

Preparation of Biomolecule Microstructures and Microarrays by Thiol–ene Photoimmobilization

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A mild, fast and flexible method for photoimmobilization of biomolecules based on the light-initiated thiol–ene reaction has been developed. After investigation and optimization of various surface materials, surface chemistries and reaction parameters, microstructures and microarrays of biotin, oligonucleotides, peptides, and MUC1 tandem repeat glycopeptides were prepared with this photoimmobilization method. Furthermore, MUC1 tandem repeat glycopeptide microarrays were success-

fully used to probe antibodies in mouse serum obtained from vaccinated mice. Dimensions of biomolecule microstructures were shown to be freely controllable through photolithographic techniques, and features down to 5 μm in size covering an area of up to 75 $\times$ 25 mm were created. Use of a confocal laser microscope with a UV laser as UV-light source enabled further reduction of biotin feature size opening access to nanostructured biochips.

Introduction

Microstructures and arrays of biomolecules ("biochips") provide a powerful platform for a wide range of applications due to their miniaturized format and the possibility for assay parallelization. Biomolecule microarrays such as DNA,^[1] peptide^[2] or protein microarrays^[3] primarily serve as tools in bioanalytical and biomedical research. Biochips displaying biomolecules in microstructures or patterns of defined geometry might find broad application in environmental monitoring, biosensing, and in tissue engineering.^[4]

Microarrays can routinely be prepared with commercially available spotting systems. For preparation of patterned biochips, in particular microcontact printing (μCP)^[5] and dip-pen nanolithography (DPN)^[6] are available. μCP allows quick bio-

molecule patterning over large areas in one step, with pattern dimensions down to 1 μm . DPN enables patterning of one or multiple biomolecules in dimensions of less than 100 nm but at a much slower rate.^[5] Light-based biomolecule immobilization methods offer an alternative and flexible approach to biochip fabrication^[7,8] with pattern size down to the nanometer range.^[9] However, the use of photocleavable protecting groups or photoactivatable reagents, for example, requires an additional reaction step either for photochemical deprotection or for surface activation and can be damaging to sensitive biomolecules if light with a wavelength of less than 300 nm is required.^[10] Therefore, biocompatible, one-step photoimmobilization methods are of general interest.

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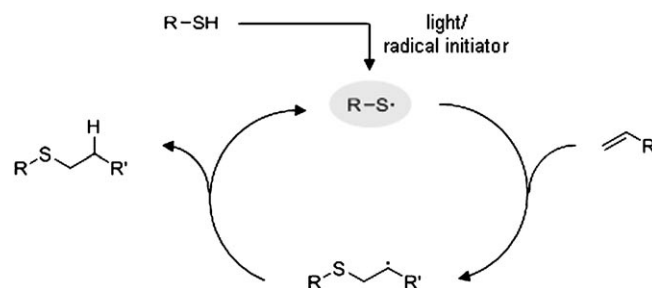
[*] Deceased.

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For the oriented, site-specific, mild, and nondenaturing immobilization of complex biomolecules on biochip surfaces, biological capture systems (such as biotin–streptavidin), affinity tags, enzymatic tags, native chemical ligation, DNA, as well as specific chemical reactions provide powerful opportunities.^[11, 12] However, due to the multifunctionality and the sensitivity of different biomolecules, for example, proteins, alternative methods that proceed under the mildest conditions and with high chemoselectivity continue to be in high demand. In addition, these novel methods should ideally proceed in one step to enable fast biomolecule immobilization and thus fast biochip generation.

Here, we report the development of the thiol–ene reaction as a method that meets these demands and additionally has the advantages characteristic of photochemical approaches (Scheme 1).^[13] It allows for fast and facile generation of both microarrays and microstructured biochips including the oriented site-directed immobilization of biotin, oligonucleotides, peptides and glycopeptides in one step. The thiol–ene reaction has previously been applied mainly in polymer chemistry,^[14] organic synthesis,^[15–18] and in biomolecule ligation.^[19] Recently, it has been used for the creation of ultrathin polymer films^[20] and modular soft-imprint materials.^[21] An initial application of this reaction for biomolecule immobilization has also been described.^[22]

prospective surface material. It was assumed that silicon oxide coatings on silicon (SiO_x/Si) gave surfaces of greater smoothness than glass, and thereby higher overall biochip quality. Silicon oxide coatings were generated on 6 inch silicon wafers either through thermal oxidation, plasma-enhanced chemical vapor deposition (PECVD), or a combination of both methods (Scheme 3 A). For an overview of the manufactured surface materials see Table 1. After oxidation or deposition, SiO_x/Si wafers were cut into pieces of $25 \times 75 \text{ mm}^2$ (microscope slide format). SiO_x/Si and glass slides were amine- or acid-functionalized by using previously reported procedures^[24] followed by derivatization to thiol- or olefin-functionalized slides in one or two steps



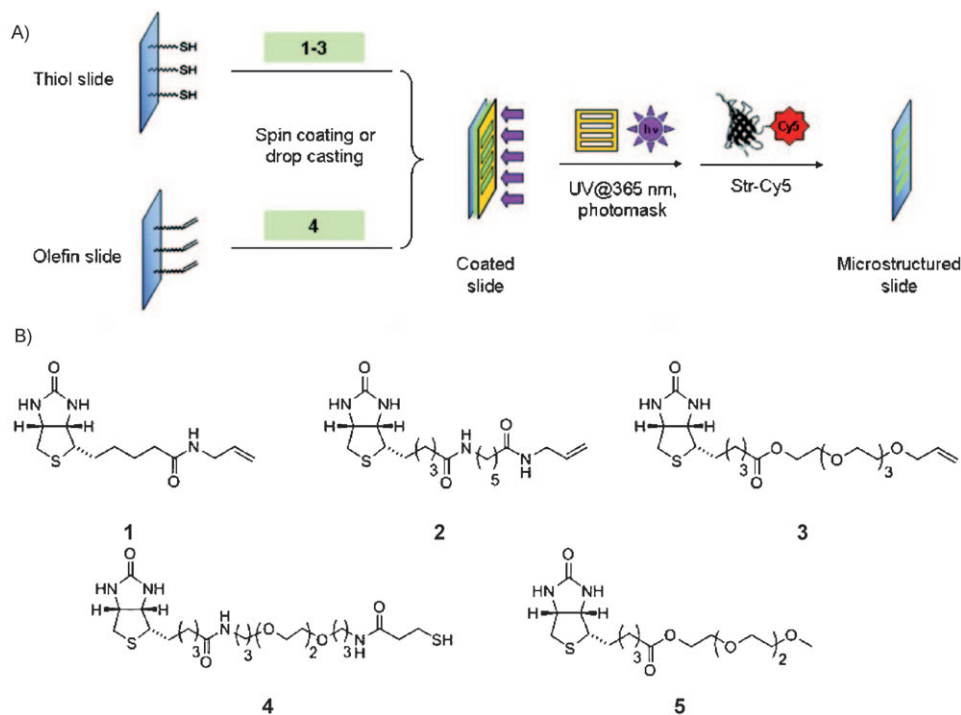
Scheme 1. Mechanism of the thiol–ene reaction. Radical formation can be initiated by either UV light or radical initiators.

