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Native Laser Lithography of His-Tagged Proteins by Uncaging of Multivalent Chelators

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Exploiting the functional diversity of proteins for fundamental research and biotechnological applications requires their functional organization into micro- and nanostructures.[1] Several powerful approaches for photolithographic and microcontact printing-based patterning of proteins into structures with a spatial resolution around the diffraction limit of light have been reported.[2] A key challenge that remains, however, is to control protein organization into microstructures *in situ*. Uncaging of caged-biotin has been successfully employed in different ways for protein micropatterning.[3] These methods, however, require either modification of target proteins with caged biotin or a sandwich-based format (caged-biotin-streptavidin−biotinylated protein) for protein immobilization. Also, a caged benzylguanine derivative has been employed for photolithography of alkylguanine-DNA-alkyltransferase-tagged proteins.[4] The slow association kinetics of this reaction, however, obstructs efficient writing of protein microstructures *in situ* as well as multiplexed protein organization. Here, we have established a rapid and versatile approach for site-specifically targeting Histidine-tagged proteins *in situ* by means of a confocal laser beam. This approach is based on a photofragmentable oligohistidine peptide, which is used for blocking the free coordination sites of Ni(II) ions bound to immobilized tris-(nitrilotriacetic acid) (tris-NTA) moieties (Figure 1a). Tris-NTA, which binds His-tagged proteins with very high affinity and specificity,[5] was coupled to glass substrates rendered biocompatible by a PEG polymer brush.[6] Upon photofragmentation of the blocking peptide by UV light, the fragments rapidly dissociate from the surface because of a dramatic loss of binding affinity due to reduced multivalency. Thus, tris-NTA moieties are efficiently uncaged and become capable of capturing His-tagged proteins. Photofragmentable oligohistidine peptides were obtained by solid phase synthesis using 3-amino-3-(2-nitrophenyl)-propionic acid (Φ) as a photocleavable building block.[7] Different sequences were tested for efficient blocking of the surface. The peptide with the sequence $(HHHΦ)_3HHH$ was found to efficiently block tris-NTA functionalized surfaces as detected by probing the sequential binding of a His-tagged protein using reflectance interference (Figure 1b). This peptide, henceforth referred to as Φ-His, was used throughout this study. Photofragmentation of Φ-His as a result of UV irradiation in a cuvette was probed by measuring the binding to a tris-NTA functionalized surface: a systematic decrease in binding rate and binding amplitude was observed as a function of irradiation time (Figure 1c) thereby establishing the UV dependent photofragmentation of Φ-His. The decrease in Φ-His binding was accompanied by a substantial increase in the subsequent binding of a His-tagged protein (Figure 1d).

Uncaging of tris-NTA bound to Φ-His was then explored by illuminating the surface through a photomask. However, insignificant binding of a His-tagged protein and irreversible loss of binding capacity were observed upon UV illumination of the surface loaded with Φ-His (data not shown). This could be explained by photodestruction of tris-NTA groups by photo-oxidation through free radicals formed during photocleavage and/or side reactions of the photocleaved nitrobenzyl groups with surface nucleophiles. To

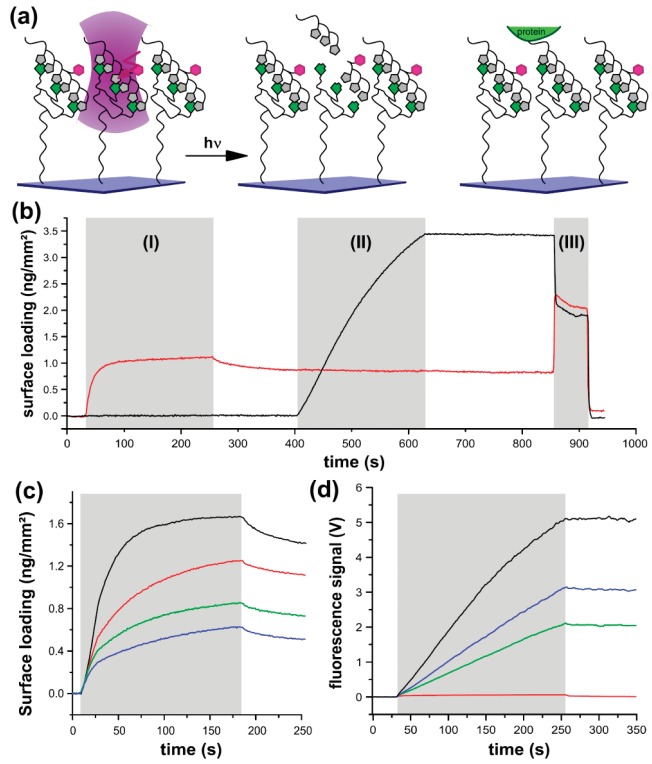

Figure 1. Blocking of tris-NTA functionalized surfaces by the Φ-His-peptide. (a) Schematic of the method: after saturating surface tris-NTA moieties with Φ-His, the peptide is locally cleaved by UV illumination either through a mask or by means of a confocal UV laser. Upon cleavage of the peptide, the multivalency of interaction with tris-NTA is substantially reduced, leading to rapid dissociation of the peptide fragments. His-tagged proteins can now bind to the free tris-NTA moieties. The Φ-His peptide is shown only partially for better clarity. (b) Blocking of surface tris-NTA groups by injection of 1 μM Φ-His-peptide (I) followed by injection of 200 nM GFP-H6 (II) and regeneration with imidazole (III, biased by a bulk refractive index signal) as detected by RIfS (red curve). For comparison, binding of GFP-H6 to the same surface without prior blocking with the Φ-His-peptide is shown (black curve). The gray bars mark the injection periods. (c,d) Photofragmentation of Φ-His in solution: (c) Binding signal for the Φ-His peptide before (black) and after UV irradiation for 100 s (red), 1000 s (green), and 10 000 s (blue). (d) Binding of 200 nM GFP-H6 to tris-NTA surfaces blocked with Φ-His-peptide before (red curve) and after irradiation for 3000 s (green) and 10 000 s (blue) as detected by TIRFS. Binding of 200 nM GFP-H6 to nonblocked surfaces is shown in comparison (black).

