Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information to

Orthogonal supramolecular protein assembly on patterned bifunctional surfaces

D. Wasserberg, J. Cabanas-Danés, V. Subramaniam, J. Huskens and P. Jonkheijm

Table of Contents

1. Materials S2
2. Synthesis S2
3. Methods S6
4. Supporting Charts, Figures and Table S11
5. Supporting References S15

1. Materials

Fmoc-L-glutamic acid α-*t*Bu-ester, Fmoc-protected amino acids and *N,N,N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and 1-hydroxy benzotriazole (HOBt) were purchased from MultiSynTech (Witten, Germany) and Novabiochem (Darmstadt, Germany), and maleimide caproic acid-hexa(histidine) (-H_6) were purchased from JPT Peptide Technologies, Germany and all solvents were of p.a. quality and purchased from Biosolve (Valkenswaard, The Netherlands). Diadamantyl-modified fluorescein (ad_2Fl) was synthesized according to a previously published procedure.[S1] All other starting components and materials were purchased from Acros (Geel, Belgium) and Sigma Aldrich (Zwijndrecht, The Netherlands). All compounds were used as received unless stated otherwise. Deuterated solvents used for NMR spectroscopy were purchased from Cambridge Isotope Laboratories and the water used was of MilliQ quality (Millipore, R = 18.2 MΩ*cm).

2. Synthesis

NTA(O*t*Bu)$_3$-NH_2

NTA(O*t*Bu)$_3$-NH_2 was synthesized according to a protocol adapted from literature.[S2] H-Lys(Z)-O*t*Bu·HCl (4.0 g, 10.7 mmol) was dissolved in 100 mL of dry dimethylformamide (DMF). Subsequently, *t*Bu-bromoacetate (6.36 mL, 43.1 mmol) and diisopropyl ethyl amine (DIPEA) (9.2 mL, 52.8 mmol) were added sequentially. After purging the flask with N_2, the solution was stirred overnight at 55°C under inert N_2 atmosphere. The volatiles were removed *in vacuo* at 60°C and the partially solidified reaction mixture was resuspended in 60 mL of cyclohexane:ethylacetate (3:1) and filtered using a sintered glass funnel. The precipitate was washed with the same solvent mixture (3 x 40 mL), the combined filtrate, then, concentrated under reduced pressure and purified by column chromatography (SiO_2, cyclohexane:ethylacetate, 3:1) to quantitatively yield 5.95 g (10.5 mmol) Lys(Z)-NTA(O*t*Bu)$_3$ (R_f = 0.50). Then, Lys(Z)-NTA(O*t*Bu)$_3$ (6.0 g, 10.5 mmol) was dissolved in methanol (300 mL) and the solution was purged with three vacuum-nitrogen cycles. Subsequently, 10% Pd/C (0.120 g) was added under N_2. The solution was mixed vigorously and stirred for 6 h under H_2 atmosphere at room temperature. After the H_2 gas had been allowed to slowly leak from the reaction mixture while being replaced by nitrogen, the Pd/C catalyst was removed by filtration over celite and the solvent was removed from the filtrate under reduce pressure to yield (O*t*Bu)$_3$NTA-NH_2 (3.68 g, 8.5 mmol, 81%). ^{1}H NMR (300 MHz, $CDCl_3$, 20°C, TMS) δ: 3.47 (dd, 4H, $((CH_3)_3COCO\underline{CH_2}))_2$N-), 3.31 (t, 1H, $((CH_3)_3COCOCH_2))_2N\underline{CH}$), 2.72 (t, 2H, $NH_2CH_2CH_2CH_2\underline{CH_2}$CHN-), 2.28 (bs, 2H, $\underline{NH_2}CH_2CH_2CH_2CH_2$CHN-), 1.65 (m, 2H, $NH_2CH_2CH_2\underline{CH_2}CH_2$CHN-), 1.50 (m, 4H, $NH_2\underline{CH_2CH_2}CH_2CH_2$CHN-), 1.45 (s, 9H, $(\underline{CH_3})_3$COCOCH-), 1.44 (s, 18H, $((\underline{CH_3})_3COCOCH_2)_2$N-); MS (ESI): m/z = 432.3 $[M+H]^+$, (calc. 431.0).

adHEG

Adamantyl hexa(ethylene glycol), adHEG, was synthesized according to a protocol adapted from literature.[S] To a solution of hexa(ethylene glycol) (12.5 mL, 50.9 mmol), 1-bromo adamantane (1.1 g, 5.1 mmol) and Et_3N (2.0 mL, 14.3 mmol) were added. The solution was stirred at 120°C overnight. After cooling the reaction mixture to room temperature dichloromethane (50 mL) was added. The solution was washed with 2 M HCl (4 x 50 mL) and once with brine (50 mL). The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to give adHEG as a brown, transparent oil (0.87 g, 2.1 mmol, 40%). MS (ESI): m/z = 417.4 $[M+H]^+$, (calc. 416.6 g/mol).

