Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supporting Information

Orthogonal coiled coils enable rapid covalent labeling of two distinct membrane proteins with peptide nucleic acid barcodes

Georgina C. Gavins, Katharina Gröger, Marc Reimann, Michael D. Bartoschek, Sebastian Bultmann, Oliver Seitz

Abstract: Templated chemistry offers the prospect of addressing specificity challenges occurring in bioconjugation reactions. Here, we show two peptide-templated amide-bond forming reactions that enable the concurrent labeling of two different membrane proteins with two different peptide nucleic acid (PNA) barcodes. The reaction system is based on the mutually selective coiled coil interaction between two thioester-linked PNA-peptide conjugates and two cysteine peptides serving as genetically encoded peptide tags. Orthogonal coiled coil templated covalent labeling is highly specific, quantitative and proceeds within a minute. To demonstrate the usefulness, we evaluated receptor internalization of two membranous receptors EGFR (Epidermal Growth Factor) and ErbB2 (Epidermal Growth Factor Recpetor 2) by first staining PNA-tagged proteins with fluorophore-DNA conjugates and then erasing signals from non-internalized receptors via toehold-mediated strand displacement.

Contents

1 Reagents for chemical synthesis

Fmoc protected amino acids were purchased from *Novabiochem* (Schwalbach, Germany). PNA monomers; Fmoc-PNA-T-OH, Fmoc-PNA-A(Bhoc)-OH, Fmoc-PNA-C(Bhoc)-OH and Fmoc-PNA-G(Bhoc)-OH were purchased from *LGC Genomics* (Teddington, UK). Fmoc-aminoethyloxyethyloxyacetic acid (Fmoc-Ahz), [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (Fmoc-AEEA) and Fmoc-L-Lys(Mmt)-OH were purchased from *Iris Biotech* (Marktredwitz, Germany). *6*-Azido-hexanoic was purchased from *Sigma-Aldrich* (St. Louis, USA). Tentagel Rink amide (TGR) Resin was from *RAPP Polymers* (Tübingen, Germany) with a loading of 0.2 mmol/g. H-Rink amide ChemMatrix® resin was purchased from *Sigma-Aldrich* (St. Louis, USA) with a loading of 0.40-0.60 mmol/g. Coupling reagents HCTU (O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate), were from *Carl Roth* (Karlsruhe, Germany), Oxyma from *Carbolutions* (Saarbrücken, Germany) and HOBt from *Angene* (Nanjing, China). PyBrOP (Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate) was from *Sigma Aldrich* (St. Louis, USA). Triisopropylsilane (TIS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPs) and triscarboxyethylphosphine (TCEP) were purchased from *Sigma Aldrich* (St.Louis, USA), piperidine and N,N-Diisopropylethylamine (DIPEA) from *Alfa Aesar* (Karlsruhe, Germany), acetic anhydride from *VWR Chemicals* (Darmstadt, Germany) and N-methylmorpholine (NMM) from *Fisher Bioreagents* (Geel, Belgium). 5,6-Carboxytetramethylrhodamine (TMR) was obtained from *ChemPep Inc.* (Wellington, USA) and coumarin 343 (C343) from *TCI* (Eschborn, Germany). Buffer components were purchased from *Carl Roth* (Triton™ X-100, ethylenediaminetetraacetic acid (EDTA)), *Fisher Bioreagents* (Tris), *VWR Chemicals* (NaCl), *Merck* (Sodium hydrogen phosphate, Tween-20 polysorbate), *Iris Biotech* (Dithiotreitol), and *Merck* (SDS, Glycerol). DMF for solid phase synthesis of peptides and PNA was purchased from *VWR Chemicals* (Radnor, USA). The ALO (2-(cyclooct-2-yn-1-yloxy)acetic acid) building block was prepared as described.[1] The S-Mmt-protected mercaptophenyl acetic was prepared as described.[2]

2 HPLC instruments and mass spectrometry

2.1 HPLC for purification of PNA

Purification of products in nanomole amounts was performed on a HPLC Elite LaChrom from *Merck-Hitachi*. Separation was carried out using a Polaris C18 column (5 µm,C18-A 100 x 4.6) from *Agilent Technologies* with a flow rate of 1.0 mL / min at 55 °C. The mobile phase was a mixture of solutions A (98.9% water, 1.0% ACN, 0.1% TFA) and B (98.9% ACN, 1.0% water, 0.1% TFA). For detection, Elite LaChrom L-2450 from *Merck Hitachi* was used, with a spectral measuring range of 200 - 350 nm.

2.2 HPLC for purification of peptides

Semi-preparative HPLC purification was performed on a 110 Series HPLC system from *Agilent Technologies.* Separation was carried out using a Polaris C18 column (5 µm, 250 x 10, pore size: 220 Å) from *Varian* at a flow rate of 6 mL / min. The mobile phase was composed as a mixture of solutions A (98.9% water, 1.0% ACN, 0.1% TFA) and B (98.9% ACN, 1.0% water, 0.1% TFA). Detection using a UV variable wavelength detector was carried out at wavelengths λ = 210 nm and λ = 260 nm.

2.3 UPLC™-MS:

Analysis of products was performed using an Aquity UPLC*™*-MS system from *Waters.* The mass spectrometry analysis was carried out using a quadrupole analyzer with electron spray ionization (ESI); detection via a diode array detector (DAD) at the wavelengths λ = 210 nm and λ = 260 nm. The stationary phase was a *Waters* Acquity UPLC™ BEH C18 column (1.7 µm, 50 x 2.1 mm). The mobile phase was a mixture of solutions A (98.9% water, 1.0% ACN, 0.1% TFA) and B (98.9% ACN, 1.0% water, 0.1% TFA), which was pumped at the flow rate of 0.5 mL / min, at 55 ° C.

2.4 FLR-UPLC™

Analysis of products by fluorescence was performed using an ACQUITY UPLC*™* Fluorescence (FLR) Detector. The stationary phase was a *Waters* Acquity UPLC™CSH C18 column (1.7 µm, 50 x 2.1 mm). The mobile phase was a mixture of solutions A (98.9% water, 1.0% ACN, 0.1% TFA) and B (98.9% ACN, 1.0% water, 0.1% TFA), which was pumped at the flow rate of 0.5 mL / min, at 55 ° C.

3 Synthesis Methods

3.1 Peptide synthesis

Peptide synthesis was carried out in a 25 or 50 µmol scale using a MultipepRS synthesizer from *Intavis* (Köln, Germany). The appropriate amount of TGR resin (0.2 mmol/g) was allowed to swell in DMF for 30 minutes prior to synthesis. Fmoc/tBu SPPS strategy was employed, using Fmoc-protected amino acids. The first deprotection step of the resin was used for Fmoc monitoring and the calculated value for reaction scale used to calculate the final yield.

Fmoc deprotection: The resin was treated with a mixture of DMF:piperidine (4:1) once for 5 minutes and again for 3 minutes before washing three times with DMF.

Coupling: 5 eq. Fmoc protected amino acid, 5 eq. Oxyma, 4.5 Eq. HCTU and 10 eq NMM in each coupling step. The following stock solutions were prepared: 0.5 M Fmoc-amino acid with Oxyma in DMF; 0.4 M HCTU in DMF; 4M NMM in DMF, and amino acids were activated immediately before coupling. Single couplings for 30 minutes were used up to the 8th amino acid, after which double couplings for 20 minutes each were used. The resin was then washed with DMF 3 times.

Capping: Unreacted terminal amines from the coupling step were capped by treatment with a solution of acetic anhydride: 2,6-lutidine: DMF (5:6:89) for 5 minutes before washing 3 times with DMF.

Mmt deprotection: Mmt was removed from the lysine side chain by repeatedly washing the resin with a DCM:TFA:TIS (95:2.5:2.5) mixture for 2 min, until the wash-out was colourless. The resin was washed with DCM five times and DMF 5 times.

5(6)-Carboxytetramethylrhodamine and coumarin343: 5(6)-Carboxytetramethylrhodamine (TMR) and coumarin 343 (C343) were coupled by hand using 4 eq. of TMR or C343, 4 eq. pyBOP and 8 eq. NMM in DMF for 30 mins. The coupling was repeated twice, and no capping step was carried out. The resin was washed for 5 times each with DMF and DCM.

N-terminal cysteine peptides: N-terminal cysteine was coupled by hand using 4eq. Boc-L-Cys(Trt)-OH, 4eq. DIPEA, 3.6 eq. HCTU for 2 x 15 mins without preactivation of the amino acid.

3.2 PNA synthesis

Synthesis of PNA oligomers was carried out in 2 or 5 µmol scale on using a ResPep synthesizer from *Intavis* (Köln, Germany). ChemMatrix resin with a loading of 0.55 mmol/g (experimentally calculated). was swollen in a syringe reactor for 30 minutes prior to synthesis. The experimentally calculated value for resin loading was used to calculate the final yield.

Fmoc deprotection:

The resin was treated with a mixture of DMF:piperidine (4:1) once for 5 minutes and again for 4 minutes before washing 5 times with DMF

Coupling:

4 eq. Fmoc-protected PNA monomer, 3.6 eq. HATU and 8 eq. NMM in DMF was used at a concentration of 0.08 M of activated PNA monomer. The activated PNA monomer was reacted for 30 minutes. Double couplings were used and followed by washing 4 times with DMF.

Capping:

Unreacted terminal amines from the coupling step were capped by treatment with a solution of acetic anhydride: 2,6-lutidine: DMF (5:6:89) for 3 minutes followed by washing 5 times with DMF.

3.3 Peptide and PNA cleavage from the resin

After synthesis, peptides and PNA oligomers were cleaved from the resin together with side group deprotection. Cleavage was carried out in syringe reactors and prior to cleavage resin was either dried under reduced pressure or rinsed 7 times with DCM.

Cleavage from TGR resin

Peptides were cleaved and deprotected using 2 x TFA:TIS:H_2O (94:3:3) for a total of 2.5 hours, before rinsing the resin once with TFA and twice with DCM for 10 minutes each.

PNA was cleaved and deprotected using 2 x TFA:TIS:H_2O (94:3:3) for a total of 4 hours.

The DCM fractions were concentrated and combined with the TFA fractions before further concentration to around 100 µL per 1µmol oligomer for PNA or 50 µL per 1µmol oligomer for peptides or. The product was precipitated in ten times the volume of cold ether (-20 C), centrifuged for 15 minutes at 4 C and the precipitate dried under compressed air. The crude product obtained was purified by HPLC and pure product fractions combined and lyophilized.

Special case-purification of Cys-P3 peptides

To obtain sufficiently pure Cys-P3-C343/TMR peptides, purified Cys-peptides were oxidised to the disulfide form by dissolving in minimal phosphate buffer (150 nM NaCl, 50 mM Na_2HPO_4 pH 8.0) with 10% MeCN. The solution was bubbled with pressurised air flow overnight, or until most of the thiol

had oxidised. The solution was diluted in 0.1% TFA and purified by preparative HPLC. The disulphide was used in the transfer reactions, and reduced in situ with TCEP.

3.4 Synthesis of thioester peptides

The donor (P2/P4) peptides were assembled on TGR resin. To the free amino terminus were coupled: 2-(2-(Fmoc-amino)ethoxy)ethoxy]acetic acid (Fmoc-AEEAc-OH), followed by Mmt-mercaptophenylacetic acid (Mmt-MPAA-OH) and finally azidohexanoic acid to generate the thioester. In between coupling steps was a capping step according to the standard protocol, followed by deprotection of Fmoc or Mmt.

Coupling of 2-(2-amino)ethoxy)ethoxy acetic acid and mercaptophenylacetic acid:

5 eq. Fmoc AEEAc-OH or Mmt-MPAA-OH; 5 eq. pyBrOP and 10 eq. DIPEA in DMF was preactivated for 4 min before coupling. After double couplings for 1 hour each, the resin was washed with DMF, DCM and finally DMF again before capping. The resin was washed with DMF 5 times and DCM 5 times.

Removal of the Mmt protecting group on MPAA:

Resin was washed repeatedly with DCM:TFA:TIS (97:2:1) for 1 minutes each, before washing 5 times with DCM and 5 times with DMF.

Coupling of azidohexanoic acid to form the thioester:

A mixture of 5 eq. azidohexanoic acid with 4.8 eq HATU and 8 eq. DIPEA in DMF was preactivated for 5 min before adding to the resin for 60mins. Coupling was repeated twice before washing with DMF 5 times and DCM 10 times.

