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Supporting Information

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for

Covalent Fluorescence Labeling of His-Tagged Proteins on the Surface of Living Cells

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Material and Methods

Crosslinking: For all crosslinking experiments UV irradiation was applied with a Stratalinker 1800 (Stratagene, USA) by placing the sample in 10 cm distance to 365 nm UV-tubes. An automatic routine deposits a dose of 120 mJ within 40 s. The dosage is calibrated internally by a photodiode. Crosslinking of solutions was performed in a droplet placed on aluminium foil from which even µL volumes could be recovered with a pipette. This treatment increased the x-link efficiency fourfold compared to crosslinking in 1.5 mL polyethylene vials.

Crosslinking on cells: Cells were allowed to attach and grow on a Fibronectin-coated chamber slide (Nunc LAB-TEK Cat. No. 155409) after transient transfection (see section on cell-culture and transfection). The NUNC chambered coverglass slide was placed in the Stratalinker 1800, at 10 cm distance from the UV bulbs. For enhanced light collecting efficiency, the bottom of the NUNC chamber was covered with an aluminium foil. Crosslinking was performed by applying a dose of 120 mJ on the sample within 40 s. After this crosslinking step, the cells were washed 3 times with medium to remove noncovalently bound probe.

Fluorescence spectroscopy: Emission spectra and kinetics were measured with a Fluorolog τ-3 spectrofluorometer (Jobin Yvon / Horiba, Germany). GFP-C-His and NTA-ASA-QSY7 In Figure 2a-b was measured in PBS supplemented with 0.005% Tween-20 using 600 µL quartz cuvettes (115-F QS, Hellma, Germany). The kinetics and K_d of NTA-ASA-TMR (Figure 2b-c) was determined in 50 mM NaPO4, 300 mM NaCl, supplemented with 0.005% Tween-20 (according to Guignet et al. 2004)[7] by using 60 µL cuvettes (26-50-F Q, Starna, UK). Slits confining the light path were opened to 2-3 nm bandwidth. Polarizer in magic angle have not been used since stray light contributions in this wavelength range were negligible. For emission, GFP was excited at 480 nm and detected in the range between 490 nm and 700 nm for kinetics exclusively at 510 nm. The Raman contribution was subtracted with a buffer measurement (S/N > 20). All measurements were performed at 22°C.

Gels: GFP-C-His was displayed with SDS-PAGE using 4-12% Bis-Tris Novex pre-cast gels (Invitrogen) run in 1xMES buffer for 1hour at 150 V constant. The gel was imaged either directly with UV excitation (365 nm) or white light after Coomassie staining (Invitrogen) using a BioSpectrumAC imaging system with integrated quantification software (UVP Cambridge UK).

Cloning: Mammalian expression vectors were produced by integrating the coding sequence of human IL-4Rα into pEGFP-N1 from Clontech via directional cloning. The hexahistidine-tag was inserted by site directed mutagenesis (GeneTailor, Invitrogen). The expressed polypeptide NHis-IL4Rac-GFP comprises the extracellular His-tagged IL-4Rα 1-297 corresponding to amino acids 1-292 Swiss-Prot P24394 with 6 inserted histidine residues at positions 27-32, a short linker GSTGRH 298-303, and eGFP amino acids 304-542 corresponding to GeneBank Accession No. U55762 (amino acids in one-letter code, numbering from N to C-terminus). As a control IL4-Rac-GFP, the receptor described above lacking the hexahistidine sequence, was used. 3-4 days after transfection, the receptor constructs were properly expressed and transported to the surface plasma membrane.

Cell maintenance: HEK cells were grown in DMEM H21 (13.38 g/l DMEM Powder (Gibco), 44 mM NaHCO3 (Gibco), 50000 IE Penicillin (30mg) (BC), 50 mg Streptomycin (Gibco), 2 mM glutamine, 10% complement inactivated fetal calf serum). For passaging the cells where washed twice with PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4*$2H_2O$), harvesting with cell dissociation solution (CDS,

Sigma, Cat. No. C5914), diluted by PBS in a 50 mL Falcon tube, and pelleted at 300 *g* for 5min at room temperature. New flasks were seeded 1:10 and 1:20 twice a week and maintained at 37°C under 5% CO_2 under an humidified atmosphere.

Transfection and preparation of the cells: Confluent HEK 293 cells were transfected in a 12-well (4 cm^2 surface area per well, Falcon) via Lipofectamine 2000 (Invitrogen, Cat. No. 11668-019) according to the manufacturers recommendation. After 12 h, the cells were transferred into Fibronectin-coated chamber slides (Nunc LAB-TEK 8-well, Cat. No. 155409). Coating was done by covering the glass with 100 µg/mL Fibronectin (BD, Cat. No. 356008) followed by incubation for 30 min at 37°C. After aspiration, the cells were directly seeded at a density of 5000 to 10000 cells per well (1 cm^2 surface area per well). Within the time range of 48 h the cells were subconfluent and sufficiently adherent to apply the staining and cross linking procedure.

Confocal microscopy: For imaging and FCS a proprietary Novartis PS03 microspectroscope was used. In a joint development effort with Evotec Technologies (Hamburg, Germany) the IX70 microscope has been modified to provide high-resolution confocal laser scanning functionalities and single-photon-sensitive signal acquisition via two fluorescence emission channels. The piezo-based 3D scanning enhancement is commercially available under the proprietary name Insight Cell (Perkin Elmer, Hamburg, Germany).

