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EDGE ARTICLE

Orthogonal, metal-free surface modification by strain-promoted azide–alkyne and nitrile oxide–alkene/alkyne cycloadditions†

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In this article we present a fast and efficient methodology for biochemical surface patterning under extremely mild conditions. Micropatterned azide/benzaldoxime-surfaces were prepared by microcontact printing of a heterobifunctional cyclooctyne oxime linker on azide-terminated self-assembled monolayers (SAMs). Strain-promoted azide–alkyne cycloaddition (SPAAC) in combination with microcontact printing allows fast and effective surface patterning. The resulting bifunctional azide/oxime substrates could successfully be used for metal-free, orthogonal immobilization of various biomolecules by 1,3-dipolar cycloadditions at room temperature. Azide-decorated areas were modified by reaction with a cyclooctyne-conjugate using SPAAC, while benzaldoxime-decorated areas were activated by *in situ* oxidation to the reactive nitrile oxides and subsequent nitrile oxide cycloaddition with alkene- and alkyne-functionalized bioconjugates. In addition, orthogonal double immobilization was achieved by consecutive and independent SPAAC and nitrile oxide cycloadditions.

Introduction

The selective immobilization of biomolecules onto surfaces is one of the largest challenges in the development of biomimetic materials and microarray technology. The optimization of biological activity and ligand binding affinity demands an attachment that is carried out in a well defined way with pre-determined orientation. Site-specific immobilization strategies have been developed during the last decades in order to fulfil these requirements.¹ Robust, covalent site-specific attachment can be achieved if the biomolecules are modified with a functional group that is reactive towards the substrate surface. Covalent immobilization of biological molecules can be regarded as a special case of bioconjugation and underlies similar demands as in the case of solution-based modifications.

The complex chemical structure of the majority of cellular molecules requires the development of chemical conjugation reactions that are affected neither by the functional groups on the target molecules nor by their biological environment. Moreover, the reactions should proceed with near quantitative yield under

mild conditions. During the last decade, much progress was made in the development of such “bioorthogonal” reactions and led to a set of conjugation principles that form central tools to probe biological processes.² Alkenes, alkynes and azides are excellent functional groups for this purpose as they are chemically inert under physiological conditions and enable site-specific modification using a bioorthogonal set of reactions. Thiol-ene additions³ and Diels–Alder cycloadditions⁴ have been successfully applied for the conjugation of alkene-functionalized biomolecules. Azides were modified by the Staudinger ligation⁵ or in combination with alkynes by the copper-catalyzed azide–alkyne cycloaddition (CuAAC).⁶ Due to the toxicity of Cu(I), strain-promoted azide–alkyne cycloadditions (SPAAC) based on cyclooctynes⁷ currently receive increasing interest and could already be successfully applied for DNA,⁸ protein⁹ and glycan¹⁰ conjugation, *in vivo* imaging¹¹ and in the field of surface functionalization.¹²

Another category of metal-free click reactions is based on cycloadditions between strong 1,3-dipoles with enhanced reactivity, such as nitrile oxides, nitrile imines and nitrones, with unsaturated hydrocarbons.¹³ Gutmiedl *et al.* demonstrated the potential of the nitrile oxide 1,3-dipolar cycloaddition in the area of bioconjugation by the derivatization of norbornene-modified DNA.¹⁴ In later work, Heaney and coworkers modified resin-supported alkyne- and cyclooctyne-functionalized DNA by reaction with nitrile oxides.¹⁵ Chemical orthogonality of azide and nitrile oxide cycloadditions has recently been demonstrated by the post-modification of heterobifunctional linkers and polymers, expanding the applicability of both reactions for molecular functionalization.¹⁶

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The investigation and development of orthogonal reactions is essential for the multistep modification of polymers, dendrimers or biomolecules as well as the preparation of multifunctional surfaces and materials. During the last few years, multifunctional surfaces have shown high potential in biological microarray technology: they have been prepared by various patterning techniques, using different types of reactions and applied for selective immobilization biomolecules, nanoparticles and dyes.¹⁷ Typically, efficient orthogonal ligation reactions rely on catalysis or photochemical triggers. In this article we demonstrate for the first time the synthesis of patterned, isoelectronic azide/nitrile oxide surfaces and their versatility for orthogonal metal-free surface immobilization by reaction with a range of alkene- and alkyne-modified (bio)molecules at ambient temperature.

