Linker Molecules Convert Commercial Fluorophores into Tailored Functional Probes during Biolabelling

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Supplementary Information

Part I: Materials, methods and synthesis

Synthesis of linker molecules

Materials. All reagents were purchased from commercial suppliers and used without further purification, unless stated otherwise. Boc-β-alanine (99%, Sigma Aldrich), tert-butyl isocyanide (98%, Sigma Aldrich), propargyl amine (99%, Acros Organics), 4-nitrobenzaldehyde (98%, Sigma Aldrich), paraformaldehyde (95%, Sigma Aldrich), 3-Azido-1-propanamine (95%, Sigma Aldrich), 2-Tributylstannylpyridine (95%, Sigma Aldrich), 2-bromo-4-pyridinecarboxaldehyde (98%, Sigma Aldrich), 3-Maleimidopropionic acid N-hydroxysuccinimidester (99%, Chempur), 3-Maleimidopropionic acid (97%, ABCR), thionyl chloride (>99%, Sigma Aldrich), cyclooctatetraene (COT) (98%, ABCR), bromine (<99.9%, Sigma Aldrich), N,N-Diisopropylethylamine (99.95%, Sigma Aldrich), magnesium turnings (98%, Sigma Aldrich), iodine (99.8%, Sigma Aldrich), trifluoroacetic acid (TFA) (99%, Sigma Aldrich), N,N-Dimethylformamide (DMF) (anhdyrous, over molecular sieves, 99.8% Acros Organics), potassium-tert-butoxide (1 M solution in THF, Sigma Aldrich), dichloromethane (DCM) (anhdyrous, over molecular sieves, 99.9% Acros Organics), methanol (p.a, 99.8%, VWR) were used as received. Tetrahydrofuran, which was used for the synthesis of COT-CO was dried with LiAlH₄ and distilled before use. Technical grade solvents were used for column chromatography.

¹H and ¹³C NMR spectra were recorded on Bruker DPX FT NMR spectrometers (600 and 151 MHz, 400 and 101 MHz or 300 and 75 MHz respectively) and are reported as follows: chemical shift δ (ppm) (multiplicity, number of protons). CDCl₃ was used as solvent. TMS was used as internal standard. Chemical shifts are reported in ppm to the nearest 0.01 ppm for ¹H and the nearest 0.1 ppm for ¹³C measurements.

High resolution ESI-TOF mass spectra were recorded on a microTOF-QII instrument from Bruker Daltonik (Bremen, Germany). Ion polarity was positive, and scan range 50-1600 m/z.

Synthesis of Cy3B-azide. The method used here was adapted from the synthesis of Cy3B-carboxyl-ethylenediaminyl-trityl.[¹] 3-Azido-1-propanamine (10 eq.), Cy3B-NHS ester (1 eq.) and TEA (66 eq.) were added, in turn, to anhydrous DMF (200 μL for 6.5 μmol Cy3B-NHS ester), and the reaction mixture was incubated 1h at room temperature. The product was purified by reversed-phase HPLC and lyophilized HPLC was done with solvent A: water; solvent B: 90% acetonitrile, 10% water; gradient 30 to 80% B in 30 min at 2 mL/min.
COT (3.20 g, 30.73 mmol, 1.0 eq) was dissolved in dry DCM (21 mL) under nitrogen atmosphere and cooled to -78°C with an ethanol dry ice bath. The solution was stirred for 10 minutes and a solution of bromine (4.91 g, 30.73 mmol, 1.0 eq) in dry DCM (9 mL) was added dropwise within 1h at -78°C. The solution was stirred for 1h at -78°C after which a solution of potassium tert-butoxide in THF (1.0 M, 43.0 mmol, 1.4 eq) was added dropwise over 1.5 h. The reaction mixture was stirred for 2h at -50°C and 1h at -10°C. The solution was poured into ice water (30 mL) in a cold separation funnel. The DCM layer was removed and the water layer was extracted with cold diethyl ether (3 x 100 mL). The organic fractions were combined and dried over magnesium sulfate, filtered, concentrated without heat, yielding the raw COT-Br as a brownish oil. Purification by column chromatography (eluent: pure pentane) afforded COT-Br (4.670 g, 83 %) as yellow oil. ¹H NMR (300 MHz, CDCl₃): δ 5.50-5.65 (m, 1H), 5.65-5.95 (m, 5H), 6.14 (br s, 1H) ppm.

Magnesium turnings (1.064 g, 43.71 mmol, 2.0 eq) were suspended in dry THF (10 mL) under nitrogen atmosphere and a crystal of iodine was added. The mixture was cooled to 0°C and a solution of COT-Br (4.006 g, 21.86 mmol, 1.0 eq) in dry THF (60 mL) was added slowly. The yellowish solution was stirred at 0°C for 1 h and 5.5 h at room temperature. The dark green mixture was cooled to 0°C and dry DMF (1.598 g, 1.68 mL, 21.86 mmol, 1.0 eq) was added slowly within 5 minutes. The resulting mixture was stirred for 20 h at room temperature after which dry THF (20 mL) and 3 M HCl (29 mL) were added to dissolve residual Mg turnings. The mixture was extracted with diethyl ether (3 x 30 mL). The combined organic layers were subsequently washed with water (3 x 30 mL), sodium hydrogen carbonate solution (2 x 30 mL), brine (2 x 30 mL), and dried over magnesium sulfate. The raw product was obtained after removal of the solvent as a brown oil. Crude 5 (2.120 g) was purified by column chromatography...
(eluent: cyclohexane, diethyl ether 2:1) yielding 5 (0.601 g, 28 %). 1H NMR (300 MHz, CDCl3): δ 5.60-6.10 (m, 6H), 6.68 (br s, 1H), 9.40 (s, 1H) ppm.

**Synthesis of compound 6.**

![Figure S3. Synthesis route of the compound 6 (2,2'-bipyridine-4-carbaldehyde precursor).](image)

The synthesis was adapted from a published work.[2] In brief, to an oven dried Schlenk flask were added Pd(PPh3)2Cl2 (0.52 g, 0.70 mmol), PPh3 (0.38 g, 1.4 mmol), 2-Tributylstannylpyridine (5.0 g, 13.5 mmol), 2-bromo-4-pyridinecarboxaldehyde (3.0 g, 16 mmol) and toluene (100 mL) under nitrogen. The resulting mixture was stirred for 72 hours under reflux. The resulting mixture was cooled to room temperature and the solvent was removed under reduced pressure. The obtained solid was dissolved in dichloromethane and washed with a saturated NH4Cl-solution. The aqueous phase was extracted with dichloromethane. The organic layers were combined, the solvent was removed under reduced pressure and the crude product was purified by chromatography to give compound 6 (0.99 g, 40 %) as a colorless solid. 1H-NMR (400MHz, CDCl3): 10.19 (s, 1H), 8.91(d, 1H),8.85 (s, 1H), 8.73 (d, 1H), 8.44 (d, 1H), 7.87 (tr, 1H), 7.73 (d, 1H), 7.38 (tr, 1H).

**Synthesis of compound 8.** Compound 3 (0.150 g, 5.0 mmol) was dissolved in methanol (21.0 mL) and stirred at 50°C until a clear solution is obtained. Upon cooling, Compound 1 (0.947 g, 5.0 mmol), Compound 2 (0.276 g, 5.0 mmol) and Compound 7 (0.416 g, 5.0 mmol) were added. The solution was stirred for 72h at room temperature after which ethyl acetate (100 mL) was added. Subsequently, the organic layer was washed with water (3 x 50 mL). The combined water layers were extracted with ethyl acetate (2 x 50 mL), the organic fractions were combined, dried with magnesium sulfate, filtered, and concentrated in vacuo yielding 8 (1.47 g, 87%) as a viscous oil.