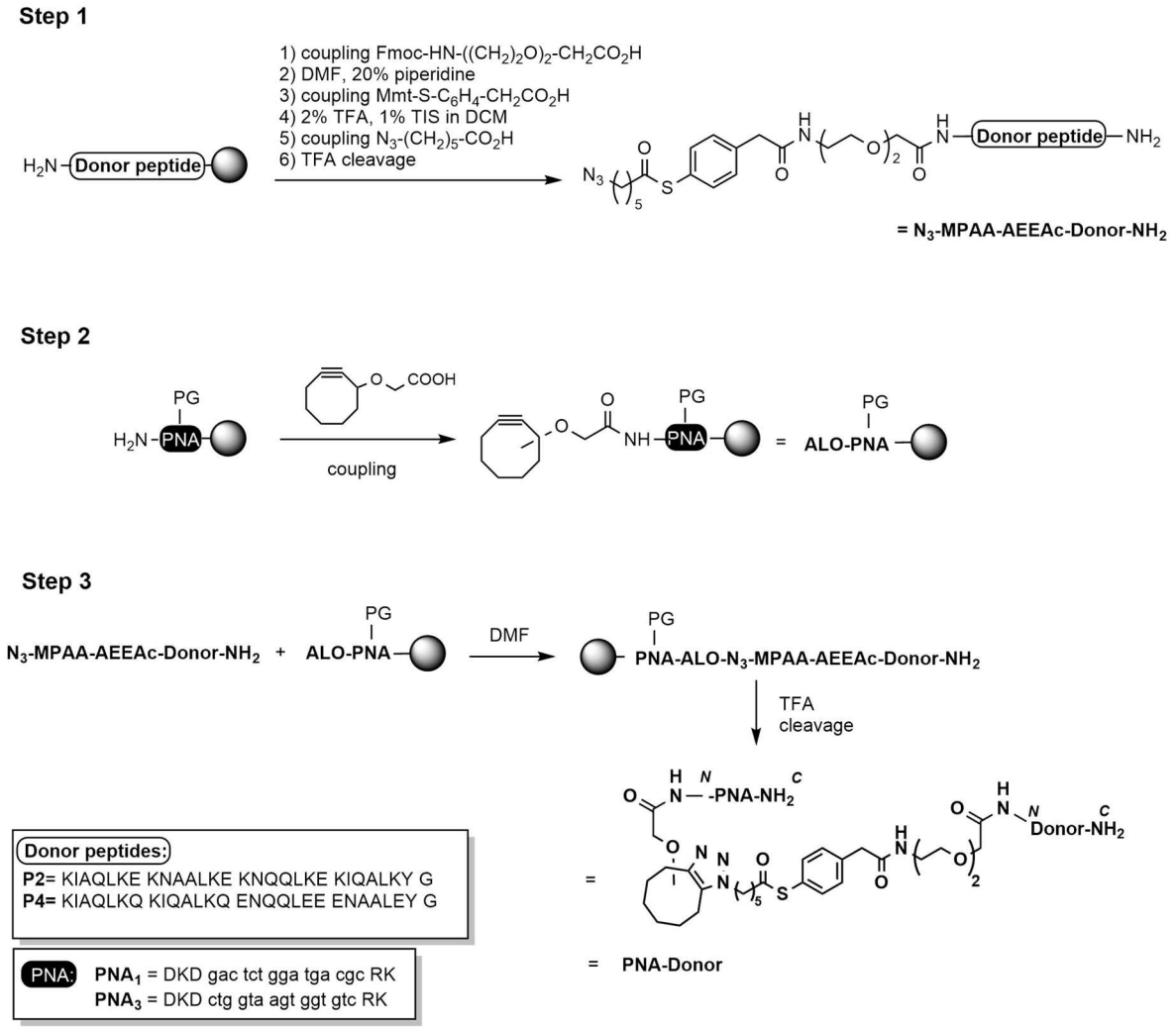
Cleavage from the resin:

After resin cleavage and protective group deprotection, it was necessary to wash the resin with DCM and TFA multiple times to maximise yield.

ALO coupling to PNA1 and PNA2:

PNA oligomers were prepared as described on Rink Amide ChemMatrix^{TM} resin with Bhoc/tBu/Pbf protective groups intact. For coupling of aryl-less cycloctyne (ALO) to the free (Fmoc deprotected) N terminus, the resin was swelled in a minimal volume of DMF. Yield of the N-terminal amine was calculated by Fmoc monitoring. Activated ALO (10 eq. ALO, 10 eq. PyBrOP, 18 eq. DIPEA, minimal DMF, 5 min activation) was added and the resin shaken for 1.5 hours. Since no retention shift was observed upon coupling ALO, and ALO is destroyed under conditions of cleavage, success of the ALO coupling was investigated by carrying out a 'test SPAAC click reaction' on a small portion of resin with

azidoacetic acid, followed by cleavage. Conversion of the crude 15mer PNA to the 'test SPAAC' product via ALO-PNA was roughly 40% in both cases after a single coupling of ALO (UPLC at 260 nM). Though not optimized this was deemed sufficient and the final SPAAC reaction was conducted. After ALO coupling, resin was washed with DMF.



Scheme S3-1 Synthetic route to thioester-linked **PNA_1-P2** and **PNA_3-P4** peptide-PNA conjugates via strain promoted azide alkyne cycloaddition.PG= protecting groups.

Strain promoted azide-alkyne cycloaddition (SPAAC):

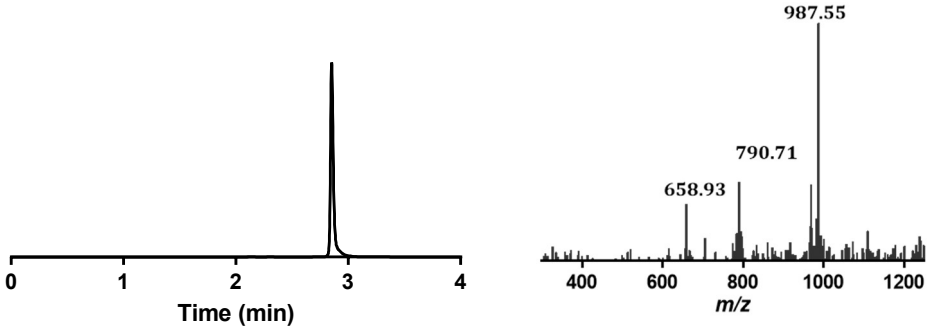
The resin bound ALO-PNA was swelled in a minimal amount of dry DMF and 1 eq. (based on N- terminal amine before ALO coupling) of purified thioester-linked azido-peptide **N_3-MPAA-AEEAc-P2** or **N_3-MPAA-AEEAc-P4** was added. The resin was shaken for 1 day at 30°C before adding another 1 equivalence of azide and shaken one more day before washing with DMF, DCM followed by cleavage from the resin. The SPAAC product had an adequately shifted LC retention factor that purification by HPLC yielded a sufficiently pure product.

4 Compound characterisation

Cys-P1-TMR, Cys-EIQALEE ENAQLEQ ENAALEE EIAQLEY G K(TAMRA)-NH_2

As previously described ([3])

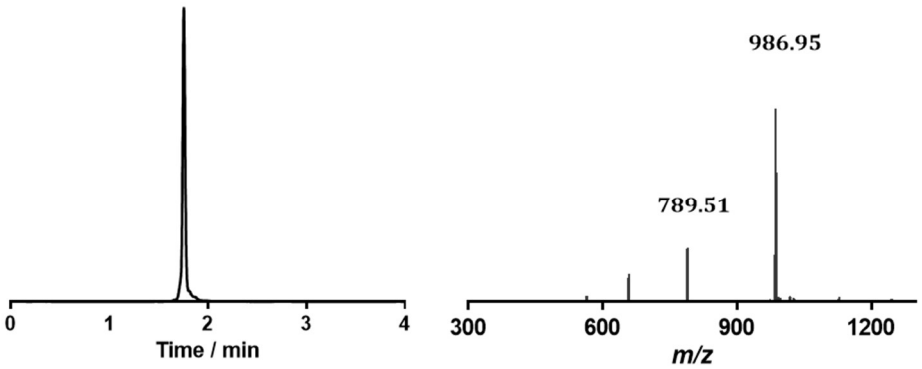
Yield: 9.9% (990 nmol). ε_{260}= 32484 $L \cdot mol^{-1} cm^{-1}$. UPLC gradient: 20-65% B in 4 min, 280 nm. ESI-MS: *m/z* = 658.93, 790.03, 987.28. ($C_{174}H_{257}N_{41}O_{62}S$: 3945.09 $gmol^{-1}$, calculated: $[M+6H]^{6+}$ =658.52; $[M+5H]^{5+}$ =790.03; $[M+4H]^{4+}$ =987.28)



Cys-P3-TMR Cys-EIQQLEE EIAQLEQ KNAALKE KNQALKY K(TAMRA)-NH_2

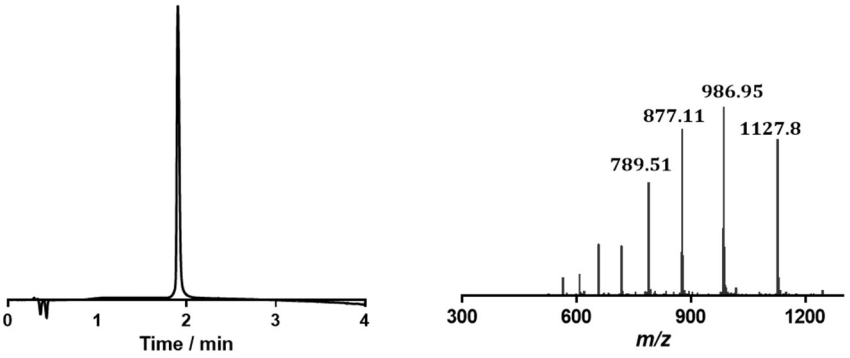
Peptide before extra purification step

Yield: 8 %. ε_{556}=95000 $L \cdot mol^{-1} cm^{-1}$. UPLC gradient: 15-45% B in 4 min, 280 nm. ESI-MS: *m/z* =986.95, 789.51 ($C_{178}H_{277}N_{45}O_{54}S$: 3940.998 $gmol^{-1}$ calculated: $[M+6H]^{6+}$ =789.20; $[M+5H]^{5+}$ =986.25)



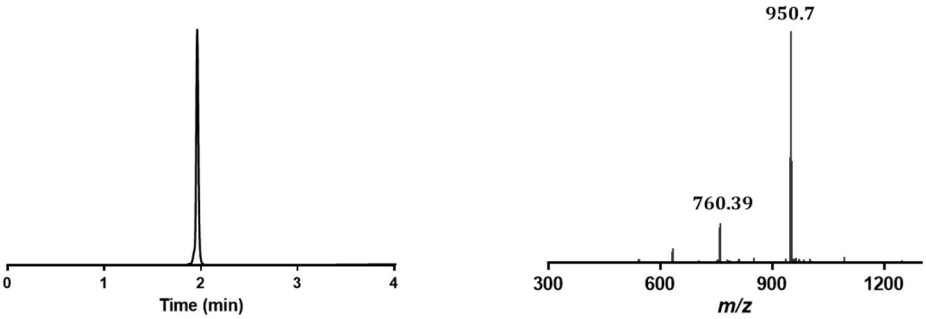
2(Cys-P3-TMR) disulfide of **(Cys-P3-TMR**)

UPLC gradient: 20-65% B in 4 min, 280 nm. ESI-MS: *m/z* =1127.8, 986.95, 877.11, 789.51. (7884.875 $gmol^{-1}$ calculated: $[M+7H]^{7+}$ =1227.41 $[M+8H]^{8+}$ =986.61; $[M+9H]^{9+}$ =877.10; $[M+10H]^{10+}$ = 789.49)



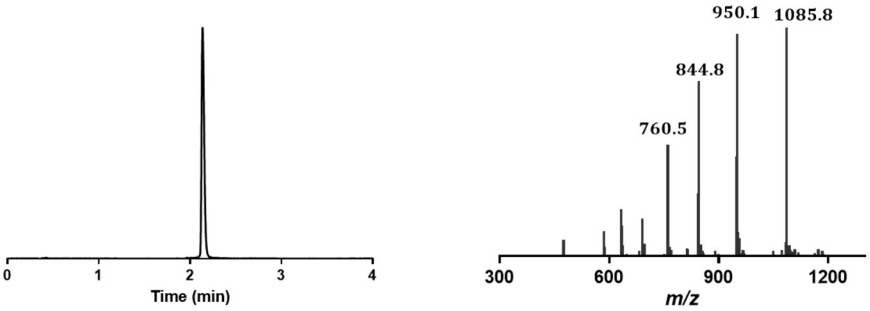
Cys-P3-C343 Cys-EIQQLEE EIAQLEQ KNAALKE KNQALKY K(C343)-NH_2

