STED controlled photobleaching for sub-diffractional optical nanopatterning

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Abstract

Laser-assisted protein adsorption by photobleaching (LAPAP) is a versatile tool to nanopattern proteins on the micrometer scale. Sub-micron patterning is, however, difficult due to diffraction. We show that, similar to stimulated emission depletion (STED) microscopy, a depleting beam can effectively suppress LAPAP and hence is apt to locally control LAPAP in order to write sub-diffractional lines of proteins. Specifically, we attach biotinylated Atto 390 to glass substrates and incubate with Alexa 555 labeled streptavidin. The Alexa 555 is subsequently imaged with STED nanoscopy. The method is currently limited by diffusion of the biotinylated Atto 390 molecules.

Stimulated emission depletion (STED) microscopy and related techniques have enabled stunning resolutions far below the diffraction barrier [1–3]. The essence is that fluorophores are actively de-excited in the outer rim of the point spread function (PSF) after excitation, but before they spontaneously emit a fluorescence photon. In 1999, it was proposed that this principle could readily be transferred to nano-localized photochemistry [4, 5], however, experimental realization in STED-inspired optical nanolithography came a decade later [6–13], but was largely restricted to multiple-photon-induced radical polymerization of acrylates and methacrylates [14]. In recent years, this principle has been expanded to non-polymerizing photochemistry using spirothiopyrans [15–17] or keto-enol switching in α-methyl benzaldehydes [18]. In the first approach, spirothiopyran is optically opened (‘switched on’) with near UV light to present the maleimide-reactive thiol group, and closed (‘switched off’) with green light. Spirothiopyran can also be used as an electrostatic crosslink in super-resolved patterning of methacrylates [19]. The second approach employs a keto-enol switch where two-photon absorption of 700 nm light induces an isomerization from the keto- into the reactive enol-form while 440 nm light switches into the Z-enol which rapidly reverts back to the inert keto-isomer [18].

In this letter, we show that it is possible to use a depleting laser beam to prevent the lithographical adsorption of the dye Atto 390 via photobleaching. This acts as an optical switch to prevent laser-assisted protein adsorption by photobleaching (LAPAP) [20–22] in the outer rim of the optical PSF and hence sub-diffractional nanopatterning can be achieved by STED-inspired inhibition of photobleaching. In LAPAP, fluorophores are intentionally bleached in order to form radicals, which subsequently bind to a surface. These surface-tethered fluorophores usually carry a functional group such as biotin, which remains active despite the photobleaching of the dye and can therefore be used for further attachment of proteins to the substrate. In recent work, we have combined STED and LAPAP in a sequential manner [23]. First, nanoscale dots were fabricated using STED-inspired nanolithography of acrylate [10]. These were subsequently functionalized with biotin using LAPAP. Here, we apply the depleting beam directly to prevent LAPAP and thus achieve nanoscale protein patterning without the intermediate acrylate nanodots. This resembles the third system to date for non-polymerizing single-reaction STED-inspired nanolithography (apart from spirothiopyran and keto-enol switching).
1. Results and discussion

Atto 390 is a coumarin-based dye, which is known to undergo singlet-triplet transitions. From the triplet state, there is a certain probability that a coumarin will turn into a radical cation with subsequent photobleaching \[24, 25\]. If they are functionalized with biotin, they can be used for LAPAP [23]. Recently, it has been shown that, after excitation with 405 nm light, the fluorescence of Atto 390 can be switched off by 532 nm light [26]. It is reasonable to assume that this depletion is due to stimulated emission (‘true STED’). However, there might be other mechanisms such as excited state absorption (ESA) towards a non-fluorescing state, as well [27, 28]. However for brevity, we will henceforth refer to the 532 nm light beam as the ‘STED-beam’. One key conclusion of our work is that whilst the fluorescence of Atto 390 can be depleted with 532 nm light, the ability to photo-pattern via LAPAP is diminished, too. Preliminary results show a reduced width of lines written with STED-LAPAP, compared to lines written with LAPAP only. The achieved line width of 170 nm is sub-diffractional. Considering reasonable radical lifetimes and diffusion coefficients, we conclude that the current line width is limited by the diffusion of the Atto 390 radicals out of the narrow effective STED-PSF before the radicals attach and stick to the substrate.

