8 STED lithography and protein nanoanchors

Abstract: Two-photon lithography (or, more general, multiphoton lithography) is apt to produce three-dimensional structures with feature sizes below the diffraction limit set out by Abbe. This is due to chemical nonlinearities which are intrinsic to polymerization. However, if one wants to write two nanometric structures close together yet prevent those structures from merging, two-photon lithography basically turns out to be bound to the diffraction limit unless some specific tricks are applied, such as the use of “forgetting starters” which require, however, particularly slow scanning speeds. An appealing means of working around this problem is provided by STED lithography, where STimulated Emission is used to Deplete (STED) the polymerization starters in the outer rim of the point spread function, very similar to the way in which it is used in STED microscopy. After reviewing STED microscopy and introducing STED lithography and its current state-of-the-art in terms of minimal structure size (54 nm) and resolution (120 nm), and after touching on photo-optical switching techniques alternative to STED, the following application for biological and physiological research will be outlined. Using STED lithography, it is possible to write nanoanchors for antibodies which carry only one antibody per nanoanchor. This allows proteins to be displayed on a substrate surface in any arbitrary pattern and with utmost control over the surface density. A discussion of some current problems and possible further directions of research will conclude this chapter.

8.1 Introduction

In 1931, Maria Göppert-Mayer proposed that two photons could be simultaneously absorbed in order to excite a molecule from its ground state to the first excited state [1]. However, it took almost 60 years until this effect was utilized by Denk, Strickler, and Webb in fluorescence microscopy in order to achieve axial resolution without confocalization being necessary [2]. The latter feature comes from the fact that the optical transfer function provides a finite value for the axial k-vector in cases of two-photon absorption, unlike the situation in one photon fluorescence microscopy [3]. Shortly after the publication of their landmark paper on two-photon microscopy, Strickler and Webb showed that two-photon excitation of a photopolymer allows high density data storage [4]. In 1997, Maruo et al. from the Kawata group adapted the method for three-dimensional, two-photon-induced polymerization lithography (2PPL) [5]. Meanwhile, 2PPL facilitates writing features with lateral sizes of 90 [6], 80 [7, 8], and 65 nm [9] when pulsed lasers are used for two-photon excitation with wavelengths of 1030, 800, and 520 nm, respectively. Due to a chemical nonlinearity [10], these feature sizes are already below the diffraction limit given by Abbe [11]. We will discuss this below...
in more detail. Further, a line width of 50 nm has been achieved by post deposition shrinking [12]. Importantly, the dimensions of isolated structures must not be confused with resolution. The latter is defined by the minimal spacing of two adjacent yet separated structures and is currently in the range of 200 nm (lateral resolution) for 2PPL [13]. This is obviously much larger than the resolution typically achieved in e-beam or ion beam lithography or with far-UV mask-based lithography. Both techniques approach a resolution of 20 nm. However, they show two decisive drawbacks: First, both use high energetic particles or photons which can be tolerated by robust solids such as semiconductor wafers, but are detrimental for biological tissue or soft plastic electronics. Second, they are intrinsically bound to structuring of surfaces, while 2PPL comprises an intrinsic three-dimensional capability. Hence, opportunities for decreasing feature size to, say, 20 nm and increasing resolution to the sub 100 nm range are highly sought after in 2PPL using low energetic visible photons and featuring intrinsic 3D capability.

