Electronic supporting information to:

Oriented Protein Immobilization using Covalent and Non-covalent Chemistry on a Thiol-Reactive Self-Reporting Surface.

Dorothee Wasserberg, Carlo Nicosia, Eldrich E. Tromp, Vinod Subramaniam,* Jurriaan Huskens,* Pascal Jonkheijm.*

Compounds

11-bromoundecyltrichlorosilane (ABCR), sodium azide (Acros), L-cysteine (Sigma), *Nα,Nα*-bis(carboxymethyl)-L-lysine (Aldrich), tetrakis(acetonitrile)copper(I) hexafluorophosphate (Aldrich), ethylenediaminetetraacetic acid (Sigma-Aldrich), γ-thiobutyrolactone (Aldrich) and 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich) were used as received.
(1S)-N-[5-[(4-mercaptobutanoyl)-amino]-1-carboxypentyl]iminodiacetic acid (thiol-NTA),[S1] tris-(benzyltriazolylmethyl)amine (TBTA),[S2] propargyl-biotin[S3] and compound **1**[S4] were prepared as described in literature. Water was of MilliQ quality. Solvents were of p.a. grade unless stated otherwise.

Substrate and Monolayer Preparation

Microscope glass slides were used for monolayer preparation. The substrates were cleaned and activated with piranha solution for 45 min (concentrated H_2SO_4 and 33 % aqueous H_2O_2 in a 3:1 ratio; handle with care!) and rinsed with water. After drying in a stream of nitrogen, the substrates were used immediately to form a silanized monolayer. The substrates were immersed in 0.1 vol.% 11-bromoundecyltrichlorosilane in dry toluene for 45 min at room temperature. Following monolayer formation, the substrates were rinsed with toluene to remove any excess of silanes with ethanol and subsequently dried in a flow of nitrogen. The bromide/azide nucleophilic substitution was carried out by reacting with a saturated solution of NaN_3 in DMF for 48h at 70 ^oC. The substrate were thoroughly rinsed with water and ethanol and gently dried in a flow of nitrogen.

Microcontact printing

Silicon masters were fabricated by photolithography. They consisted of micrometer-sized features (hexagonally arranged 10 µm diameter circular features separated by 5 µm or 5 µm wide lines separated by 15 or 20 µm) and were treated with 1H,1H,2H,2H-perfluoredecyltrichlrorosilane to facilitate separation of the PDMS from the master. Stamps were prepared by casting a 10:1 (v/v) mixture of poly(dimethylsiloxane) and curing agent (Sylgard 184, Dow Corning) onto a silicon master. After overnight curing at 60 ^oC, the stamps were oxidized by oxygen plasma for 10 s (power tuned to 50 mA) and subsequently inked by covering the stamp with an inking solution (1.5 mM **1** or propargyl-biotin (in CH_3CN), 0.5 mM $Cu(I)(CH_3CN)_4PF_6$ and 0.5 mM TBTA (CH_3CN/EtOH=2/1) (catalyst mixture), prepared by mixing 75 µL of 2 mM solution of **1** or propargyl-biotin in CH_3CN and 25 µL of 2 mM of catalyst mixture). After 4 min incubation, the stamps were dried in a stream of nitrogen and brought into conformal contact with the substrate for 60 min. The stamps were changed for each new printing, and the same inking procedure was used. After stamp removal, the printed substrates were rinsed with ethanol, sonicated in acetonitrile for 2 min, rinsed again with ethanol, gently dried with nitrogen and imaged with a fluorescence microscope.

Reactive contact printing of thiols

After immobilization of **1**, the platform was further reacted with thiols via a printing procedure. In the case of the immobilization of cysteine or thiol-NTA, an oxidized PDMS stamp was inked with a 10 mM thiol-NTA solution in PBS (pH 7.5) for 30 min. Stamps were gently dried in a stream of nitrogen and brought into conformal contact with substrates patterned with **1** for 10 min. The PDMS was carefully removed and substrates were washed via sonication in PBS and water.