The proposed mechanism for STED controlled LAPAP for bio-nanopatterning is depicted in Scheme 1. A substrate is passivated with bovine serum albumin (BSA) and incubated with biotinylated Atto 390, which is locally photobleached after two-photon absorption within the red PSF using 100 fs pulses of 780 nm wavelength (scheme 1(a), left). After washing, only those biotinylated Atto 390 stick to the surface, which turned into radical ions due to photobleaching. The biotin remains active and binds streptavidin (SA) upon incubation with SA-Alexa 555 (scheme 1(a), right). The red emitting Alexa 555 serves as a reporter that LAPAP and the binding of SA have been successful. Scheme 1(b) depicts the simultaneous application of the 780 nm two-photon excitation of Atto 390 together with a donut-shaped, 532 nm, CW depletion beam (scheme 1(b), left). The latter inhibits LAPAP, most probably by inhibiting the formation of radical cations and hence shrinks the effective PSF for LAPAP lithography. The result is a narrowed area where SA-Alexa 555 is attached to the surface (scheme 1(b), right).

In order to test whether LAPAP can be optically suppressed, we write lines using 5.5 ± 0.5 mW of 780 nm light (all powers measured in front of the objective lens with NA = 1.46) for two-photon excitation. Additionally, we apply a green (532 nm) CW laser focus with an ordinarily shaped PSF confocalized with the 780 nm PSF. This should prevent LAPAP, in a similar manner to the suppression of Atto 390 fluorescence using 405 nm one-photon excitation and 532 nm for STED as shown by Luo et al [26]. Figure 1(a) shows the Alexa 555 signal of the biotinylated Atto 390 LAPAP lines after ‘development’ with Alexa 555 labelled streptavidin (532 nm CW excitation, confocal detection). From left to right, an increasing power of the
Figure 1. Inhibition of two-photon excited LAPAP by 532 nm STED. (a) Excitation and depletion laser powers are varied to determine the optimum parameters. For both laser foci, ordinary PSFs are used and confocalized. With increasing depletion laser power, the line visibility is reduced; scale bar: 5 µm. (b) Alexa 555 intensity vertically integrated along three lines in the dashed box in (a), as a function of the applied STED power. Solid line: exponential decay with 25% offset and a decay constant of $P_{\text{sat}} = 3.0 \pm 0.2 \, \text{mW}$. (c) Scheme of possible ways to prevent Atto 390 fluorescence and radical formation: STED and ESA act on both fluorescence and radical formation, TTA acts only on radical formation.

532 nm depletion laser was applied, starting with 0 mW, increasing in steps of 1 mW up to 5 mW and further increasing in steps of 3 mW up to 59 mW. The Alexa 555 reporter signal becomes fainter with increasing power of the depletion laser. Figure 1(b) shows the line-integrated Alexa 555 signal as a function of the depletion power (averaged over three runs with two-photon excitation powers of 5.0, 5.5 and 6.0 mW, background corrected). The Alexa 555 signal rapidly drops down to about 25% of its original value. The solid line is an exponential fit with a 1/e decay constant of $3.0 \pm 0.2 \, \text{mW}$. This constitutes the saturation power $P_{\text{sat}}$ in the improvement factor $\beta$ for resolution in STED microscopy [29]

$$\beta = \frac{1}{\sqrt{1 + P_{\text{max}}/P_{\text{sat}}}} \quad (1)$$

where $P_{\text{max}}$ is the maximally applied power in the donut (see below). Some similarities but also some discrepancies are apparent compared to the work by Luo et al [26]. Also Luo et al observed a rapid drop of Atto 390 fluorescence with a residuum of 22% at 60 mW, slowly decaying further to 10% when applying up to 550 mW STED power. However, they reported $P_{\text{sat}} = 14 \, \text{mW}$ for fluorescence depletion while we measured a 4.7 times smaller saturation power. Using a similarly high NA objective lens, this discrepancy could be explained by the fact that in our case, some effect in addition to STED is active to optically prevent the oxidation of Atto 390. Stimulated emission as well as ESA within the singlet system can empty the S1 state as depicted in figure 1(c). Both diminish fluorescence and inter-system crossing (ISC) with the same efficiency. Hence, we consider it very likely that in the coumarin-based Atto 390, an additional depletion channel is active in the triplet system, such as triplet–triplet absorption (TTA), which hinders the formation of cationic radicals. Pump–probe spectroscopy [27] or electronically gated spectroscopy [28] could possibly separate these different contributions, however, this is beyond the scope of this initial report.