6-aminohexyl-1-adamantane carboxamide

1,6-Hexanediamine (5.81 g, 50.0 mmol) was dissolved in 180 mL CH_2Cl_2 and, subsequently, Et_3N (0.77 mL, 5.55 mmol) was added. The reaction mixture was cooled to 0°C and a solution of adamantane carboxylic acid chloride (1.0 g, 5.04 mmol) in 20 mL CH_2Cl_2 was added dropwise during the course of 1 h. The reaction mixture was stirred for 30 min at 0°C, left to slowly warm to room temperature (RT) and quenched with 150 mL of a saturated aqueous $NaHCO_3$ solution. The resulting aqueous phase was, then, washed 3 times with (50 mL) CH_2Cl_2 and the combined organic phases were washed three times with water and once with brine. The solution was dried over $MgSO_4$, concentrated *in vacuo* and the product, 6-aminohexyl-1-adamantane carboxamide (0.9 g, 3.22 mmol, 64%), was analyzed with NMR spectroscopy.
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 3.1 (t, 2H), 2.7 (t, 2H), 2.0-1.7 (m, 15H), 1.4-1.2 (m, 8H).

Fmoc-glutamic acid adamantane hexane amide-carboxylic acid α -*t*Bu-ester

To a solution of Fmoc-L-glutamic acid α-*t*Bu-ester (1.56 g, 3.66 mmol) in 30 mL DMF, N,N'-diisopropylcarbodiimide (DIC) (1.54 g, 12.2 mmol) and HOBt (1.65 g, 12.2 mmol) were added under Ar atmosphere. The reaction mixture was stirred for 30 min at RT for the activation of the carboxylic acid to take place. 6-aminohexyl-1-adamantane carboxamide (0.85 g, 3.05 mmol) was dissolved in 10 mL DMF and added to the reaction mixture, which was stirred at RT for 2 h. The progress of the reaction was followed by NMR (measured by the intensity of the free amine peak) and stopped after approximately 2 h, at which time all the free amine had been consumed. The solvent was removed *in vacuo* and the resulting residue was dissolved in 150 mL ethylacetate:hexane 1:1 and washed 3 times with sat. NH_4Cl (30 mL) in order to remove DMF, any residual traces of DMF were, then, removed *in vacuo*. The crude product was, subsequently, dissolved in methylene chloride and washed 3 times with sat. $NaHCO_3$ (50 mL), twice with water (50 mL) and once with brine. The organic phase was dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column

chromatography (SiO_2, chloroform:methanol, 95:5) and resulted in 1.7 g (2.50 mmol) of pure product corresponding to 82% yield.

^{1}H-NMR (300MHz, $CDCl_3$): δ (ppm) 7.8-7.3 (m, 8H), 5.6 (d, 2H), 5.5 (m, 1H), 4.4 (t, 1H), 4.4 (t, 1H), 4.2 (m, 2H), 3.1 (t, 4H), 2.0-1.7 (m, 15H), 1.43 (s, 9H), 1.4-1.2 (m, 8H). MS (ESI-TOF): m/z; 686.4 g/mol $[M+H]^+$ and 708.4 g/mol $[M+Na]^+$ (calc. 685.89 g/mol).

Fmoc-glutamic acid adamantanehexaneamide-carboxylic acid

1.65 g (2.40 mmol) Fmoc-glutamic acid adamantanehexaneamide-carboxylic acid α-tBu ester was dissolved in 30 mL CH_2Cl_2, to which 30 mL TFA were added. The solution was stirred for 4 h at RT and subsequently, the reaction mixture was concentrated *in vacuo*. The resulting solution was washed 3 times with water, once with brine and dried over $MgSO_4$. All residual CH_2Cl_2 was removed *in vacuo* and the obtained yellowish solid (1.3 g, 2.04 mmol, 85%) was analyzed by NMR spectroscopy and MS spectrometry and stored at -20°C until further use.
^{1}H-NMR (300MHz, $CDCl_3$): δ (ppm) 7.8-7.3 (m, 8H), 5.6 (d, 2H), 5.5 (m, 1H), 4.4 (t, 1H), 4.4 (t, 1H), 4.2 (m, 2H), 3.1 (t, 4H), 2.0-1.7 (m, 15H), 1.4-1.2 (m, 8H).
MS (ESI-ToF): m/z; 630.3 $[M+H]^+$ and 652.2 $[M+Na]^+$ (calc. 629.8).