Peptide before extra purification step
Yield: 11 % . ε_{437}= 44000 $L{\cdot}mol^{-1}cm^{-1}$. LC gradient: 20-65% B in 4 min, 280 nm. ESI-MS: *m/z* =950.7, 760.39. ($C_{169}H_{270}N_{44}O_53S_1$: 3798.287 $gmol^{-1}$ calculated: $[M+5H]^{5+}$ =760.66 $[M+4H]^{4+}$ =950.57)



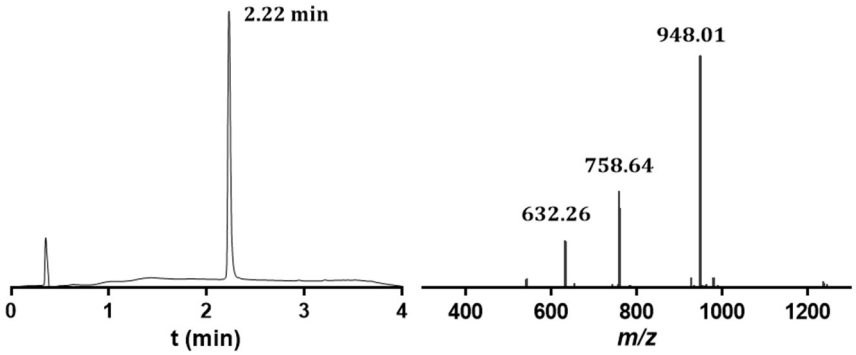
2(Cys-P3-C343) disulfide of **Cys-P3-C343**

UPLC gradient: 20-65% B in 4 min, 280 nm. ε_{437}= 88000 $L{\cdot}mol^{-1}cm^{-}$ESI-MS: *m/z* =1085.8, 950.1, 844.8, 760.5. (7594.559 $gmol^{-1}$ calculated: $[M+7H]^{7+}$ =1085.94; $[M+8H]^{8+}$ =950.32; $[M+9H]^{9+}$ =844.84; $[M+10H]^{10+}$ = 760.46)



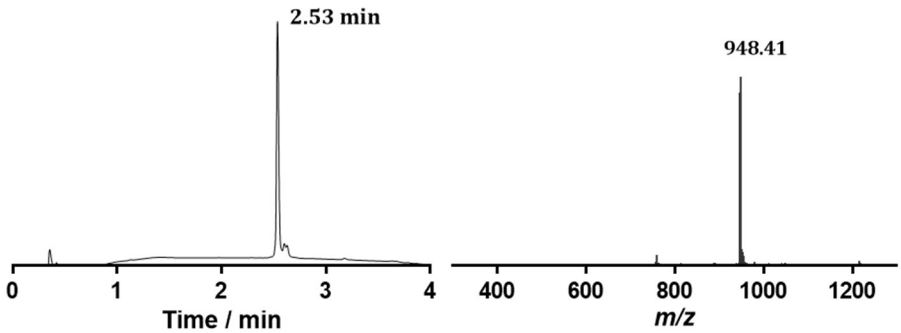
N_3-MPAA-AEEAc-P2, $N_3(CH_2)_5$(CO)-MPAA-AEEAc-KIAQLKE KNAALKE KNQQLKE KIQALKY G-NH_2

Yield: 9% . ε_{214}= 41008 L·mol^{-1}cm^{-1} . LC gradient: 3-80% B in 4 min, 210 nm. ESI-MS: *m/z* = 948.01, 758.64, 632.2 ($C_{170}H_{288}N_{48}O_{47}S$: 3788.48 gmol^{-1} calculated: $[M+4H]^{4+}$ = 948.1, $[M+5H]^{5+}$ =758.7; $[M+6H]^{6+}$ =632.4)



N_3-MPAA-AEEAc-P4, $N_3(CH_2)_5$(CO)-MPAA-AEEAc- KIAQLKQ KIQALKQ ENQQLEE ENAALEY G-NH_2

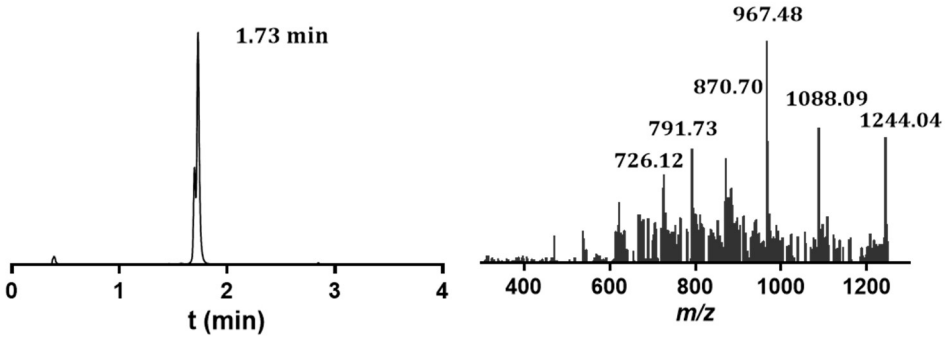
Yield: 12%. ε_{214}= 41394 L·mol^{-1}cm^{-1}. LC gradient: 3-80% B in 4 min, 210 nm. ESI-MS: *m/z* = 948.41 ($C_{166}H_{270}N_{46}O_{53}S$: 3787.9 gmol^{-1}, calculated: $[M+4H]^{4+}$ = 948.6).



PNA_1-P2, NH_2- ^{C}KR cgc agt agg tct cag DKD ^{N} -ALO-$N_3(CH_2)_5$(CO)-MPAA-AEEAc-KIAQLKE KNAALKE KNQQLKE KIQALKY G-NH_2

Yield: 3% (9.2 nmol from 300 nmol scale). ε_{260}= 154423 L·mol^{-1}cm^{-1}. LC gradient: 3-80% B in 4 min, 280 nm. ESI-MS: *m/z* = 791.73, 870.70, 967.48, 1088.09, 1244.04 ($C_{367}H_{547}N_{147}O_{103}S$, 8698.33 g·mol^{-1}, calculated: $[M+7H]^{7+}$= 1243.6, $[M+8H]^{8+}$= 1088.3, $[M+9H]^{9+}$=967.5, $[M+10H]^{10+}$ =870.8, $[M+11H]^{11+}$= 791.8)

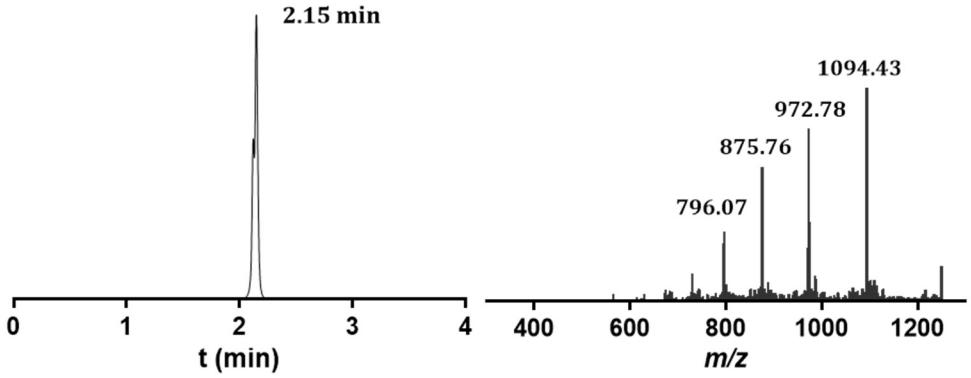
*note that the two peaks correspond to the isomers formed from SPAAC



PNA$_3$-P4, NH_2-^{C} KR ctg tgg tga atg gtc DKD ^{N}-ALO-$N_3(CH_2)_5$(CO)-MPAA-AEEAc- KIAQLKQ KIQALKQ ENQQLEE ENAALEY G-NH_2

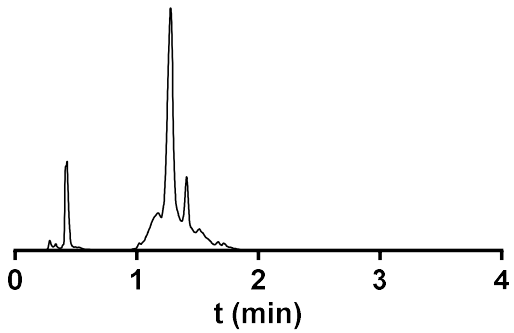
Yield: 6% (33 nmol from 600 nmols scale). ε_{260}= 156423 L·mol^{-1}cm^{-1}. LC gradient: 3-80% B in 4 min, 280 nm. ESI-MS: *m/z* = 1094.43, 972.78, 875.76, 796.07 ($C_{369}H_{549}N_{145}O_{106}S$, 8744.26 g·mol^{-1}, calculated: $[M+8H]^{8+}$ = 1094.0, $[M+9H]^{9+}$ =972.6, $[M+10H]^{10+}$ =875.4, $[M+11H]^{11+}$ = 795.9)

*note that the two peaks correspond to the isomers formed from SPAAC



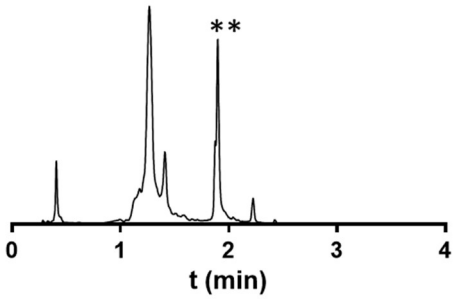
Crude obtained after test cleavage of ALO-PNA$_1$ ALO-^{N}D K D gac tct gga tga cgc R K ^{C}-NH_2

LC gradient: 3-80% B in 4 min, 280 nm. *ALO is unstable to the cleavage conditions used here*



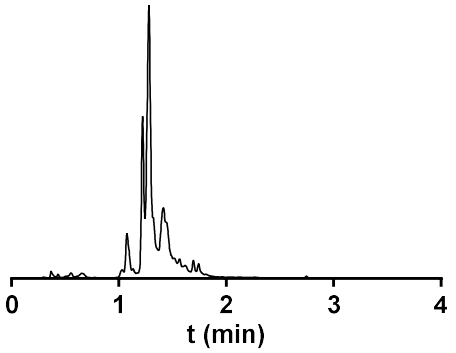
Crude SPAAC product PNA_1-P2

LC gradient: 3-80% B in 4 min, 280 nm ***SPAAC product as two isomers*



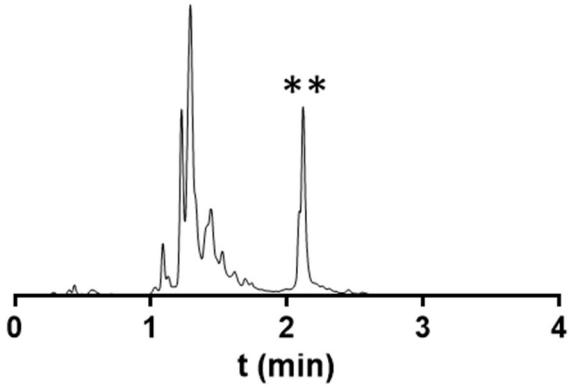
Crude resin coupling ALO-PNA_3 ALO-^{N}D K D ctg gta agt ggt gtc R K ^{C}-NH_2

LC gradient: 3-80% B in 4 min, 280 nm. *ALO is unstable to the cleavage conditions used here*



Crude SPAAC product PNA_3-P4

LC gradient: 3-80% B in 4 min, 280 nm ***SPAAC product as two isomers*



5 Transfer reactions

5.1 Separate transfer reactions

Reaction time courses of transfer reactions between PNA_1-P2 or PNA_3-P4 (PNA Donors) and Cys-P1-TMR, Cys-P3-TMR or Cys-P3-C343 (Cys-Acceptors) were analysed by Fl-UPLC™. TMR= 5(6)-Carboxytetramethylrhodamine; C343= Coumarin 343.Reactions were carried in phosphate buffer (200 mM Na_2HPO_4, 50 nM NaCl, 1 mM TCEP, 0.1% CHAPS, pH 7.2, 30°C). 200 nM Cys-Acceptor was shaken for 10 minutes in buffer before addition of 1200 nM (6 eq) PNA donor. 10 µL aliquots were taken at different time points and diluted by 50 % upon addition to 4% TFA (in water) pre-added to plastic UPLC-MS inserts. Reaction progress was observed by Fl-UPLC™ (TMR: Ex 550 nm, Em 580 nm; C343: Ex 420 nm, Em 500 nm) with a gradient of 15-45% eluent B in A where (eluent A= 98.9% H_20, 1% MeCN, 0.1% TFA ; eluent B= 98.9% MeCN, 1% H_20 0.1% TFA) unless stated otherwise.