In a next step, we inserted a 2π vortex phase mask into the 532 nm depletion beam in order to form a donut around the excitation focus and wrote lines using 5.5 mW of 780 nm two-photon excitation power.
and 33 mW of 532 nm depletion power. After writing, the lines were again incubated with Alexa 555 tagged streptavidin (c.f. Scheme 1(b)). Imaging of the Alexa 555 was then carried out in the STED microscopy mode of the setup, using 10 µW of 532 nm for excitation and 30 mW of 660 nm CW for STED. Figure 2 shows some exemplary lines written with 780 nm two-photon excitation only (figure 2(a)), or with 780 nm excitation and 532 nm depletion (figure 2(b)).

Figure 2(c) shows a line profile (green) taken at the dashed horizontal line in figure 2(a). This reveals the local line width of a two-photon LAPAP line. However, the line width varies slightly in the vertical direction, mostly due to diffusion of the Atto 390 radical cations before they attach on the glass substrate. In order to quantify this effect, we vertically integrated a complete line (dashed box in figure 2(a)) and plotted the resulting profile (see black line in figure 2(c)). Compared to the local line width, this integrated line width is slightly broadened from 200 to 250 nm. Figure 2(d) shows the same two profiles, but now from a STED-LAPAP line indicated by the dashed line and the dashed box in figure 2(b). As the local line width of 70 ± 5 nm (green line) resembles the resolution limit of our CW-STED microscope using 532 nm CW excitation and 660 nm depletion [23, 30], we conclude, that the narrow width of this line profile is the profile of presumably only one streptavidin molecule. However, it is apparent from the lines in figure 2(b) that there is quite some scatter of the attachment of streptavidin along the line. Again, we plot in figure 2(d) the vertically integrated profile of the line in the dashed box in figure 2(b). The integrated intensity profile now measures W = 170 ± 5 nm. This width resembles a convolution of three widths, that of the effective STED-PSF of the read out microscope PSF_read = 70 nm, the effective PSF_write which was used for writing with STED-LAPAP, and broadening by lateral diffusion d of the Atto 390 cationic radicals out of the STED-LAPAP focus prior to the immobilization at the substrate. In a rough estimate, the three contributions add quadratically to the square of the overall measured line width W and hence

$$W = \sqrt{PSF_{\text{write}}^2 + PSF_{\text{read}}^2 + d^2}$$

(2)

The PSF_write can be estimated with equation (1) using $P_{\text{sat}} = 3.0$ mW, $P_{\text{max}} = 11$ mW (the intensity in the donut is reduced to 1/3 of the intensity of an ordinary PSF used for figure 1) and the two-photon (hence a pre-factor $1/\sqrt{2}$) [31] diffraction limited PSF at 780 nm and using NA = 1.46. This leads to:

$$PSF_{\text{write}} = \beta \frac{1}{\sqrt{2}} \frac{780 \text{ nm}}{2 \cdot 1.46} = 88 \pm 6 \text{ nm}.$$  

(3)

We therefore assume a spread by diffusion of $d = 128 \pm 8$ nm. This is a reasonable value when compared to diffusion of dye molecules in air-saturated aqueous solutions present during the writing step. The average two-dimensional (lateral) diffusion radius can be estimated from $d/2 = \sqrt{4Dt}$ where $D$ is the diffusion coefficient and $t$ is the lifetime of the cationic Atto 390 radical in aerated aqueous solution. We calculated a hydrodynamic radius $R$ for biotinylated Atto 390 using the Chem3D software from ChemOffice2016 v. 16 and find $R \approx 1.1 \pm 0.1$ nm. Using the Stokes–Einstein equation and the viscosity of water at 20°C $\eta = 1.0 \cdot 10^{-3}$ Pa·s, we determine a diffusion coefficient

$$D = \frac{k_B T}{6\pi \eta R} = 195 \pm 20 \mu \text{m}^2/\text{s}.$$  

(4)
With these numbers, we retrieve a lifetime of the Atto 390 radical cations of $t = 5.3 \pm 0.9 \mu s$, which is a very reasonable value for coumarin-type radical cations in aerated aqueous solution which was found to lie between 2 and 8 $\mu s$ [24, 32].

