Electronic Supplementary Material (ESI) for Chemical Science.
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1. General experiment procedures

Materials

H-Rink amide ChemMatrix resin (35-100 mesh, with a loading of 0.4-0.6 mmol/g) was obtained from Sigma-Aldrich. TentaGel R RAM (with a loading of 0.2 mmol/g) was purchased from RAPP POLYMERE (Tuebingen, Germany). NovaSyn TGR resin was purchased from Novabiochem/ Merck Biosciences. Oxyma was obtained from Luxembourg Bio-technologies (Ness Ziona, Israel). HCTU, PyBOP were purchases from Chemcube (Bochum, Germany). PNA monomers, such as Fmoc-PNA-T-OH, Fmoc-PNA-A(Bhoc)-OH, Fmoc-PNA-C(Bhoc)-OH, Fmoc-PNA-G(Bhoc)-OH were obtained from Link Technologies Ltd (Bellshill, Scotland). Fmoc-protected amino acids were purchased from Iris Biotech GMBH (Marktredwitz, Germany). NIR-664-iodoacetamide dye was obtained from Santa Cruz Biotech. RNA oligonucleotides were purchased from Biomers GmbH (Ulm/Donau, Germany). Water was purified with a Milli-Q Ultra Pure Water Purification system of Membrapure Company (Germany). Column chromatography was carried out with SDS 60 ACC silica gel, checked by TLC with Merck silica gel 60 F254 plates. Mass spectra were measured by using an Applied Biosystems QStar XI spectrometer (ESI+).

HPLC analysis and purification

Before purification, the crude PNA probes were dissolved in water/acetonitrile (1:1) containing 0.1% TFA, filtered to remove insoluble residues if necessary. The semi preparative HPLC was carried out with an Agilent 1100 series instrument with C18 column (C18 A 5µ 250x100, pore size 220Å from Macherey-Nagel GmbH) using eluent A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and eluent B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in a linear gradient (from 3% A to 60 % in 30 min) with a rate of 6 mL/min at 260 nm. The desired fractions were combined, checked by HPLC-MS and dried by a lyophilizer machine to give a white powder.

Fluorescence spectroscopy

Fluorescence emission measurement was performed by using a Cary Eclipse Fluorescence spectrometer. FIT-probes and target RNA with specified concentrations were added to 10 mm quartz cuvettes with 1.0 mL phosphate buffer (10 mM Na_2HPO_4, 100 mM NaCl, pH 7). Samples were allowed to equilibrate for about 10 minutes prior to measurement. Settings for TO-labeled probes: λ_{ex} = 485 nm, λ_{em} = 500-700 nm, $slit_{ex}$ = 5 nm, $slit_{em}$ = 5 nm; settings for JO-labeled or TOJO-labeled probes: λ_{ex} = 500 nm, λ_{em} = 510-700 nm, $slit_{ex}$ = 5 nm, $slit_{em}$ = 5 nm. For TO, JO, and TOJO PNA probes, quantum yields were measured by using Fluorescein as a standard, with excitation at 475 nm. For TOJO-NIR667 or TO-NIR667 binary system, quantum yields were measured by using ATTO 490 LS as a standard (purchased from ATTO-TEC GmbH, Siegen, Germany) with excitation at 500 nm.

UV-Vis spectroscopy

Absorption spectra (400-700 nm, 1 nm steps) were measured by using Cary 100 UV-Visible spectrophotometer.

2. FIT-PNA synthesis

Preloading H-Rinkamide Chemmatrix resin with Fmoc-Gly-OH

H-Rink amide Chemmatrix resin (200 mg) was swollen in 5 mL of DMF in a 10 mL frit-fitted plastic syringe for 15 min. After thoroughly washing with DMF, DCM and DMF, a reactive mixture in 5 mL DMF containing Fmoc-Gly-OH (18 mg, 0.06 mmol), PyBOP (46 mg, 0.06 mmol), NMM (19 µL, 0.12 mmol) was added to the resin. The coupling step was carried out at room temperature under agitation. After 5 hours, the reactive mixture was discarded and the resin thoroughly washed with DMF, DCM, DMF. Capping was performed twice by treatment with Ac_2O/2, 6-lutidine/DMF (5:6:89, v:v:v) for 10 min. The Fmoc loading was determined by UV-vis absorbance measurement of the amount of the piperidine-fulvene (ε_{301} = 7800 $cm^2 \cdot mol^{-1}$) adduct obtained after treatment of a resin aliquot with DMF/piperidine.. With this procedure, the loading of the resin usually is in the range of 0.2-0.3 mmol/g.

Fmoc-based solid phase PNA synthesis and amino acid coupling

The PNA probes were prepared in a 1 µmol scale with glycine-loaded H-Rink amide Chemmatrix resin (0.2-0.3 mmol/g). The automated synthesis was performed by using a ResPep synthesizer (Intavis, Germany). For coupling of standard PNA monomers, 4.0 equiv. PNA monomer, 3.8 equiv. HCTU and 8.0 equiv. NMM were dissolved in DMF (0.1 M final concentration of building block). Coupling was performed at RT for 30 min and repeated once. Fmoc deprotection was carried out by two consecutive treatments of the resin with piperidine/DMF (1:4, v/v, 200 µl) for 5 min. The resin was washed the resin with DMF (7 x 20 µl). For capping, the resin was treated for 10 min with 200 µL of a mixture of $A_{C2}O$/2, 6-lutidine/DMF (5:6:89, v: v: v). The procedure was repeated once. After the PNA couplings, the resin was washed with DCM prior to TFA cleavage (TFA/m-cresol/H_2O/TIPS, 90/5/5/2), which was performed at room temperature for 120 min. The crude PNA probes were precipitated upon addition of cold diethyl ether and collected as a white powder by centrifugation. Coupling of Fmoc-aeg(TO)-OH (or Fmoc-aeg(JO)-OH) was performed by a reactive mixture in 55 µl DMF containing 3.4 mg Fmoc-aeg(TO)-OH (or Fmoc-aeg(JO)-OH), 4.25 mg PyBOP, 2 mg PPTS (pyridinium p-toluenesulfonate), 2.75 µl NMM. The coupling time was extended to 4 hours or overnight and the coupling temperature was set to 28 °C. Coupling of Fmoc-Lys(Boc)-OH was performed by using a reactive mixture containing Fmoc-Lys(Boc)-OH (3 mg, 6.0 equiv.), HCTU (2.5 mg, 6 equiv.), HOBt (1 mg, 6.0 equiv.), NMM (1.32 µL, 12.0 equiv.) for 1 h. Coupling of Fmoc-aeg(Boc)-OH, was performed by a reactive mixture containing Fmoc-aeg(Boc)-OH (3 mg, 6.0 equiv.), PyBOP (3.4 mg, 6.0 equiv.), NMM (2.1 µL, 12.0 equiv.) for 1 h. Coupling of Fmoc-aeg(Boc)-OH, was performed by a reactive mixture containing Fmoc-aeg(Boc)-OH (3 mg, 6.0 equiv.), PyBOP (3.4 mg, 6.0 equiv.), NMM (2.1 µL, 12.0 equiv.) for 1 h.

Note: For coupling of GPNA monomers the resin was treated at 37 ^oC for 30 min with a 50 µl NMP mixture containing 2.5 equiv. monomer, 2.5 equiv. PyBOP and 5.0 equiv. NMM. α-GPNA monomers were synthesized according to the reported papers (S. Pothukanuri, Z. Pianowski, N. Winssinger, *Eur. J. Org. Chem.* **2008**, 3141-3148; Z. Pianowski, K. Gorska, L. Oswald, C. A. Merten, N. Winssinger, *J. Am. Chem. Soc.* **2009**, *131*, 6492-6497) Fmoc-

aeg(TO)-OH and Fmoc-aeg(JO)-OH were prepared as described previously described (L. Bethge, D. V. Jarikote, O. Seitz*, Bioorg. Med. Chem.* **2008**, *16*, 114-125)

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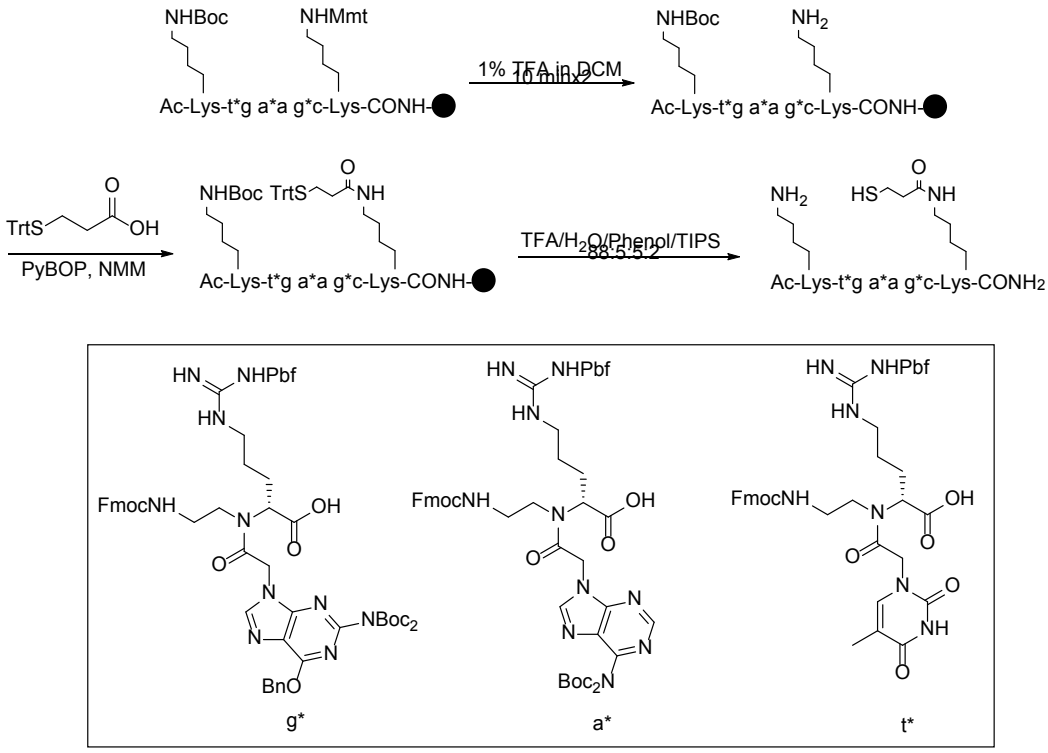
Mass spectrometric data of TO, JO, TOJOaeg PNA FIT probes