1H NMR (400 MHz, CDCl3): δ 1.27-1.29 (d, 9H), 1.35 (s, 9H), 2.25-2.31 (d, 1H), 2.42 (tr, 1H), 2.62 (tr, 1H), 3.25-3.45 (m, 2H), 3.93-3.94 (d, 2H), 4.11 (s, 1H), 4.23 (s, 1H), 5.28 (m, 1H), 6.05-6.13 (d, 1H) ppm.

13C NMR (101 MHz, CDCl3): δ 28.5, 28.7, 33.4, 33.7, 36.1, 36.2, 38.7, 50.4, 51.0, 51.5, 51.9, 73.0, 73.6, 77.8, 78.7, 79.2, 156.0, 166.9, 167.7, 172.4 ppm.

HRMS [M(8+Na)] calculated for C17H29N3O4 362.2056 u, found 362.2640 u.
Synthesis of compound 9. Compound 4 (0.756 g, 5.0 mmol) and 1 (0.947 g, 5.0 mmol) were dissolved in methanol (21.0 mL) before 7 (0.416 g, 5.0 mmol) and 2 (0.276 g, 5.0 mmol) were added. The solution was stirred for 72 h at room temperature after which ethyl acetate (100 mL) was added. Subsequently, the organic layer was washed with water (3 x 50 mL). The combined water layers were extracted with ethyl acetate (2 x 50 mL), the organic fractions were combined, dried with magnesium sulfate, filtered, and concentrated in vacuo yielding 9 (2.04 g, 88%) as a viscous oil.

9 (1.903 g) was further purified by column chromatography (eluent: pentane, ethyl acetate (3:1, 2:1 then 1:1)). Yield: 1.579 g (83%).

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 1.30\) (s, 9H), 1.35 (s, 9H), 2.04, 2.22 (s, 1H), 2.72 (br s, 2H), 3.39 (tr, 2H), 4.00-4.10 (m, 2H), 5.19 (br s, 1H), 6.11-6.15 (d, 2H), 7.48-7.50 (d, 2H), 8.11-8.13 (d, 2H) ppm.

\(^{13}\)C NMR (101 MHz, CDCl\(_3\)): \(\delta 28.4, 28.5, 34.0, 35.6, 36.2, 52.0, 60.3, 72.8, 78.5, 79.2, 123.7, 130.2, 142.4, 147.7, 155.9, 167.5, 173.1\) ppm.

HRMS [M(9+Na)] calculated for C\(_{23}\)H\(_{32}\)N\(_4\)O\(_6\) 483.22 u, found 483.2890 u.

Synthesis of compound 10. Compound 1 (0.804 g, 4.25 mmol) was dissolved in methanol (10.0 mL) and a solution of 5 (0.562 g, 4.25 mmol) in methanol (8.0 mL) was added before 7 (0.353 g, 4.25 mmol) and 2 (0.234 g, 4.25 mmol) were added. The solution was stirred for 72 h at room temperature after which ethyl acetate (100 mL) was added. Subsequently, the organic layer was washed with water (3 x 50 mL). The combined water layers were extracted with ethyl acetate (2 x 50 mL), the organic fractions were combined, dried with magnesium sulfate, filtered, and concentrated in vacuo yielding 10 (0.920 g, 49%) as a viscous oil.

10 (0.833 g) was further purified by column chromatography (eluent: pentane, ethyl acetate (1:1)). Yield: 0.210 g (25%).

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 1.28\) (s, 9H), 1.36 (s, 9H), 2.27 (s, 1H), 2.37-2.80 (m 2H), 3.37 (m, 2H), 4.00-4.40 (m, 2H), 5.21 (br s, 1H), 5.28 (s, 1H), 5.60-6.20 (m, 7H) ppm.

\(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta 28.5, 28.7, 34.2, 35.6, 36.3, 51.8, 63.1, 67.2, 73.1, 79.1, 79.9, 131.2, 131.7, 132.1, 132.2, 132.4, 132.6, 133.7, 137.5, 156.0, 168.0, 173.6 ppm.

Synthesis of compound 11. Compound 1 (1.894 g, 10.0 mmol) and 2,2'-bipyridine-4-carbaldehyde (6, 1.84 g, 10.0 mmol) were dissolved in methanol (42.0 mL) and 7 (0.832 g, 10.00 mmol) and 2 (0.551 g, 10.0 mmol) were added. The solution was stirred for 72 h at room temperature after which ethyl acetate (200 mL) was added. Subsequently, the organic layer was washed with water (3 x 50 mL). The combined water layers were extracted with ethyl acetate (2 x 20 mL), the organic fractions were combined, dried with magnesium sulfate, filtered, and concentrated in vacuo yielding 11 (3.180 g, 86%) as a sticky solid. 11 (was further purified by column chromatography (eluent: pentane, ethyl acetate (3:1, 2:1 then 1:1)). Yield: 3.6 g (73%).
$^1$H NMR (400 MHz, CDCl$_3$): 1.40 (s, 9H), 1.42 (s, 9H), 2.10 (s, 1H), 2.54-2.84 (m, 2H), 3.39-3.50 (m, 2H), 4.17-4.20 (m, 2H), 6.19 (s, 1H), 7.33-7.37 (m, 2H), 7.81-7.86 (m, 2H), 8.37-8.39 (m, 2H), 8.66-8.70 (m, 2H) ppm

$^{13}$C NMR (100 MHz, CDCl$_3$): 29.93, 30.73, 34.61, 36.20, 39.32, 53.14, 59.98, 61.15, 72.83, 77.94, 79.29, 121.39, 121.56, 123.37, 123.94, 136.66, 144.46, 148.55, 149.30, 154.78, 155.59, 156.54, 167.26, 172.95 ppm

HRMS [M+11$^+$] calculated for C$_{17}$H$_{29}$N$_3$O$_4$ 493.2689 u, found 494.2787 u.

**Synthesis of compounds 12-15.** The compounds 8 (0.699 g, 2.06 mmol, 1.0 eq), 9 (0.700 g, 1.52 mmol, 1.0 eq), 10 (0.209 g, 0.47 mmol, 1.0 eq) and 11 (1.0 g, 2.06 mmol, 1.0 eq) were dissolved in DCM (36 mL - 18 mL each compound). 40 eq of TFA were added slowly and the mixture was stirred for 2.5 h at room temperature. The solvent was removed in vacuo and the raw product was taken up in DCM and the solvent was evaporated again. This process was repeated three times. The crude products 12, 13, 14 and 15 were used in the following reaction without further purification and characterization.

**Synthesis of compound 16.** 3-Maleimidopropionic acid (1.100 g, 6.50 mmol, 1.0 eq) was dissolved in DCM (68 mL). Thionyl chloride (7.74 g, 65.0 mmol, 10 eq) and DMF (52 drops, cat.) were added followed by 110 minutes of stirring. Compound 16 was obtained as a greyish solid after removal of the solvents and was used in subsequent reactions without further purification. Full conversion was proven by TLC using ethyl acetate/ pentane (1:1) as eluent. Yield: 0.96 g (79 %).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 3.20 (tr, 2H), 3.80 (tr, 2H), 6.69 (s, 2H) ppm.