Results and discussion

Surface patterning was achieved by microcontact chemistry.¹⁹ This technique is based on the use of microstructured elastomeric stamps to locally transfer reactive molecules onto a substrate surface and induce a chemical reaction in the area of contact. Oxidized PDMS stamps with a dot pattern (approx. 10 μm dots in diameter) were inked with cyclooctyne oxime (**1**) and applied on undecylazide-modified glass and silicon surfaces (Fig. 1A). The induced microcontact SPAAC reaction ($\mu\text{C-SPAAC}$) leads to highly effective, metal-free chemical patterning of the surface and stable attachment of the linker molecule within 20 min of

reaction time. The patterned azide/oxime substrates are highly versatile for post-modifications as they allow orthogonal reactions either by (i) the oxidation of oximes to nitrile oxides using oxidizing agents such as diacetoxy iodobenzene (DIB)^{20,16c} and reaction of the generated nitrile oxides with a variety of unsaturated hydrocarbons (Fig. 1B) or by (ii) SPAAC in areas with the remaining azide groups (Fig. 1C).

AFM measurements verified the immobilization of the linker (**1**) in contact areas with the stamp in height (Fig. 1D) and also in phase (Fig. 1E). The height difference between azide and oxime terminated areas was found to be approximately 1 nm, fitting with the expected length of the immobilized molecule. Moreover, printing of the cyclooctyne oxime linker (**1**) leads to a change of surface wetting properties. Water condensation experiments visualize the hydrophilic pattern ($\theta_{\text{static}} = 64 \pm 3$) in good contrast to the hydrophobic azide monolayer ($\theta_{\text{static}} = 83 \pm 2$) (Fig. ESI-1†). Surface analysis of azide and oxime-terminated monolayers on silicon was additionally carried out by X-ray photoelectron spectroscopy (XPS). The C1s carbon signal of the oxime-terminated surface consists of a peak caused by C–C carbons that are not connected to a heteroatom (285 eV) and of characteristic shoulders, which represent carbon atoms in a C–O/C–N bond (286.5 eV), in an amide (288 eV) and in a carbamate group (290 eV) (Fig. ESI-2A†). Furthermore, the two N1s signals of the internal and the two neighbouring azide nitrogens vanish after immobilization of oxime (**1**) and a single peak (400 eV) for the triazole, amide and carbamate nitrogens is formed, indicating the consumption of azide groups during μCP (Fig. ESI-2B†).

The quality of the azide/oxime pattern and its chemical composition were imaged by time-of-flight secondary ion mass spectrometry (ToF-SIMS). The immobilization of the linker (**1**) was verified by the formation of several oxygen-containing fragments like $\text{C}_2\text{H}_5\text{O}^+$, $\text{C}_7\text{H}_6\text{NO}^+$ and $\text{C}_8\text{H}_6\text{NO}_2^+$ in the positive ion mode (Fig. 2A and ESI-3†). The generation of the aromatic ions C_7H_7^+ , $\text{C}_7\text{H}_4\text{N}^+$, $\text{C}_7\text{H}_6\text{NO}^+$ can be attributed to the presence of the terminal benzaldoxime on the surface. The fragment $\text{C}_8\text{H}_6\text{NO}_2^+$ represents exactly the benzaldoxime with attached carbonyl group. Moreover, the immobilization of the cyclooctyne oxime linker (**1**) was visualized indirectly by the Si^+ signal. Si^+ cations are preferentially observed in the interspaces, since the thicker organic layer within the dotted structure leads to enhanced shielding of the underlying SiO_2 against the primary ion beam. Analysis of the oxime pattern was additionally carried out in the negative ion mode (Fig. ESI-4†). Also in this case, characteristic oxygen-rich fragments like CNO^- , $\text{C}_2\text{H}_3\text{O}^-$ and $\text{C}_2\text{H}_3\text{O}_2^-$ and aromatic fragments like $\text{C}_7\text{H}_4\text{N}^-$ and $\text{C}_7\text{H}_6\text{NO}^-$ verified successful immobilization.

