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Supporting Information

Absolute Quantification of Drug Vector Delivery to the Cytosol

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Experimental Procedures

HPLC purifications and UPLC-MS analyses

HPLC purifications were done using a Water Xbridge BEH 300 Prep C18 5 µm 30*150 mm column with a Waters 2545 Quaternary Gradient Module, a Waters 2998 Photodiode Array Detector, and a Waters FlexInject. Eluants used were: 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile. Pure fractions were freeze-dried using an ALPHA 2-4 LDplus freeze-dryer from CHRIST, with a Chemistry-HYBRID pump RC 6 from Vacuubrand.

Samples were analyzed with a Waters UPLC-MS comprised of an ACQUITY UPLC H-Class sample manager, an ACQUITY UPLC PDA eLambda Detector, and a Single Quadrupole Detector 2 for positive and negative Electron Spray Ionization (ESI) mass spectra. An ACQUITY UPLC BEH C18 1.7 µm 2.1x50 mm column was used. Solvents were: A - 0.1 % formic acid in Milli-Q water; B - 0.1 % formic acid in acetonitrile.

Cycles were: 0.2 min 5 % B for accumulation at the head of the column, followed by 2.3 min linear gradient from 5 % to 95 % B, 0.5 min 100 % B for washing, and 1 min at 5 % B for column equilibration.

Compound purities were calculated from UPLC analyses.

Synthesis of Maleimide-BG-biotin B, C and D

The compounds were synthesized through manual solid-phase peptide synthesis (SPPS), with the synthesis of Fmoc-E(OtBu)-K(Mtt)-K(biotin)-Rink amide resin for **B**, Fmoc-E(OtBu)-K(Mtt)-O2Oc-K(biotin)-Rink amide resin for **C**, and Fmoc-E(OtBu)-O2Oc-K(Mtt)-O2Oc-O2Oc-K(biotin)-Rink amide resin for **D** (see Supplementary Fig. S5). Fmoc-O2Oc-OH is a commercial PEG2 amino acid from Iris Biotech. Fmoc deprotection conditions: 10 mL 20 % piperidine in DMF 1 time 5 min and 2 times 10 min, followed by 5 washes with DMF for 1 min, and 1 DCM wash for 1 min. Coupling conditions: 2 eq amino acid, 4 eq DIPEA, and 2 eq PyBOP in DMF for 30 min, followed by three washes 1 min with DMF, and 2 washes with DCM for 1 min. After deprotection of the last amino acid, capping was performed with 10 eq DIEA, 5 eq acetic acid, and 5 eq PyBOP in DMF for 30 min. The Mtt protecting group was then removed by successive 1 % TFA in DCM 1 min treatments, followed by DCM washes. After having been dried, the resin for **B** and **C** was coupled under anhydrous conditions to 2.5 eq Mal-NHS with 5 eq DIPEA in DMF for 1 h, followed by DMF washes. For **D**, an elongation with one Fmoc-O2Oc-OH was done prior to coupling with Mal-NHS under the same conditions as described for **B** and **C**. Each peptide was then cleaved for 90 min from the resin in 95/2.5/2.5 TFA/TIS/H_2O, followed by precipitation in ether, and three ether washes. For **D**, the crude peptide was purified by HPLC with a 15 min 30 mL/min gradient from 15 to 30 % acetonitrile with 0.1 % formic acid (FA) in water 0.1 % FA. For **B** and **C**, crude peptides were directly used for the next step. SPPS yields: 43 % for **B**, 45 % for **C,** and 40 % for **D**.

Each peptide (**B**: 33 mg crude, 40 µmol; **C**: 41 mg crude, 42 µmol; **D**: 76 mg purified, 54 µmol) was coupled in DMF under argon with 2 eq BG amine in presence of 2 eq PyBOP and 5 eq DIPEA for 2 h. The gradient used for purification was for 15 min at 30 mL/min from 10 % to 35 % of acetonitrile 0.1 % FA in water 0.1% FA for **B**, and from 15 % to 35 % for **C** and **D**. After freeze-drying, 3.8 mg **B** were recovered as a white powder (yield 9 %, purity 96 %, MS m/z $C_{49}H_{67}N_{15}O_{11}S$ $[M+H^+]^+$ calculated: 1074.5, found: 1075.0; $[M-H^+]^-$ calculated: 1072.5, found: 1072.4). 15 mg **C** were recovered as a white powder (yield 29 %, purity 96 %, MS m/z $C_{55}H_{78}N_{16}O_{14}S$ $[M+H^+]^+$ calculated: 1219.6, found: 1219.6; $[M+2H^+]^{2+}$ calculated: 610.3, found: 610.9; $[M-H^+]^-$ calculated: 1217.6, found: 1218.2). To ensure good recovery from the flask, **D** was solubilized after freeze-drying in a small volume of water/acetonitrile 0.1 % FA mixture and freeze-dried again, yielding 37.9 mg **D** as a white solid (yield 42 %, purity 98 %, MS m/z $C_{73}H_{111}N_{19}O_{23}S$ $[M+H^+]^+$ calculated: 1654.8, found: 1655.4; $[M+2H^+]^{2+}$ calculated: 827.9, found: 828.3; $[M-H^+]^-$ calculated: 1652.8, found: 1653.4; $[M-2H^+]^{2-}$ calculated: 825.9, found: 826.2).

SUPPORTING INFORMATION

BG-biotin synthesis

BG-biotin, which was used to evaluate the efficiency of SNAP-tag reaction in NG-SNAP cell lysate, was synthetized similarly to Mal-BG-biotin molecules **B**, **C** and **D** (see above). First, the dipeptide Ac-Glu-Lys(biot) was synthetized using manual SPPS on a Rink amide resin. SPPS yield: 98%.

40 mg of crude dipeptide Ac-Glu-Lys(biot) (73 µmol, MW= 542.65 g/mol) were then coupled in DMF under argon with 1 eq BG amine, 2 eq PyBOP and 5 eq DIPEA for 1 h. The resulting product was purified by HPLC, yielding 19 mg of product as a white powder, out of which 9 mg were purified again by HPLC (20 min gradient from 5 % to 100 % acetonitrile 0.1 % TFA in water 0.1% TFA). 7 mg BG-Biotin were recovered from the second purification as a white powder (yield 25 %, purity 93 %, MS m/z $C_{36}H_{50}N_{12}O_7S$ $[M+H^+]^+$ calculated: 795.4, found: 795.9; $[M+2H^+]^{2+}$ calculated: 398.2, found: 398.5; $[M-H^+]^-$ calculated: 793.4, found: 793.4).

Preparation of STxB-BG-biotin conjugates

An engineered recombinant STxB with an additional C-terminal cysteine was purified as previously described[1]. The additional C-terminal cysteine is the only free cysteine per monomer, allowing controlled conjugation of one maleimide-containing molecule per monomer.

STxB conjugate **A** was obtained by first conjugating 1 equivalent commercial EZ-Link Maleimide-PEG2-biotin (Thermofisher Scientific) to the C-terminal cysteine of STxB-Cys at 5 mg/mL by overnight reaction at 21 °C against PBS 10 mM EDTA with 10 % DMSO. Excess Maleimide-PEG2-biotin was then removed by overnight dialysis in PBS at 4 °C. A second conjugation step on lysine positions was then done at 1 mg/mL STxB-biotin with 1 equivalent BG-NHS ester (BG-GLA-NHS, New England BioLabs Inc) by overnight reaction at room temperature in PBS with 10 % DMSO. Conjugate A was purified by overnight dialysis against PBS at 4 °C.

The STxB conjugates **B**, **C** and **D** were obtained by overnight reaction at 21 °C under agitation at 750 rpm of recombinant STxB-Cys at 1.5 mg/mL with 3 equivalents of **B**, **C** or **D** in PBS with 10 % DMSO. The conjugates were purified from excess maleimide-PEG2-biotin, BG-NHS ester or molecules **B**, **C** or **D** with Zeba Spin desalting columns (0.5 mL, 7 kDa Thermo Scientific).

For A, B, C and D, conjugate formation was confirmed by UPLC-MS analyses (Supplementary Fig. S6). Small aliquots were flash-frozen and stored at -20 °C. The concentration of STxB-Cys conjugates was determined by measuring absorbance at 280 nm with baseline correction at 340 nm using a Nanodrop 2000c (Thermo Scientific). The extinction coefficient was calculated by the addition of the extinction coefficient of STxB monomer calculated from Gill and von Hippel coefficients[2] (ε= 8370 $M^{-1}cm^{-1}$ for STxB monomer) to the extinction coefficient of BG (ε= 7100 $M^{-1}cm^{-1}$ for BG[3]) leading to ε= 15470 $M^{-1}cm^{-1}$ for STxB-BG-biotin monomer. All concentrations mentioned in the main article correspond to STxB pentamer.

Cys-TAT and Cys-TAT-PEG_6-GFWFG synthesis

Fmoc-NH-PEG_6-CH_2CH_2COOH (CAS: 882847-34-9) was purchased from ChemPep Inc.