PNA	Sequence	Found	Calc.
TO_4	NH_2-a a a c t g a a g c a **TO** g g t Gly-NH_2	4355	4355
JO_6	NH_2-a a a c t g a a g **JO** a g g g t Gly-NH_2	4381	4380
JO_7	NH_2-a a a c t g a a **JO** c a g g g t Gly-NH_2	4340	4339
JO_8	NH_2-a a a c t g a **JO** g c a g g g t Gly-NH_2	4356	4356
JO_9	NH_2-a a a c t g **JO** a g c a g g g t Gly-NH_2	4356	4356
JO_{10}	NH_2-a a a c t **JO** a a g c a g g g t Gly-NH_2	4339	4340
JO_{11}	NH_2-a a a c **JO** g a a g c a g g g t Gly-NH_2	4365	4364
JO_{12}	NH_2-a a a **JO** t g a a g c a g g g t Gly-NH_2	4379	4380
TO_4JO_6	NH_2-a a a c t g a a g **JO** a **TO** g g t Gly-NH_2	4521	4520
TO_4JO_7	NH_2-a a a c t g a a **JO** c a **TO** g g t Gly-NH_2	4480	4479
TO_4JO_8	NH_2-a a a c t g a **JO** g c a **TO** g g t Gly-NH_2	4496	4496
TO_4JO_9	NH_2-a a a c t g **JO** a g c a **TO** g g t Gly-NH_2	4496	4495
TO_4JO_{10}	NH_2-a a a c t **JO** a a g c a **TO** g g t Gly-NH_2	4481	4480
TO_4JO_{11}	NH_2-a a a c **JO** g a a g c a **TO** g g t Gly-NH_2	4506	4505
TO_4JO_{12}	NH_2-a a a **JO** t g a a g c a **TO** g g t Gly-NH_2	4519	4520
$TO_4JO_6X_7$	NH_2-a a a c t g a a **X JO** a **TO** g g t Gly-NH_2	4332	4329
$TO_4JO_7X_8$	NH_2-a a a c t g a **X JO** c a **TO** g g t Gly-NH_2	4308	4306
$TO_4JO_8X_9$	NH_2-a a a c t g **X JO** g c a **TO** g g t Gly-NH_2	4324	4323
$TO_4JO_9X_{10}$	NH_2-a a a c t **X JO** a g c a **TO** g g t Gly-NH_2	4304	4305
$TO_4JO_{10}X_9$	NH_2-a a a c t **JO X** a g c a **TO** g g t Gly-NH_2	4305	4305
$TO_4JO_{11}X_7$	NH_2-a a a c t **JO** a a **X** c a **TO** g g t Gly-NH_2	4291	4289
$TO_4JO_{11}X_6$	NH_2-a a a c t **JO** a a g **X** a **TO** g g t Gly-NH_2	4328	4328
$TO_4JO_{11}X_5$	NH_2-a a a c t **JO** a a g c **X TO** g g t Gly-NH_2	4304	4305
$TO_4JO_{11}X_{12}$	NH_2-a a a **X JO** g a a g c a **TO** g g t Gly-NH_2	4352	4354
$TO_4JO_{12}X_{11}$	NH_2-a a a **JO X** g a a g c a **TO** g g t Gly-NH_2	4352	4354
$TO_7JO_{12}X_{13}$-F	NH_2-g t c **X JO** g c c g **TO** t g a t t a Gly-NH_2	4553	4555
TO_7JO_{12}-F	NH_2-g t c c **JO** g c c g **TO** t g a t t a Gly-NH_2	4705	4706
$TO_7JO_{13}X_{12}$-F	NH_2-g t c **JO X** g c c g **TO** t g a t t a Gly-NH_2	4553	4555
TO_7JO_{13}-F	NH_2-g t c **JO** g g c c g **TO** t g a t t a Gly-NH_2	4745	XX4746
TO_7-F	NH_2-g t c c g g c c g **TO** t g a t t a Gly-NH_2	4581	4580
TOJO_1	NH_2-a g **X JO** t g a g **TO** c c t t c Gly-NH_2	3996	3998
TO_1	NH_2-a g g g t g a g **TO** c c t t c Gly-NH_2	4005	4006

X = aminoethylglycine (see Figure 2)

3. Synthesis of NIR667-labeled PNA

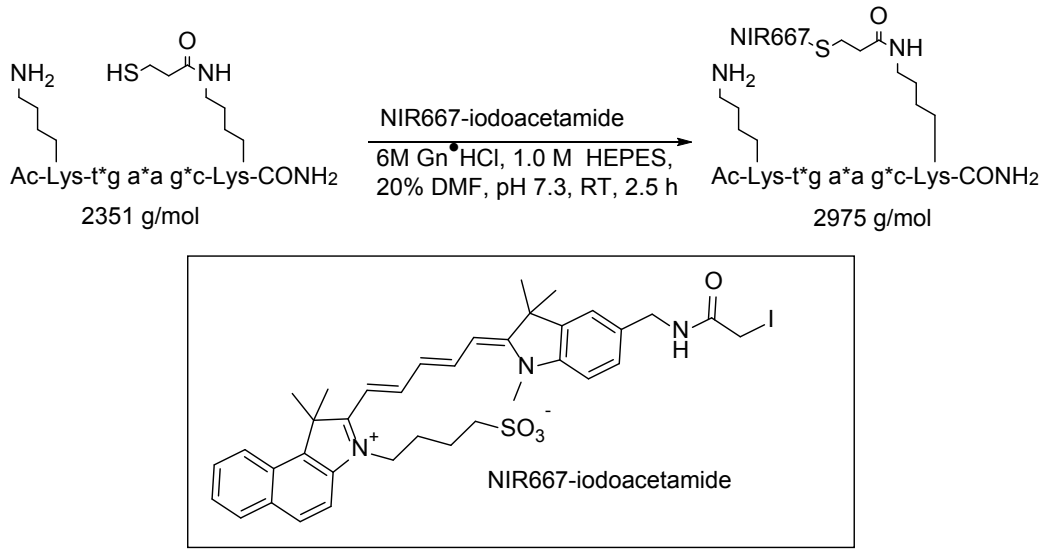
Synthesis of thiol-modified PNA



Scheme 1. Fmoc-based SPPS of thiol-modified PNA

Thiol-modified PNA of 1 μmol scale was synthesized by Fmoc-based solid phase peptide synthesis as shown in Scheme 1. Mmt of the side chain of Lys was deprotected by 1% TFA in DCM for 10 min x 2 until the yellow color of the Mmt cation completely disappeared. The coupling of 3-(tritylthio)propanoic acid was performed at room temperature. Guanidine-based peptide nucleic acid monomers were prepared according to the method reported by Nicolas Winssinger et al.,[1,2] and their structures are shown in Scheme 1. The resin was treated with TFA/m-cresol/H_2O/TIPS (90/5/5/2) for 120 min and the precipitate obtained after ether addition was analyzed by LC-MS.

Modification of thiol-modified PNA with NIR667-iodoacetamide



Scheme 2. Coupling of NIR667-iodoacetamide to the thiol-modified PNA

HEPES buffer was prepared by mixing 1.72 g Gn•HCl and 0.72 g HEPES with Millipore water to a final volume of 3.0 mL, followed by adjusting the pH value to 7.2 by addition of 0.1 N NaOH. The crude thiol-modified PNA (approx. 200 nmol) was dissolved in 160 µL HEPES buffer. NIR667-iodoacetamide (0.5 mg, 660 nmol) was dissolved in 40 µL DMF. The two solutions were combined and the mixture agitated for 2 hours at room temperature. Completion of the reaction was confirmed by LC-MS. Purification by semi preparative HPLC and subsequent lyophilization afforded a blue powder.

Sequence and mass of NIR664-modified PNA

NIR_2a: ^{Ac}Lys-tgaagc-Lys(COCH$_2$CH$_2$S-NIR664)^{CONH2}(2677 g/mol)
NIR_2b: ^{Ac}Lys-t*ga*ag*c-Lys(COCH$_2$CH$_2$S-NIR664)^{CONH2}(2975 g/mol)
NIR_2c: ^{Ac}Lys-ctgaagc-Lys(COCH$_2$CH$_2$S-NIR664)^{CONH2}(2929 g/mol)
NIR_2d: ^{Ac}Lys-actgaagc-Lys(COCH$_2$CH$_2$S-NIR664)^{CONH2}(3203 g/mol)

4. Absorbance and fluorescence emission spectra of TO-PNA, JO-PNA, TO/JO-PNA and TO/JO/X-PNA

TO_4

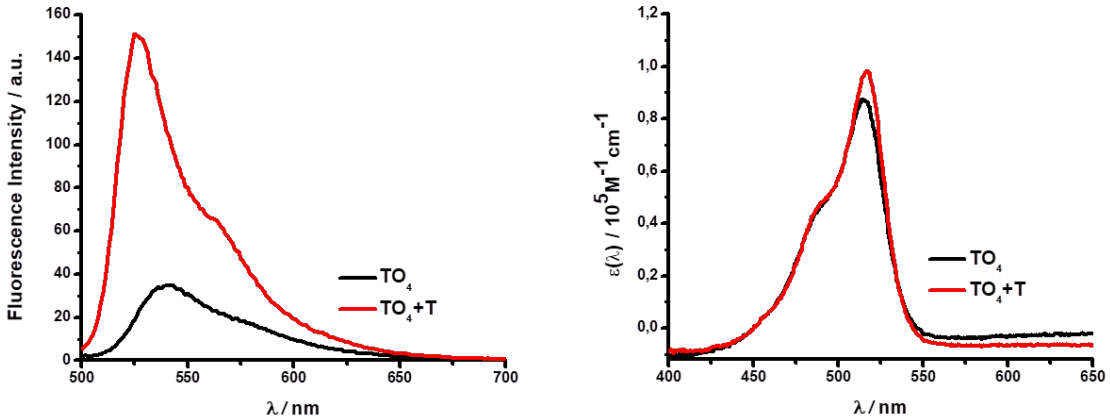
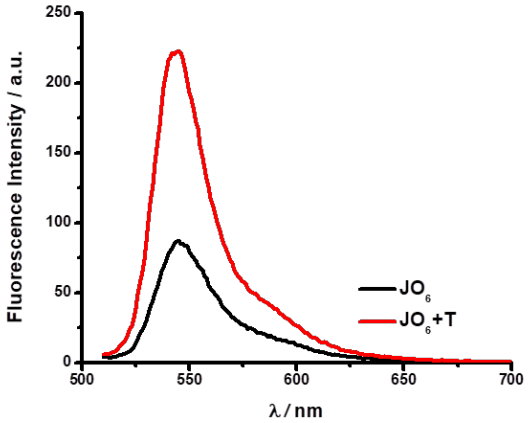
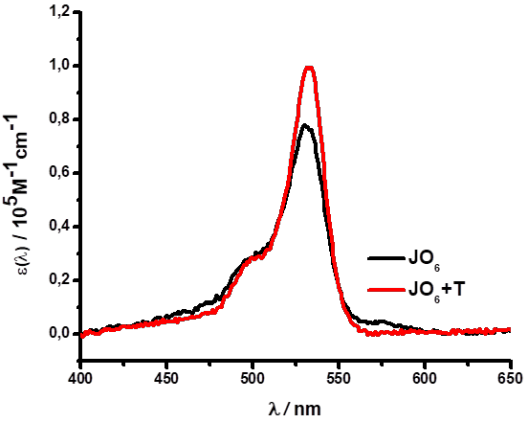
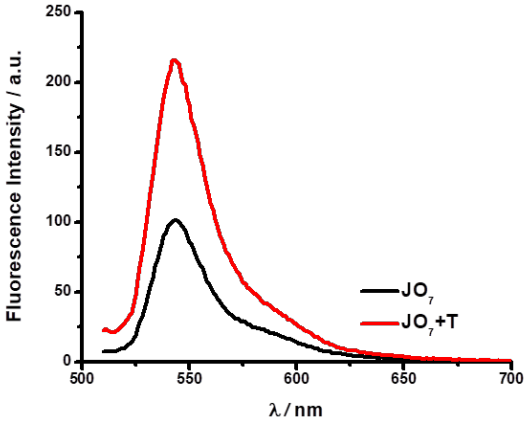


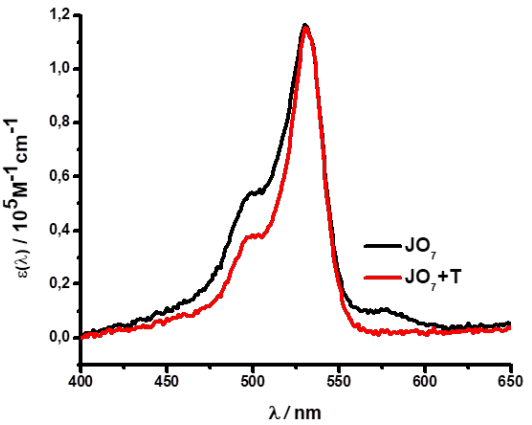
Figure S1: Emission spectra (left) and absorption spectra (right) and of **TO_4** in absence (black) and presence of complementary RNA target (red). Conditions: 0.25 µM probe and 2 eq. Glyα2-RNA target, in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), in 1 mL cuvette (path length = 1 cm); λ(ex) = 485 nm, λ(em) = 500-700 nm, slit(ex) = 5 nm, slit(em) = 5 nm, 37 °C.

