Supporting Information

Quantum yield optimized fluorophores for site-specific labeling and super-resolution imaging

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Within this supporting information, following topics are addressed:

Synthesis of *tris*NTA-fluorophore conjugates with "zero" prolyl residue spacer

Trivalent N-nitrilotriacetic acid (NTA) will hereafter be referred to as *tris*NTA. Amino-*tris*(N-nitrilotriacetic acid) fluorophore conjugates were synthesized similar to a previously published route[1]. In the present paper, these conjugates were used as *tris*NTA-fluorophore conjugates with a "zero" prolyl residue spacer. The structures of *tris*NTA-**OG488** (**1**) and *tris*NTA-**ATTO565** (**2**) are presented in Figure S1.

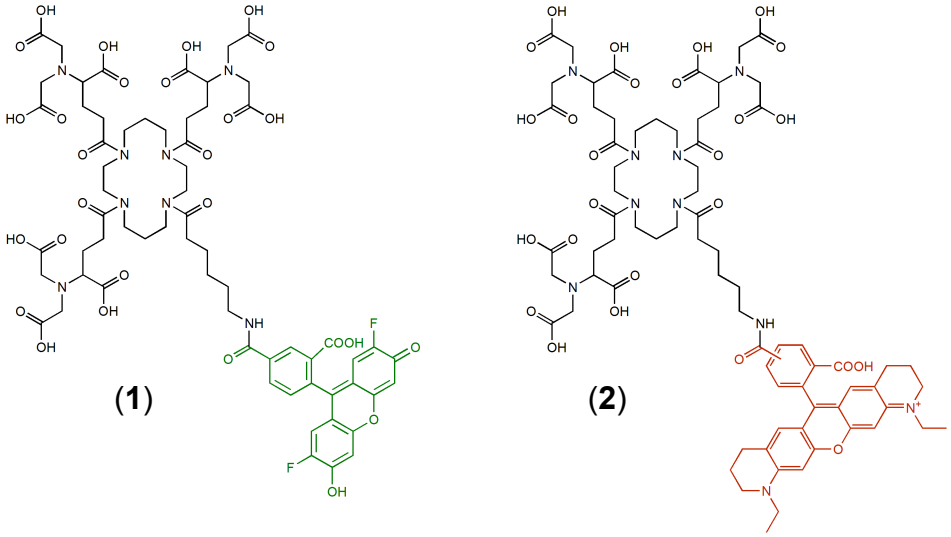


Figure S1: Structure of *tris*NTA-**OG488** (**1**) and *tris*NTA-**ATTO565** (**2**)

General experimental strategies for peptide synthesis

Coupling reagents and amino acid derivatives were purchased from Bachem (Weil am Rhein, Germany). All other reagents and solvents were purchased from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany) and were used as received. Reactions on solid support were performed with an automated peptide synthesizer. RP-HPLC analyses and separations were conducted on a Jasco instrument using Grace Vydac columns (semi-preparative (218TP1025) 25 x 250 mm, 10 µm C18, 2 mL/min). Linear H_20/CH_3CN gradients (0.2% TFA) were applied. Educts and products are detected at 220 nm. Mass spectra (ESI or MALDI) were recorded at in-house services.

Peptide synthesis was carried out using TCP-resin following standard Fmoc-strategy[2]. Fmoc-Xaa-OH was attached to the TCP resin with DIPEA in anhydrous DCM at room temperature for 1.5 h. After filtration, the remaining trityl chloride groups were capped by a solution of DCM, MeOH, DIPEA for 15 min. The resin was

filtered and washed thoroughly with DCM (2x), DMF (3x) and MeOH (5x). The loading capacity was determined by weight after drying the resin under vacuum. The resin-bound Fmoc peptide was treated with 20% piperidine in NMP (v/v) for 15 min and a second time for 10 min. The resin was washed with NMP (5x). A solution of Fmoc-Xaa-OH, TBTU, HOBt, DIPEA in NMP was added to the resin-bound free amine peptide and shaken for 30 min at room temperature. The coupling was repeated another time and the resin was washed with NMP (5x). The iterative application of TBTU/HOBt coupling and Fmoc deprotection was used to synthesize resin-bound $P_xC(Trt)A$-peptides with x = 4, 8 and 12.