Results and Discussion

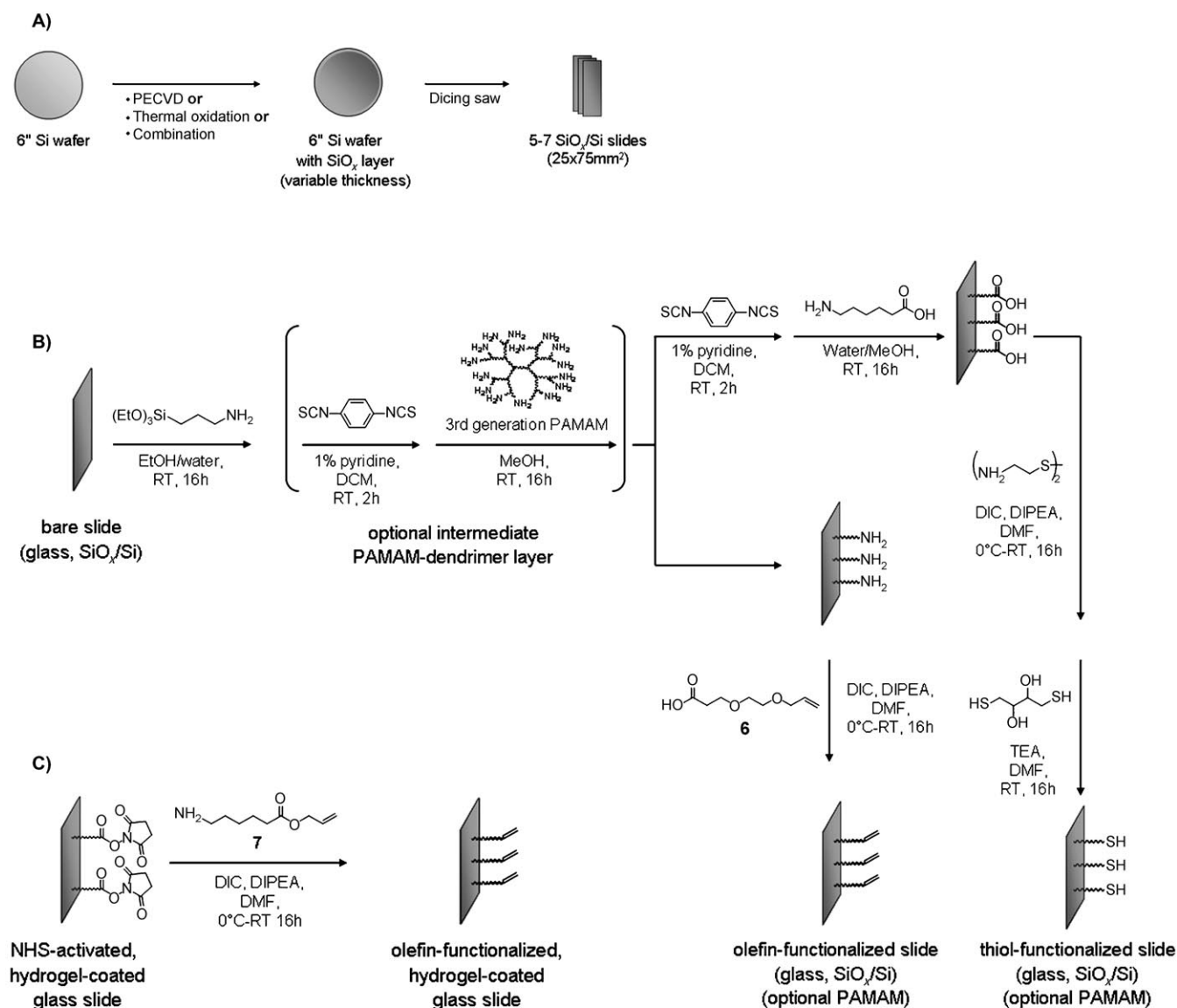
Photomicrostructuring setup

To identify suitable parameters for efficient photochemically triggered thiol–ene immobilization on solid supports, functionalized biotin compounds 1–4 were synthesized as initial probes (Scheme 2B, for synthesis see the Supporting Information and Scheme S1). Biotin is a well-established model system for the development of surface modification techniques, and biotin-modified surfaces can easily be further functionalized by using streptavidin as molecular bridge for immobilization of biotinylated biomolecules.^[23] Olefin-functionalized probes 1–3 were used for photoimmobilization on thiol-functionalized surfaces, while thiol-probe 4 was synthesized to investigate the immobilization of thiol-modified compounds on olefin-functionalized surfaces.

Glass and silicon-oxide-coated silicon slides were chosen as



Scheme 2. Photopatterning with the thiol–ene reaction. A) Thiol- or olefin-functionalized SiO_x/Si or glass slides with an optional layer of PAMAM dendrimers were coated with biotin probes 1–4 by using spin coating or drop casting (see Figure S1 for a graphic depiction of these processes). Coated slides were exposed to UV light at 365 nm through a photomask to obtain biotin microstructures ($10 \text{ mW}/\text{cm}^2$). After washing and blocking, slides were incubated with Cy5-labeled streptavidin. Microstructures of biotin–streptavidin–Cy5 were detected with a microarray fluorescence scanner. B) Biotin derivatives employed in the development of the thiol–ene photoimmobilization.



Scheme 3. Preparation of thiol- or olefin-functionalized slides for use in the thiol-ene photoimmobilization. A) SiO_x/Si slides were obtained from silicon wafers either through thermal oxidation or PECVD or a combination of both. Coated wafers were cut into slides with a dicing saw. B) Preparation of thiol- and olefin-functionalized SiO_x/Si or glass slides. Optionally, a layer of PAMAM-dendrimers is introduced. C) Preparation of olefin-functionalized, hydrogel-coated glass slides.

Table 1. Slide materials employed for slide preparation. Various layer thicknesses of silicon dioxide on silicon were obtained by controlled thermal oxidation or PECVD. Layers of different oxide types were also generated by thermal oxidation followed by PECVD.

SiO _x layer thickness [nm]	SiO _x layer type
–	glass
–	hydrogel
1000	SiO _x (thermal oxidation) on Si
100	SiO _x (PECVD) on Si
250	
500	
1000	
200/1000	SiO _x (PECVD)/SiO ₂ (thermal oxidation) on Si
1000/1000	

(Scheme 3B). Optionally, an intermediate layer of third-generation-poly(amidoamine) (PAMAM) dendrimers was introduced, to improve loading, stability, and hydrophilicity of the biochip surface and to reduce nonspecific adsorption of biomolecules.^[24,25] Hydrogel-coated glass slides were also tested (Scheme 3B and C; for slide functionalization protocols see the Experimental Section).

The photomicrostructuring process is depicted in Scheme 2A. Biotin probes 1–4 were spin coated or drop cast onto thiol- or olefin-functionalized slides (for details on spin coating or drop casting, see the Materials and Methods section and Figure S1 in the Supporting Information) and subsequently exposed to UV-light through a photomask in a photolithography

