CHEMBIOCHEM

Supporting Information

Comparing Agent-Based Delivery of DNA and PNA Forced Intercalation (FIT) Probes for Multicolor mRNA Imaging

Jasmine Chamiolo,[a] Ge-min Fang,[a, b] Felix Hövelmann,[a] Dhana Friedrich,[c, d] Andrea Knoll,[a] Alexander Loewer,[c, d] and Oliver Seitz*[a]

cbic_201800526_sm_miscellaneous_information.pdf

Content

1. General Information

Materials:

Reagents were purchased from Sigma-Aldrich, Acros Organics or TCI-Europe. Synthetic RNA targets were obtained from IDT (Leuven, Belgium). The Fmoc-protected amino acids were acquired from Iris Biotech (Marktredwitz, Germany) and the PNA monomers from Link Technologies Ltd. (Lanarkshire, Scotland). HCTU and trifluoroacetic acid, *N,N*-diisopropylethylamine were obtained from Roth (Karlsruhe, Germany), DMF from VWR (Pennsylvania, USA), Oxyma from Carbolution (Saarbrücken, Germany). 3´-Spacer-C3-CPG (1 µmol, pore size 500 Å), 5´-thiol modifier C6 S-S CE, 3´-Palmitate-SynBase CPG and phosphoramidites (DNA and 2´-OMe) were purchased from Link Technologies Ltd. (Lanarkshire, Scotland). LNA was acquired from Lexiqon (Vedbeak, Denmark) and Ribotask Aps (Langeskov, Denmark). DNA synthesis reagents were purchased from EMP-Biotech (Berlin, Germany).

HPLC purification and analysis:

The crude peptides and PNA were purified by using a preparative HPLC 1100 system from Agilent (Santa Clara, USA) and Polaris C18 A 250x21 mm, 5 µm columns from Varian (Palo Alto, USA) with a binary mixture of the solvents A (98.9% H_2O, 1% MeCN, 0.1% TFA) and B (98.9% MeCN, 1% H_2O, 0.1% TFA) at 6 mL/min flow rate and a linear gradient. The PNA conjugates were purified using a LaChrome Elite system from Hitachi (Tokyo, Japan) and a Polaris 5 C18-A 250x4.6 mm column at a flow rate of 1 mL/min at 55°C. PNA, Peptides and conjugates were analyzed by Acquity ultraperformance liquid chromatography system from Waters (Eschborn, Deutscland) and a BEH130 C18 (2.1x50mm, 1.7µm) column with a binary mixture of the solvents A (98.9% H_2O, 1% MeCN, 0.1% TFA) and B (98.9% MeCN, 1% H_2O, 0.1% TFA) at 50°C and 0.5 mL/min flow rate in a linear gradient shown in table 2.

Oligonucleotides and oligonucleotide conjugates were purified by reversed-phase HPLC (DMT-on and -off) by using a 1105 Gilson HPLC and a YMC Triat C18 (150x10 mm, 5 µm) column at 8 mL/min flow rate or a YMC Triat C18 (250 x 4.6 mm, 5µm) at 1.5 mL/min flow rate and 55°C with a binary mixture of A (0.1 M TEAA buffer, pH = 7.5, aq.) and B (acetonitrile). Oligonucleotides and oligonucleotide conjugates were characterized on the YMC Triat C18 (250 x 4.6 mm, 5µm) column.

MALDI-TOF MS:

MALDI-TOF mass spectra were measured by using a Shimadzu Axima Confidence instrument in positive mode. The THPTA matrix was used (28 mg/mL 2',4',6'-trihydroxyacetophenonmonohydrate und 6 mg/mL citric acid diammonium salt in MeCN/H_2O (1:1)).