Coupling of carboxy-*tris*NTA to $P_xC(Trt)A$ TCP resins

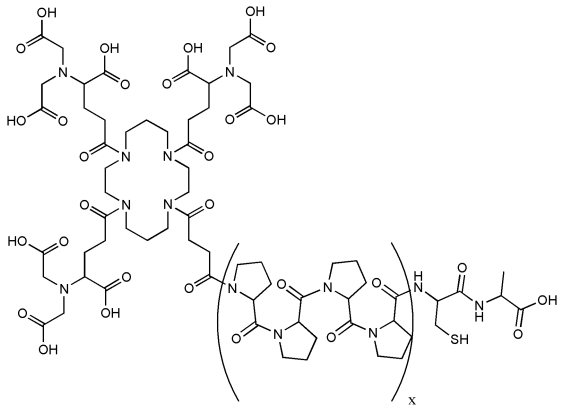
Carboxy-*tris*NTA(OtBu) was synthesized as previously described[1]. After swelling of the resin with NMP for 15 min, the resin-bound free amino termini of the $P_xC(Trt)A$-peptides were reacted with carboxy-*tris*NTA(OtBu) by incubating under agitation in a solution of carboxy-*tris*NTA(OtBu) (4 equiv), TBTU (4 equiv), HOBt (4 equiv), DIPEA (6.4 equiv) in NMP for 16 h at room temperature. The resin was then washed with NMP (3x) and DMF (5x). The coupling of carboxy-*tris*NTA(OtBu) was repeated until no primary amines could be detected (quantitative coupling reaction can be verified via Kaiser test [Fluka][3] or acetaldehyde/chloranil test[4]). Typically, the coupling had to be repeated three times according to the above outlined procedure.

Cleavage from the resin and final deprotection

Peptides were cleaved from the resin with a mixture of TFA, Phenol, TIPS, EDT and Milli-Q water (95:1.25:1.25:1.25:1.25 v/v/v/v/v) for 4-5 h at room temperature under agitation. After cleavage, deprotected *tris*NTA-P_x peptides were separated by filtration from the resin. The resin was then washed with a small amount of TFA and the solvent of the peptide solution was almost completely removed under reduced pressure. After evaporation of the solvent, the products were precipitated three times with cold ether and dried. Finally, products were purified via RP-HPLC and characterized via MALDI-MS (Table S1).

Compound	# of Pro	formula	$MW_{cal.}$	MALDI-MS [m/z]
*tris*NTA-P_4CA (**3**)	**4**	$C_{67}H_{99}N_{13}O_{30}S$	1598.6	1600.7
*tris*NTA-P_8CA (**4**)	**8**	$C_{87}H_{127}N_{17}O_{34}S$	1987.1	1988.6
*tris*NTA-P_{12}CA (**5**)	**12**	$C_{107}H_{155}N_{21}O_{38}S$	2375.6	2376.5

Table S1: Calculated and experimentally confirmed molecular mass for *tris*NTA-P_x peptides. Structures of peptides are shown in Figure S2.



x = 1 → *tris*NTA-P_4CA (**3**)
x = 2 → *tris*NTA-P_8CA (**4**)
x = 3 → *tris*NTA-P_{12}CA (**5**)