Cys-TAT (CYGRKKRRQRRR peptide sequence) and Cys-TAT-PEG_6-GFWFG (CYGRKKRRQRRR-PEG_6-GFWFG peptide sequence) were obtained using automated SPPS with a Rink amide resin at 25 µmol synthesis scale on a Prelude peptide synthesizer (Gyros Protein Technologies, Inc). Deprotection was done with 2 x 2 mL 2 min 20 % piperidine in NMP, followed by 3 x 3 mL 30 s NMP washes. Coupling steps were done twice 10 min with 1300 µL 200 mM amino acid in NMP, 1000 µL 250 mM HCTU in NMP and 500 µL 1 M NMM in NMP, followed by 2 x 3 mL 30 s NMP washes. Capping was done 5 min with 2 mL 250 mM acetic anhydride in NMP and 500 µL1 M NMM in NMP, followed by 3 x 3 mL 30 s NMP washes. Cleavage was performed with 5 mL TFA/thioanisole/anisole/TIS/H_2O (82.5/5/5/2.5/5)

for 2 h under stirring. The cleavage solution was then precipitated in 40 mL cold diethyl ether. After 3 washes with cold diethyl ether, the precipitate was dried and dissolved in 10 % acetic acid and lyophilized.

For Cys-TAT, crude peptide obtained was purified by HPLC with a run for 12 min at 30 mL/min of water with 0.1 % formic acid, followed by a gradient from 0 to 100 % acetonitrile 0.1 % formic acid 30 mL/min for 10 min. Pure fractions from the different reactors were combined. To ensure good recovery from the flask, Cys-TAT was solubilized after freeze-drying in a small volume of water 0.1 % FA and freeze-dried again, yielding from 3 reactors at 25 µmol scale 57,7 mg Cys-TAT as a white powder (yield 46 %, purity 96 %, MS m/z $C_{67}H_{124}N_{34}O_{14}S$ $[M+H^+]^+$ calculated: 1662.0, found: 1662.6; $[M+2H^+]^{2+}$ calculated: 831.5, found: 831.8; $[M+3H^+]^{3+}$ calculated: 554.7, found: 555.3; $[M+4H^+]^{4+}$ calculated: 416.2, found: 416.6; $[M+5H^+]^{5+}$ calculated: 333.2, found: 333.2; $[M+6H^+]^{6+}$ calculated: 277.8, found: 278.0).

For Cys-TAT-PEG_6-GFWFG, crude peptide (from one reactor at 25 µmol scale: 44 mg obtained, yield 68 %, purity 90 %, MS m/z $C_{115}H_{187}N_{41}O_{26}S$ $[M+2H^+]^{2+}$ calculated: 1296.2, found: 1297.0; $[M+3H^+]^{3+}$ calculated: 864.5, found: 864.8; $[M+4H^+]^{4+}$ calculated: 648.6, found: 648.9) was directly used for BG-Biotin conjugate preparation.

Preparation of BG-biotin-TAT and BG-Biotin-TAT-PEG_6-GFWFG conjugates

TAT conjugate B: 10.5 mg Cys-TAT (6.3 µmol, MW 1662.0 g/mol) was reacted with 1.1 eq **B** for 3 h in 4.4 mL of a solution comprising 70 % 0.1 M phosphate buffer pH 7 and 30 % acetonitrile, sparged with argon prior to reaction to remove oxygen. The reaction mixture was then acidified with formic acid just before 0.2 µM filtration and HPLC purification with a gradient from 5 % to 50 % of acetonitrile 0.1 % FA in water 0.1 % FA for 15 min at 30 mL/min. To ensure good recovery from the flask, TAT conjugate B was solubilized after freeze-drying in a small volume of water and acetonitrile with 0.1 % FA and freeze-dried again. 4.6 mg of BG-biotin TAT conjugate B was recovered as a white powder (yield 27 %, purity 99 %, MS m/z $C_{116}H_{191}N_{49}O_{25}S_2$ $[M+2H^+]^{2+}$ calculated: 1369.1, found: 1369.1; $[M+3H^+]^{3+}$ calculated: 913.1, found: 913.2; $[M+4H^+]^{4+}$ calculated: 685.0, found: 684.6; $[M+5H^+]^{5+}$ calculated: 548.2, found: 548.3; $[M+6H^+]^{6+}$ calculated: 457.0, found: 456.9; $[M+7H^+]^{7+}$ calculated: 391.9, found: 391.8).

TAT conjugate C: 10 mg Cys-TAT (6 µmol, MW 1662.0 g/mol) was reacted at 5 mg/mL with 1.5 eq **C** for 4 h in a solution comprising 80 % 0.1 M phosphate buffer pH 7 and 20 % acetonitrile, sparged with argon prior to reaction to remove oxygen. The reaction mixture was then acidified with formic acid just before HPLC purification with a gradient from 5 % to 30 % of acetonitrile 0.1 % FA in water 0.1 % FA for 25 min at 30 mL/min. After freeze-drying, 6.8 mg of BG-biotin TAT conjugate C was recovered as a white powder (yield 39 %, purity 99 %, MS m/z $C_{122}H_{202}N_{50}O_{28}S_2$ $[M+2H^+]^{2+}$ calculated: 1441.7, found: 1441.4; $[M+3H^+]^{3+}$ calculated: 961.5, found: 961.5; $[M+4H^+]^{4+}$ calculated: 721.4, found: 721.6; $[M+5H^+]^{5+}$ calculated: 577.3, found: 577.2; $[M+6H^+]^{6+}$ calculated: 481.2, found: 481.3; $[M+7H^+]^{7+}$ calculated: 412.6, found: 412.6).

TAT conjugate D: 10.2 mg Cys-TAT (6.1 µmol, MW 1662.0 g/mol) was reacted with 1.2 eq **D** for 3 h in 2 mL of a solution comprising 80 % 0.1 M phosphate buffer pH 7 and 20 % acetonitrile, sparged with argon prior to reaction to remove oxygen. The reaction mixture was then acidified with formic acid just before HPLC purification with a gradient from 5 % to 50 % of acetonitrile 0.1 % FA in water 0.1 % FA for 15 min at 30 mL/min. To ensure good recovery from the flask, TAT conjugate D was solubilized after freeze-drying in a small volume of water and acetonitrile with 0.1 % FA and freeze-dried again. 14.3 mg of BG-biotin TAT conjugate D was recovered as a white powder (yield 70 %, purity 99 %, MS m/z $C_{140}H_{235}N_{53}O_{37}S_2$ $[M+2H^+]^{2+}$ calculated: 1659.4, found: 1659.4; $[M+3H^+]^{3+}$ calculated: 1106.6, found: 1106.5; $[M+4H^+]^{4+}$ calculated: 830.2, found: 830.4; $[M+5H^+]^{5+}$ calculated: 664.4, found: 664.4; $[M+6H^+]^{6+}$ calculated: 553.8, found: 554.0; $[M+7H^+]^{7+}$ calculated: 474.8, found: 474.9).

TAT-PEG_6-GFWFG conjugate C: 14 mg crude Cys-TAT- PEG_6-GFWFG was reacted for 1 h with 1.2 eq **C** in 3 mL of a solution comprising 70 % 0,1 M phosphate buffer pH 7 and 30 % acetonitrile, sparged with argon prior to reaction to

remove oxygen. The reaction mixture was then acidified with formic acid just before HPLC purification with a gradient from 15 % to 45 % of acetonitrile 0.1 % FA in water 0.1 % FA for 20 min at 30 mL/min. After freeze-drying, 5.6 mg of BG-biotin TAT-PEG_6-GFWFG conjugate C was recovered as a white powder (yield 27 %, purity 97 %, MS m/z $C_{170}H_{265}N_{57}O_{40}S_2$ $[M+2H^+]^{2+}$ calculated: 1906.2, found: 1906.3 $[M+3H^+]^{3+}$ calculated: 1271.1, found: 1271.4; $[M+4H^+]^{4+}$ calculated: 953.6, found: 953.4; $[M+5H^+]^{5+}$ calculated: 763.1, found: 763.5; $[M+6H^+]^{6+}$ calculated: 636.1, found: 636.6; $[M+7H^+]^{7+}$ calculated: 545.3, found: 545.3; $[M+8H^+]^{8+}$ calculated: 477.3, found: 477.4).

Stock solutions of TAT conjugates were prepared in PBS and were stable for at least 2 months upon storage at -20 °C as shown by UPLC-MS analyses. The concentration was determined by measuring absorbance at 280 nm with baseline correction at 340 nm using a Nanodrop 2000c. The extinction coefficient was calculated by the addition of the extinction coefficient of TAT calculated from Gill and von Hippel coefficients[2] (ε= 1280 $M^{-1}cm^{-1}$ for TAT) to the extinction coefficient of BG (ε= 7100 $M^{-1}cm^{-1}$ for BG[3]) leading to ε= 8380 $M^{-1}cm^{-1}$ for BG-biotin-TAT.

For the comparison of TAT versus TAT-PEG_6-GFWFG, the stock concentration was calculated from the weight of the added compounds instead of absorbance measurement to avoid any bias due to approximated ε difference between TAT and TAT-PEG_6-GFWFG.

Plasmid generation

The mNeonGreen-SNAPf-N1 plasmid was generated using a RDPPVA-linker between mNeonGreen and SNAPf. mNeonGreen was amplified by PCR using the primer pair of FW-KpnI 5' GCA GCG GGT ACC GAT GGT GAG CAA GGG CG and RV-BamHI 5' GCT GAG GGA TCC CGC TTG TAC AGC TCG TCC and introduced into pSNAPf-N1. Sequencing validated correct insertion of the PCR fragment.

Cell culture

Cells were cultured at 37 °C under 5 % CO2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10 % heat-inactivated fetal bovine serum, 0.01 % penicillin-streptomycin, 4 mM glutamine and 5 mM pyruvate (complete medium). The stable cell lines NG and NG-SNAP were cultured as above in presence of 0.2 mg/mL of Geneticin® (G418 Sulfate, ThermoFisher Scientific).