In our setup, either argon ion (488 nm) or helium/neon (543 nm) laser light was fiber-coupled into the microscope (IX70, Olympus). The excitation beam passes the beam splitting plate (reflection 10%) and is directed onto the back aperture of a high-numerical objective (Olympus Uplan 60w NA1.2). The generated fluorescence is collected by the same objective, passes the beam splitting plate (transmission 90%) and focused by the tube lens onto the confocal pinhole (40 µm). In the detection path, a color splitting dicroic mirror (550 DLRP, Omega Optical, Brattleboro, Vt) splits the fluorescence into the GFP- (band pass 515DF30) and TMR-specific (band pass 585DF20) color channels, which are separately detected by single-photon sensitive avalanche photodiodes (SPMC-AQR-13-FC, Perkin Elmer). For imaging a laser power between 10 and 20 µW (488 nm) and 40-60 µW (543 nm) was applied.

Molecular brightness determination of NTA-ASA-TMR 5: The molecular brightness of the unconjugated dye TMR-COOH and NTA-ASA-TMR **5** in PBS buffer was measured via a modified confocal microscope (IX70, Olympus). The samples were

excited by means of a 543 nm HeNe laser (1676P, JDS Uniphase). The laser power was less than 200 W at the sample plane. A high numerical aperture (NA) water immersion objective was used (UApo 40, NA 1.15, Olympus) to focus and collect the laser and fluorescence light, respectively. The confocal pinhole aperture was 70 micrometer. The fluorescence light was isolated between 560 – 620 nm and detected via single photon sensitive photodetectors (Avalanche Photodiodes). The acquired data was subject to fluorescence correlation spectroscopy (FCS) using PerkinElmer's proprietary software FCS++ and a one-component fitting model (PSF 3D Gaussian, axis ratio = fixed).

Image evaluation: The binary image files generated by the MIPS software (Evotec Technologies, Hamburg, Germany) contain the number of photon counts accumulated within the residence time of the laser focus (pixel time, PT) at a certain position in the sample. The binary files are a proprietary image format and have been converted into TIF images by a script (Acapella, Evotec Technologies). The PTs used for imaging were 0.5 or 1 ms. Thus, dividing the counts for each pixel by PT in ms, converts the absolute number of photons to kHz intensities. The molecular brightness (kHz per particle) for free eGFP and NTA-ASA-TMR was determined by fluorescence correlation spectroscopy (FCS) in medium. The molecular brightness of the eGFP tagged receptors and free eGFP is the same as determined by FCS at the membrane (not shown). A background due to autofluorescence in the medium was taken into account according to $N_{cor} = G(0)*(1+U/F)^{-2}$, where $G(0)$ is the intercept of the correlation curve, U the background intensity for a certain laser power and F the average background subtracted signal. Dividing the intensity images by the molecular brightness returns particles per pixel. A typical volume of 0.25 femtoliter was assumed as a good estimate for the effective observation volume based on FCS of a fluorescent standard: AlexaFluor488 in PBS showed a translational diffusion time of t_d = 40 µs; using the diffusion coefficient for Fluorescein $D_t \sim 2.6*10^{-10}$ m^2/s, axis ratio of k = 5 and $V_{eff} = \kappa (4\ p\ t_d\ D_t)^{3/2}$. The red shifted 543 nm laser line was assumed to scale with a factor of $1.37 = (543/488)^3$.

The average concentrations at the surface membrane were determined with a image calculation script (MatLab Version 7.2.0.232 (R2006a), MathWorks Inc., USA). The region of interest (ROI) was segmented by threshold settings and manual corrections, a global background fluorescence from the medium was subtracted.

Chemical Syntheses

HCl.H-Glu(OBzl)-OBu was purchased from Senn Chemicals. The succinimidyl ester of TMR was purchased from Fluka, the succinimidyl ester of QSY 7 from Invitrogen. Boc-Lys(Z)-OH was purchased from Novabiochem. The photocrosslinker NHS-ASA (N-Hydroxysuccinimidyl-4-azidosalicylic acid) was purchased from Pierce. All other chemicals were obtained either from Fluka or Sigma Aldrich.

Analytical Tools

HPLC

Analytical HPLC of non-complexed compounds: Analytical reversed phase (RP) HPLC: Agilent 1100 Series System (Quat pump G1311A, degasser G1322A, multi-wavelengths and fluorescence detector DAD G1315B and FLD G1321A). Column: Zorbax C_{18} (4.6 x 150 mm, 3.5 µm).

Analyses were performed using a linear gradient of A (95% H_2O, 5% MeCN, 1% TFA) and B (95% MeCN, 5% H_2O, 1% TFA).

The following gradient was used:

t (min)	0	20	25	30
B (%)	5	45	95	5

at a flow rate of 1 mL/min with UV detection at 220 nm, and/or 555 nm for the TMR or 560 nm for the quencher QSY7 .

Analytical HPLC of Ni^{2+} complexed compounds: Ni^{2+} complexed compounds were analyzed by using the gradient described above, but with A: ammonium acetate ($AcONH_4$) in water (400 mg/L) and B: MeCN.

Retention times (t_R) are given in minutes.