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reduce these reactions, we treated the surface with a known electron scavenger, 1,4-benzoquinone. Binding of 1,4-benzoquinone to the surface was observed by label-free detection (Supporting Information). Thus, protein binding experiments did not require the presence of this compound in solution.

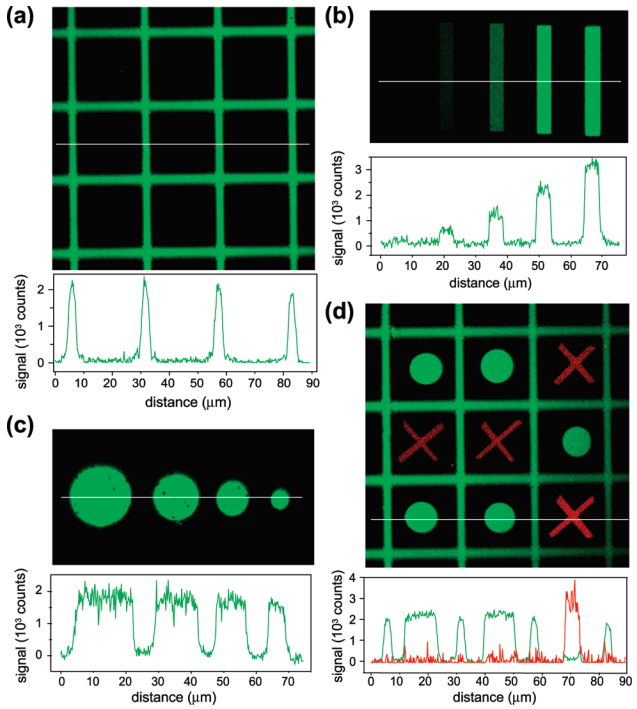

Figure 2. Protein patterning by spatially resolved uncaging of surface tris-NTA blocked with Φ-His peptide. (a) CLSM image of a tris-NTA surface blocked with Φ-His, which was uncaged by UV illumination through a photomask followed by incubation of GFP-H6. (b) Protein patterning by laser lithography: After uncaging different ROIs by scanning with the confocal beam of a 405 nm laser for different numbers of iterations (1, 3, 10, 30, and 100), the surface was incubated with GFP-H6. (c) Activity of proteins immobilized by laser lithography: after uncaging circular ROIs with different diameters, unlabeled IFNAR2-H10 was immobilized, followed by incubation of ^{AT488}IFNα2. (d) Multiplexed protein immobilization by combining illumination through a photomask and multiplexed *in situ* laser lithography using GFP-H6 and ^{Dy649}MBP-H6 for sequentially "writing" protein structures into the grid. The cross sections show the intensity across the indicated lines.

Indeed, uncaging of tris-NTA by photofragmentation of complexed Φ-His was possible after treatment with 1,4-benzoquinone: under these conditions, strong binding of GFP-H6 into microstructures was observed after UV-illumination of the surface through a photomask (Figure 2a). Protein binding was specific to His-tagged proteins, and immobilized proteins could be quantitatively removed by imidazole (Supporting Information).

Uncaging of surface tris-NTA groups was also possible by laser lithography using a 405 nm laser in a confocal fluorescence microscope: Upon scanning regions of interest (ROI) with the laser, specific binding of His-tagged proteins to these areas was detected (Figure 2b). Depending on the number of iterations, different levels of protein binding were observed until saturation was reached. Microstructures close to the diffraction limit of light (~300 nm fwhm) could be obtained by this method (Supporting Information). To confirm the activity of immobilized proteins, the extracellular domain of IFNAR2 fused to a decahistidine-tag (IFNAR2-H10) was immobilized by laser lithography. Specific targeting of this protein into the scanned ROIs was confirmed by injection of its ligand Interferon-α2, which was labeled with ATTO 488 (^{AT488}IFNα2). Binding of ^{AT488}IFNα2 was detected exclusively in the prescanned ROIs only after incubation of IFNAR2-H10 (Figure 2c).

In addition to the flexibility to freely design protein patterns by laser lithography, the method also enables iterative writing of different proteins. To demonstrate this capability, we sequentially targeted two different proteins with different fluorescence labels *in situ*. To this end, a grid obtained by mask illumination followed by immobilization of GFP-H6 was sequentially decorated with GFP-H6 and DY-649 labeled maltose binding protein with a hexahistidine tag (^{Dy649}MBP-H6). The overlay of the green and red fluorescence channels is shown in Figure 2d. The high fidelity of protein targeting into different ROIs is confirmed by intensity profiles of the red and green channels.

These examples demonstrate the versatile capabilities of our approach for iterative "writing" of different recombinant proteins into functional microstructures under physiological conditions. The high specificity of tris-NTA toward His-tagged proteins enables for direct protein lithography from crude cell lysates (Supporting Information). By combination with microfluidics, multiplexed protein organization into complex microstructures will be feasible. Another characteristic feature of our approach is the noncovalent nature of the caging/uncaging mechanism, which is based on modulation of the multivalency of the interaction. Thus, proteins and photocleavable peptides can be removed by imidazole, enabling complete erasure of a protein pattern and multiple use of the substrate. With the His-tag being by far the most frequently used affinity tag for protein purification and the relatively simple compounds used for surface modification, highly generic application of the technique can be envisioned.

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Supporting Information Available: Description of the methods and several control measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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