5.1.1 Hydrolysis of double transfer product PP'

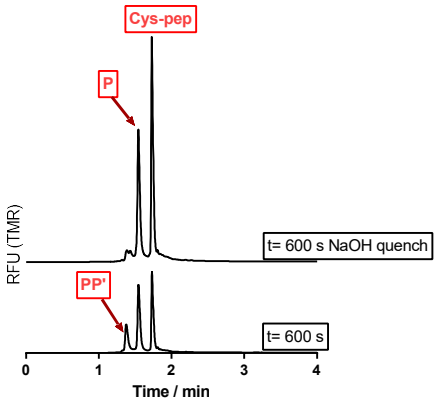


Figure S 5-1 Fl-UPLC™ traces of the transfer reaction between PNA_1-P2 (1200 nM) and Cys-P1-TMR (200 nM). Reactions were quenched as described or with addition of NaOH (0.1M) for 15 minutes before injection into UPLC with a gradient of 10-70% eluent B in A. Upon addition of base, PNA_1-Cys(PNA_1)-P1-TMR (PP') is converted to PNA_1-P1-TMR (P).

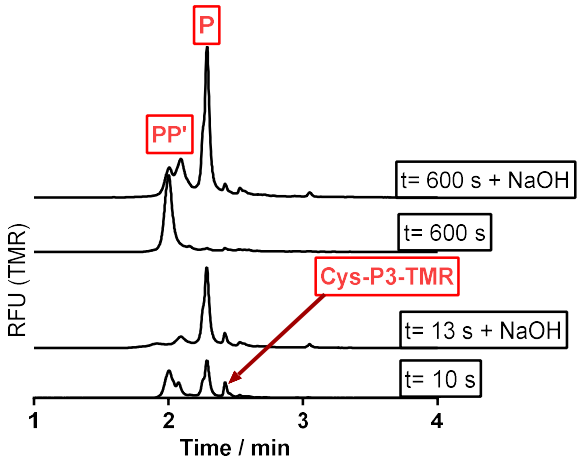


Figure S 5-2 FI-UPLC™ traces of the transfer reaction between PNA_3-P4 (1200 nM) and Cys-P3-TMR (200 nM). Reactions were quenched as described or with addition of NaOH (0.1M) for 15 minutes before injection into UPLC. Upon addition of base, PNA_3-Cys(PNA_3)-P3-TMR (PP') is converted to PNA_3-P3-TMR (P).

5.1.2 Transfer reaction of PNA1-P4 with Cys-P3-C343

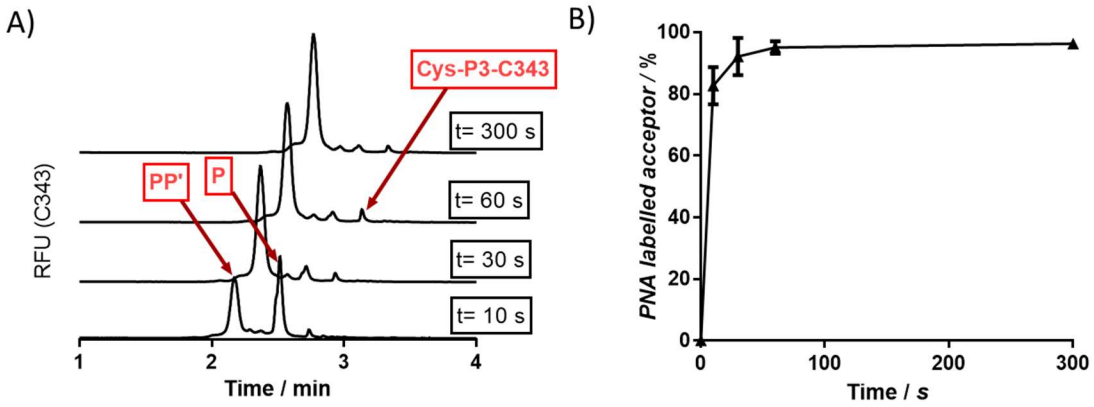


Figure S 5-3 Exemplary FI-UPLC™ traces of the transfer reaction between PNA_3-P4 (1200 nM) and Cys-P3-C343 (200 nM). B) Overall yield of PNA transfer reaction calculated from FI-UPLC experiments as shown in A. PNA labelled acceptor = PNA_3-Cys-P3-C343 (P) + PNA_3-Cys(PNA_3)-P3-TMR (PP'). Independent replicates n=3.

5.1.3 UPLC-MS analysis

Transfer reactions were carried out as described with 2 µM of peptides, quenched to a final concentration of 1% TFA by addition of 10% TFA in water after 5 seconds, and measured by UPLC-MS with a 4 min gradient of 10-50 % eluent D in C (eluent D= 98.9% H_20, 1% MeCN, 0.1% formic acid ; eluent C= 98.9% MeCN, 1% H_20 0.1% formic acid.) Absorption spectra were recorded at 260 nm.

Note,formic acid was introduced in the eluents to improve electrospray ionization. However, this affects UPLC properties by causing tailing.

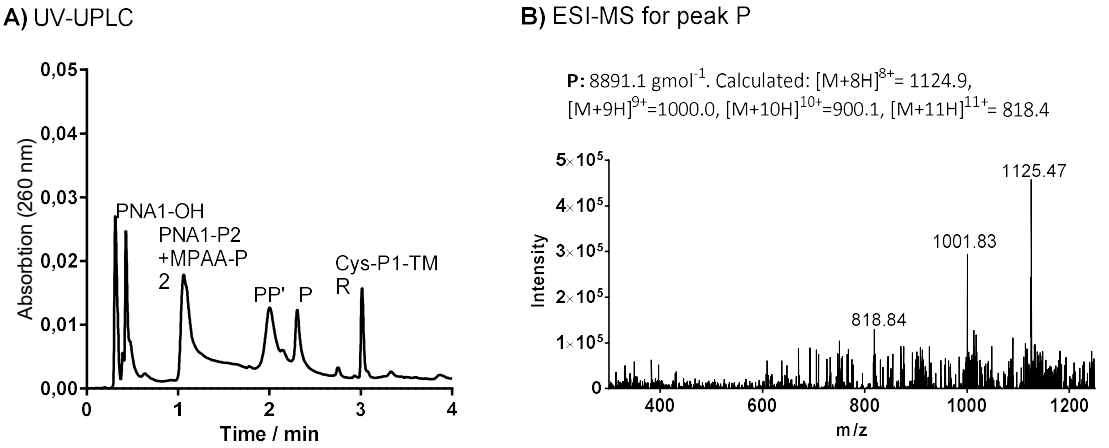


Figure S 5-4 A) UV-UPLC analysis of the transfer reaction between PNA_1-P2 (2000 nM) and Cys-P1-TMR (2000 nM). Detection at 260 nm. B) ESI mass spectrum extracted for peak P.

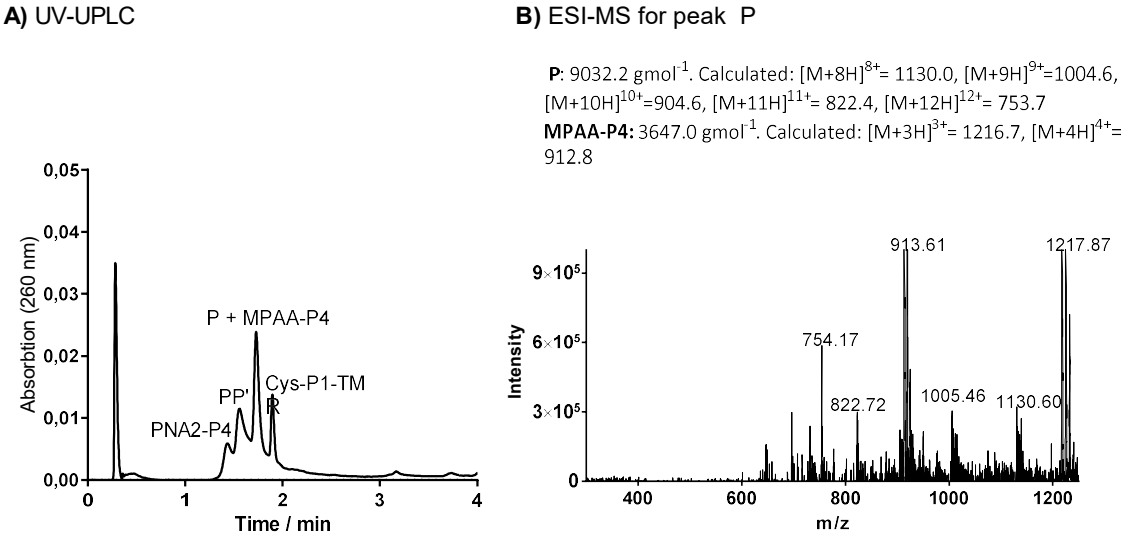


Figure S 5-5 A) UV-UPLC analysis of the transfer reaction between PNA_3-P4 (2000 nM) and Cys-P3-TMR (2000 nM). Detection at 260 nm. B) ESI mass spectrum extracted for peak P. Note, MPAA-P4 (i.e. the thiol leaving group in PNA_3-P3) and P have similar Rf. ESI-MS spectra shows mass of both P and MPAA-P4.

5.2 Orthogonal dual transfer reaction

One pot transfer between PNA Donors PNA_1-P2 / PNA_3-P4 and Cys-P1-TMR / Cys-P3-C343 were analysed by Fl-UPLC™. TMR= 5(6)-Carboxytetramethylrhodamine; C343= Coumarin 343. Reactions were carried in phosphate buffer (200 mM Na_2HPO_4, 50 nM NaCl, 1 mM TCEP, 0.1% CHAPS, pH 7.2, 30°C). Both Cys-peptides (Cys-P1-TMR / Cys-P3-C343; 200 nM) were incubated for 10 minutes in buffer before addition of PNA donors (PNA_1-P2 / PNA_3-P4; 1200 nM). 10 µL aliquots were taken at time points and diluted by 50 % upon addition to 4% TFA (in water) pre-added to plastic UPLC-MS inserts. Reaction

progress was observed by FI-UPLC™ with two runs of each aliquot; once measuring TMR: Ex:550 nm, Em:580 nm and once C343: Ex:420 nm, Em:500 nm) with a gradient of 15-45% MeCN in H_20 with 0.1% TFA.

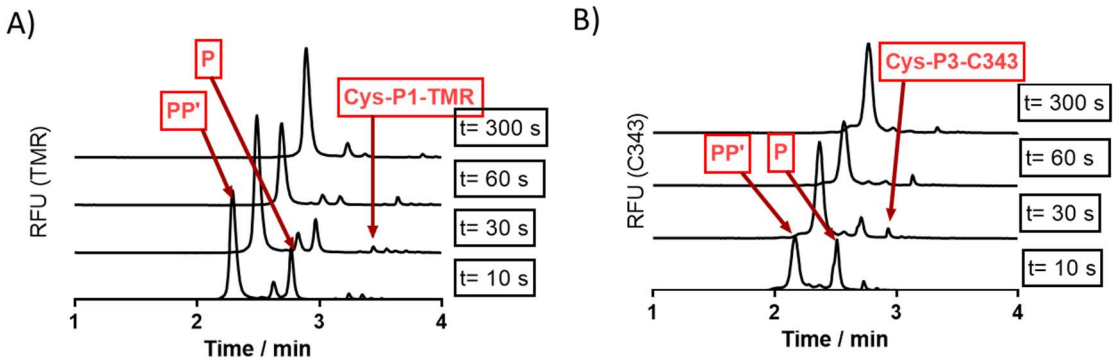


Figure S 5-6 Exemplary FI-UPLC™ traces of one pot dual transfer reaction between PNA_1-P2 (1200 nM) / PNA_3-P4 (1200 nM) and Cys-P1-TMR (200 nM) / Cys-P3-C343 (200 nM). A) Read-out in TMR channel (Ex 550 nm, Em 580 nm). B) Read-out in C343 channel (Ex 420 nm, Em 500 nm). Transfer was repeated in triplicate with similar results.