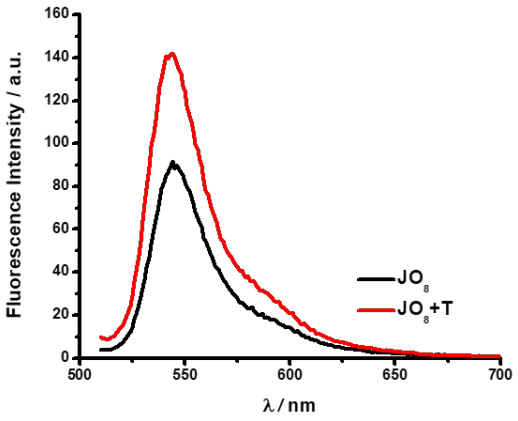
JO-PNA

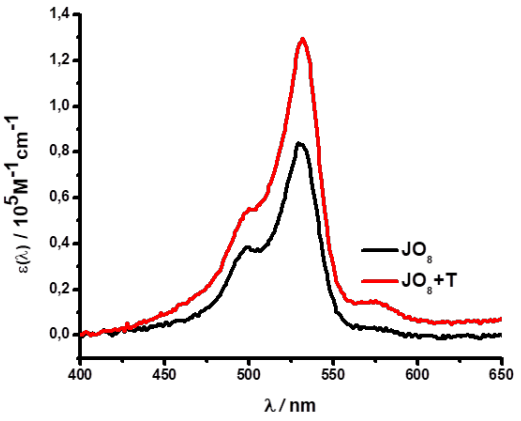

Fluorescence Intensity / a.u.
λ / nm
JO6
JO6+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
JO6
JO6+T


Fluorescence Intensity / a.u.
λ / nm
JO7
JO7+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
JO7
JO7+T


Fluorescence Intensity / a.u.
λ / nm
JO8
JO8+T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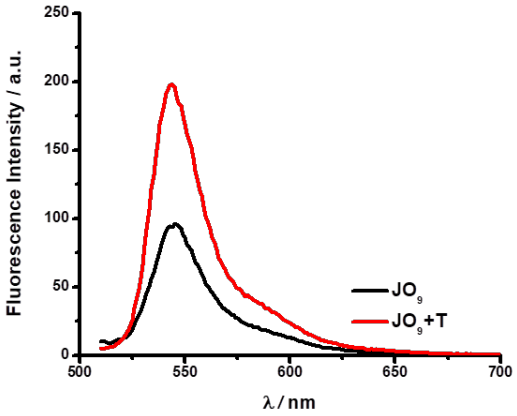
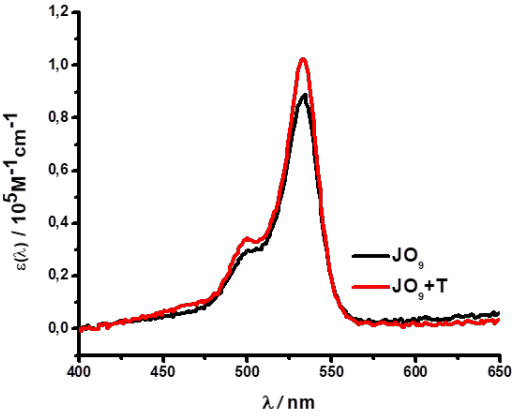
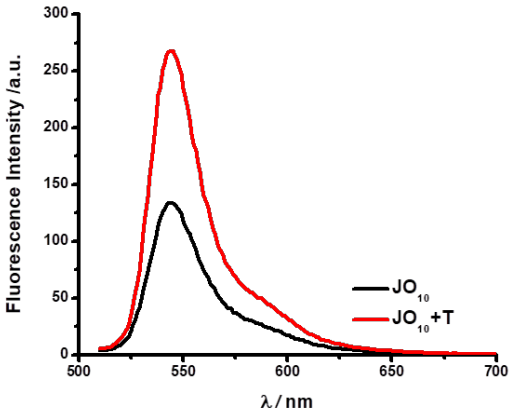
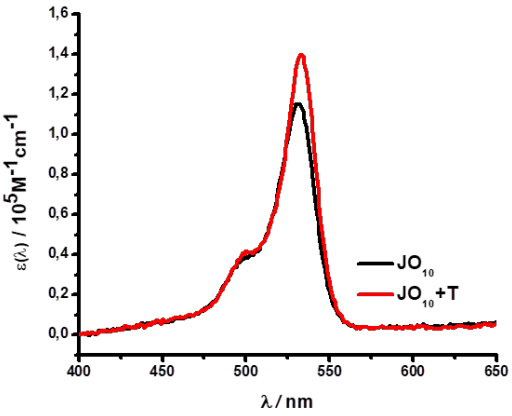
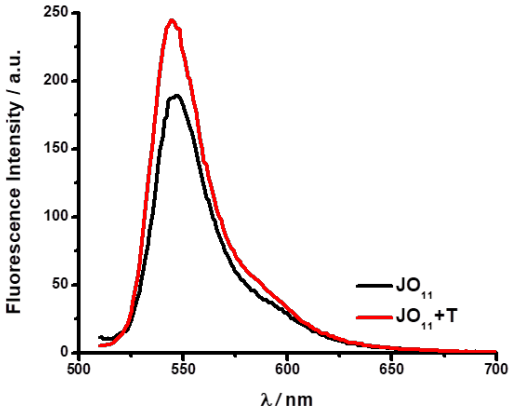
λ / nm
JO8
JO8+T

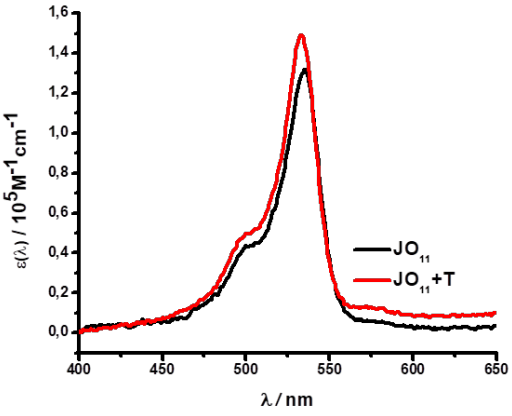

Fluorescence Intensity / a.u.
λ / nm
JO9
JO9+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
JO9
JO9+T


Fluorescence Intensity /a.u.
λ / nm
JO10
JO10+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
JO10
JO10+T


Fluorescence Intensity / a.u.
λ / nm
JO11
JO11+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
JO11
JO11+T

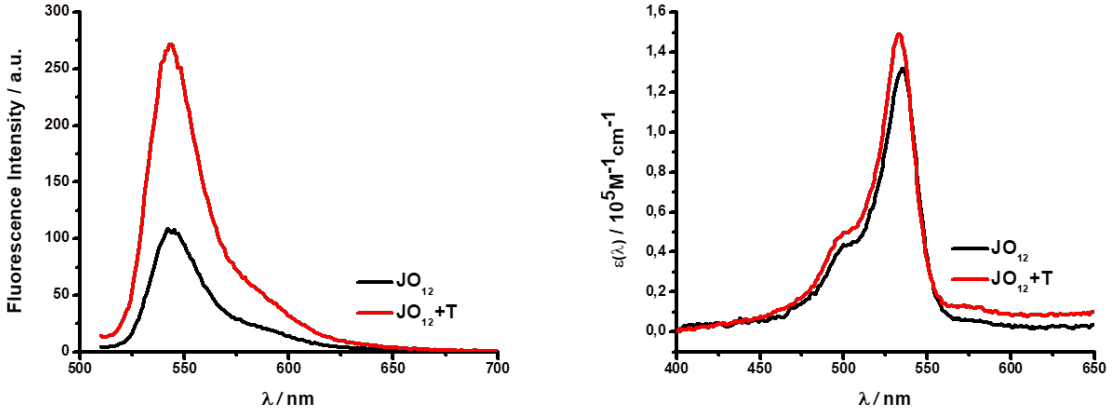
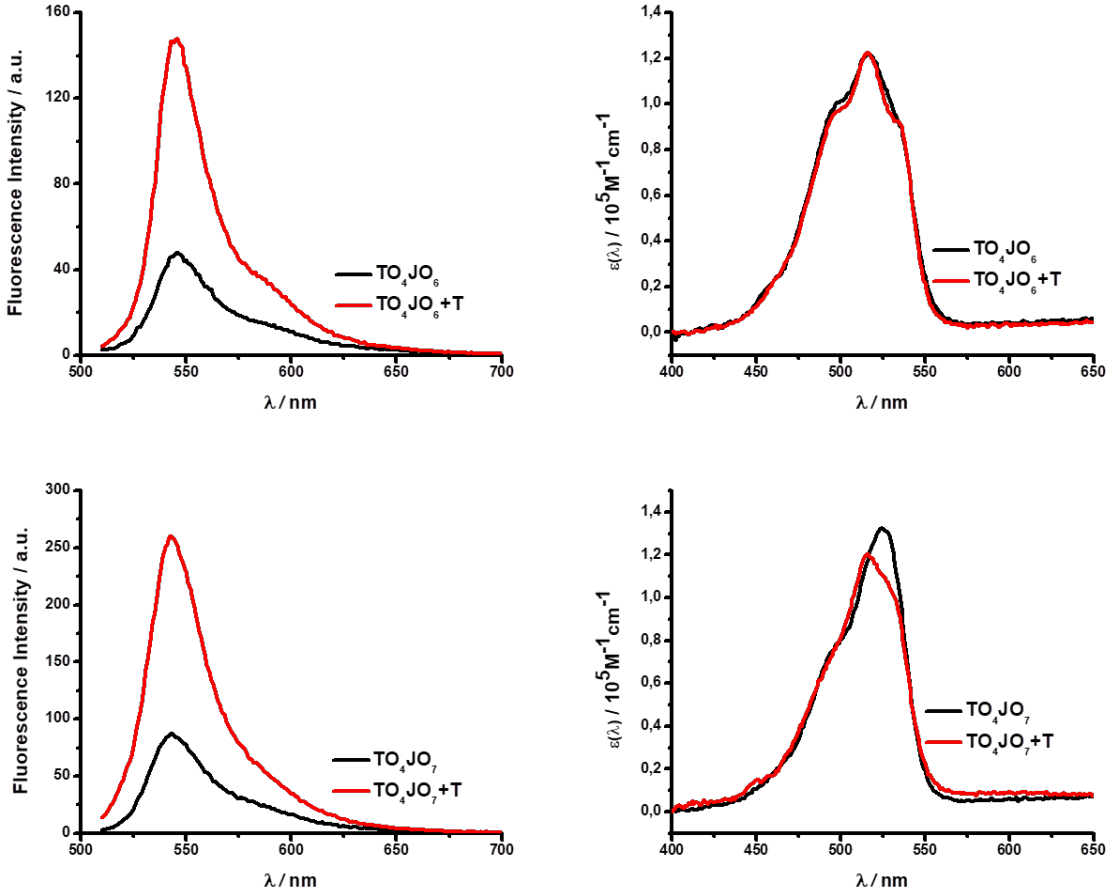
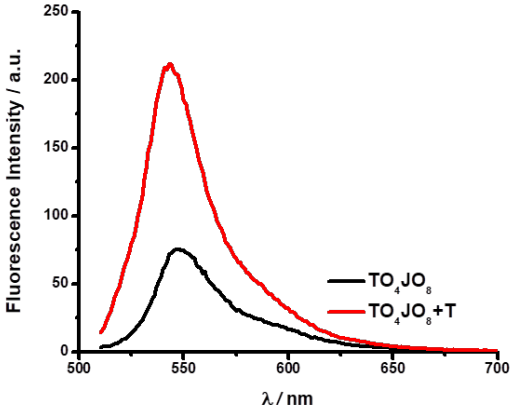
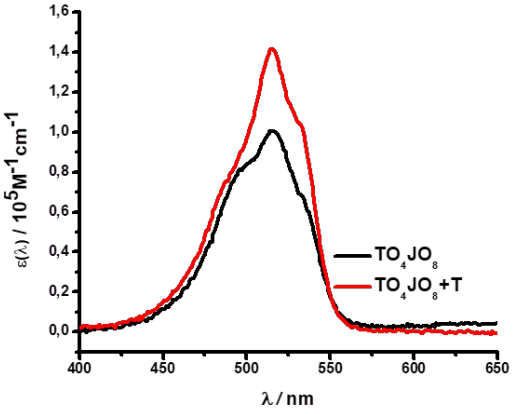


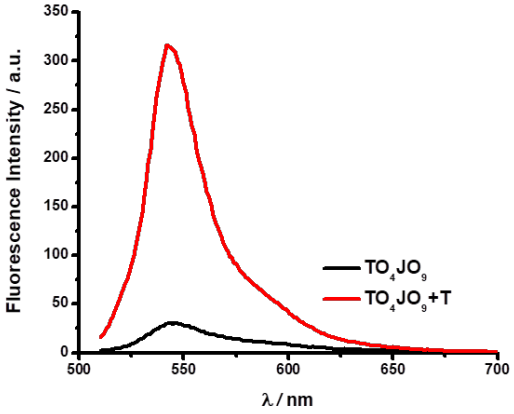
Figure S2: Emission spectra (left) and absorption spectra (right) of JO-PNA probes in absence (black) and presence of complementary RNA target (red). Conditions: 0.25 µM probe and 2 eq. Glyα2-RNA target, in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), in 1 mL cuvette (path length = 1 cm); λ(ex) = 500 nm, λ(em) = 510–700 nm, slit(ex) = 5 nm, slit(em) = 5 nm, 37 °C.