Generation of NG and NG-SNAP cell lines

Subconfluent HeLa cells were trypsinized, centrifuged at 300 g for 5 min, and rinsed with PBS. 6 million cells were resuspended in a 250 µL electroporation buffer (Ingenio Electroporation solution, Mirus Cat. No. MIR50111) in presence of 10 µg of plasmid. Electroporation was done in a 4 mm gap electroporation cuvette at 0.2 kV with High Cap set at 975 µF. Cells were resuspended in 10 mL of complete DMEM and seeded in a 10 cm dish. After 24 hours, G418 selection at 0.5 mg/mL was performed. Cells were sorted after 2 weeks of G418 treatment by flow cytometry on FACS-ARIA (BD Biosciences) according to mNeonGreen fluorescence intensity, STxB-Cy3 binding intensity and SNAP-tag ligand BG-647-SiR labelling efficiency. For monoclonal mNeonGreen-SNAP-tag (NG-SNAP) cell line generation, cells were seeded at a very low concentration in 96 well plates and grown from a single cell prior to clone selection with the above-mentioned criteria.

BG-647-siR labelling

The day before the experiment, cells were seeded on glass lamellae in 4-well plates, 80,000 cells/well. BG-647-SiR (SNAP-Cell® 647-SiR, New England BioLabs) was incubated for 30 min at 37 °C on cells at a concentration of 3 µM in complete DMEM. The cells were washed three times with PBS supplemented with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS^{++}), prior to incubation with fresh medium for 30 min at 37 °C. Cells were rinsed with PBS, fixed for 15 minutes at

room temperature with 4 % paraformaldehyde (PFA), and quenched with 50 mM NH_4Cl for at least 30 min. The lamellae were washed in water and mounted on slides using Fluoromont G.

Intracellular trafficking assay

The day before the experiment, cells were seeded on glass lamellae in 4-well plates, 80,000 cells/well.

The day of the experiment, cells were incubated 30 min at 4 °C in 500 µL of 40 nM STxB in ice-cold complete medium for binding, followed by 3 washes with PBS^{++}. Complete medium at 37 °C was added onto cells, which were then incubated 50 min at 37 °C for synchronized internalization. Cells were washed 3 times with PBS^{++}, fixed with 4 % PFA in PBS during 15 min, washed once with 50 mM NH_4Cl, and incubated with 50 mM NH_4Cl for at least 30 min to quench the PFA. Cells were washed 3 times with PBS/BSA/Saponin (PBS/1.0 % bovine serum albumin/0.1 % saponin), and permeabilized at room temperature for 30 min in PBS/BSA/Saponin. Lamellae were incubated on 30 µL of antibody dilution into PBS/BSA/Saponin for 30 min at room temperature, then washed 3 times with PBS/BSA/Saponin. The primary antibodies were a mouse monoclonal anti-STxB antibody (13C4, used at 1/250 dilution), and a home-made rabbit polyclonal antibody against the Golgi marker giantin (used at 1/100 dilution). The secondary antibodies were Cy3-coupled anti-mouse and Alexa647-coupled anti-rabbit IgGs, used at 1/100 dilution each. Alexa Fluor® 647 streptavidin (Invitrogen™) was used at 1/200 dilution. Lamellae were washed in water and then deposited on slides with 6 µL of Fluoromont G. Polymerization was allowed for 30 min at 37 °C.

Microscopy

Images were acquired on an inverted Nikon Ti2 microscope with High Definition 1K Resonant Scanner and motorized *XY* stage (for Ti-E/Ni-E) fitted with a confocal A1R system, using 60x and 100x oil immersion objectives. Nikon NIS-Elements software was used for image acquisition, its Denoise.ai functionality to remove image shot noise, and Fiji ImageJ software (National Institutes of Health)[4] for image processing.

Western Blot

Samples and molecular weight markers (Page Ruler Plus Prestained Protein Ladder from Thermo Scientific) were run on 4-20 % Mini Protean TGX precast gels from Biorad in 1X Tris/Glycine/SDS buffer from Biorad. Proteins were then transferred for 6 min 30 s onto 0.2 µm Amersham™ Protran® nitrocellulose blotting membrane, using a Pierce G2 Fast Blotter instrument and 1-Step Transfer Buffer from Thermo Scientific. Membranes were blocked at room temperature for 30 min in blocking buffer: 5 % defatted milk in TBS-Tween (50 mM Tris, 150 mM NaCl, 0.1 % Tween, pH 7.4). Membranes were incubated with the primary antibody at 1/1000 dilution in blocking buffer for 1 h at room temperature, then washed 3 times 5 min with TBS-Tween. Membranes were incubated for 45 min at room temperature with HRP-secondary antibody from Jackson ImmunoResearch at 1/5000 dilution in blocking buffer, then washed 3 times 5 min with TBS-Tween. Membranes were revealed with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and imaged using a Fusion S Solo instrument (Vilber). Primary antibodies used: polyclonal rabbit anti-SNAP-tag antibody (Genscript, Cat. No. A00684) and monoclonal mouse anti-mNeonGreen antibody (Chromotek Cat. No. 32F6).

Evaluation of SNAP-tag reaction efficiency

NG-SNAP cell lysate at 500,000 cells/mL (except mentioned otherwise in the figure legend, in 1 % NP40, 100 mM NaCl, 50 mM Tris pH 7.4, cOmplete™ EDTA-free Protease Inhibitor Cocktail from Roche) was incubated for 1 h at 37 °C under agitation at 750 rpm with 1 mM DTT with or without 2.4 µM BG-biotin. Excess BG-biotin was then removed from the reaction mixture using with Zeba Spin columns 7 kDa equilibrated in lysis buffer. 70 µL streptavidin Dynabeads™

M-280 (Invitrogen, ThermoFisher Scientific) were washed 3x with lysis buffer, then incubated with 200 µL purified reaction mixture per condition for 2 h at room temperature under gentle rotation. The supernatant was recovered prior to bead washes 3 times 5 min with lysis buffer. Elution from beads was done for 10 min at 95 °C in 300 µL Laemmli buffer 1X. Laemmli buffer 3X was added to input and supernatant samples to reach 1X concentration, and these samples were boiled as well for 10 min at 95 °C. The efficiency of streptavidin pull-down was then evaluated by Western Blot.

Evaluation of mNeonGreen immunoprecipitation efficiency

25 µL mNeonGreen-Trap magnetic agarose beads (Chromotek, Cat. No. ntma-200) were rinsed 3 times with lysis buffer (1 % NP40, 100 mM NaCl, 50 mM Tris pH 7.4, cOmplete™ EDTA-free Protease Inhibitor Cocktail from Roche) and incubated for 1.5 hours at room temperature under gentle rotation with NG-SNAP cell lysate at 500,000 cells/mL. Supernatant was collected and beads washed 3 times 3 min under gentle rotation with 500 µL of lysis buffer. Absorbed proteins were eluted from the beads by incubating the beads for 10 min at 95 °C in 350 µL Laemmli buffer 1X. The efficiency of mNeonGreen immunoprecipitation was then evaluated by Western Blot.

Relative quantification of membrane translocation to the cytosol

See Supplementary Fig. S3. NG-SNAP or NG cells were seeded the day before the experiment at 200,000 cells/well in 24-well plates. STxB-BG-biotin or BG-biotin-TAT conjugates were incubated for 4 h at 37°C on cells at the mentioned concentration in complete medium. The cells were then blocked for 30 min at 37 °C with 10 µM SNAP-Cell® block (New England Biolabs) in complete medium. After 3 washes with PBS^{++} to remove unbound STxB, cells were lysed for 30 min on ice with 350 µL lysis buffer (1 % NP40, 100 mM NaCl, 50 mM Tris pH 7.4, cOmplete™ EDTA-free Protease Inhibitor Cocktail Roche). Cells were mechanically detached, and cell lysate was centrifuged at 17,000 g 4 °C 15 min to eliminate aggregates and DNA. The supernatant was recovered for subsequent mNeonGreen immunoprecipitation. When high concentrations of TAT (above 10 µM) were used for incubation, supernatants were all diluted 10 times in lysis buffer prior to immunoprecipitation (except for TAT-PEG_6-GFWFG 20 µM and 25 µM, which were diluted 100 times) to avoid a too fast ELISA development.

mNeonGreen immunoprecipitation: 10 µL beads/condition of mNeonGreen-Trap magnetic agarose beads from Chromotek were washed three times with 0.5 mL lysis buffer + 1 % BSA for blocking. 300 µL cell lysate supernatant were added onto the beads and incubated by end-over-end rotation for 90 min at room temperature. The beads were then washed 3 times with 0.5 mL lysis buffer.

Streptavidin-HRP incubation: Beads were incubated for 45 min end-over-end rotation with 0.5 mL streptavidin/HRP (dilution 1/5000; Roche, cat. No. 11089153001) in blocking buffer (1 % Triton X-100, 0.1 % SDS, 2 % BSA, 50 mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.4). The beads were then washed 3 times 5 min with RIPA buffer (10 mM Tris, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS, 140 mM NaCl, 0.1 % sodium deoxycholate, pH 8.0) and then once in PBS before ELISA development.

ELISA development: PBS was removed from the beads. 200 µL of ELISA solution (prepared from 25 mL 50 mM phosphate, 27 mM citrate buffer pH 5, 10 µL 30 % H_2O_2, and one 10 mg tablet of o-phenylenediamine dihydrochloride, cat. No. P8287 Sigma-Aldrich) were added on the beads, and the tubes were put under gentle end-over-end rotation until a yellow color appeared (5-15 min). The reaction was then quenched by addition of 50 µL 6 M H_2SO_4 solution. Bead supernatants were transferred to a 96-well plate and the absorbance was read at 490 nm.

