Supporting Information

Boron-Containing Probes for Non-optical High-Resolution Imaging of Biological Samples

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SUPPORTING INFORMATION

1. Materials and General Methods for Chemical Synthesis

Reagents. Starting materials and reagents were of the highest grade available from commercial sources and were used as delivered. Fmoc-protected amino acids, coupling reagents and Sieber amide resin were purchased from Iris Biotech (Marktredwitz, Germany) and Carl Roth (Karlsruhe, Germany). Star635-NHS ester and Star635 maleimide were obtained from Abbeior (Göttingen, Germany). Two non-conjugated FluoroTag®-Q anti-green fluorescent protein (GFP) nanobodies addressing independently two epitopes of GFP (here named as GFP-Nanobody 1, GFP-Nb#1 and GFP-Nanobody 2, GFP-Nb#2) were supplied from NanoTag Biotechnologies (Göttingen, Germany). Flash column chromatography on silica was performed using Macherey-Nagel Silica Gel 60 with a particle size of 0.063–0.2 mm (Düren, Germany). Thin layer chromatography (TLC) was conducted on aluminum backed plates of silica gel 60 F254 (layer thickness: 0.20 mm) from Merck (Darmstadt, Germany) to detect UV-active spots by fluorescence quenching at 254 nm and 366 nm. Non-fluorescence quenching substances containing boron were visualized by staining with 0.5% (w/v) PdCl₂ in 10% acq. HCl/aceton and subsequent careful heat-drying.

Solvents. Anhydrous solvents (toluene, acetonitrile) of extra dry or puriss. absolute grade (over molecular sieves) were obtained from Acros-Organics (Geel, Belgium) and Sigma Aldrich (Schnelldorf, Germany). Deuterated dimethyl sulfoxide (DMSO) and chloroform were purchased from Deutero (Kastellaun, Germany). Technical solvents were distilled prior to use in flash column chromatography. Acetonitrile and methanol (MeOH) for high-performance liquid chromatography (HPLC) in respective grade and all other solvents of the grade puriss. p.a. were purchased from commercially available sources and were used as supplied. Water for HPLC (milliQ H₂O) and buffers was purified using MilliQ Advantage A10 from Merck Millipore (Darmstadt, Germany). Buffers were degassed by stirring under vacuum and kept under argon atmosphere afterwards. The pH of buffers was adjusted with a pH meter from Hanna Instruments (Vöhringen, Germany) before every use.

Reactions. All air- and water-sensitive reactions were conducted under inert atmosphere (argon or nitrogen). Therefore, glass equipment utilized for large scale reactions was flame-dried before use. A purge-and-refill technique was applied for small reactions, which were performed in non-heatable tubes.

Instruments. ¹H-, ¹³C- and ¹¹B-nuclear magnetic resonance (NMR) spectra were performed using a Bruker Avance III 400 (AV-401). Chemical shifts are quoted in ppm (TMS = 0 ppm). The resonances of the rest protons of deuterated solvents were taken as internal standards. ¹³C- and ¹¹B-NMR spectra were recorded as broadband ¹H-decoupled spectra. Electrospray-ionization (ESI) mass spectra were recorded with a maXis and a micrOTOF spectrometer from Bruker. Matrix-assisted laser desorption/ionization (MALDI) spectra were recorded on a MALDI TOF Autoflex Speed from Bruker. The values are given as mass per charge (m/z). Microwave-mediated manual solid phase peptide synthesis (SPPS) was realized on a CEM Discover microwave instrument, while automatized SPPS was performed on a CEM LibertyBlue (Kamp-Lintfort, Germany). Reverse-phase HPLC was performed on a JASCO (Groß-Umstadt, Germany) with a two pump system PU-2080Plus, a multi wavelength detector MD-2010Plus with an analytical or preparative cell, a 3-line degasser DG-2080-SS, and an interface LC-Net II/ADC using a Nucleodur® RP C-18 analytical HPLC column (250 x 4.6 mm, 5 µm) and a C-18 semipreparative HPLC column (250 x 10 mm, 5 µm) from Macherey-Nagel (Düren, Germany). Reverse-phase HPLC runs were recorded on ChromNav as system software and carried out using a linear gradient of 0.1% acq. TFA (solvent A) and CH₃CN/0.1% TFA (solvent B) in 30 min. Flow rates were taken as 1 mL/min for the analytical, and 3 mL/min for the semi-preparative purpose. The UV absorbance of peptides could be detected at three different wavelengths (215 nm, 254 nm and 280 nm) simultaneously. For the detection of compounds having Star635 fluorophores, its respective major absorption wavelength was used for detection instead of 280 nm. Size-exclusion (SE-) HPLC was performed on an Äkta pure 25 instrument from GE Healthcare Life Science with internal Unicorn 7.0 software using Superdex 75 Increase 10/300 GL, a column from GE Healthcare Life Science (Amersham, United Kingdom). Isocratic elution with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) as mobile phase with a flow rate of 0.8 mL/min was applied. The samples were eluted within 1.5 column volume, which corresponds to 23.562 mL and detected by UV-absorbance at 280 nm and 635 nm (fluorophore-conjugated probes). Freeze-drying of compounds from aqueous solutions containing minimal amounts of MeOH or acetonitrile was performed using a Christ-Alpha 2-4 lyophilizer attached to a high vacuum pump and a Christ RCV-2-18 ultra Centrifuge (Osterode am Harz, Germany). UV-Spectra for determining the concentration of fluorophore-labeled peptides were recorded with a Thermo Scientific Nanodrop 2000c (Waltham, Massachusetts, USA). Gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were visualized using an Amersham Imager 600 from GE Healthcare Life Science.
2. Materials and General Methods for Biochemical and Cellular Experiments