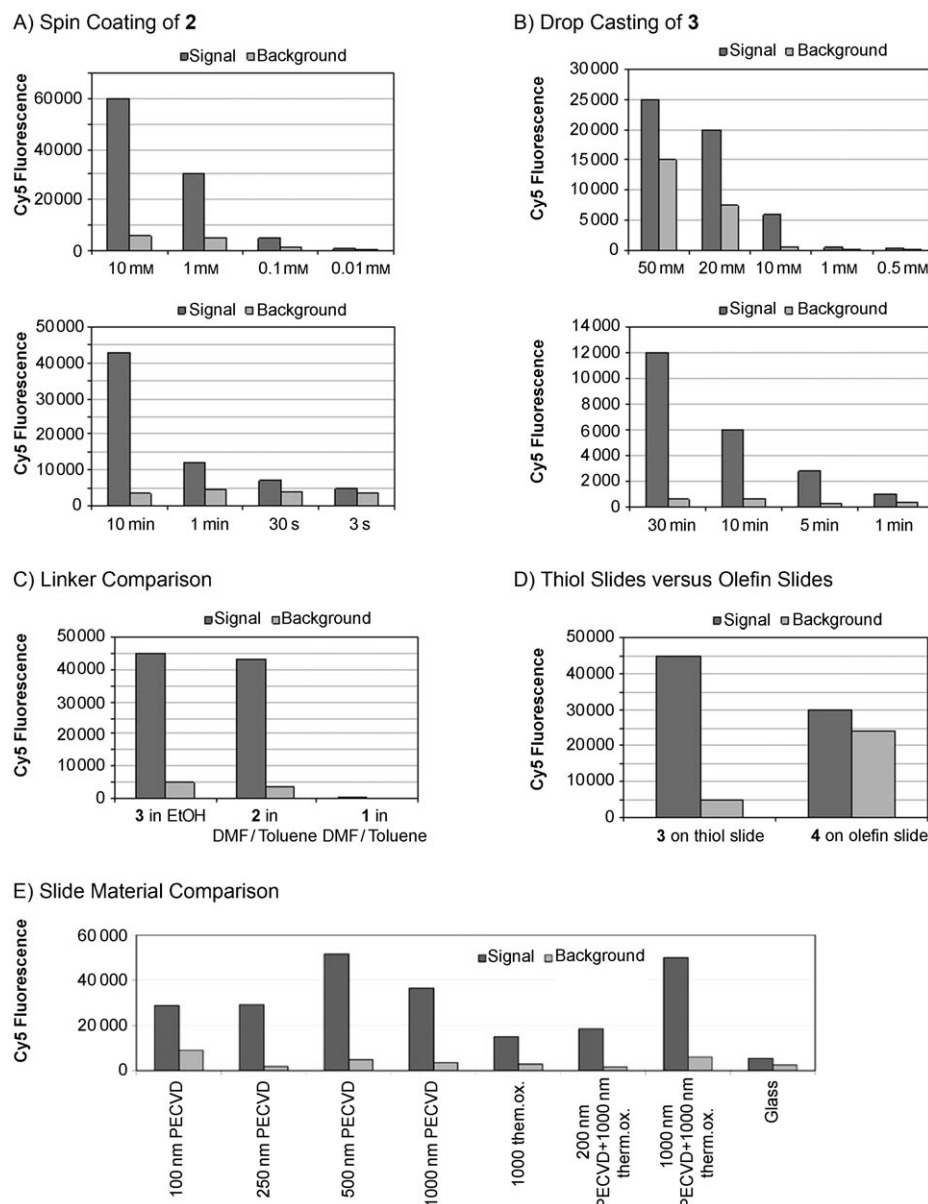


Figure 1. Results of thiol-ene photomicrostructuring experiments. A biotin probe concentration of 10 mM, a UV exposure time of 10 min and SiO_x/Si slides with PECVD-deposited SiO_x in a thickness of 1000 nm were used unless stated otherwise. A) Results obtained after spin coating of **2** in 3:1 DMF/toluene on thiol-PAMAM-functionalized SiO_x/Si slides and photoimmobilization. B) Results obtained following drop casting of **3** in ethylene glycol on thiol-PAMAM-functionalized SiO_x/Si slides and photoimmobilization. C) Comparison of olefin-functionalized biotin probe linkers **1**, **2** and **3** deposited through spin coating on thiol-PAMAM-functionalized SiO_x/Si slides followed by photoimmobilization (**1** and **2** in 3:1 DMF/toluene, **3** in EtOH). D) Comparison of thiol- or olefin-PAMAM-functionalized SiO_x/Si slides by spin coating probes **3** or **4** (dissolved in EtOH) onto a thiol- or olefin-functionalized surface and photoimmobilization. E) Comparison of different thiol-functionalized slide materials for performance in photomicrostructuring of **2** (spin coating of **2** in DMF/toluene 3:1).

apparatus to induce the photoreaction and immobilization. Immobilized biotin was then allowed to bind Cy5-conjugated streptavidin, which was detected with a fluorescence microarray scanner.

Photomicrostructuring

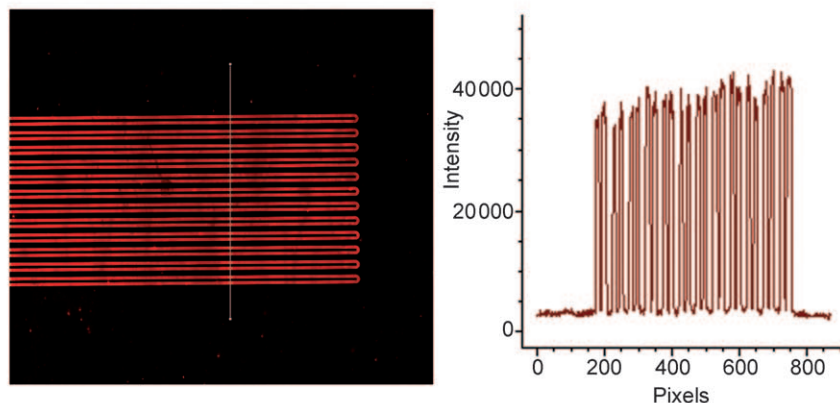
Key results of the initial investigation on thiol-ene photomicrostructuring are summarized in Figure 1. To investigate differ-

ent probe linkers, the photoimmobilization efficiency of olefin-functionalized probes **1**, **2** and **3** was compared. Probe **2**, bearing an aliphatic olefin linker (dissolved in DMF/toluene 3:1), and probe **3**, bearing a tetraethylene-glycol-based olefinic linker, (dissolved in EtOH) were spin-coated on thiol-PAMAM-functionalized SiO_x/Si slides (PECVD-deposited SiO_x in 1000 nm thickness) and photoimmobilized by exposure to UV light at 365 nm for 10 min. Both probes gave comparably high line intensities with an *S/N* ratio (determined as the ratio between the fluorescence intensity of microstructure lines and the background) of about 10:1. Therefore, the linker type is less important for photoimmobilization efficiency or streptavidin binding. Probe **1** was immobilized through the same procedure, however line intensity was low and close to the detection limit. This might be due to the short distance between the biotin heterocycle and the slide surface, which might sterically impair streptavidin binding.

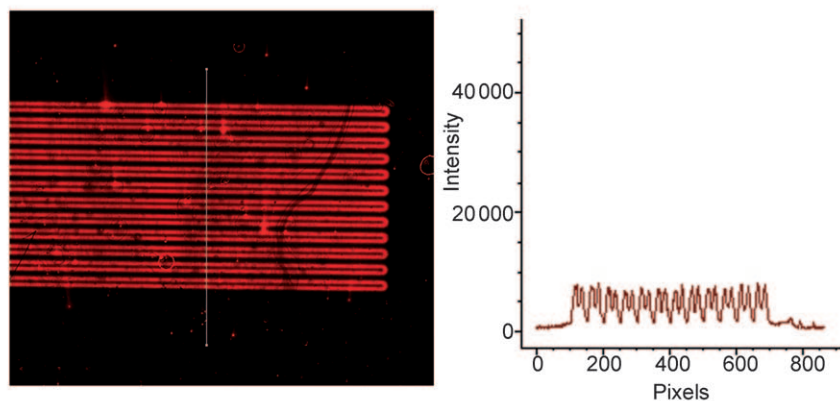
Thiol-functionalized probe **4** performed poorly when photoimmobilized on olefin-PAMAM-functionalized SiO_x/Si slides subsequent to spin coating. While line intensities were comparable to those obtained with spin-coated olefin-functionalized probe **3** on thiol-PAMAM-functionalized SiO_x/Si slides, high background levels were observed leading to a maximal *S/N* ratio of only about 1.3:1 in comparison to about 10:1 for **3**.

We observed that the surface composition of the underlying solid support had a considerable impact on the photoimmobilization efficiency. Following spin coating of probe **2** on thiol-PAMAM-functionalized SiO_x/Si slides (500 nm PECVD-deposited SiO_x) or thiol-PAMAM-functionalized glass slides and photoimmobilization, SiO_x/Si as support material led to about tenfold higher line intensity than glass, which is the standard material for biochips (Figure 1 E). Comparison of spin coating and photoimmobilization of probe **2** on thiol-functionalized SiO_x/Si slides with and without

A) With PAMAM



B) Without PAMAM



C) Comparison

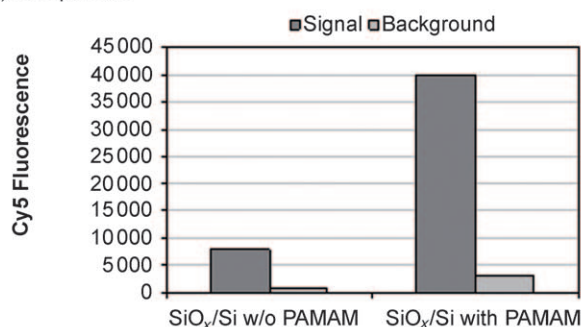


Figure 2. Influence of the PAMAM-dendrimer functionalization on thiol-ene photoimmobilization efficiency. Probe **2** was spin coated onto a thiol-functionalized SiO_x/Si slide with or without an intermediate PAMAM dendrimer layer, photoimmobilized and detected with streptavidin-Cy5. A) Fluorescence image of microstructures created on a thiol-PAMAM-functionalized SiO_x/Si slide. The white line denotes a cross-section, the histogram of which is shown on the right. B) Biotin microstructures as in A) but on a thiol-functionalized SiO_x/Si slide without PAMAM dendrimers. C) Graphical comparison of A) and B).

PAMAM dendrimers, revealed that the dendrimers increased line intensity by a factor of five and also led to a more homogeneous surface (Figure 2). This is attributed to higher surface loading resulting from a high density of functional groups on the outer dendrimer surface.^[24,25] Silicon-oxide layer thickness and the oxidation/deposition method also had a marked effect on line intensity (Figure 1E). Spin-coating of probe **2** on various thiol-PAMAM-functionalized SiO_x/Si surfaces followed by photoimmobilization led to maximal line intensity for a PECVD-generated SiO_x layer with a thickness of 500 nm. A decrease in

intensity was observed for thicker, that is, 1000 nm, or thinner, that is, 250 nm and 100 nm, SiO_x layers on Si. Optical interference could be a possible explanation for this observation.^[26] When comparing PECVD-generated SiO_x and SiO_x generated through thermal oxidation, both with a layer thickness of 1000 nm, the PECVD-generated SiO_x/Si surface achieved twofold higher line intensity. It is assumed that free silanol groups are generated in the case of PECVD-generated SiO_x ; these silanol groups facilitate subsequent surface functionalization and hence enhance surface loading. Oxygen-plasma treatment has been shown to increase hydrophilicity of SiO_2 and TiO_2 surfaces; this hints at formation of free hydroxyl groups, and furthermore led to higher degrees of surface functionalization with aminopropyltriethoxysilane (APTES).^[27] Surfaces consisting of combined layers of PECVD- and thermal-oxidation-generated SiO_x on Si did not lead to any improvements in line intensity.