Cloning of TagRFP and TFP variants for high-level expression

The following primers were used for PCR amplification of *TagRFP* using pTagRFP-C (purchased from Evrogen JSC) as DNA template: 5'-cgcggatccaatgagcgagctgattaaggagaacatgca-3' containing a unique BamHI restriction site (underlined) and 5'-cgcgaattccttgtgccccagtttgctag-3' containing a unique EcoRI restriction site (underlined). The PCR product was purified and digested with BamHI and EcoRI restriction enzymes (NEB) and ligated into pRSETB plasmid (Invitrogen) digested with the same restriction enzymes. pRSETB contains an N-terminal hexahistidine-tag (His_6-tag) for nickel-affinity purification. The resulting plasmid, pRSETB-TagRFP, was first transformed into *E. coli* (XL10 gold, Stratagene) using standard procedures in the presence of ampicillin (100 mg/L). pRSETB-TagRFP plasmid was transformed into *E. coli* BL21 pLysS using standard procedures in the presence of ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Single colony transformants were selected and the pre-culture was grown overnight at 37 ^{o}C. This pre-culture was used to inoculate 2 L of LB medium containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) at 37 ^{o}C with shaking until an OD_{600} = 0.6 was reached. The culture was cooled to 16 ^{o}C before protein expression was induced with IPTG (isopropyl-D-thiogalactopyranoside) to a final concentration of 1 mM and incubated overnight at 16 ^{o}C. Cells were harvested by centrifugation at 4000x*g* at 4 ^{o}C for 20 min. The resulting cell pellet was resuspended for 20 min in BugBuster reagent with benzonase nuclease (Novagen) according to the supplier's instructions. The lysate was cleared by centrifugation at 16000x*g* for 30 min at 4 ^{o}C. Ni-NTA agarose beads (QIAGEN) were added to the protein supernatant at a 10:1 v/v ratio, respectively and incubated at 4 ^{o}C for at least 1 h with slow but continuous mixing. The agarose beads were filtered and washed with wash buffer (20 mM Tris buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with elution buffer (20 mM Tris buffer, 300 mM NaCl, 1 M imidazole, pH 8.0). The purified protein fractions (~ 30 µM) were subsequently desalted using PD10 columns (GE Healthcare) into 0.1 x PBS (0.8 mM phosphate buffer (PB), 14.4 mM NaCl, 0.27 mM KCl, pH 7.4), aliquoted, snap-frozen in liquid nitrogen and stored at -80 ^{o}C. Proteins were characterized using SDS- and native PAGE, UV-Vis, steady state- and time-resolved fluorescence spectroscopy and MALDI-TOF mass spectrometry.[S5]

For the insertion of a second, C-terminal His_6-tag, the *TagRFP* gene was amplified using 5'-cgcggatccaatgagcgagctgattaaggagaacatgca-3' (BamHI restriction site is underlined) and 5'-gcggaattcttagtggtggtggtggtggtgcttgtgccccagtttgcta-3' (EcoRI restriction site is underlined) as forward and reverse primers, respectively, and pRSETB-TagRFP as DNA template. After PCR purification, the gene product and pRSETB-TagRFP were digested sequentially, first with EcoRI, then BamHI according to the manufacturer's instructions. DNA ligations were performed using T4-ligase (NEB) at 16 ^{o}C overnight and the resulting pRSETB-TagRFP-His_6 plasmid was transformed into *E. coli* XL10 gold competent cells according to standard procedures.

pRSETB-TagRFP-His_6 plasmid was transformed into *E. coli* BL21 pLysS in the presence of ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Single colony transformants were selected and the pre-culture was grown overnight at 37 ^{o}C. The pre-culture was used to inoculate 2 L cultures of *E. coli* BL21 pLysS cells, which were grown at 37 ^{o}C to OD_{600} = 0.6 before protein expression was induced upon addition of IPTG to a final concentration of 1 mM (left overnight, 16 ^{o}C). The cells were harvested by centrifugation (4000x*g*, 20 min) and lysed using BugBuster (as described above). Protein purification and characterisation of the N- and C-terminal (doubly) His_6-tagged TagRFP (His_6-TagRFP-His_6) was carried out as described for His_6-TagRFP.[S5]