**Synthesis of compound 18-20.** The compounds 12 (0.728 g, 2.06 mmol, 1.0 eq), 13 (0.721 g, 1.52 mmol, 1.0 eq), and 14 (0.215 g, 0.47 mmol, 1.0 eq) were dissolved in DCM and cooled to 0°C. Subsequently, N,N-Diisopropylethylamine (10 eq) was added slowly followed by freshly prepared 16 (1.0 eq). The solution was stirred for 72h at room temperature and the reaction mixture was washed with water (2 x 11 mL). The combined aqueous layers were extracted with DCM (2 x 11 mL) and the combined organic fractions were dried with magnesium sulfate, filtered, and concentrated in vacuo yielding the raw products 18-20.

18 was purified by column chromatography (eluent: ethyl acetate, ethanol (1:0 to 1:1) yielding 18 (0.090 g, 23 %).
\[ ^1H \text{NMR (400 MHz, CDCl}_3): \delta 1.27-1.30 \text{ (d, 9H), 2.20, 2.28 (tr, 1H), 2.34-2.45 (m, 3H), 2.60 (tr, 2H), 3.40-3.55 (m, 2H), 3.74 (m, 2H), 3.91, 3.94 (s, 1H), 4.09, 4.21 (d, 2H), 5.70 (br s, 1H), 6.40 (br s, 1H), 6.63 (s, 2H) ppm.} \]

\[ ^13C \text{NMR (151 MHz, CDCl}_3): \delta 28.7, 32.6, 34.4, 34.9, 35.0, 35.2, 36.0, 38.7, 50.1, 50.8, 51.6, 52.0, 70.0, 73.7, 77.6, 78.7, 134.2, 166.7, 167.5, 170.0, 170.1, 170.6, 172.4, 172.6 ppm.} \]

HRMS [M(18)+H]\(^+\) calculated for \(C_{19}H_{27}N_4O_5\) 391.1981 u, found 391.1992 u.

\(19\) was purified by column chromatography (eluent: pure ethyl acetate) yielding \(19\) (0.185 g, 31 %).

\[ ^1H \text{NMR (400 MHz, CDCl}_3): \delta 1.31 (s, 9H), 2.04 (s, 1H), 2.42 (tr, 2H), 2.72 (tr, 2H), 3.40-3.64 (m, 2H), 3.68-3.84 (m, 2H), 4.07 (s, 2H), 5.81 (s, 1H), 6.07 (s, 1H), 6.33 (m, 1H), 6.64 (s, 2H), 7.51 (d, 2H), 8.16 (d, 2H) ppm.} \]

\[ ^13C \text{NMR (101 MHz, CDCl}_3): \delta 28.4, 33.6, 34.3, 34.9, 35.0, 35.6, 52.3, 60.5, 73.0, 78.4, 79.2, 123.9, 130.3, 134.2, 142.1, 147.9, 167.3, 169.7, 170.5, 173.3 ppm.} \]

HRMS [M(19)+H]\(^+\) calculated for \(C_{25}H_{30}N_5O_7\) 512.2145 u, found 512.2147 u.

\(20\) was purified by column chromatography (eluent: ethyl acetate, ethanol 10:1), yielding \(20\) (0.197 g, 81 %).

\[ ^1H \text{NMR (300 MHz, CDCl}_3): \delta 1.28 (s, 9H), 2.20, 2.29 (s, 1H), 2.40 (tr, 2H), 2.70 (tr, 2H), 3.40-3.55 (m, 2H), 3.75 (tr, 2H), 3.94-4.40 (m, 2H), 5.24 (s, 1H), 5.60-6.04 (m, 7H), 6.44 (m, 1H), 6.63 (s, 2H) ppm.} \]

\[ ^13C \text{NMR (151 MHz, CDCl}_3): \delta 28.6, 33.6, 34.3, 34.8, 35.1, 35.7, 51.9, 63.5, 67.1, 73.2, 79.8, 131.0, 131.7, 132.0, 132.2, 132.6, 134.2, 137.5, 167.8, 169.6, 170.5, 173.7 ppm.} \]

HRMS [M(20)+H]\(^+\) calculated for \(C_{27}H_{33}N_4O_5\) 493.2451 u, found 493.2457 u.

**Synthesis of compound 21.** 2.31 g (4.62 mmol, 1.0 eq) of \(15\) was dissolved in DCM (20 mL) and cooled to 0°C. Subsequently, \(N, N\)-Diisopropylethylamine (10 eq) was added slowly followed by \(17\) (1.0 eq). The solution was stirred for 72h at room temperature and the reaction mixture was washed with water (2 x 11 mL). The combined aqueous layers were extracted with DCM (2 x 11 mL) and the combined organic fractions were dried with magnesium sulfate, filtered, and concentrated in vacuo yielding the raw products.

\(21\) was purified by column chromatography (eluent: dichloromethane, methanol (15:1, 10:1 then 9:1), yielding \(21\) (0.624 g, 25 %).
**Synthesis of compound 22.** 0.52 g (1.09 mmol, 1.0 eq) of 13 was dissolved in DCM (12.5 mL). Subsequently, diisopropylethylamine (1.41g, 10.9 mmol, 10 eq) was added and the mixture was cooled to 0°C before acryloyl chloride (0.20 g, 2.18 mmol, 2.0 eq) was added dropwise. The solution was stirred for 18 h at room temperature and the crude product was concentrated in vacuo. 22 was purified by column chromatography (eluent: ethyl acetate, pentane (3:1), yielding 22 (0.25 g, 56 %).

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.30 (s, 9H), 2.04 (s, 1H), 2.63 (m) & 2.77 (tr, 2H), 3.47-3.70 (m, 2H), 4.06 (m, 2H), 5.55 (d, 1H), 5.95-6.12 (m, 3H), 6.17 (d, 1H), 6.56 (tr, 1H), 7.48 (d, 2H), 8.13 (d, 1H) ppm.

$^{13}$C NMR (100 MHz, CDCl$_3$): 28.6, 33.5, 35.2, 35.8, 52.2, 53.11, 60.6, 73.1, 78.5, 123.9, 126.6, 130.3, 136.91, 130.9, 142.3, 147.8, 165.6, 167.5, 173.3 ppm.

HRMS [M(22)+H]$^+$ calculated for C$_{21}$H$_{27}$N$_4$O$_5$ 415.1981 u, found 415.1985 u.
Part II: Chemical characterization of synthesized compounds

Figure S4. $^1$H NMR spectrum of COT-Br.

Figure S5. $^1$H NMR spectrum of 5.
Figure S6. $^1$H NMR spectrum of 8.

Figure S7. $^{13}$C NMR spectrum of 8.
Figure S8. $^1$H NMR spectrum of 9.

Figure S9. $^{13}$C NMR spectrum of 9.
Figure S10. $^1$H NMR spectrum of 10.

Figure S11. $^{13}$C NMR spectrum of 10.
Figure S12. $^1$H NMR spectrum of 18.

Figure S13. $^{13}$C NMR spectrum of 18.
Figure S14. $^1$H NMR spectrum of 19.

Figure S15. $^{13}$C NMR spectrum of 19.
Figure S16. $^1$H NMR spectrum of 20.

Figure S17. $^{13}$C NMR spectrum of 20.
Figure S18. $^1$H NMR spectrum of 6.

Figure S19. $^1$H NMR spectrum of 11.  