In 1994, Hell and Wichmann [14] came up with an idea to break the restrictions set to resolution in optical microscopy by Abbe’s diffraction limit [11]. They proposed that the resolution in fluorescence microscopy will not be bound to diffraction if one switches off the fluorescence in the outer rim of a diffraction-limited point spread function (PSF) of the excitation beam quick enough so that the fluorophores cannot emit a photon. Depletion after excitation but before emission is actually possible because the average fluorescence lifetime of a typical fluorophore is in the range of 1 nanosecond, which leaves plenty of time to bring the molecule from the excited state down to the ground state [14]. One possibility (but by far not the only one) to inhibit spontaneous emission is depletion of the excited state via stimulated emission. After initial experimental corroboration of the idea, which showed a resolution in all three directions in the range of 100 nm [15, 16], an effective PSF with a lateral width of 8 nm was realized using far field fluorescence STED microscopy [17], a value which was unimaginable 20 years ago. In consequence, Stefan Hell received the Nobel Prize for Chemistry for his achievements in the year 2014. The original method of de-exciting the molecules in the outer rim of the excitation focus, stimulated emission depletion (STED), gave the name to a whole field of sub-Abbe resolution microscopy. Many applications have been shown within the last decade, most of them in the field of nanoscopic physiology and cell biology [18–28], but some applications in materials science were also reported [29–31].

Similar to the further development of two-photon microscopy [2] into 2PPL [4, 5], it was already postulated in the first experimental reports of STED microscopy that a confined effective excitation volume can also be applied to spatially control photochemical reactions on the nanometer scale [15, 16]. STED and STED-inspired 2PPL have been reported experimentally recently [13, 32–37].
8.2 STED microscopy

Before turning to STED lithography, one should briefly recall what is required to build a STED microscope. In the traditional STED configuration (Fig. 8.1 (a)) [16], an excitation laser is required in the UV-Vis range, which matches the excitation spectrum of the fluorophores. Commonly, a pulsed laser in the nano- to femtosecond regime is applied in order to guarantee a temporally defined excitation, although STED microscopy with continuous wave (CW) excitation and CW STED have been reported [38], though with some compromise in resolution. A typical choice for a pulsed excitation laser is the tunable output of an optical parametric oscillator (OPO) with built-in second harmonic generation, or simply a suitable picosecond semiconductor laser [39, 40]. Traditionally, the STED beam stems from a Ti:Sa femtosecond laser. In this case, the STED pulse needs to be stretched to some tens of picoseconds at least in order to achieve effective depletion of the excited singlet state via STED, because a femtosecond pulse would only lead to a 50/50 equal distribution between the effective emission level and a higher vibronic level of the ground state due to reabsorption [41]. Alternatives to Ti:Sa lasers for the STED beam are Raman lasers [42], fiber lasers [40], or even pulsed laser diodes [43]. CW lasers have been used as STED lasers, as well, with some compromise in resolution [38]. Nevertheless, full resolution can be regained by temporal gating of the detection of fluorescence if at least the excitation is pulsed [44, 45].

The excitation pulse must be expanded in a telescope and the transversal mode profile is cleaned (if necessary) by a pinhole in order to illuminate the rear aperture with a plane wave. This is mandatory to achieve the smallest possible diffraction limited excitation-PSF. The mode of the STED laser is cleaned by a pinhole and expanded as well. In order to produce a high-quality donut shaped STED-PSF in the focus, a phase pattern needs to be imprinted on the wave front of the STED beam, which is then Fourier-transformed by the objective lens into a hole-shaped STED-PSF in the focus. When only a lateral improvement of the resolution is desired (specifically if all fluorophores of interest are in one plane only, such as in total internal reflection (TIRF) STED microscopy [46]), the $2\pi$ spiral phase mask is superior [19]. When a three-dimensional improvement is desired, an annular phase mask is the choice, as shown in Fig. 8.1 (a) [16]. Instead of these static phase masks where the phase is imprinted onto the wave front of the STED beam via varying optical path length, dynamic phase control using spatial phase modulators has recently been applied in some STED setups [18, 47, 48]. This dynamic phase control bears the advantage that aberrations can be corrected which could otherwise deteriorate the central zero of intensity of the STED-PSF. However, dynamic phase control systems are more complicated to handle and more expensive than static phase masks.