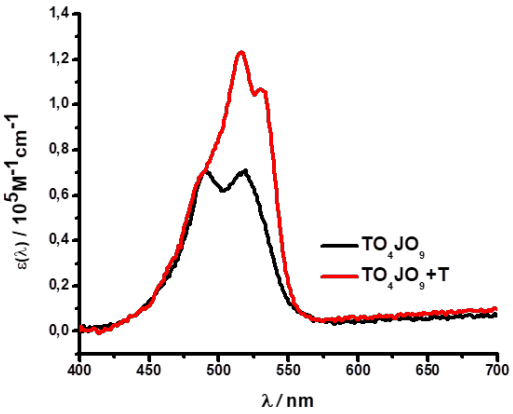
TOJO-PNA

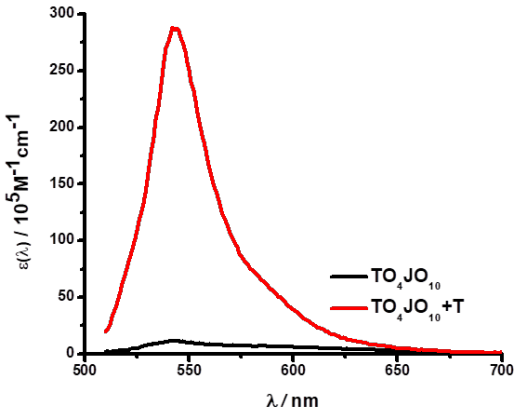


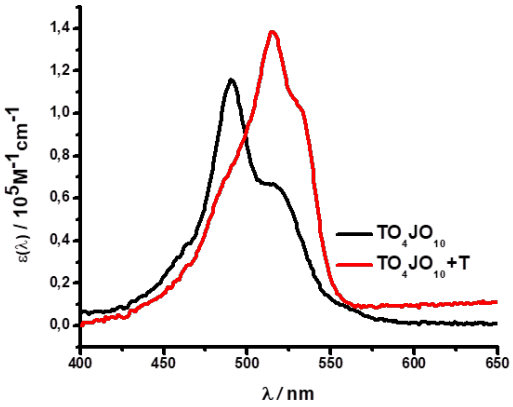

Fluorescence Intensity / a.u.
λ / nm
TO4JO8
TO4JO8+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO8
TO4JO8+T


Fluorescence Intensity / a.u.
λ / nm
TO4JO9
TO4JO9+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO9
TO4JO9+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO10
TO4JO10+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO10
TO4JO10+T

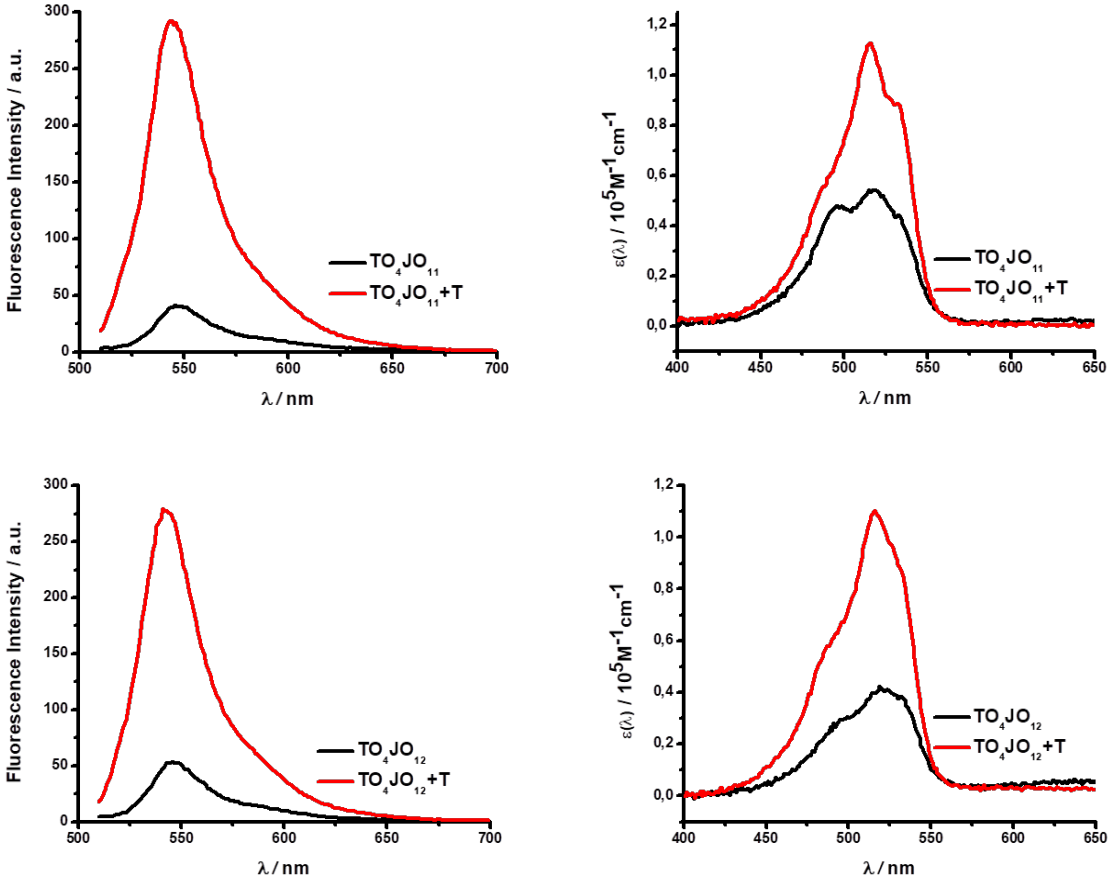
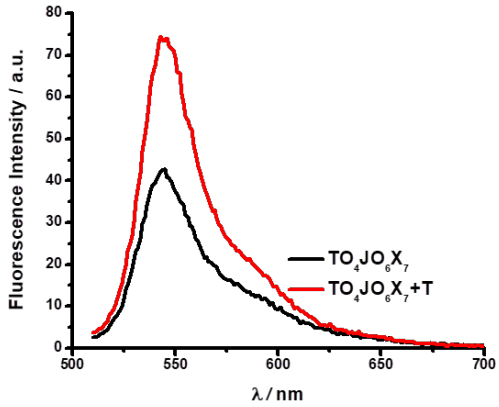
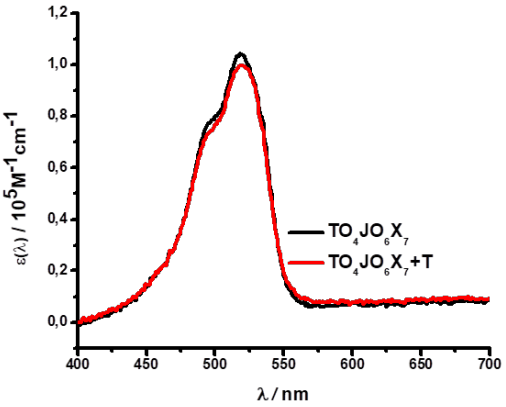
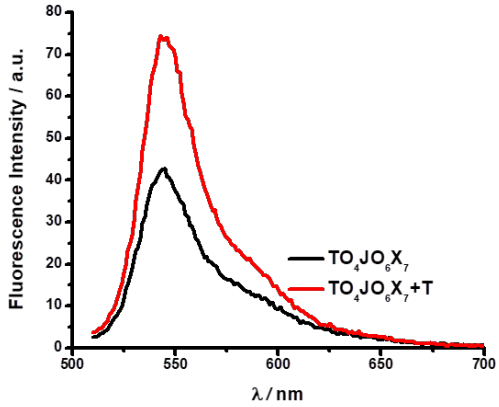


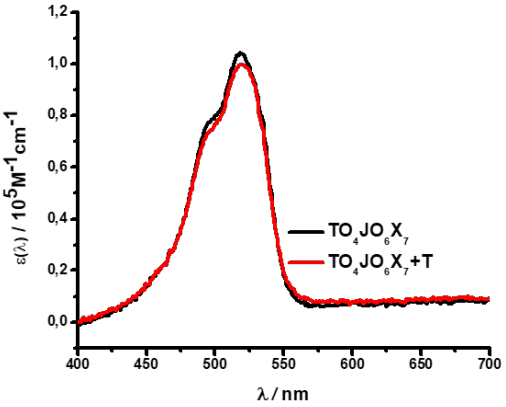
Figure S3: Emission spectra (left) and absorption spectra (right) of TO/JO-PNA probes in absence (black) and presence of complementary RNA target (red). Conditions: 0.25 µM probe and 2 eq. Glyα2-RNA target when added, in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), in 1 mL cuvette (path length = 1 cm); λ(ex) = 500 nm, λ(em) = 510-700 nm, slit(ex) = 5 nm, slit(em) = 5 nm, 37 °C.

TO/JO/X-PNA


Fluorescence Intensity / a.u.
λ / nm
TO4JO6X7
TO4JO6X7+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO6X7
TO4JO6X7+T


Fluorescence Intensity / a.u.
λ / nm
TO4JO6X7
TO4JO6X7+T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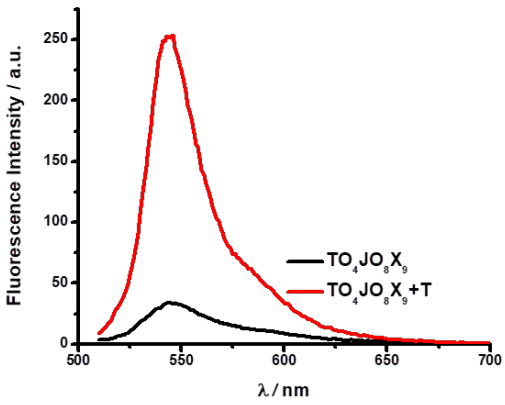
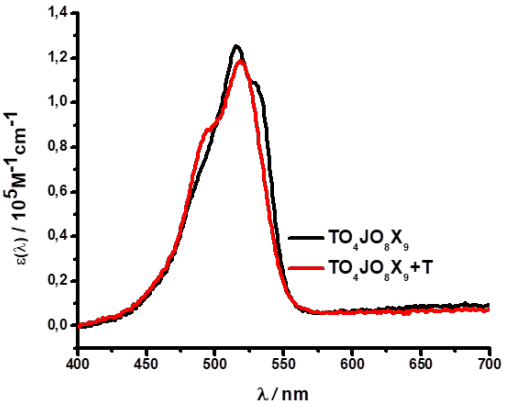
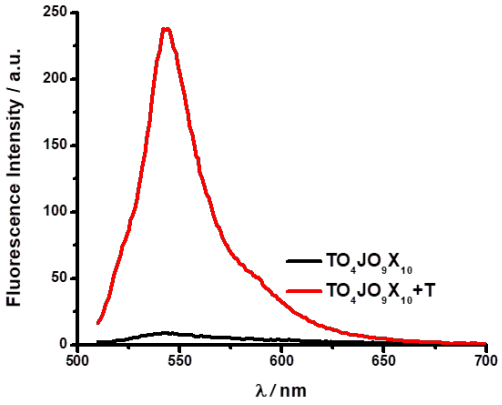
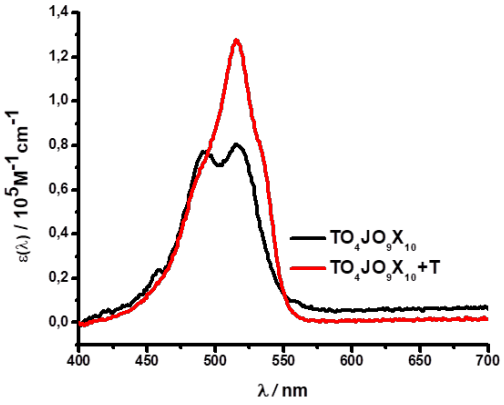
λ / nm
TO4JO6X7
TO4JO6X7+T