By site-directed mutagenesis (QuikChange Multi kit, Stratagene Technologies), selected mutations were introduced into pRSETB-TagRFP (both, singly and doubly His_6-tagged TagRFP) using the following primers: C222S forward: 5'-ggctgtggccagatactccgacctccc-3'; reverse: 5'-gggaggtcggagtatctggccacagcc-3' and C114S forward: 5'-gcctccaggacggctccctcatctacaac-3' and reverse: 5'-gttgtagatgagggagccgtcctggaggc-3'.
The mutant variants were expressed and purified under the same conditions as for the wild-type protein, to yield His_6-TagRFP and His_6-TagRFP-His_6. Mutations C222S and C114S yielded mutants '^{14}TagRFP' containing no accessible cysteine residues. Characterisation by SDS- and native PAGE, UV-Vis, steady state and time-resolved fluorescence spectroscopy and MALDI-TOF mass spectrometry were carried out and the mutants showed no discernible differences in their photophysical properties to the wild type, thus indicating that their overall structure upon site-directed mutagenesis is retained.[S5]

For monomeric teal fluorescent protein TFP, the following primers were used for PCR amplification using pET15b-TFP (kindly provided by Rik Rurup, University of Twente, The Netherlands) as DNA template: **5'-**ctccacggatccatggtgagcaagggcgag-3' containing a unique BamHI site (underlined) and 5'-ctccacgaattccttgtacagctcgtccatgc-3' containing a unique EcoRI restriction site (underlined). The PCR product was cloned into pRSETB as described above resulting in pRSETB-TFP, lacking a His_6-tag due to a frameshift just before the inserted gene. Site-directed mutagenesis was carried out to yield pRSETB-^{G174C}TFP using the above protocol and the following primers: forward 5'-tgctggagggctgcggccaccac-3' and reverse 5'-gtggtggccgcagccctccagca-3'.
Purification of TFP and ^{G174C}TFP were carried out using an Äkta FPLC system with a 5 mL ResourceQ anion exchange chromatography column (GE Healthcare) according to the manufacturer's instructions using sterile filtered wash buffer (40 mM PB, pH 8.0) and elution buffer (40 mM PB, 1 M NaCl pH 8.0). Both proteins were characterized using the same techniques as mentioned above.[S5]

Protein Immobilization
Substrates patterned with **1**, further reacted with thiol-NTA and then incubated in a solution of 1 µM ^{14}TagRFP and 2 µM $NiCl_2$ in PBS buffer for 30 min. Substrates were rinsed with PBS containing 0.01% of surfactant Tween-20 for 1 h, with water and dried in a stream of nitrogen. The selectivity and reversibility was tested via incubation in a 10 mM EDTA solution in PBS overnight. Substrates patterned with **1** were incubated in a 20 µM cysteine-containing ^{G174C}TFP (or the native TFP or the ^{G174C}TFP conjugate with Ellman's reagent) overnight. Samples were then rinsed in PBS containing 0.01% of surfactant Tween-20 for 2 h, with water and dried in a stream of nitrogen prior to fluorescence microscopy imaging.

The free thiol group (after overnight reduction using 1 mM DTT and its subsequent removal with Zebaspin desalting columns, MWCO 7000, Thermo Fisher) of the cysteine-containing ^{G174C}TFP was converted to disulfide groups by the use of the Ellman's reagent (5,5'-dithiobis-(2- nitrobenzoic acid) or DTNB). 20 μM Solutions of ^{G174C}TFP and, as negative control, TFP were reacted overnight with 2 mM DTNB in 40 mM PB, pH 7.8, and subsequently desalted using Zebaspin desalting columns to remove by-products (in particular to separate the protein from 5-thio-2-nitrobenzoic acid that, as it contains a free thiol group, would react with the thiol-sensitive coumarin on the surface). Substrates patterned with propargyl-biotin were incubated in a 0.6 μM Cy5-labelled streptavidin in PBS (pH 7.4) for 30 min and washed with PBS containing 0.01% of Tween-20 for 1 h.

