Breaking the diffraction resolution barrier in far-field microscopy by molecular optical bistability

Mariano Bossi, Jonas Fölling, Marcus Dyba, Volker Westphal and Stefan W Hell
Department of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, 37070 Göttingen, Germany
E-mail: hell@nanoscopy.de

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Abstract. We demonstrate the breaking of the diffraction resolution barrier in far-field fluorescence microscopy by photoswitching ensembles of optically bistable organic molecular markers from a non-fluorescent to a fluorescent state and back. The photoswitching is accomplished by an isomerization reaction of a photochromic compound serving as a reversible energy acceptor of a fluorescent compound. The surpassing of the diffraction barrier with power levels of only a few hundred W cm\(^{-2}\) of continuous wave irradiation is evidenced both in the effective point spread function and in the fluorescence images of test samples.

For a long time it has been commonly accepted that the resolution of a far-field optical microscope is limited by diffraction [1]. This notion has led to the development of near-field optics [2] as well as of x-ray microscopy [3], but like all microscopy techniques, they have their own limitations. Whereas near-field optics is confined to imaging surfaces, x-ray microscopy requires intense x-ray sources and sophisticated diffractive lenses. Moreover, unlike far-field fluorescence microscopy, none of them readily fulfils the needs of biology where molecular specificity and rapid three-dimensional (3D) imaging are imperative. In fact, the non-invasive 3D-imaging of cellular substructures, such as protein aggregations inside intact cells, virtually necessitates the use of visible focused light. Therefore the breaking of the diffraction barrier in far-field fluorescence microscopy is not only academically appealing, but also enormously relevant to other areas of science.
Following Abbe [1], in a standard far-field fluorescence microscope the minimum distance \( \Delta x \) at which two features can still be separated is given by 
\[
\Delta x = \frac{\lambda}{2n \sin \alpha},
\]
where \( \lambda \), \( n \) and \( \alpha \) are the wavelength of light, the refractive index of the media, and the semiaperture angle of the lens, respectively. The first concept implying the separation of identical fluorescent molecules at arbitrarily small distance with far-field optics has been stimulated emission depletion (STED) microscopy [4]. In STED microscopy, the diffraction barrier is broken by a nearly exponential de-excitation of the fluorescent state of a marker by a light beam featuring a local intensity zero. Usually this is accomplished by overlapping the diffraction-limited spot of the excitation beam of a scanning microscope with a red-shifted doughnut-shaped beam for the molecular de-excitation, i.e. for ‘switching off’ the fluorescence ability of the molecule by stimulated emission [5]. In a STED-microscope, the resolution basically follows
\[
\Delta x \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + I/I_{\text{sat}}}}.
\]
(1)
Here \( I \) is the intensity at the doughnut crest and \( I_{\text{sat}} \) gives the intensity value at which the population of the excited state has been depleted to \( 1/e \). \( I_{\text{sat}} \) is readily calculated as \( (\sigma \cdot \tau)^{-1} \) with \( \sigma \) denoting the cross-section for stimulated emission and \( \tau \) being the fluorescence lifetime [6]–[8]. Commonly used fluorophores have \( \sigma \) and \( \tau \) values of \( \sim 10^{-17} \text{ cm}^{-2} \) and \( \sim 1 \text{ ns} \), respectively, thus \( I_{\text{sat}} \) typically amounts to \( 10^{26} \text{ photons} (\text{s cm}^2)^{-1} \) corresponding to about 30 MW cm\(^{-2}\) in the visible range. Applying \( I \gg I_{\text{sat}} \) obviously leads to \( \Delta x \rightarrow 0 \), i.e. the resolution limit is basically the size of the molecule [6]. Equation (1) and the ability of STED microscopy to provide <20 nm resolution has been confirmed in single molecule experiments [7] and in the imaging of subcellular structures [8], although the large intensities required for STED call for pulsed lasers.