# ethyl acetate
Figure S20. $^{13}$C NMR spectrum of 11.

Figure S21. $^1$H NMR spectrum of 21.
Figure S22. $^{13}$C NMR spectrum of 21. # ethyl acetate, § N-hydroxysuccinimide

Figure S23. $^1$H NMR spectrum of 22.
Figure S24. $^{13}$C NMR spectrum of 22.
Mass spectrometry data of linker compounds used for labelling

Chemical Formula: C₁₀H₂₆N₄O₅
Exact Mass: 390.1903

Figure S25. ESI MS of 18. m/z = 391.1992 Da [M(18)+H], m/z = 413.1810 Da [M(18)+Na], m/z = 429.1560 Da [M(18)+K], m/z = 781.3904 Da [2*M(18)+H], m/z = 803.3741 Da [2*M(18)+Na].
Figure S26. MALDI-TOF MS of 19. m/z = 512.2147 Da [M(19)+H], m/z = 534.1977 Da [M(19)+Na], m/z = 550.1713 Da [M(19)+K], m/z = 1023.4228 Da [2*M(19)+H], m/z = 1045.4062 Da [2*M(19)+Na], m/z = 1061.3789 Da [2*M(19)+K]
Figure S27. ESI-MS of 20. m/z = 493.2457 Da [M(20)+H], m/z = 515.2284 Da [M(20)+Na], m/z = 531.2021 Da [M(20)+K], m/z = 985.4857 Da [2*M(20)+H], m/z = 1007.4697 Da [2*M(20)+Na], m/z = 1023.4453 Da [2*M(20)+K].
Figure S28. ESI-HRMS of 21. [M(21)+H], m/z = 545.2530 Da, [M(21)+H+CH₃OH], m/z = 577.2764 Da
Figure S29. ESI-MS of 22. m/z = 415.1985 Da [M(22)+H], m/z = 437.1809 Da [M(22)+Na], m/z = 453.1548 Da [M(22)+K], m/z = 851.3740 Da [2*M(22)+Na], m/z = 867.3475 Da [2*M(22)+K]
Part III: Biological samples and labelling procedures

Protein labelling with linker molecules and dyes. Reactive maleimide- and azide-containing fluorophore derivatives of sulfo-Cy5, SiR, sulfo-Cy3, and TMR were obtained from Lumiprobe (Germany). Reactive maleimide- and azide-containing fluorophore derivatives of Atto647N were obtained from ATTOTEC. CuSO$_4$, BTTES (water-soluble ligand for copper(I)-catalyzed click reaction) and sodium ascorbate were purchased from Sigma Aldrich. SATP (N-Succinimidy l S-Acetyltiopropionate) was obtained from Thermofisher.

Model protein for in vitro photostability analysis: GlnPQ-SBD2. ABC transporters are found in all kingdoms of life and are divided into the two subpopulations of ABC importers and ABC exporters. These transporters are integral membrane proteins and are for example involved in uptake of nutrients, intercellular communication, drug excretion and lipid translocation. In general, ABC importers consist of three types of protein domains: transmembrane domains (TMDs), cytoplasmic nucleotide binding domains (NBDs) and substrate-binding proteins (SBPs) or substrate-binding domains (SBDs). In Lactococcus lactis, SBD2 is part of the ATP-binding cassette (ABC) importer GlnPQ.

Isolated SBD2 protein containing single cysteine substitution (pBAD 10x His-tag SBD2 / Thr369Cys (GlnP), from hereon out abbreviated as SBD2-T369C) was obtained as described in ref.[3] Briefly, SBD2-T369C was purified via Ni$^{2+}$ affinity chromatography and stored at -20 °C as aliquots of 17.8 mg mL$^{-1}$ in 50 mM KPi, pH 7.4, 50 mM KCl and 50% glycerol plus 1 mM dithiothreitol (DTT).

In this work, SBD2 was used as the model protein to characterize the fluorescence and photostability properties of the designed probes. Even though many other proteins could have been applied, the well-characterized SBD2 protein provides a good starting point since this is a well-studied protein in our research group. In previous research, Gouridis and coworkers studied the structure of the protein and its mechanism of binding to its substrate glutamine.[4] Since this data, as well as an appropriate mutant for labeling, were already available, it provides good means to function as a model for linker development studies. SBD2 derivatives were labelled with maleimide-fluorophores according to ref.[3]

Overexpression of SBD2-T369C protein.

The SBD2-T369C overexpression plasmid was made by PCR. In pBAD vector a stop codon was inserted at the C-terminus of His$_{10}$ SBD2 / Thr369Cys (Figure S25) before the epitope and a C-terminal his-tag.
Escherichia coli BL21-pLysS cells were freshly transformed with the SBD2-T369C plasmid and grown in 2 L LB medium (100 mg/ml carbenicillin and 50 mg/ml chloramphenicol) at 37 °C under aerobic conditions. At OD₆₀₀ between 0.6 and 0.8, the culture was induced with 10% arabinose and shaken for 1.5-2.0 h. Then the cells were harvested by centrifugation at 4°C for 20 min at 6,000 rpm. From this step onwards, all the processes were done at 4 °C and all solutions were kept at 4 °C. All cell pellets were collected in one 50 mL falcon and resuspended in a 50 mL falcon with buffer A (50 mM Tris-HCl, pH 8.0, KCl 1 M, imidazole 10 mM, glycerol 10%) with additional 1 mM dithiothreitol (DTT) and 200 µM phenylmethylsulfonyl fluoride. The falcon was shaken gently overnight at 4°C.

**Purification of SBD2-T369C protein**

The above falcon was put with ice in a Tip sonicator (on/off alternative) to disrupt the cells. The cell lysate was fractionated by centrifugation (centrifugation at 4°C for 30 min with 5,000 rpm and centrifugation at 4°C for 1 h with 40,000 rpm in vacuum), and the pellet was discarded. Ni²⁺-Seapharose fast flow resin (GE Healthcare) was equilibrated with 10 column volumes of buffer A with 1 mM DTT, and the supernatant of the previous centrifugation was gravity loaded to the column. The resin-bound protein was sequentially washed with 10 column volumes of buffer A with 1 mM DTT, then buffer B (50 mM Tris-HCl, pH 8.0, KCl 50 mM, imidazole 20 mM, glycerol 10%) with 1 mM DTT, and lastly eluted with buffer C (50 mM Tris-HCl, pH 8.0, KCl 50 mM, imidazole 250 mM, glycerol 10%) with 1 mM DTT. The eluted sample through of the column was concentrated (Vivaspín, ~5 mg/mL), dialyzed with SnakeSkin membrane in buffer D (50 mM Tris-HCl, pH 8.0, KCl 50 mM) supplemented with 1 mM DTT and mixed gently at 4°C overnight. Then, dialysis buffer D was exchanged with buffer E (50 mM Tris-HCl, pH 8.0, KCl 50 mM, glycerol 50%) containing 1 mM DTT. Protein overexpression and purification was verified with SDS page (RotiBlue staining) and Western Blot. The protein concentration was calculated by absorbance at 280 nm (extinction coefficient 28 880 L mol⁻¹ cm). Then the protein was divided into aliquots and stored at −20 °C.
Prior to experiments, all proteins were further purified with size-exclusion chromatography on a Superdex-200 column (GE Healthcare) equilibrated in 50 mM KPi, pH 7.4, and 50 mM KCl.

