Electronic Supplementary Material (ESI) for ChemComm.

Content

1. General Information

Materials:
Chemicals were purchased from *Sigma-Aldrich* (St. Louis, USA), *TCI Deutschland GmbH, Link Technologies* (Bellshill, Scotland) or *Carl Roth* (Karlruhe, Germany). Synthetic oligo ribonucleotides were obtained from *Biomers GmbH* (Ulm, Germany). Aqueous solutions were made of water purified using a *Milli-Pore* purification device.

HPLC purification and analysis
Semi preparative and analytical HPLC were carried out on a 1105 HPLC System (*Gilson*, Limburg, Germany). A Waters *X-Bridge BEH130 C18 (10x150 mm, 5 µm)* column at a flow rate of 8 mL/min at 55 °C, linear gradient (gradient 1: 15-40% B, gradient 2: 5-40%B) was used for purification. The mobile phase consist of a binary mixture of A (0.1 M triethylammonium acetate buffer, pH = 7.4, aq.) and B (acetonitrile).
For analytics, a Waters *X-Bridge BEH130 C18 (4.6x250 mm, 5 µm)* column at a flow rate of 1.5 mL/min at 55 °C, 5-50% B in 10 min was used.

MALDI-TOF mass spectrometry
MALDI-TOF mass spectra were measured on a Shimadzu Axima Confidence (*Shimadzu,* Kyoto, Japan) in positive mode. For measurements, HPA Matrix was used (1:1 mixture of 50 mg/mL 3-Hydroxy picolinic acid in acetonitrile/H_2O, 1:1 and 50 mg/mL diamonium citrate in acetonitrile/H_2O, 1:1).

2. DNA-Synthesis & optical Properties

DNA FIT probe synthesis
DNA FIT probes were assembled by using a *Bioautomation MerMade-4* (Irving, Texas). 3'-C3-Spacer CPG (1 µmol, 500 Å pore size), DNA phosphoramidites (dT, dG(DMF), dC(Bz), dA(Bz)) and 2'-O-Me-building blocks were purchased from *Link Technologies*). DNA synthesis reagents from *Carl Roth* and *EMP-Biotech* (Berlin, Germany*)*. LNA phosphoramidites were obtained from *Exiqon* (Vedbak, Denmark). All phosphoramidites were used according to manufacturer's instructions. The synthesis of QB containing building blocks was described elsewhere. [1] The quality of each coupling step was monitored by measuring the absorbance of DMT cleavage solutions. The synthesis was programmed to yield oligomers carrying the terminal DMT protective group „trityl-on".
After synthesis the resulting CPGs were dried under reduced pressure and transferred to 2 mL tubes. 1 mL of aqueous ammonia (32%) was added and the tubes were agitated for 2 h at 55 °C. The volatiles were removed and the remaining resin was filtered off. The crude product was purified by RP-HPLC (gradient 1). DMT cleavage was carried out using 300 µL of 80% aqueous AcOH for 30 min at room temperature. The Oligos were precipitated with *i*PrOH and ammonium acetate and purified by RP-HPLC again (gradient 2). Finally, the DNA FIT probes were precipitated and dissolved in water (Milli-Pore), characterization and purity were determined by MALDI-TOF mass spectrometry and UV traces of RP-HPLC.

Fluorescence spectroscopy:
Fluorescence emission spectra were measured by a *Varian Cary Eclipse* fluorescence spectrometer (*Agilent Technologies*, Santa Clara, CA, USA) in 10 mm quartz cuvettes in phosphate buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) and buffer fluorescence corrected. FIT probes and target RNA were added as specified. Prior to measurement, samples were allowed to equilibrate for 2 min. The spectra are the average of 3 measurement cycles. Quantum yields were assessed by using ATTO 590 (*ATTO-Tec GmbH*, Siegen, Germany) as standard. All fluorescence measurements were corrected

according to their concentration in the cuvette (determined by UV-Vis absorption measurements). The fluorescence measurements were reproducible within 5-10% error.

UV-Vis spectroscopy: Probe concentrations were measured on a *Jasco V750* UV-Vis spectrometer (*Jasco*, Germany). Complete absorption spectra (800-220 nm, 1 nm steps) were measured directly before or after the fluorescence experiments in the same cuvettes.

To determine the melting temperatures 0.5 µM probe and 0.5 µM synthetic RNA target in 1 mL phosphate buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) were heated 3 times from 20 °C to 90 °C at a heating rate of 1 °C/min and the absorption at 260 nm was recorded. The resulting curves were averaged and the first derivative was calculated.

3. **Analytical Data**

Table S1: Analytical data of FIT probes

FIT Probe	Sequence	Yield	MALDI-TOF-MS		Retention Time (a)
		[%]	calc. $[M+H]^+$	found $[M+H]^+$	[min]
ED-OMe- 1	AATAAACTGAA-QB-C_LA-C3	38	4890	4892	8.9
ED-OMe- 2	AATAAACTGAA-QB-C_LAG-C3	56	5250	5251	9.8
ED-OMe- 3	AATAAACTGAA_L-QB-CA-C3	57	4876	4876	9.1
UE-OMe- 1	AATAAACTGAG-QB-C_LA-C3	42	4905	4903	10.7
UE-OMe- 2	AATAAACTGAG-QB-C_LAG-C3	52	5266	5266	9.7
UE-OMe- 3	AATAAACTGAG_L-QB-CA-C3	55	4891	4890	9.7