**Materials.** Cell culture reagents and substances were purchased either from Sigma Aldrich (now Merck, Darmstadt, Germany) or AppliChem GmbH (Darmstadt, Germany), unless stated otherwise. The transfection reagent Lipofectamine® 2000, Opti-MEM, and the Click-iT® Cell Reaction Buffer Kit were obtained from Life Technologies (now Thermo Fisher Scientific, Darmstadt, Germany). The clickable unnatural amino acid (UAA) propargyl-L-lysine (PRK) was purchased from Sirius Fine Chemicals SiChem GmbH (Bremen, Germany). Click-iT® L-homopropargylglycine (HPG) and Hoechst staining solution were acquired from Thermo Fisher Scientific GmbH (Dreieich, Germany).

**Cells.** Three different cell lines were utilized for this study: baby hamster kidney (BHK), cell line derived from monkey kidney (COS-7) and human embryonic kidney (HEK)293 cells. The cells were maintained in culture in Dulbecco’s Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Darmstadt, Germany) to which a final concentration of 10% tryptose phosphate (only for BHK), 5% fetal calf serum, 2 mM L-glutamine, 60 U/mL penicillin, and 60 U/mL streptomycin were added.

**Constructs.** The Amber stop codon-containing Syntaxin1-(yellow fluorescent protein) YFP vector DNA[1] and TOM70-eGFP (mentioned in the rest of manuscript as TOM70-GFP) were produced in-house. The vector pCMV tRNA/RS<sup>WT</sup> was provided by Lemke laboratory.
3. Synthesis of Carborane-Tags

3.1 BorEncode

Supporting Scheme S1: Synthesis of BorEncode for click-based strategy.

Fmoc-Lys(Fmoc)-Asp(O"Bu)-Glu(O"Bu)-Lys(N<sub>2</sub>)-Asp(O"Bu)-Glu(O"Bu)-Lys(Boc)-Gly-Sieber amide resin (1)

C<sub>60</sub>H<sub>72</sub>N<sub>15</sub>O<sub>17</sub> [1030.66] (for cleaved and fully deprotected peptide)

Manual Fmoc-based SPPS of compound 1 was performed under microwave irradiation starting with a 50 µmol scale on Sieber amide resin (0.76 mmol/g). The resin was swollen in a BD syringe for 1 h in N,N-dimethylformamide (DMF)/dichloromethane (DCM) (1:1) followed by washing with DMF (3×), DCM (3×) and N-methyl-2-pyrrolidin (NMP) (3×). Each coupling cycle was started by microwave supported Fmoc-deprotection, applying 20% piperidine in NMP 0.1 M 1-hydroxybenzotriazole (HOBr) (2×, 30 sec, 50 W, 50 °C and 3 min, 50 °C, 25 W) followed by successive washing with DMF (3×), DCM (3×) and NMP (3×). The following amino acid building...
blocks were used for synthesis: Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(OBu)-OH, Fmoc-Asp(OBu)-OH, Fmoc-Lys(N2)-OH, and Fmoc-Lys(Fmoc)-OH. For pre-activation and coupling, the respective Fmoc-protected amino acid (5.0 eq), O-(benzotriazole-1-y)-N,N',N'-tetramethyluronium hexafluorophosphate (HTBU) (4.5 eq) and HOBT (5.0 eq) were dissolved in NMP (1.75 mL). Directly before transferring the coupling cocktail to the resin, N,N-diisopropylethylamine (DIEA) (10 eq) was added to activate the mixture.

Single coupling was performed for all amino acids except the first amino acid (double coupling). Each coupling was carried out by microwave irradiation (10 min, 50 °C, 25 W) followed by washing with DMF (3×), DCM (3×), NMP (3×). After the final coupling cycle, the resin was washed with DMF, DCM, NMP, DCM, MeOH, DCM (3× each), dried in vacuo and stored at -21 °C until been further processed.

Test cleavage revealed H-Lys-Asp-Glu-Lys(N2)-Gly-Asp-Glu-Lys-Gly-NH2: Analytical data: HPLC (C-18, analytical, gradient 5 → 90% B in 30 min): tR = 9.22 min. m/z (ESI) = 515.8 [M + 2H]2+, 1031.5 [M + H]−, 1028.5 [M − H]−, m/z (HR-ESI-MS) = calculated: 515.7492 [M+2H]2+, 1030.4912 [M+H]−, 1028.4767 [M − H]−, found: 515.7495 [M+2H]2+, 1030.4910 [M+H]−, 1028.4725 [M + Na]−.