Preparative HPLC of non complexed compounds: Preparative reversed phase (RP) HPLC: *Agilent* 1100 Series preparative system (Prep pump G1361A, multiwave-lengths detector MWD G1365B). Column: Agilent prep C_{18} (21.2 x 150 mm, 10 µm).

Crude products were purified using the following linear gradient of A (95% H_2O, 5% MeCN, 1% TFA) and B (95% MeCN, 5% H_2O, 1% TFA) at a flow rate of 20 mL/min with UV detection at the corresponding wavelengths:

t (min)	0	20	25	30
B (%)	5	45	95	5

Preparative HPLC of Ni^{2+} complexed compounds: Ni^{2+} complexed compounds were purified using the same gradient, flow, and UV detection as above but with A: ammonium acetate ($AcONH_4$) in H_2O (400 mg/L) and B: MeCN.

Retention times (t_R) are given in minutes.

General Synthesis Procedures

Coupling reactions using TMR, QSY7 and ASA succinimidyl ester derivatives: The compound containing the free amine was dissolved in dry DMF to a final concentration of 7-40 mg/mL . DIPEA (6 equiv) was then added to the solution followed by addition of the dye or photocrosslinker (1 equiv) dissolved in DMF. The reaction mixture was then stirred for 2 h at RT. After removing the solvents, the labeled compound was purified by preparative HPLC and analyzed by MS.

Complexation with Ni^{2+}: The compound foreseen to be complexed was dissolved in H_2O to a final concentration of 5-10 mg/mL and treated with a 0.1 M solution of $NiCl_2$ (1 equiv). The mixture was stirred for 2 h at RT. Finally, the Ni^{2+} complex was purified by preparative HPLC and analyzed by MS.

3-([2-(2-Benzyloxycarbonylamino-ethylcarbamoyl)-ethyl]-*tert*-butoxycarbonyl-amino)-pentanedioic acid di-*tert*-butyl ester (2): Compound **1** (synthesized according to previously published methods by Lata et al. 2005[27] 1 equiv, 4.63 mmol, 2 g) was dissolved in dry DMF (150 mL) at 0° C, followed by addition of (2-amino-ethyl)carbamic acid benzyl ester (1.5 equiv, 6.95 mmol, 1.6 g). HATU (1 equiv, 1.762 g, 4.63 mmol) and DIPEA (6 equiv, 27.8 mmol, 4.76 mL) were added to the solution so obtained. This mixture was stirred overnight at RT under Ar atmosphere. $NaHCO_3$ saturated solution (40 mL) was added to the mixture, which was stirred for 10 min. Then AcOEt (60 mL) and H_2O (40 mL) were added. The aqueous phase was washed with AcOEt (2 x 50 mL). The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography using silica gel (cyclohexane/ethyl acetate, 2:3) to give the title compound **2** as a grey solid (2.33 g, yield 83%). R_f= 0.4 (cyclohexane/ethyl acetate, 2:3); MS (70 eV, EI): *m/z* 630.4 $[M+Na]^+$.

3-((2-[2-(6-Benzyloxycarbonylamino-2-*tert*-butoxycarbonylamino-hexanoylamino)ethyl-carbamoyl]ethyl)-*tert*-butoxycarbonyl-amino)-pentanedioic acid di-*tert*-butyl ester (3): Pd/C (20 mg) was added to a solution of **2** (2.3 g, 3.78 mmol) in MeOH (100 mL). The reaction mixture was vigorously stirred overnight under H_2 atmosphere at RT. Pd/C was then filtered off over celite and the volatiles were removed under reduced pressure yielding the Z-deprotected intermediate 3-([2-(2-Amino-ethylcarbamoyl)-ethyl]-*tert*-butoxycarbonyl-amino)-pentanedioic acid di-*tert*-butyl ester (768 mg, 43%). MS (70 eV, EI): *m/z* 474.3 $[M+H]^+$; 496.3 $[M+Na]^+$; ^{1}H NMR (500 MHz, CD_3OD): d 1.45(s, 18H, *t*Bu); 1.47 (s, 9H, *t*Bu); 1.91 (m, 3H, CH_2); 2.41 (m, 2H, CH_2CONH); 2.72 (t, ^2J = 6.29, 2H, CH_2NH_2); 3.25 (td, ^2J = 6.3, ^3J = 1.98, 2H, $CH_2CH_2NH_2$); 3.33 (t, *J* = 4.9, 1H, NCH); 3.58 (s, 4H, $N(CH_2)_2$); ^{13}C NMR DEPT 135 (500 MHz, $CDCl_3$): d 27.2 (CH_2); 28.4 (CH_3); 28.4 (CH_3); 33.3 (CH_2); 43.0 (CH_2); 54.94 (CH_2); 66.1 (CH); 82.1 (CO); 82.6(CO); 172.4 (CO); 173.2 (CO); 175.9 (CO).