Automated solid phase peptide synthesis

Peptides of the following sequences, end-capped with maleimido caproic acid, were synthesized using a Syro1 (MultiSynTech) and standard Fmoc-chemistry in solid phase peptide synthesis (SPPS)[S3]: the triply adamantane substituted -GE(ad)RE(ad)DE(ad)K and the corresponding adamantine-free reference peptide -GEREDEK. Rink Amide MBHA resin was used as solid phase and all amino acids carried Fmoc-protected α-amino groups. Each amino acid was dissolved in N-methyl-2-pyrrolidone (NMP, 0.3 M), stabilized with 0.3 M HOBt and all reactions were carried out at RT. Extensive washing steps with NMP were carried out after each reaction in order to prevented cross-contaminations between steps.
In short, syntheses were carried out from C- to N-terminus as follows:
A solution containing an Fmoc-protected amino acid was added to the reaction vessel containing the amine-terminated Rink amide resin. Coupling of the free carboxylic acid group of the Fmoc-protected amino acid to the free amine of the resin was done using HBTU and DIPEA as coupling reagents and reaction time was set to 90 min. This resulted in the Rink amide resin terminated with the newly coupled amino acid displaying its Fmoc-protected amines on the resin. In order to increase yields, all couplings with natural amino acids were done twice (double couplings); all others once (single couplings). After coupling, Fmoc cleavage was carried out using first 40%, then 20% piperidine in NMP. After Fmoc-cleavage the next amino acid was added and the cycle was repeated for each amino acid until the sequence had been completed. The resin was washed with NMP, CH_2Cl_2 and MeOH each three

times and dried for at least 4 h *in vacuo*. Subsequently, the peptide was cleaved from the resin as well as deprotected in a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water (TFA:TIS:H_2O, 95:2.5:2.5) overnight. The resin was rinsed three more times with pure TFA and the combined fractions were concentrated *in vacuo*. From this mixture the peptide was precipitated three times using cold diethyl ether and a centrifugation step (8000xg, 2 min) and the combined precipitated fractions were re-dissolved in water (mixed with acetonitrile containing ~1% TFA for the adamantane substituted peptide) and lyophilized. Peptides (re-dissolved in acetonitrile/water mixtures) were purified using reverse-phase HPLC (Waters HPLC system with photoarray detector, reverse phase C18 column (Xterra, Waters), gradients of water:acetonitrile containing 0.1% TFA), subsequently lyophilized and analyzed by mass spectrometry.

MS (ESI): -GE(ad)RE(ad)DE(ad)K m/z 1836 g/mol $[M+H]^+$ (calc. 1835 g/mol).

MS (ESI): -GEREDEK m/z 1056 g/mol $[M+H]^+$ (calc. 1054 g/mol).

TFP, including $1H_6$TFP

For Teal Fluorescent Protein (TFP), the following primers were used for PCR amplification using pET15b-TFP (kindly provided by W.F. Rurup, University of Twente, The Netherlands) as DNA template: 5'-ctccacggatccatggtgagcaagggcgag-3' containing a unique BamHI restriction site (underlined) and 5'-ctccacgaattccttgtacagctcgtccatgc-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, resulting in pRSETB-TFP. Site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent) was carried out to yield pRSETB-^{G174C}TFP using the same protocol and the following primers: forward 5'-tgctggagggctgcggccaccac-3' and reverse 5'-gtggtggccgcagccctccagca-3'.

The resulting plasmids, pRSETB-TFP and pRSETB-^{G174C}TFP, were each transformed into E. coli (XL10 gold, Stratagene) using standard procedures in the presence of ampicillin (100 mg/L) for amplification and further mutagenesis. pRSETB-TFP and pRSETB-^{G174C}TFP plasmids were also transformed into E. coli BL21 pLysS using standard procedures in the presence of ampicillin (100 mg/L) and chloramphenicol (34 mg/L) for protein expression: Single colony transformants were selected and pre-cultures were grown overnight at 37°C. These pre-cultures were each used to inoculate 2 L of LB medium containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) at 37°C with shaking until an O.D.600 = 0.6 was reached. The cultures were cooled to 16°C before protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubated overnight at 16°C. Cells were harvested by centrifugation at 4000xg at 4°C for 20 min. The resulting cell pellets were resuspended for 20 min in BugBuster reagent with benzonase nuclease (Novagen) according to the supplier's

instruction. The lysate was cleared by centrifugation at 16000xg for 30 min at 4°C. Purification of TFP and ^{G174C}TFP was 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 wash (40 mM PB, pH 8.0) and elution buffers (40 mM PB, 1 M NaCl pH 8.0). The purified TFP and ^{G174C}TFP fractions (~30 µM) were subsequently rebuffered using PD10 columns (GE Healthcare) into 0.1xPBS (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°C. Both expressed variants lack an N-terminal H_6-tag encoded in pRSETB, due to a frameshift just before the inserted gene. An additional TFP variant was produced following above procedures: **1H$_6$TFP**, including the N-terminal H_6-tag, used as reference. The proteins were characterized using SDS- and native PAGE (Figure S1), UV-Vis, steady state- and time-resolved fluorescence spectroscopy and MALDI-ToF mass spectrometry (see below).
Both proteins were characterized using SDS- and native PAGE, steady state and time-resolved fluorescence spectroscopy and mass spectrometry (Table S1).