The influence of probe concentration and UV exposure time on the photoimmobilization was investigated for two different application methods, spin coating and drop casting. Thiol-PAMAM-functionalized SiO_x/Si slides were used as surface material. With probe **2** the influence on the photoimmobilization subsequent to spin coating was investigated. Increasing probe concentration and UV exposure time led to an increase in line intensity and the S/N ratio. A maximal S/N ratio of about 10:1 was observed for the

highest probe concentration of 10 mM and the longest UV exposure time of 10 min. Probe **3** was employed to investigate the influence of probe concentration and UV exposure time of the photoimmobilization subsequent to drop casting. Due to its low vapor pressure, ethylene glycol was used as solvent for **3** to prevent evaporation of the thin liquid film formed during drop casting. As in the case of spin coating of probe **2**, line intensity as well as the S/N ratio increased with increasing probe concentration and UV exposure time. A maximal S/N ratio of about 10:1 was observed at a probe concentration of 10 mM.

Further increases in probe concentration led to strongly elevated background levels and lower *S/N* ratios, possibly due to side reactions unrelated to light exposure. By extending UV exposure time from 10 min to 30 min, an increase in the *S/N* ratio from about 10:1 to 20:1 was observed.

Despite the similar results for the *S/N* ratios observed for spin coating or drop casting, there were differences in the absolute line intensities. At a probe concentration of 10 mM and an exposure time of 10 min, drop casting typically resulted in line intensities that were ten times lower than those recorded for spin coating. This is attributed to a lower apparent probe concentration at the slide surface. Spin coating leads to a highly concentrated, dried probe film in close proximity to the reactive thiol surface. Drop casting causes the photoreaction to occur at the solid–liquid interface between slide surface and probe solution and hence relies on diffusion to bring probe molecules into close proximity to the reactive thiol moieties; this limitation might negatively affect photoimmobilization efficiency. Despite this disadvantage, drop casting consumes far smaller amounts of probe when compared to spin coating (20 μL versus 700 μL probe solution); this makes it attractive for immobilization of biomolecules available in only small amounts. Furthermore, unreacted probe was easier to remove by slide washing following drop casting-based photoimmobilization because the probe molecules stay in solution at all times. This could be advantageous for immobilization of sensi-

tive biomolecules such as proteins, which are prone to denaturation upon drying on a slide surface.

The flexibility of thiol–ene photomicrostructuring with regard to covered area and microstructure variety is demonstrated in Figure 3. Biotin microstructures were generated by spin coating probe 2 on a thiol-PAMAM-functionalized SiO_x/Si slide followed by photoimmobilization. Patterns of varying line widths ranging from 5 μm to 90 μm across the entire slide area (75 $\times$ 25 mm²) were achieved. Furthermore, the numerical characters generated with the photomask indicate that non-line-shaped features can also be readily generated.

Thiol–ene photomicrostructuring of biomolecules with higher complexity than biotin was previously demonstrated with a phosphopeptide.^[22] Furthermore, thiol–ene-photomicrostructured biotin surfaces served as templates for the indirect immobilization of biotinylated proteins through an intermediate layer of streptavidin. Immobilization of the tetrameric protein streptavidin proceeds through two of four possible biotin binding sites leaving the two remaining biotin binding sites facing away from the surface and accessible for protein immobilization.^[28] In this way, calf-intestine alkaline phosphatase (CIAP) and the Ras GTPase, which holds a prominent position in the Ras-MAP-kinase signaling pathway,^[29] were immobilized and shown to be active.^[22]

Biotin nanostructuring

In previous investigations, the preparation of biotin nanostructures with a line-width of 650 nm was achieved with a confocal laser microscope connected to a UV laser source.^[22] While these results that submicrometer features are attainable by thiol–ene photoimmobilization, the approach relies on spin coating as application method. As described above, spin coating is not the preferred process for immobilization of sensitive biomolecules, such as proteins, which are prone to denaturation upon drying of the slide surface. Therefore, as an extension of the method, drop casting was also investigated for sub-micrometer photostructuring. Circular thiol-PAMAM-functionalized glass coverslips with a diameter of 25 mm were obtained with the functionalization procedure described for SiO_x/Si and glass microscope slides (Scheme 3B). A thiol-functionalized coverslip was fixed in a microscope sample holder, 10 mM 3 in 3:1 water/ethylene glycol was applied as a drop on the coverslip surface, and a second, unfunctionalized coverslip was fixed on the same holder to obtain a millimeter-thick film between the two glass surfaces. With a confocal laser microscope, the functionalized coverslip surface was then exposed to UV laser light at 405 nm by continuously scanning line shapes on the surface for different exposure times ranging from 20 s to 5 min. Following washing, blocking and incubation with Cy5-labeled streptavidin, the obtained structures were analyzed with the same microscope. Line-shaped features were clearly visible on the slide surface with longer UV exposure times leading to higher line intensity (Figure 4A). Although the line width could not be reduced to less than 1.5 μm , the results demonstrate the feasibility of the approach, which is compatible to sensitive biomolecules such as proteins.

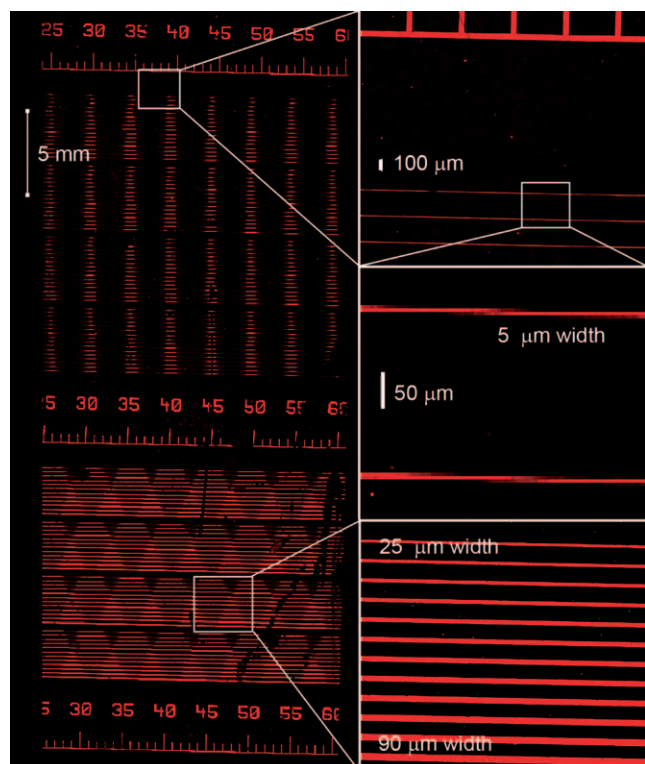


Figure 3. Flexibility of thiol–ene photoimmobilization in terms of surface coverage. Line structures with a line-width ranging from 5 μm to 90 μm were generated across the whole area of the slide surface (75 $\times$ 25 mm²) of a thiol-PAMAM-functionalized SiO_x/Si slide by thiol–ene-photomicrostructuring of 2 (deposited through spin coating).

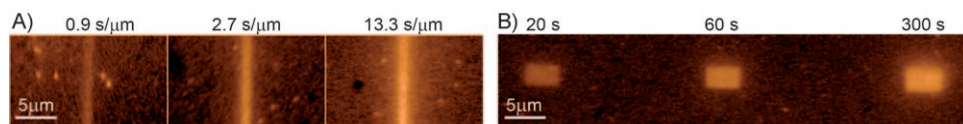


Figure 4. Reduction of microstructure size. Shown are lines and microstructures obtained by thiol-ene photoimmobilization in solution (drop casting) by using a confocal laser microscope connected to a UV laser (405 nm). Biotin probe **3** was immobilized on a thiol-PAMAM-functionalized glass coverslip through UV laser illumination for various scan times. Immobilized biotin was subsequently detected with streptavidin-Cy5.

Optical near-field techniques might enable further size reduction.^[30,31] In an analogous experiment, rectangular features of $7 \times 3.5 \mu\text{m}^2$ were generated to demonstrate the flexibility of the approach with regard to biomolecule pattern shape (Figure 4, right).

Fabrication of microarrays

To further investigate the scope of thiol-ene photoimmobilization, we applied this method for the preparation of biomolecule microarrays. For microarraying experiments, commercially available glass slides were employed as surface material, which are compatible with established procedures and equipment. Slides were functionalized as described above with an intermediate layer of PAMAM dendrimers. Olefin- or thiol-functionalized biomolecule samples were spotted in different concentrations and buffers by using a piezo-controlled nanospotter and exposed to UV light with a photolithography instrument (Figure 5A). 20% glycerol was added to samples spotted on thiol- or olefin-PAMAM-functionalized glass slides to prevent drying of the spots on the slide, which would hinder later removal of unreacted biomolecules.