SA-Lys(SA)-Asp(OBu)-Glu(OBu)-Lys(N2)-Gly-Asp(OBu)-Glu(OBu)-Lys(Boc)-Gly-Sieber amide resin (2)

The fully-protected peptide on resin 1 (14.0 µmol, 0.76 mmol/g) was swollen for 1 h at room temperature (RT) in DMF/DCM (1:1). Then, the Fmoc- groups were cleaved with 20% piperidine in NMP + 0.1 M HOBt (2 × 20 min) followed by washing with DMF (3×), DCM (3×), NMP (3×). Succinic anhydride (SA) (14.0 mg, 140 µmol, 10 eq) was dissolved in NMP (586 µL) and treated with DIEA (24.4 µL, 18.1 mg, 140 µmol, 10 eq). After short mixing, the clear solution was transferred to the resin. The resin was shaken for 1 h at RT and washed afterwards with DMF, DCM, NMP (3× each). The SA functionalization reaction was repeated once followed by final washing with NMP, DCM, NMP, MeOH and DCM (3× each). The resin was dried in vacuum and used in the following step.

Test cleavage revealed (SA)-Lys(SA)-Asp-Glu-Lys(N2)-Gly-Asp-Glu-Lys-Gly-NH2: Analytical data: HPLC (C-18, analytical, gradient 5 → 90% B in 30 min): tR = 10.57 min. m/z (ESI) = 626.8 [M + H + Na]2+, 1253.5 [M + H]−, 1252.5 [M + Na]−, 613.7 [M − 2H]3−, 1228.5 [M − H]−, m/z (HR-ESI-MS) = calculated: 1230.52333 [M+H]−, 1252.5052 [M + Na]−, 613.7507 [M − 2H]3−, 1228.5087 [M − H]−, found: 1230.5229 [M + H]−, 1252.5046 [M + Na]−, 613.7541 [M − 2H]3−, 1228.5141 [M − H]−.

o-CB-SA-Lys(o-CB-SA)-Asp-Glu-Lys(N2)-Gly-Asp-Glu-Lys-Gly-NH2 (3)

Under argon atmosphere the peptide on resin 2 (12.0 µmol, 0.75 mmol/g) was swollen for 1 h at RT in anhydrous DMF (250 µL) and DIEA (12.5 µL, 9.31 mg, 72.0 µmol, 6.0 eq). HATU (13.7 mg, 36.0 mg, 3.0 eq) in dry DMF (125 µL) was given to the solution and incubated for 30 min. Aminobuty1-ortho-carborane hydrochloride (o-CB)2 (13.5 mg, 51.7 µmol, 4.3 eq) was dissolved in dry DMF (125 µL), subsequently added to the resin and gently shaken overnight under ambient temperature. The resin was washed with DMF, DCM, MeOH and DCM (3× each). The cleavage of compound 3 from the solid support was performed by treating the resin with 20% cleavage cocktail (TFA/H2O/TIS, 95:2.5:2.5, v/v/v/v) in DCM (3 × 10 min). The collected fractions were mixed with concentrated cleavage cocktail (1:1) and incubated for an additional 0.5 h. The resulting solution was concentrated under an argon stream, followed by precipitation using cold Et2O. The resulting suspension was centrifuged at -4 °C. The supernatant was discarded and the

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1 The azido amino acid (5)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidoheptanoic acid, Fmoc-Lys(N2)-OH, was prepared as described in literature.[2] [3]  
2 Aminobuty1-ortho-carborane hydrochloride (o-CB) was synthesized according literature-known procedures.[4]  
3 Additional incubation time with concentrated cleavage cocktail is required to remove protecting groups completely.
peptide pellet was washed with cold Et₂O (3×) and dried in vacuo. The crude peptide was purified by HPLC followed by freeze-drying. The compound 3 (4.45 mg, 2.74 µmol, 23%) was obtained as a white solid.

Analytical data: HPLC (C-18, analytical, gradient 30 → 90% B in 30 min): t₀ = 19.78 min. m/z (ESI) = 824.5 [M + H + Na]⁺, 1626.0 [M + H]⁺, 811.6 [M − 2H]²⁺, 1624.1 [M − H]⁻. m/z (HR-ESI-MS) = calculated: 824.5120 [M + H + Na]⁺, 1626.0348 [M + H]⁺, 811.5065 [M − 2H]²⁺, 1624.0202 [M − H]⁻. found: 824.5138 [M + H + Na]⁺, 1626.0378 [M + H]⁺, 811.5108 [M − 2H]²⁺, 1624.0253 [M − H]⁻.

**α-CB-SA-Lys(α-CB-SA)-Asp-Glu-Lys(NH₂)-Gly-Asp-Glu-Lys(Star635)-Gly-NH₂, BorEncode**

C₁₀₅H₁₅₀B₂F₃N₂₀O₂₀S₂ [2502.85]

Peptide 3 (1.10 mg, 677 nmol, 2.68 eq) was dissolved in dry DMF (300 µL) and DIEA (3.54 µL, 20.3 µmol, 80 eq). The mixture was stirred after addition of Star635-NHS ester (250 µg, 253 nmol, 1.0 eq) in dry DMF (25 µL) for 21 h at ambient temperature and under light exclusion. Afterwards, the crude mixture was diluted in acetonitrile/H₂O followed by HPLC purification and freeze-drying. The product BorEncode (633 µg, 253 nmol, quant.) was obtained as a blue solid.