ad$_3$TFP and E$_3$TFP

To ^{G174C}TFP, possessing a single accessible cysteine, thiol-reactive maleimide-functionalized peptides were conjugated. The heptapeptide -GEREDEK was used to yield reference E$_3$TFP, while -GE(ad)RE(ad)DE(ad)K was used to yield ad$_3$TFP, where E(ad) is an adamantyl ester of glutamic acid, E, see above. Conjugations were carried out by first reducing the cysteines using 10 mM phospate buffer (PB), 0.1 mM dithiothreitol (DTT) (pH 8.0) for 30 min and removing the DTT again (zebaspin, Thermo Scientific) and by then incubating a 10:1 mixture of maleimide:protein (~20 µM) in 10 mM PB (pH 8.0) in the dark for 24 h at RT. The reactions were, subsequently, quenched upon addition of a 10-fold molar excess of DTT with respect to the maleimide for 30 min at RT after which the samples were rebuffered (Zeba spin, Thermo Scientific) into 0.1xPBS at least three times to remove any residual peptide, dye and DTT. TFP, containing no cysteines, was mixed with maleimido-Atto488 dye to serve as negative controls for the conjugation. Characterisation was performed as described above and conjugation yields were typically between 30–50%.

3. Methods

Surface plasmon resonance and modeling

Surface plasmon resonance (SPR) measurements were performed using a Resonant Technologies GmbH (Germany) RT2005 SPR setup in Kretschmann configuration. Glass substrates covered with a 50 nm gold layer were used. On the gold, β-cyclodextrin (βCD) was self-assembled into a monolayer via Au-sulfide interactions (see below) and attached to a 70 µL volume microfluidic cell mounted on a prism which in turn was mounted on a double

goniometer head, with which the angle of incidence of the exciting laser on the prism (Schott, LaSFN9) could be controlled. Light from a 2 mW HeNe laser of 633 nm wavelength passed through the prism and onto the substrate. The intensity of the reflected light from the substrate was measured by a large-area photodiode. The gold-on-glass substrate was optically matched to the prism using index matching oil (Cargille; series B; n_D^{25} = 1.700±0.002). The resonance angle was determined by continuously scanning through the surface plasmon resonance dip and fitting the minimum during binding experiments in a flow-cell. Phosphate buffered saline (PBS) (with 0.01%v/v Tween20 and 0.1 mM adHEG for TFPs) was used as a running buffer at a continuous flow rate of 20 µL/min.

Experimental data was fitted using a multivalent binding model.[S4] The competitive equilibrium for the formation of the surface complex of HEG-Ad was taken into account for the calculation of the theoretical maximum SPR angle shift for each concentration. The effective concentration, which accounts for the immobilization of a second and third adamantane group after the first adamantane was immobilized, was set to 0.1 M.[S4] Using a binding constant of $2.6 \cdot 10^4$ M^{-1} for Ad-HEG[S4] a value of K_i,TFP = $1.8 \cdot 10^3$ M^{-1} was determined and an overall stability constant for the trivalent surface complex of K = $5.4 \cdot 10^7$ M^{-1}. From the analysis of the binding species, we learned that the concentration at which the binding curve reaches a saturation plateau, the surfaces is covered for 40% with a trivalent species and smaller contributions of the mono and divalent complexes while 40% of the surface is still covered by Ad-HEG, indicating that replacement by a higher stability complex is difficult.

NMR spectroscopy and mass spectrometry measurements

NMR measurements were carried out on either a Varian Unity INOVA 300 MHz or a Bruker Avance 600 MHz UltraShield and mass spectrometry of small molecules and peptides on a Waters micromass LCT spectrometer in ESI mode, while protein masses were determined using an Applied Biosystems Voyager-DE-RP MALDI-ToF mass spectrometer with cinnamic acid as matrix (see Table S1).

Preparation of βCD monolayers on gold

SPR sensors (1 inch, Ssense) were cleaned using a mixture of H_2SO_4:H_2O_2(30%) 3:1 (this mixture reacts violently with organic compounds and should be handled with care) for 30 s, quenched, then rinsed with water, dried in a stream of N_2 and subsequently immersed in a solution of 5 µM βCD-hepta(ε-amino di(dodecyl)thioether) in 2:1 chloroform:EtOH and left to assemble for at least 16 h under Ar at 60°C. Samples were kept for a maximum of 1 week in the reaction mixture under argon atmosphere at 60°C. Samples were only removed from the reaction mixture prior to a measurement, washed with chloroform, EtOH, water, EtOH, 3 times, in this order, dried under a stream of N_2 and immediately used for measurements.