2. Synthesis

Synthesis of PNA FIT probes and peptides

PNA and peptides were synthesized on a Tentagel®R RAM resin from *Novabiochem* (Schwalbach, Germany) in a 10 µmol scale. For the maleimide-containing peptides, the resin was allowed to swell in

DMF/piperidine (4:1 [v/v]) for 15 min and then washed five times with DMF. Afterwards 4 eq of Fmoc-(Mmt)-Lys-OH, 4 eq Oxyma, 3.9 eq HCTU, 6 eq. DIPEA in DMF (0.2 M) were coupled 2 x 45 min at room temperature. The subsequent assembly of the peptide chain was performed by using an automatized peptide synthesizer from *Intavis* (Köln, Deutschland) and coupling Fmoc-protected amino acids according to manufacturer´s instructions. The removal of the Mmt-group was performed by treatment with 1% TFA in DCM, which was followed by a manual coupling of 6-maleinimidohexaonicacid. To release peptides the resin was treated with TFA/TIS/thioanisole (90/4/6) for 1.5h. The TFA extract was concentrated using an argon stream. For precipitation, the remaining solution was added to ice-cold ether. The precipitate was purified by preparative HPLC. For the synthesis of the DTNP-peptides, cysteine was coupled as *N*-terminal amino acid. The peptides were cleaved from the resin upon treatment of TFA/TIS/H_2O/EDT (95.5/2/2/0.5) for 1.5h followed by a precipitation in ice-cold ether. The HPLC purified material (1 eq) was modified in a reaction with 2,2'-dithiobis(5-nitropyridine) (20 eq) in acetic acid/ water (3:1, 12 mM). After 12 h shaking the crude conjugate was purified by preparative HPLC. The synthesis of FIT-PNA was performed according to manufacturer's instructions. For installing the N-terminal thiol function, Fmoc-6-Ahx-OH and subsequently 3-tritylthio-propionic acid were manually coupled. For detachment, the PNA-resin was treated with TFA/TIS/H_2O/EDT (95.5/2/2/0.5). The TFA extract was concentrated using an argon stream. The remaining solution was added to ice-cold ether. The precipitate was purified by preparative HPLC.

Synthesis of DNA FIT probes

Automated DNA synthesis was performed by using a *Bioautomation MerMade 4* synthesizer (Irving, Texas). All phosphoramidites were used according to manufacturer´s instructions. The yield of every coupling step was monitored by measuring the absorbance of the DMTr cleavage solution. The synthesis was programmed to yield the oligonucleotide carrying the DMTr protection group ("trityl-on"). Dyes and dye nucleotides were synthesized as described in former publications.[1] After completion of chain assembly the CPG was dried under vacuum. To remove the protecting groups and release the oligonucleotide the solid support was treated with 80% aqueous ammonia for 2.5 h at 55°C. The volatiles where removed under reduced pressure, the solid dissolved in water and the resulting solution filtered prior to HPLC purification. Afterwards, the DMTr group was removed upon treatment with 300 µL of 80% aqueous AcOH for 15 min at room temperature. The detritylation mixture was treated with 1 mL *i*PrOH and the resulting precipitate again purified by HPLC.

Synthesis of DNA/PNA-Peptide Conjugates via conjugate addition to maleimide

The Thiol-PNA was used for maleimide-ligation directly after HPLC purification. To reduce the disulfide protecting group DNA-based probes the oligonucleotide was dissolved (200µM) in a degassed TCEP buffer (5 mM TCEP, 10 mM NaCl, 10 mM $NaHPO_4$, pH 6.5) and shaken at room temperature overnight. Afterwards the oligonucleotide was precipitated with 10% vol. 3 M ammonium acetate and 1 mL isopropanol. To prevent the oxidation of the thiol the probe was dried under argon. The thiol-oligonucleotide was dissolved (800 µM) in ligation buffer (10 mM NaCl, 10 mM NaH_2PO_4, pH 6.5) with 4 eq of maleimide peptides. The mixture was stirred for 2h. Urea buffer (0.1 M KH_2PO_4, 0.3 M KBr, 8

M Urea, 1 mM TCEP, pH 8) was added. The mixture was the stirred for 30 min. After filtration over a NAP column the conjugates were purified by HPLC.