Analytical data: HPLC (C-18, analytical, gradient 35 → 90% B in 30 min): t₀ = 20.8–22.8 min. m/z (ESI) = 1274.4 [M + 2Na]²⁺, 624.8 [M − 4H]⁴⁺, 840.4 [M + Na − 4H]³⁻. m/z (HR-ESI-MS) = calculated: 857.4225 [M + 3Na]³⁺, 1274.6391 [M + 2Na]²⁺, 624.8177 [M + 4H]⁴⁺, 840.7533 [M + Na − 4H]³⁻, found: 857.4246 [M + 3Na]³⁺, 1274.6406 [M + 2Na]²⁺, 624.8203 [M + 4H]⁴⁺, 840.7557 [M + Na − 4H]³⁻.

Supporting Scheme S2: Testing BorEncode in a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction by using its precursor 3 and propargyl-L-lysine in the presence of copper(I).

**BorEncode-PRK (4)**

C₇₀H₁₂₅B₂₀N₉O₁₄S [1853.11]

BorEncode (167 µg, 103 nmol, 1.0 eq), PRK-HCl (27.3 µg, 103 nmol, 1.0 eq), CuSO₄·5H₂O (20.0 µg, 80.1 nmol, 0.78 eq) and sodium ascorbate (20.0 µg, 101 nmol, 0.98 eq) were dissolved under argon atmosphere in degassed EtOH/H₂O (4:1) and stirred overnight at room temperature. The reaction mixture was analyzed by analytical HPLC. The new formed product 4 was confirmed by HR-ESI-MS.

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¹ HPLC chromatogram of crude product showed two peaks in a 1:1 ratio (product/side product). The in situ HATU-activated carboxylic acids of SA in compound 2 can either react with two amino-ortho-carborane to the desired product 3 or the newly formed α-CB-mono-functionalized amide-nitrogen can attack the second, still activated succinic acid to form a 16-membered ring (HR-ESI-MS confirmed, data not shown here).
3.2 BorLink

Supporting Scheme S3: Synthesis of the thiol-reactive BorLink.
Automated Fmoc-based solid phase peptide synthesis (SPPS) of peptide 5 was performed with a 100 µmol scale on a Sieber amide resin (0.65 mmol/g). Before the resin was swollen for 1 h in DMF/DCM (1:1). Each coupling cycle was started by microwave supported Fmoc-deprotection, applying 20% piperidine in DMF 0.1 M HOBt (2×, 30 sec, 50 W, 50 °C and 3 min, 50 °C, 25 W) followed by washing steps. The following amino acid building blocks were used for synthesis: Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(O^Bu)-OH, Fmoc-Asp(O^Bu)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Lys(Fmoc)-OH. For pre-activation and coupling, the respective Fmoc-protected amino acid, O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU) in DIEA/DMF was applied. Single coupling was performed for all amino acids except the first amino acid (double coupling). Each coupling was carried out by microwave irradiation (10 min, 50 °C, 25 W) except for cysteine (10 min, 20 W, 40 °C). Afterwards, the resin was washed with DMF, DCM, NMP, DCM, MeOH, DCM (5× each), was dried in vacuo and stored at -21 °C until further processed.

Test cleavage revealed H-Lys-Asp-Glu-Cys-Gly-Asp-Glu-Lys-Gly-NH₂. Analytical data: HPLC (C-18, analytical, gradient 0 → 90% B in 30 min): tᵣ = 8.45 min. m/z (ESI) = 490.2 [M + 2H]²⁺, 979.4 [M + H]⁺, 488.3 [M – 2H]²⁻, 977.5 [M – H]. m/z (HR-ESI-MS) was calculated: 490.2111 [M + 2H]²⁺, 979.4149 [M + H]⁺, 1001.3969 [M + Na]⁺, found: 490.2115 [M + 2H]²⁺, 979.4149 [M + H]⁺, 1001.3961 [M + Na]⁺.

The peptide on resin 5 (25.0 µmol, 0.65 mmol/g) was swollen for 1 h at RT in DMF/DCM (1:1). Then the Fmoc-protecting groups were cleaved with 20% piperidine in NMP + 0.1 M HOBt (2×, 20 min) followed by washing with DMF (3×), DCM (3×), NMP (3×). Succinic anhydride (SA) (25.0 mg, 250 µmol, 10 eq) was dissolved in NMP (1000 µL) and treated with DIEA (43.5 µL, 32.3 mg, 250 µmol, 10 eq). After short mixing, the clear solution was given to the resin. The resin was shaken for 1 h at RT and purified by washing with DMF (3×), DCM (3×), NMP (3×). The coupling of succinic anhydride was repeated followed by a final washing step with NMP (3×), DCM (3×), MeOH (3×), DCM (3×). The resin was dried in vacuum and was ready for the next step.

Test cleavage revealed (SA)-Lys(SA)-Asp-Glu-Cys-Gly-Asp-Glu-Lys-Gly-NH₂. Analytical data: HPLC (C-18, analytical, gradient 0 → 90% B in 30 min): tᵣ = 10.67 min. m/z (ESI) = 601.2 [M + H + Na]³⁺, 1201.4 [M + H]⁺, 588.3 [M – 2H]²⁻, 1177.5 [M – H]. m/z (HR-ESI-MS) was calculated: 601.2181 [M + H + Na]³⁺, 1201.2091 [M + 2Na]⁴⁺, 1179.4470 [M + H]⁺, 1201.4290 [M + Na]⁺, found: 601.2173 [M + H + Na]³⁺, 1201.4272 [M + Na]⁺.