Orthogonal functionalization of azide/oxime surfaces was initially carried out by *in situ* generation of the nitrile oxide by DIB and subsequent reaction with biotin alkyne (**2**) to form isoxazole linkages. To induce the nitrile oxide–alkyne cycloaddition (NOAC), patterned glass surfaces were incubated with solutions of the alkyne (50 mM) and DIB (100 mM) in methanol for 2 h. Remaining azides were subsequently decorated by reaction with cyclooctyne tetraethylene glycol^{2c} and the surfaces exposed to DyLight 405-labeled streptavidin. Fluorescence microscopy verified the attachment of the biotin-binding streptavidin ($K_a = 10^{15} \text{ M}^{-1}$)²¹ exclusively in areas with the

Fig. 1 Surface modification by metal-free click chemistry. Immobilization of cyclooctyne oxime linker (**1**) by $\mu\text{C-SPAAC}$ (A). Surface modifications by nitrile oxide cycloadditions (B). Surface modification by SPAAC (C). AFM analysis of the azide/oxime-patterned surface in height (D) and phase (E).

Fig. 2 ToF-SIMS analysis of patterned silicon substrates. Analysis of an azide/oxime-patterned silicon substrate in the positive ion mode (A) and analysis of the surface after immobilization of biotin alkyne (2) by NOAC in the negative ion mode (B) (mc: maximum counts, tc: total counts).

Fig. 3 Fluorescence microscopy image of protein binding after orthogonal, metal-free surface modifications. Immobilization of biotin alkyne (2) by NOAC and streptavidin binding (A). Immobilization of β -D-galactose norbornene conjugate (3) via nitrile oxide-alkene cycloaddition and binding of peanut agglutinin (B). Immobilization of cyclooctyne mannose conjugate (4) by SPNOAC (C) and by SPAAC (D) with subsequent binding of concanavalin A.

immobilized nitrile oxide precursor (1) (Fig. 3A). Immobilization of the biotin residue was additionally investigated by ToF-SIMS. In contrast to the azide/oxime-modified surface, SIMS analysis of the same surface after reaction with biotin alkyne (2) showed significant formation of sulphur containing anions such as S⁻, HS⁻ and ³⁴S⁻ within the dotted pattern (Fig. 2B and ESI-5†).

In addition to alkynes, nitrile oxides also react with alkenes to give isoxazolines. Especially the strained norbornene shows high reactivity and quickly undergoes strain-promoted nitrile oxide-alkene cycloaddition reactions. In order to demonstrate the suitability of this reaction for surface functionalization, β -D-galactose norbornene conjugate (3) was synthesized and reacted with azide/oxime patterned glass substrates in the presence of DIB. Binding to TRITC-labeled peanut agglutinin (PNA) after blocking remaining azides with cyclooctyne tetraethylene glycol confirmed successful immobilization of the carbohydrate within the printed pattern (Fig. 3B). We note that a high surface coverage of carbohydrate is required for multivalent lectin binding. In addition to norbornenes, also non-strained alkenes react with nitrile oxides. Functionalization of the pattern with carboxylic acid groups *via* nitrile oxide reaction with 5-pentenoic acid and subsequent attachment of PAMAM G4 dendrimers by peptide chemistry proved the successful cycloaddition. Immobilized PAMAM G4 dendrimers were detected by fluorescence microscopy after labelling with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (Fig. ESI-6†).

We also investigated the potential of the exceptionally fast strain-promoted nitrile oxide-alkyne cycloaddition (SPNOAC) for surface immobilization. Azide/oxime patterned glass substrates were exposed to dilute solutions of α -D-mannose cyclooctyne conjugate (4) (1 mM) and DIB in methanol. After 10 min the surfaces were washed and remaining azides reacted with cyclooctyne tetraethylene glycol. Exposure to FITC-labeled concanavalin A (ConA) and fluorescence microscopy analysis showed that in spite of the short reaction time a high density of carbohydrate residues was obtained in the dotted pattern (Fig. 3C). The mannose density within the azide-modified areas can be assumed to be negligible low, since no binding of ConA occurred in the interspaces.