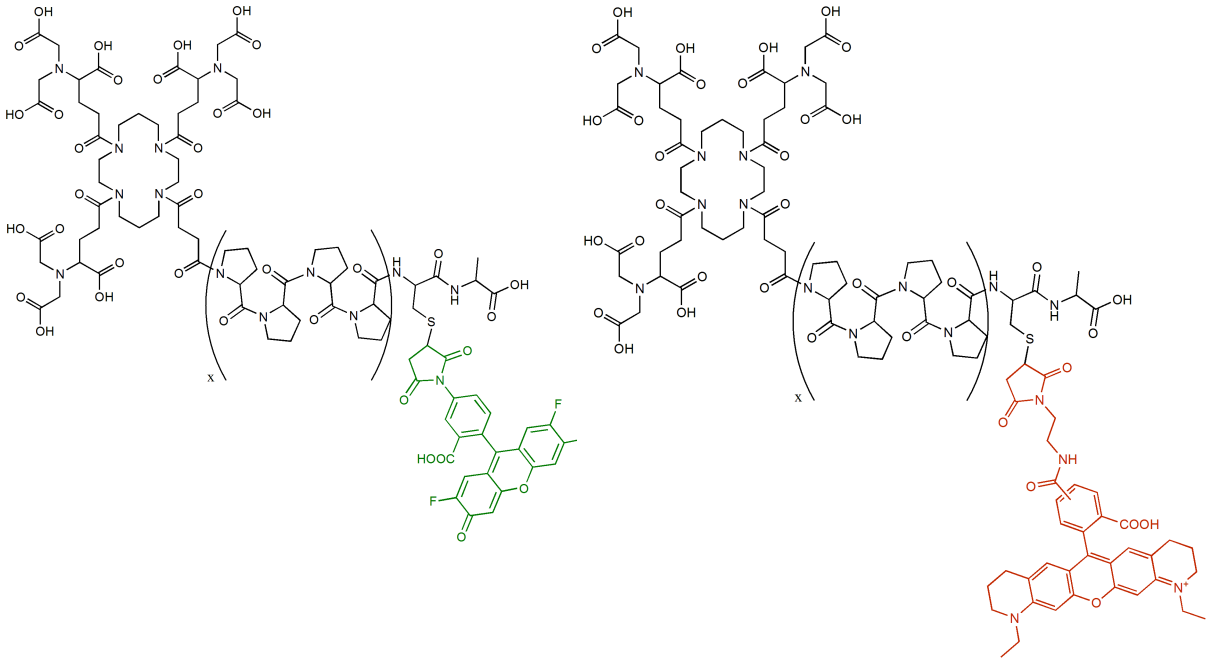
Figure S2: Structure of *tris*NTA-P_x peptides (PPII-helix peptides)

Fluorescence labeling of *tris*NTA-P_x peptides

Single cysteines in the *tris*NTA-P_xCA peptides were labeled using maleimide derivatives of Oregon Green 488 (OG488), ATTO565 or ATTO647N (see www.invitrogen.com and www.atto-tec.com for detailed protocols). Subsequently, the *tris*NTA-P_x fluorophores were purified via C_{18}-RP HPLC and characterized via $MALDI^-$- or ESI^--MS (Table S2).

Compound	# of Pro	sum formula	$MW_{cal.}$	MS [m/z]
*tris*NTA-P_4C(**OG488**)A (**6**)	4	$C_{91}H_{110}F_2N_{14}O_{37}S$	2061.9	2061.4
*tris*NTA-P_4C(**ATTO565**)A (**7**)		$C_{104}H_{136}N_{17}O_{36}S^+$	2232.4	2229.3
*tris*NTA-P_8C(**OG488**)A (**8**)	8	$C_{111}H_{138}F_2N_{18}O_{41}S$	2450.4	2450.4
*tris*NTA-P_8C(**ATTO565**)A (**9**)		$C_{124}H_{164}N_{21}O_{40}S^+$	2620.8	2617.4
*tris*NTA-P_{12}C(**OG488**)A (**10**)	12	$C_{131}H_{166}F_2N_{22}O_{45}S$	2838.9	2839.2
*tris*NTA-P_{12}C(**ATTO565**)A (**11**)		$C_{144}H_{192}N_{25}O_{44}S^+$	3009.3	3010.7

Table S2: Calculated and experimentally confirmed molecular mass for *tris*NTA-P_x-fluorophore peptides.



x = 1 → *tris*NTA-P_4C(**OG488**)A (**6**)
x = 2 → *tris*NTA-P_8C(**OG488**)A (**8**)
x = 3 → *tris*NTA-P_{12}C(**OG488**)A (**10**)

x = 1 → *tris*NTA-P_4C(**ATTO565**)A (**7**)
x = 2 → *tris*NTA-P_8C(**ATTO565**)A (**9**)
x = 3 → *tris*NTA-P_{12}C(**ATTO565**)A (**11**)

Figure S3: Structure of OG488 (left) and ATTO565 (right) labeled *tris*NTA-P_xCA.