Figure 8.1 (b) shows, as a historic example, the first ever published effective PSF of a three-dimensional STED microscope [16]. A 48 nm bead, doped with the dye LDS 751, was imaged, whereby each forward and backward scanning line was sorted into two different images. While the STED beam was switched off during one scan direction
Fig. 8.1: (a) First ever realization of a 3D STED microscope. A normal shaped green PSF excites the fluorophores, while a hole shaped red PSF depletes the fluorophores in the outer rim. The hole shaped STED PSF is realized by an annularly shaped $\lambda/2$ plate in the STED beam path. (b) A dye doped bead imaged in confocal (left) and in STED-confocal mode (right). In 2000, effective STED PSF measured approximately 100 nm in all 3 directions. Meanwhile, sub 10 nm PSFs are realized in STED microscopy. Taken from [16]. Copyright 2000, National Academy of Sciences, USA.

(hence detecting a “classical” confocal image), it was switched on in the other scan direction. This way, both images shown in Fig. 8.1 (b) were taken, line by line, at the same time, excluding bleaching. In those days (around the year 2000), STED microscopy showed an effective PSF of 100 nm in all three directions, which was clearly below the Abbe limit. Due to systematic improvements, modern STED microscopy has now reached sub 10 nm effective PSFs and resolution [17].
8.3 STED lithography

Figure 8.2(a) shows a typical setup for STED-2PPL lithography. Femtosecond laser pulses of 780 nm are used for multiphoton excitation of the photostarters, and STED is performed via a continuous wave (CW) 532 nm laser. Similar to a STED microscope, both beams are expanded by a telescope and pinholes in the foci of the telescopes are used to clean the transversal modes. A phase plate is used to produce the donut shaped STED beam. If only a mere lateral improvement of feature sizes and resolution is desired, a $2\pi$ phase spiral is used as a phase plate (PP) and a $\lambda/4$ wave plate is applied to match the helicity of the polarization to the orientation of the phase spiral. In order to start with the utmost resolution already without STED, a high-end alpha-Plan Apochromat, 100×, NA = 1.46 oil immersion lens is applied. Stage scanning is performed via a capacitively coupled three axes piezo stage which is mounted on top of a mechanical three axes micrometer driven stage. An avalanche photodiode (APD) is used to monitor the (weak) back-reflected beams transmitting the two dichroic mirrors before the objective lens in order to adjust and properly overlap the two beams.

A 40/60 mixture of the acrylates pentaerythritol tetraacrylate (PETTA) and pentaerythritol triacrylate (PETA), including 300–400 ppm monomethyl ether hydroquinone, was used as a photoresist [37]. 0.25 wt % of 7-Diethylamino-3-thenoylcoumarin (DETC) was added as a photosensitive starter of radical polymerization. It has recently been shown that at least one more photon seems to be required to start radical polymerization when DETC is used as a starter, although two 780 nm photons are needed to excite fluorescence of DETC [49]. Despite this fact of an effective multiphoton excitation of DETC, the more common term “2PPL” will be used instead of “MPPL” as an acronym for multiphoton polymerization. Whatever the excitation mechanism might be, DETC can be readily depleted by 532 nm photons from a CW laser via STED [50]. The wavelength of the depleting STED laser is in the red wing of the fluorescence emission spectrum of DETC as shown in Fig. 8.2(b). Figure 8.2(c) and (d) show the lateral point spread functions of the multiphoton excitation beam and the STED beam, respectively, imaged by back reflection from a gold nanoparticle. Figure 8.2(e) shows a line written by 2PPL only (without STED). Due to an intrinsic chemical nonlinearity [10], this line already shows a line width of 87 nm, below the diffraction limit. Applying the STED beam in addition, the line width shrinks to 54 nm, as shown in the SEM image of Fig. 8.2(f).