2. Conclusion

In summary, we have shown that LAPAP with the coumarin dye Atto 390 can be optically prevented using 532 nm laser light. Probably both STED of the excited singlet state as well as ESA within the triplet system contribute to prevent the formation of Atto 390 radical cations. When the 532 nm laser is applied in a donut shape around the two-photon excitation focus, lines of about 170 nm width can be written. In a detailed analysis, we deduce that the size of the sub-diffractional effective STED-LAPAP writing focus (88 nm), the STED read out focus (70 nm) and the diffusion of the radical cations (128 nm) contribute to the measured line width. Hence, STED-LAPAP has the potential of sub-diffractional patterning, specifically when diffusion can be restricted in the future. Ultimately, coumarins could be fixed to the substrate, for instance using a silane bond, and be locally turned into radicals using an effective STED focus. This sub-diffractional pattern of radicals may then be used for nano-localized redox-reactions. LAPAP is mostly aimed for patterning proteins in cell-biological studies. Reducing the size of the binding sites for proteins with STED will lead to a much better control of the number of individual proteins or the surface densities of triggering peptides [33] or chemokines [34]. Examples of physiological applications could be the integrin activation for focal adhesion [35,36], studies on T cell organization and signaling [37,38], or investigations of lipid organization in ordered domains in living cells [39]. In a recent work, we studied proteins on supported lipid bilayers diffusing around proteins fixed on acrylate anchors [40]. It would be intriguing to replace the acrylate anchors by directly attached proteins using STED-LAPAP. Our results are also relevant for understanding how STED could be useful for preventing photo-oxidation of dyes, specifically of coumarins, in bioimaging.

3. Experimental section

Glass substrates (Menzel Gläser No.1.5, Germany) are passivated with 3% aq. solution of bovine serum albumin (BSA, $\geq 99\%$, Sigma Aldrich GmbH, Germany). After washing the excess of BSA, 20 $\mu l$ of 100 $\mu g \text{ ml}^{-1}$ Atto 390 biotin (ATTO-TEC, Germany) in distilled water was drop casted onto the glass substrate. Photobleaching is initiated using 780 nm ultra-short laser pulses (FemtoRay780, 50 MHz repetition rate, 100 fs pulse duration, Menlo Systems GmbH, Germany). For depletion, a 532 nm continuous wave laser (Verdi-V5, Coherent, USA) was used. In case of LAPAP-depletion experiments, an ordinarily shaped PSF was used for the 532 nm beam. In case of sub-diffractional line writing, the 532 nm depletion beam was shaped into a donut using a $2\pi$ phase spiral (RPC Photonics, USA) and a $\lambda/4$ plate. Both beams were focused through an oil immersion objective lens (Zeiss $\alpha$-plan Apochromat, 100 $\times$, numerical aperture $NA = 1.46$). Excitation and depletion powers were measured in front of the objective lens. Avalanche photo diodes were used for aligning the foci in case of adjusting the STED lithography setup (APD-SPCM-AQRH, PerkinElmer Optoelectronic Inc. USA) and for collection of the fluorescence signal when the setup was used as a STED microscope (Micro Photon Devices s.r.l, Italy). For imaging, the Alexa 555 labelled streptavidin (ThermoFisher Scientific Inc. USA) was excited using 10 $\mu W$ of the 532 nm laser beam (in an ordinarily shaped PSF) and the Alexa 555 was depleted using 30 mW of a 660 nm donut shaped STED beam (Laser Quantum Opus, Germany). A three axes piezo stage (P-562.3 CD, Physik Instrumente PI, Germany) with a bidirectional positioning accuracy of 2/2/4 $\mu m$ and a travel range of 200x200x200 $\mu m$ was used for sample scanning. For sample positioning, recording of images and controlling the writing process, a custom written LabView (National Instruments Corporation, USA) program was used. Further details of the setup can be found in [23].

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Note

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[37] Schwarzenbacher M, Kaltenbrunner M, Brameshuber M, Hesch C, Paster W, Weghuber J, Heise B, Sonnleitner A, Stockinger H and Schütz G J 2008 Micropatterning for quantitative analysis of protein-protein interactions in living cells Nat. Methods 5 1053–60
[38] Pi F, Dillard P, Alameddine R, Bernard E, Wahl A, Ozerov I, Charrier A, Limozin I. and Sengupta K 2015 Size-tunable organic nanodot arrays: a versatile platform for manipulating and imaging cells Nano Lett. 15 5178–84
[39] Sevcsik E, Brameshuber M, Fölser M, Weghuber J, Honigmann A and Schütz G J 2015 GPI-anchored proteins do not reside in ordered domains in the live cell plasma membrane Nat. Commun. 6 6969
[40] Buchegger B, Kreutzer J, Axmann M, Mayr S, Wöllhoven R, Plochberger B, J. J and Klar T A 2018 Proteins on supported lipid bilayers diffusing around proteins fixed on acrylate anchors Anal. Chem. 90 12372–6