SUPPORTING INFORMATION

Inhibitor treatments

Glycosphingolipid depletion: Cells were pre-treated for 6 days with 5 µM of the glucosylceramide synthase inhibitor Genz-123346 in DMEM with 0.01 % penicillin-streptomycin, 4 mM glutamine, 5 mM pyruvate, 5 % foetal bovine serum instead of the 10 % that are usually used, 200 µg/mL geneticin.

ATP depletion: Cells were pre-incubated for 30 min in PBS^{++}, 10 mM 2-deoxyglucose-D-glucose, and 10 mM NaN_3, while control cells were pre-incubated in PBS^{++}, and 5 mM glucose. STxB incubation and SNAP-Cell® blocking were done in the same respective media. The incubation with STxB was done for 90 min and not 4 h to decrease incubation times under ATP depletion conditions.

Acidification: Cells were pre-treated for 30 min with 100 nM bafilomycin A1 in complete medium, followed by incubation with STxB-BG-biotin and SNAP-Cell® blocking also in presence of 100 nM bafilomycin A1.

Absolute quantification of membrane translocation to the cytosol

NG-SNAP cells were seeded the day before the experiment at 500,000 cells/well in 6 well plates. Wells for cytosolic amount, total amount, standard curve (multiple wells required to have enough non-treated lysate) and an additional well for cell counting were used. The day of the experiment, cytosolic quantification, total quantification, and standard curve samples needed to be prepared in parallel (see Supplementary Fig. S10).

Lysis buffer: 5 % Elugent, 1 M NaCl, 50 mM Tris pH 7.4, cOmplete™ EDTA-free Protease Inhibitor Cocktail (PIC).

Dilution buffer: 50 mM Tris pH 7.4 with PIC for STxB; 50 mM Tris pH 7.4, 1 M NaCl with PIC for TAT (in order to keep the high salt concentration, which is important to avoid TAT binding to cellular nucleic acids and proteins[5]).

Cell count (to be done just before lysis for the standard curve, cytosolic amount, and total amount wells): The counting well was washed 2x with PBS^{++}, then 1x in PBS, prior to detachment with 0.3 mL trypsin/EDTA for 5 min at 37 °C. The cells were then resuspended with the addition of complete DMEM and counted using a Malassez counting chamber.

Non-treated NG-SNAP lysate preparation for standard curve and total amount lysate dilutions: Cells were washed 3x with 1 mL PBS^{++}. On ice, 300 µL cold lysis buffer was added per well. The cells were mechanically detached and recovered in 1.5 mL Eppendorf tubes. After 10 min incubation on ice, the cells were sonicated at 4 °C with a Bioruptor® Pico (Diagenode), 10 cycles 30 s ON 30 s OFF. Dilution buffer was then added to reach 500,000 cells/mL lysate concentration, according to the number of cells per well as per the counting well. Lysate was centrifuged for 20 min at 4 °C 17,000 g. 1 mM DTT was added to the supernatant, and the resulting solution (= non-treated NG-SNAP lysate) was kept on ice.

Blocked NG-SNAP lysate preparation for cytosolic amount lysate dilution (used to dilute cytosolic amount wells incubated with TAT concentrations above 10 µM): Cells were incubated for 30 min at 37 °C with 10 µM SNAP-Cell® block in complete medium and then lysed similarly to non-treated NG-SNAP lysate preparation (except for DTT addition).

Cytosolic and total amount quantification: The day of the experiment, the cells were incubated for 4 h at 37 °C with STxB or TAT conjugates at the mentioned concentration in complete medium. Cytosolic wells were then incubated for 30 min at 37 °C with 10 µM SNAP-Cell® block in complete medium, while total amount wells were incubated with complete medium only. Cells were washed 3x with 1 mL PBS^{++} and lysed similarly to non-treated NG-SNAP lysate preparation (except for DTT addition).

Cytosolic lysates were kept on ice until the other samples were ready. When high concentrations of TAT or TAT-PEG_6-GFWFG were used on cells, cytosolic lysates were diluted in blocked NG-SNAP lysate (1/5 dilution for 10 µM TAT and TAT-PEG_6-GFWFG; 1/10 dilution for 20 µM TAT and 1/100 dilution for TAT-PEG_6-GFWFG).

For total amount lysates, several dilutions (1/250, 1/500, 1/1000) were prepared in non-treated NG-SNAP lysate to make sure that the absorbance reading of one of them was in the range of those of the standard curve. The total amount dilutions were then incubated for 1 h at 37 °C 750 rpm for the reaction with SNAP-tag to occur.

Standard curve: Known dilutions from 0 to 30 pM of STxB-BG-biotin or 0 to 200 pM BG-biotin-TAT were prepared in non-treated NG-SNAP cell lysate. These were then incubated for 1 h at 37 °C 750 rpm for the reaction with SNAP-tag to occur.

mNeonGreen immunoprecipitation, Streptavidin-HRP incubation, and ELISA development steps were performed simultaneously for cytosolic, total and standard curve samples. The protocol for these steps was described above in the "Relative quantification of membrane translocation to the cytosol" section. The only change was that the mNeonGreen immunoprecipitation was performed at 4 °C and not room temperature to ensure limited degradation prior to absolute quantification.

Controls for STxB extraction conditions

NG-SNAP cells were seeded the day before the experiment at 500,000 cells/well in 6-well plates. The day of the experiment, cells were incubated for 4 h in presence or absence (negative control cells) of 40 nM STxB-Cy3. The cells were washed 3 times with PBS^{++}. Positive and negative control cells were mechanically detached in 50 mM Tris pH 7.4, pelleted by centrifugation, and kept on ice. Lysis control cells were mechanically detached on ice in 0.3 mL 5 % Elugent, 1 M NaCl, 50 mM Tris pH 7.4 with PIC, and recovered in Eppendorf tubes. After 10 min incubation on ice, the cells were sonicated at 4 °C with a Bioruptor® Pico (Diagenode), 10 cycles 30 s ON 30 s OFF. Dilution buffer (50 mM Tris pH 7.4 with PIC) was then added to reach 500,000 cells/mL lysate concentration, according to the number of cells per well counted from the counting well (similarly to the protocol in the "Quantitative analysis of translocation" section). Lysates were centrifuged for 15 min at 4 °C 17,000 g, and supernatants were removed. Pellets were washed with 1 mL 50 mM Tris pH 7.4 with PIC, and recentrifuged 15 min at 4 °C 17,000 g. Positive, negative and lysis control pellets were then imaged in the Cy3 channel of a ChemiDoc instrument (Biorad).

Data analysis and figures

Prism software (GraphPad) was used for statistical analysis and graph plotting, Fiji ImageJ software (National Institutes of Health)[4] for microscopy and Western Blot image processing, and Adobe Illustrator to draw figures.