The thus obtained intermediate (1 equiv, 1.37 mmol, 652.2 mg) was dissolved in dry DMF (40 mL) at 0° C, followed by addition of Boc-Lys(*Z*)-OH (1.5 equiv, 2.06 mmol, 785.7 mg). HATU (1 equiv, 1.37 mmol, 523.5 mg) and DIPEA (6 equiv, 8.26 mmol, 1.41 mL) were added. This mixture was stirred overnight at RT under Ar atmosphere. $NaHCO_3$ saturated solution (25 mL) was added to the mixture, which was stirred for 10 min. The aqueous phase was extracted with DCM (2 x 20 mL). The combined organic phases were then washed with NH_4Cl saturated solution (20 mL), and NaCl saturated solution. The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography using silica gel (cyclohexane/ethyl acetate, 1:4) to give the title compound **3** as white foam (831.3 mg, yield 72%). R_f = 0.3 (cyclohexane/ethyl acetate, 1:4), MS (70 eV, EI): *m/z* 859.6 $[M+Na]^+$; ^{1}H NMR (500 MHz, $(CD_3)2O$): d 1.36 (m, 2H, $NHCHCH_2CH_2$); 1.44 (s, 9H, *t*Bu); 1.47 (s, 9H, *t*Bu); 1.45 (s, 18H, 2 x *t*Bu); 1.52 (m, 2H, CH); 1.65 (m, 2H, NHCHCH); 1.89 (m, 2H, $NHCOCH_2$); 2.39 (td, ^2J = 7.44, ^3J = 1.34, 2H, COCH); 3.13 (t, ^2J = 6.82, 2H, CH_2NHCO); 3.28 (m, 4H, $NHCH_2CH_2NH$); 3.33 (t, ^2J = 5.89, $NHCHCH_2$); 3.44 (m, 4H, 2x NCH_2CO); 3.94 (m, 1H, NHCH); 5.06 (s, 2H, OCH_2Ph); 7.31 (m, 5H, 5 arom. H); ^{13}C NMR DEPT 135 (500 MHz, $CDCl_3$): d 24.1 (CH_2); 27.6 (CH_2); 28.4 (CH_3); 28.49 (CH_3); 28.7 (CH_3); 30.5 (CH_2); 32.9 (CH_2); 33.3 (CH_2); 39.9 (CH_2); 40.0 (CH_2); 54.9 (CH_2); 56.2 (CH); 66.1 (CH); 67.3 (OCH_2Ph); 80.6 (OC); 82.1

(CO); 82.6 (CO); 128.763 (PhCH); 138.45 (Ph); 157.92 (CO); 158.94 (CO); 172.394 (CO); 173.2 (CO); 175.5 (CO); 175.9 (CO).

(*S*)-4-{2-[(*S*)-6-(4-Azido-2-hydroxy-benzoylamino)-2-*tert*-butoxycarbonylamino-hexano-ylamino]-ethylcarbamoyl}-2-(bis-*tert*-butoxycarbonylmethyl-amino)butyric acid *tert*-butyl ester (4): Pd/C (70 mg) was added to a solution of compound **3** (793.6 mg, 0.95 mmol) in MeOH (25 mL). The reaction mixture was vigorously stirred 3 h under H_2 atmosphere at RT. Pd/C was then filtered off over celite and the volatiles were removed under reduced pressure yielding the Z-deprotected intermediate 3-((2-[2-(6-Amino-2-*tert*-butoxycarbonylamino-hexanoylamino)-ethylcarbamoyl]-ethyl)-*tert*-butoxycarbonyl-amino)-pentanedioic acid di-*tert*-butyl ester as a colourless oil (653 mg, 98%). MS (70 eV, EI): *m/z* 702.5 $[M+H]^+$; 725.5 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): d 1.37(m, 2H, CH_2); 1.47(s, 27H, 3x tBu); 1.6 (m, 2H, CH_2); 1.89 (m, 2H, $_2CH_2$); 2.39 (t, ^2J = 7.51, 2H, CH_2); 2.65 (t, ^2J = 7.03, 2H CH_2); 3.24 (m, 2H, 2H, CH_2); 3.33 (t, ^2J = 3.43, 1H, CH); 3.44 (d, J = 6.02, 2H, CH_2COO); 3.94 (m, 1H, NHCH); ^{13}C NMR DEPT 135 (400 MHz, CD_3OD): d 24.6 (CH_2); 27.6 (CH_2); 28.9 (CH_3); 33.2 (CH_2); 33.5 (CH_2); 33.8 (CH_2); 40.3 (CH_2); 40.5 (CH_2); 42.4 (CH_2); 55.3 (CH_2); 56.6 (CH); 66.5 (CH); 82.6 (C); 83.1 (C); 172.8 (CO); 173.6 (CO); 175.5 (CO); 176.3 (CO).

This intermediate was reacted with NHS-ASA following the general coupling procedure described above in the general synthesis procedures section. After preparative HPLC purification, the title compound **4** was obtained. (18 mg, 55 %). MS (70 eV, EI): *m/z* 885.6 $[M+Na]^+$.

Ni^{2+} complexed NTA-ASA-TMR (5): The *tert*-Butyl and Boc protecting groups of compound **4** (17 mg, 19.6 µmol) were removed by treatment with a solution of TFA/TIS/H_2O (95:2.5:2.5, 5 mL) for 1 h at RT. The volatiles were removed under reduced pressure and the crude product was purified by preparative HPLC to give the unprotected NTA-ASA intermediate (10 mg, 85 %). t_R = 10.5 min; MS (70 eV, EI): *m/z* 595.3 $[M+H]^+$.

TMR was then coupled to this NTA-ASA intermediate (4 mg, 6.7 µmol) by reaction with TMR-NHS, proceeding as described above in the general synthesis procedures section. Preparative HPLC yielded the uncomplexed NTA-ASA-TMR intermediate (4.3 mg, 65%). t_R = 18.5 min; MS (70 eV, EI): *m/z* 1007.4 $[M+H]^+$.