Fluorescence Intensity / a.u.
λ / nm
TO4JO8X9
TO4JO8X9+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO8X9
TO4JO8X9+T


Fluorescence Intensity / a.u.
λ / nm
TO4JO9X10
TO4JO9X10+T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λ / nm
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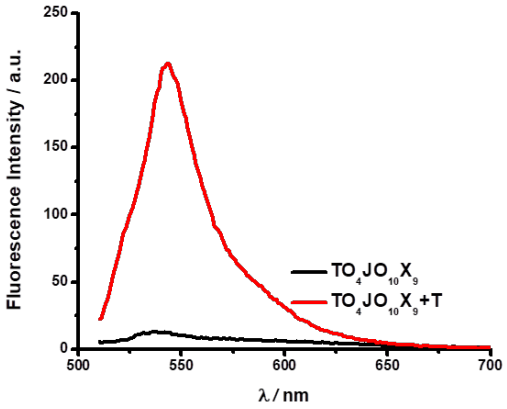
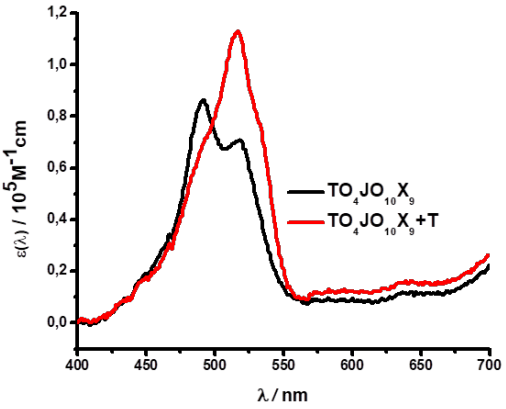
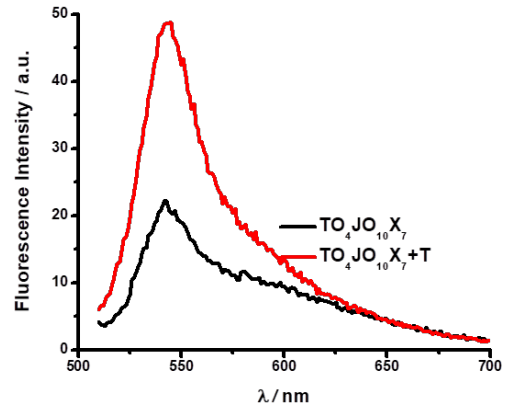
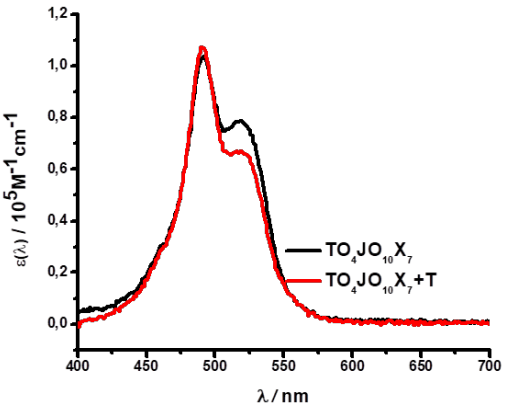
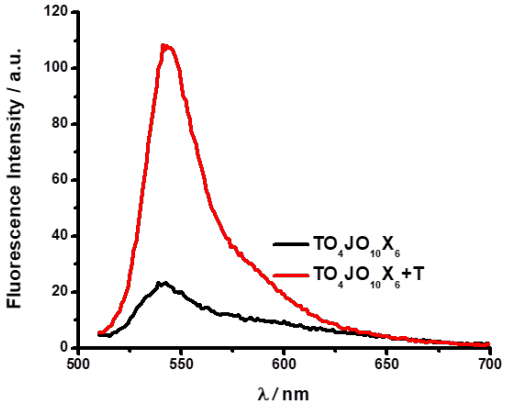
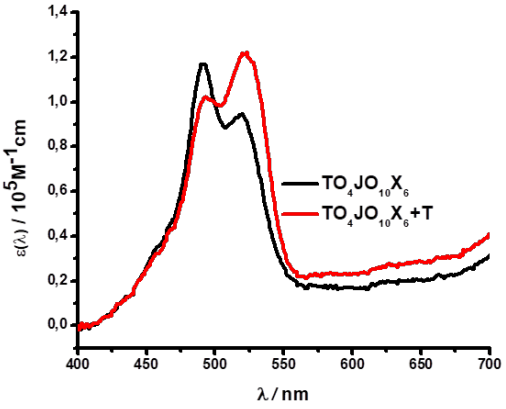

Fluorescence Intensity / a.u.
λ / nm
TO4JO10X9
TO4JO10X9+T


ε(λ) / 10^5M^-1cm
λ / nm
TO4JO10X9
TO4JO10X9+T


Fluorescence Intensity / a.u.
λ / nm
TO4JO10X7
TO4JO10X7+T


ε(λ) / 10^5M^-1cm^-1
λ / nm
TO4JO10X7
TO4JO10X7+T


Fluorescence Intensity / a.u.
λ / nm
TO4JO10X6
TO4JO10X6+T


ε(λ) / 10^5M^-1cm
λ / nm
TO4JO10X6
TO4JO10X6+T

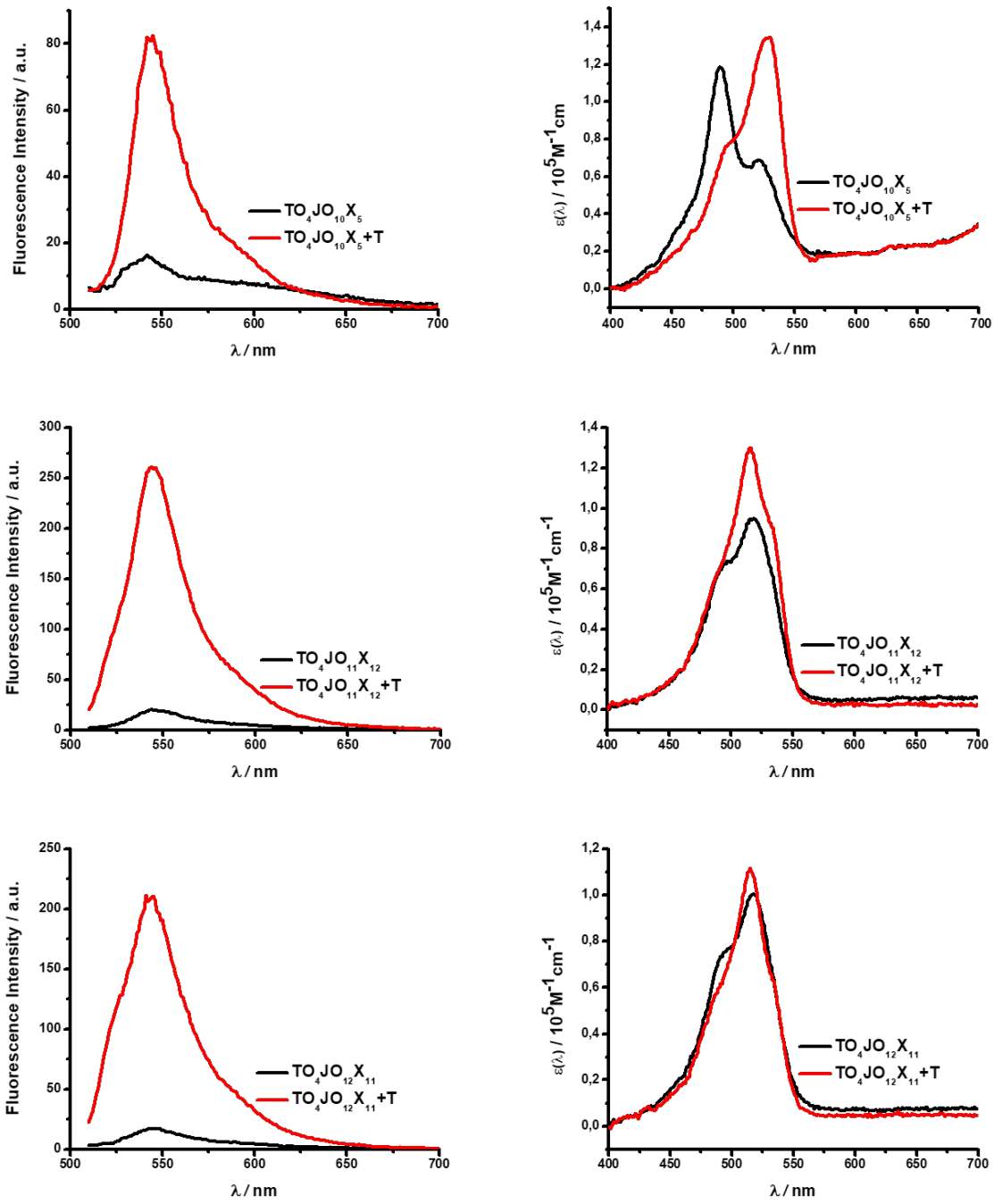


Figure S4: Emission spectra (left) and absorption spectra (right) of TO/JO/X-probes in absence (black) and presence of complementary RNA target (red). Conditions: 0.25 µM probe and 2 eq. Glyα2-RNA target, in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), in 1 mL cuvette (path length = 1 cm); λ(ex) = 500 nm, λ(em) = 510-700 nm, slit(ex) = 5 nm, slit(em) = 5 nm, 37 °C.