Fluorescence Microscopy

Fluorescence microscopy images were taken using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition. For UV excitation and blue emission imaging a standard DAPI Olympus filter cube was used to image the fluorogenic platform. A standard Cy5 Olympus filter cube was used to image emission of Cy5-labelled streptavidin and Olympus filter cubes with appropriate band pass and long pass filters for TFP (excitation 460 nm, emission 490 nm) and TagRFP (excitation 555 nm, emission 580 nm) imaging were used. All fluorescence microscopy images were acquired on dry samples using a 20x Fluorplan objective from Olympus. Filter settings used: λ_{exc}=350 nm, λ_{em}=420 nm (FB); λ_{exc}=460 nm, λ_{em}=520 nm (FG); λ_{exc}=510-550, λ_{em}=590 nm, (FR=FR1); λ_{exc}=650 nm, λ_{em}=670 nm (FR2).

Mass Spectra

ESI-ToF-MS spectra were recorded using a LCT Mass spectrometer (Waters/Micromass). Protein masses were determined using a BioSystems, VoyagerII MALDI-ToF mass spectrometer using sinapinic acid as matrix.

NMR

^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity (300MHz) spectrometer. ^{1}H and ^{13}C chemical shifts values, δ (in ppm) at 300 MHz and 75 MHz, respectively, are reported using the residual solvent as internal standard.

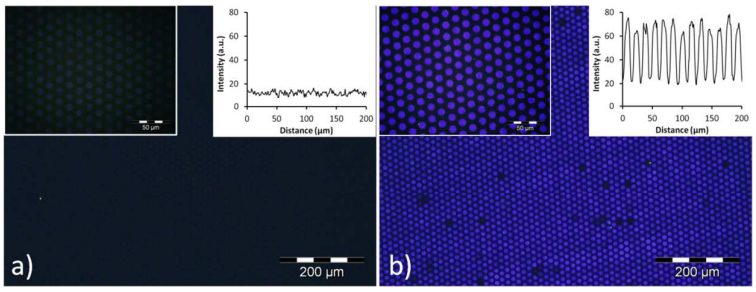

Figure S1. Fluorescence images (inserts show intensity profiles) a) before and b) after printing a 10 mM cysteine solution on the fluorogenic platform. λ_{exc}=350 nm, λ_{em}=420 nm (FB).

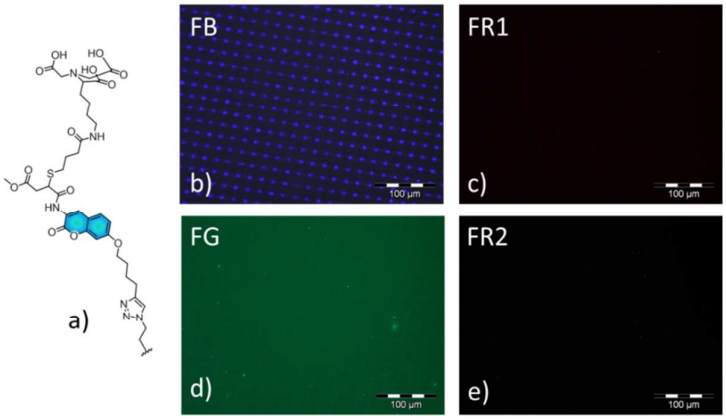

Figure S2. a) Molecular structure of immobilized thiol-NTA. b-e) Fluorescence images of the surface using different settings: λ_{exc}=350 nm, λ_{em}=420 nm (FB); λ_{exc}=460 nm, λ_{em}=520 nm (FG); λ_{exc}=510-550, λ_{em}=590 nm, (FR=FR1); λ_{exc}=650 nm, λ_{em}=670 nm (FR2).