However, it has also been shown [6, 9] that the idea underlying STED microscopy can be generalized to encompass any molecular mechanism that switches the fluorescence on and off. Grouped under the name REversible Saturable OpticaL Fluorescent Transitions (RESOLFT), the resolution of these concepts also follows equation (1) and hence is not limited by diffraction. In particular, it has been predicted that photoswitching between a fluorescence ‘on’ and an ‘off’ state in photochromic (so-called photoactivatable) fluorescent proteins and of photochromic organic compounds should lead to molecular scale resolution [6, 9]. Since, in this case, the fluorescence ‘switching’ is due to atomic rearrangements (isomerism) within the fluorescent moiety of the marker, the ‘on’ and ‘off’ isomeric states feature typical lifetimes \( \tau \gg 1 \text{ ms} \), rendering the molecule optically bistable. The >6 orders of magnitude longer \( \tau \) now leads to values of \( I_{\text{sat}} \) that are lower than those encountered in STED microscopy by the same factor [6, 9]. Following (1), the same values \( I/I_{\text{sat}} \) are achieved at orders of magnitude lower values of \( I \), thus making a RESOLFT type of microscopy with optically bistable markers very appealing [6, 9].

Initial implementations of protein photoswitching have indeed confirmed the possibility of breaking the diffraction barrier with only a few W cm\(^{-2}\) of illumination intensity [10]. More recently, subdiffraction imaging was demonstrated by switching molecules individually, which enable nanoscale separation by localizing each molecule individually [11, 12]. Despite the enormous potential of the latter approaches, breaking the diffraction barrier in an ensemble retains its attractiveness because being an ensemble technique, it is less prone to sample background and possibly more easily adaptable to 3D imaging. In this paper, we now provide first evidence for the viability of RESOLFT microscopy with photoswitchable synthetic organic markers.
Since the relocation of atomic groups is antagonistic to efficient fluorescence emission, there are not many photochromic organic compounds that are also fluorescent. In this study, we circumvented this problem by using one of the isomers as an energy acceptor from an excited fluorophore acting as a donor. The most general solution is to covalently link a fluorescent moiety to a photochromic compound [13, 14]. Nonetheless, even with a resonance energy transfer (RET) efficiency of 100%, incomplete conversions of the photochromic compound would undesirably lead to residual fluorescence.

For test purposes, however, it is efficient to disperse a concentrated mixture of both compounds in a polymer film [15]. Therefore, in a proof-of-principle study, using a mixture of compounds has the advantage that the amount of the individual components can be controlled, so that the modulation of the fluorescence can be made less sensitive to the efficiency of the photochromic conversion. The most important difference between the two cases is the dependence of the fluorescence signal on the optical power driving the photoswitching. In the case of a covalently linked RET pair, the fluorescence signal decreases linearly with the concentration of the acceptor. Thus, in a first approximation, the fluorescence decays exponentially with the applied intensity. In the case of a dispersed mixture, the relationship is more complex. In a simple approximation, the Perrin model [16] predicts an exponential dependence of the fluorescence with the concentration of molecular acceptors, which also depends exponentially on the intensity of the converting light. Specifically, in the case where several acceptors are present for each donor a faster fluorescence saturation is predicted. Therefore, in this study, we used a mixture of Coumarine 6 (C6) as the fluorescent dye and a photochromic compound belonging to the furyl-fulgides family, with the commercial name Aberchrome 670 (Ab670), embedded in a poly(methyl methacrylate) (PMMA) matrix. The furyl fulgides are characterized by the absence of thermal processes, so that the forward and reverse reactions between the two forms, called the open form (OF) and the closed form (CF) is driven only by light. The formula of both isomers is depicted in figure 1.

Optical bistability is very valuable in a RESOLFT type of approach, since the time taken for switching an ensemble between the two states is solely determined by the intensity of the light applied. If there is a spontaneous interchange rate with a constant $k_s$, then the rate (for transferring the molecule from one state to the other) has to be faster than the characteristic time $\tau_s = 1/k_s$ [6, 9]. In this case, one would require stronger intensities to counteract the spontaneous transitions. Besides, only a dynamic equilibrium could be installed, i.e. a complete conversion to the non-emitting state cannot be reached. In contrast, Ab670 is a thermally stable molecular switch.