5. Fluorescence emission spectra of FIT-PNA probes for the repeat sequence tag

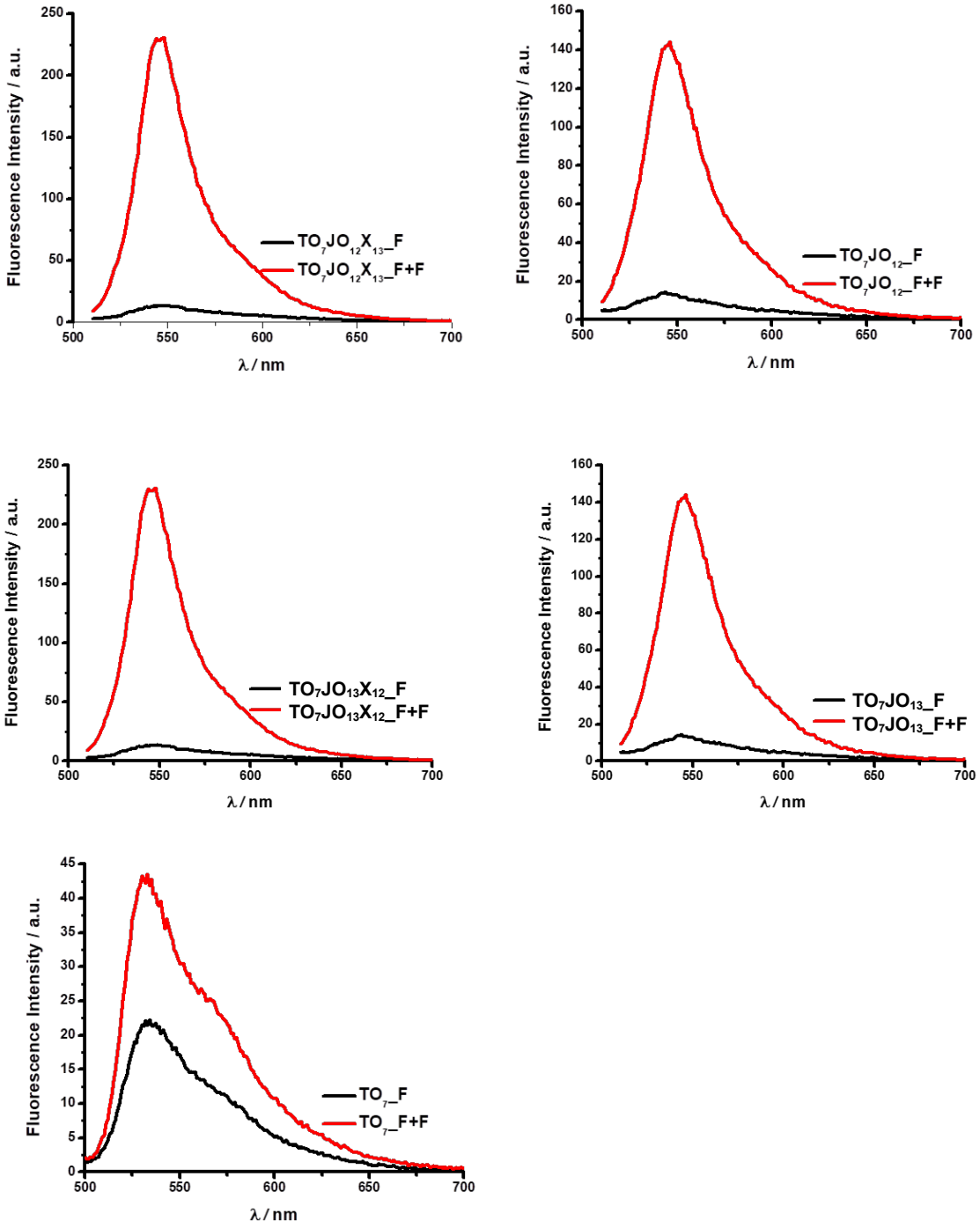


Figure S5: Emission spectra of TO/JO/X-F-, TO/JO-F- and TO-F-probes in absence (black) and presence of complementary RNA target **F** (red). Conditions: 0.25 µM probe and 4 eq. F-RNA target, in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), in 1 mL cuvette (path length = 1 cm), 37 °C; for TOJO probes, λ(ex) = 500 nm, λ(em) = 510-700 nm, slit(ex) = 5 nm, slit(em) = 5 nm; for TO probe, λ(ex) = 485 nm, λ(em) = 495-700 nm, slit(ex) = 5 nm, slit(em) = 5 nm.

6. Cell line/cloning for experiments with the $TO_7JO_{12}X_{13}$_F probe

To express a repeat sequence of F-RNA together with a mCherry open reading frame under control of an inducible promoter, we employed oligo condensation and MultiSite-Gateway recombination cloning (Invitrogen). First, we designed a synthetic 22 nt long RNA sequence. Unique sequence identity was verified by *BLAST* search in the human genome. We then synthesized complementary oligonucleotides (5' GATCCTAATCAACGGCCGGACGTGCATCTAATCAACGGCCGGACGTGCATA 3') containing two repeats of this binding sequence, phosphorylated them with polynucleotide kinase and hybridized them to form double stranded oligonucleotides with overhangs compatible with BamHI and BglII restriction sites. We polymerized these oligonucleotides in the presence of both restriction enzymes using T4 ligase, purified products of 300-500 bp length by agarose gel electrophoresis and cloned them into a custom gateway donor vector containing a stop codon in front of a multiple cloning site. This resulted in a product with 9 repeats of the binding sequence in head-to-tail orientation, which was validated by sequencing. We repeatedly re-inserted this fragment using a terminal BamHI restriction site as well a BglII/XmaI sites to finally obtain a vector with 45 repeats of the binding sequence. To generate an expression plasmid we used previously generated donor vectors containing the mCherry ORF, as well as a CMV promoter followed by Tet-operators (CMV-TO). We then generated a fusion construct by three-fragment recombination (LR Clonase Plus, Invitrogen) using all three donor plasmids and a custom destination vector vector containing R4 and R3 recombination sites for the Multiside-Gateway system as well as an FRT site for *Flp-In*^{TM} recombination. Stable cell lines were generated using *Flp-In*^{TM} *293 T-Rex cells*^{TM} by co-transfection of a plasmid expressing Flp recombinase and the reporter plasmid as described previously by Spitzer et al (Methods in Enzymology, Vol 529 99-124, 2013). Hek 293 T-Rex cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL Zeocine to select for the FRT recipient site and 15 µg/mL Blasticidine to select for Tet-R gene expression. 24h after transfection cells were selected for integration of the reporter plasmid using 100 µg/mL Hygromycin B until distinct colonies had formed. Individual clones were isolated, cultured and validated in time course experiments (see below). Inducible RNA expression was measured by quantitative real-time PCR using primers specific for the mCherry ORF. Protein translation from induced tagged RNA was validated by Western Blot as well as time-lapse microscopy imaging of mCherry fluorescent protein (Fig. S6).

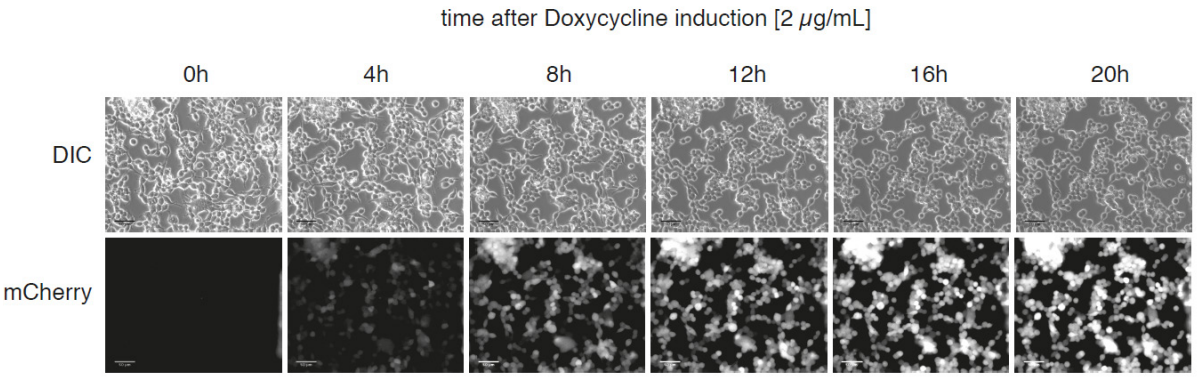


Figure S6: Widefield microscopy to assess expression of mCherry in HEK-Flp-In cells. A) differential interference contrast. B) Fluorescent microscopy in the mCherry channel (Excitation: 560/40 nm, Emission: 630/75 nm) at 20-fold magnification and different time after addition of doxycycline. Scale bar is 50 µm.

7. Fluorescence microscopy for RNA imaging with the $TO_7JO_{12}X_{13}$_F probe

Prior to imaging experiments with live cells, the cells were subcultured and plated in µ-slide 8 well Ibidi (Ibidi, Munich, Germany). For FISH–type experiments, the cells were plated on cover glass (TED PELLA, Redding, USA) treated with 0.01% poly-D-lysine (Sigma-Aldrich, Saint Louis, USA) with a confluency of 80%.

Images were acquired with an inverted Olympus IX83 microscope (Olympus, Hamburg, Germany) with a 60x/1.35 UPLSAPO oil objective (Olympus, Hamburg, Germany) and an Orca Flash 4.0 V2 camera (Hamamatsu, Ammersee, Germany). A JC12V100W halogen lamp (Traydon, Frechen, Germany) for bright field images and a LED lamp pE-4000 (Cool LED, Andover, UK) with a 500/24 nm filter for the PNA probes and a 350/50 nm for DAPI (AHF, Pfrondorf, Germany) was used.

8. FISH with the TO7JO12X13_F probe

Cells were washed twice with DPBS (Gibco, Thermo Scientific, Rockford, USA) and then fixed with 4 % *p*-formaldehyde (Thermo Fisher Scientific, Rockford, USA) for 10 min at RT. After washing twice with DPBS, the cells were permeabilized by applying 70% ethanol (Applichem, Darmstadt, Germany) at 4 °C overnight. The ethanol was removed and the cells were rinsed twice with DPBS. Afterwards the cells were incubated with the *FISH* hybridization buffer (20 nM Tris (Roth, Karslruhe, Germany) pH 7.4 supplemented with 60% formamide (EMD Millipore, Billerica, USA) and heated up to 90°C for 3 min. The hybridization buffer was removed, followed by treating cells with 100 nM of FIT-PNA probe in hybridization buffer at 37°C. After 1 h, the hybridization buffer was replaced by DPBS. Finally, the cells were covered by a glass cover, and placed on mounting medium (Vector laboratories, Burlingarne, USA) for subsequent imaging experiments.

9. Live cell imaging with the $TO_7JO_{12}X_{13}$_F probe

Streptolysin *O* (Sigma-Aldrich, Munich, Germany) was aliquoted in ~350 U /10 µL RNAse free water and supplemented with 10 µM TCEP (Sigma-Aldrich, Munich, Germany) and incubated for 30 min at 37°C. The cells were washed once with DPBS + 1 mM $MgCl_2$ and then incubated with 30 U SLO and 600 nM of FIT probes in DPBS + 1 mM $MgCl_2$ for 10 min at 37°C. Afterwards, the cells were washed twice with DPBS + 1 mM $MgCl_2$ and incubated with recovery medium (DEMEM with 2 mM ATP and 2 mM GTP (Sigma-Aldrich, Munich, Germany)) for 2 h at 37°C. Then cells were washed once again with DPBS + 1 mM $MgCl_2$. Cell nucleus was stained with Hoechst in PBS for 5 min. Before fluorescence, the cells were washed with DPBS + 1 mM $MgCl_2$.

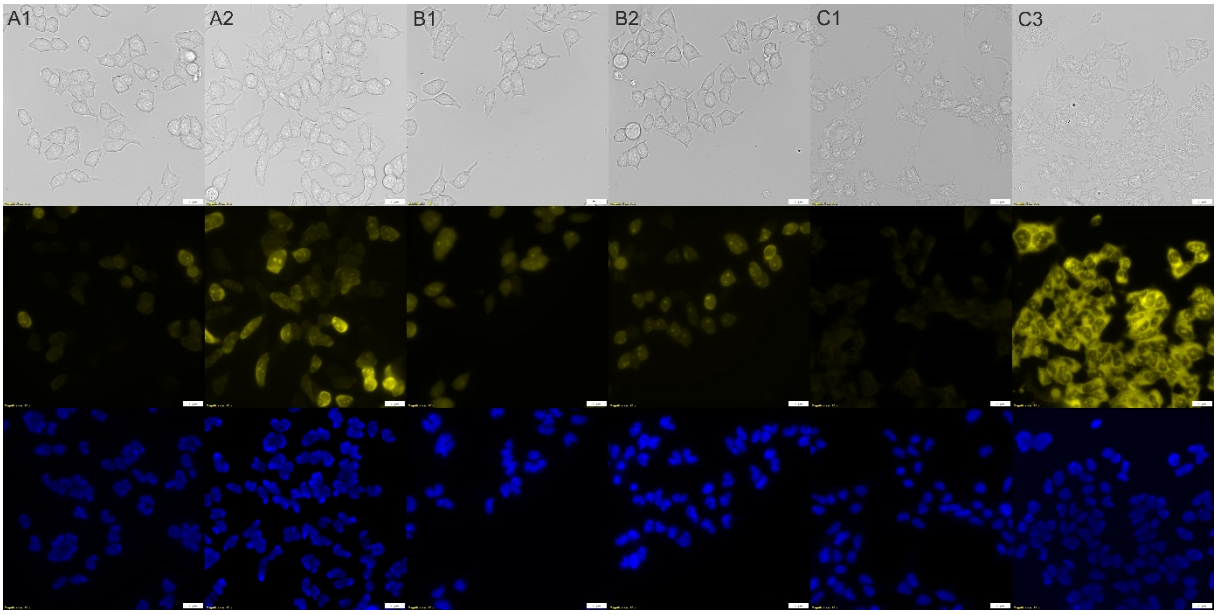


Figure S7. Flp-In™ 293 T-REx™ 45 x F cells measured with a wide field microscope. A) live cell imaging with 600 nM **$TO_7JO_{12}X_{13}$_F** 1) without doxycycline, 2) after 1h doxycycline treatment; B) live cell images with 600 nM **TO_7_F** probe 1) without doxycycline, 2) after 1 h doxycycline treatment; C) FISH with 100 nM of 1) **TO_7_F** probe, 2) **$TO_7JO_{12}X_{13}$_F** probe after 15 min of doxycycline treatment with 2 ms exposure time. Images shown with nucleus staining (DAPI stain). Scale bars are 20 µm.