As we will discuss in more detail in Section 8.5, the feature size (in this case: line width) must not be confused with resolution. As shown in Fig. 8.2(e), it is easily possible to write individual lines with sub-Abbe line widths using 2PPL, however, to the best of our knowledge, a true resolution below the Abbe diffraction limit is not expected using 2PPL unless a photoresist is “completely forgetting” as outlined by Fischer and Wegener [51]. In order to determine the lateral resolution of STED-2PPL, double lines with a nominal distance of $\delta$ were written on a glass substrate. The AFM images of such double lines are shown in Fig. 8.3 [37]. One clearly sees that double lines with
Fig. 8.2: (a) Setup for STED-lithography. Two-photon excitation at 780 nm and depletion at 532 nm. PH: pinholes for mode purification; PP: $2\pi$ spiral phase plate to create a donut beam; objective lens: 100×, NA = 1.46; APD: avalanche photodiode. (b) Spectra of the photoinitiator DETC in PETA. (c) Measured excitation PSF and (d) depletion PSF, lateral ($x$, $y$)-cross-sections in the focal plane (measured via back-reflection from a gold nanoparticle, diameter 50 nm). (e), (f) SEM images of solitary polymerized lines written with (e) ordinary 2PPL, and (f) STED-2PPL. Taken from [37] with permission. Copyright 2013 The Optical Society of America.

Fig. 8.3: AFM images of double lines, written at nominal distances $\delta$ of 100, 120, 160, and 200 nm. Adjacent lines with 120 nm line distance are clearly resolved. Reprinted with permission from [37]. Copyright 2013 The Optical Society of America.
distances down to $\delta = 120\,\text{nm}$ can be written and appear as double lines with clear spacing in between, while the double lines with a nominal distance of $\delta = 100\,\text{nm}$ have merged into one thick line. It was therefore concluded in 2013 that the lateral resolution of STED lithography was $120\,\text{nm}$ [37]. This is clearly below the diffraction limit considering the writing wavelength of $780\,\text{nm}$ and the depleting wavelength of $532\,\text{nm}$, however, one should think about further improvements. Nevertheless, there is justified hope that resolution in STED lithography will further improve, in the same way that resolution in STED microscopy has improved from the $100\,\text{nm}$ range in its earliest experimental reports [15, 16] down to below $10\,\text{nm}$ [17].

In order to achieve a three-dimensionally improved structure size with STED, an annular phase mask needs to be applied. This creates a “bottle” shaped STED PSF, as shown in the inset of Fig. 8.4 [52]. Again, a 40/60 mixture of PETTA and PETA, including 300–400 ppm monomethyl ether hydroquinone was used as a photoresist, and $0.25\%$ of DETC was added as a photosensitive starter. Figure 8.4(a) shows two vertical poles and one horizontal bar on top written with 2PPL to provide a frame for STED-2PPL written horizontal rungs using different STED-intensities. Between the two poles, it was intended to write five thin rungs applying STED-intensities of $0$ to $8.4\,\text{mW}$ in steps of $2.1\,\text{mW}$. The diameters of the rungs continuously decrease with increasing STED power from bottom to top for the first four rungs. The fifth rung did not appear, possibly because the STED power became too large and/or because the zero in the center of the hole-shaped STED-PSF became distorted when the focus was scanned deeper into the resist. Figure 8.4(b) shows a close-up of the fourth rung, which displays a thickness of only $53\,\text{nm}$. Assuming a

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**Fig. 8.4:** (a) Scanning electron microscopy (SEM) image showing two vertical poles and a horizontal bar on top written with 2PPL to provide a frame for STED-2PPL written horizontal rungs using different STED-intensities. (b) Close-up of the rung written with $4.24\,\text{mW}$ excitation and $6.27\,\text{mW}$ STED power, showing a thickness of $53\,\text{nm}$. Inset: calculated ordinary shaped two-photon excitation PSF ($780\,\text{nm}$) and hole shaped STED PSF ($532\,\text{nm}$). From [52]; The Royal Swedish Academy of Sciences. Reproduced by permission of IOP Publishing. All rights reserved.
circular cross-section of the rung, it can be concluded that the axial feature size is also in the range of 53 nm. This is clearly below the diffraction limit, when using 780 nm for two-photon excitation and 532 nm for STED.