In the solutions and film casts prepared for this study, Ab670 is initially in the OF, absorbing at 350 nm. Irradiation of this isomer with UV light produces a ring closing reaction to the CF, responsible for the broad absorption band in the visible spectral region (450 – 600 nm). The CF can be converted back to the OF with visible light. The spectra of a PMMA film doped with Ab670 before irradiation and in the photostationary state after UV irradiation (366 nm light) are shown in figure 1, along with the fluorescence emission spectrum of C6 in the film. Based on absorption and HPLC experiments, we calculated that in the photostationary state in PMMA, the conversion to the CF was $\sim$75%. The emission spectrum of C6 substantially overlaps with the absorption band of the CF, while the overlap with the absorption spectrum of the OF is minor. Hence, unlike the OF isomer, the CF is a good RET acceptor.

The RESOLFT microscopy experiment reported here is based on this pronounced difference in the spectral overlap. The fluorophore was excited at the isosbestic point of the photochromic
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Figure 1. Photoswitching the photochromic molecule Aberchrome 670 between an OF and CF. Absorption spectra of a PMMA film doped with Aberchrome 670 in the OF (black line) and in the photostationary state under irradiation with 366 nm light (red line; ratio OF : CF = 25 : 75). Irradiation leads to substantial changes in the absorption spectrum in the 450–550 nm wavelength range where it overlaps with the emission spectrum of Coumarine 6 (C6) in PMMA (green line). The working principle of the photochromic RET system is sketched in the upper scheme. The arrows on the lower axis indicate the wavelengths of the lasers used in the setup (see figure 2(a)) to drive Ab670’s photoisomerization.

system (405–415 nm), in order to minimize the perturbation of the latter while reading out the fluorescence signal. To analyse the performance of this photochromic–fluorophore system, PMMA films containing 0.33 and 33 mmol kg\(^{-1}\) of polymer of C6 and Ab670 respectively were prepared. The films were irradiated with UV and visible light from a mercury lamp, and studied by conventional absorption and fluorescence spectroscopy. The C6 fluorescence was completely quenched after irradiation with UV light (366 nm), and the initial state was recovered after irradiation with visible light (546 nm). This reversible phenomenon was observed for several cycles with no sign of fatigue, as shown in figure 2.

Figure 2 also shows the kinetics of this switching process under UV irradiation. The fluorescence at the emission maximum of C6 and the absorbance at 522 nm are plotted against the irradiation time/energy of 366 nm light. The absorbance at 522 nm directly represents the concentration of the CF. Two important features can be extracted from these curves. Firstly, the characteristic time for the decay of the fluorescence is faster than that for the generation of the CF, implying that more than one RET acceptor is generated for each donor. Secondly, the observed saturation of the residual fluorescence with the illumination dose makes this molecular system a potential candidate for a RESOLFT superresolution microscopy.

We note that considering randomly distributed donor and acceptors in a frozen diffusion, the critical values \(R_0\) and \([A]_0\) obtained for this system (C6–CF) are 36 Å and 9.5 \(\times\) 10\(^{-3}\) M respectively, where \(R_0\) is the Förster radius and \([A]_0\) is the critical concentration of acceptors [17]. Trivial energy transfer processes can be excluded since the absorbance of the samples was below 0.02.

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Figure 2. Kinetics of a PMMA film doped with C6 and Ab670 under irradiation with 366 nm light. Normalized maximum emission of C6 at 485 nm (green, left axis), and absorption of the closed isomer of Ab670 at 522 nm (red, right axis). The inset shows the fluorescence signal after repeated irradiation with UV (black symbols) and visible light (green symbols), for 5 consecutive cycles.