5.3 Time course of single and one pot PNA transfer

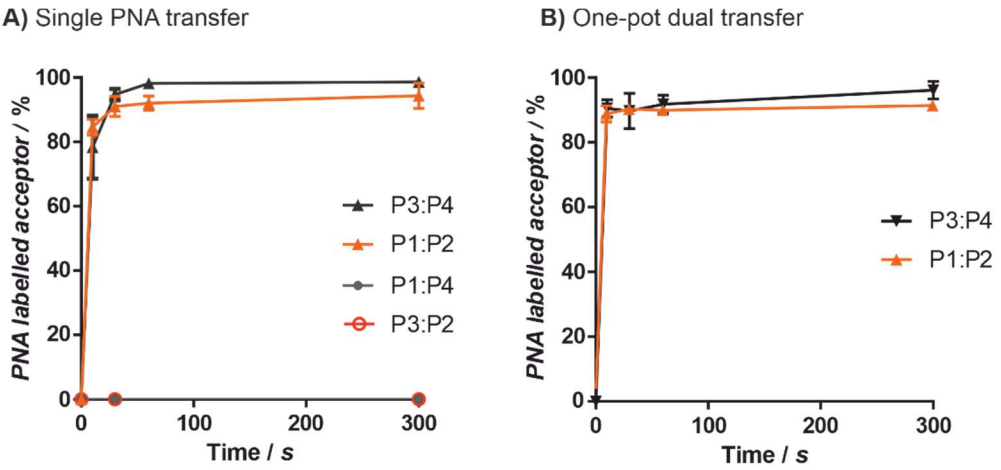


Figure S 5-7 Time course of PNA_1 or PNA_3 labelling of Cys-P1-TMR and Cys-P3-C343 with PNA_1-P2 or PNA_3-P4 performed in A) separate vials or in B) one pot. Conditions: as described in A) 5.1 and B) 5.2. Overall yield of PNA transfer reaction calculated from FI-UPLC experiments shown in Figure 1. Independent replicates n=3 or n=4 for P3:P4 reaction in A).

6 DNA and DNA Sequences

Oligonucleotides were purchased from *Biomers* (Ulm, Germany).

Table S 6-1 DNA and PNA Sequences. Bold-faced nucleotides are designed gaps and remain unpaired after hybridization. Toehold portion of DNA is underlined. * after the name denotes the reverse complement.

Name	**Function**	**DNA/PNA sequences** (5'->3' or N->C)
PNA_1	PNA tag (EGFR)	Asp Lys Asp gac tct gga tga cgc Arg Lys- NH_2
PNA_3	PNA tag (ErbB2)	Asp Lys Asp ctg gta agt ggt gtc Arg Lys- NH_2
Atto565-DNA_1	imager (EGFR)	ATTO565-GCG TCA TCC AGA GTC
Cy7-DNA_3	imager (ErbB2)	Cy7- GAC ACC ACT TAC CAG
DNA_1-DNA_5*-1-DNA_5*	adapter I (EGFR)	GCG TCA AGA GTC GCC AGT AAT AGA TCC GTG GTT C **A** GCC AGT AAT AGA TCC GTG GTT C
Atto565-DNA_5-TOE1	erasable imager I (EGFR)	Atto565- GAA CCA CGG ATC TAT TAC TGG C AT AGC ACA
TOE1*-DNA_5*	DNA eraser I (EGFR)	TGT GCT AT GCC AGT AAT AGA TCC GTG GTT C
DNA_3-1-DNA_4*-1-DNA_4*	adaptor III (ErbB2)	GAC ACC ACT TAC CAG **A** CCT ACG TTC GAA GTC TAA GCA **A** CCT ACG TTC GAA GTC TAA GCA
DY751-DNA_4-TOE2	erasable imager III (ErbB2)	DY751-TGC TTA GAC TTC GAA CGT AGG TGG AAT CA
TOE2*-DNA_4*	DNA eraser III (ErbB2)	TGA TTC CA CCT ACG TTC GAA GTC TAA GCA

The following complexes were used for erasable DNA hybridisation:

Atto565-complex I (EGFR): DNA_1-DNA_5*-1-DNA_5*: 2 x Atto565-DNA_5-TOE1

DY751-complex III (ErbB2): DNA_3-1-DNA_4*-1-DNA_4*: 2 x DY751-DNA_4-TOE2

6.1 Predicted Melting Temperature of DNA-PNA duplexes

Orthogonal PNA strands and DNA adaptors were designed with the help of OligoAnalyzer tool, to avoid hairpins or cross reactivity (IDT) (OligoAnalyser® program, IDT, Coralville, Iowa, USA. Accessed 10 December, 2018. https://www.idtdna.com/SciTools.)

PNA/DNA 15mer duplex melting temperatures ($T_{m(PNA/DNA)}$) were predicted using according to the literature: First the analogous DNA-DNA duplex melting temperature was determined using the nearest neighbor approach assuming 1 mM salt concentration[4] followed by application of an empirical formula to predict $T_{m(PNA/DNA)}$ from $T_{m(DNA/DNA)}$. At 200 nM oligonucleotides predicted melting temperatures were as follows:

$T_{m(pred.)}$ PNA_1/DNA_1= 65.8°C; $T_{m(pred.)}$ PNA_3/DNA_3= 64.3°C

7 Cloning of Cys-Acceptor tagged proteins

The DNA sequence for both EGFR and ERBB2 code an N terminal signal peptide (EGFR: UniProt Q504U8, amino acids 1-24; ERBB2: UniProtKB - P04626, amino acids 1-22) which are cleaved off during biosynthesis. The DNA sequence of the Cys-Acceptor peptide (Cys-P1 and Cys-P3) in both cases was inserted after the signal peptide (EGFR: after amino acid 24; ERBB2: after amino acid 22). This places the cysteine directly at the N-terminus of the mature protein. After biosynthesis and cleavage of the signal peptide, the mature protein offers an N terminal cysteine.

7.1 Cloning of Cys-P1-EGFR-eYFP

Cloning of Cys-P1 peptide into EGFR-eYFP-N1 vector, and subsequently into the PiggyBac donor plasmid (Figure S 7-1) was previously described [3]

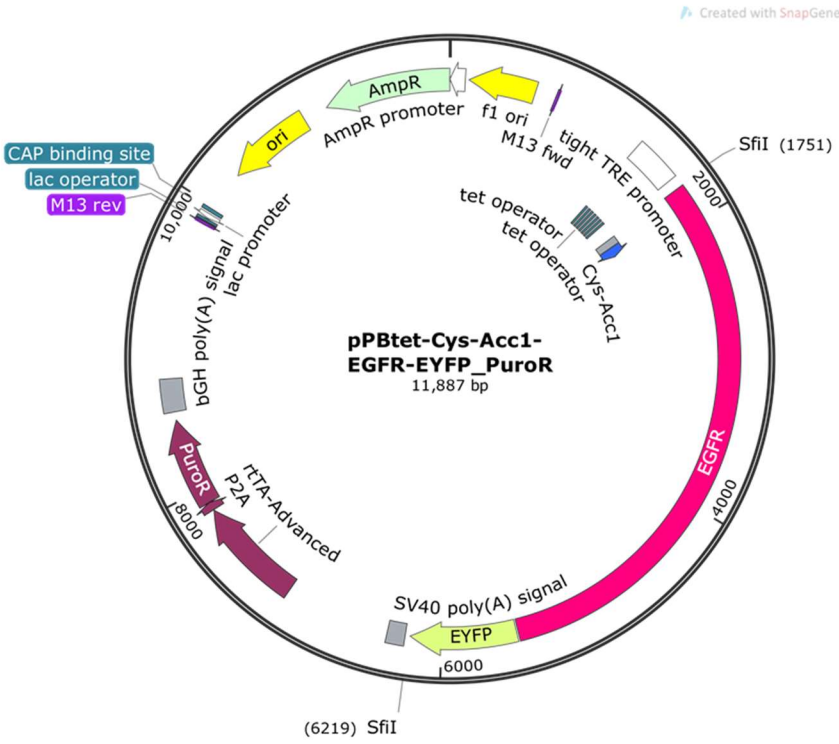


Figure S 7-1 Plasmid map of pPBtet-Cys-P1-EGFR-eYFP-PuroR donor vector used for PiggyBac transposition.

7.2 Cloning Cys-P3-ErbB2-eCFP

Cloning of Cys-P3 peptide sequence onto ErbB2 (HER2) was analogous to cloning of Cys-P1-EGFR-eYFP. A vector containing ErbB2-eCFP-pcDNA3.1(+)Zeo was kindly donated by Prof. Thorsten Wohland (Centre for Bioimaging Sciences, National University of Singapore) (Figure S 7-2A). The gene for Cys-P3

was ordered from GeneScript (Piscataway, NJ, USA) and cloned by GeneScript into the plasmid ErbB2-eCFP-pcDNA3.1(+)Zeo directly after the C terminal of the signal peptide (Figure S 7-2B)

ErbB2 Signal peptide (N → C): MELAALCRWG LLLALLPPGA AS

Cys-P3 Peptide (N → C): C EIQQLEE EIAQLEQ KNAALKE KNQALKY GG

Inserted DNA sequence of Cys-P3 Peptide (5' → 3'): TGC GAG ATC CAG CAG CTG GAG GAG GAG ATC GCC CAG CTG GAG CAG AAG AAC GCC GCC CTG AAG GAG AAG AAC CAG GCC CTG AAG TAC GGC GGC

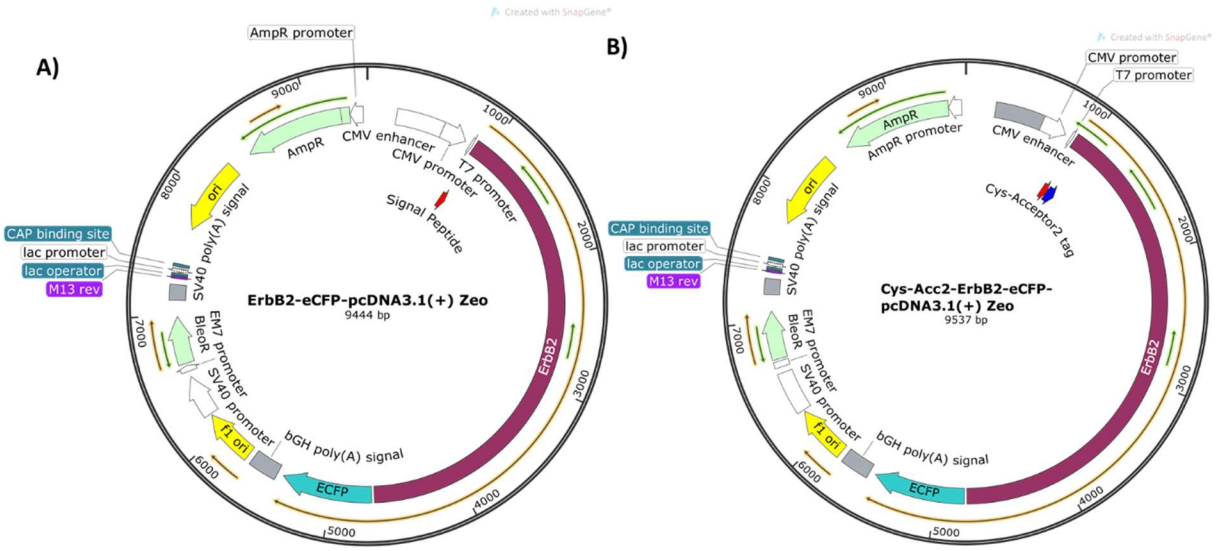


Figure S 7-2 **A)** Plasmid map of ErbB2-eCFP-pcDNA3.1(+)Zeo kindly donated by Prof. Thorsten Wohland**.B)** Plasmid map of Cys-P3-ErbB2-eCFP-pcDNA3.1(+)Zeo.Cys-P3 peptide was cloned directly before the signal peptide by *GeneScript* (Piscataway, N.J.,USA).

Cys-P3-ErbB2-eCFP was inserted into the PiggyBac plasmid pPBtet-3xFLAG-IRES-DsRed-Express-PuroR donor vector[5] by GenScript (Piscataway, NJ, USA). Cys-P3-ErbB2-eCFP was amplified with the addition of two unique SfiI restriction sites directly before and after the gene, before cloning into the pPBtet-3xFLAG-IRES-DsRed-Express-PuroR donor vector using the SfiI restriction endonuclease to generate the vector pPBtet-Cys-P3-ErbB2-eCFP-PuroR (Figure S 7-3Figure S 7-1) used for generation of the stable cell line.