10. TO- and TO/JO-PNA FIT-probes in a binary system with NIR664-labeled PNA.

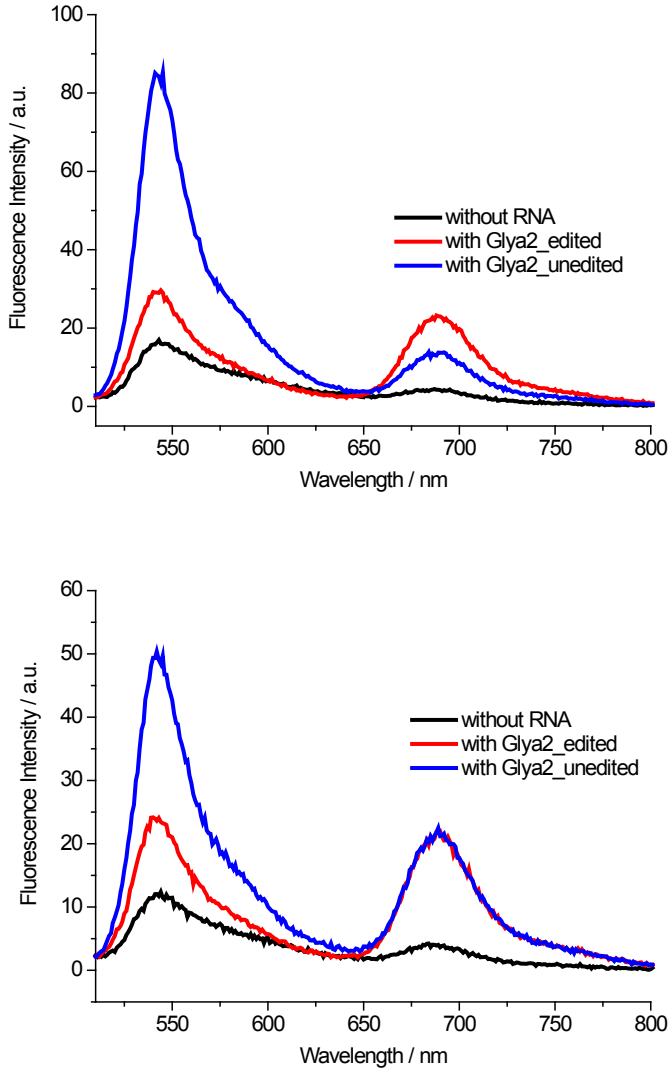


Figure S8: Emission spectra of a binary probe system composed of **TOJO_1** and NIR-PNA-7mer Ac-Lys-ctgaagc-Lys($COCH_2CH_2S$-NIR664)-$CONH_2$ (top) or NIR-PNA-8mer Ac-Lys-actgaagc-Lys($COCH_2CH_2S$-NIR664)-$CONH_2$ (bottom) in absence of RNA (black), in presence of complementary RNA target **glyR_ed** (red) or single mismatched RNA target **glyR_uned** (blue). Conditions: 0.5 µM **TOJO_1**, 0.5 µM **NIR_probe** and 0.17 µM **glyR_ed** or **glyR_uned** in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), 37 °C, λ(ex) = 500 nm; slit(ex) = 5 nm, slit(em) = 5 nm.

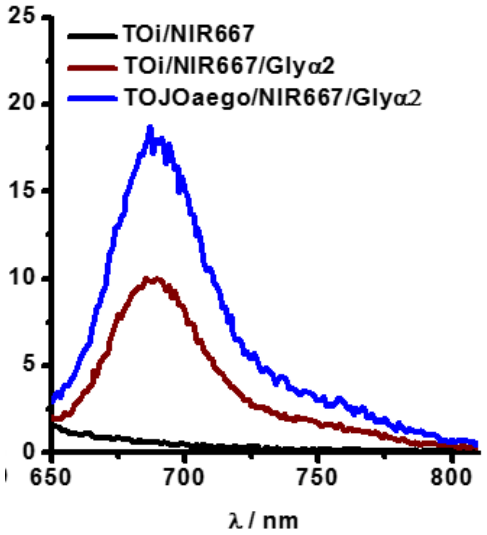


Figure S9: Emission spectra of a binary probe system composed of **NIR_2b** and either **TOJO_1** (blue) or **TO_1** (brown) after addition of complementary RNA target **glyR_ed**. Conditions: 0.5 µM **TO_1** or **TOJO_1**, 0.5 µM **NIR_2b** and 0.17 µM **glyR_ed** in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.3-7.5), 37 °C, λ(ex) = 500 nm for **TOJO_1**, 485 nm for **TO_1**; slit(ex) = 5 nm, slit(em) = 5 nm.

11. Cloning for experiments with the TOJO_1/NIR664_2b probes.

Molecular cloning of the constructs encoding EGFP-MS2 binding protein (K. Valegard, J. B. Murray, N. J. Stonehouse, S. van den Worm, P. G. Stockley and L. Liljas, *J Mol Biol*, 1997, **270**, 724-738.) followed by 9x-MS2-hairpin-repeat-tagged GlyR α2 edited or non-edited cDNA was performed using standard techniques. The 2A self-processing peptide was then inserted in frame using fusion PCR (touch-up protocol: annealing: 4 58°C, 4 60°C, 31 62°C) and oligonucleotides 5'-GGTCCACGTGCGGAGATGTTGAAGAAAATCCAGGTCCAAGCATCACGGAAACAG GAATG-3' and 5'-TCCAGCACGTGAGCAAACTACCTCTGCCCTCTATCGATACCG CGGTACCTACCT-3'. The PCR product was digested with PmlI (catalog no. R0532S, New England Biolabs) and self-ligated with T4 DNA ligase (catalog no. K1422, Thermo Fisher Scientific). The resulting expression constructs coded for EGFP-MS2 binding protein (for visualization of the GlyR-coding mRNA) followed the 2A peptide and the GlyR-coding ORFs that were tagged with 9x repeats of the MS2 hairpin sequences 5'-AAAAAACAUGAGGAUUACCCAUGUAAAAA-3' (Fig. S10).

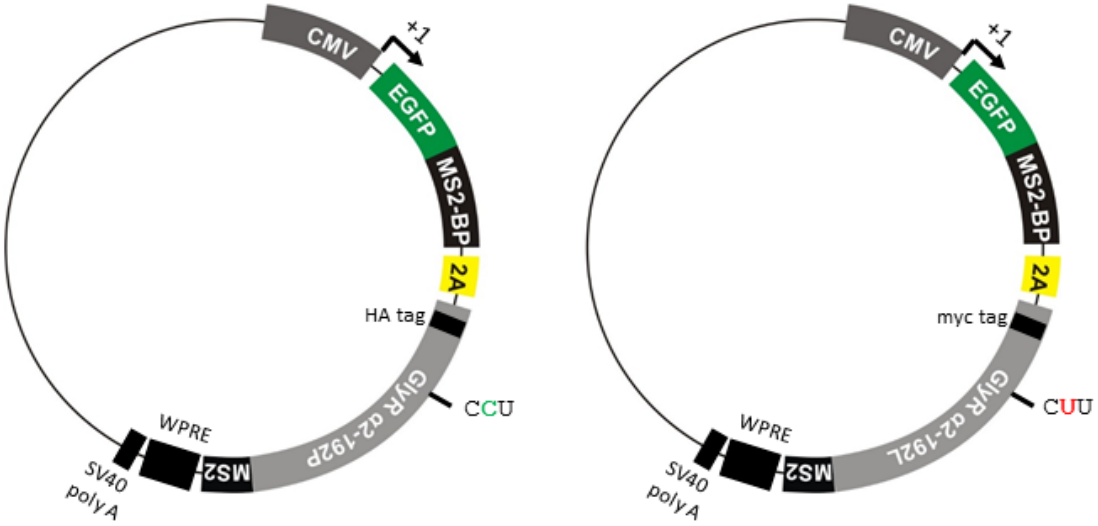


Figure S10: Maps of plasmids used for transfection of HEK293T cells depict molecular GlyR target construct design. The two expression plasmids were derived from pEGFP-N1 vector by molecular cloning. Transcription of mRNA coding for EGFP-MS2-binding protein and unedited GlyR α2-192P (left map) or edited GlyR α2-192L (reight map) is driven by the cytomegalovirus (CMV) promotor (transcription start at "+1"). The self-processing viral 2A peptide coding sequence (*Thosea asigna* virus) allows bicistronic expression of EGFP-MS2-BP and GlyR proteins as they separate coding frames. The positions of epitope tag coding sequences (HA or myc) used for immunochemistry (Figure S11) are indicated. The Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) and SV40 poly A consensus sequences were inserted downstream of the 9x MS2 hairpin repeats in the 3' UTR to enhance stability of transcribed mRNA.

12. Cell culture and transfection for experiments with the TOJO_1/NIR664_2b probes.

HEK293T cells were cultured in T25 culture flasks containing 5 ml of DMEM (catalog no. 41965-062, Gibco) supplemented with 4.5 g/liter glucose, 10% FCS (catalog no. 1050064, Life Technologies), and 1% penicillin/streptomycin (catalog no. 15140122, Life Technologies) at 37 °C and 5% CO2 in a humidified incubator. Cell passaging was performed every 3–4 days at an average confluence of 80–90%. One day before transfection, 50,000 HEK293T cells were seeded onto 24-Well plates, each well containing 1 ml of DMEM/FCS/penicillin/streptomycin to reach 60–70% confluence for transfection of the EGFP-MS2 binding protein_2A_MS2-tagged GlyR-coding plasmids with ViaFect™ transfection reagent (catalog no E4981, Promega) according to the manufacturer's instructions. The TOJO_1 / NIR664_2b probes were applied the day after transfection of GlyR-coding plasmids using streptolysin-O mediated permeabilisation as described in chapter 9. Briefly, streptolysin-O (SLO, catalog no. S5265, Sigma-Aldrich) at ~300 U/10 µl RNAse free water and supplemented with 10 µl of 20 µM TCEP (catalog no. HN95, Carl Roth) was incubated for 30 min at 37°C and applied to cells that were washed once with PBS (catalog no. 70011044, Life Technologies) supplemented with 1 mM $MgCl_2$ (catalog no. T888.1, Carl Roth) and then incubated with 30 U SLO and 600 nM of FRET probes in PBS + 1 mM $MgCl_2$ (200 µL) for 10 min at 37°C. Then, cells were washed twice with PBS supplemented with 1 mM $MgCl_2$ and incubated with 200 µL recovery medium (DMEM with 2 mM ATP and 2

mM GTP (catalog no. A6419 and catalog no. G8877, Sigma-Aldrich) for 2 h at 37 °C. Cells were then washed once again with PBS supplemented with 1 mM $MgCl_2$ fixed using an ice-cold 1:1 mixture of 8% paraformaldehyde and 8% sucrose in phosphate-buffered saline (15 min at room temperature). Coverslips were mounted on microscope slides using Vectashield medium with DAPI (catalog no. H-1200, Vector Laboratories).