8.4 STED related lithography

Figure 8.5 compares several methods of improving resolution in 2PPL via optical switching. The upper panel shows the “true” STED method, where an intense STED beam is spectrally tuned to the red side of the fluorescence emission line in order to quench the excited molecules of the photostarter back down to the ground state before they undergo intersystem crossing (ISC). Hence, the population of the triplet system is avoided and the triggering of the polymerization consequently hindered as this triggering is supposed to be initiated by molecules in the triplet state (in the case of radical polymerization). The middle panel of Fig. 8.5 shows an alternative: one could actually allow for ISC if the radical polymerization is hindered by a further excitation within the triplet system, the so-called up-conversion quenching [53]. This is effectively the case if the polymerization starter ITX is used [50, 54], or if the starter DETC is quenched by yellow or red light [54] (instead of 532 nm which predominantly causes STED in the case of DETC [50]). Nevertheless, the exact mechanism is not yet fully understood. For instance, it could as well be the case that an excited state absorption within the singlet system takes place and a higher lying singlet state $S_n$ is not effective for polymerization. It is, however, probable that this kind of excited state absorption is responsible for the early observation of sub-Abbe sized features using malachite green carbinol base as photostarter [33, 55]. In this case, volume elements (voxels) axially confined down to 40 nm have been reported, but without lateral improvement of the feature size and resolution was not quantified. As the triplet states are rather long-lived [55], it has been speculated that this technique is less suitable for fast patterning using a donut shaped depletion PSF [51].

The lower panel in Fig. 8.5 shows another idea of how to improve structure size and resolution in 2PPL lithography. In this technique, the photostarter is not manipulated by a second laser beam. Instead, a second type of photoactivatable molecule is mixed into the resist which, once photo-excited by another wavelength than that used for the starter, may either de-excite the starter molecules directly or stop chain growth of the polymer in the outer rim of the starter-PSF. In a radical polymerization resist, a photoactivatable radical scavenger would be suitable. Two groups claim to have experimentally achieved sub-Abbe feature size and resolution based on this start/stop technique [32, 35, 56]. However, these publications have recently been deeply questioned [57] by some of the authors who were also in the original claims. They raised deep doubts on the effectiveness of photo-excited stoppers which ideally should only be present in the donut shaped ring around the excitation focus but not within the hole of the STED PSF. The authors of [57], however, argued that the stoppers, activated in
the donut, diffuse into the hole of the STED PSF very quickly, in fact in a shorter time than it is supposed to take for the polymer to solidify. Nevertheless, very recent work shows that the voxels solidify within less than a millisecond [58]. This, in turn, questions the basic assumption of a slow (1 second) time scale for solidification assumed by the authors who scrutinized the start/stop technique. More research is definitely needed in order to find out why, indeed, sub-Abbe feature sizes and resolutions could be reported in [32, 35, 56].
Tab. 8.1: Overview of STED and STED-inspired 2PPL results for structure size and resolution. Note that the results for “starter & stopper” are currently under discussion [57, 58].

<table>
<thead>
<tr>
<th>Technique</th>
<th>Group</th>
<th>Structure Size</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>true STED</td>
<td>Wegener¹</td>
<td>170 nm axial</td>
<td>175 nm lateral</td>
</tr>
<tr>
<td></td>
<td>Klar²</td>
<td>55 nm lateral</td>
<td>120 nm lateral</td>
</tr>
<tr>
<td></td>
<td>Excited state</td>
<td>Fourkas³</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Wegener⁴</td>
<td>65 nm lateral</td>
<td>—</td>
</tr>
<tr>
<td>polymer. starter &amp; stopper</td>
<td>Scott⁵</td>
<td>110 nm lateral</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Gu⁶,⁷</td>
<td>10 nm lat.</td>
<td>52 nm lateral</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 Phot. Start)</td>
<td>80 nm axial</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 nm lat.</td>
<td>(2 Phot. Start)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1 Phot. Start)</td>
<td></td>
</tr>
</tbody>
</table>


An overview of the values of feature sizes and resolutions so far achieved is given in Tab. 8.1. The start/stop technique using two-photon absorption to excite the starter and one-photon absorption to excite the stopper seems to be superior; however, as mentioned above, these results need to be taken with care.