DNA sequence inserted before ATG start codon of Cys-P3-ErbB2-eCFP: GGC CTC TGA GGC C

DNA sequence inserted before TAA stop codon of Cys-P3-ErbB2-eCFP: GGC CTG TCA GGC C

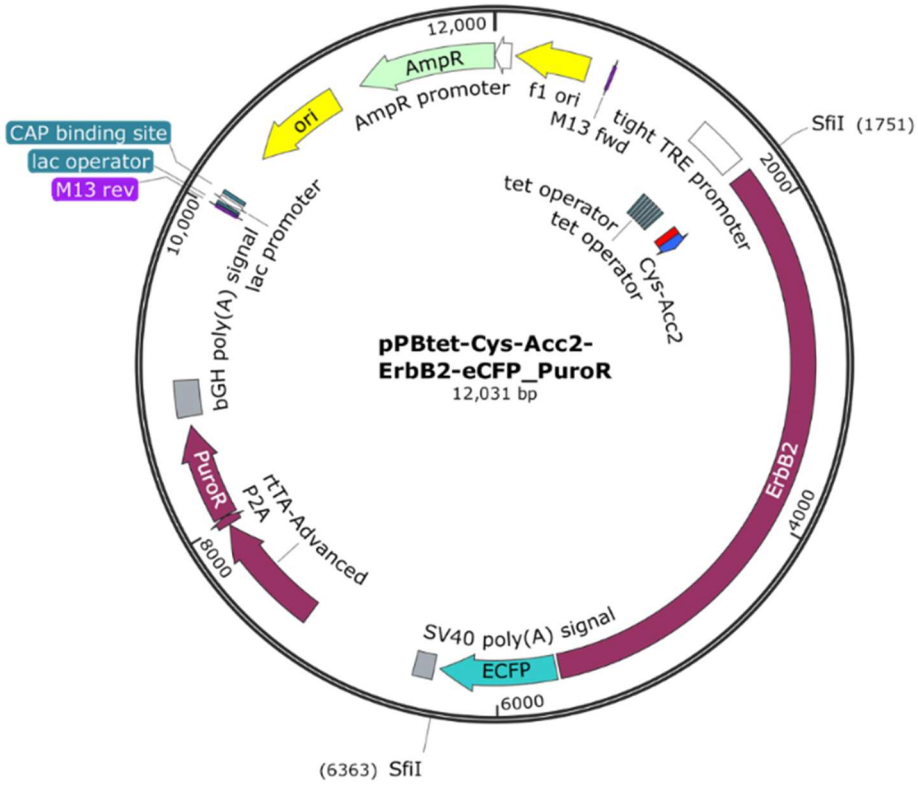


Figure S 7-3 Plasmid map of pPBtet-Cys-P3-ErbB2-eCFP-PuroR donor vector used for PiggyBac transposition.

8 Cell culture and stable cell production

8.1 Reagents and media for cell culture and fluorescence microscopy

Nutrient Mixture F-12 Ham **(**N6658), Fetal Bovine Serum (FBS) superior, L-Glutamine, penicillin, streptomycin, doxycycline, trypsin, TRIS and EDTA were purchased from *Merck* (Darmstadt, Germany). Trypsin / EDTA (0.25%), OptiMEM, Phosphate buffered saline (PBS, 100 mM phosphate, 150 mM sodium chloride), Dulbecco's phosphate buffered saline (DPBS; calcium, magnesium, 14040141), Hanks balanced salt solution (HBSS, calcium, magnesium, no phenol red 14025092), Opti-MEM, Puromycin Dihydrochloride, Lipofectamine®2000, Lipofectamine®3000, Poly-D-Lysine (0.1 mg/mL), Hoechst 33342 (0.5mg/mL), were purchased from *Thermo Fischer Scientific* (Waltham, U.S.A.). Triton™ X-100, Salmon Sperm DNA sodium salt, BSA (Albumin Fraction V) was purchased from *Carl Roth* (Karlsruhe, Germany). 8-well µ-slides were acquired from *ibidi GmbH* (Martinsried, Germany). Fluorescence labeled and non-labeled oligonucleotides were purchased from *Biomers* (Ulm, Germany).

8.2 CHO cell culture

CHO cells were cultured in Hams F12 from *Thermo Fischer Scientific* with a freshly added 2mM L-Glutamine, and penicillin / streptomycin (10,000 units/ mL), puromycin (8 µg/mL) and 10 % FBS at 37 °C and 5% CO_2. For plating cells were washed with PBS and detached with 1 mL 0.25% trypsin / 0.02% EDTA solution for 2 min at 37 °C. Cells were centrifuged and fresh medium was exchanged.

8.3 Generation of stable doxycycline inducible Cys-P1-EGFR-eYFP and Cys-P3-ErbB2-eCFP single and double positive CHO cell lines by PiggyBac transposition

Generation of stable doxycycline inducible Cys-P1-EGFR-eYFP single positive CHO clone 12.3 was described previously.[3] Stable doxycycline inducible single positive Cys-P3-ErbB2-eCFP (clone 17.1) and double positive Cys-P1-EGFR-eYFP/ Cys-P3-ErbB2-eCFP (clone 6.2) CHO cell lines were generated accordingly. Briefly, wild-type CHO cells (CHO Cell Line from Chinese hamster ovary, 85050302, Merck*)* were transfected in a 6-well plate with either 1.88 µg Cys-P3-ErbB2-eCFP donor plasmid and 0.62 µg PiggyBac transposase (System Biosciences, #PB200PA-1) vector (single positive clone 17.1) or 1.0 µg Cys-P1-EGFR-eYFP donor plasmid, 1.0 µg Cys-P3-ErbB2-eCFP donor plasmid, and 0.5 µg PiggyBac transposase vector (double positive clone 8.3) using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Two days after transfection, cells were transferred to puromycin selection (8 µg/mL) for 13 days before fluorescence-activated cell sorting (FACS). To establish stable CHO clone 17.1 and 6.2, transfected CHO cells were subjected to two rounds of FACS: First, eCFP or eYFP/eCFP negative uninduced cells were sorted to ensure tight doxycycline dependent expression of Cys-P3-ErbB2-eCFP and Cys-P1-EGFR-eYFP/ Cys-P3-ErbB2-eCFP, respectively. Second,

cells were treated for 18 h with doxycycline (100 ng/mL), 10,000 cells sorted based on thresholded levels of Cys-P3-ErbB2-eCFP and Cys-P1-EGFR-eYFP/P3-ErbB2-eCFP expression, and plated at clonal density into a p100 cell culture dish without doxycycline. After 5 days, single colonies were picked, transferred to a 96-well plate, and expanded. Before starting experiments, stable CHO clones 17.1 and 6.2 were analysed by flow-cytometry for homogeneous levels of Cys-P3-ErbB2-eCFP and Cys-P1-EGFR-eYFP/ Cys-P3-ErbB2-eCFP expression after doxycycline induction (100 ng/mL) for 18 h (Figure S8-2). To maintain expression of the pPBtet-Cys-P3-ErbB2-eCFP-PuroR and pPBtet-Cys-P1-EGFR-eYFP-PuroR cassette, all stable CHO clones were continuously cultured with puromycin (8 µg/mL).

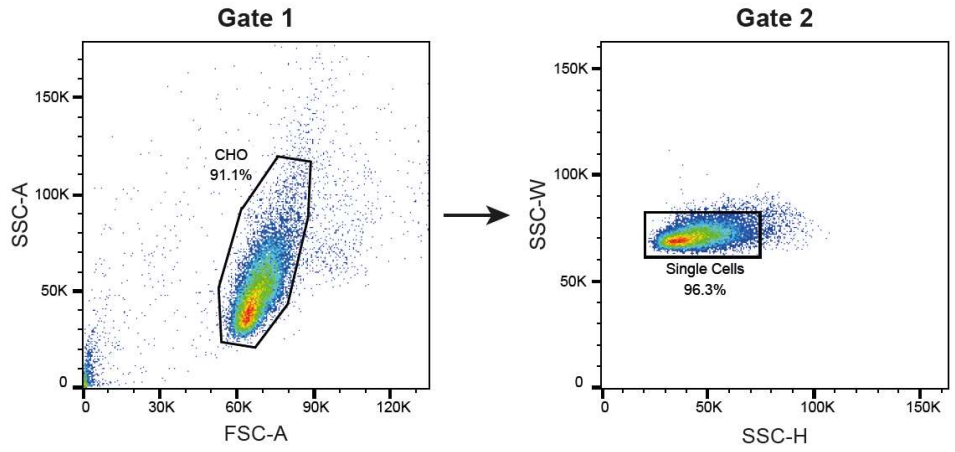


Figure S 8-1 Exemplified gating strategy displaying wild-type CHO (wtCHO). Debris (FSC-A, SSC-A; Gate 1) and doublets (SSC-H, SSC-W; Gate 2) were excluded and 18,000 – 19,000 single cells per sample analyzed for eYFP and/or eCFP fluorescence intensity

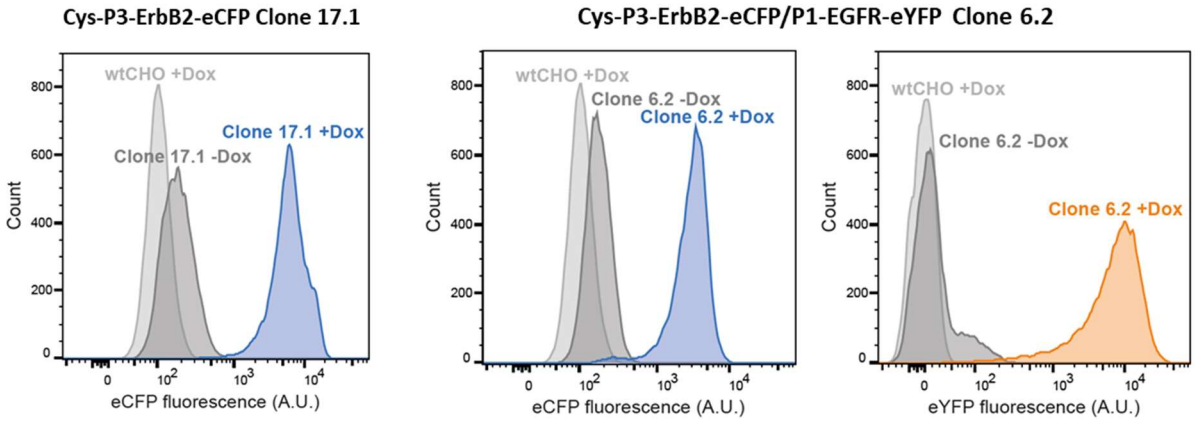


Figure S 8-2 Expression of Cys-P3-ErbB2-eCFP only (stable CHO clone 17.1) or Cys-P3-ErbB2-eCFP/Cys-P1-EGFR-eYFP (stable CHO clone 6.2) was analyzed by fluorescence flow cytometry in the absence of doxycycline (-Dox) and 18 h after induction with 100 ng/mL doxycycline (+Dox). wtCHO +Dox are plotted as negative control. Fluorescence intensity of eCFP/eYFP is indicated in arbitrary units (A.U.).

9 Fluorescence microscopic analysis

Fluorescence microscopy was performed by using an IX83 microscope from *Olympus* using a 60x lens. Transfected cells were imaged in five different channels

Hoechst33342: λ_{ex} = 350 ± 25 nm, λ_{em} = 460 ± 25 nm; CFP: λ_{ex} =438 ± 12 nm, λ_{em} 483 ± 12 nm; YFP: λ_{ex} = 500 ± 12 nm λ_{em} 545 ± 20 nm; Atto565(TRITC): λ_{ex} = 575 ± 12 nm λ_{em} 628 ± 20 nm CY7/DY751: λ_{ex} = 710 ± 37 nm, λ_{em} 810 ± 40 nm. *Olympus* CellSens software and ImageJ was used for image processing.