Nickel-loading of *tris*NTA-P_x-fluorophores and purification of Ni-*tris*NTA-P_x-fluorophores via IEC

After C_{18}-RP HPLC purification, *tris*NTA-P_x-fluorophores were incubated with a 15-fold molar excess of Ni^{2+} ions in 10 mM Hepes buffer, pH 7.0. The Ni-*tris*NTA-P_x-fluorophores were allowed to incubate for 30 min before the solution was applied to a 1 mL HiTrap™ Q HP column (GE Healthcare). By washing with 5 column volumes, the excess of nickel ions was removed. A flow rate of 2 mL/min was chosen for washing while a linear gradient from 0 to 100% over 8 column volumes at 1 mL/min was used for elution. Beforehand, the ion exchange column was equilibrated with the running buffer (10 mM Hepes; pH 7.0). Running buffer supplemented with 1 M NaCl served as elution buffer. Final concentrations were determined by VIS spectroscopy of the attached fluorophores.

Concentration of Ni-*tris*NTA-P_x-fluorophores

Concentrations were determined via absorbance measurements (Lambert-Beer's law). Following extinction coefficients were used according to the suppliers (see www.invitrogen.com and www.atto-tec.com):

Fluorophore	Absorbance max.	Extinction coefficient
Oregon Green 488	492 nm	ε_{492} = 8.28 x 10^4 L / (mol cm)
ATTO565	563 nm	ε_{563} = 1.20 x 10^5 L / (mol cm)
ATTO647N	644 nm	ε_{644} = 1.50 x 10^5 L / (mol cm)

Table S3: Molar extinction coefficients for fluorescent probes

Structural details regarding the PPII-helix element

PPII-helix distances were calculated based on PPII-helix conformation. PPII-helices are known as spectroscopic rulers employed to confirm the R^6-distance dependency of FRET efficiency between donor/acceptor pairs[5]. PPII-helices are water soluble, left-handed all-trans helices with three amino acid residues per turn corresponding to a pitch of 9 Å[6,7]. Up to a length of 12 prolyl residues, the PPII-helix might be considered as a rigid rod[8].

Spectroscopic characterization of *tris*NTA-P_x-fluorophores

Fluorescence spectra were recorded using a Fluorolog III (Horiba) fluorescence spectrometer. In addition, time dependent (kinetic) measurements at fixed wavelength were conducted. To prevent adsorption of the probes to the wall of the cuvette 0.01%, Tween20 was added to the HBS buffer (20 mM Hepes, 150 mM NaCl, pH 7.5). Dye conjugates were analyzed according to Table S4. The concentration of Ni-*tris*NTA-P_x fluorophores was adjusted to 20 nM. Spectra of nickel free *tris*NTA-P_x fluorophores were obtained after incubation with 120 µM EDTA over night.

Fluorophore conjugate	Excitation wavelength	Emission wavelength (max.)
Oregon Green 488	488 nm	515 nm
ATTO565	563 nm	592 nm
ATTO647N	644 nm	670 nm

Table S4: Excitation and emission wavelength used for spectroscopic characterization of the *tris*NTA-P_x-fluorophores

Specific interaction between *tris*NTA-P_x-fluorophores and His-tagged protein

We have chosen His_6-tagged maltose binding protein (MBP-H_6) as model protein to demonstrate specific and stable interaction to *tris*NTA-P_{12}C(**OG488**)A (**10**) via size exclusion chromatography.