8.5 Resolution in microscopy and lithography

Let us briefly review the terms “localization” and “resolution” in microscopy and compare them to “feature size” and “resolution” in two-photon lithography. See Fig. 8.6 for some illustrations to help distinguish these terms. Suppose the observer has prior knowledge that only one observable entity (such as a single fluorophore) is located within the PSF. It can then be located with infinite precision, provided the total number \( N_{\text{Phot}} \) of detected photons is infinite, because the localization accuracy of a PSF is proportional to \( 1/\sqrt{N_{\text{Phot}}} \) (Fig. 8.6, upper row left). This fact is used in stochastically blinking microscopy techniques such as PALM, STORM, dSTORM, and the like [59–64]. In contrast to localization, resolution deals with the question of whether two objects with identical physical properties (same emission wavelength, same or random polarization etc.) can be distinguished. Two typical situations are frequently addressed, the Rayleigh criterion[65], with a pronounced minimum in the intensity distribution of the image (Fig. 8.6 upper row right), or the Sparrow criterion[66], where the minimum is just about to appear (Fig. 8.6 upper row middle).
In the case of two-photon lithography (2PPL), the size of a solitary feature plays a similar role to that of the localization accuracy in microscopy; compare the left column in Fig. 8.6, upper and middle panels. “Solitary” means that the next neighboring features are well separated by several wavelengths at least. An infinitely small feature size could in principle be attained if the threshold of polymerization (gray horizontal line) is close to the peak of the PSF [10, 67]. The polymerization threshold is defined as the illumination intensity which causes just enough polymerization (say 80%), such that the written structure withstands the development (washing) steps. Indeed, this resembles a strong chemical type of nonlinearity because of the binary distinction between areas polymerized sufficiently to withstand washing and the minor exposed areas which do not withstand washing. Strictly speaking, this is a “yes or no” function, the most nonlinear function possible and clearly, this is apt to provide sub-Abbe feature sizes. In reality, however, an infinitely thin feature size is hard to reach, because
this would require pushing the polymerization threshold to the upmost tip of the PSF, or in turn lowering the illumination intensity such that it just exceeds the polymerization threshold. In practice, a solitary line written in such conditions would not be continuous if the threshold was pushed too close to the limit of the PSF [51]. This can be easily understood because both the top of the PSF and the polymerization threshold fluctuate. The intensity of the PSF fluctuates because, for instance, the excitation laser fluctuates or because there are differently absorbing species between the objective lens and the focus while scanning. The polymerization threshold fluctuates as well because it depends, for instance, on the local density of polymerization starters, which in practice will show spatial inhomogeneity. For these reasons, feature sizes below 100 nm are difficult to achieve using 2PPL.

But let's now turn to the term “resolution” in 2PPL: in the case of the Sparrow criterion (Fig. 8.6, middle row, central panel), the polymerized structures are broader compared to the solitary lines (assuming an “non-forgetting” resist [51]). When a minimum develops in the sum of both PSFs by further separating them, e.g. up to the Rayleigh limit (Fig. 8.6, middle row, right), it becomes possible for the polymerization threshold to lie in between the two maxima and the central minimum of the summed illumination intensity. Both polymerized features (grey shaded areas) are now fully separated due to the binary nonlinearity of polymerization while the optical image in microscopy would not show a zero of intensity in the center (Fig. 8.6 upper row).

The lower row of Fig. 8.6 depicts the situation of STED-assisted 2PPL. In comparison to ordinary 2PPL, STED-2PPL shows a substantially narrowed effective PSF. Obviously, the sharpened effective PSF leads to an improvement of the minimal feature size (Fig. 8.6, lowest row, left), as compared to the feature size achievable by 2PPL. The reason is that due to the sharper effective PSF in the case of STED-2PPL, the requirement to push the polymerization threshold to the upmost tip of the PSF is relaxed. Mathematically speaking, the bending or the second derivative of the effective PSF of STED-2PPL is larger. Finally, STED-2PPL allows for true sub-Abbe resolution of two neighboring features as depicted in the lower right panel of Fig. 8.6.