Under argon atmosphere the peptide 6 (22.0 µmol, 0.65 mmol/g) was swollen in a reaction tube at RT for 1 h in anhydrous DMF (400 µL) and DIEA (22.9 µL, 17.0 mg, 132 µmol, 6.0 eq). HATU (22.3 mg, 58.6 mg, 2.7 eq) in dry DMF (200 µL) was given to the solution and incubated for 30 min. Aminobutyl-ortho-carborane hydrochloride (o-CB)² (27.6 mg, 110 µmol, 4.98 eq) was dissolved in dry DMF (200 µL), subsequently added to the reaction tube, which was gently shaken overnight under ambient temperature. The resin was transferred to a BD syringe and washed with DMF (3×), DCM (3×), MeOH (3×) and DCM (3×). The cleavage of the carboranyl peptide 7 from the solid support was performed by treating the resin with 20% cleavage cocktail (TFA/EDT/H₂O/TIS, 95:2.5:2:5:1, v/v/v/v/v) in DCM (3 × 20 min). The collected fractions were incubated for an additional 1 h with concentrated cleavage cocktail (1:1).³ The resulting solution was concentrated under an argon stream, followed by precipitation using cold Et₂O. The resulting suspension was centrifuged at -4 °C. The supernatant was discarded and the peptide pellet was washed with cold Et₂O.

S8
Analytical data: HPLC (C-18, analytical, gradient 30 → 90% B in 30 min): t_R = 18.60 min. m/z (ESI) = 799.0 [M + H + Na]^{2+}, 1575.0 [M + H]^{+}, 1572.9 [M - H]. m/z (HR-ESI-MS) = calculated: 787.9847 [M + 2H]^{2+}, 798.9756 [M + H + Na]^{2+}, 1574.9620 [M + H], 785.9701 [M – 2H]^{2+}, 1572.9475 [M – H], found: 787.9842 [M + 2H]^{2+}, 798.9752 [M + H + Na]^{2+}, 1574.9619 [M + H], 785.9700 [M – 2H]^{2+}, 1572.9459 [M – H].

**Supporting Information**

The crude peptide was purified by HPLC and freeze-dried afterwards. The compound 7 (14.6 mg, 9.24 µmol, 42%) was obtained as a white solid.

The compound 7 (3.70 mg, 2.35 µmol, 4.79 eq) was suspended in degassed PBS (pH 7.26, 50 mM sodium phosphate buffer + 100 mM NaCl, 600 µL). Then, Abberior® Star635-maleimide (500 µg, 491 µmol, 1.0 eq) in dry DMF (50 µL) was added to the solution. After stirring for 2.5 h at ambient temperature and under light exclusion, the reaction mixture was stored overnight at +4 °C. Afterwards, the crude product was purified by HPLC. The compound 8 (1.08 mg, 417 nmol, 85%) was obtained as a blue solid after lyophilisation.

Analytical data: HPLC (C-18, analytical, gradient 45 → 90% B in 30 min): t_R = 14.41–16.23 min. m/z (ESI) = 887.3 [M + 3Na]^{3+}, 1308.4 [M + Na + H]^{2+}, 863.1 [M – 3H]^{3–}, 1295.1 [M – 2H]^{2+}. m/z (HR-ESI-MS) = calculated: 887.0833 [M + 3Na]^{3+}, 1308.1394 [M + Na + H]^{2+}, 863.0879 [M – 3H]^{3–}, 1295.1338 [M – 2H]^{2+}, found: 887.0832 [M + 3Na]^{3+}, 1308.1395 [M + Na + H]^{2+}, 863.0868 [M – 3H]^{3–}, 1295.1327 [M – 2H]^{2+}.

To the peptide 8 (1.05 mg, 407 nmol, 1.0 eq) a solution of DIEA (1.09 µL, 6.26 µmol, 15.4 eq) in dry DMF (290 µL) was transferred under argon atmosphere, followed by the addition of succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (1.35 mg, 4.04 µmol, 9.92 eq) in dry DMF (100 µL). After stirring for 10 h at ambient temperature and under light exclusion, the reaction mixture was diluted with 30% acetonitrile and TFA (2 µL)³ and purified by HPLC. The product BorLink (883 µg, 314 nmol, 75%) was obtained as a blue solid after lyophilisation.

Analytical data: HPLC (C-18, analytical, gradient 45 → 90% B in 30 min): t_R = 17.29–19.43 min. m/z (ESI) = 1404.8 [M – 2H]^{2+}, 1417.7 [M + Na + H]^{2+}. m/z (HR-ESI-MS) = calculated: 938.1314 [M + 3H]^{3+}, 1406.6935 [M + 2H]^{2+}, 1404.6789 [M – 2H]^{2+}, found: 938.1324 [M + 3H]^{3+}, 1406.6925 [M + 2H]^{2+}, 1404.6860 [M – 2H]^{2+}.