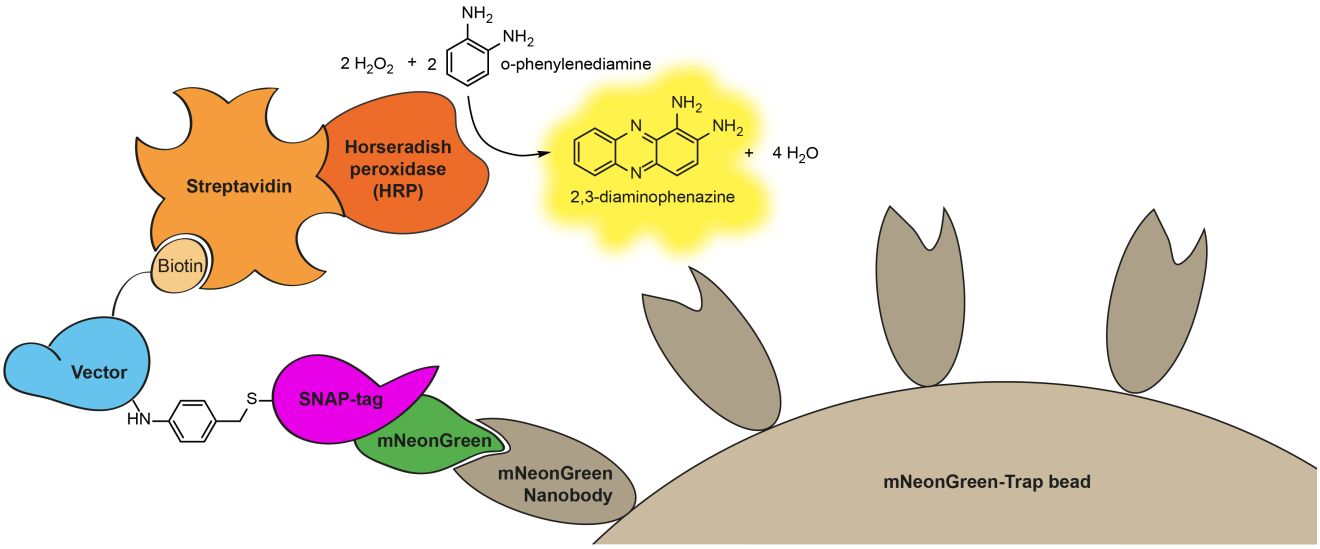
SUPPORTING INFORMATION

Supplementary Tables and Figures

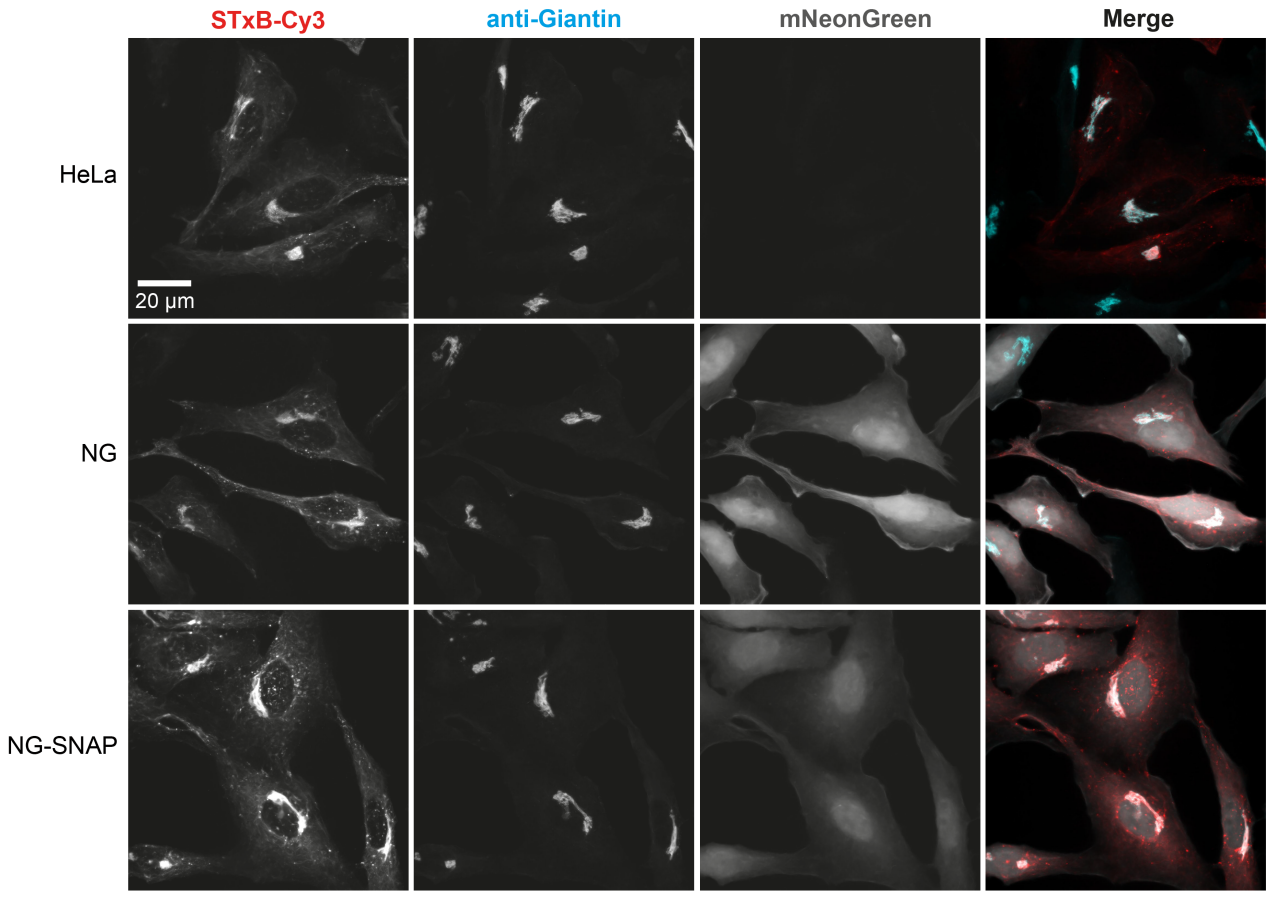
Supplementary Table S1. Available assays to assess cytosolic arrival (table adapted from Ref. [6]). Assays that measure total cellular uptake, comprising cytosolic and intracellular compartments-trapped materials, are not listed here.

Method	Ref.	Tag	Detection	Advantages	Disadvantages
Environment-sensitive fluorescence or luminescence	[7–12]	pH sensitive dye (fluorescent at neutral pH)[8] or tags sensitive to reducing conditions: fluorescence-quencher construct[9–11] or luciferin conjugate[7,12] with disulphide bond linkers	Fluorescence or luminescence spectroscopy	Small tag. Easy read-out.	Not specific to the cytosol (other intracellular compartments have neutral pH and/or reducing conditions).
Fluorescence correlation spectroscopy (FCS)	[13,14]	Dye	Fluorescence microscopy	Small tag. Absolute quantification of concentration.	Necessitates either cell lysis and cytosol isolation through ultracentrifugation[14], or live-cell microscopy and identification of areas without strong punctate signal which are interpreted as cytosolic[13]. Cytosol isolation is prone to experimental artefacts (impurities, leakage from compartments…). Distinction between cytosol and small diffraction limited intracellular compartments is basically impossible by conventional fluorescence microscopy. Furthermore, both methods will underestimate the cytosolic amount if the vector remains associated with the cytoplasmic membrane leaflet after translocation to the cytosolic compartment (e.g., STxB, see Ref. [15]).
Split protein complementation	[16–18] with GFP [19] with Luciferase [20] with NanoLuc Luciferase	Peptide	Fluorescence or luminescence spectroscopy	Easy read-out.	Relatively large tag (16 aa for split-GFP, 285 aa for Split-luciferase, 11 aa for split NanoLuc without counting the linker between the tag and the vector) that could affect the vector translocation to the cytosol. Limited by the affinity to the complementary cellular target (non-covalent binding). Split-GFP: Low sensitivity (cellular treatments with vector concentration above 10 µM required[16,17]). Addition of streptavidin-binding peptide 2 to the tag improves sensitivity, but the resulting tag is very large – 55 aa, thus not applicable to small vectors[18]. Split-GFP induces furthermore a delay in signaling due to the long time required for maturation of the chromophore[21].
Farnesylation	[22]	C-terminal CaaX motif	SDS-PAGE	Small tag. Specific covalent modification in the cytosol.	Sensitivity limited by the detection method (SDS-PAGE).
Deubiquitination	[23]	Ubiquitin	SDS-PAGE	Specific cleavage of ubiquitin in the cytosol.	Large tag (76 aa) that can affect the vector translocation to the cytosol. Sensitivity limited by the detection method (SDS-PAGE).
Enzyme delivery	[24]	Enzyme (β-lactamase), which cleaves the FRET substrate CCF2-AM accumulated specifically in the cytosol.	Fluorescence	Easy read-out.	Huge tag (30 kDa). No signal amplification thus limited sensitivity.
FACS pulse width analysis	[25]	Fluorophore	Flow cytometry	Small tag. Easy read-out.	No quantification possible. Fluorophore may diffuse inside the cell and is likely not strictly cytosol specific. Limited to vectors which remain associated to membranes after translocation into the cytosol.

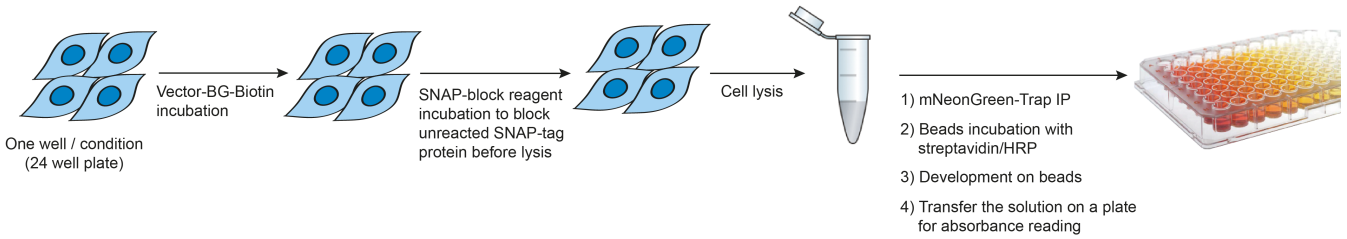
Method	Ref.	Tag	Detection	Advantages	Disadvantages
Fluorogenic probe sensitive to enzymatic activities	[26–28]	Masked fluorophore needing reaction by cytosolic enzymes to fluoresce. The enzyme is either exogenous but stably expressed in the cytosol (such as β-galactosidase[26] or β-lactamase) or endogenous (cytosolic esterases[27] or protein tyrosine phosphatases[28]).	Fluorescence	Small tag. Easy read-out.	No signal amplification thus limited sensitivity.
Biotin-ligase assay (BirA)	[29]	Avi and HA tag peptides	Western Blot	Allows to quantify both cytosolic amount (with biotin labelling of the avi-tag by BirA) and total amount (with the HA tag).	Sensitivity limited by the Western Blot read-out.
Glucocorticoid receptor (GR) based assays	[30,31]	Dexamethasone, which upon binding to GR in the cytosol leads to GR translocation to the nucleus (which can be monitored by microscopy using a fusion GR-eGFP protein[31]) and to GR induced transcription of a reporter (eGFP[31] or Luciferase[30])	Luminescence or Fluorescence	Small tag. Cytosol-specific. Easy read-out.	Not possible to get absolute quantification from the GR induced transcription of a reporter due to non-linear transcriptional amplification. Limited sensitivity for monitoring of GR-eGFP translocation to the nucleus since no signal amplification.
Expression of a reporter protein	[32,33]	Splice-correcting single-stranded complementary RNA[32]. Or Cre recombinase[33].	Luminescence or fluorescence	Sensitivity. Cytosol-specific.	Large tags that can affect vector translocation to the cytosol. Not possible to get absolute quantification due to non-linear transcriptional or translational amplification.
Chloroalkane penetration assay (CAPA)	[34]	Chloroalkane (reacts covalently with cytosolic localized HaloTag).	Fluorescence	Small tag.	Indirect quantification (quantification of unreacted HaloTag through the addition of fluorescent haloalkane). The signal is inversely proportional to the extent of translocated molecules, which limits the sensitivity of the assay.
Visualization of membrane disruption	[35–38]	None	Fluorescence	No tag. Easy read-out.	Indirect measurement of membrane integrity (leakiness with calcein release experiment[36–38] or membrane damage using galectin-9 sensor[35]). Does not enable to quantify cytosolic arrival of the vector per se.
$SNAP_{Switch}$	[39]	Construct containing a fluorophore and a quencher separated by a benzylguanine. Upon SNAP-tag (expressed in the cytosol) reaction on the benzylguanine, the fluorophore is released from the quencher and becomes fluorescent.	Fluorescence	Small tag. Easy read-out. Cytosol specific.	Does not provide absolute quantification. No signal amplification thus limited sensitivity.
NanoClick	[40]	Azide (reacts covalently with a DBCO labeled NanoLuc-HaloTag cytosolic protein)	Fluorescence (BRET signal)	Very small tag. High throughput. Easy read-out.	Indirect quantification (quantification of unreacted NanoLuc-HaloTag-DBCO through the addition of an azide dye and measurement of the BRET signal between the dye and the NanoLuc moiety). The signal is inversely proportional to the extent of translocated molecules, which limits the sensitivity of the assay.