Synthesis of PNA-disulfide-CPP-conjugates

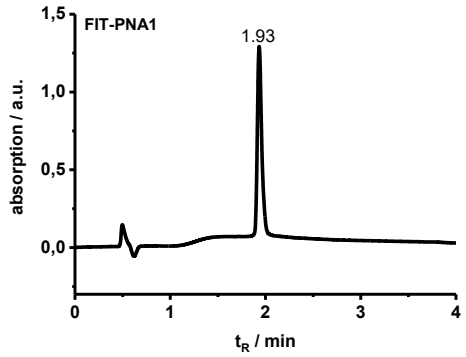
To 1 eq. of thiol-PNA (100 µM) in buffer (1 M HEPES, 6 M Gn-HCl, pH 6.8) were added 4 eq of DTNP-peptide. The mixture was stirred for 1h at 25°C. Subsequently, the mixture was filtrated trough a NAP column prior to HPLC purification.

Table S1 Analytical data of the DNA probes.

No.	Sequence (X = Ser(TO, QB, BO))	R_T/ min	HPLC gradient	MALDI-TOF-MS Calculated $[M]^+$ / found
FIT-DNA1	GCCGXT_LGAUUA	8.85	10% - 50% B in 10 min	3875/ 3873
Pen_FIT-DNA1	^{Ac}*N*-RQIKIWFQNRRMKWKKK* -S- GCCGXT_LGAUUA	21.0	10% - 40% B in 33 min	6679/ 6672
SAP(E)_FIT-DNA1	^{Ac}*N*-$(VELPPP)_3K^*$-S- GCCGXT_LGAUUA	29.5	10% - 40% B in 30 min	6349/ 6341
Palm_FIT-DNA1	$C_{22}H_{44}N_1O_4P_1$- GCCGXT_LGAUUA	17.7	30% - 70% B in 20 min	4270/ 4270
FIT-DNA2	AUGCACGXC$_L$CG	8.95	10% - 50% B in 10 min	3868/ 3862
FIT-DNA3	UUUUUUUTXT_LTUUUUUUU	8.80	10% - 50% B in 10 min	5934/ 5936

Table S2 Analytical data of the PNA probes. (X = $(CH_2)_2CO$-$NH(CH_2)_5CO$)