Protein labeling with linker molecules and azide fluorophores. Briefly, purified proteins (100 μg) were treated with DTT (100 eq, 30 min), to fully reduce oxidized cysteines. After dilution of the protein sample to a DTT concentration of 1 mM, the reduced protein was bound to a Ni²⁺-Sepharose resin (GE Healthcare) and washed with ten column volumes of Tris-HCl buffer (pH 7.6, 25 mM) (buffer A). Simultaneously, the linker molecule dissolved in DMSO (1 μL, 0.1 M) was diluted in 1 mL buffer A and applied to the protein bound to the Ni²⁺-Sepharose resin. The resin was incubated overnight and kept at 4 °C (under mild agitation). After incubation, unreacted linker molecule was removed by sequential washing with buffer A. In the meantime, click solution was prepared with the final components of CuSO₄ (0.3 mM), ligand BTTES (0.9 mM), Azide-fluorophore (0.05 mM) and sodium ascorbate (2 mM). The ‘click’ solution was then loaded on the column and first incubated overnight at 4°C and subsequently for 1 h at room temperature under mild agitation, wrapped in aluminum foil. Next, the column was drained, washed with buffer A, then the protein was eluted with imidazole solution (1 mL, 0.5 M in Tris-HCl buffer). The eluted sample was then centrifuged to remove aggregates (5 min, 13.2 krpm, 4°C) and purified using an ÄKTA system (Superdex 75 GL column, buffer A). Labelling efficiency for corresponding dye was further determined by measuring the maximum absorbance increase of the dye (Figure 2). The relevant fractions obtained from the ÄKTA system was then stored at 4°C until further characterization with a microscope.

Protein labeling efficiency. To determine the overall labeling efficiency, the Lambert-Beer law (equation 1) was used, where A is the measured absorbance which is equal to the logarithm of the inverse transmission I/I₀, c the molar concentration, l path length and episolon the wavelength-dependent extinction coefficient of the sample.[⁵] Using the proportionality of the absorbance and molar concentration, the labeling efficiency (LE) can be calculated according to equation 2. In this formula, ε of the protein was calculated based on the amino acid sequence; the ε values of the dyes were used as reported by the supplier; the absorbance value (A) was calculated from the integrated area under the absorbance peak in the chromatogram for each wavelength (as shown in Table 1), which was obtained from the size-exclusion chromatography curve.

\[
A = \varepsilon \cdot c \cdot d = \log \frac{I_0}{I}
\]

\[
LE = \sum 100 \cdot \frac{A_{\text{fluorophore}}}{A_{\text{protein}}} \cdot \frac{\varepsilon_{\text{protein}}}{\varepsilon_{\text{fluorophore}}}
\] equation (1)

\] equation (2)
Table 1: Values of spectroscopic parameters used for label efficiency calculations based on https://web.expasy.org/cgibin/protparam/protparam for SBD2 and supplier information for the respective dyes b,c,d (https://de.lumiprobe.com/p/tamra-azide-5; https://www.lumiprobe.com/p/sulfo-cy3-azide; https://www.lumiprobe.com/p/sulfo-cy5-azide)

|         | SBD2 | TAMRA | sCy3 | sCy5 |
|---------|------|-------|------|------|
| \( \lambda_{\text{absorption}} \) (nm) | 280  | 541   | 548  | 646  |
| \( \varepsilon \) (L mol\(^{-1}\) cm\(^{-1}\)) | 28880 | 84000 | 162000 | 271000 |

DNA labelling with linker molecules and dyes. Synthetic oligomers (P1: HS-5’-TAA TAT TCG ATT CCT CTG GAC G-3’, P2: Biotin-5’-CGT CCA GAG GAA TCG AAT ATT A-3’) were received in HPLC-purified quality from IBA or Eurofins (Germany). dsDNA-thiol was achieved form the hybridization of P1 and P2 by a typical DNA annealing protocol. In brief, mix 10 µL of 100 µM P1 with 10 µL complementary 100 µM P2 in 80 µL annealing buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH = 7.6), heat the mixed solution to 95°C for 5 min and cool down to 4°C in 1°C/min steps.

dsDNA-thiol labelling with maleimide-fluorophores. The dsDNA-thiol was incubated with TCEP at the ratio of 1:100 in PBS buffer for 30 min. After removing the excess DTT by PD-10 desalting columns with Sephadex G-25 resin, Maleimide-fluorophores (25-fold molar excess) were added and the mixture vortexed thoroughly. The sample was then incubated overnight and dsDNA-dye conjugate was further purified by PD-10 desalting columns to remove unbound dyes.

dsDNA-thiol labelling with linker molecules then clicked by azide-fluorophores. The dsDNA-thiol was incubated with DTT at 1:100 molar ratio in PBS buffer for half an hour. After removing the excess DTT by PD-10 desalting columns, linker molecules (25-fold molar excess) were applied to the dsDNA-thiol solution and was vortexed thoroughly for 2 h. Unlabeled linker molecules were separated from dsDNA by PD-10 desalting columns. The purified dsDNA-linker was then incubated with a mixture of click solution (CuSO\(_4\) (0.3 mM), ligand BTTES (0.9 mM), azide-fluorophore (0.05 mM) and sodium ascorbate (2 mM) for 3 h, followed by removal of unreacted reagents from the click reaction via PD-10 desalting columns.

Labelling of living E-coli cells. E. coli K12 cells were grown overnight in LB medium and subsequently diluted 1000 times in LB medium. The cells were grown until the culture reached an OD\(_{600}\) value of 0.6 after which the cells were centrifuged at 3000 rcf for 5 minutes and resuspended in PBS buffer. The thiol-reactive dye maleimide-Cy3B (GE Healthcare) was added to a final concentration of 1 mM and incubated while protected from light at room temperature for 1 hour. Next, the cells were centrifuged at 2000 rcf for 10 minutes and the cell pellet was resuspended in PBS. In the case of linker molecules, the well grown cells were incubated with linker molecules for 1 h and then washed three times by centrifugation at 2000 rcf for 10 minutes. The pellet was resuspended in PBS and treated with a mixture of azide-fluorophores (50 µM), CuSO\(_4\) (100 µM), BTTES (300 µM) and sodium ascorbate (500 µM) in PBS for half an hour.
**GFP-binding protein (GBP) labelling.** GBP is a specific binder for fluorescent proteins based on a 13-kDa GFP binding fragment derived from a llama single chain antibody.\[^6\] It was kindly provided by Jeannette Koch (from AG Prof. Dr. Heinrich Leonhardt, LMU München). It was initially modified with SATP reagents according to the instructions from the distributor of ThermoFisher. SATP reagent creates the requisite protected sulfhydryls modification of the antibody necessary for conjugation with the maleimide-activated compounds. After the medication of SATP reagent, the nanobody was incubated with DTT (100 eq, 30 min) to fully reduce oxidized thiol. Upon the Pd-10 desalting column’s purification to remove DTT, the nanobody was labelled by linker and/or fluorophores similar to the typical protein labelling. Each step of the purification was done on Pd-10 column and we selected the best labelling fraction via UV-visible absorption monitoring among several collected ones. The labelled protein was characterized by UV-visible absorption spectra (Figure S31).