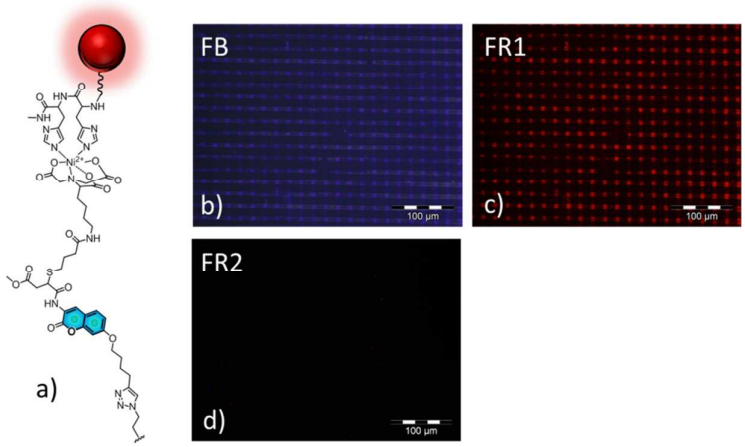

Figure S3. a) Molecular structure of immobilized thiol-NTA complexed with His_6-tagged protein. b-d) Fluorescence images of the surface using different settings: λ_{exc}=350 nm, λ_{em}=420 nm (FB); λ_{exc}=510-550, λ_{em}=590 nm, (FR=FR1); λ_{exc}=650 nm, λ_{em}=670 nm (FR2).

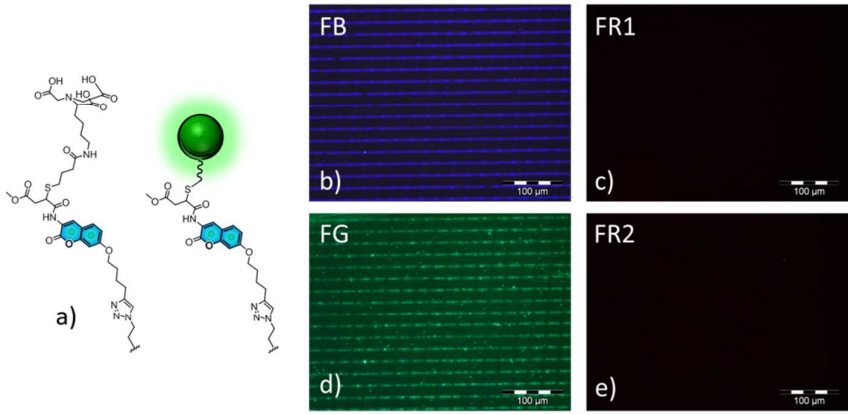

Figure S4. a) Molecular structure of immobilized thiol-NTA followed by immobilization of 20 µM ^{G174C}TFP reported by the fluorogenic reaction. b-e) Fluorescence images of the surface using different settings: λ_{exc}=350 nm, λ_{em}=420 nm (FB); λ_{exc}=460 nm, λ_{em}=520 nm (FG); λ_{exc}=510-550, λ_{em}=590 nm, (FR=FR1); λ_{exc}=650 nm, λ_{em}=670 nm (FR2).

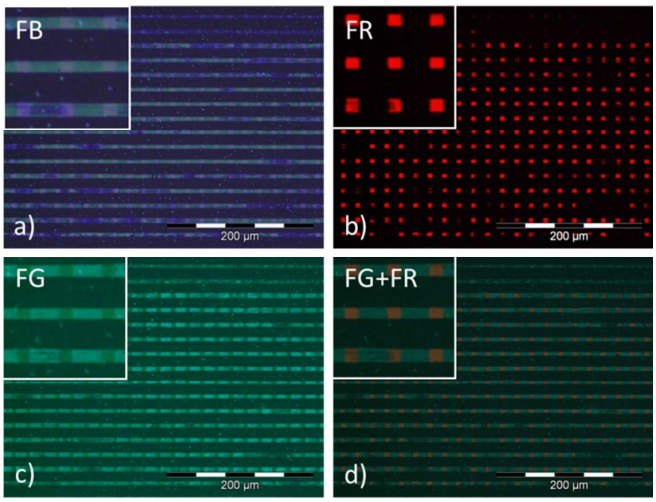

Figure S5. Immobilized thiol-NTA followed by immobilization of 1 µM **His_6-^{14}TagRFP-His_6** (1 h, with Ni^{2+}) and subsequently with 20 µM **^{G174C}TFP** (overnight). a-d) Fluorescence images of the surface using different settings: λ_{exc}=350 nm, λ_{em}=420 nm (FB); λ_{exc}=460 nm, λ_{em}=520 nm (FG); λ_{exc}=510-550, λ_{em}=590 nm, (FR=FR1); and overlay.