In order to image with subdiffraction resolution, subdiffraction effective point-spread-functions (PSF_{EFF}), i.e. fluorescent spots, are generated through the combined action of beams that are scanned across the specimen: (1) a regularly focused excitation beam ($\lambda_{EX} = 415$ nm) producing PSF_{EX} for reading out the fluorescence, (2) a beam ($\lambda_{OFF} = 375$ nm) featuring a node at the centre ($x = 0$) delivering PSF_{OFF} that switches OF $\rightarrow$ CF and hence generates RET acceptors at the periphery of the excitation beam, and (3) a focal spot PSF_{ON} at $\lambda_{ON} = 543$ nm driving the reverse reaction CF $\rightarrow$ OF to restore the initial condition.

The experimental arrangement is sketched in figure 3(a). The focusing was accomplished with an oil immersion objective lens of 1.4 numerical aperture (PL APO 100 $\times$ 1.4–0.7, Leica Microsystems, Wetzlar, Germany). The excitation light was linearly polarized and provided by a continuous wave diode laser (DL100, Toptica Photonics AG, Gräfelfing, Germany). The collected fluorescence light was imaged onto an optical fibre of 62.5 $\mu$m diameter acting as the pinhole. A dichroic mirror and a bandpass filter in front of the detector selected the fluorescence emitted in the range 470–530 nm. The lasers driving the photochromic switch were collinearly aligned by means of dichroic mirrors. Light at $\lambda_{OFF}$ was provided by a diode laser operating at 375 nm (iPulse-375, Toptica Photonics AG, Gräfelfing, Germany) and the reverse switching ($\lambda_{ON}$) was accomplished using a green He–Ne laser (25LGPI93-230, Melles Griot, Carlsbad, CA, USA). The lasers were polarized in the $y$-direction, perpendicular to the direction of resolution enhancement. PSF_{OFF} is prepared by applying a $\pi$-phase shift in a half-plane of the entrance pupil such that it features a nodal $yz$-plane. Thus, in our initial experiments, the PSF_{EFF} is squeezed along the $x$-axis only. The $\pi$-phase to realize the central node of PSF_{OFF} was produced with a spatial light modulator (X8267-3558, Hamamatsu Photonics K K Hamamatsu City, Japan) whose screen was projected into the entrance pupil of the objective lens. Figure 3(b) shows the calculated PSF_{OFF} together with the PSF_{EX} measured using 80 nm silver beads. Line profiles in the $x$-direction along the dotted line of these PSFs are shown in figure 3(d) (black and blue curves respectively). The temporal succession of the three laser pulses is controlled with a programmable pulse generator (9500 Series, Quantum Composers, Bozeman, MT, USA). The He–Ne laser was modulated by means of an acousto optic tunable filter -AOTF- (AA.AOTF.nC-TN1003, AA Optoelectronic, St. Remy, France), and the detector was gated simultaneously with...
Figure 3. (a) Schematic of the microscope set-up. (b) Measured focal spot of excitation light PSF_{Exc} (top) and calculated depletion spot (bottom) featuring a nodal yz-plane. (c) Fluorescence depletion curve measured in a thin sample; logarithmic representation. (d) Effective focal spot PSF_{EFF} (purple line), calculated from the depletion curve in (c) and the PSFs in (b) exhibits a FWHM of 48 nm. The measured image profile of a single groove along the x-direction (full red line) and the expected one (red dotted line), compares well, corroborating the 48 nm FWHM of the PSF_{EFF}. X-profile for the excitation PSF and for the depletion PSF is indicated as a blue and black line, respectively.

The excitation laser. Sample scanning was performed with a piezo scanning stage, controlled with a home made software that was also used for image acquisition.