Preparation of dual βCD/NTA- and βCD/PEG- line-patterned glass substrates using NIL

βCD/NTA-line-patterns

4 inch borofloat (Schott, Borofloat 33) wafers were immersed in a mixture of H_2SO_4:H_20_2(30%) 3:1 (this mixture reacts violently with organic compounds and should be handled with care) for 10 min, quenched, then rinsed with water, dried under a stream of N_2 and subsequently spin-coated (Speedline, p6700) with a 6%w/v 350 kDa poly(methyl methacrylate) (PMMA) solution in toluene for 30 s at 3000 rpm. To remove residual solvent, the wafer was baked at 120°C for 10 min. NIL was carried out using a Peltier temperature controlled (Julabo FP 50) HP Specac NIL setup at 200°C and 1 ton for 10 min. As imprint master a 4 inch 1H,1H,2H,2H-perfluorodecyltrichlorosilane (PFDTS, AB111155, ABCR) coated Si-wafer with 24 1x1 cm large patterns of 10 to 25 µm-wide ridges separated by 5 µm-wide 0.5 µm deep trenches was used. In detail, the patterned imprint master was brought into contact with the spin-coated PMMA layer on a borofloat wafer, cushioned with Kunze heat conducting cushioning foil (KU-TXE100) on one side and 4 layers of aluminium foil on the other side and sealed with aluminium foil. This stack was heated to 200°C, without applying pressure, then the pressure was carefully increased to the equivalent of 1 ton and these conditions were kept constant for 10 min requiring occasional re-adjustment of the pressure. Subsequently, the heating was switched off, while keeping the pressure constant. Upon cooling to 100°C the pressure was left to decrease simultaneously with the temperature. Upon reaching 80°C the stack was removed from the NIL setup, disassembled and residual layer removal was carried out using an SPI supplies, Plasmaprep II oxygen-plasma cleaner. The progress of residual layer removal was monitored for each of the 1x1 cm patterns individually using an Olympus BH2 light microscope in transmission mode with a halogen lamp as light source. Upon completion of removing the residual layer (with only 5 µm-wide ridges of PMMA remaining) the substrates were silanized by overnight chemical vapor deposition of N-[3-(trimethoxysilyl)propyl]ethylenediamine TPEDA *in vacuo*. To remove excess silane, the samples were rinsed thoroughly with ethanol. The resulting amine-terminated line-pattern was functionalized with phenyl diisothiocyanate (DITC). To this end, the samples were immersed in a 0.04 M solution of DITC in ethanol for 2 h at 50°C under argon. After the reaction, the samples were rinsed with ethanol and dried under a stream of N_2. The ITC-terminated monolayer was then functionalized with NTA(tBu)$_3$-NH_2. To this end, the samples were incubated in a 1 mM solution of NTA(tBu)$_3$-NH_2 in ethanol at 50°C for 2 h under argon. All remaining PMMA was stripped from the borofloat substrates by 30 min sonication in 1 L of aceton, rinsing with aceton and drying with N_2. Subsequently, the as yet unfunctionalized (5 µm-wide) lines were silanized by overnight chemical vapor deposition of TPEDA and DITC as described above. Subsequently, the ITC-terminated lines were reacted with β-CD by incubating the samples in a 1 mM solution of β-cyclodextrin-

heptaamine[S5] in water at 50°C for 2 h under Ar. Samples were then washed with water and dried under a stream of N_2. To remove the *t*Bu-protecting groups the samples were immersed in (TFA) overnight, rinsed with copious amounts of water and dried under a stream of N_2. The resulting bi-functional NTA/CD line-patterns could be stored under N_2 for a maximum of 4 weeks prior to use.

βCD/PEG- line-patterns

In order to fabricate βCD/PEG-line-patterns the same procedure as described above was used for functionalization of the first set of lines. The fabrication of NTA/PEG, line-patterns, has been described previously.[S6] βCD/PEG line-patterns were fabricated similarly to the dual line-patterns (see above) by letting β-cyclodextrin-heptaamine react with DITC during the functionalization of the first (wider) set of lines. After the stripping of the remaining PMMA, the second set of lines (5 µm wide) was functionalized with a solution of 100 µL PEG-(trimethoxy)silane (AB111226, ABCR) in 60 mL dry toluene by leaving the samples to react overnight at room temperature under Ar. After washing the samples with toluene they were dried under a flow of N_2. The resulting mono-functional βCD/PEG-line-patterns could be stored under N_2 for a maximum of 4 weeks prior to use.

Protein immobilization on (patterned) substrates

NTA-layers were incubated with a 1 mM $NiCl_2 \cdot 6H_2O$ solution in water for 30 min after which the samples were rinsed briefly with water. Similarly, βCD-layers were incubated with a 1 mM adamantane hexa(ethylene glycol) (adHEG) solution in PBS. The solution was removed with a pipette from the surface and the sample was rinsed three times with PBS containing 1 mM adHEG. The concentration of adHEG was kept constant in all buffers used in all following incubation steps. For the subsequent immobilization of proteins, samples were incubated with the appropriate protein and/or dye solution generally at a concentration of 1 µM overnight, using humidity chambers, unless stated otherwise. Protein and/or dye solutions were then removed with a pipette and retained for further use. Samples were, then, washed on an orbital shaker (80 rpm) in the appropriate buffers for 2-4 h (first immobilization) or overnight (second immobilization, see text). Samples were then rinsed with the respective wash buffer, subsequently with water and dried under a stream of N_2. Samples were imaged using an Olympus IX70 inverted fluorescence microscope (see below).