Biotin microarrays

By analogy to the procedure described for photomicrostructuring, biotin microarrays were employed to identify suitable experimental conditions for thiol-ene photoimmobilization. Biotin probes **3** and **4** and the negative control **5** were spotted as three subarrays on thiol- and olefin-PAMAM-functionalized glass slides. Spotting was carried out in triplicate in concentrations ranging from 0.1 mM–50 mM. After exposure to UV light at 365 nm for 10 min and washing, biotin microarrays were blocked against unspecific protein adsorption and immobilized biotin was detected by using streptavidin-Cy5.

The results are depicted in Figure 5C and D. The fluorescence intensity values observed for **3** and **4** were concentration-dependent, comparable to the results of the photopatterning experiments. Control compound **5** showed negligible immobilization. Microarray spots generated from **4** on the olefin-PAMAM-functionalized glass surface were of higher quality than microarray spots obtained from **3** on the thiol surface (Figure 5F); this is opposite to the results obtained in the photomicrostructuring experiments. Thiol-PAMAM-functionalized glass led to inhomogeneous spot shape and large spot footprints (Figure 5F, top), potentially resulting in a decrease in microarray density and data quality. Olefin-PAMAM-functionalized

glass showed more homogeneous and well-defined spots (Figure 5F, bottom). Possibly, the higher hydrophilicity of thiol-functionalized surfaces as compared to olefin-functionalized surfaces is responsible for this result. The maximal fluorescence intensities observed for both approaches were comparable.

Olefin-PAMAM-functionalized glass slides were therefore used for the immobilization of thiol-functionalized biomolecules in subsequent microarray experiments.

Oligonucleotide microarray generation

For preparation of an oligonucleotide microarray, thiol-functionalized 24-mer oligonucleotide **8**, 5'-functionalized with a C6-alkylthiol linker, and an analogous negative control **9** without the thiol linker were employed (Figure 5B). **8** and **9** were spotted in triplicate and in three subarrays per slide in varying concentrations ranging from 100 μM –0.8 μM on olefin-PAMAM-functionalized glass slides. After exposure to UV light at 365 nm for 10 min, slides were washed and blocked against unspecific adsorption of DNA and immobilized oligonucleotides were detected by hybridization with a complementary, 5'-Cy5-labeled, 24-mer oligonucleotide. Results are depicted in Figure 5E. Oligonucleotide **8** showed concentration-dependent immobilization while the negative control **9** showed negligible immobilization. This result clearly demonstrates and confirms that direct immobilization of highly functionalized biomolecules is possible through thiol-ene photoimmobilization.

Glycopeptide microarray generation

To investigate the use of the thiol-ene reaction for immobilization of complex bioconjugates, a set of glycosylated or biotinylated, thiol-functionalized, mucin-derived MUC1 tandem repeat peptides as well as acetylated or amine-functionalized control peptides was synthesized following procedures reported previously (Scheme 4, for synthesis see the Supporting Information and Scheme S2–S4).^[32–34] Mucins are glycoproteins produced by glandular epithelial cells. They protect as well as lubricate the epithelium and are also involved in cell signaling.^[35–37] Although the functions of the mucin MUC1 are not entirely clear, it is attributed a role in tumor progression and metastasis as it is overexpressed in many cancers.^[38,39] Furthermore, its complex glycosylation patterns vary from healthy to cancer cells^[40] because aberrant glycosylation with short saccharides and premature sialylation leads to exposure of the extracellular tandem repeat peptide backbone. In this way, cancer epitopes dependent on both the glycan and peptide structure are formed.^[41–44] Therefore, glycopeptide- or glycoprotein microarrays might be powerful tools for deciphering these aberrant glycosylation patterns, which would enhance diagnostics and facilitate glycoprotein vaccine development.^[45–47]

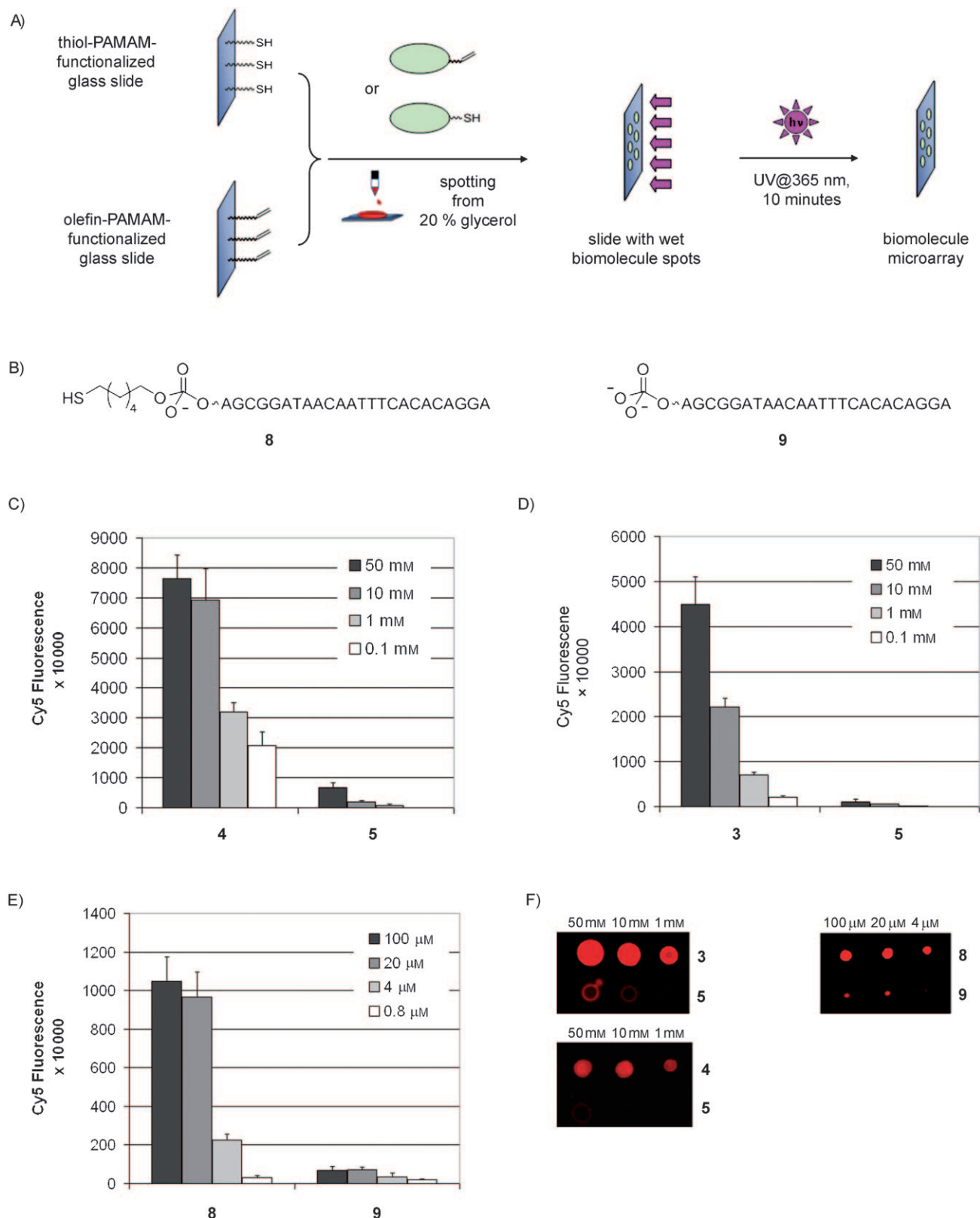
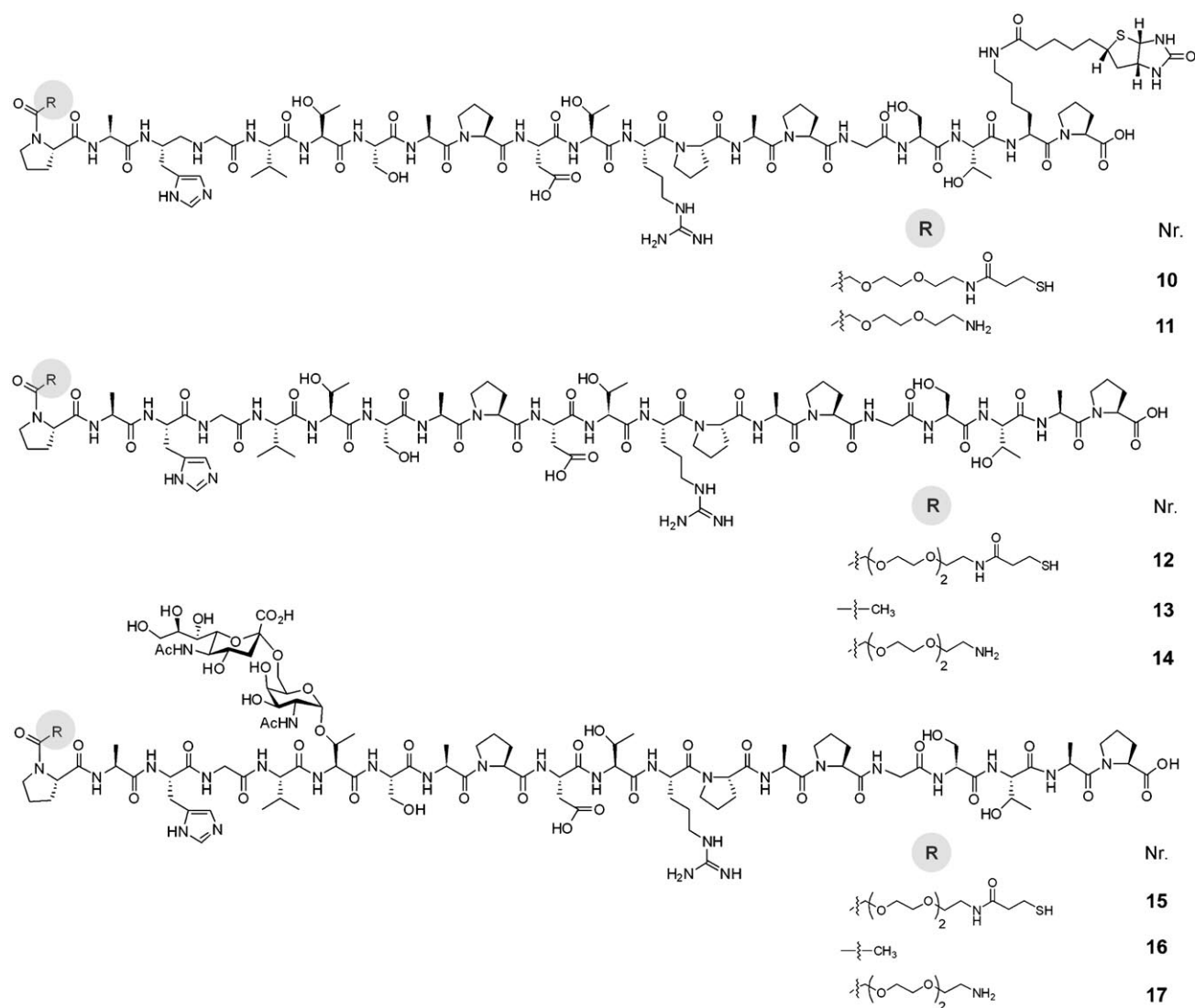