### 8.6 Nanoanchors for single antibody display

We have used STED-2PPL in order to write arrays for proteins as a first application in biological and physiological research. This is a first step towards templates for proteins which could mimic the native surroundings and stimulate specific cell responses. Such templates may help study cellular dynamics and interactions, though ex vivo, in an environment similar to the natural habitat [68–70]. For instance, micropatterned substrates have been used to investigate cell proliferation, differentiation, and migration [68, 71–74]. Nanoconfined, specific ligand display serves as an important tool for such studies [75–77]. Templates or scaffolds have been written for proteins using different techniques. Nanoparticle self-assembly [69, 70, 78], dip-pen nanolitho-
STED lithography and protein nanoanchors

graphy [79–81], nanoimprint lithography [82], or electron beam lithography [83, 84] were used to construct two-dimensional nanostructures, which serve as binding sites for proteins. Although all these techniques have their advantages, they also have distinct limitations, for example high cost (e-beam) or restrictions in structure geometry, specifically in self-assembled structures where only periodic patterns are possible, and all of them are hardly extendable into the third dimension. In contrast, 2PPL allows free-form patterning of docking sites for proteins even in 3D. And of course, STED is apt to reduce structure size and enhance resolution also in the case of protein scaffolds as outlined below.

In a pilot study we used a (80/20) mix of the two acrylate monomers SR499 (Sartomer, Colombes Cedex, France) and PETA, including 300–400 ppm mono-methyl ether hydroquinone, and again added 0.25 wt % of DETC as a photosensitive starter [85]. Two-photon excitation powers of 4.85 mW and depletion powers of 25 mW (entering the objective lens) were applied to write the smallest features.

Figure 8.7 summarizes the results [85]. We wrote periodic arrays of $32 \times 32$ patches with a spacing of 5 μm between the patches, some written with low resolution using conventional 2PP lithography and some written with high resolution using STED-2PP lithography. The protein-adherent acrylate patches were written on glass slides which were passivated with PEG-silan prior to writing. This ensured that the substrate was inert against protein adhesion while the acrylate patches (due to the content of SR499) adhered proteins. Figure 8.7 (a) shows, from left to right, an SEM image of one such array, a fluorescence image of another array in the green channel of a fluorescence microscope, and an image taken with the red channel after incubation of the patches with anti-CD59 antibodies (αCD59), stained with Atto655. The patches are visible in the green channel because the photostarter DETC is a coumarin dye molecule and hence the patches show auto-fluorescence. However, the patches are invisible in the red channel prior to incubation with the stained antibodies. Hence, auto-fluorescence can be used to find the arrays in the fluorescence microscope quickly, while the red channel can be used to monitor protein loading.

The sketch on the left of Fig. 8.7 (b) shows two situations whereby the larger patches, written with ordinary 2PPL carry several αCD59, while the patches written by STED-2PPL are supposed to carry only one αCD59 each. The fluorescence strength per patch, averaged over one array and normalized to the fluorescence strength of single αCD59 antibodies, shows quick saturation after only a few incubation steps (right graph in Fig. 8.7 (b)). In the case of the 2PPL written patches, an average of $8.28 \pm 1.22$ antibodies per 2PP fabricated patch is found (blue line). In contrast, the STED-2PPL patches carry on average only $1.16 \pm 0.11$ antibodies (red line) [85]. Hence, the STED-2PPL patches were termed nanoanchors.