Fluorescence Microscopy

Fluorescence microscopy images were recorded using an Olympus inverted microscope IX71 equipped with a mercury burner U-RFL-T lamp as light source and a digital Olympus DR70 color

camera for image acquisition. Fluorescence micrographs were acquired using a 20x Fluorplan objective from Olympus and exposure times of 1-3 s.

Fluorescence Spectroscopy

Steady state and time-dependent Spectroscopy. Absorption spectra of all fluorescent proteins, their mutants and conjugates were recorded using a Perking Elmer LAMBDA850 UV/Vis spectrophotometer. Fluorescence spectra and lifetime data of all fluorescent proteins, their mutants and conjugates were recorded using a JobinYvon-Horiba Fluoromax4 fluorimeter including a TCSPC system for time-dependent measurements with pulsed LEDs for excitation at 488 nm or 561 nm. Results are summarized in Table S1.

4. Supporting Figures

Chart S1 Structures of E_3TFP and $3H_6$TagRFP
Table S1 Fluorescence characteristics and mass spectrometry data of protein variants
Figure S1 Specific assembly of protein variants on dual substrates
Figure S2 Influence of adHEG and βCD competitors on the assembly of dual protein patterns
Figure S3 Selfsorting during assembly of dual patterns from a mix of protein and dye
Figure S4 Permutations of assembly of dual protein patterns
Figure S5 Influence of Tween20 and adHEG on the specific assembly of proteins

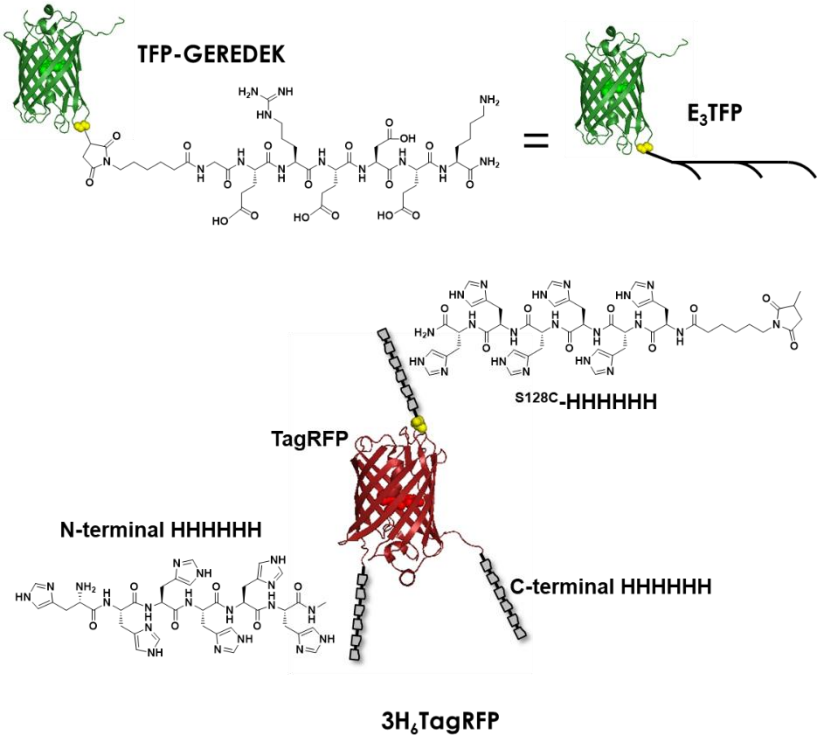

Chart S1. Structure of adamantane free, maleimide-functionalized heptapeptide (-GEREDEK) conjugated to the single accessible cysteine residue (yellow) at position G174C of TFP mutant ^{G174C}TFP (E_3TFP). Structure of mutant ^{S128C}TagRFP modified with an N- and a C-terminal hexahistidine-tag (HHHHHH) and a single accessible cysteine residue (yellow) at position S128C, to which a maleimide-functionalized hexahistidine-tag (-HHHHHH) was conjugated ($3H_6$TagRFP).

Table S1: steady-state (absorption and emission maxima in nm) and time-resolved (fitted single exponential lifetimes in ns) fluorescence and MALDI-ToF MS (calculated mass in g/mol and measured data in m/z) data of protein variants.