The C6 fluorescence generated in the subdiffraction sized spot is measured by an avalanche photodiode detector after passing a pinhole amounting to about 1.4 times the backprojected Airy disk. The fluorophores that are spatially located off the centre have a very large probability to perform a radiation-less energy transfer to a CF isomer of Ab670 and thus a very low probability to emit a photon, confining the fluorescence to a subdiffraction area around the centre. The width of this area is reduced by applying larger light doses (higher intensities or longer times). The system has to be refreshed before the next scanning step is performed at \( \lambda_{ON} \). The diameter of the main maximum of PSF_{ON} is selected large enough to cover the whole area initially irradiated with \( \lambda_{OFF} \). By probing the fluorescence before and after generating the CF isomers, two images are
obtained simultaneously pixel by pixel: a ‘standard’ and a subdiffraction resolution ‘RESOLFT’ image. Thus, a typical laser pulse sequence used was: first, a refreshing pulse with $\lambda_{\text{ON}}$ to ensure the whole system is in the emitting state; second, an excitation pulse with $\lambda_{\text{EX}}$ to record the standard image; third a depleting pulse of $\lambda_{\text{OFF}}$; and finally a second excitation pulse to record the RESOLFT image.

To estimate the subdiffraction PSF$_{\text{EFF}}$, we used grooves produced on a glass cover-slip by ion beam etching (Fraunhofer Institute IISB, Erlangen, Germany) that were about $\sim 8 \mu$m long, 600 nm deep and 90 nm wide. The grooves were spin-coated with the mixed-doped PMMA films in such a way that only their bottom part was covered with the mixture. With the excitation laser, we produced a PSF$_{\text{EX}}$ of 230 nm FWHM in the $x$-direction, which is larger than the width of the grooves and comparable to the intergroove distance. Figure 3(c) shows the depletion of the fluorescence resulting from the photoswitching OF $\rightarrow$ CF. The measurement shows that, unlike the one measured in a film, there is a component of fluorescence light that cannot be completely quenched. This component is partly due to contributions from C6 molecules that are outside the focal plane in the $z$-direction where fewer CF isomers are created. Nonetheless, figure 3(c) shows a strongly nonlinear decline in the fluorescence that can be exploited for breaking the diffraction barrier.

Adding the ‘switch-off beam’ PSF$_{\text{OFF}}$ with a peak energy of 0.09 J cm$^{-2}$ indeed leads to a line profile (red full line in figure 3(d)) featuring a FWHM of 112 nm, which is half as narrow as the 230 nm observed for PSF$_{\text{EX}}$ (blue line in figure 3(d)). We note however that the FWHM of the line profile does not represent the obtained resolution, because the measured profile constitutes the convolution of the object function of the groove with the effective PSF$_{\text{EFF}}$. The latter can be calculated according to equation (2):

$$\text{PSF}_{\text{EFF}}(x) = \text{PSF}_{\text{EX}}(x) \cdot \text{FS}(I_{\text{DEPL}} \cdot t_{\text{IRR}}) = \text{PSF}_{\text{EX}}(x) \cdot \text{FS}(\text{PSF}_{\text{OFF}}(x)),$$

where all PSF$_{XX}$ have been defined above, and FS is the remaining fluorescence signal after irradiation with depleting (UV) light of intensity $I_{\text{DEPL}}$ during a time $t_{\text{IRR}}$. The function FS is the depletion curve shown in figure 3(c). The spatial filtering effect of the comparatively large detection pinhole used in our study and hence the detection PSF arising from the use of a pinhole can be ignored, since their effect on the lateral extent of PSF$_{\text{EFF}}$ is negligible. The resulting PSF$_{\text{EFF}}$ is shown in figure 3(d) featuring a FWHM of 48 nm. If we now calculate the anticipated image as a convolution of the object function (known from electron microscopy) with this PSF$_{\text{EFF}}$, we obtain the anticipated image function of the groove (dotted red line in figure 3(d)). Comparison with the actually measured image function shows that the two are in excellent agreement, thus confirming the $\sim 50 \text{nm} \ (\sim \lambda/8)$ lateral resolution observed in this experiment. Application of higher intensities does not lead to narrower image profiles of the grooves because the width of the grooves is larger than the PSF$_{\text{EFF}}$. Nonetheless, the experiment clearly showed that the effective PSF could be squeezed significantly below the diffraction barrier.