(a) Analytical HPLC

Table S2: Spectroscopic data

FIT probe	RNA target	E= F_{ds}/F_{ss}	S	Br	Tm
ED-OMe-01	GlyR α2 192L, ED (match)	41.2	7.1	9.8	48.8
	GlyR α2 192P, UE (mismatch)	5.8		2.7	40.8
ED-OMe-02	GlyR α2 192L, ED (match)	15.0	6.3	15.0	50.4
	GlyR α2 192P, UE (mismatch)	2.4		4.6	42.1
ED-OMe-03	GlyR α2 192L, ED (match)	63.3	26.4	4.3	45.4
	GlyR α2 192P, UE (mismatch)	2.4		0.6	37.0
UE-OMe-01	GlyR α2 192L, ED (mismatch)	28.4	3.1	14.6	43.9
	GlyR α2 192P, UE (match)	87.5		16.5	56.3
UE-OMe-02	GlyR α2 192L, ED (mismatch)	8.1	1.6	26.0	47.0
	GlyR α2 192P, UE (match)	12.8		24.5	60.5
UE-OMe-03	GlyR α2 192L, ED (mismatch)	8.0	1.9	8.1	39.0
	GlyR α2 192P, UE (match)	15.3		6.0	47.3

Conditions: 0.2 µM probe and 4 eq. RNA target for measurement of F_{ss} and F_{ds} or 0.5 µM probe and 4 eq. RNA target for measurement of quantum yield Φ, extinction coefficient ε and melting temperature Tm, respectively, buffer: PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) at 37 °C. λex = 560 nm, λem = 605 nm (F_{ss} and F_{ds}) and λem = 570-750 nm (Φ_{ds}). S = Selectivity = E_{match}/E_{mm}), ε = extinction coefficient at 560 nm in $mL*mmol^{-1}*cm^{-1}$, Br = Brightness = $\varepsilon*\Phi_{ds}/1000$ in $mL_*mol^{-1}{}_*cm^{-1}$, Tm in °C.

Fluorescence Spectra

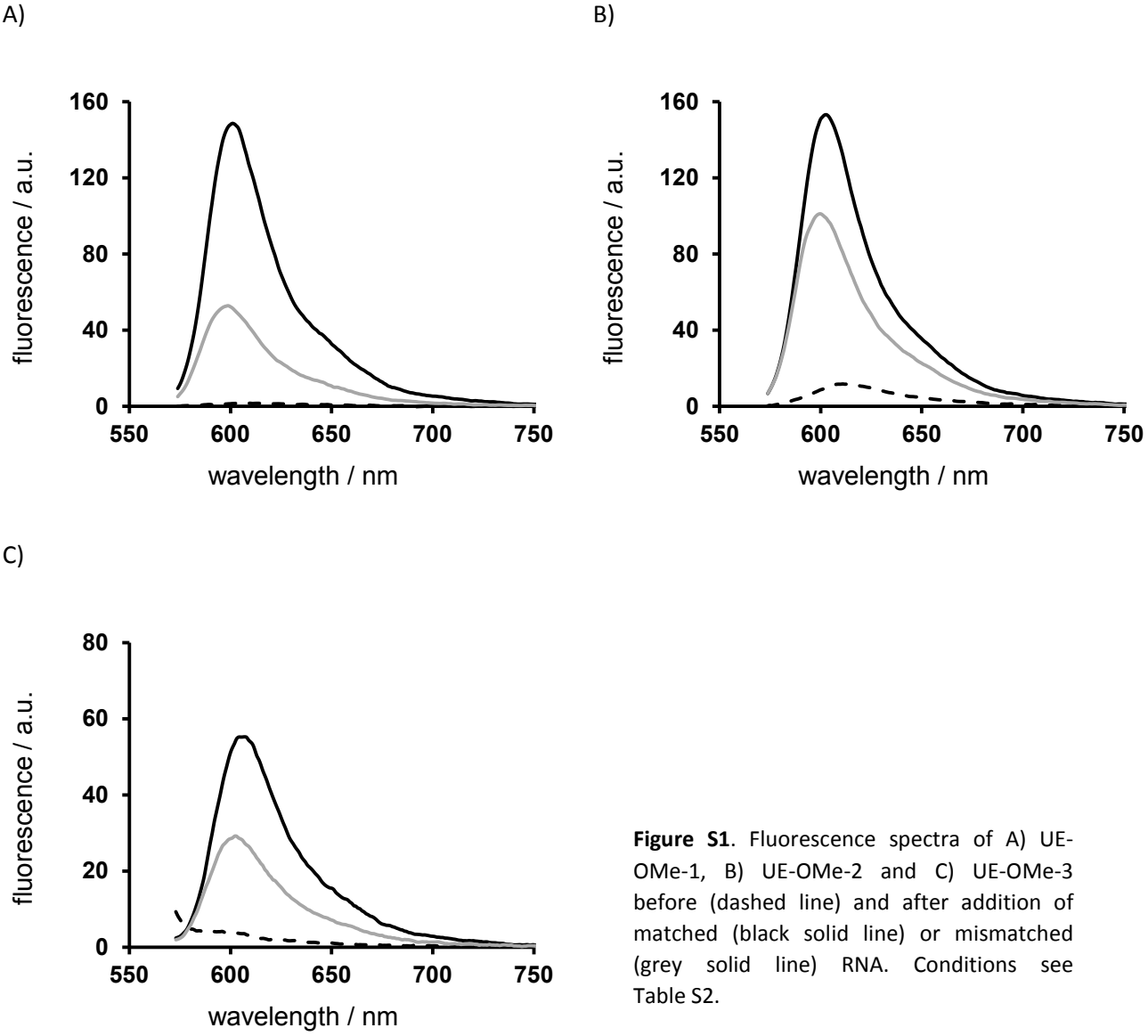


Figure S1. Fluorescence spectra of A) UE-OMe-1, B) UE-OMe-2 and C) UE-OMe-3 before (dashed line) and after addition of matched (black solid line) or mismatched (grey solid line) RNA. Conditions see Table S2.