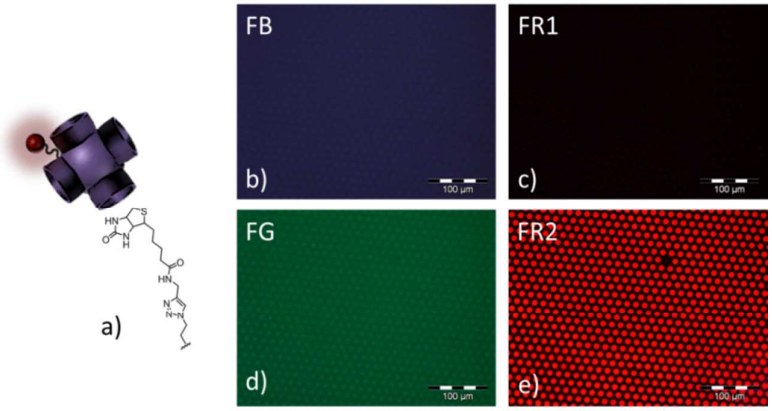

Figure S6. a) Molecular structure of immobilized alkyne-biotin followed by immobilization of Cy5-labeled streptavidin (0.6 µM, 30 min, PBS). b-e) Fluorescence images of the surface using different settings: λ_{exc}=350 nm, λ_{em}=420 nm (FB); λ_{exc}=460 nm, λ_{em}=520 nm (FG); λ_{exc}=510-550, λ_{em}=590 nm, (FR=FR1); λ_{exc}=650 nm, λ_{em}=670 nm (FR2).

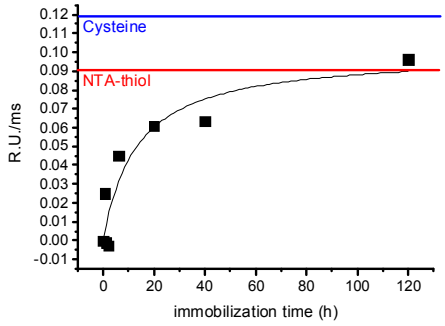

Figure S7. Fluorescence intensities of coumarin after immobilization of cysteine[S4] for 1 h (blue line), NTA-thiol for 1 h (red line) and ^{G174C}TFP (for various time points, squares) through the fluorogenic thio-Michael addition (λ_{exc}=350 nm, λ_{em}=420 nm).

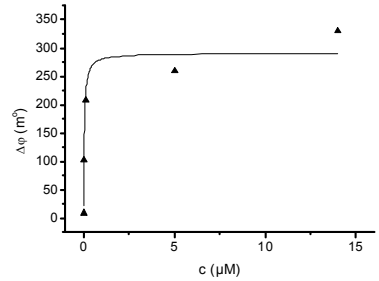

Figure S8. SPR sensograms of His_6-^{14}TagRFP-His_6 on full NTA monolayers on gold.

References

(S1) Du Roure, O.; Debiemme-Chouvy, C.; Malthête, J.; Silberzan, P. *Langmuir* **2003,** *19*, 4138-43.

(S2) Chan, T.R.; Hilgraf, R.; Sharpless, K.B.; Fokin, V.V. *Org. Lett.* **2004**, *6*, 2853-5.

(S3) He, B.; Velaparthi, S.; Pieffet, G.; Pennington, C.; Mahesh, A.; Holzle, D.L.; Brunsteiner, M.; van Breemen, R.; Blond, S.Y.; Petukhov, P.A. *J. Med. Chem*. **2009**, *52*, 7003-13.

(S4) Nicosia, C.; Cabanas-Danés, J.; Jonkheijm, P.; Huskens, J. *ChemBioChem.* **2012**, *13*, 778-82.

(S5) Wasserberg, D.; Cabanas-Danés, J.; Huskens, J.; Subramaniam, V.; Jonkheijm, P. *manuscript in preparation.*