³ Subsequent addition of TFA is important, when bringing the reaction solution to aqueous environment. Otherwise the basic character will hydrolyse the maleimide moiety over time.
3.3 BorLink Nanobodies

**Supporting Scheme S4:** Synthesis of BorLink-GFP Nanobodies 1 and 2.

**General synthetic procedure**

The freeze-dried nanobody (0.5 mg, ~35 nmol, 1.0 eq) was resuspended in degassed milliQ-H_2O (200 µL). Degassed tris(hydroxymethyl)aminomethane (Tris) buffer (22.2 µL, 1 mM, pH = 7.5) was added to the solution to get a final buffer concentration of 100 mM Tris-buffer. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in degassed milliQ-H_2O (4 mg/mL, 12.3 µL, 50.2 µg, 175 nmol, 5 eq) was added to the nanobody solution and incubated for 30 min at ambient temperature. The buffer was changed to degassed PBS by passing through a column prepacked with Sephadex® G25. Afterwards, the resulting solution was cooled to 0 °C followed by the addition of BorLink in anhydrous DMSO (10 mM, 10 µL, 100 nmol, 2.86 eq). The reaction mixture was incubated for 2 h at 0 °C under argon atmosphere excluding light. Then, the reaction mixture was purified by SE-HPLC followed by analysis of the fractions by SDS PAGE. The BorLink-Nanobodies, isolated as blue solutions, were aliquoted and shock-frozen in liquid nitrogen. Afterwards the stock solutions were stored at −21 °C by excluding light and avoiding several freeze-thaw cycles.
4. Biochemical and Cellular Experiments

4.1 Click Chemistry Approach

Cells. For the application of BorEncode to label proteins via click chemistry, baby hamster kidney (BHK) fibroblasts were grown in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% tryptose phosphate, 5% fetal bovine serum (FBS), 2 mM L-glutamine, 60 U/mL penicillin and 60 U/mL streptomycin and passaged every 3–4 days.

HPG-Treatment. In order to label a large number of proteins with BorEncode, cells were grown on 18 mm glass coverslips (CS; ~50,000 cells/CS). The culture medium was replaced with methionine free DMEM, supplemented with 2 mM L-glutamine, 500 µM L-cysteine, 60 U/mL penicillin and 60 U/mL streptomycin 1 h before starting the experiment. Click-iT® L-homopropargyl glycine (HPG) was added to cells in a final concentration of 50 µM. Cells were incubated for 88 h at 37 °C and 5% CO₂, thereby incorporating HPG into every newly synthesized protein, then washed with cold PBS and fixed with 4% PFA in PBS for 30 min at RT.

Incorporation of UAA Into Proteins of Interest. BHK fibroblasts were cultured on CS (~20,000 cells/CS) in antibiotic free culture medium. 24 h after seeding, the UAA PRK was added to the cells in a final concentration of 250 µM. Furthermore, cells were transfected with two DNA vectors using Lipofectamine® 2000 reagent following the manufacturer’s instructions. Briefly, the DNA encoding the protein of interest (POI), which contains the Amber stop codon, and the vector for the expression of the tRNA/RSWT were equilibrated together in Opti-MEM for 5 min at RT. Lipofectamine was equilibrated separately in Opti-MEM, then added to the DNA constructs, mixed and incubated for 20 min at RT. Afterwards the DNA/Lipofectamine mixture was added to the cells and incubated for 18–24 h at 37 °C and 5% CO₂ to allow protein expression with incorporation of the PRK. Medium was exchanged to normal BHK culture medium ~4 h before fixation. Cells were briefly washed with cold PBS and fixed with 4% PFA in PBS for 30 min at RT.

Click Reaction. PFA-fixed cells were quenched in 100 mM NH₄Cl in PBS for 20 min at RT before they were permeabilized with 0.1% Triton-X 100 in PBS. Blocking of unspecific epitopes was performed by incubating samples in 5% BSA, 5% tryptone/peptone (Carl Roth GmbH, Karlsruhe, Germany) and 0.1% Triton-X 100 in PBS for 30 min at RT. Directly before reaction, cells were washed with 3% BSA in PBS and incubated in freshly prepared click solution in a dark humidified chamber for 30 min at RT. The click solution was composed of milliQ-H₂O, Click-iT® Cell Reaction Buffer Kit, containing Click-iT® reaction buffer (component A), 1.3 mM CuSO₄ (component B), Click-iT® reaction buffer additive (component C, all applied according to the manufacturer’s instructions) and 10 µM BorEncode. After the reaction cells were washed with 5% tryptone/peptone in PBS for 15 min.

Hoechst Labeling and Embedding for Epifluorescence Microscopy. After click reaction and washing cells with 5% tryptone/peptone in PBS, cell nuclei were labeled by 5 min incubation in 2 µM Hoechst in PBS at RT. Afterwards, samples were washed with PBS for a minimum of 30 min and embedded in Mowiol 4-88 mounting medium (Carl Roth GmbH, Karlsruhe, Germany).

4.2 Immunocytochemical Approach

Transfection. COS-7 and HEK293 cells were cultured in complete DMEM medium containing 10% FCS, 4 mM L-glutamine and 100 U/mL penicillin and streptomycin. For the experiment, ~500,000 cells per CS in DMEM without antibiotics were seeded into 12 well plates containing PLL-coated coverslips and incubated 3–6 h at 37 °C in a humid atmosphere with 5% CO₂. The cells were transfected following the instructions of the manufacturer using Lipofectamine® 2000 and the vector for the protein of interest, TOM70-GFP. The transfection was carried out by separately incubating the plasmids and the Lipofectamine® 2000 for 5 min in Opti-MEM Gibco®, then mixing the two solutions and waiting for further 20–25 min before applying them to the cells. The cells were allowed to express the proteins of interest for 12–19 h at 37 °C.