NTA-ASA-TMR (4 mg, 4 µmol) was then complexed with Ni^{2+} following the method described above in the general synthesis section. Preparative HPLC yielded the final product NTA-ASA-TMR **5** (1.2 mg, 28 %). t_R = 9.8 min; MS (70 eV, EI): *m/z* 555.3 $[M+2Na]^{2+}$.

Ni2+ complexed NTA-ASA-QSY7 (6): QSY7 was coupled to the unprotected NTA-ASA intermediate (4 mg, 6.7 µmol) from above, proceeding as described in the general synthesis procedures section. Preparative HPLC yielded the uncomplexed NTA-ASA-QSY7, (6.2 mg, 75%). t_R = 10.5 min; MS (70 eV, EI): *m/z* 618.1 $[M+2H]^{2+}$; 1234.6 $[M+H]^{+}$.

NTA-ASA-QSY7 (5.5 mg, 4.5 µmol) was then complexed with Ni^{2+} following the method described in the general synthesis procedures section, using a mixture of MeOH/H_2O (1:1) as solvent. Purification by preparative HPLC yielded the final product NTA-ASA-QSY7 **6** (2.7 mg, 47 %). t_R = 23 min; MS (70 eV, EI): *m/z* 658.8 $[M+Na+H]^{2+}$

Reference for compound **1**:

[27] "High-Affinity Adaptors for Switchable Recognition of Histidine-Tagged Proteins" S. Lata, A. Reichel, R. Brock, et al. *J.Am.Chem.Soc.* **2005**, *127* 10205-10215.

Spectroscopic characterization of NTA-I

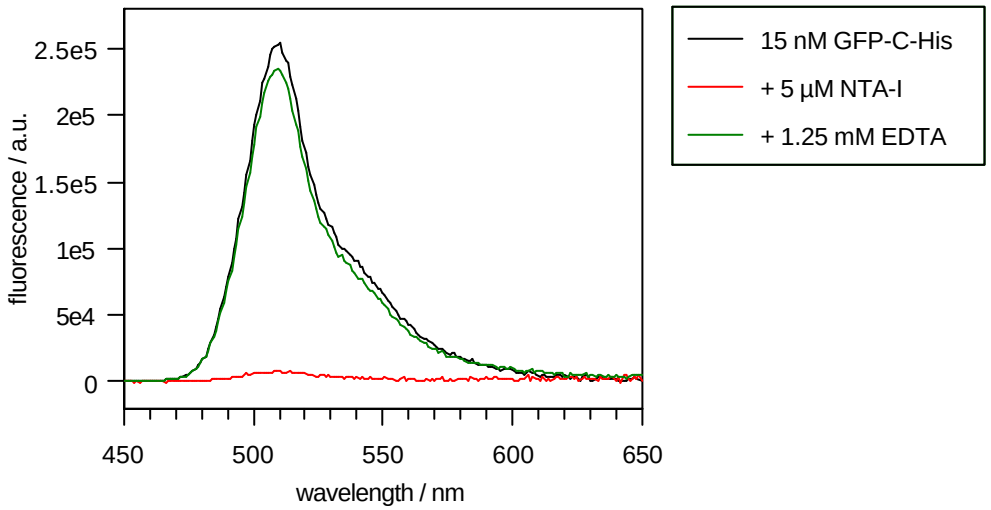

Figure S1. Reversible binding of NTA-I to GFP-C-His in presence and absence of EDTA

The binding of NTA-I (5 µM) to GFP-C-His (15 nM) is almost completely reversed upon addition of a large excess of EDTA (1.25 mM).