Melting curves

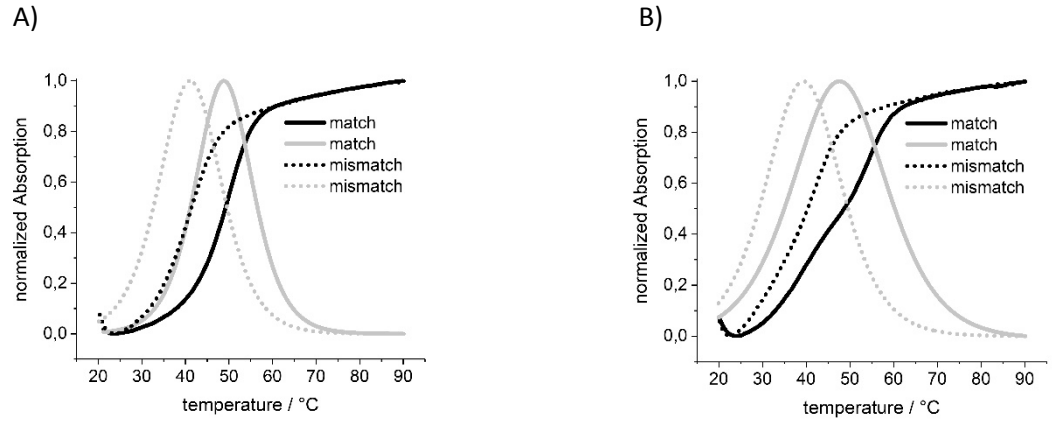


Figure S2. Representative melting curves of FIT probes A) ED-OMe-1 and B) UE-OMe-3 in the presence of 1 eq. match (black, solid) or mismatch (black, dashed) mRNA. Grey curves represent the 1st derivative.

4. Determination of the detection limit (LOD)

The limit of detection of the probes ED-OMe-1 or ED-OMe-3 was estimated in vitro by measuring the fluorescence of the probes in the presence of different concentrations of match or mismatch RNA (Fig. S3 A). Calibration curves were generated by linear curve fitting (Fig. S3 B).

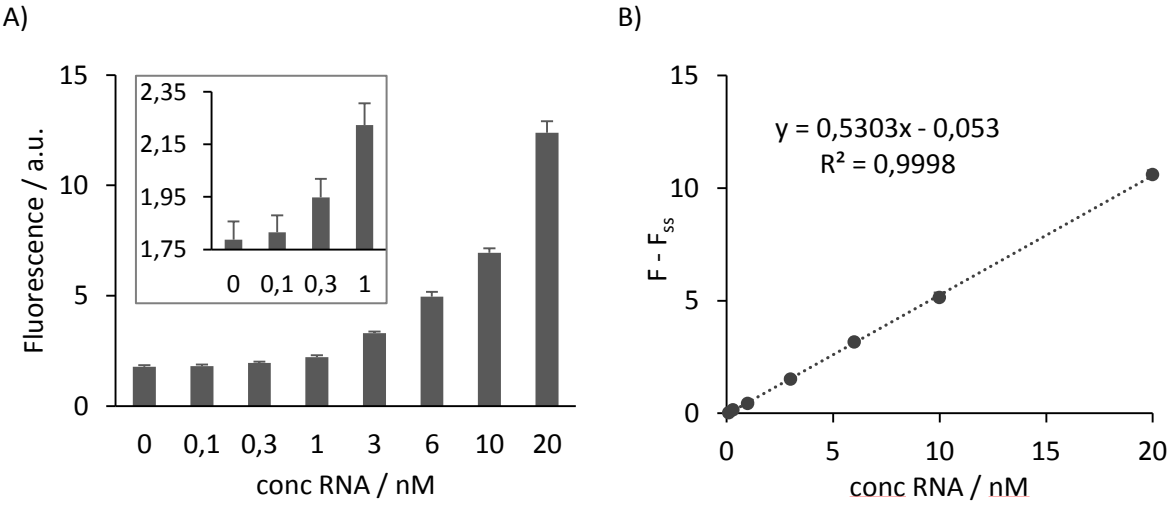


Figure S3. Concentration dependence of fluorescence signals of probe ED-OMe-1 in the presence of match target RNA. Conditions: 200 nM probe in 1 mL buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.0), 37°C, λex = 560 nm, λem = 605 nm. Error bars indicate the standard deviation of 3 independent measurements. F = fluorescence of probe-target complex, F_{ss} = fluorescence of unbound probe.

Using the formula LOD = 3 · SD / b [2], where SD means the standard deviation of the single strand fluorescence of the probe (blank) and b represents the slope of the corresponding calibration curve (Fig. S3B), the limit of detection was calculated (Table S3).

Table S3: Limit of detection

Probe	LOD with match target [nM]	LOD with mismatch target [nM]
ED-OMe-1	0.39	4.2
ED-OMe-3	1.6	11.6

Conditions: see Figure S3

5. Cell preparation

Molecular cloning

Molecular GlyR-coding constructs were described recently [3], but the 192P- and 192L-coding constructs used in this study both contain an HA epitope tag and 8x MS2 hairpin repeats.

Cell Culture and Transfection

HEK293T cells (from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, DSMZ No ACC 635) were cultured in T25 culture flasks containing 5 mL of DMEM (catalogue no. 41965-062, *Gibco*) supplemented with 4.5 g/L glucose, 10% FCS (catalogue no. 1050064, *Life Technologies*), and 1% penicillin/streptomycin (catalogue no. 15140122, *Life Technologies*) at 37 °C and 5% CO_2 in a humidified incubator. Cell passaging was performed every 3–4 days at an average confluence of 80–90%. Two or 3 days before transfection, 300,000 HEK293T cells were seeded onto 35-mm culture dishes containing 1.5 mL of DMEM/FCS/penicillin/streptomycin to reach 70–80% confluence for transfection of plasmids encoding eGFP-MS2_2A_HA-GlyR $\alpha2^{192L}$ or eGFP-MS2_2A_c-myc-GlyR $\alpha2^{192P}$ with ViaFect™ transfection reagent (catalogue no. E4981, *Promega*). Based on the manufacturer's instructions, a protocol for the transfection of FIT probes was developed. The mixture contained 9 nM probe solved in Milli-Q® H_2O, 15 µL DMEM and 1 µL Viafect and was incubated for 15 min at room temperature before addition to the cells. For FIT probe assessment, plasmids coding for the GlyR were transfected 24 h prior to FIT probe transfection.