Immunostaining. Fixation was performed using 4% PFA in PBS. This was followed by quenching for 30–40 min in 100 mM glycine in PBS and a brief PBS wash before the samples were permeabilized and blocked with 2.5% BSA and 0.1% Triton X-100 in PBS for

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Hoechst Labeling and Embedding for Epifluorescence Imaging Purpose. The cell nuclei were labeled using 4 μM Hoechst in PBS for 5 minutes. This was followed by washing with PBS, high-salt PBS and PBS (5–10 min each) and mounting in Mowiol 4-88.

4.3 Plastic Embedding and Thin Sectioning

For nanoSIMS measurements, the samples were fixed with 4% PFA (+ 0.2% glutaraldehyde for the immunocytochemical approach) in PBS for 20 min, then quenched for 15 min with 100 mM NH₄Cl in PBS (+ 100 mM glycine in PBS for the immunocytochemical approach). Afterwards the samples were washed 3× for 5 min with PBS. The embedding in medium grade LR White resin (London Resin Company Ltd, Berkshire, England) was based on a previously described procedure. In short, samples were partially dehydrated in 30% EtOH (in milliQ-H₂O) for 10 min, and in 50% aq. EtOH (in milliQ-H₂O) 3× for 10 min. The samples were then incubated for 1 h in a 1:1 mixture of LR White and 50% aq. EtOH (in ddH₂O). Afterwards, the samples were transferred into pure LR White for 1 h. Finally, the cells were covered with BEEM® capsules (BEEM Inc., West Chester, PA, USA) and embedded in LR White plus LR White accelerator (London Resin Company Ltd) for 30 min on a pre-cooled metal-plate and afterwards for 90 min at 60 °C. The resulting samples were sectioned at a thickness of 200 nm using an EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). The thin sections were placed on silicon wafers (SIEGERT WAFER GmbH, Aachen).

5. Imaging

Epifluorescence Microscopy. To illustrate control conditions of BorEncode application (shown in Supporting Figure S2), an Olympus IX 71 inverted fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 20× objective (Olympus) was used. Images were acquired by a CCD camera (FView II, Olympus) and processed with the CellF software (Olympus). A 377/50 excitation filter, a 409 long pass beam splitter, and a 447/60 emission filter were used to detect Hoechst, and to visualize GFP a 480/40 HQ excitation filter, a 505 LP Q beam splitter, and 527/30 HQ emission filter were employed. To visualize BorEncode fluorescence, a 620/60 HQ excitation filter, a 660 LP Q beam splitter, and a 700/75 HQ emission filter were used. All filters were purchased from AHF (Tübingen, Germany).

For the immunostaining approach, proof-of-principle images (shown in Supporting Figure S3 A to D and F) were taken with an inverted microscope Nikon Ti Eclipse (Nikon Corporation, Chiyoda, Tokyo, Japan) equipped with a 0.75 NA/20× air and 100× oil objective and Nikon DS-Qi2 camera (Nikon GmbH, Düsseldorf, Germany) controlled via the NIS-Elements AR software (version 4.20; Nikon). Overview images (shown in Figure 2–4) of embedded slices to aid identification of probe-positive cells were taken with the same microscope using a 0.75 NA/40× air objective.

Confocal and STED Microscopy. Confocal and stimulated emission depletion (STED) images (shown in Supporting Figure S2 E and S3 E) were obtained employing a multicolor microscope (Abberior Instruments, Göttingen, Germany) based on a IX83 inverted microscope (Olympus) equipped with a UPLSAPO 100x 1.4 NA oil immersion objective (Olympus). BorEncode and BorLink were visualized by excitation with a 640 nm laser followed by signal detection between 650 and 720 nm. Depletion for BorEncode fluorophores was effected by 775 nm depletion laser (set to 40% of max. power of 1.2 W). To obtain the signal of the fluorescent protein (FP) reporter in the POI 485 nm excitation laser was employed, and signal was detected between 525–575 nm.

Secondary Ion Mass Spectrometry (SIMS) Measurement and Data Analysis. The SIMS analysis was performed with a nanoSIMS 50L instrument ( Cameca, France) equipped with a Hyperion II dual polarity oxygen source. The primary current of ∼30 pA (primary aperture D1=2) was used to produce secondary ions from the samples. The secondary ions were then focused via the ion optics, mass-separated by a magnetic sector for potential isobars, and were detected using parallel electron multipliers. Prior to each

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6 The required concentrations were determined by performing dilution series from both BorLink-GFP-Nb stocks and immunostaining experiments respectively, followed by the analysis of the epifluorescence images.
measurement, an implantation of O\(^+\) ions was performed with primary current intensity \(\sim 190\) pA \((D1=1)\) on an area larger than the imaged area in order to obtain the sputter steady state. Images with the raster size from 20×20 \(\mu\)m to 40×40 \(\mu\)m and 512×512 pixels were acquired though many planes, with a dwell-time of 6 ms/pixel. The following ions of interest were detected: \(^{11}\)B\(^+\), \(^{23}\)Na\(^+\), \(^{39}\)K\(^+\), and \(^{40}\)Ca\(^{2+}\), which are mentioned in the rest of the manuscript as \(^{11}\)B, \(^{23}\)Na, \(^{39}\)K, and \(^{40}\)Ca, respectively. Each ion images shown in all figures of the manuscript are the accumulation of five consecutive image layers.