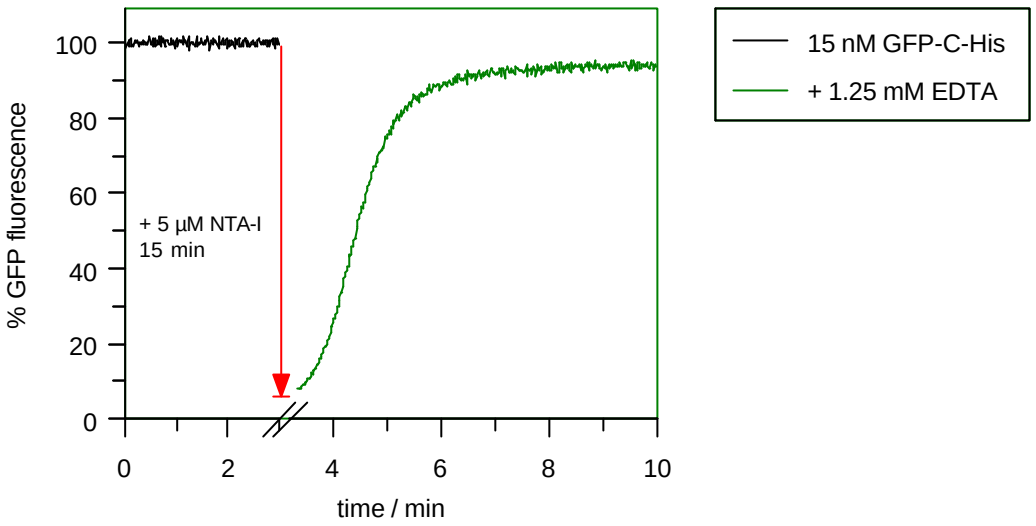

Figure S2. Unquenching kinetics of NTA-I binding to GFP-C-His

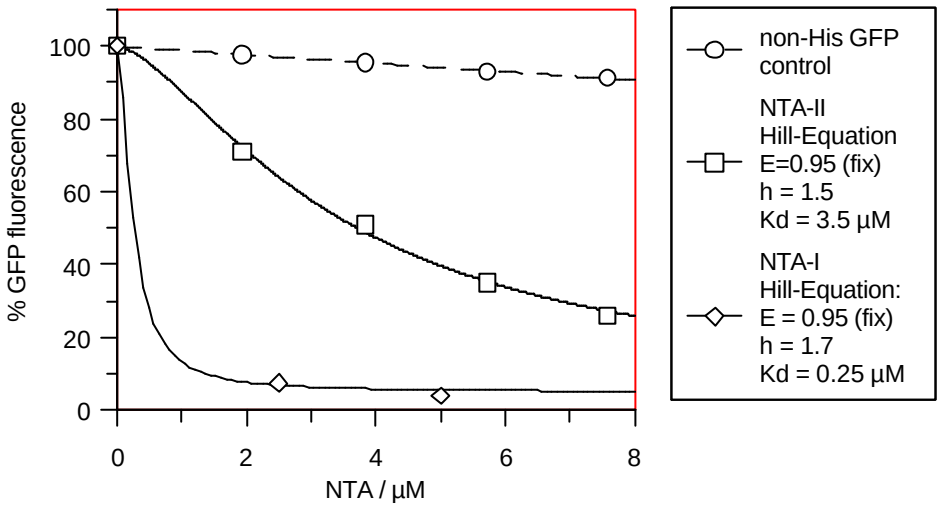

Figure S3. Determination of dissociation constants for NTA-I and NTA-II.

In order compare the data of compounds 5 and 6 to the previously published reversible probes NTA-I and NTA-II, the dissociation constants for NTA-I and NTA-II binding to C-His-GFP were determined using the method of Guignet et al. [7].

Table S1. Molecular brightness determination for NTA-ASA-TMR

	N	t_{diff} [µs]	trip [%]	Ttrip [µs]	CPP [kHz]
TMR-COOH	1.37	61	26.2	2.1	52
stdv.	0.01	1	0.8	0.2	1
NTA-ASA-TMR	1.25	68	13.1	3.9	36
stdv.	0.03	3	0.1	2.4	1

Detailed results from the molecular brightness determination of NTA-ASA-TMR and TMR-COOH. *N* particle number, t_{diff} diffusion time, trip triplet state fraction, Ttrip triplet time, CPP counts per particle.

Image calibration via FCS

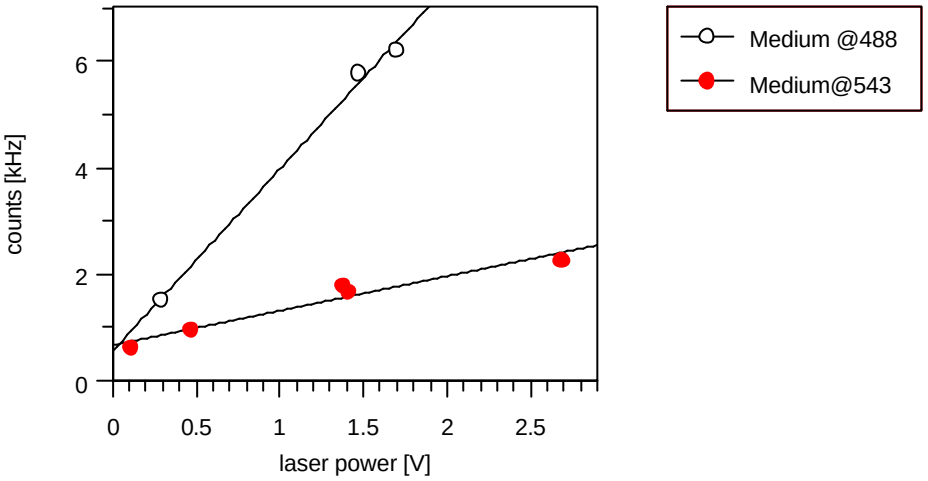

Figure S4: Calibration curves for background autofluorescence of cell-medium.

Background fluorescence from cell-medium at different laser power values [V] was collected under the identical experimental setting as used for the measurements described in Figure 3 and Table 1.

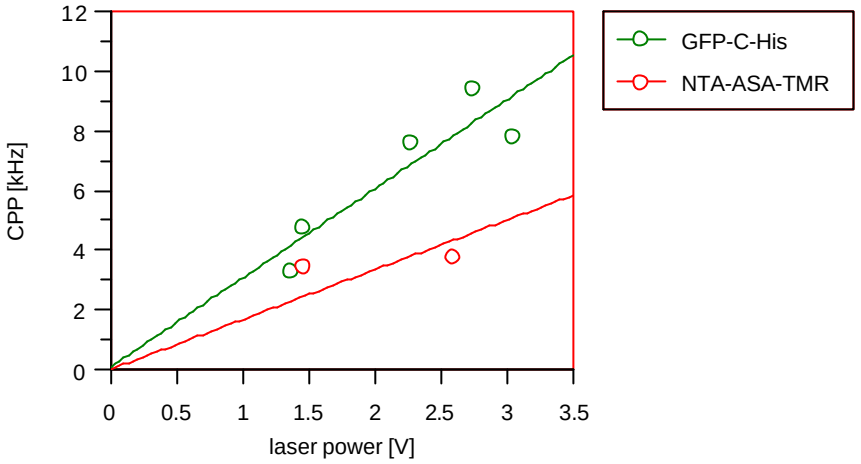

Figure S5: Calibration curves for particle countrates of GFP-C-His and NTA-ASA-TMR.