![Figure S31. UV-VIS absorption spectra of labelled GFP-binding protein (GBP) by (A) maleimide-functionalized ATTO647N and (B) azide-functionalized ATTO647N using linker 20.](image)

**Microscopy, spectroscopy and sample preparation**

**In vitro sample preparation and surface immobilization of proteins/DNA.** For single molecule investigations, labelled proteins and labelled DNA were immobilized on a PEG-functionalized coverslip using biotin-streptavidin interaction. For proteins, this was achieved via the protein-his-tag and a biotinylated penta-His antibody, whereas DNA was directly tagged with a biotin. For PEG-functionalization, cover slides (1.5H Marienfeld Superior) were cleaned by sonication in 2% Hellmanex, MQ water, acetone and 1 M KOH subsequently, for 10, 5, 10, 10 min, respectively, then dried under nitrogen stream and treated with oxygen plasma for 10 minutes. The slides were then immediately incubated with a 99:1 solution of mPEG\(^{3400}\)-silane (abcr, AB111226)/biotin-PEG\(^{3400}\)-silane (Laysan Bio Inc) in Toluene overnight to generate
mPEG/biotin–coated surface. Afterwards, slides were sonicated in ethanol and MQ water (10,10 minutes respectively), dried under nitrogen stream and stored under vacuum. For single molecule experiments, the slide was incubated with a 0.2 mg/mL streptavidin in PBS solution for 5 min using Grace Bio-Labs SecureSeal™ hybridization chambers. Unbound excess streptavidin was then washed away using PBS buffer. For protein immobilization 10 nM of biotinylated penta-His antibody (QIAGEN) was then incubated for 10 min on the slide followed by washing away unbound excess of antibodies using PBS (this step was omitted for the labelled DNA samples). 0.5 nM of SBD2-T369C or 0.05 nM dsDNA was incubated for 5 and 1 min, respectively. The treated chambered cover slides lead to a typical surface coverage of fluorophore-labelled protein or dsDNA as shown in Fig 4 and Supplementary Figs 3–11. The imaging buffer containing 25 mM Tris-HCl (pH 7.4), 50 mM NaCl for protein and PBS for dsDNA with an oxygen scavenging system (pyranose oxidase at 3 U/mL, catalase, at final concentration of 90 U/mL and 40 mM glucose) was injected into the chamber for single-molecule data acquisition. Chambers where then sealed using Silicone Isolators™ Sheet Material (Grace Bio-labs). All single-molecule experiments were carried out at room temperature (22 °C).

**Single-molecule TIRF microscopy and data analysis.** Widefield fluorescence imaging for photostability measurements was conducted on an inverted microscope (Olympus IX-71 with UPlanSApo 100, NA 1.49, Olympus, Germany) in objective type TIRF) configuration as described before.[7] Images were collected with an electron multiplying CCD camera (C9100-13 (Hamamatsu, Japan), in combination with a Dual Line Laser Rejectionband ZET488/640 (AHF Analysentechnik)). Excitation intensities from a 647 nm diode laser (Cube (Coherent, Germany), filtered by ZET640/10 (Chroma, USA)) were estimated to be 90 W cm⁻² at the sample location unless stated otherwise. A movie was typically recorded for 400-500 s with an exposure time of 100 ms at room temperature. For data acquisition of blinking fluorophores, as well as respective negative controls, a Flat-top TIRF similar to [8] was used using an inverted microscope (Olympus IX-73 with UPlanSApo 100, NA 1.49, Olympus, Germany) in objective type TIRF), a 640nm laser (OBIS FP 640 Coherent, Germany, filtered by ZET640/10 (Chroma, USA)), a triple line dichroic with a dual-line emission filter (F73-435_T2, ZET488/640 respectively, (both AHF Analysentechnik)) and a back-illuminated sCMOS camera (pco.edge 4.2bi (PCO, Germany)). Fluorescent time traces were extracted from movies using an ImageJ plugin provided by the Duderstadt group.[9] For photostability and photoblinking measurements, Fluorophores were selected from the first frame or from an image containing the mean of the 20 brightest values of each pixel respectively. Fluorophores were selected based on a discoidal averaging filter criterium. Namely, if the mean counts of the inner 3x3 Pixels were at least 4 Standard deviations of background signal above the mean background signal. For this, a disk with 3 pixel width surrounding the 3x3 Pixel around the peak of the fluorophore was selected as background. Neighbouring peaks closer than 8 pixels were excluded from analysis. Timetraces were extracted from the same 3x3 Pixel...
areas and background corrected. Background was determined using a disc with a width of 2 pixels around the fluorophore. Timetraces involving fluorophores that bleach during the acquisition time of recorded movies show a drop of signal to zero counts, showing that background correction worked as expected.

Photophysical parameters shown in Figure 4 were extracted from timetraces using custom made python code using free open source python libraries. A two-state Hidden-Markov model using Viterbi-algorithm in combination with a thresholding approach was used to determine on and off states of fluorophores. A thresholding was used to circumvene problems of fitting two states in case of non-bleaching fluorophores. States were processed as “on” states if the mean counts during the respective state were above threshold. Thresholds were chosen for each timetrace automatically as 6 times the standard deviation of the detected background signal per pixel of fluorophore, which results in a total of 54 standard deviations. As output, plots of each timetrace with marked on and off states were saved and accuracy of detection was verified manually. The mean photon countrate for each trace was calculated as the mean of all timepoints of its on-states divided by exposure time and photon-conversion factor. SNR of each trace was determined as its mean counts of all timepoints in its on-state divided by the standard deviation of all timepoints in its on-states. Datasets contained multiple movies of multiple labelling repetitions. For summarization, each fluorophore was weighted by the inverse of the number of evaluated timetraces in the labelling-repeat to ensure no bias towards labelling-repeats with more fluorophores. Mean Photon rates and mean SNR were calculated as a weighted average over all evaluated timetraces. Standard deviations of Mean Photon rates and SNR were calculated via the biased weighted variance. For Photobleaching analysis as shown in Figure 4, the binary information about on- and off-states of each fluorophore from all movies were collected at each timepoint, summed for all molecules and normalized to the maximum. This was done for molecules from each labelling repeat separately. A monoexponential fit was then done with $Ae^{-t/\tau} + B$, while B was limited to not exceed 0.2. Mean bleaching times and standard deviations in Figure 4 refer to mean and standard deviations between fits from the different labelling-repeats. We would like to note, that due to very slow bleaching, B tends to increase even for very long observation times of 30 minutes, which decreases resulting $\tau$, leading to underestimation of $\tau$ in Figure 4 especially for 20-ATTO647N. Mean total Photon counts were calculated by multiplying the mean count rate with the mean photobleaching time. Standard deviations of total photon counts were calculated using standard error progression.

Timetraces that were detected as “on” for less than 20 frames were excluded from the evaluation since we could show that such signals come from fluorescent artifacts on the slide (data not shown). Based on this scheme, less than 3 fluorophores per movie were excluded. To rule out variation in fluorophore intensity due to lower excitation power from gaussian-shaped laser profile, we excluded fluorophores that were closer that 20 Pixels to the border of the illumination.
Photoblinking was evaluated from timetraces. First, timetrace intensities were histogramed and a two-level gaussian distribution was fit to the histogram, estimating means and occurrence of on- and off-states, being used as priors for a Hidden-Markov Model using Viterbi algorithm. Changes in states as detected from Hidden-Markov model were then analyzed. Duration of each on- and off-state were extracted, excluding the final state if it was an off-state since such states cannot be distinguished from photobleaching. From the remaining state lengths, Duty cycle was calculated as the total on-time divided by the sum of total on-time and off-time. Next, the blinking frequency was calculated as the amount of completed blinking cycles divided by the time from the beginning of the movie until the end of the last detected on-state. The resulting Switching Frequency - Duty cycle diagrams allow visualization of the correlation between the two quantities, which are key parameters for STORM-type super-resolution microscopy, since both values limit the minimal resolvable distance between labels. In the presented histograms, the column with the lowest blinking frequencies divides into completely non-blinking fluorophores (high Duty Cycle) and fluorophores that e.g. start “on”, transverse into a long dark state and subsequently turn “on” again. The presented way of evaluating the blinking dynamics may however lead to misleading determination of the blinking frequency in cases with multi modal blinking behavior, where fluorophores exhibit e.g. periods of fast blinking alternating with long lasting non-fluorescent-periods (e.g. Figure S38B, bottom right trace).