Figure 5. Biotin and oligonucleotide microarrays obtained with thiol–ene photoimmobilization. A) Biomolecule microarray-generation process. Olefin- or thiol-functionalized biomolecules were spotted on thiol-PAMAM- or olefin-PAMAM-functionalized glass slides respectively. 20% glycerol was added to prevent spot drying on the slide surface. Photoimmobilization was carried out through exposure to UV light at 365 nm for 10 min. B) Thiol-functionalized 24-mer oligonucleotide **8** and the negative control **9** used for microarray preparation. C) Quantitative evaluation of a microarray of **4** and the negative control **5** on an olefin-PAMAM-functionalized glass slide. D) Quantitative evaluation of a microarray of **3** and **5** as negative control on a thiol-PAMAM-functionalized glass slide. E) Quantitative evaluation of a microarray of oligonucleotide **8** and the negative control **9** on an olefin-PAMAM-functionalized glass slide. F) Fluorescence images of the microarrays C)–E).



Scheme 4. Structures of the MUC1 tandem repeat peptides and glycopeptides with the sialyl- T_N moiety used for thiol-ene photo immobilization.

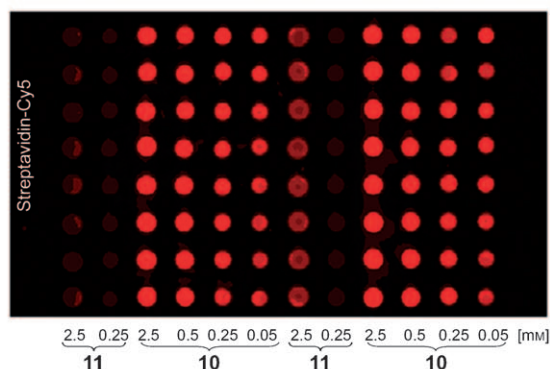
To determine appropriate immobilization conditions, biotinylated MUC1 tandem repeat peptide **10** and control peptide **11** were spotted in concentrations from 2.5–0.05 mM on olefin-PAMAM-functionalized glass slides. Following exposure to UV light at 365 nm for 10 min, immobilized glycopeptide was detected with Cy5-labeled streptavidin (Figure 6A). Spots of thiol-functionalized peptide **10** are clearly visible, while spots of the amine control **11** are only faint.

After this initial step, we investigated whether the MUC1 tandem repeat peptide could successfully be recognized by antibodies on the microarray surface. To this end, thiol-functionalized peptide **12** (that is, the nonbiotinylated variant of peptide **10**) was spotted on olefin-functionalized, hydrogel-coated glass slides (Scheme 3C) together with the acetylated control peptide **13**. For the photoimmobilized MUC1 peptides, the olefin-functionalized, hydrogel-coated glass slides gave better results than olefin-PAMAM-functionalized glass slides in cases in which nonlabeled monoclonal antibodies and serum antibodies were detected by employing a labeled secondary

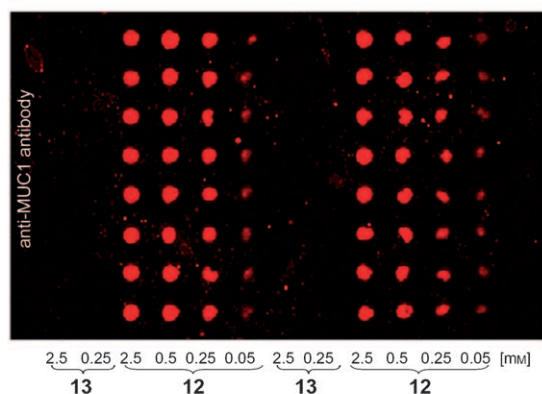
antibody. Regular buffer lacking glycerol was used for spotting on olefin-functionalized, hydrogel-coated glass slides. After exposure to UV light at 365 nm for 10 min, the array was incubated with a monoclonal primary antibody (SM3, from deglycosylated mucin)^[48,49] known to recognize the MUC1 tandem repeat backbone. The antibody was in turn detected with a Cy5-labeled secondary anti-mouse antibody. The results shown in Figure 6B and Figure 7A demonstrate concentration-dependent immobilization and successful detection of the MUC1 peptide by the antibody.

As a more challenging example, MUC1 tandem repeat peptide **15**, which is glycosylated with the tumor-associated sialyl- T_N antigen in the HGVT part, was synthesized.^[50] The glycopeptide was spotted together with the acetylated control **16** on olefin-functionalized, hydrogel-coated glass slides (Scheme 3C) and exposed to UV light at 365 nm for 10 min. The obtained glycopeptide microarray was then incubated with mouse serum obtained from mice immunized with glycopeptide **17** conjugated to tetanus toxoid protein.^[51] A Cy5-labeled anti-

A) Biotinylated MUC1 Peptides (10, control 11)



B) MUC1 Peptides (12, control 13)



C) MUC1 Glycopeptides (15, control 16)

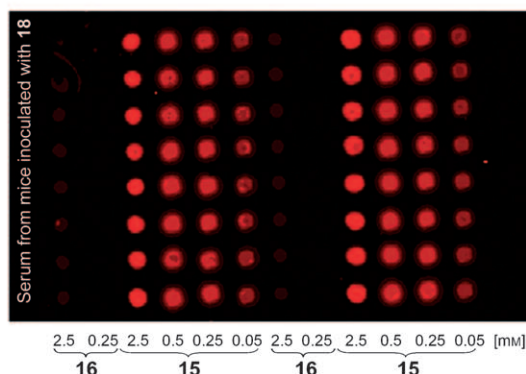


Figure 6. MUC1 tandem repeat peptide and glycopeptide microarrays generated via thiol–ene photoimmobilization. A) Microarray of peptide 10 and control peptide 11 obtained after spotting on olefin-PAMAM-functionalized glass slides and exposure to UV at 365 nm for 10 min. The biotinylated peptides were visualized with Cy5-labeled streptavidin (two repeats of columns: 2.5 mm, 0.25 mm control peptide 11; 2.5 mm, 0.5 mm, 0.25 mm, 0.05 mm peptide 10). B) Microarray of peptide 12 and control peptide 13 obtained as in A) on olefin-functionalized, hydrogel-coated glass slides. Immobilized peptides were detected with a primary monoclonal mouse antibody against the MUC1 backbone and a Cy5-labeled secondary anti-mouse antibody (two repeats of columns of control peptide 13 and peptide 12 with concentrations as in A). Right: Quantitative evaluation. C) Microarray of glycopeptide 15 and control glycopeptide 16 obtained as in A) on olefin-functionalized, hydrogel-coated glass slides. The microarray was incubated with serum of mice immunized with a conjugate of glycopeptide 17 and tetanus toxoid protein.^[51] Bound antibodies were detected with a secondary, Cy5-labeled anti-mouse antibody (two repeats of columns of control peptide 16 and glycopeptide 15 with concentrations as in A). Right: Quantitative evaluation.

mouse secondary antibody was used to detect bound primary antibody from the serum. (Figure 6C and Figure 7B). In the fluorescence image, successful, concentration-dependent immobilization of the glycopeptide 15 as well as binding of antibodies from the mouse serum is visible. The control peptide 16 shows close to no immobilization. Taken together, these and the previous results demonstrate that microarrays of highly complex biomolecules, which are suitable for biological assays, can be readily manufactured with the thiol–ene reaction.