**Data Analysis.** Images were processed using the Open MIMS Image plugin\(^7\) from FiJi and ImageJ (NIH, Bethesda, USA). Matlab routines (the Mathworks Inc, Natick, MA) were used for all data analysis in this manuscript. In brief, the average signal intensity in circular regions of interest (ROI) selected manually by an experimenter was calculated, and plotted in the different figures, after being normalized to the background intensities. The background was determined in similar ROIs selected by the experimenter outside the cells. For BorLink-Nb cell samples 150 transfected and 150 non-transfected ROIs were selected, for BorEncode cell samples 60 ROIs each, and for BorEncode-HPG 120 ROIs each.

### 6. Supporting Figures

![Supporting figure](image-url)

**Supporting Figure S1.** SDS PAGE of BorLink-GFP-Nanobodies 1 and 2 after SE-HPLC purification. The expected mass range of \(~18\) kDa is indicated by the SDS gel. FI-Red: Fluorescence, Epi-RGB (red), light 630 nm, filter Cy5. Marker = PageRuler protein ladder (ThermoScientific, Germany).

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\(^7\) The plugin has been developed at the National Resource for Imaging Mass Spectrometry (NRIMS, now Bringham and Women’s Hospital Center for Nanolmagin, BWH CNI).
Supporting Figure S2. General observations from fluorescence micrographs of BHK cells labeled with BorEncode. A) The incorporation of HPG into every newly synthesized protein enables click reaction with BorEncode to fluorescently label every cell. HPG here replaces methionine, and is therefore incorporated in every newly secreted protein, which renders the cells highly reactive to BorEncode. B) PRK is incorporated into POI, which also expressed a FP-reporter domain. BorEncode specifically labeled the POI via CuAAC, illustrated by colocalization of the BorEncode (red) and the FP (green) labeling in the overlay (Hoechst is shown in blue). C) Adding PRK to cells in the absence of the expressed POI resulted in no click reaction, as expected. D) In the absence of PRK (no UAA) no reaction occurs, as expected, confirming the specificity of the labeling. E) BorEncode applied for super resolution microscopy (STED) show precise labeling of the POI, which nicely overlaps with the confocal scan of FP signal. For better visualization brightness and contrast of some images were scaled; all images from the same color channel are scaled identically. Scale bar A) to D) 50 µm, and E) 10 µm.
Supporting Figure S3. Fluorescence micrographs of HEK293 (A to E) and COS-7 (F) cells transfected with Tom70-GFP and immunostained with BorLink-GFP-Nanobodies.

Two anti-GFP nanobodies (in short, GFP-Nb) were conjugated to BorLink, and were used to label cells transfected with a construct that places GFP on mitochondria (TOM70-GFP).

A) BorLink-GFP-Nb#1 shows specific labeling of GFP. The co-localization of BorLink (red) and GFP (green) is shown in the overlay (Hoechst is shown in blue). B) BorLink-GFP-Nb#2 also stains GFP specifically. C) and F) The same is observed when both nanobodies are used. D) No labeling in absence of POI. E) A confocal image of an immunostaining performed using both BorLink-GFP-Nbs #1 and #2 confirms the excellent specificity of the labeling.

A) to D) and F) indicate epifluorescence images, while E) shows confocal images. Scale bar: A) to D) 100 µm, E) 10 µm, and F) 20 µm.
Supporting Figure S4. Line scans on the ion images of a BHK cell labeled with BorEncode for determination of lateral resolution. (a, b, c) Ion images of $^{11}$B, $^{23}$Na, and $^{39}$K, respectively. The line scan was performed along the arrow in each selected zoom-in images. (d, e, f) Corresponding line scans of $^{11}$B, $^{23}$Na, and $^{39}$K.
Supporting Spectra S1: HPLC chromatogram of compound 3 (top) and HR-ESI-MS spectra of compound 3, [M + H]^+ selected as example (bottom).
Supporting Spectra S2: HPLC chromatogram of BorEncode (top) and HR-ESI-MS spectra of BorEncode, $[M - 4H]^4-$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635 under applied conditions.
Supporting Spectra S3: Analysis of the test reaction of BorEncode precursor 3 and PRK in the presence of copper(I) catalyst. HPLC chromatogram (top) showed a newly formed peak, while the peak belonging to the starting material 3 decreased. The formed triazole-conjugate 4 was confirmed by HR-ESI-MS (bottom, [M + 2H]^{2+} selected as example).
Supporting Spectra S4: HPLC chromatogram of compound 7 (top) and HR-ESI-MS spectra of compound 7, [M + H + Na]^{2+} selected as example (bottom).
Supporting Spectra S5: HPLC chromatogram of compound 8 (top) and HR-ESI-MS spectra of compound 8, [M + Na + H]^{2+} selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635 under applied conditions.
Supporting Spectra S6: HPLC chromatogram of BorLink (top) and HR-ESI-MS spectra of compound BorLink, [M – 2H]2– selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635 under applied conditions.
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Author Contributions

S.K. and S.O.R. designed the project. S.K. performed all chemistry work. K.A.S. and S.K. performed the click reactions. S. K. and F. O. performed the immunolabeling reactions. F.O. obtained the nanobodies. S. J. performed plastic embedding and thin sectioning. N.T. N. P. designed and supervised all SIMS work. P.A.G. and N.T. N. P. performed the SIMS imaging. P.A.G., S.O.R. and N.T. N. P. analyzed the images. All authors participated in writing the manuscript.

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