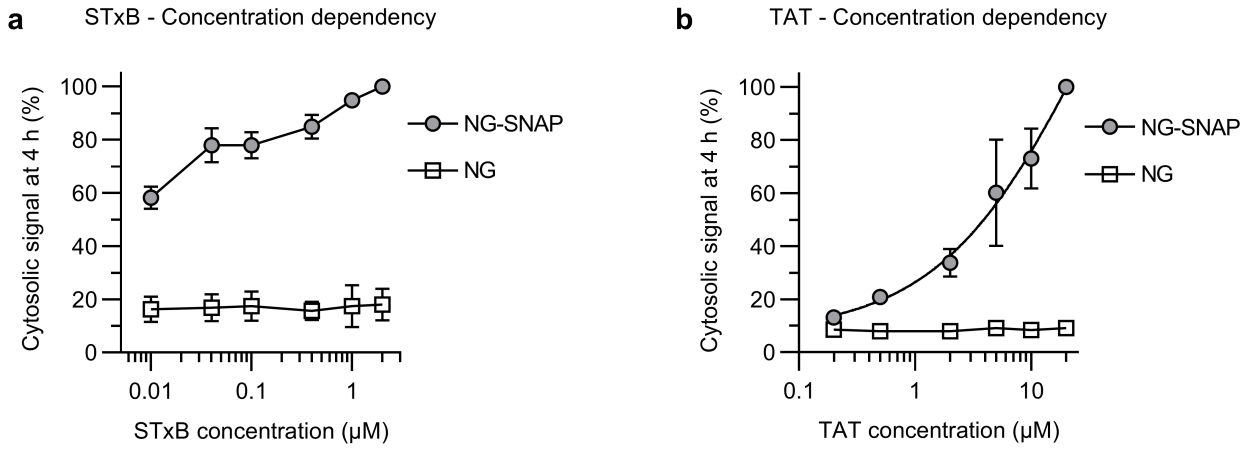
Supplementary Figure S1. Schematic representation of the ELISA development strategy on beads in the Cyto-SNAP assay.



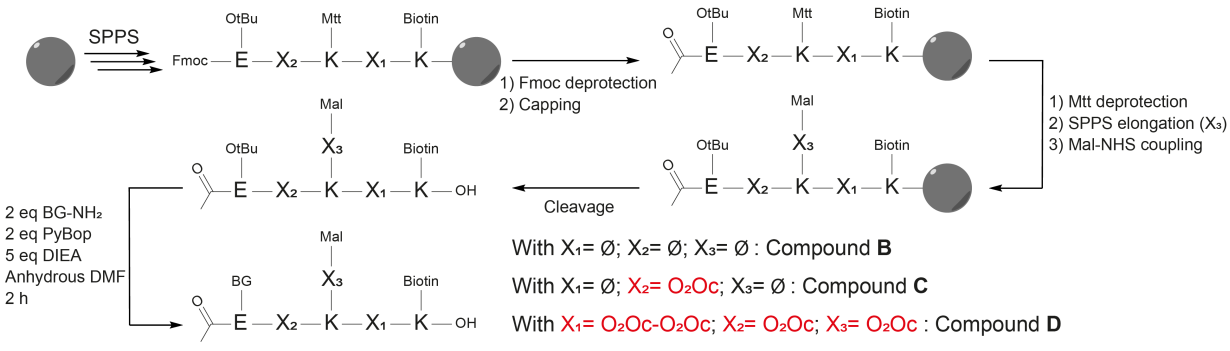
Supplementary Figure S2. STxB intracellular trafficking assay in parental, NG, or NG-SNAP HeLa cells. 40 nM STxB-Cy3 was bound to cells for 30 min at 4 °C, followed by PBS washes and incubation for 45 min at 37 °C for synchronized internalization. Golgi was labelled with an anti-giantin antibody and AF647-tagged secondary antibody. Retrograde trafficking of STxB-Cy3 to the Golgi was indistinguishable in all cell lines. Merge: STxB-Cy3 in red, anti-Giantin in cyan, and mNeonGreen in grey.



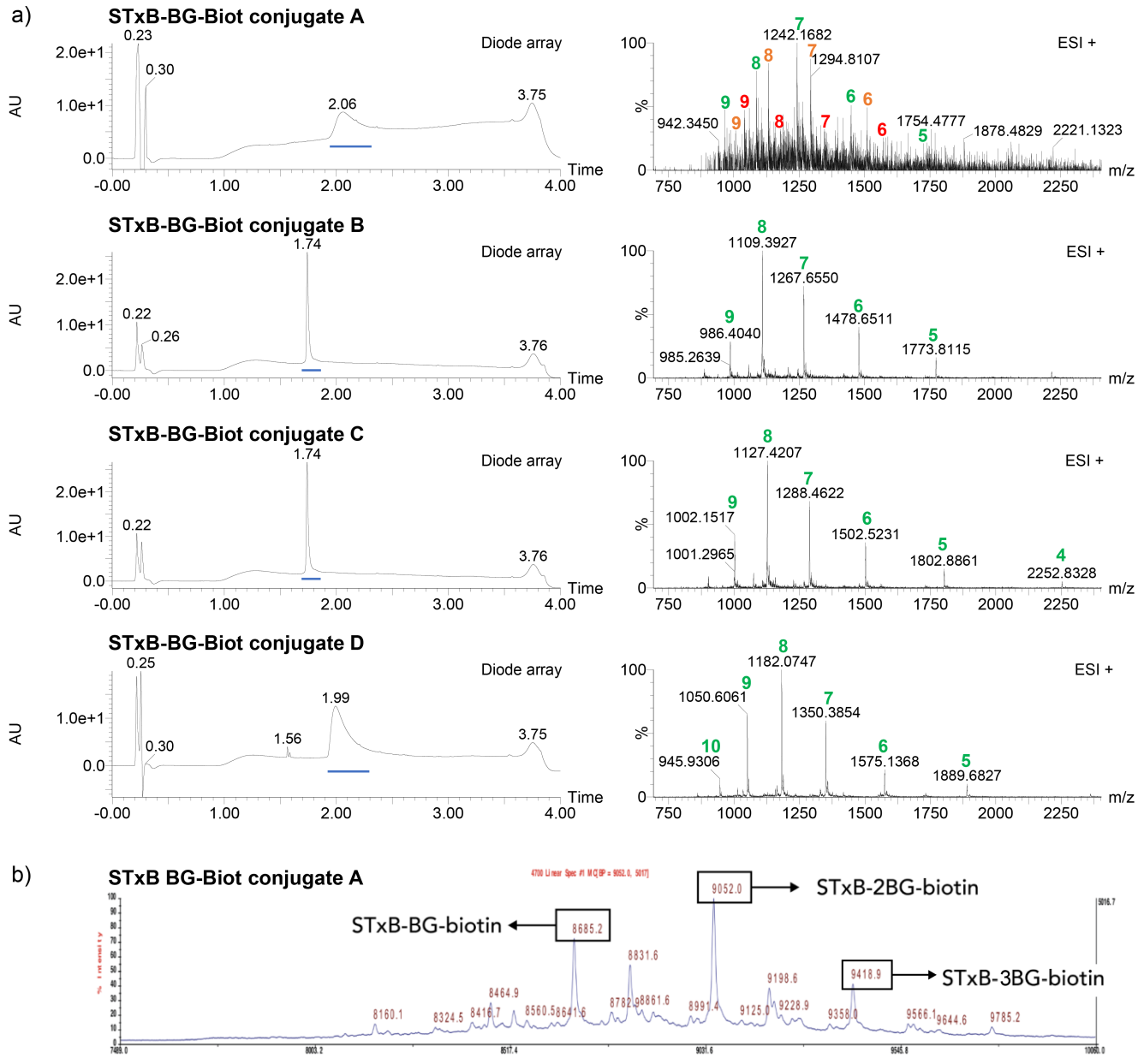
Supplementary Figure S3. Procedure for the relative quantification of membrane translocation to the cytosol in the Cyto-SNAP assay. BG: benzylguanine; IP: immunoprecipitation; HRP: Horseradish peroxidase.