Conclusions

We have developed a novel method for immobilization and patterning of biomolecules by employing the light-induced thiol–ene reaction as key immobilization method. By means of this transformation, biotin microstructures and microarrays of biomolecules such as biotin, oligonucleotides, MUC1 tandem repeat peptides and glycopeptides could successfully be generated in as little as ten minutes reaction time, under mild conditions and in one step. The activity of highly functionalized biomolecules such as glycopeptides was not affected by the photoimmobilization. Furthermore, antibodies from vaccinated mice were successfully probed from serum with MUC1 glycopeptide microarrays obtained through thiol–ene photoimmobilization. The use of light renders the method very flexible with regard to structure size and shape, as could be demonstrated with biotin microstructures ranging in size from 650 nm^[22] to over 90 μ m on surfaces as large as a microscope slide (75 $\times$ 25 mm²). These results demonstrate the wide applicability of the method and indicate its potential for use in the biological sciences.

Experimental Section

General procedures: Solvents were purchased from Fisher Scientific/Biosolve/Acros and were used as received: dichloromethane (DCM), dimethylformamide (DMF), ethanol (EtOH), methanol (MeOH). Materials used were from the following suppliers: cystamine dihydrochloride, diisopropylcarbodiimide (DIC), diisopropylethylamine (DIPEA) (Aldrich), dithiothreitol (DTT), triethylamine (TEA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Aldrich/Fluka/Sigma), hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) (Novabiochem), 6 inch silicon wafers (Si-Mat, Landsberg am Lech, Germany, diameter 150 mm, thickness 675 μ m, doped P/Bor, front side polished, back side etched; with or without 1000 nm thermally oxidized SiO₂), amine-reactive *N*-hydroxysuccinimide (NHS)-functionalized, hydrogel-coated glass slides (Xantec Bioanalytics, Münster, Germany), GeneFrames (ABgene, Epsom, UK), streptavidin, streptavidin-Cy5 (Invitrogen), MB grade herring sperm DNA (Roche), milk powder (Oxoid, Basingstoke, UK), oligonucleotides 8 and 9 (Eurofins MWG, Ebersberg, Germany). TRIS buffer A consisted of TRIS (20 mM, pH 7.5), MgCl₂ (5 mM), DTE (2 mM). TETBS buffer consisted of TRIS (20 mM, pH 7.5), NaCl (150 mM), EDTA (5 mM), Tween-20 (0.05%). MESTBS buffer consisted of TRIS-HCl (20 mM, pH 7.5), NaCl (150 mM), milk

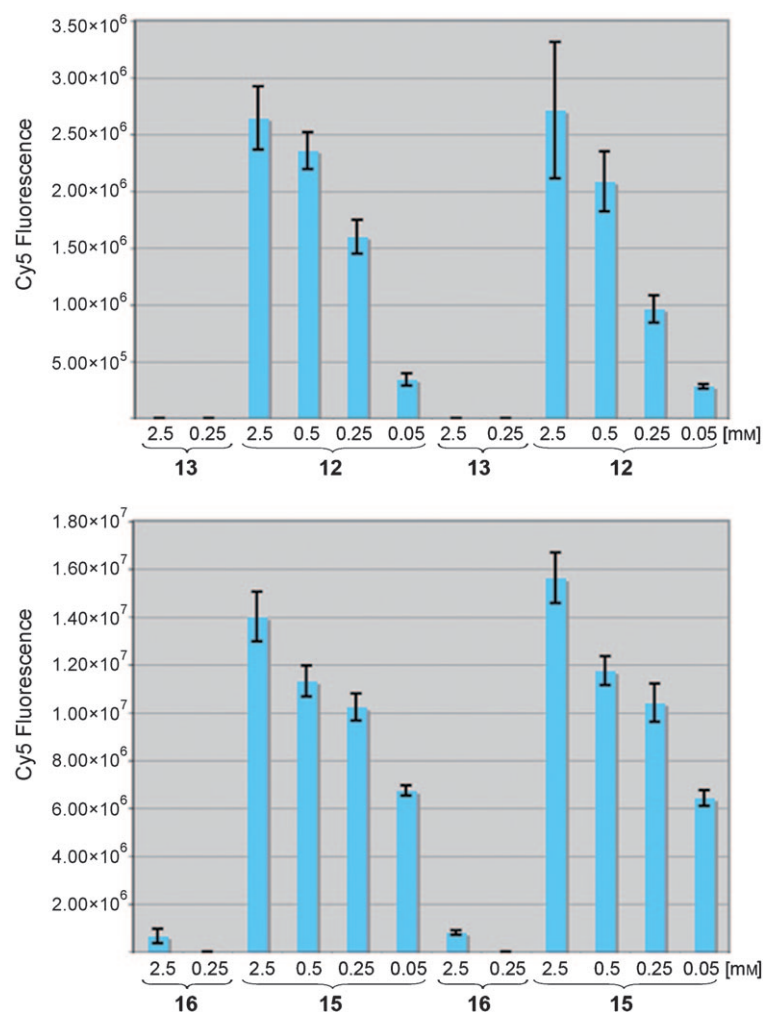


Figure 7. A) Quantitative evaluation of the microarray found in Figure 6B. B) Quantitative evaluation of the microarray found in Figure 6C.

powder (4.5%), EDTA (5 mM), NaN₃ (0.2%), DNA MB grade (1 mg mL⁻¹, Roche).

Slide preparation: For illustration of the whole slide-preparation procedure see Scheme 3. PECVD-coatings of silicon oxide in defined thicknesses were deposited on the polished side of bare 6 inch silicon wafers or silicon wafers bearing 1000 nm silicon oxide (thermally oxidized) with a PECVD machine (Oxford Instruments, Abingdon, UK) by using N₂O (400 sccm; standard cubic cm per minute) and SiH₄ (60 sccm) containing Ar (5%) at a pressure of 1400 mTorr and at a temperature of 390 °C. Deposition duration varied according to SiO_x-layer thickness (calibrated to 1 min per 50 nm SiO_x-layer thickness). For SiO_x/Si slide preparation, coated silicon wafers were cut into standard microscope slide format (25 × 75 mm²) with a semi-automatic dicing saw (ESEC 8004) using a diamond dicing blade (2.187–10–45 H, Minitron). SiO_x/Si or glass slides were functionalized by Chimera Biotec following published procedures to obtain terminally acid- or amine-functionalized slides with an optional layer of third-generation PAMAM dendrimers.^[24]

Thiol-functionalized SiO_x/Si or glass slides: For an illustration of the whole slide-preparation procedure see Scheme 3. Terminally carboxylic-acid-functionalized SiO_x/Si or glass slides (25 × 75 mm², with or without PAMAM layer) were mounted on a custom-made

teflon holder on top of a magnetic stirring bar inside a glass reactor and submerged in DMF (60 mL) under argon atmosphere and with stirring. The assembly was cooled to 0 °C. DIC (18.8 μL, 0.12 mmol) was added and the assembly was stirred for 15 min at 0 °C. Cystamine dihydrochloride (32.4 mg, 0.14 mmol) was dissolved in water (40 μL), DIPEA (49.0 μL, 0.29 mmol) and DMF (1 mL) were added and the mixture was added to the slide solution at 0 °C. The assembly was stirred overnight letting it warm to room temperature. The solution was decanted, the slides were rinsed with DMF inside the reactor and stirred for 15 min with DMF/water (3:1, 60 mL). After decantation, the slides were rinsed once more with DMF. For reduction, the reactor was filled with a solution of DTT (185 mg, 1.20 mmol) and TEA (83 μL, 0.60 mmol) in DMF (60 mL) and the assembly was stirred overnight at room temperature. The solution was decanted, the slides were rinsed with DMF and stirred in DMF (60 mL) for 15 min at room temperature. After decantation, the slides were rinsed with DMF and stirred in DCM (60 mL) for 15 min. Following decantation, slides were dried in vacuo for one hour and stored under argon in the dark. Thiol-PAMAM-functionalized glass coverslips (25 mm diameter) were prepared in analogy.

