Biofunctionalization of Sub-Diffractionally Patterned Polymer Structures by Photobleaching

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Supporting Information

ABSTRACT: Stimulated emission depletion (STED) nanolithography allows nanofabrication below the diffraction limit. Recently, it was applied to nanoanchors for protein fixation down to the single molecule level. We now combined STED nanolithography with laser-assisted protein adsorption by photobleaching (LAPAP) for optical and selective attachment of proteins to subdiffractional structures. In turn, STED was used for imaging of fluorescently tagged streptavidin to reveal protein binding to STED-lithographically patterned acrylate structures via LAPAP. Protein localization down to 56 nm spots was achieved using all-optical methods at visible wavelengths.

KEYWORDS: laser-assisted protein adsorption by photobleaching, two-photon absorption, two-photon lithography, nanolithography, stimulated emission depletion, biofunctionalization

Laser-assisted protein adsorption by photobleaching (LAPAP) is an important tool for light-induced surface patterning using commercially available fluorophores which are intentionally bleached in order to form radicals which subsequently bind to a surface. The surface tethered fluorophores, although bleached, carry a functional group, usually biotin, which can then be used for further attachment of proteins to the substrate. However, the smallest achievable dimensions of such protein patterns are limited by diffraction. An alternative method for optically patterning surfaces is multiphoton lithography (MPL), which is even apt to create three-dimensional structures due to the intrinsic optical sectioning capability of multiphoton absorption (MPA).

Due to a chemical nonlinearity, MPL is already capable of achieving subdiffractional structure sizes down to 100 nm in the lateral direction, however it is hard to achieve subdiffractional resolution. A method to write even smaller structures is adding stimulated emission depletion (STED) or STED-inspired techniques, methods which were derived by transferring super-resolving concepts from STED microscopy to lithography. Feature sizes of around 56 nm and a resolution of 120 nm can be achieved using STED lithography.

As a proof of concept, we demonstrate a route for selective, optically triggered biofunctionalization of polymeric nanostructures prepared via MPL and STED lithography using LAPAP. Photobleaching of the LAPAP fluorophore generates free radicals that react and bind to acrylate nanostructures. Importantly, we initiate LAPAP by MPA in the same setup and by the same laser which is also used as excitation laser in STED lithography. The sample is left in place after MPL or STED lithography in order to perform MPA-LAPAP.

attachment of the molecules is selective to those nanostructures that are illuminated by the 780 nm MPA laser beam a second time, whereas unilluminated nanostructures, or the glass substrate surrounding an illuminated nanostructure, are not functionalized.

A short description of the protocol is sketched in Figure 1 (more details are given in the Supporting Information). Prior to fabrication, the glass substrates were cleaned with peroxymonosulfuric acid and treated with 1 mM 3-(trimethoxysilyl) propyl methacrylate, unless otherwise mentioned. This provides a substrate where MPL structures adhere tightly. An acrylate photoresist comprising pentaerythritol triacrylate (PETA, Sigma-Aldrich, Austria) with 0.25 wt % of the photoinitiator 7-diethylamino-3-thenoylcoumarin (DETC, Acros Organics, Belgium) was dropcasted (Figure 1a). Next, nanodots were written by MPL applying 4.8 mW of a 780 nm 100 fs laser (all powers measured in front of the objective lens) for 1.2 ms. Optionally, a second continuous wave (CW) laser beam of 532 nm wavelength and 25 mW power was additionally applied, shaped in a donut like fashion in order to deplete the photoinitiator in the outer rim of the point spread function (PSF) (STED lithography, Figure 1b). After rinsing with acetone (but keeping the sample in place on the scanning stage), only the illuminated structures remained because PETA is a negative tone photoresist (Figure 1c). Next, the LAPAP fluorophore, dissolved in phosphate buffered saline (PBS, 200 μg/mL), was added (Figure 1d). Unless otherwise mentioned, we used Atto 390 biotin as the LAPAP.
fluorophore. 1.5 mW of the 780 nm, 100 fs laser pulses were applied a second time in order to initiate LAPAP via MPA (Figure 1e). After rinsing with PBS, only the photobleached LAPAP fluorophores, which were attached to the acrylate nanodots, remained. The surrounding glass substrate was also illuminated by the 780 nm laser, because the diffraction-limited 780 nm PSF is larger than the nanostructures. However, the LAPAP fluorophores attached selectively only to the acrylate nanostructures (Figure 1f). The biotin remains active despite the bleaching of the Atto 390 chromophoric part.1,2 Subsequent incubation with streptavidin, labeled with the "revealing" fluorophore Alexa 555 (Figure 1g), and repeated washing with pure PBS, finally leaves protein functionalized nanostructures (Figure 1h).