9.1 Labelling of Cys-P1-EGFR-eYFP and Cys-P3-ErbB2-eCFP on stable CHO cells

Prior to cell seeding 8-well µ-slides (ibidi, ibiTreat) were coated with 0.01% poly-D-lysine. After 10 min incubation the solution was removed and the slides were allowed to dry. Cys-P1-EGFR-eYFP, Cys-P3-ErbB2-eCFP or Cys-P1-EGFR-eYFP/Cys-P3-ErbB2-eCFP stable CHO cells (20,000) were seeded and incubated in 200 µL Hams F12 media (10% FBS) overnight at 37°C. Cells were induced with 0.1 µg/mL doxycycline by addition of 100 µL Hams F12 media (10% FBS, 0.3 µg/mL doxycyline) overnight. Cells were starved for 4 hours in 0% FBS Hams F12 Media. Prior to labelling the cell nuclei were stained with Hoechst 33342 in HBSS for 10 min. After removing and washing with HBSS, 100 nM PNA_1-P2 or PNA_3-P4 or both in HBSS was added for 4 min. Cells were washed with HBSS prior to addition of 200 nM Atto565-DNA_1 or Cy7-DNA_3 or both in HBSS. After 4 min cells were washed with HBSS and fluorescence microscopy was performed (Figure 2, Figure S 9-1 ,Figure S 9-2).

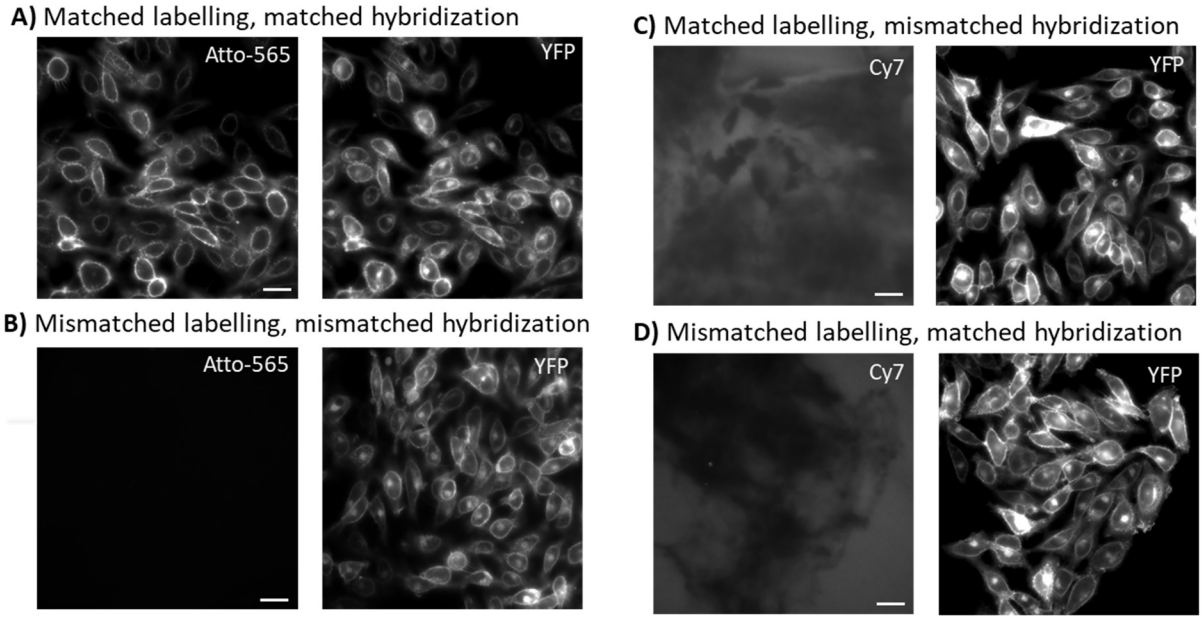


Figure S 9-1 Fluorescence microscopy images of CHO cells expressing Cys-P1-EGFR-eYFP after labelling with A) 'P1-matched' PNA_1-P2 and staining with 'PNA_1-matched' DNA_1-Atto565; B) 'P1-mismatched' PNA_3-P4 and staining with 'PNA_3-mismatched' DNA_1-Atto565; C) 'P1-matched' PNA_1-P2 and staining with 'PNA_1-mismatched' DNA_3-Cy7; D) 'P1-mismatched' PNA_3-P4 and staining with 'PNA_3-matched' DNA_3-Cy7. Excitation times: Cy7; 500 ms, Atto565: 500 ms, YFP:

200 ms, CFP 150 ms. Scale bar=20 µm. Experiments were repeated three times with similar results.

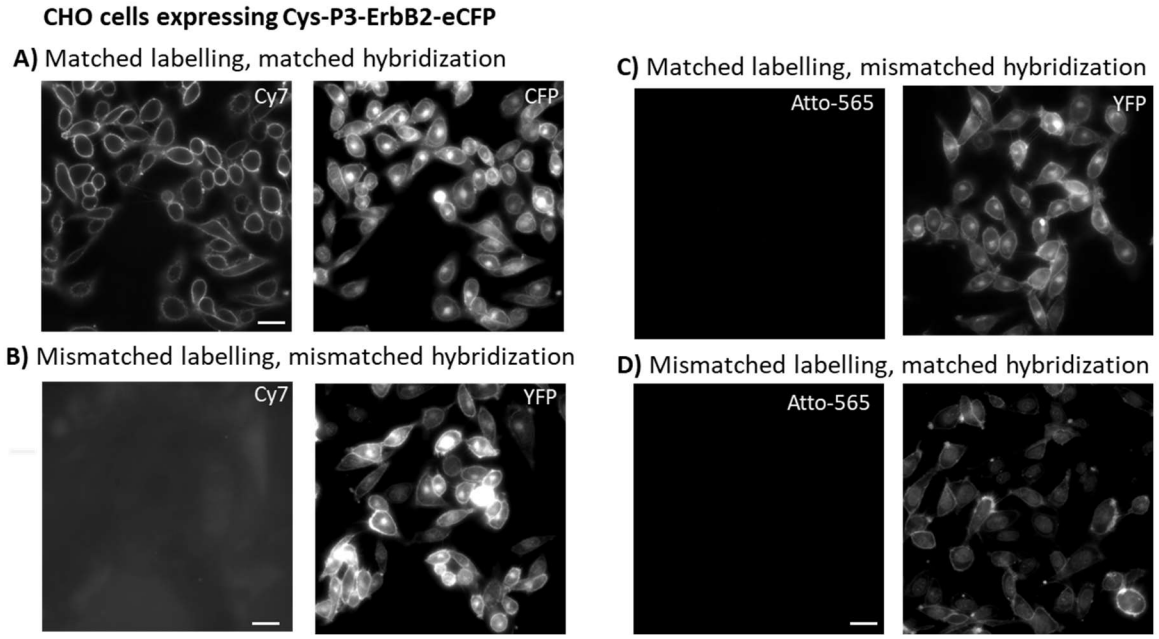


Figure S 9-2 Fluorescence microscopy images of CHO cells expressing Cys-P3-ErbB2-eCFP after labelling with A) 'P3-matched' PNA_3-P4 and staining with 'PNA_3-matched' DNA_3-Cy7; B) 'P3-mismatched' PNA_1-P2 and staining with 'PNA_1-mismatched' DNA_3-Cy7; C) 'P3-matched' PNA_3-P4 and staining with 'PNA_3-mismatched' DNA_1-Atto565; D) 'P3-mismatched' PNA_1-P2 and staining with 'PNA_1-matched' DNA_1-Atto565. Excitation times: Cy7; 500 ms, Atto565: 500 ms, YFP: 200 ms, CFP 150 ms. Scale bar=20 µm. Experiments were repeated three times with similar results.

9.1.1 Colocalization analysis

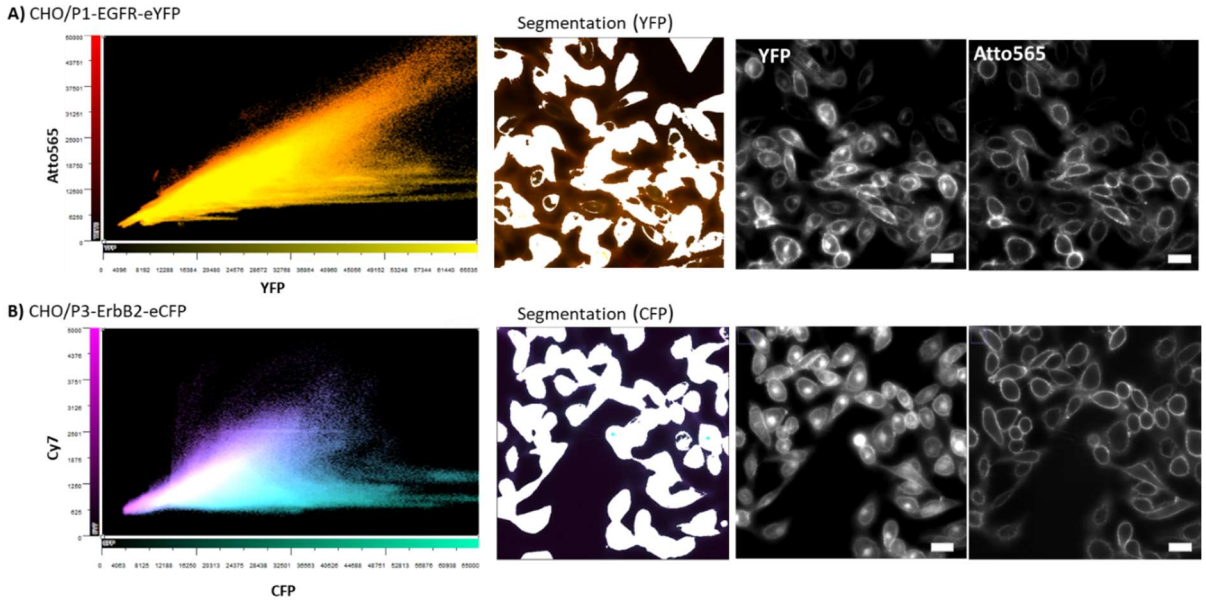


Figure S 9-3 Colocalization scatterplot obtained using *CellSens* software.for images shown in Figure 2A. Image was segmented according to the YFP/CFP signal before analysis

9.2 Quantifying internalization using erasable DNA strands

Cys-P1-EGFR/Cys-P3-ErbB2 CHO cells (30,000) were seeded in in 200 µL Hams F12 media (10% FBS) on 8-well µ-slides (ibidi, ibiTreat) and incubated overnight at 37°C. Cells were induced with 0.035 µg/mL doxycycline by media change for 20 hours. Cells were starved for 4 hours in serum free Hams F12 media with 0.035 µg/mL doxycycline. HBSS-BB and DPBS were prewarmed in a water bath at 37°C prior to experiment. After removing media and washing with once with DPBS, 100 nM of both PNA_1-P2 and PNA_3-P4 (from 50 µM stocks in 0.1% in TFA) in DPBS was added for 4 min an incubator (37°C, 5% CO_2). For a negative control, in one well no PNA thioester was added. Cells were washed with HBSS-BB (HBSS-Blocking buffer: 0.1 mg/mL salmon sperm DNA, 0.2% BSA in HBSS with calcium, magnesium) prior to addition of DNA complexes (Atto565-complex I and DY751-complex III; each 200 nM in HBSS-BB). After 4 minutes at room temperature cells were washed with HBSS-BB before addition of serum free HamsF12 media with either epidermal growth factor (EGF, 100 nM), Geldanamycin (GA) 3 µM nothing (negative control and no PNA control). The cells were returned to the incubator for 20 minutes before addition of 50 µM erasure DNAs to make up a final concentration of 1 µM. After 4 minutes, media was removed and replaced with eraser DNAs in HBSS-BB (1 µM) for a further 4 minutes before washing twice with HBSS-BB and leaving in HBSS-BB for microscopy.

Atto565-complex I: DNA_1-3-DNA_5*-1-DNA_5*: 2 x Atto565-DNA_5-TOE1
DY751-complex III: DNA_3-1-DNA_4*-1-DNA_4*: 2 x DY751-DNA_4-TOE2

DNA eraser I: TOE1*-DNA_5*
DNA eraser III: TOE2*-DNA_4*

9.2.1 Internalization analysis

To quantify internalisation of EGFR and ErbB2, two photos from the same well plate (2 viewpoints) were taken per experiment for each condition and analysed using Olympus *CellSens* software. (excitation times Atto565,Cy7: 600 ms; CFP, YFP: 400 ms). For each photo, automatic segmentation was applied to the YFP channel and regions of interest (ROIs) were manually created based on the segmentation results. Every cell which was clearly segmented was counted, and 50 cells from each photo were taken. In the event that more than 50 cells were counted per photo, 50 cells were picked via random number generation in Microsoft Excel. In the event that less than 50 cells were counted, the number was made up to 50 from the second photo, also chosen by random number generation. 100 cells were therefore collected per condition per experiment. For each ROI a measure of brightness was given in RFU (relative fluorescence units) and from this a mean fluorescence value calculated for

each population. Figure S 9-4 shows results from one replicate. From these mean fluorescence values, a 'background' value was subtracted. These were cells which had been treated with the relevant DNA, but not PNA and represented the nonspecific binding/internalisation of DNA-fluorophore to cells, or non specific binding to the 8-well plate. The calculated means were then expressed as a relative to the negative control ie cells treated with only media.