In order to demonstrate chemical orthogonality of nitrile oxide-alkene/alkyne cycloadditions to SPAAC, azide/oxime-patterned surfaces were reacted with solutions of α -D-mannose cyclooctyne conjugate (4) in DMF (50 mM) *without* the addition of DIB. After 3 h, the surfaces were cleaned and exposed to FITC-labeled ConA. Fluorescence microscopy verified that immobilization of ConA and therefore also of mannose residues exclusively took place in azide-modified areas (Fig. 3D).

Both types of cycloadditions are orthogonal and could be used for selective double-functionalization of azide/oxime-patterned surfaces. In a first experiment β -D-galactose norbornene conjugate (3) was immobilized *via* nitrile oxide cycloaddition, followed by the immobilization of α -D-mannose cyclooctyne conjugate (4) *via* SPAAC. Fluorescence microscopy after exposure to a mixture of FITC-labeled ConA and TRITC-labeled PNA verified attachment of galactose residues within the dotted pattern (Fig. 4A, B) and of mannose residues in the interspaces (Fig. 4B, C). In another experiment, α -D-mannose cyclooctyne conjugate (4) was immobilized on azide-terminated areas by SPAAC while biotin alkyne (2) was reacted by nitrile oxide

Fig. 4 Fluorescence microscopy images of TRITC-labeled PNA and FITC-labeled ConA on azide/oxime-patterned surfaces after sequential immobilization of β -D-galactose norbornene conjugate (**3**) and α -D-mannose cyclooctyne conjugate (**4**) (A, B, C). Fluorescence microscopy images of DyLight 405-labeled streptavidin and FITC-labeled ConA on azide/oxime-patterned surfaces after sequential immobilization of α -D-mannose cyclooctyne conjugate (**4**) and biotin alkyne (**2**) (D, E, F). Fluorescence microscopy analysis of TRITC-labeled streptavidin attached to biotin residues that were immobilized by μ C-NOAC of biotin alkyne (**2**) on an azide/nitrile oxide-patterned surface (G).

cycloaddition in the dotted areas. Exposure of the surface to a mixture of DyLight 405-labeled streptavidin and FITC-labeled ConA again verified complete orthogonality, as streptavidin was exclusively immobilized on the dot-pattern (Fig. 4D, E) and ConA in the interspace (Fig. 4E, F).

In addition to the functionalization of azide/oxime-patterned surfaces by *in situ* generation of nitrile oxides and reaction from solution with alkenes and alkynes, we could also convert the oximes into nitrile oxides in a first step and bring them to reaction in a subsequent printing step. Conversion of oximes into nitrile oxides was carried out by immersion of the surfaces in methanolic solutions of DIB (100 mM) for 10 min. The surfaces were washed with methanol and dried to give the azide/nitrile oxide patterned surfaces. In order to demonstrate the orthogonal reactivity of the 1,3-dipolar groups, biotin alkyne (**2**) was immobilized within 10 min in lines by microcontact NOAC (μ C-NOAC) on the patterned surface. Remaining azides and nitrile oxides were decorated subsequently by a reaction with cyclooctyne tetraethylene glycol. Exposure of the surface to TRITC-labeled streptavidin and fluorescence microscopy showed that immobilization of biotin residues occurred

exclusively in nitrile oxide-modified areas (dots) and in the contact area with the stamp (lines) (Fig. 4G).

Conclusions

In summary, we have described the preparation of patterned azide/oxime and azide/nitrile oxide surfaces that can be orthogonally modified by different types of 1,3-dipolar cycloadditions. Strained and non-strained alkenes and alkynes were successfully immobilized in the absence of any metal catalyst. Both SPAAC and all types of nitrile oxide cycloadditions studied here can be carried out under mild conditions with high yields. Moreover, the reactions proceed readily in an aqueous environment at room temperature, which makes azide/nitrile oxide-modified surfaces of high interest for the immobilization of a wide variety of alkene- and alkyne-modified biomolecules.

Materials and methods

Materials

11-Bromoundecyl-trichlorosilane was purchased from ABCR. Glass slides were prepared from IDL microscope slides (Interessengemeinschaft der Laborfachhändler) by cutting them into pieces of $2.6 \times 1.4 \text{ cm}^2$. Silicon wafers (B-doped, 1-0-0 orientation, 20–30 $\Omega \text{ cm}$) were kindly donated by Siltronic AG (Burghausen, Germany). Milli-Q water was prepared by using a PureLab UHQ deionization system (Elga). Fluorescein isothiocyanate (FITC)-labeled concanavalin A (ConA) and bovine serum albumin (BSA) were purchased from Sigma Aldrich. Tetramethylrhodamine isothiocyanate (TRITC)-labeled peanut agglutinin (PNA) was delivered by Vector Laboratories. DyLight 405-conjugated- and tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin were purchased from Thermo Fisher Scientific.