Protein variant	Absorption max (nm)	Emission max (nm)	Fluorescence lifetime (ns)	Mass calc. (g/mol)	MALDI TOF MS Mass meas. (m/z)
$1H_6$TagRFP	555	579	2.4	30277	30000
$3H_6$TagRFP	N/A	N/A	N/A	32164	31700
$1H_6$TFP	466	494	3.3	30677	30916
^{G174C}TFP	466	494	3.3	26953	26700
E_3TFP	N/A	N/A	N/A	28007	27200
Ad_3-TFP	N/A	N/A	N/A	28788	28300

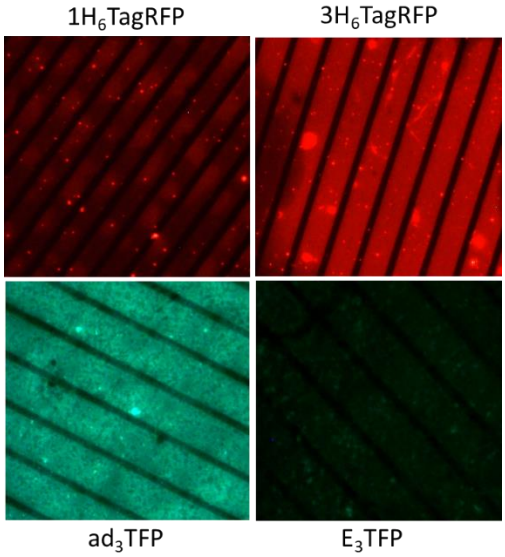

Fig. S1 Representative fluorescence images of NIL-patterned dual substrates with fluorescent proteins assembled following optimized procedures. Top row: narrower, 5 µm wide lines are PEG-silane; wider lines are Ni(II)NTA, after incubation with 100 nM $1H_6$TagRFP (top, left) and 100 nM $3H_6$TagRFP (top, right) and subsequent washing overnight with PBS containing 5%v/v Tween20. Bottom row: narrower, 5 µm wide lines are PEG-silane; wider lines are βCD, after incubation with 5 µM ad_3TFP (bottom, left) and 5 µM reference compound E_3TFP (bottom, right) and subsequent washing overnight with PBS containing 0.1 mM adHEG and 10 mM βCD. All images were recorded at 2 s exposure time and are shown with comparable intensities for each row.

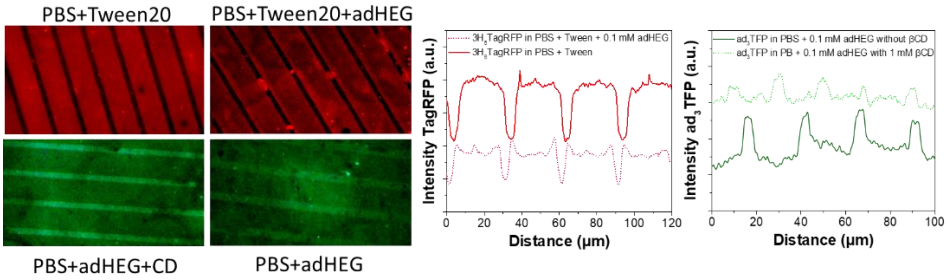

Fig. S2 Representative fluorescence images (far left and left columns) of NIL-patterned bifunctional βCD-Ni(II)NTA substrates (narrower, 5 µm wide lines are βCD; wider lines are Ni(II)NTA) after incubation with 1 µM $3H_6$TagRFP (top row, red channel), then, with 500 nM ad_3TFP (bottom row, green channel) and subsequent washing overnight with PBS containing 5%v/v Tween20 (top row) without (top, left) or with (top, right and bottom row) 0.1 mM adHEG, or containing additional 1 mM βCD (bottom left). Top and bottom row images were recorded at 500 ms and 3 s exposure time, respectively, and are shown with comparable intensities for each row. Plots of fluorescence intensity profiles (offset) of top row fluorescence images in left and far left column (right) as well as of fluorescence intensity profiles (offset) of bottom row fluorescence images in left and far left column (far right).

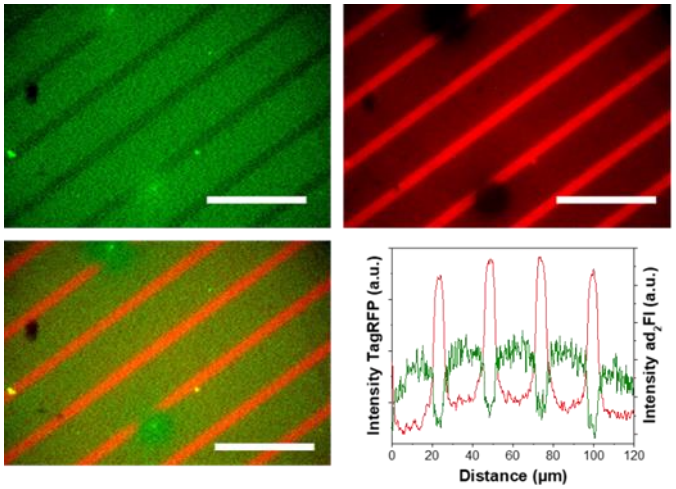

Fig. S3 Representative fluorescence image overlays of green and red channel images of photolithography-patterned bifunctional βCD-Ni(II)NTA substrates (narrower, 5 µm wide lines are Ni(II)NTA; wider lines are βCD) after incubation with a mixture of 2 µM ad_2Fl and 1 µM $1H_6$mRFP (the plasmid pRSETB-mRFP was a generous gift from Kirsten Leijenhorst-Groener) and subsequent rinsing. All solutions were in PBS containing 5%v/v Tween20. White scale bars are 50 µm.