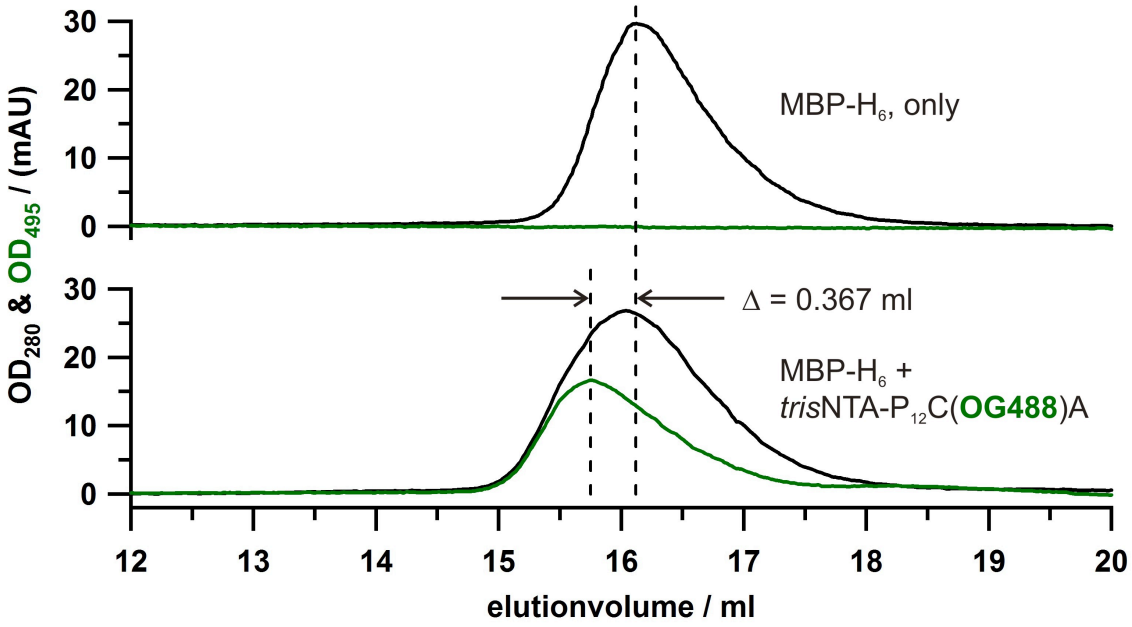


Figure S4: MBP-H_6 (0.5 µM) and *tris*NTA-P_{12}C(**OG488**)A were mixed in a molar ratio of 2:1. Subsequently, the mixture was applied to a Superdex 200 PC 3.2/30 column (GE Healthcare) with a flow rate of 70 µL/min. Black chromatograms indicate the protein absorbance at 280 nm while *tris*NTA-P_{12}C(**OG488**)A is monitored via the absorbance at 495 nm. If the His-tagged protein is incubated with the *tris*NTA-P_{12}-fluorophore (dashed grey line), the protein peak is shifted to a slightly larger retardation volume, indicating a slightly larger gyration radius based on the labeling by the *tris*NTA-P_{12}-fluorophore. The coinciding absorbance at 280 (black) and 495 nm (green) with a shift to a slightly larger molecular mass clearly confirms specific and stable complex formation. Noteworthily, no free *tris*NTA-P_{12}C(**OG488**)A could be detected due to the excess of protein, thus demonstrating that all *tris*NTA-P_{12}C(**OG488**)A molecules are engaged with MBP-H_6 in a 1:1 stoichiometry. No complex formation of the His-tagged protein and *tris*NTA-P_{12}C(**OG488**)A was observed in the presence of $EDTA^{2-}$ or imidazole (not shown).

In vivo visualization of *tris*NTA-P_x-fluorophores in living neurons

Wide-field fluorescence images: The wide-field fluorescence images were acquired using an Olympus IX 70 epifluorescence microscope equipped with a 100x/1.4 NA objective, a mercury bulb, and appropriate filter sets. Images were acquired with a CCD camera (cascade 512B, Photometrics, Roper scientific). Resolution, one pixel equals 156 nm with 512 x 512 pixels for the total image.

STED and confocal images: The confocal and STED fluorescence images were acquired using an inverted STED Leica DMI6000 TCS SP5 AOBS microscope. For the confocal images, the 635 nm line of a pulsed diode laser was used for the excitation. For the STED images, excitation was performed via the 635 nm line of a pulsed diode laser and depletion was performed using an infrared Laser Spectra-Physics MaiTai tuned at 750 nm. Resolution: one pixel equals 32.9 nm with 1024 x 256 pixels for the total image.

References with regard to this Supporting Information:

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Complete reference 18 of main manuscript:

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