To experimentally verify the scheme outlined above, we produced three sets of 10 × 10 nanodots (pitch 2 μm). Figure 2a shows a 532 nm light scattering confocal image of the sample. Two of the sets (lower left and lower right) have been fabricated using MPL and one set (upper right) was fabricated using STED lithography. The upper left quarter was left blank as a reference and to prove that no permanent adhesion takes place during the incubation steps. The nanostructures prepared by MPL have diameters of 160 ± 2 nm as revealed by the scanning electron micrograph shown in Figure 3a. The nanodots fabricated via STED lithography have diameters of 56 ± 5 nm (Figure 3b). In the green channel of a fluorescence confocal scanning microscope, the nanodots show an intrinsic fluorescence due to the DETC in the photoresist,23 (Figure 2b). While the whole sample was incubated with Atto 390 biotin (cf. Figure 1d), only the two right patterns were illuminated a second time with 780 nm in order to induce LAPAP (cf. Figure 1e). After incubation with Alexa 555 streptavidin and washing (cf. Figure 1g,h), it becomes apparent in the red channel of the confocal microscope that the streptavidin only adheres to the right patterns (Figure 2c).
lower left pattern was not functionalized with biotin, because the MPA-LAPAP step was not applied.

Fluorescence readout as presented in Figure 2c cannot answer the question whether the glass substrate surrounding the nanodots might be covered with photobleached Atto 390 biotin and subsequently with streptavidin, as well. This might be possible because the LAPAP PSF is diffraction limited and hence the PSF is much wider than the 56 nm dots produced with STED lithography. Further, the confocal read-out is diffraction limited, as well. To achieve a more-detailed characterization of the functionalized nanodots, we took STED microscopy images. In this case, the Alexa 555 was excited with 532 nm and depleted with 660 nm laser light. Figure 3c shows a deconvolved STED image of the Alexa 555 signal from four dots (for raw STED images, see the Supporting Information). Figure 3d shows a line scan across the lower right of the four dots (yellow line in Figure 3c). Averaging over various STED-written dots, a full width of half-maximum of 65 ± 5 nm is obtained, which matches well with the size of the nanodots obtained by SEM (Figure, 3c). Although the effective MPA-PSF size of the 780 nm LAPAP beam is about 200 nm and hence approximately 4 times wider than the size of a STED nanodot, LAPAP is effective only on the acrylate nanodots, not on the glass surface surrounding the nanodot.

The goal of a further study was to investigate alternatives to Atto 390 biotin for LAPAP. In case of Abberior STAR 470 SXP biotin, the 780 nm, 100 fs pulsed laser was used for MPA-LAPAP, the same way as for Atto 390. In case of Alexa 546 biocytin, LAPAP was initiated by the 532 nm CW laser, usually used for STED in the STED lithography experiments, however the 2π phase plate was removed such that the 532 nm PSF was of an ordinary shape. We wrote several horizontal acrylate lines with MPL. Perpendicular to the horizontal acrylate lines, vertical scans initiating LAPAP were performed, each with a different LAPAP fluorophore in the subsequently applied PBS buffers. The left-hand side of Figure 4a shows a sketch of the horizontal MPL written acrylate lines and the vertical functionalization with the three different fluorophores. To improve passivation, a lipid bilayer was spread after MPL and prior to LAPAP in order to passive the glass substrate. In detail, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles were spread according to Huppa et al., in order to cover the glass slide around the acrylate structures but not the acrylate lines themselves.

After LAPAP functionalization, the acrylate lines were again incubated with the revealing fluorophore Alexa 555 streptavidin which binds to both biotin and biocytin. The right-hand side of Figure 4a shows the fluorescence of the revealing Alexa 555. Figure 4b presents vertical cross cuts along each of the three LAPAP scan lines (a horizontal cross cut is shown in the Supporting Information). It is apparent that Atto 390 is more effective for 780 nm MPA-LAPAP than Abberior STAR 470 SXP, corresponding to the larger absorbance at 390 nm of Atto 390 compared to the absorbance of Abberior STAR 470 SXP (cf. Supporting Information). Both were used at the same mass concentration of 200 μg/mL and with the same MPA power of 3.0 mW in front of the objective lens and the same scanning speed of 5 μm/s. The two-photon (3.0 mW) excited LAPAP efficiency of Abberior STAR 470 SXP is of the same order as the one photon LAPAP efficiency of Alexa 546 using 300 μW of 532 nm CW laser light. The selective attachment of different kinds of LAPAP fluorophores onto acrylate nanostructures using different colors of light holds promise for protein multiplexing.
In summary, selective functionalization of subdiffractive acrylate nanostructures fabricated with MPL and with STED-lithography was achieved using multiphoton induced LAPAP. Radicals created during the LAPAP process attack spare acrylate groups of the photoresist but do not adhere to the glass substrate. Hence, LAPAP functionalization is specific to the nanostructures, as revealed by STED nanoscopy. In addition, and to the best of our knowledge, the three fluorophores Atto 390, Abberior STAR 470 SXP and Alexa 546 have not been reported before as suitable LAPAP fluorophores. LAPAP can be performed with the same setup as STED lithography, without the need of changing the sample stage and timely retrieval of the nanostructures. Further, MPA-LAPAP is in principle capable of three-dimensional patterning due to the intrinsic optical sectioning capability of MPA. With this respect, it also ideally fits with three-dimensional MPL and STED lithography. It would be of interest, but needs to be left for a future study, whether STED could also be applied to MPL-written collagen or fibrin structures, which subsequently could be functionalized using MPA-LAPAP.  

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b11777.

Detailed description of the setup for MPL and STED lithography, LAPAP and confocal microscopy; setup for STED nanoscopy; used reagents; structure and absorption and emission spectra of the fluorophores; composition of photoresist, MPA-LAPAP protocol, additional data (PDF)

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**Notes**
The authors declare no competing financial interest.

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