As a further proof that there was only one antibody per nanoanchor in most cases, we performed dSTORM measurements [86]. Figure 8.7 (c) shows two nanoanchors, one of which displays only one fluorescing spot (with 26 nm localization diameter), while the other nanoanchor shows two such spots. On approx. 80 % of the dots we
Fig. 8.7: (a) Rectangular array of STED-2PPL fabricated nanoanchors, spaced 5 μm apart. Left: SEM image; middle: auto-fluorescence of the DETC polymerization starter in the green channel of a fluorescence microscope; right: Atto655 fluorescence of the labeled IgG antibodies attached to the nanoanchors, imaged by the red channel of a fluorescence microscope. (b) Left: sketch of many IgGs on 2PPL-written islands and single IgGs on STED-2PPL-written nanoanchors. Right: Fluorescence signal per island, normalized to the fluorescence strength of a single IgG as a function of incubation steps. 2PPL-written islands carry 8 to 9 IgG, while STED-2PPL written nanoanchors carry only 1.2 IgGs on average. (c) Zoomed out are two typical dSTORM images of nanoanchors. In about 20% of all cases, two fluorescence spots are detected, in about 80% of all cases, only a single localized spot is detected. (d) On/off blinking of a spot containing only one IgG. Parts (b)–(d) reprinted and adapted with permission from [85]. Copyright 2013 American Chemical Society.
observed single fluorescent spots. Still, as the localization accuracy was 26 nm, several fluorophores could be hidden inside this spot. However, on-off blinking experiments showed unambiguously that only single fluorophores were present in most cases (Fig. 8.7(d)). We further know [85], that more than 90% of the antibodies are loaded with fluorophores, so we can safely conclude that about 80% of the nano-anchors are loaded with only one antibody. Additional evidence was achieved by applying a probability density fit algorithm [87, 88] estimating the average number of fluorescing species per patch. Details can be found in Wiesbauer et al. [85].

8.7 Conclusion/Further directions

Since its proposition 20 years ago, STED nanoscopy has become a routine technique in bio-imaging. A multitude of physiological questions has been addressed using STED microscopy, specifically in the fields of neurophysiology and the physiology of membrane-based proteins, and the Nobel Prize in Chemistry was co-awarded for these achievements in 2014. One can safely say that the diffraction limit implied by Abbe lost its significance in fluorescence microscopy and a resolution below 10 nm has been achieved using visible light. The transformation of STED microscopy toward STED lithography has been illustrated in this chapter. Similar to STED-microscopy, STED is not the only way to deplete photostarters of polymerization. In the family of STED-related microscopy, stimulated emission, ground state depletion, optical activation of fluorescence-quenchers, and photochromic switching are techniques which can be utilized to force dye molecules into an off-state. In STED inspired lithography, stimulated emission, up-conversion from excited states or, potentially, optically activatable polymerization-stoppers can be applied. STED nanoscopy has meanwhile reached sub 10 nm resolution. Until now, the resolution in STED- and STED-inspired-lithography is an order of magnitude worse, though clearly below the diffraction limit. However, it should be recognized that STED microscopy took 10 years to come down from 100 nm resolution in the early experimental reports to sub 10 nm resolution. STED lithography and its relatives are only 4 years old and should still be considered in its infancy. Above, one field of applications, producing nanoscale patterns for protein adhesion down to the single protein level [85], has been outlined.

Other applications, shown by the Wegener group, are in the field of photonic devices: photonic crystals with stop bands in the visible range [13, 89, 90], including polarization sensitive devices [91], and a carpet cloak [36, 92]. Further, the Min Gu group reported ultra-high density data storage [93, 94].

Some challenges need to be tackled in the future. In order to push resolution further down in STED and STED related lithography, materials need to be discovered which allow for nanoscale structuring on the one hand but are stiff enough to withstand the washing process on the other. Most importantly, photoresists must not shrink during the washing or drying process. Only negative tone resists have
been used in STED- and STED-inspired lithography so far. A positive tone resist will certainly open new fields of applications. Further, STED lithography has merely been restricted to radical polymerization. Finding alternative classes of polymerization and alternative polymerization starters is top on the agenda for further development of STED lithography.

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