Ratiometric quantification of mRNA and protein signals in transfected HEK293T cells was performed to verify expression and translation of the bicistronic mRNA. The detection of mRNA and protein signal according to eGFP-MS2 coat protein and using an anti-HA antibody (catalogue no. 11867423001, Roche, diluted 1:100) in combination with a secondary antibody conjugated to DyLight649 (catalogue no. 712495153, *Jackson ImmunoResearch*, diluted 1:200), respectively, within different compartments of the same cell provides evidence in support of mRNA expression and translation as well as functionality of the 2A peptide.[3]

6. RNA isolation and RT-qPCR

RNA Isolation and RT-qPCR

Two days after transfection of eGFP-MS2_2A_HA-GlyR $\alpha2^{192L}$, eGFP-MS2_2A_c-myc-GlyR $\alpha2^{192P}$ or eGFP cell density was determined before total RNA was isolated using TRIzol reagent (catalog no. 15596018, *Invitrogen*). To eliminate residual plasmid DNA, the sample was treated with RNase free DNase (catalog no. 11119915001, *Roche*). Ten units were incubated (20 min, 37 °C) with 50 µg RNA sample and purified using RNeasy Protect Mini Kit (catalog no. 74124, *Qiagen*) according to manufacturer's instructions. Complementary DNA was obtained by reverse transcription (Superscript II, catalog no. 18064014, *Invitrogen*) of 2 µg RNA with an equimolar mixture of 3'-anchored poly-T oligonucleotides (T18V, T15V, T13V). DNA-free isolation was verified by PCR amplification (RedTaq® Polymerase, catalog no. D4309, *Sigma-Aldrich*). For this purpose, different combinations of oligonucleotides covering non-transcribed and transcribed regions of the plasmids were used: Oligonucleotides matched either CMV promotor region of the non-transcribed plasmid region and transcribed target GlyR α2 cDNA (5'- GGAGGTCTATATAAGCAGAGC-3' and 5'-GTGAAACTTGACCTCAATGCAG-3') (Figure S4 "pCMV"), the housekeeping gene GAPDH (5'-ATGGCACCGTCAAGGCTGAG-3' and 5'-CGACGCCTGCTTCACCACC-3') to verify presence of mRNA in general (Figure S4 "GAPDH"), or the transcribed region of the target GlyR α2 cDNA (5'-ATGAACCGGCAGCTAGTGAA-3' and 5'-GTGAAACTTGACCTCAATGCAG-3') (Figure S4 "GlyR"). Purified plasmid DNA was used to check presence or absence of DNA in the three different mRNA preparations.

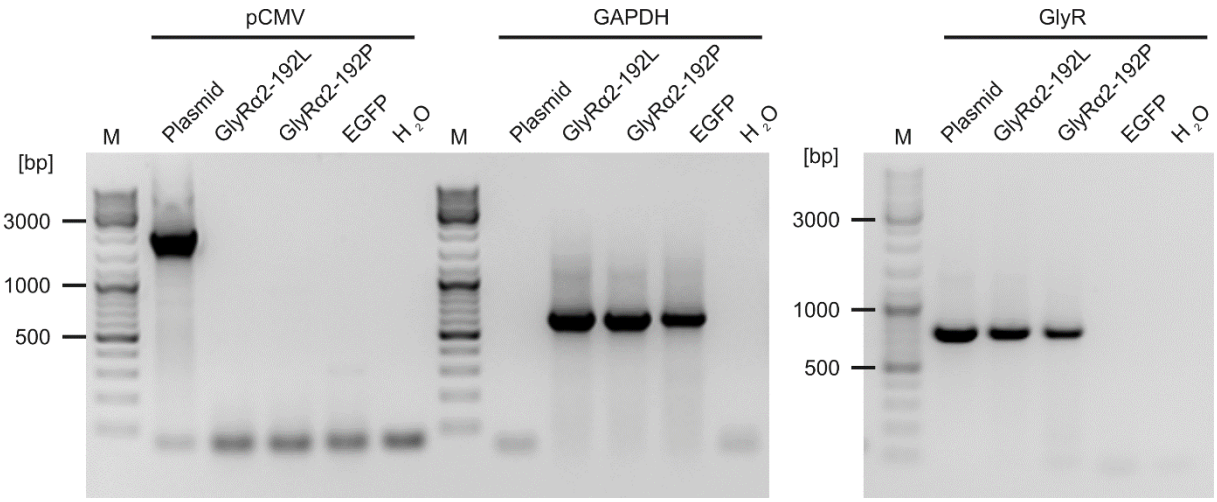


Figure S4: Agarose gel electrophoresis of PCR products obtained using oligonucleotides as indicated above. Absence of PCR product ("pCMV") in mRNA preparations ("GlyR α2-192L", "GlyR α2-192P", "EGFP") proves DNA-free RNA isolation. Functionality of the PCR was verified using purified plasmid DNA ("Plasmid"). Successful amplification of the house keeping gene GAPDH only in mRNA preparations of transfected HEK cells expressing GlyR-coding mRNA or eGFP-coding mRNA indicates successful mRNA isolation. Finally, selective amplification of GlyR α2-coding sequences from purified plasmid DNA and DNA-free RNA preparations indicates selectivity of the GlyR α2-specific oligonucleotides. M: molecular weight marker gene ruler (GeneRuler DNA Ladder Mix, catalog no. SM0331, *Thermo Fisher Scientific*)); bp.: base pairs.