Methods

Preparation of poly(dimethylsiloxane) (PDMS) stamps. PDMS stamps were prepared by mixing sylgard 184 silicone elastomer base with the curing agent in a 10 : 1 ratio (Dow Corning) and casting the mixture on a patterned silicon master. Curling was achieved overnight in an oven at 80 °C. Patterned areas were cut out with a knife and the resulting stamps were oxidized in an UV-ozone (PSD-UV, Novascan Technologies Inc.) for 55 min directly before printing. Flat, non-patterned PDMS stamps were prepared by following the same procedure with the difference that a flat silicon master was used.

Preparation of undecylazide-terminated glass and silicon substrates. Undecylazide-modified substrates were prepared following a known procedure with small modifications.¹⁸ Glass and silicon substrates were cleaned by sonication in pentane, acetone and Milli-Q water. The surfaces were treated with a freshly prepared piranha solution ($\text{H}_2\text{SO}_4 : \text{H}_2\text{O}_2$ 3 : 1, CAUTION!) for 30 min. After washing the substrates thoroughly with Milli-Q water, the surfaces were dried in a stream of Ar and immersed in a solution of 11-bromoundecyltrichlorosilane (0.1% vol.) in toluene for 40 min. The substrates were washed with DCM, ethanol and milli-Q water and dried.

The bromine-functionalized surfaces were immersed into a saturated solution of NaN_3 in DMF for 48 h at 70 °C. Finally, the surfaces were again washed with Milli-Q water and ethanol and dried in a stream of Ar.

Preparation of patterned azide/oxime-terminated glass and silicon substrates by μC -SPAAC. Oxidized PDMS stamps were covered with a solution of cyclooctyne oxime linker (**1**) in ethanol (10 mM, 20 μL). After 1 min, the stamps were blow dried in a stream of Ar and carefully placed on the undecylazide-modified substrate surfaces. Printing was carried out for 20 min at rt. The stamps were removed from the surfaces and the substrates washed with Milli-Q water, ethanol and DCM, sonicated in ethanol and dried in a stream of Ar.

Orthogonal ligation reactions

Immobilization of biotin alkyne (2**) and galactose norbornene conjugate (**3**) by NOAC.** Azide/oxime-patterned substrates were modified with approximately 2 mm thick, flat PDMS squares that had a hole in the middle. The PDMS was positioned in such a way that the patterned areas of the substrate surfaces were not covered by the PDMS and accessible through the hole. A freshly prepared mixture of DIB in methanol (300 mM, 10 μL) and biotin alkyne (**2**) in methanol (75 mM, 20 μL) or of DIB in methanol (300 mM, 10 μL) and galactose norbornene conjugate (**3**) in methanol (75 mM, 20 μL) was applied on the patterned surfaces. The reaction chambers were sealed with microscopy cover slides. After 2 h, the PDMS was removed and the surfaces cleaned by washing with DCM, Milli-Q water and ethanol.

Immobilization of mannose cyclooctyne conjugate (4**) by SPNOAC.** Immobilization of mannose cyclooctyne conjugate (**4**) by SPNOAC was carried out as described in the case of the non-strained alkyne (**2**) with the difference that 1.5 mM solutions of the cyclooctyne in methanol (20 μL) were mixed with 300 mM solutions of DIB in methanol (10 μL) and applied on the patterned substrates surfaces. The reaction time was reduced to 10 min.

Immobilization of mannose cyclooctyne conjugate (4**) by SPAAC.** Immobilization of mannose cyclooctyne conjugate (**4**) was carried out by covering the substrate surfaces with solutions of the cyclooctyne in DMF (50 mM). The solutions were covered with a cover slide and incubated for 3 h. Finally, the surfaces were washed with Milli-Q water and ethanol and dried in a stream of Ar.