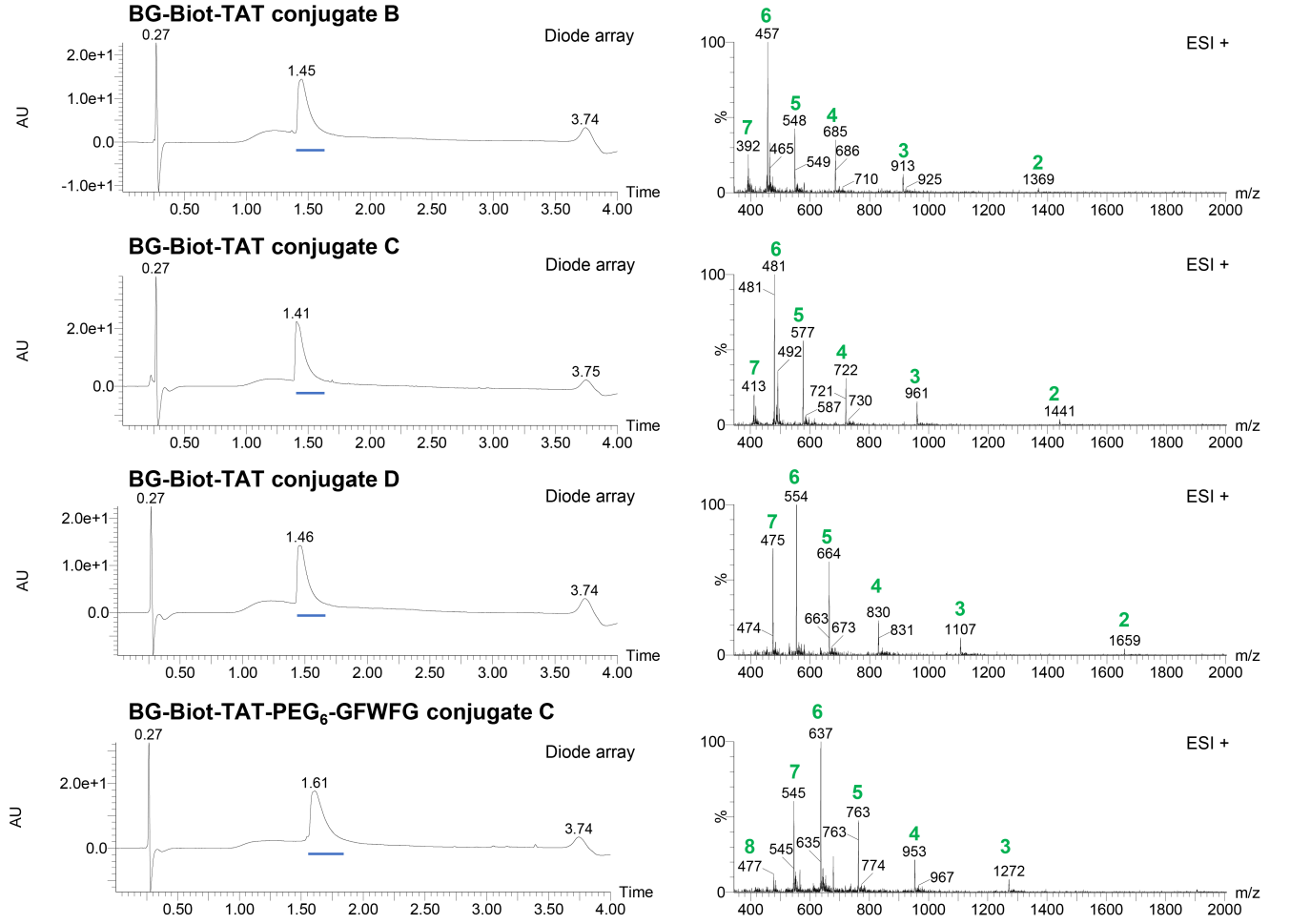
Supplementary Figure S4. STxB conjugate C (a) and TAT conjugate C (b) translocation to the cytosol at higher concentrations.



Supplementary Figure S5. Synthesis scheme for compounds B, C and D using solid-phase peptide synthesis (SPPS).

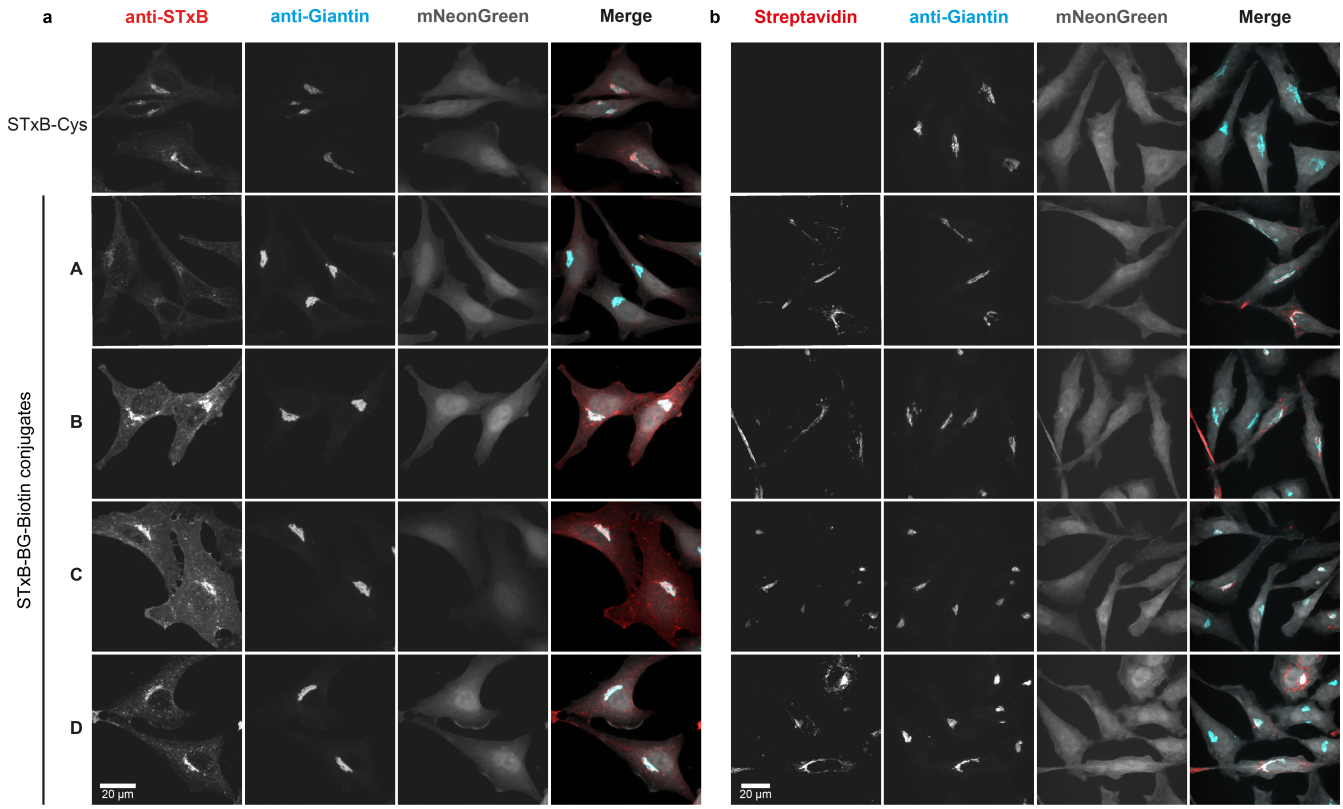


Supplementary Figure S6. a) UPLC-MS analyses of STxB conjugates. Left side: Chromatograms. Right side: ESI+ mass spectra of the portions underlined in blue on the left side. The electrospray ionization leads to a multiple charged ion distribution that is detected by the mass spectrometer. Peaks corresponding to the expected product are annotated with their corresponding positive charge in green. For STxB-BG-biotin conjugate A, peaks annotated in orange correspond to STxB linked to maleimide-biotin and 2 BG-NHS, while peaks annotated in red correspond to STxB linked to maleimide-biotin and 3 BG-NHS. b) MALDI-TOF analysis of STxB BG-biotin conjugate A, shown in addition to the UPLC-MS analysis for easier visualization of the multiple BG modifications of STxB.