Calibration curves for the brightness of GFP-C-His and NTA-ASA-TMR (in counts per particle, (CPP)) were collected at different laser power values [V] under the identical experimental setting as used for the measurements described in Figure 3 and Table 1.

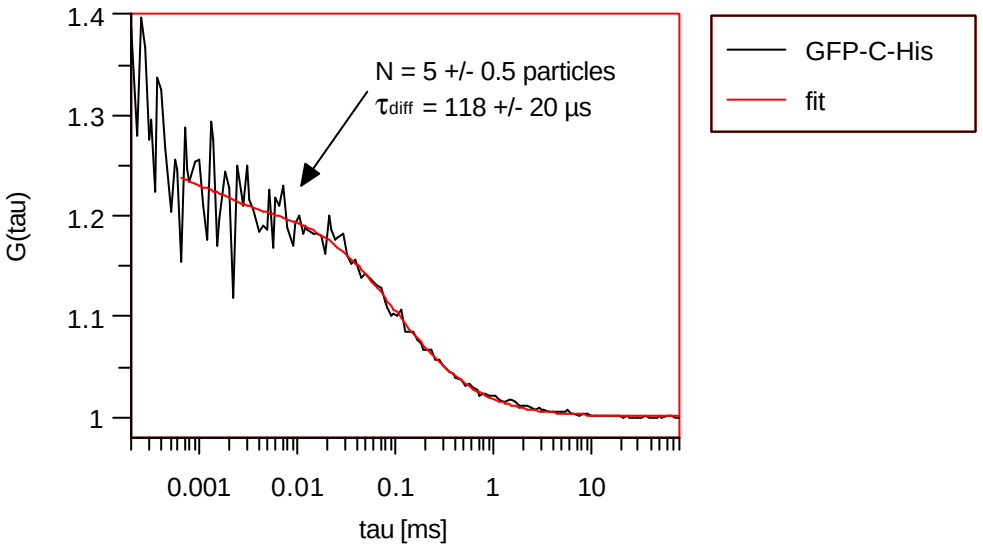

Figure S6: Analysis of GFP-C-His.

In order to obtain calibration parameters for the confocal volume, FCS measurements of GFP-C-His were carried out under the identical experimental setting as used for the measurements described in Figure 3 and Table 1.

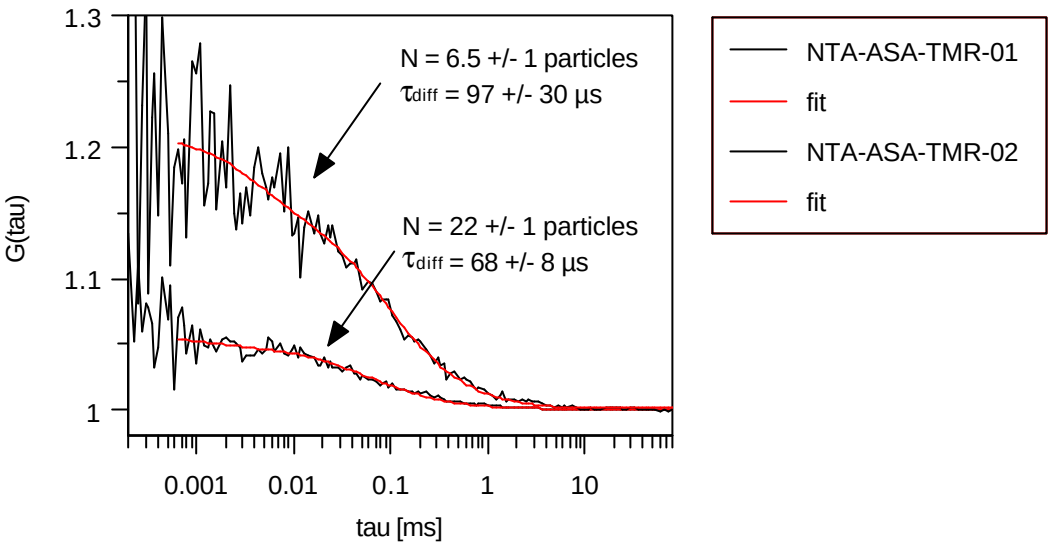

Figure S7: FCS analysis of NTA-ASA-TMR.

In order to obtain calibration parameters for the confocal volume, FCS measurements of NTA-ASA-TMR were carried out under the identical experimental setting as used for the measurements described in Figure 3 and Table 1.