Preparation of patterned azide/nitrile oxide surfaces and immobilization of biotin alkyne (2**) by μC -NOAC.** Azide/oxime-modified surfaces were treated for 10 min with a freshly prepared solution of DIB in methanol (100 mM). The surfaces were washed with methanol and dried. Oxidized PDMS stamps were covered with a solution of biotin alkyne (**2**) in ethanol, incubated for 1 min, blow dried and immediately placed on the freshly prepared azide/nitrile oxide surfaces. Printing was carried out for 10 min at rt. The stamps were removed and the surfaces thoroughly washed with Milli-Q water and ethanol and blow dried. Directly after printing, remaining azide and nitrile

oxide groups were blocked by reaction with cyclooctyne tetraethylene glycol.

Protein binding

Blocking of azide and nitrile oxide groups by reaction with cyclooctyne tetraethylene glycol. Remaining azide and nitrile oxide groups were decorated with cyclooctyne tetraethylene glycol by dropping a small volume of the cyclooctyne solution in DMF (50 mM) on the substrate surfaces. The solutions were covered with a cover slide and incubated for 3 h. Finally, the surfaces were washed with Milli-Q water and ethanol and dried in a stream of Ar.

PNA and ConA binding. Substrate surfaces were incubated for 30 min in a solution of bovine serum albumin (BSA, 3% wt.) in phosphate buffered saline (1 $\times$ PBS, pH = 7.5) and washed with PBS (2 $\times$ 5 min). Subsequently, the substrates were covered with a solution of fluorescence-labeled lectin (FITC-labeled ConA or TRITC-labeled PNA, $c = 20 \mu\text{g mL}^{-1}$) in HEPES buffer (20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl_2). In the case of ConA binding experiments, MnCl_2 was added to a concentration of 1 mM. After 30 min, the surfaces were washed with the same buffer without lectin, rinsed with distilled water and carefully dried with a tissue.

Streptavidin binding. Substrate surfaces were incubated for 30 min in a solution of bovine serum albumin (BSA, 3% wt.) in phosphate buffered saline (PBS, pH = 7.5) and washed with PBS (2 $\times$ 5 min). Subsequently, the substrates were covered with a solution of streptavidin (TRITC-labeled or DyLight 405-labeled streptavidin, $c = 100 \text{ nM}$) in PBS buffer (pH = 7.5). After 30 min, the surfaces were washed with the same buffer without streptavidin, rinsed with distilled water and carefully dried with a tissue.

Simultaneous binding of PNA and ConA. Substrate surfaces were incubated for 30 min in a solution of BSA (3% wt.) in PBS (pH = 7.5) and washed with PBS (2 $\times$ 5 min). Subsequently, the substrates were covered with a solution of FITC-labeled ConA and TRITC-labeled PNA in HEPES buffer. The lectin solution was prepared by mixing a solution of TRITC-labeled PNA in HEPES buffer (100 μL , $c = 50 \mu\text{g mL}^{-1}$ protein, 20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl_2) with a solution of FITC-labeled ConA in HEPES buffer (100 μL , $c = 50 \mu\text{g mL}^{-1}$ protein, 20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl_2 , 1 mM MnCl_2). After 30 min, the surfaces were washed with HEPES buffer (20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl_2 , 1 mM MnCl_2), rinsed with distilled water and carefully dried with a tissue.

Simultaneous binding of streptavidin and ConA. Substrate surfaces were incubated for 30 min in a solution of BSA (3% wt.) and PBS (pH = 7.5) and washed with PBS (2 $\times$ 5 min). Subsequently, the substrates were covered with a solution of FITC-labeled ConA and DyLight 405 labeled streptavidin in buffer. The protein solution was prepared by mixing a solution of FITC-labeled ConA in HEPES buffer (100 μL , $c = 50 \mu\text{g mL}^{-1}$ protein, 20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl_2 , 1 mM

MnCl₂) with a solution of DyLight 405-labeled streptavidin in PBS buffer (50 µL, c = 500 nM, pH = 7.5) and diluting the mixture by the addition of HEPES buffer without protein (100 µL, 20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂). After 30 min, the surfaces were washed with HEPES buffer (20 mM HEPES, pH = 7.5, 0.15 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂), rinsed with distilled water and carefully dried with a tissue.

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