microscope. To this end, we alternated site-directed and error-prone mutagenesis while maintaining the key amino acids of EGFP (that is, F64L and S65T) we concomitantly introduced A206K to ensure that the protein remained a monomer.

The amino acid exchange Q69L was sufficient to make EGFP(A206K) reversibly switchable, but the resulting on–off contrast was low. Although it makes the protein switchable, we avoided the mutation E222Q because it seemed to reduce the number of cycles. After analysing ~30,000 clones, we identified EGFP(Q69L/V150A/V163S/S205N/A206K) (Supplementary Fig. 2) that could be reversibly switched on at λ = 405 nm and off at 491 nm, and named it reversibly switchable EGFP (rsEGFP).

At equilibrium, rsEGFP adopts a bright on-state fluorescence quantum yield of 0.36; extinction coefficient ε = 47,000 M⁻¹ cm⁻¹ (Supplementary Table 1). In the on-state, rsEGFP exhibits a single absorption band peaking at 491 nm (Fig. 1a), corresponding to the ionized state of the phenolic hydroxyl of the chromophore. The pK₅ of the chromophore is 6.5 (Supplementary Fig. 3). Absorption at 490 nm yields fluorescence peak at 510 nm and, in a competing process, switches rsEGFP off (Figs 1a–c). Prolonged irradiation of a pH 7.5 solution of purified rsEGFP at ~490 nm reduces the rsEGFP fluorescence to 1–2% of its initial value. The off-state exhibits a single absorption band at 396 nm, corresponding to the neutral state of the chromophore (Fig. 1b). Excitation at this band switches the protein from the off-state to the on-state by continuous irradiation at 405 nm (2 kW cm⁻²) and at 491 nm (0.6 kW cm⁻²). The duration of off-switching at 491 nm was chosen such that the fluorescence reached a minimum; irradiation with 405 nm was chosen so that the proteins were fully switched. The relaxation of rsEGFP embedded in PAA from the off-state into the fluorescent equilibrium state at 22 °C. The black line is a stretched exponential fit with a stretching factor of ~0.6 accounting for inhomogeneous spectral broadening or the involvement of multiple dark states. Fluorescence per switching cycle normalized to the initial fluorescence, with the same light intensities and switching durations as in c, f. Photobleaching of rsEGFP and dronpa embedded in a PAA layer were kept in their on-states by continuous irradiation at 405 nm (1 kW cm⁻²), while fluorescence was probed by irradiation at 491 nm (3 kW cm⁻²).

Figure 1 | Properties of rsEGFP. a, Absorption (red dashed line), excitation (solid black line) and fluorescence (dotted green line) spectrum of rsEGFP in the fluorescent equilibrium state at pH 7.5. b, Absorption spectra obtained at different time points during irradiation with 405-nm light. c, Switching curves of dronpa (blue) and rsEGFP (red) immobilized in PAA using the same intensities. Switching was performed by alternating irradiation at 405 nm (2 kW cm⁻²) and at 491 nm (0.6 kW cm⁻²). The duration of off-switching at 491 nm was chosen such that the fluorescence reached a minimum; irradiation with 405 nm was chosen so that the proteins were fully switched. d, Relaxation of rsEGFP embedded in PAA from the off-state into the fluorescent equilibrium state at 22 °C. The black line is a stretched exponential fit with a stretching factor of ~0.6 accounting for inhomogeneous spectral broadening or the involvement of multiple dark states. e, Fluorescence per switching cycle normalized to the initial fluorescence, with the same light intensities and switching durations as in c, f. Photobleaching of rsEGFP and dronpa embedded in a PAA layer were kept in their on-states by continuous irradiation at 405 nm (1 kW cm⁻²), while fluorescence was probed by irradiation at 491 nm (3 kW cm⁻²).
Rewritable data storage

To analyse whether immobilized rsEGFP could be used for repeated short-term data storage, we coated a microscope slide with a 1-μm thin layer of rsEGFP (~0.03 mM) in PAA. Switching and reading by illumination at 405 nm and 491 nm in a scanning confocal set-up provided an on-off contrast of ~50:1. We translated the text of 25 Grimm’s fairy stories (http://www.gutenberg.org/files/11027/11027.txt) into 7-bit binary ASCII code (‘0’: off; ‘1’: on) and wrote and read the 370,000 letters into a 17 μm × 17 μm region in 6,596 frames, each comprising 41 letters (287 bits) (Fig. 2). Individual bits were written 0.5 μm in diameter with 1 μm centre-to-centre spacing, corresponding to a DVD storage density. Discriminating ‘0’ from ‘1’ by a simple threshold entailed 7-bit errors within the entire data set. After ~6,600 read/write cycles in the same region, the average fluorescence of the ‘1’ was reduced by ~35% (Supplementary Fig. 8). Hence, the same rsEGFP layer can be used for ~15,000 read/write processes.