For control of presence and integrity of the GlyR target mRNAs in the transfected HEK293T cells, we performed immunochemistry to demonstrate target mRNA translation into GlyR proteins (Fig. S11). Transfected HEK293T cells were fixed using an ice-cold 1:1 mixture of 8% paraformaldehyde and 8% sucrose in phosphate-buffered saline (15 min at room temperature). Cells were permeabilized with 0.12% Triton X-100 for 4 min at room temperature. Primary antibodies were applied for 1 h at room temperature in PBS-gelatine (0.1%). Myc-tagged GlyRs were stained using a mouse monoclonal antibody (catalog no. 13-2500, 1:100, Thermo Fisher Scientific), and HA-tagged GlyRs were stained using a rat monoclonal antibody (catalog no. 11867423001, 1:100, Roche). Primary antibodies were visualized using secondary CY5 coupled donkey polyclonal antibody directed against mouse IgG (catalog no. 715-175-151, 1:200, Jackson ImmunoResearch) and secondary DyLight649 coupled donkey polyclonal antibody directed against rat IgG (catalog no. 712-495-153, 1:200, Jackson ImmunoResearch). The secondary antibodies were applied for 45 min under same conditions. Coverslips with immunostained HEK293T cells were mounted on microscope slides using Vectashield medium with DAPI (catalog no. H-1200, Vector Laboratories). Fluorescence intensities corresponding to EGFP and HA or myc epitope tag signals were measured as integrated pixel intensities within circular (15 μm diameter) regions of interest centered on transfected cells.

13. Fluorescence imaging of GlyR-coding mRNA in HEK293T cells with TOJO_1/NIR664_2b probes

mRNA corresponding to transfected GlyRα2 was visualized according to EGFP fluorescent signals of the EGFP-MS2 binding protein that tracks the MS2-tagged GlyRα2 mRNA. Imaging was performed with an epifluorescence microscope (Olympus BX51, Olympus). Fluorescent signals were detected by appropriate filters (DAPI: U-NSP100v2 and EGFP: U-NSP101, and a FRET filter cube consisting of exciter HQ470/40x, emitter HQ667/30m and beam splitter Q497/LP; Chroma Technology Corp.). A 14-bit cooled CCD camera (Spot PURSUIT, Visitron Systems) and the Metamorph software (Universal imaging) were used for image acquisition and quantification of pixel intensities using line scans as described previously (A. Winkelmann, N. Maggio, J. Eller, G. Caliskan, M. Semtner, U. Haussler, R. Juttner, T. Dugladze, B. Smolinsky, S. Kowalczyk, E. Chronowska, G. Schwarz, F. G. Rathjen, G. Rechavi, C. A. Haas, A. Kulik, T. Gloveli, U. Heinemann and J. C. Meier, *J. Clin. Invest.*, 2014, **124**, 696-711). Image acquisition parameters (exposure times) were adjusted and kept constant throughout so that FRET-signals were marginally detected in the mismatch condition. Also, exposure time for acquisition of EGFP signals (GlyR-coding mRNA) was kept constant throughout in match (edited mRNA) and mismatch (unedited mRNA) conditions.

Statistical analysis: Normal distribution of data was assessed using Shapiro-Wilk test. As normal distribution was not given Mann-Whithney Test was used for statistical analysis of the correlation between GlyRα2 mRNA and FRET probe signal (software OriginPro 2016, Origin Lab). $P<0.05$ was considered significant. Asterisks used to indicate statistical significance were defined as follows: n.s. (not significant) = $p > 0.05$; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

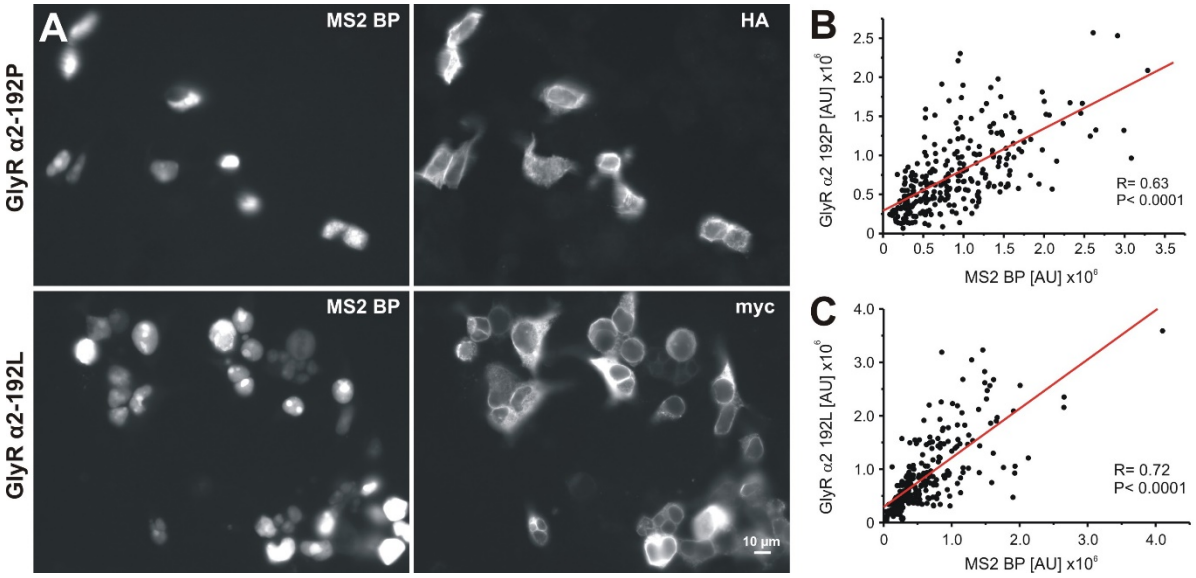


Figure S11: (A) Protein signals corresponding to EGFP-MS2-binding protein and protein signals corresponding to GlyR $\alpha2^{192P}$ (revealed using immunochemistry against human influenza hemagglutinin [HA] epitope tag) or GlyR $\alpha2^{192L}$ (revealed using immunochemistry against myc epitope tag). (B,C) EGFP and HA or myc signals significantly and positively correlate as revealed by linear regression analysis. Each dot corresponds to integrated signal intensity region measurements of EGFP and HA or myc signals in one cell. 332 and 283 transfected cells expressing GlyR $\alpha2^{192P}$ (B) or GlyR $\alpha2^{192L}$ (C), respectively, were analyzed. The slight difference between R values likely corresponds to use of different antibodies (anti-HA vs. anti-myc) for the detection of GlyR proteins. However, as HA and myc epitope tag sequences are located upstream of the edited position this experiment indicates integrity of the targeted GlyR mRNAs. Scale bar: 10 µm.

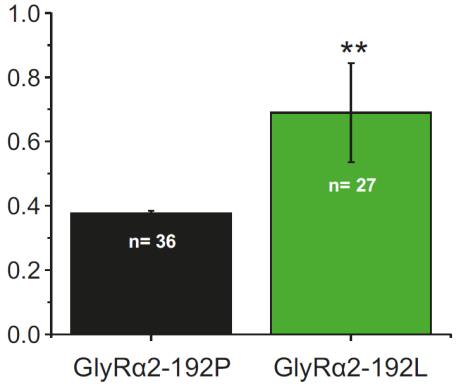


Figure S12: The ratio of emission in the FRET channel (excitation: HQ470/40x, emission: HQ667/30m, beam split: Q497/LP) and in the eGFP channel (excitation: HQ470/40x, emission: HQ522/40m, beam split: Q497/LP) for cells expressing GlyR $\alpha 2^{192P}$ mRNA (= unedited nRNA) or $\alpha 2^{192L}$ mRNA (= edited mRNA) obtained by using the **TOJO_1** / **NIR664_2b** probes.

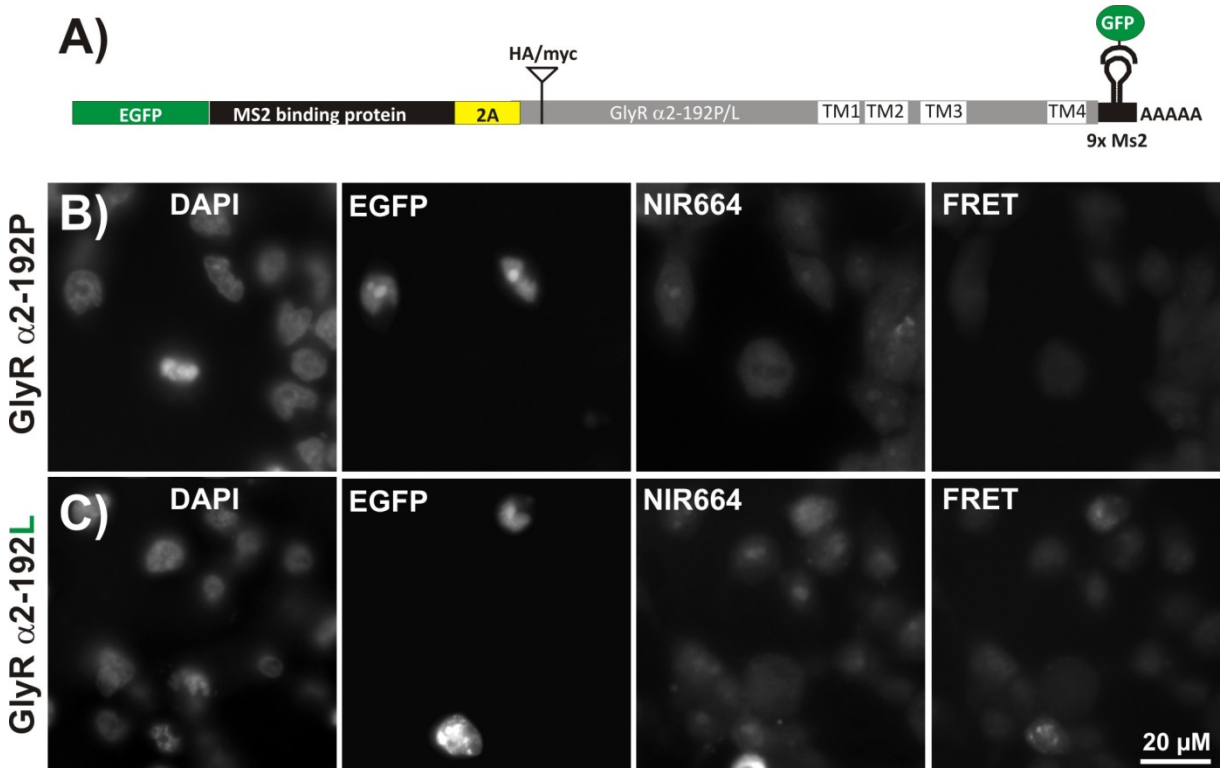


Figure S13. A) Design of the MS2 reporter construct for in vivo analysis. EGFP-MS2 binding protein (gene 1, green) and the GlyR target (gene 2, gray) α2192P (unedit) or α2192L (edit) including MS2 hairpin repeats in 3'-UTR can be expressed at almost equimolar ratio due to use of the 2A self-processing peptide (yellow) inserted in between. B) Fluorescence microscopy images of transiently transfected HEK293 cells in gray scale measured in the DAPI channel (excitation: 350/50x, emission: 470/30m, beam split: 400), eGFP channel (excitation: HQ470/40x, emission: HQ522/40m, beam split: Q497/LP), NIR664 channel (excitation: ET630/20x, emission: HQ667/30m, beam split: Q647/LP) and

FRET channel (excitation: HQ470/40x, emission: HQ667/30m, beam split: Q497/LP). Conditions: 600 nM **TOJO_1**+**NIR664_2b**, 30 units SLO, 2 h recovery, then fixation, imaging at 25°C.