RT-qPCR was carried out by using 1 µl cDNA solution of each sample as template employing GlyR α2 specific primers (forward: 5'-GGGTACACCATGAATGACCTGA-3', reverse: 5'-TGCTTTGTGCAATAGCCAAGTT-3'). This resulted in the amplification of a 125 bp sequence of the transfection product. Amplification, detection, and data analysis were performed with the iQ5 Real-Time PCR Detection System (*Bio-Rad*, Hercules, CA, USA) in a volume of 20 µL in 96-well plates.

cDNA was amplified by the following conditions: 400 nM each, 1 µl SYBRGold, diluted 1:100 (catalog no. S11494, *Thermo Fisher Scientific*, Waltham, MA, USA), initial activation of 'Hot Start' *Taq*-DNA-Polymerase (catalog no. 01-8120, *VWR*, Germany) at 95 °C for 4 min followed by 40 cycles of denaturation (95 °C, 10 s), annealing (59 °C, 20 s) and elongation (72 °C, 20 s). Fluorescence was measured during the annealing step. For the negative control cDNA was replaced by water.

Calculation of copy number / cell

For quantification of copies, a calibration curve was generated with samples of different concentrations (0.1 – 10^{-5} ng/µL) of a synthetic DNA oligomer (129 bp, MW = 40007 g/mol, sequence according to the target of interest). After curve fitting (Fig. S4), the resulting equation allows the calculation of copy number /µl cDNA by Ct values. The copy number is equal to the starting amount times N_A (Avogadro´s number, 6.022×10^{23} molecules/mole) divided by the molecular weight of the amplicon (40007 g/mol).

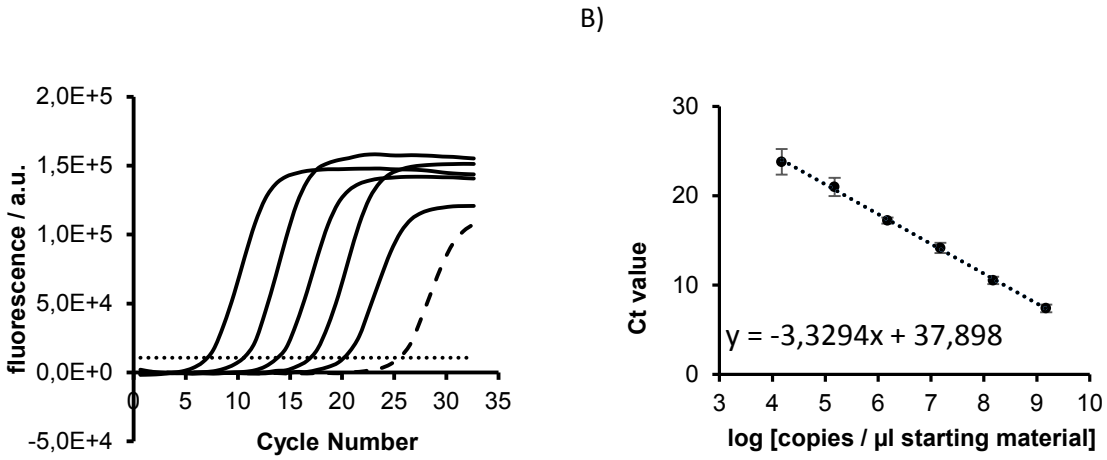


Figure S5: Quantitative PCR analysis. A) Amplification of a 129 bp DNA target encoding for GlyR α2A in a 10-fold dilution series (0.1 – 10^{-5} ng). B) Logarithmic presentation of the calibration curve with result of curve fitting.

From the total amount of extracted RNA of a given cell number 4 µg were reverse transcribed *in vitro* and diluted to 117 µL. The calibration curve and transfection rate allows the inference of specific mRNA copies per transfected cell:

$$No.of\frac{copies}{cell} = exp10\left(\frac{CT-37.9}{-3.3}\right) * 117 * \frac{1}{4\mu g} total\ RNA * \frac{1}{No.of\ cells} * \frac{1}{transfection\ rate}$$

Table S4: Results of quantitative RT-PCR

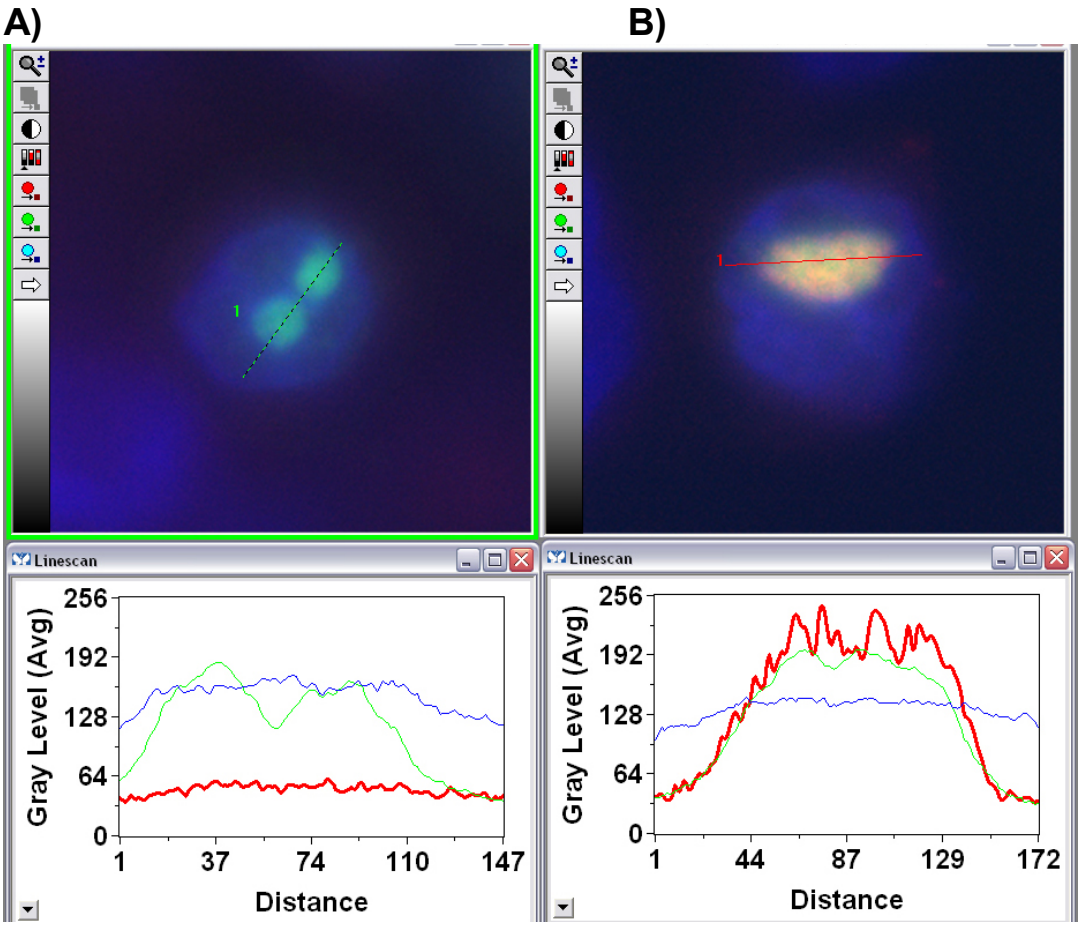
Batch-No.	No. of cells	Total RNA [µg]	Transfection rate [%]	copies/cell
1	4,405,625	30.76	23.0	3086
2	2,536,750	71.29	20.59	4475
3	2,365,000	69.60	11.63	6354