Olefin-functionalized SiO_x/Si or glass slides: For illustration of the whole slide-preparation procedure see Scheme 3. To obtain olefin-functionalized SiO_x/Si or glass slides, terminally amine-functionalized SiO_x/Si or glass slides (25 × 75 mm²) were introduced into a glass reactor as described above and submerged in DMF (60 mL) under argon atmosphere. The assembly was cooled to 0 °C and DIPEA (49.0 μL, 0.29 mmol) was added. A solution of the allyl acid linker 6 (25 mg, 0.14 mmol) in DMF (1 mL) was cooled to 0 °C and DIC (22.5 μL, 0.14 mmol) was added. The mixture was incubated for 15 min at 0 °C and transferred to the slide solution at 0 °C. The assembly was stirred overnight, letting it warm to room temperature. The solution was decanted, the slides were rinsed with DMF and stirred for 15 min in DMF/water (3:1, 60 mL). For glycopeptide microarrays, slides were

reacted for 1.5 h with HOBt (0.20 g, 1.48 mmol), DIPEA (2.25 mL, 12.9 mmol) and acetic anhydride (4.75 mL, 50.3 mmol) in DMF (100 mL) to cap unreacted amine groups followed by stirring in DMF (60 mL) for 15 min. After decantation, the slides were rinsed once more with DMF and stirred for 15 min in DCM (60 mL). The slides were dried in vacuo for one hour and stored under argon and in the dark.

Photomicrostructuring with spin coating: A solution of biotin-probe solution (700 μL, 0.01–10 mM, 1 and 2 in 3:1 DMF/toluene, 3 and 4 in EtOH) was applied with a pipette onto a SiO_x/Si or glass slide (thiol-functionalized for 1–3, olefin-functionalized for 4; optional PAMAM functionalization) fixed in a custom-made slide holder in a standard spin-coating apparatus (WS-400B, Laurell Tech-

nologies, North Wales, PA, USA). The slide was then spun at high speed following a solvent-specific program to obtain a dry film of sample on the slide surface (for DMF/toluene 3:1: 500 rpm for 5 s, 1000 rpm for 80 s, 0 rpm for 20 s; for EtOH: 1250 rpm for 40 s, 0 rpm for 20 s). The slide was exposed to UV light at 365 nm in a photolithography apparatus (MA56, Süss MicroTec, Garching, Germany) through a photomask with a line pattern of either constant line width of 100 μm or a decreasing line width from 100 to 5 μm (chromium on glass, ML&C GmbH). After exposure, the slide was again fixed in the spin coating apparatus and exposed to a constant flow of DMF from a spray bottle while running the solvent-specific centrifugation program (about 200 mL DMF). The process was repeated with MeOH. The slide was then further cleaned in water in an ultrasonic bath for 3×10 min.

Photomicrostructuring with drop casting: A solution of biotin probe (20 μL , 0.5 to 50 mM, **3** and **4** in ethylene glycol) was applied with a pipette onto a SiO_2/Si slide (thiol-PAMAM-functionalized for **3**, olefin-PAMAM-functionalized for **4**) and subsequently covered with a photomask to produce a thin liquid film between the two surfaces mediated by capillary forces. The assembly was then exposed to UV light as described for the spin-coating process. After exposure, the photomask was removed from the slide while submerged in water to prevent drying of the slide surface. The slide was rinsed with solvent and cleaned in an ultrasonic bath for 3×10 min in water.

Biotin microstructure analysis: Slides bearing biotin microstructures were placed into a moisture chamber on a circular shaker while still wet, covered with TETBS buffer (1 mL) and shaken for 10 min at ~ 120 rpm. Slides were blocked with MESTBS buffer for 30 min while shaking. Slides were rinsed with TETBS, covered with 100 nm streptavidin-Cy5 in TETBS (1 mL) and shaken for 10 min. For washing, slides were shaken (2×5 min) with TETBS (1 mL). Slides were rinsed with water and dried under a stream of argon. Fluorescence was detected with a microarray scanner (GenePix 4000B, Molecular Devices). Histograms were generated with the software GenePix Pro.

Microarray fabrication: Microarrays were created with a noncontact nanoplotter (NP1.2, GeSIM, Großberkmannsdorf, Germany). Three subarrays were spotted per slide. Biotin probes **3–5** in TBS buffer containing glycerol (20%) and TCEP (5 mM) were spotted in triplicate in concentrations ranging from 0.1 mM to 50 mM on glass slides (thiol-PAMAM-functionalized for **3** and **5**, olefin-PAMAM-functionalized for **4** and **5**) while oligonucleotides **8** and **9** were spotted in triplicate on olefin-PAMAM-functionalized glass slides in concentrations ranging from 0.8 μM –100 μM from TBS buffer containing glycerol (20%) and TCEP (5 mM).

MUC1 peptides **10–13** and glycopeptides **15** and **16** were spotted in concentrations ranging from 0.05 mM–2.5 mM from PBS buffer containing glycerol (20%) on olefin-PAMAM-functionalized glass slides or from PBS-T buffer containing Tween-20 (0.05%) and TCEP (1 equiv) on olefin-functionalized, hydrogel-coated glass slides. Following spotting, the slide bearing the microarray was exposed to UV light at 365 nm for 10 min in a photolithography apparatus (Süß Microtec MA56). Follow-up washing procedures were specific to the type of biomolecule immobilized as described below.

Biotin and oligonucleotide microarray analysis: Biotin or oligonucleotide microarrays were placed into a moisture chamber on a circular shaker, covered with TETBS buffer (1 mL) and shaken for 10 min at ~ 120 rpm. This step was repeated twice. For blocking, slides were covered with blocking solution (1 mL, MESTBS for biotin probes, TETBS with 1 mg mL^{-1} DNA MG grade (Roche) for

oligonucleotides) and shaken for 30 min. After rinsing, slides were covered with the detection solution (1 mL, 100 nm streptavidin-Cy5 in TETBS for biotin probes, 0.1 nm labeled oligonucleotide in TETBS with 1 g L^{-1} DNA MB grade (Roche) for oligonucleotides), shaken for 1 h and again washed with buffer for 3×10 min as before. Slides were dipped into a beaker with water and dried under a stream of argon. Fluorescence was detected with a microarray scanner (General Electric Typhoon Trio+). Obtained microarray images were evaluated and quantified with the software ImageQuant.

Glycopeptide microarray analysis: Glycopeptide microarrays were washed with water and dried under a stream of argon. Three GeneFrames (25 μL volume when covered) were placed onto the three obtained subarrays to minimize consumption of antibody or serum. Microarrays were washed with PBS buffer containing Tween-20 (0.05%, 80 μL , PBS-T) for 10 min and blocked for 2×15 min with blocking buffer (80 μL ; 3% BSA, 0.2% Tween-20 in PBS buffer) to avoid unspecific protein binding. The microarrays were washed again with PBS-T buffer (80 μL) for 10 min and then shaken for 45 min with streptavidin-Cy5 (100 nm, 50 μL) or primary antibody (1:100 or 1:400) or serum^[51] (1:4000) and then washed 3×10 min with PBS-T buffer. If nonlabeled monoclonal antibody or serum was used, a second incubation with a Cy5-labeled, secondary anti-mouse antibody was carried out followed by washing with PBS-T buffer for 3×10 min. Microarrays were washed with water and dried. Fluorescence was detected on a Typhoon Trio+ scanner (General Electric) and analyzed and quantified with the software ImageQuant.

Photonanostructuring with drop casting: A thiol-PAMAM-functionalized glass coverslip (diameter 25 mm) was mounted on the sample holder of an LSM510 meta microscope (Carl Zeiss) and fixed with silicon grease. A solution of **3** (100 μL , 10 mM) in water/ethylene glycol (3:1) was pipetted onto the center of the fixed slide to obtain a droplet. A second glass coverslip (25 mm diameter) was fixed on the sample holder above and in parallel to the first glass slide in such a way as to come into contact with the droplet and thus create two parallel glass-liquid interfaces. After applying oil and focussing on the functionalized glass surface with a oil objective (60 $\times$, Carl Zeiss), the surface was exposed to light from a UV laser at 405 nm (30 mW, 460 kW cm^{-2}). Lines and pattern writing was carried out with the microscope software. The unfunctionalized glass slide was removed, the functionalized glass slide was rinsed with water and incubated with 100 nm streptavidin-Cy5 in PBS buffer (100 μL) for 10 min. The slide was rinsed with PBS buffer, one droplet of PBS was applied and a second glass slide was fixed as described above to create two parallel glass-liquid interfaces. Biotin structures were visualized with laser excitation at 632.5 nm using the same objective.

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Keywords: biochips • immobilization • microarrays • photochemistry • thiol–ene reaction

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