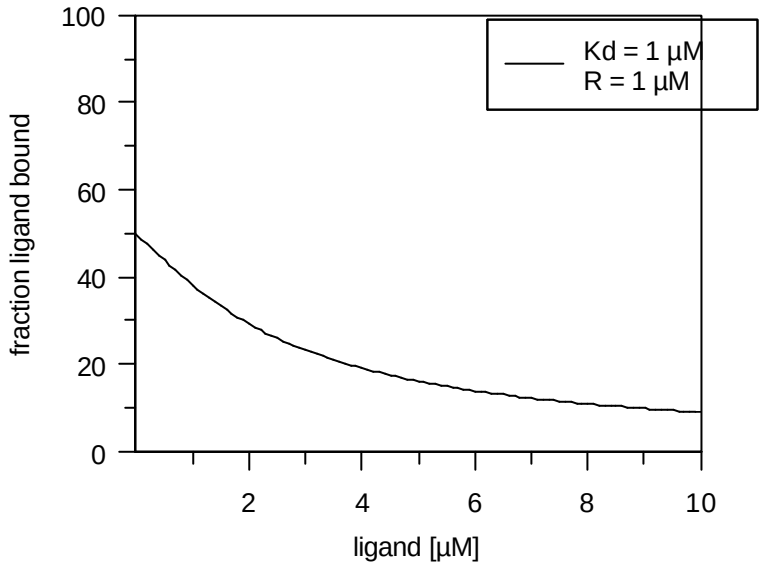

Figure S8. Simulation of fraction ligand bound for NTA-ASA-TMR.

The fraction of ligand, NTA-ASA-TMR bound to NHis-IL4Rac-GFP as function of the total ligand concentration present in the sample was simulated based on the solution of the quadratic equation, describing a 1:1 protein-ligand complex formation at equilibrium with a K_d of 1 µM and at receptor concentration of 1µM.

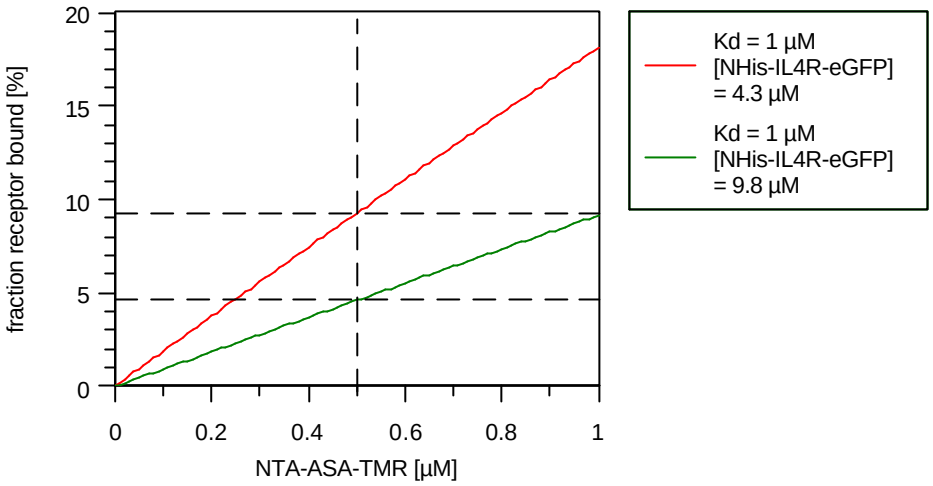

Figure S9. Simulation of fraction receptor bound for NHis-IL4Rac-GFP.

The fraction of receptor, NHis-IL4Rac-GFP, bound to NTA-ASA-TMR as function of total receptor concentration in the sample was simulated based on the solution of the quadratic equation, describing a 1:1 protein-ligand complex formation at equilibrium with a K_d of 1 µM and at a receptor concentration of 4.3 µM (red line, corresponding to cell 1 from Figure 3 and 9.8 µM (green line, corresponding to cell 2 from Figure 3).

Table S2. Detailed results obtained from the quantification of fluorescence intensities on the surface of transfected and non-transfected cells.

clone	NTA-ASA-TMR [µM]	area	mean [cnts/px]	back-ground [cnts/px]	PT [ms]	laser power [V]	CPP [kHz]	particles / px	c [µM]	S/N	fraction bound [%]
NHis-IL4R-eGFP	0,5	membrane receptor	1560	2	1	0,78	2,40	648	4,30		
NHis-IL4R-eGFP	0,5	membrane tag	297	14	1	1,48	2,48	114	0,56	5,0	10,4
NHis-IL4R-eGFP	0,5	mebrane, non-transfected	69,9	14	1	1,48	2,48	23	0,11		
NHis-IL4R-eGFP	0,5	membrane receptor	1760	1	1	0,37	1,19	1480	9,83		
NHis-IL4R-eGFP	0,5	membrane tag	436	22	1	2,37	3,96	105	0,51	5,2	4,2
NHis-IL4R-eGFP	0,5	mebrane, non-transfected	101	22	1	2,37	3,96	20	0,10		
IL-4Rac-eGFP	2,6	membrane receptor	562	1	0,5	0,35	1,13	995	6,61		
IL-4Rac-eGFP	2,6	mebrane, transfected	172	15	0,5	1,60	2,67	118	0,58	1,2	1,4
IL-4Rac-eGFP	2,6	mebrane, non-transfected	146	15	0,5	1,60	2,67	98	0,48		

Given a confocal volume of 0.25 fL, a concentration of 1 µM equals ~ 3 500 molecules/µm².