7. Fluorescence microscopy

Fluorescence imaging of FIT probes in HEK293T cells

HEK293T cells obtained after two subsequent transfections with i) plasmid and ii) FIT probe) were 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 (catalogue no. H-1200, *Vector Laboratories*).

Transfected GlyR α2-expressing cells were visualized according to EGFP fluorescent signals of the eGFP-MS2 coat protein tracking the MS2 tagged GlyR α2 mRNA. Imaging was performed with an epifluorescence microscope (Olympus BX51, *Olympus*, Tokio, Japan) under UPlan FL x20 objective with a numerical aperture of 0.50. Fluorescent signals were detected by appropriate filters (DAPI: U-NSP100v2 and FITC: U-NSP101 Chroma Technology Corp. and custom made mCherry filter set including exciter XF1206 572AF15, emitter XF3304 605WB20 and beam splitter QMAX_DI580LP; Omega Optical). A 14-bit cooled CCD camera (Spot PURSUIT, Visitron Systems) and the Metamorph imaging software (Universal imaging) were used for image acquisition.



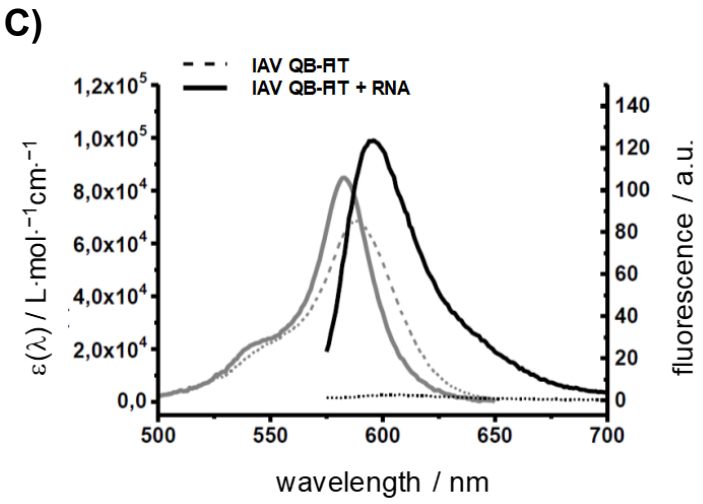
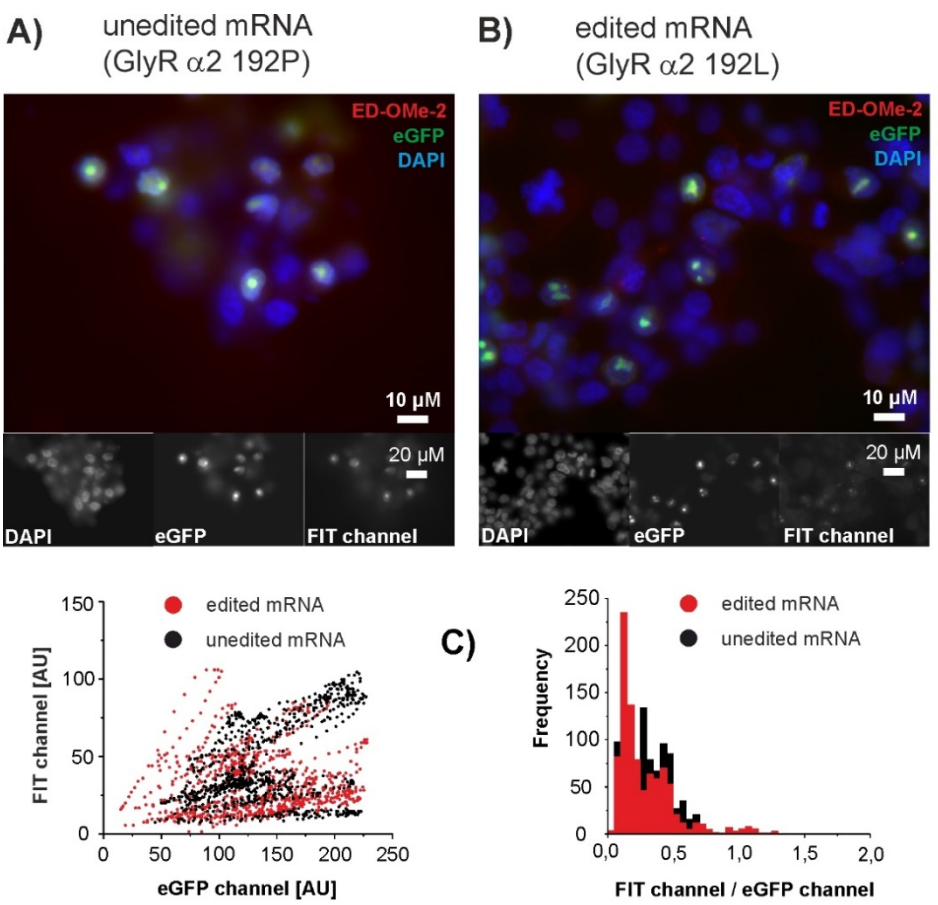