RESOLFT nanoscopy of living samples

Next, we implemented a scanning confocal set-up with a 405 nm (ultraviolet) beam for switching the rsEGFP on, a 491 nm (blue) beam for eliciting fluorescence, and a doughnut-shaped 491 nm beam for the off-switching (Supplementary Fig. 9). We fused rsEGFP to the amino-terminus of the bacterial actin homologue MreB and expressed the fusion protein in E. coli bacteria. Living bacteria on agar-coated slides were recorded by first irradiating each pixel for 100 μs with ultraviolet light (1 kW cm⁻²), thus activating most of the rsEGFP in the focal volume. Then the doughnut-shaped blue beam (1 kW cm⁻²) was applied for 10–20 ms to switch all the rsEGFP molecules off, except those located within 6/2 distance from the doughnut centre. Lastly, the rsEGFP fluorescence was read out for 1–2 ms by the 491 nm beam (~1 kW cm⁻²). The sequence was repeated for each sample pixel.

The double-helical cytoskeletal structure of rsEGFP–MreB is more clearly revealed by RESOLFT than by its confocal counterpart (Fig. 3a). The RESOLFT image of a typical filament showed a full-width half-maximum (FWHM) of ~70 nm. Because this value seemed to be determined by the thickness of the filament itself, a more accurate upper limit for the resolution d is obtained by imaging the finer keratin-19–rsEGFP intermediate filament network in living mammalian cells (Fig. 3b, c). Line profiles from recorded data gave a FWHM of 40 nm corresponding to a 5–6-fold all-optical resolution improvement over confocal microscopy (Fig. 3c). To investigate its applicability to living brain tissue, we locally injected viral particles carrying a lifeact–rsEGFP construct into a cultured organotypic hippocampal brain slice. Lifeact is a 17-amino-acid-long peptide with high affinity to filamentous actin. RESOLFT revealed fine morphological differences between the spines protruding from a dendrite (Fig. 3d). A profile through a spine neck showed a FWHM of <80 nm. Electron microscopy of similar samples demonstrated that this value is close to the actual size of the spine necks themselves, suggesting a resolution d substantially <80 nm. Repeated imaging revealed dynamic changes over 5 min (Fig. 3d). Altogether, the resolution is comparable to that provided by STED.
on similar structures\textsuperscript{10}, but here it is obtained with light intensities lower by about a million times.

**RESOLFT optical data storage**

For investigating subdiffraction resolution writing, an rsEGFP layer was prepared as previously outlined. The writing entailed (1) an ultraviolet beam (405 nm, 1 kW cm\textsuperscript{−2}) applied for 100 µs to switch rsEGFP on, (2) a 2-ms break for equilibration, (3) a doughnut-shaped blue beam (491 nm, 0.5 kW cm\textsuperscript{−2}) lasting 20 ms confining the on-state within d/2 around the doughnut centre, and (4) an \(\sim\)2-ms 532 nm beam (\(\sim\)900 kW cm\textsuperscript{−2}) for transferring on-state rsEGFP to a permanent off (bleached) state (Fig. 4a) (Supplementary Fig. 10a). Lastly, the rsEGFP molecules located outside this region were switched back on, which is critical for writing another feature within subdiffraction proximity.

We wrote nine patterns of 3 \(\times\) 3 bit fields in an rsEGFP layer, with 250 nm centre-to-centre separation between individual bits (Fig. 4b), both in the conventional and in the RESOLFT mode. Whereas conventional writing and/or confocal reading blurred the data, the bits were fully discernible when both writing and reading were performed by RESOLFT. We wrote and read the data down to distances of 200 nm between the individual bits (Supplementary Fig. 10b). Hence this scheme allowed storing and reading out bits \(\sim\)4 times more densely than by regular focusing. The structures could be read 5–10 times.