Figure 4 shows a pair of images of a set of grooves with a decreasing groove separation distance from left to right. The comparison between the standard image and the RESOLFT counterpart image confirms the increase of resolution in the latter case along the $x$-direction. The increase becomes more apparent after applying a linear deconvolution with the PSF$_{\text{EFF}}$ using a regularized Tikhonov–Miller algorithm [18], enhancing the contribution of the higher spatial frequencies in the image. For fair comparison, the standard reference image was also deconvolved (with the PSF$_{\text{EX}}$). The deconvolved RESOLFT image exhibits significantly more details than the
Figure 4. Standard ((a): raw data; (c): linearly deconvolved) and subdiffraction RESOLFT ((b): raw data; (d): linearly deconvolved) images of a set of grooves with an average width of 90 nm and decreasing intergroove distance from left to right. Both raw data (b) and linearly deconvolved images (d) prove an increase of resolution by molecular photoswitching. (e) Normalized line profile along the marked area for the deconvolved images (standard: black line; RESOLFT: red line).

references. The resolution increase by molecular photoswitching is also confirmed in the line profiles in figure 4 featuring an increase in contrast \((I_{\text{peak}} - I_{\text{valley}})/(I_{\text{peak}} + I_{\text{valley}})\) from 0.08 to 0.56, for the two grooves with the smallest separation distance (the two rightmost ones). For the calculation of the above values, the average \(I_{\text{peak}}\) value was used in the standard profile. The power of the depleting laser transmitted by the objective lens was 75 nW, corresponding to an intensity of 185 W cm\(^{-2}\) in the focal plane. The power of the excitation laser and the one to drive the reverse reaction \((\lambda_{\text{ON}})\) were 8 nW and 16 \(\mu\)W, respectively.

As a control experiment, we occasionally removed the phase-plate of the UV laser so that PSF\(_{\text{OFF}}\) turned into a regular circular spot. Consequently, most of the fluorescence signal was quenched and no resolution gain was observed, as expected. In a further control experiment, the sample was rotated by 90\(^\circ\) so that the PSF\(_{\text{EFF}}\) was squeezed in the direction parallel to the grooves. Again, no resolution improvement was observed.

The pixel dwell time in our images was arbitrarily set to about one millisecond, to perform the reading of a complete (standard and RESOLFT) image in a reasonable time (on the minute timescale). The intensities applied were thus defined by the time required to saturate the transition \(\text{OF} \rightarrow \text{CF}\). The intensity can be increased, when faster times are required, for example in the case of diffusing objects. The scanning may be speeded up to the microsecond range and still the illumination will require only a few kW cm\(^{-2}\). Using such low intensities also allows for parallelizing the image formation by applying arrays of holes or lines, an approach often referred to as structured illumination [19]. Note that the physical approach reported herein does not require
the use of a pinhole or single beam scanning, as neither does the RESOLFT scheme or STED microscopy in general [4]. As long as the local zeros are further apart in the focal plane than the minimal distance that can be classically resolved by imaging the zero pattern on a camera, parallelized reading out of the fluorescence with a camera is possible [6]—at expense of the background suppression. A reduction in the applied intensity can be accomplished by resorting to molecules with larger effective cross sections. However, the primary goal in the design of new compounds remains to increase the number of switching cycles that can be performed in the molecule. Since the number of pixels in the volume of the depleting PSF defines a lower limit of the number of cycles the molecules should resist before suffering photodamage, a higher fatigue resistance (i.e. larger number of isomerization cycles) will be required for 2D and 3D imaging with ultra-high resolution.

In conclusion, we have demonstrated the breaking of the diffraction resolution barrier in far-field microscopy using the isomerization reaction of photochromic organic markers. The use of low power lasers makes the technique a low cost alternative for fluorescence microscopy with resolution beyond the diffraction limit.

A further highly intriguing prospect is the possibility to extend the concept to the optical writing of nanoscale structures with arbitrarily small separations that are not limited by diffraction. For this purpose, the photochromic moiety should be locked in a third state defining the structure to be written [9, 20]. The success of this endeavour would be highly relevant to information storage at higher densities, a major technological goal that has been pursued for several decades.

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