Confocal ALEX smFRET experiments. Single-molecule ALEX confocal microscopy experiments were performed at room temperature (21 °C) using a home-built confocal microscope described in more detail by Van der Velde et al. [11] The different ALEX smFRET experiments were subsequently carried out with 50 pM double-labeled DNA samples in PBS imaging buffer (pH 7.4) with 2 mM Trolox in some cases, as described in detail by Gouridis et al. [3] Before every experiment, the alignment of the microscope was confirmed with two dsDNA-fluorophore Gold standards in PBS imaging buffer with 1 mM Trolox (50 pM DNA Cy3B-Atto647N, 13 base pairs and 23 base pairs, E*: 0.58-0.67 and E*: 0.25-0.28 respectively). The applied laser powers per experiment are written under Figure 5 in the manuscript. In general, all samples were measured over the course of 0.5 hour.

Widefield fluorescence imaging of E-coli cells. Labelled cells were centrifuged at 3000 rcf for 5 minutes and resuspended in PBS buffer. 3 μL of the labelled cell solution (OD = 0.6) was transferred onto a microscope coverslide and covered by an agarose gel pad. The cells’ bright-field images were observed under Zeiss Axio Observer Z1 microscope equipped with a Hamamatsu OrcaR2camera. A Plan-Apochromat 100 × 1.4 Oil Ph3 objective (Zeiss) was used and the fluorescence was visualized with filter set 63 HE. ImageJ was used to analyse the micrographs.
Confocal and STED microscopy for cellular imaging (including image analysis)

Cell culture. HeLa-H2B-GFP\textsuperscript{[12]} cells and HeLa cells expressing H2B-mRFP-EGFP-alpha-Tubulin\textsuperscript{[13]} were cultured in DMEM (Sigma Aldrich) medium supplemented with 10% FCS and antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin) at 37 °C and 5% CO\textsubscript{2}. Cells tested negative for mycoplasma contamination.

Immunostaining. Immunodetection was performed on cells grown to 60–80% confluency on 8-well glass bottom slides (Ibidi). H2B-GFP and EGFP-alpha-Tubulin was detected with either GFP-Binder-Atto647N or with GFP-Binder-20-Atto647N.

Cells were washed with PBS (pH = 7.4 w/o Ca/Mg) and fixed in 4% paraformaldehyde in PBS for 10 min followed by 2 × 5 min washing with PBS. The samples were permeabilized with 0.5% Triton X-100 for 10 min and subsequently washed 2 × 5 min with 0.02% Tween. Cells were incubated for 1 h in blocking buffer containing 0.02% Tween and 2% BSA Albumin Fraction V (Sigma Aldrich). Nanobodies were diluted in blocking buffer and incubated for 1 h followed by washing with 2 × 5 min with 0.02% Tween. Imaging chambers were filled with oxygen scavenging solution (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose in PBS) and sealed with Silicone Isolator Sheet Material (Grace Bio-labs) to prevent oxygen diffusion from air. Concentrations refer to % in PBS buffer unless stated otherwise.

STED and confocal microscopy. Super-resolution and Confocal microscopy images were acquired using a 2C STED 775 QUAD Scan microscope (Abberior Instruments) with a 100x 1.4NA UPlanSApo oil immersion objective lens (Olympus) and immersion oil with refractive index of RI = 1.518. Confocal and STED were performed with a 640nm excitation laser. For STED, an additional 775nm laser for depletion was used. Images were acquired with a pixel size of 20 nm. For Photobleaching analysis, 10 patches of 2.8 µm x 2.8 µm from 3-5 different cells were acquired for each repeat. 10 frames were acquired for each patch with a dwell-time of 10 µs per pixel and a line accumulation of one or 10 in the case of confocal and STED respectively. Those measurements were repeated 4 times for each imaging condition with freshly grown and labelled cells.

Image analysis. Images were pre-processed by cropping the borders by 20 pixels to avoid boundary effects. After background subtraction, intensity measurements for each single patch were normalized to their maximal mean intensity and fitted using a monoexponential decay \( I(t)=Ae^{-t/\tau}+B \) with \( \tau \) the bleaching time. Bleaching times of all measurements were compared between Atto647N labelled and 20-Atto647N labelled cells. Statistical significance between the results of each condition were inferred through a two-sided Welch’s t-test for the null hypothesis that both labels have identical mean \( \tau \). For 27.7 µW and 56.6 µW confocal excitation intensity, as well as 95.5mW
STED-laser intensity with 27.7 µW and 56.6 µW confocal excitation intensity, this resulted in p-values of 2.4*10⁻⁶, 5.9*10⁻¹⁰, 7.1*10⁻⁶ and 6.7*10⁻⁴, respectively. The specified laser intensities were measured separately in the back focal plane of the objective.

STED resolution and peak separation (Figure 5C, right) of intensity profile indicated in Figure 5C was determined through Least-Square fitting using a double-Lorentzian function

\[
\frac{A_1}{\pi \gamma_1} \left( \frac{\gamma_1^2}{(x - \mu_1)^2 + \gamma_1^2} \right) + \frac{A_2}{\pi \gamma_2} \left( \frac{\gamma_2^2}{(x - \mu_2)^2 + \gamma_2^2} \right) + B.
\]

Here, A₁, A₂ are the respective peak amplitudes, μ₁ and μ₂ the respective Peak Positions, the FWHM as γ₁/(2*ln(2)) and γ₂/(2*ln(2)) (where ln depicts the natural logarithm) and B an offset value. Confocal Data in Figure 5C (right) was analysed by a least-square fit with a gaussian function

\[
A \cdot e^{-0.5 \frac{(x-\mu)^2}{\sigma^2}} + B
\]

with A the amplitude, μ the peak position, σ² the variance and B an offset value.
Part IV: Supplementary data and figures

Supporting Movies

Supporting Movie 1. Fluorescence microscopy of commercial sCy5 maleimide, 20-sCy5 and 21-sCy5 on SBD2 in direct comparison over a period of 5 minutes.

Supporting Figures

Figure S32. ÄKTA chromatograms of control experiment of SBD2-T369C incubated with azide-functionalized dyes in presence of linker molecules. The black and blue curves show absorbance at 280 nm (protein absorbance), the red and green curves show absorption at 548 nm (sCy3 absorbance maximum) and at 541 nm (TMR absorbance maximum). Both were incubated in presence of linker molecules but in click reaction condition.

Figure S33. ÄKTA chromatograms of SBD2- T369C labelled by (A) maleimide-functionalized dyes, (B) 19 and then azide-functionalized dyes (C) 20 and then azide-functionalized dyes. In each graph, one curve shows absorbance at 280 nm (protein absorbance), the other curve shows absorption at the dyes’ absorbance maximum. The dyes used are sCy3, TMR, sCy5 and Atto647N.
Figure S34. (A) Synthesis route of the linker compound 22 with the acrylamide functional group at bioactive site. (B) AKTA chromatogram of SBD2-T369C protein labelling via 22 then clicked by sCy5-azide. Compound 22 is an analogue of compound 19. Compound 19 labels the protein with maleimide and 22 with acrylamide. Both bioconjugation reactions proceed via a thiol-Michael addition.