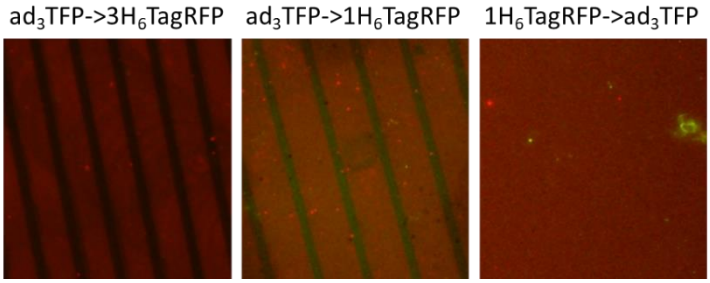

Fig. S4 Representative fluorescence image overlays of green and red channel of NIL-patterned bifunctional βCD-Ni(II)NTA substrates (narrower, 5 µm wide lines are βCD; wider lines are Ni(II)NTA) after incubation with, left: first 500 nM ad_3TFP, then with 100 nM $3H_6$TagRFP; middle: first with 500 nM ad_3TFP, then with 100 nM $1H_6$TagRFP; or right: first with 100 nM $1H_6$TagRFP, then with 100 nM ad_3TFP. PBS was used as wash buffer for ad_3TFP containing 0.1 mM adHEG and 1 mM βCD; PBS containing 5%v/v Tween20 and 0.1 mM adHEG was used for TagRFP variants.

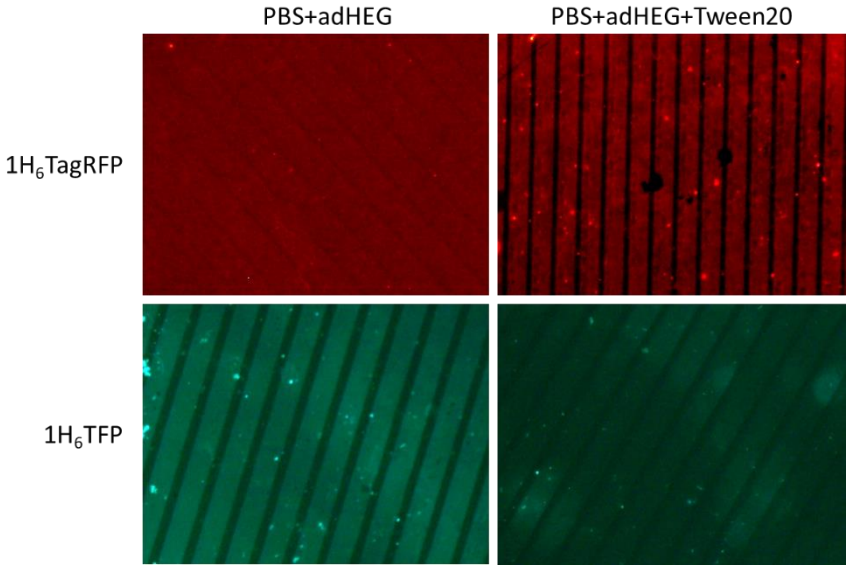

Fig. S5 Representative fluorescence images of NIL-patterned bifunctional βCD-Ni(II)NTA substrates (narrower, 5 µm wide lines are βCD; wider lines are Ni(II)NTA) after incubation with 5 µM $1H_6$TagRFP (top row, red channel), or 5 µM $1H_6$TFP (bottom row, green channel) and subsequent washing overnight with PBS containing 1 mM adHEG, without (left column) or with 5%v/v Tween20 (right column). Bottom row images were recorded at 1 s and top row images at 2 s (left) 200 ms (right) exposure time, and are shown with comparable intensities for each row.

5. Supporting References

S1 A. Mulder, S. Onclin, M. Peter, J. P. Hoogenboom, H. Beijleveld, J. ter Maat, M. F. Garcia-Parajo, B. J. Ravoo, J. Huskens, N. F. van Hulst and D. N. Reinhoudt, *Small*, 2005, **1**, 242-253.

S2 S. Lata and J. Piehler, *Nat. Protoc.*, 2006, **1**, 2104-2109.

S3 R. B. Merrifield, *J. Am. Chem. Soc.*, 1963, **85**, 2149.

S4 M. L. W. Ludden, A. Mulder, K. Schulze, V. Subramaniam, R. Tampe and J. Huskens, *Chem.-Eur. J.*, 2008, **14**, 2044-2051.

S5 P. R. Ashton, R. Königer, J. F. Stoddart, D. Alker and V. D. Harding, *J. Org. Chem.*, 1996, **61**, 903.

S6 D. Wasserberg, J. Cabanas-Danes, J. Prangsma, S. O'Mahony, P. A. Cazade, E. Tromp, C. Blum, D. Thompson, J. Huskens, V. Subramaniam and P. Jonkheijm, *ACS Nano*, 2017, **11**, 9068-9083.