**Discussion and conclusion**

The many-switching cycles afforded by the fluorescence protein rsEGFP reported here has facilitated live-cell RESOLFT microscopy, a super-resolution microscopy that is similar to STED microscopy in usability but operates at \(\sim\)10\textsuperscript{6} times lower levels of light. Multiphoton-induced optical damage\textsuperscript{45} can therefore be virtually excluded. The fundamental reduction in optical intensity required for the on–off switching stems from the fact that the fluorescence capability of the molecule is not modulated by disallowing the population of its nanosecond fluorescent state, but rather by toggling it between two long-lived ground states, one in which the fluorophore remains dark when exposed to the excitation light.

RESOLFT is readily combined with confocal imaging, which increases its use in scattering living samples. In fact, the imaging of neuronal spines in living organotypical brain slices testifies this potential. Although the recording time reported here is still of the order of most other super-resolution techniques\textsuperscript{13,21} and slower than the fastest biological STED recordings\textsuperscript{5}, by gathering the signal from typically many molecules located at predefined positions, RESOLFT has all the prerequisites for fast imaging. Scanning with arrays of doughnuts or zero-intensity lines (so-called structured illumination\textsuperscript{14,15,66}) and detection by a camera will reduce the number of scanning steps required to cover large fields of view and facilitate low-intensity video-rate imaging. The maximum recording speed is determined by the time it takes to establish the disparity of (on–off) states in space, that is, by the switching kinetics, which probably can be improved by further mutagenesis. Note that the switching is not restricted to changes in brightness (on–off) only. Other reversible transitions between disparate states may also prove suitable for RESOLFT imaging, such as states yielding differences in emission wavelengths, lifetime or polarization.

Photonswitching between long-lived states also pose challenges, because in the process the molecule can assume transient (dark) states, such as triplet states, which depend on the molecular microenvironment. In this regard, STED maintains a unique advantage because it entails just basic optical transitions between the ground and the fluorescent state; no atom relocation, spin flip or change in chemical bond is required to switch the fluorescence capability of the molecule — just light. Therefore, switching fluorescence by STED is nearly universal and instantaneous.

The switching stamina of rsEGFP also enabled writing and reading of patterns of both subdiffraction size and spacing \(d\), which has so far been difficult for direct far-field optical writing. In our study, the smallest obtainable structure size was co-determined by the fact that the 532-nm light moderately bleached the off-state proteins too, thus reducing the writing contrast. However, this initial demonstration should spur on new advancements in this field, because current nanowriting efforts are dominated by concepts that resort to much shorter wavelengths of electromagnetic radiation at which focusing should become possible at ultralow light levels.

The resolution demonstrated here is similar to or even exceeds the resolution attained until now by STED in living cells\textsuperscript{6,10}. Although in both methods the resolution can be continually increased by increasing \(I_m/I_s\) in STED microscopy this strategy will reach practical limits due to the intensities required. Using a threshold intensity \(I_t\) that is lower by many orders of magnitude, switching between long-lived states overcomes these limits and, as we have demonstrated here, offers a pathway to lens-based optical imaging and writing at molecular dimensions.

**METHODS SUMMARY**

**Protein generation and screening.** Site-directed mutagenesis was performed with the QuikChange Site Directed Mutagenesis Kit (Stratagene) or a multiple-site approach using several degenerative primers. The proteins were expressed from the high-copy expression vector pQE31 (Qiagen) and expressed in E. coli.

**Viral transfection.** A modified Senliki Forest Virus containing the pSCA-Lifeact-rsEGFP vector construct was injected into the slice cultures using a patch pipette. Imaging was performed within 16–48 h after incubation.

**Data storage.** A layer containing immobilized rsEGFP was prepared by mixing 24.5 µl purified proteins (0.09 mM) with 17.5 µl Tris·HCl pH 7.5, 30 µl acrylamide (Rotiphorese Gel 30, Roth), 0.75 µl 10% ammonium persulfate and 1 µl 10%
TEMED. About 10 μl of this solution was placed on a glass slide and a cover slip was pressed onto the sample to attain a thin layer. Custom MATLAB (The MathWorks) programs allowed automated generation of the voltages and signals for moving the sample and for generating the desired laser pulses. Images were also taken using the software Inspector (http://www.inspector.de).

**RESULTS**

We implemented a home-built confocal microscope with a normally focused beam for generating fluorescence plus a doughnut-shaped beam for switching rEFP off (both at 491 nm wavelength). The beams were circularly polarized, superimposed in the focal plane and applied sequentially. The 495 nm beam for switching rEFP on was also circularly polarized. The fluorescence emitted between 500–560 nm was imaged on the opening of a multimode fibre and detected by a counting avalanche photodiode. The same set-up was used for writing, which was most specific at 532 nm.

Full details of methods used are available in Supplementary Information.

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