Figure 35. UV-VIS absorption spectra of functionalized oligonucleotides: double stranded DNA (P1 annealing with P2) labelled by fluorophores (A) ATTO647N and (B) SiR, either directly via fluorophore-maleimide group or via linker-fluorophore click reaction. The labelled DNA showing the characteristic peak of DNA in the UV range and the visible absorption of the dye. Control shows DNA with thiol labelled by azide dyes in absence of linker compounds.
Figure S36. AKTA chromatograms of SBD2-T369C labelled by (A) azide-functionalized sCy5, (B) 18 and then azide-functionalized sCy5 (C) 21 and then azide-functionalized sCy5.
Figure S37. Representative single fluorescence time traces of SBD2-T369C protein labelled with azide-functionalized sCy5 linked via compound 18 (A) in oxygen scavenging buffer (2.5 mM protocatechuic acid (PCA), 50 nM protocatechuate-3,4-dioxygenase (PCD)) and (B-C) in presence of (B) 5 μM CuSO₄ and (C) 10 μM EDTA in oxygen scavenging buffer (2.5 mM protocatechuic acid (PCA), 50 nM protocatechuate-3,4-dioxygenase (PCD) and 2 mM Trolox in 10mM MOPS, pH 7.0).
Figure S38. (A) Schematic view of SBD2-T369C labelled by azide-functionalized ATTO647N using our linker labelling protocol for single-molecule characterization and normalized decay of number of single emitters. Representative single fluorescence time traces of SBD2-T369C protein labelled with azide-functionalized sCy5 linked via compound 21 in presence of (B, C) 5 μM CuSO₄ with the exposure time 20 ms (B) and exposure time of 100 ms (C) and (D) 10 μM EDTA in oxygen scavenging buffer (2.5 mM protocatechuic acid (PCA), 50 nM protocatechuate-3,4-dioxygenase (PCD) and 2 mM Trolox in 10mM MOPS, pH 7.0).
Figure S39. Blinking properties of SBD2-T369C protein labelled with azide-functionalized sCy5 linked via compound 18/21 in presence of Cu$^{2+}$ (5 µM, A) or EDTA (10 µM, B) in oxygen scavenging buffer (2.5 mM protocatechuic acid (PCA), 50 nM protocatechuate-3,4-dioxygenase (PCD) and 2 mM Trolox in 10mM MOPS (pH 7.0). All data was recorded with 100 ms exposure time.
**Figure S40.** Further details of photophysical characterization of SBD2-T369C labelled by maleimide-functionalized ATTO647N in different conditions. (A) Schematic view of immobilized ATTO647N-maleimide-functionalized SBD2-T369C for single-molecule characterization. (B) Normalized decay of number of single emitters and representative fluorescence time traces of respective single emitters (C-E) of maleimide-functionalized Atto647N on protein in (C) absence and (D) presence of 2 mM TX or (E) 2 mM COT respectively. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, Tris-HCl, pH 7.4). Laser power: 90W/cm².
Figure S41. Further details of photophysical characterization of SBD2-T369C labelled by azide-functionalized ATTO647N using our linker labelling protocol. (A) Schematic view of the labelled protein and the experimental strategy for single-molecule surface immobilization. (B) Normalized decay of number of single emitters and (C-E) representative fluorescence time traces of respective single emitters of azide-functionalized ATTO647N labelled on protein through linker compounds (C) 18, (D) 19 and (E) 20. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, Tris-HCl, pH 7.4). Laser power: 90W/cm².
Figure S42. Further details of photophysical characterization of oligonucleotides labelled by maleimide-functionalized Atto647N in different conditions. (A) Schematic view of the maleimide-functionalized Atto647N on the protein and the experimental strategy for single-molecule surface immobilization. (B-E) Normalized decay of number of single emitters and (B) representative fluorescence time traces of respective single emitters (C-E) of maleimide-functionalized ATTO647N on DNA in (C) absence and (D,E) presence of (D) 2 mM TX and (E) 2 mM COT. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, PBS, pH 7.4). Laser power: 90W/cm².
Figure S43. Further details of photophysical characterization of oligonucleotides labelled by azide-functionalized ATTO647N using our linker labelling protocol. (A) Schematic view of the labelled protein and the experimental strategy for single-molecule surface immobilization. (B) Normalized decay of number of single emitters and (C-D) representative fluorescence time traces of respective single emitters of azide-functionalized ATTO647N labelled on DNA through linker compounds (C) 19 and (D) 20. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, PBS, pH 7.4). Laser power: 90W/cm².
Figure S44. Further details of photophysical characterization of SBD2 labelled by maleimide-functionalized sCy5 in different conditions. (A) Schematic view of the maleimide-functionalized sCy5 on the protein and the experimental strategy for single-molecule surface immobilization. (B) Normalized decay of number of single emitters and (C-E) and representative fluorescence time traces of respective single emitters of maleimide-functionalized sCy5 on protein in (C) absence and presence of (D) 2 mM TX and (E) 2 mM COT. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, Tris-HCl, pH 7.4). Laser power: 90W/cm².
Figure S45. Further details of photophysical characterization of SBD2 labelled by azide-functionalized sCy5 using our linker labelling protocol. (A) Schematic view of the labelled protein and the experimental strategy for single-molecule surface immobilization. (B) Normalized decay of number of single emitters and (C-D) representative fluorescence time traces of respective single emitters of azide-functionalized sCy5 labelled on protein through linker compounds (C) 19 and (D) 20. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, Tris-HCl, pH 7.4). Laser power: 90W/cm².
Figure S46. Further details of photophysical characterization of oligonucleotides labelled by maleimide-functionalized sCy5 in different conditions. (A) Schematic view of the maleimide-functionalized Atto647N on the protein and the experimental strategy for single-molecule surface immobilization. (B) Normalized decay of number of single emitters and (C-E) representative fluorescence time traces of respective single emitters of maleimide-functionalized sCy5 on DNA in (C) absence and presence of (D) 2 mM TX and (E) 2 mM COT. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, PBS, pH 7.4). Laser power: 90W/cm².
Figure S47. Further details of photophysical characterization of oligonucleotides labelled by azide-functionalized sCy5 using our linker labelling protocol. (A) Schematic view of the labelled protein and the experimental strategy for single-molecule surface immobilization. (B) Normalized decay of number of single emitters and representative fluorescence time traces of respective single emitters of (C,D) azide-functionalized sCy5 labelled on DNA through linker compounds (C) 19 and (D) 20. All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, PBS, pH 7.4). Laser power: 90 W/cm².
**Figure S48.** Further details of photophysical characterization of oligonucleotides labelled by azide-functionalized SiR using different labelling protocol. (**A**) Schematic view of the labelled protein and the experimental strategy for single-molecule surface immobilization. (**B**) Normalized decay of number of single emitters and representative fluorescence time traces of respective single emitters of (**C**) maleimide-functionalized SiR labelled on DNA (**D**) azide-functionalized sCy5 labelled on DNA through linker compounds 18 (**D**) and 20 (**E**). All measurements were done in oxygen scavenging buffer (3 U/mL of pyranose oxidase, 90 U/mL of catalase and 40 mM glucose, PBS, pH 7.4). Laser power: 250 W cm$^{-2}$. 

**A**

**B**

**C**

**D**

**E**

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Figure S49. ES histograms of the confocal smFRET experiments for the application of the photostabilizing ability of linker 20 for fluorophores labelling on dsDNA in comparison to labelled by maleimide fluorophores at different laser powers (G: green laser, R: red laser). Oligonucleotide P1 (5′-Cy3B- TAA TAT TCG ATT CCT CTG GAC G-3′) annealed with oligonucleotide P2 (5′-CGT CCA GAG GAA T*CG AAT ATT A-3′ (T* = T labelled by ATTO647N) in (A) absence and (B) presence of the photostabilizer trolox (TX, 2 mM) in PBS pH 7.4. (C) Oligonucleotide P1 (5′-Cy3B- TAA TAT TCG ATT CCT CTG GAC G-3′) annealed with oligonucleotide P2 (5′-CGT CCA GAG GAA T*CG AAT ATT A-3′ functionalized with 20 at T* and then clicked by azide-functionalized ATTO647N in PBS pH 7.4. In all cases, the same image settings and data processing were used at the according laser intensities.
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