Figure S6: Representative line scans for fluorescence microscopy images of HEK293T cells that were transfected with plasmids encoding edited (192L) GlyR α2 mRNA and treated with AAAACGACC-QB-T_LGTTTCTACT, **IAV QB-FIT** (A) or **ED-OMe-1** (B). Delivery: 9 nM of probes. Blue: DAPI, Green: eGFP, Red: FIT probe. Distance is measured in pixels with 12,7185 pixel/μm C) Absorption spectra (grey) and fluorescence spectra (black) of FIT probe **IAV QB-FIT** (designed to target vRNA from influenza A virus) before (dashed) and after (solid) hybridization with complementary RNA. Conditions: 0.5 μM probe and 2.5 μM complementary RNA target in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7, 37 °C); λ_{ex} = 560 nm.

IAV QB-FIT provides 43-fold enhancement of fluorescence emission upon hybridization with complementary RNA and is, therefore, as responsive to the presence of complementary target as **Ed-OMe-1**. However, IAV QB-FIT does not find a target inside HEK293 cells and the emission signal remains low outside and inside of GlyR α2 mRNA-containing regions.


A)
unedited mRNA
(GlyR α2 192P)
B)
edited mRNA
(GlyR α2 192L)
ED-OMe-2
eGFP
DAPI
10 µM
20 µM
DAPI
eGFP
FIT channel
edited mRNA
unedited mRNA
FIT channel [AU]
eGFP channel [AU]
C)
Frequency
FIT channel / eGFP channel

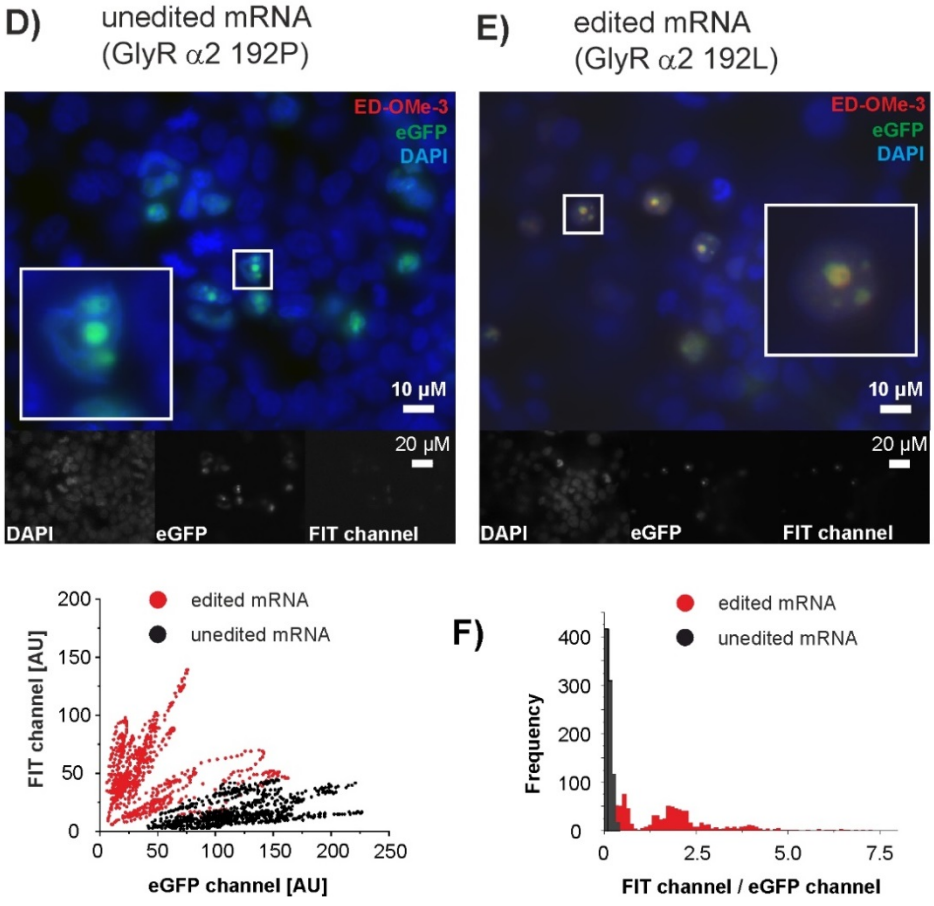
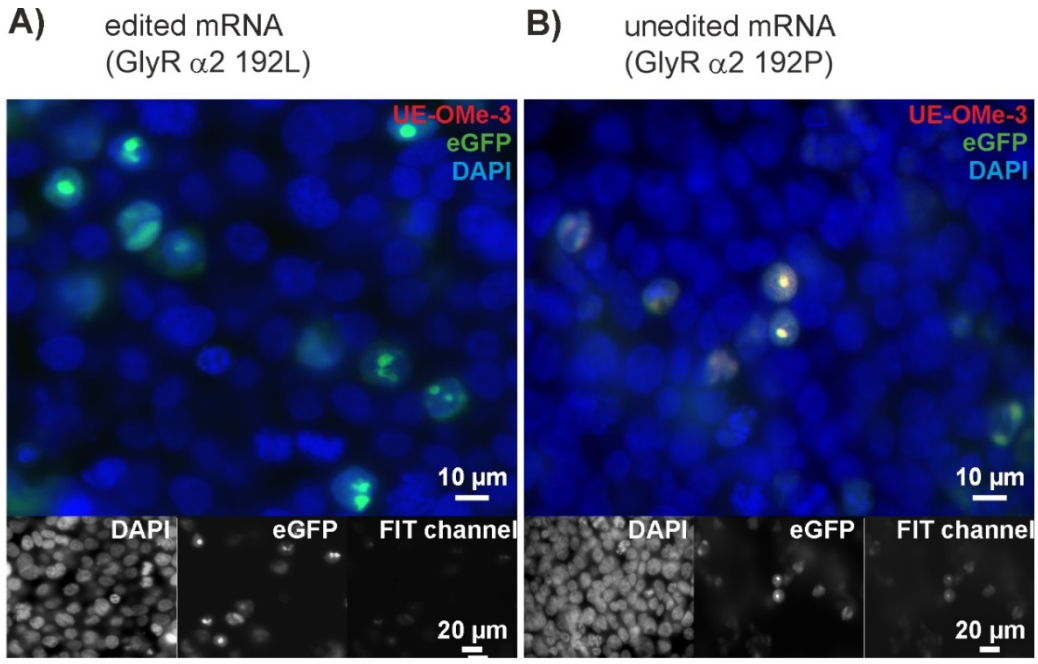