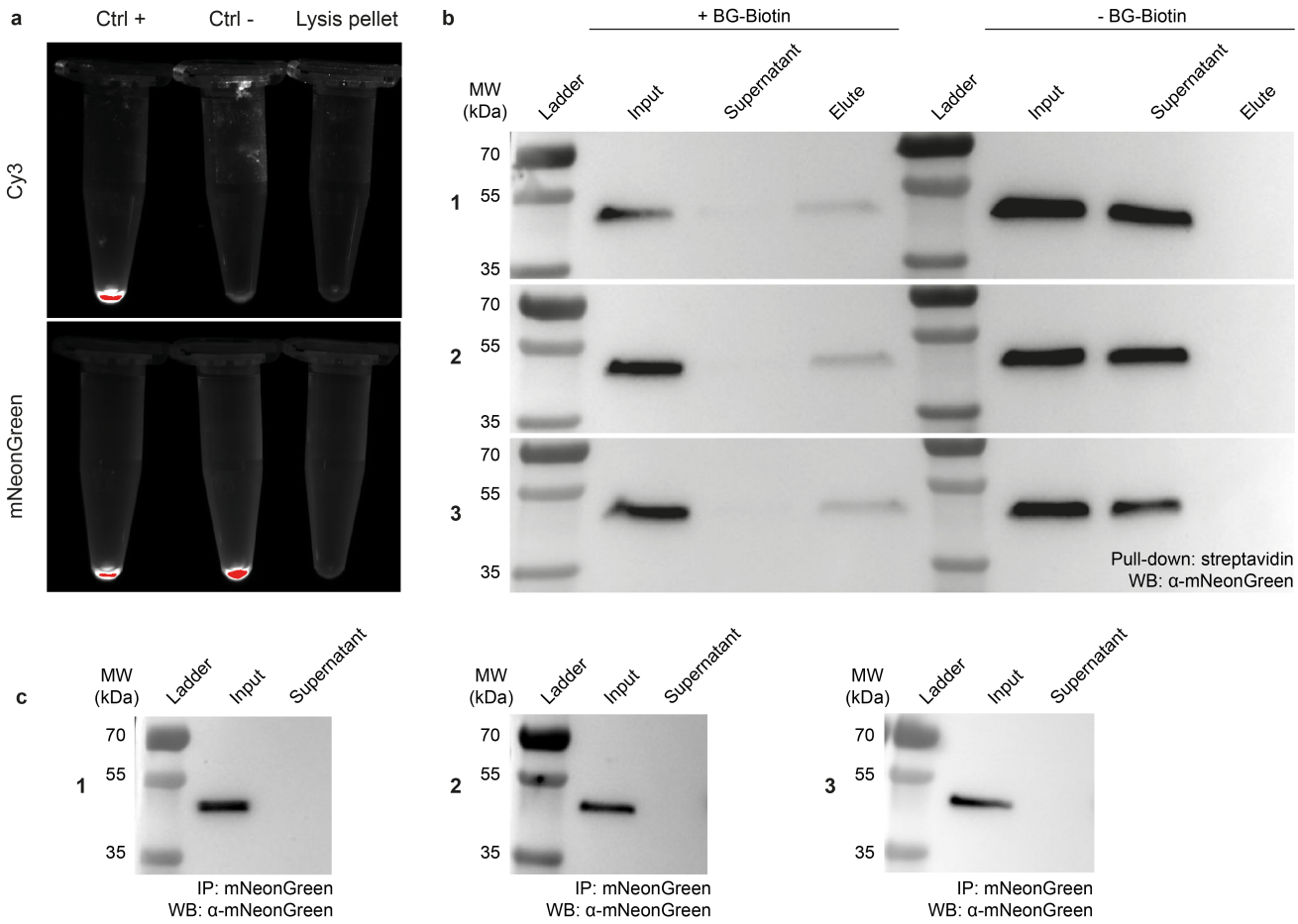
Supplementary Figure S7. UPLC-MS analyses of TAT conjugates. Left side: Chromatograms. Right side: ESI+ mass spectra of the portions underlined in blue on the left side. The electrospray ionization leads to a multiple charged ion distribution that is detected by the mass spectrometer. Peaks corresponding to the expected product are annotated with their corresponding positive charge in green.

SUPPORTING INFORMATION

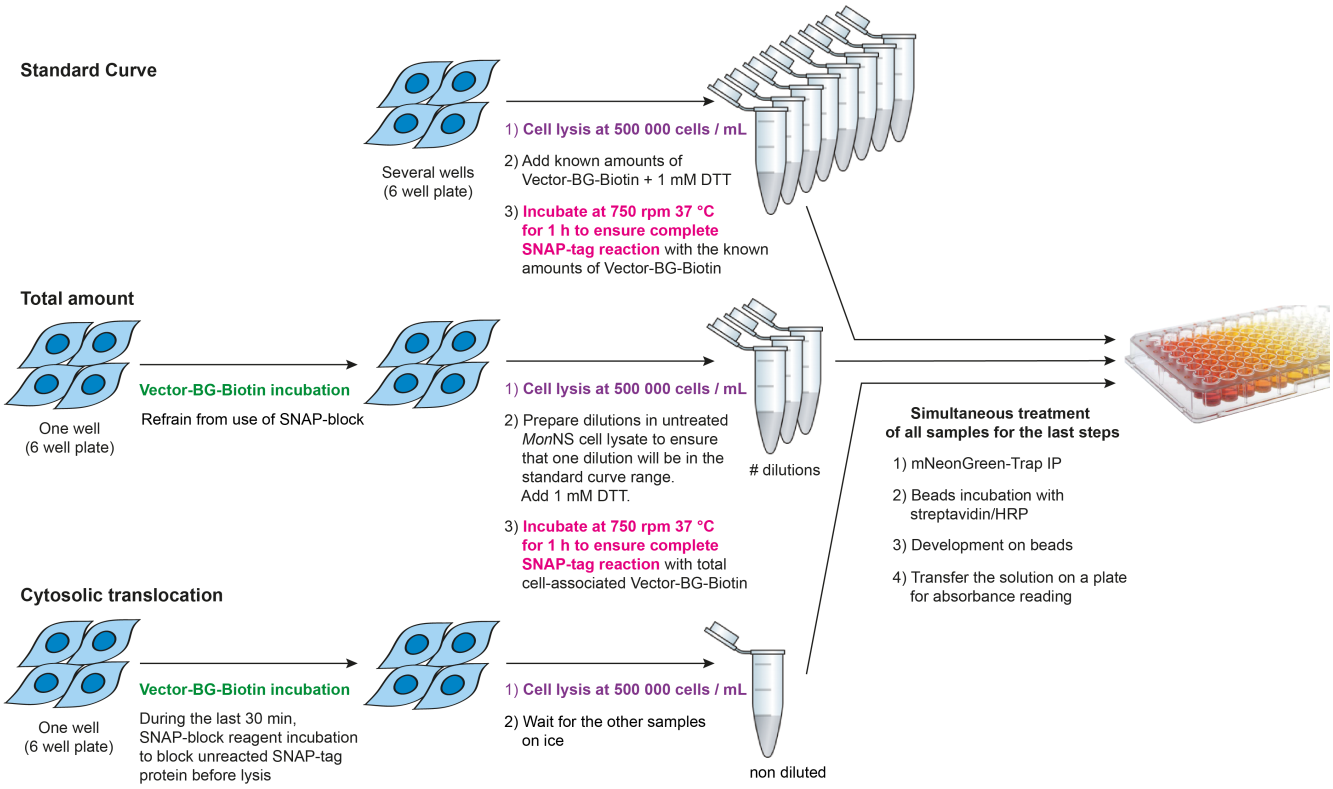


Supplementary Figure S8. – Intracellular trafficking of the STxB-BG-biot conjugates on NG-SNAP cells. 40 nM of the different STxB conjugates were incubated for 30 min at 4 °C with NG-SNAP cells, followed by washes and incubation for 45 min at 37 °C for synchronized internalization. The Golgi apparatus was labelled by immunofluorescence with an anti-giantin antibody. STxB was either labelled with an anti-STxB antibody (in a), or with fluorescent streptavidin to label the biotin present on STxB-BG-biotin conjugates (in b). All conjugates are efficiently transported from the plasma membrane to the Golgi apparatus. The reduced labelling intensity of STxB conjugate A by anti-STxB 13C4 antibody is likely due to reduced affinity to the antibody due to the multiple BG addition on lysines. STxB conjugates A indeed shows a normal labeling intensity when labeled with streptavidin. Merge: anti-STxB or streptavidin in red, anti-giantin in cyan, and mNeonGreen in grey.

SUPPORTING INFORMATION



Supplementary Figure S9. Controls for cell lysis conditions used for absolute quantification of TAT and STxB translocation to the cytosol. a) Efficient STxB extraction upon lysis. Ctrl +: NG-SNAP pellet after 4 h incubation with 40 nM STxB-Cy3. Ctrl -: NG-SNAP cell pellet without STxB-Cy3 incubation. Lysis pellet: Pellet obtained after lysis of cells incubated with 40 nM STxB-Cy3 for 4 h. Lysis conditions: 5 % Elugent, 1 M NaCl, 50 mM Tris pH 7,4 + PIC, 10 min on ice, followed by Bioruptor® Pico sonication 10 cycles 30 s ON / 30 s OFF. b) SNAP-tag reacts efficiently in the different lysis buffers used. NG-SNAP cells were lysed in different lysis buffers (1, 2 or 3) and incubated for 1 h at 37 °C with 2 µM BG-biotin ligand. After removal of non-reacted BG-biotin, streptavidin beads were added for pull-down. Western blot analysis shows that the cell lysate was depleted of the mNeonGreen-SNAP-tag proteins, which were bound to the beads. c) mNeonGreen immunoprecipitation (IP) is complete in the different lysis buffer used. 1: Lysis in 1 % NP40, 100 mM NaCl, 50 mM Tris pH 7.4 (buffer used for relative quantification analyses). 2: Lysis in 5 % Elugent, 1 M NaCl, 50 mM Tris pH 7.4 + PIC followed by dilution in 50 mM Tris pH 7.4 (buffer used for absolute quantification of STxB translocation to the cytosol). 3: Lysis in 5 % Elugent, 1 M NaCl, 50 mM Tris pH 7.4 + PIC followed by dilution in 1 M NaCl, 50 mM Tris pH 7.4 (buffer used for absolute quantification of TAT translocation to the cytosol).



Supplementary Figure S10. Procedure for the absolute quantification of membrane translocation to the cytosol in the Cyto-SNAP assay. Three different sample types need to be prepared in parallel: The standard curve, the quantification of total cell-associated amount of a given vector (note that the SNAP-tag quenching step is omitted in this case), and the quantification of cytosolic amount (SNAP-block is used for the quenching of unreacted SNAP-tag). The steps of mNeonGreen immunoprecipitation and ELISA development on beads are then performed simultaneously for all samples.

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Author Contributions

M.L., A.B., C.W., L.J., E.D. and F.S. contributed to the conception of the work. M.L., A.B., S-K.B., J.H. and C.W. performed the experiments and interpreted the data along with L.J. A.B. drafted the manuscript and C.W., M.L., L.J., and F.S. revised it substantively.