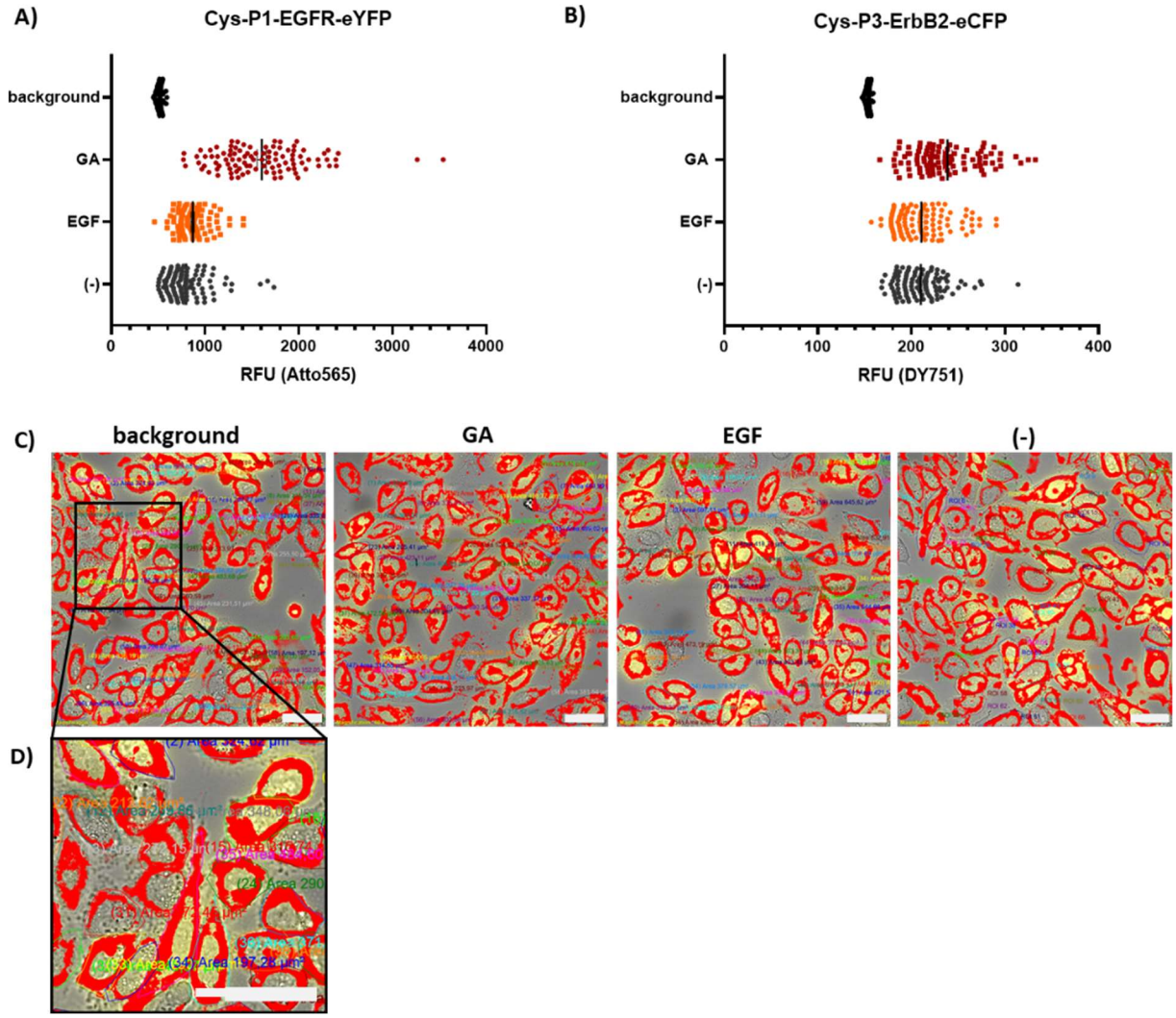


Figure S 9-4 **Internalization analysis methodology.** A) and B) Scatter plots from a single replicate from experiment shown in Figure 3. Each dot represents one ROI (region of interest) which represents one cell. The brightness (RFU) of the ROI in an indication of internalization in response to the given conditions. For each condition 100 cells were counted, from 2 photos (viewpoints) on the same well. Scatter plots show mean with SEM. C) Examples of automatic segmentation (red) and manual picking of ROIs. Pictures show brightfield images (grey) overlayed with YFP signal(yellow), segmentation and ROIs (mulitcoloured). Scale bar=30 µm D) Zoomed in image of black square in (C). Experiments were repeated 3 times with similar results and combined data shown in Figure 3.

CHO Cys-P1- EGFR-eYFP/ErbB2-eCFP

Imager DNA only; no PNA tagging

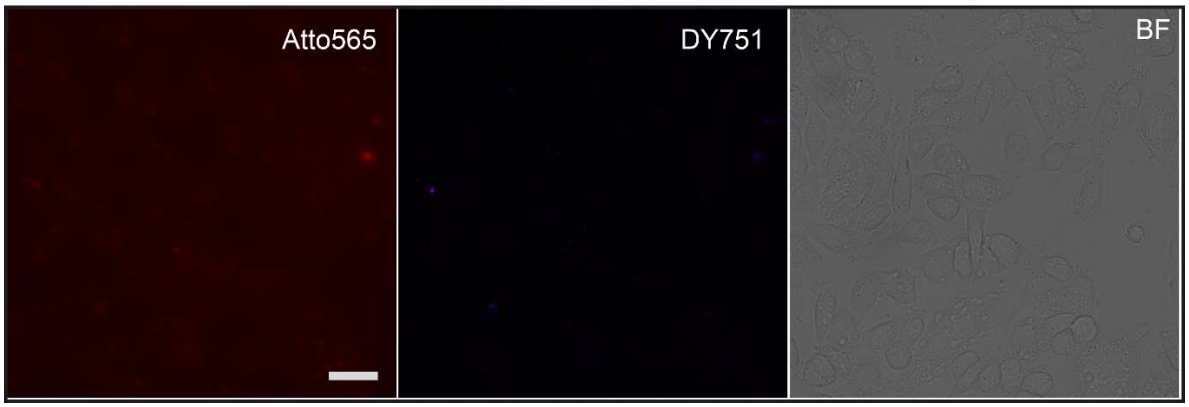


Figure S 9-5 Control for experiment shown in Figure 3B for unselective DNA staining. Conditions: DPBS, 4 min: Atto565-Complex I and DY751-Complex III (200 nM in HBSS-BB, 4min); 4 min serum free media at 37 °C; erasure: 4 min DNA erasers I and III (1µM) at 37°C then wash with 1 µM in HBSS-BB, 4 min; wash with HBSS-BB). BF= Brightfield.

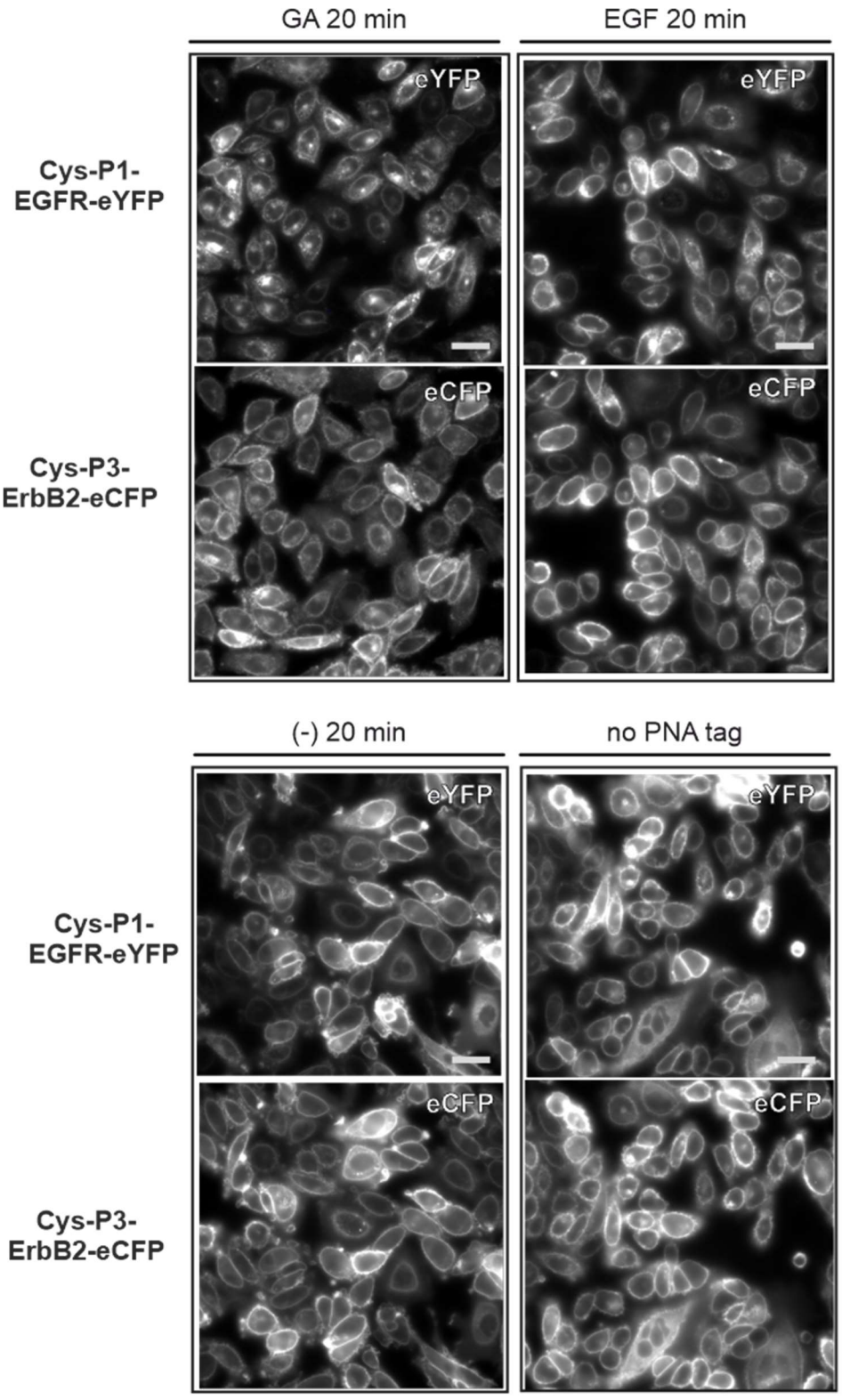


Figure S 9-6 CFP and YFP channel images from fluorescence microscopy experiment shown in Figure 3B and Figure S9-5. Imaging were used for internalization analysis of Cys-P1-EGFR-eYFP and Cys-P3-ErbB2-eCFP in stable CHO cells. Excitation time eCFP/eYFP: 400 ms.

References

[1] A. Roloff, Templatgesteuerte Reaktionen von Peptidnukelinsäuren, Dissertation, Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I **2014**.

[2] S. Mourtas, D. Gatos, V. Kalaitzi, C. Katakalou, K. Barlos, *Tetrahedron Letters* **2001**, *42*, 6965-6967.

[3] G. C. Gavins, K. Gröger, M. D. Bartoschek, P. Wolf, A. G. Beck-Sickinger, S. Bultmann, O. Seitz, *Nature Chemistry* **2021**, *13*, 15-23.

[4] a) J. SantaLucia, H. T. Allawi, P. A. Seneviratne, *Biochemistry* **1996**, *35*, 3555-3562; b) A. Panjkovich, F. Melo, *Bioinformatics* **2004**, *21*, 711-722.

[5] S. M. Stief, A.-L. Hanneforth, S. Weser, R. Mattes, M. Carlet, W.-H. Liu, M. D. Bartoschek, H. Domínguez Moreno, M. Oettle, J. Kempf, B. Vick, B. Ksienzyk, B. Tizazu, M. Rothenberg-Thurley, H. Quentmeier, W. Hiddemann, S. Vosberg, P. A. Greif, K. H. Metzeler, G. Schotta, S. Bultmann, I. Jeremias, H. Leonhardt, K. Spiekermann, *Leukemia* **2019**.