D)
unedited mRNA
(GlyR α2 192P)
E)
edited mRNA
(GlyR α2 192L)
ED-OMe-3
eGFP
DAPI
10 µM
20 µM
DAPI
eGFP
FIT channel
edited mRNA
unedited mRNA
FIT channel [AU]
eGFP channel [AU]
F)
Frequency
FIT channel / eGFP channel

Figure S7: Fluorescence microscopy images of HEK293T cells that were transfected with plasmids encoding A), D) unedited (192P) or B), E) edited (192L) GlyR α2 mRNA and treated with ED-OMe-2 (A, B) or ED-OMe-3 (D, E). Delivery: 9 nM of probes. Colored images show overlays of the DAPI (blue), eGFP (green) and FIT probe channels (red). C), F) Cell-based correlation of mRNA fluorescence signals (eGFP) corresponding to unedited GlyR $\alpha2^{192P}$ (black) or edited GlyR $\alpha2^{192L}$ (green) mRNA and FIT probe ED-OMe-2 (C) or ED-OMe-3 (F). Lines for line scans of fluorescence intensity measurements were projected within areas of interest in cell nuclei so that they covered peak signals. Fluorescence intensities were analysed pixel wise. Raw data (C left, F left) and the corresponding histogram (C right, F right) shows cell-based distributions of the ratios between FIT probe signals corresponding to GlyR $\alpha2^{192L}$ mRNA (red pixels / bars) or GlyR $\alpha2^{192P}$ mRNAs (black pixels / bars).



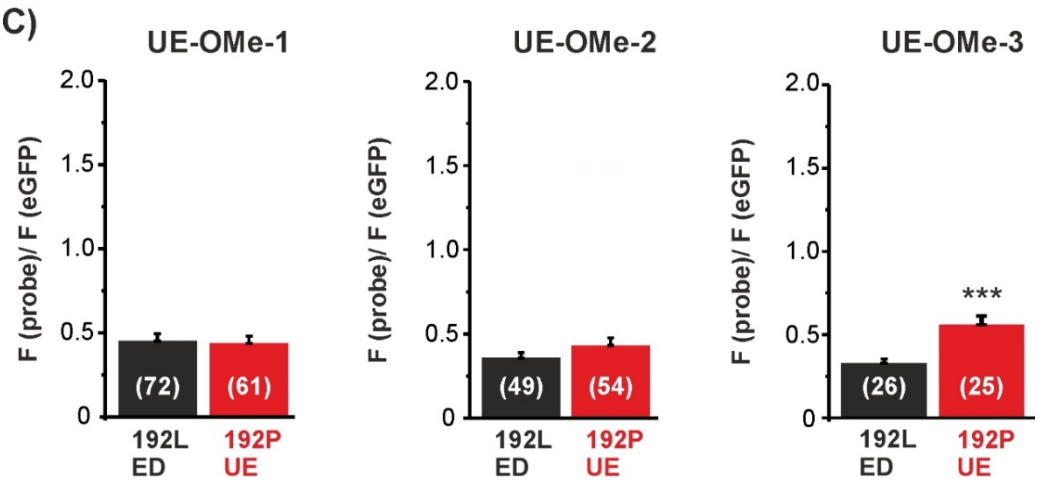


Figure S8. Fluorescence microscopy images of HEK293T cells treated with FIT probe UE-OMe-3 after transfection with plasmids encoding A) edited (192L) or B) unedited (192P) GlyR α2 mRNA. Colored images show overlays of DAPI (blue), EGFP (green) and FIT probe channels (red). C) Quantitative analysis given as QB/EGFP fluorescence ratios of three different FIT probes complementary to unedited mRNA. Lines for line scans of fluorescence intensity measurements were projected within areas of interest in cell nuclei so that they covered peak signals. Fluorescence intensities were analysed pixel wise, all data given as ±SD. The number of analysed cells is indicated in brackets.

Statistics

Fluorescence intensities were measured pixel wise using Metamorph line scan tool in regions within the nucleus where GlyR α2 mRNA hotspots were detected. One-way ANOVA analysis followed by post hoc Bonferoni test was used for statistical analysis (software Origin41, Origin Lab). $P<0.05$ was considered significantly different.

References

[1] F. Hövelmann, I. Gaspar, J. Chamiolo, M. Kasper, J. Steffen, A. Ephrussi, *Chemical science*. 2016, **7,** 128-35.

[2] A. Shrivastava, V.B. Gupta, ChronYoungSci 2011, **2**, 21

[3] G. Fang, J. Chamiolo, S. Kankowski, F. Hövelmann, D. Friedrich, A. Löwer, J.C. Meier, O. Seitz, *Chem. Sci*., 2018, **9**, 4794