No.	Sequence (X = Ser(TO))	R_T/ min	UPLC gradient	ESI-MS calculated / found
FIT-PNA1	HS-X-g c c g Aeg(TO) t g a t t a	1.93	3% - 60% B in 4 min	$[M]^{3+}$ 1126.3/ 1125.3
Pen_FIT-PNA1	^{Ac}*N*-RQIKIWFQNRRMKWKKK* -S-X- g c c g Aeg(TO) t g a t t a	1.94	3% - 80% B in 4 min	$[M]^{5+}$ 1197.5/ 1197.7
SAP(E)_FIT-PNA1	^{Ac}*N*-$(VELPPP)_3K^*$-S-X-g c c g Aeg(TO) t g a t t a	2.35	3% - 80% B in 4 min	$[M]^{3+}$ 1131.5 / 1131.8
Transp_FIT-PNA1	^{Ac}*N*-LIKKALAALAKLNIKGLLYGASNLTWGC*-S-X-g c c g Aeg(TO) t g a t t a	2.84	3% - 80% B in 4 min	$[M]^{6+}$ 1075.3/ 1075.3
Pep2_FIT-PNA1	^{Ac}*N*-KETWFETWFTEWSQPKKKRKVC*- S-X-g c c g Aeg(TO) t g a t t a	2.30	3% - 80% B in 4 min	$[M]^{6+}$ 1049.1/ 1048.9
MPG_FIT-PNA1	^{Ac}*N*-GALFLGFLGAAGSTMGAWSQPKKRKVC*-S-X- g c c g Aeg(TO) t g a t t a	2.58	3% - 80% B in 4 min	$[M]^{6+}$ 1055.2/ 1054.9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absorption / a.u.
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0,5
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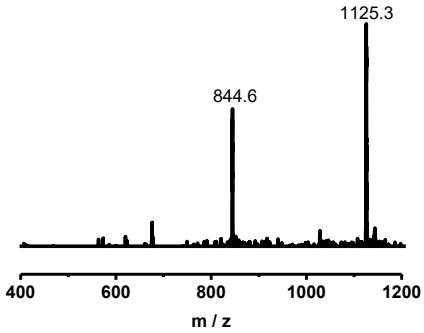
0
1
2
3
4
t_R / min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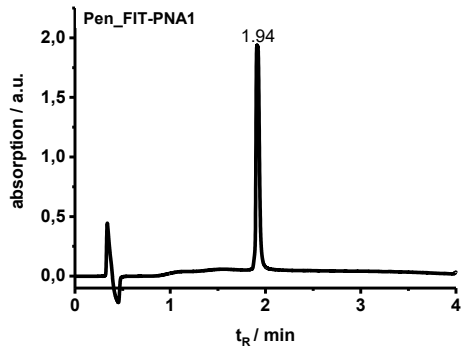
600
800
1000
1200
m / z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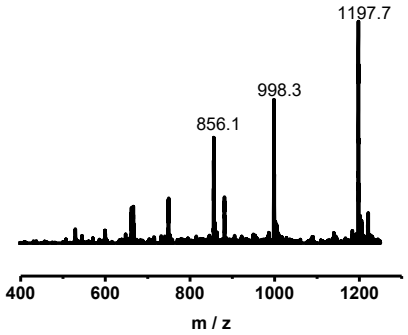
4
t_R / min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856.1
400
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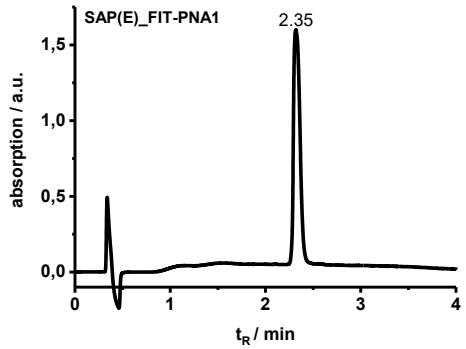
1000
1200
m / z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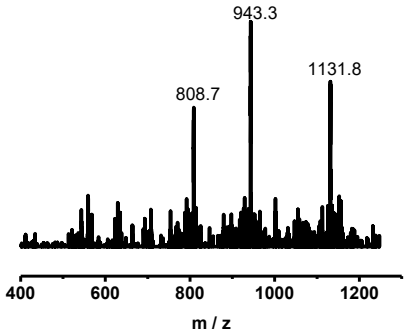
3
4
t_R / min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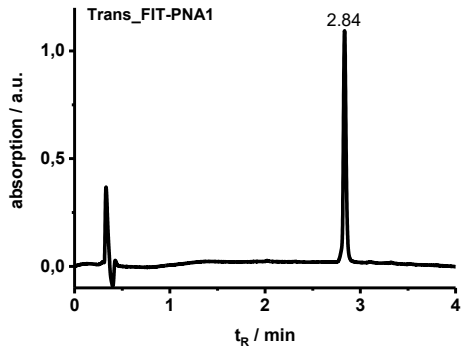
1200
m / z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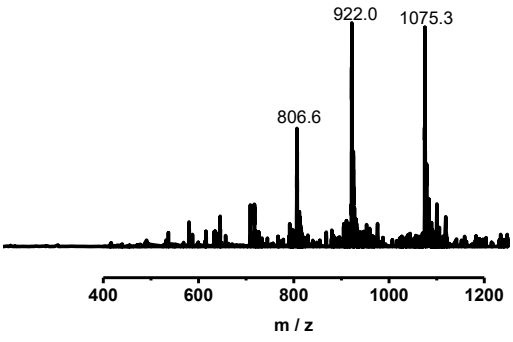
2
3
4
t_R / min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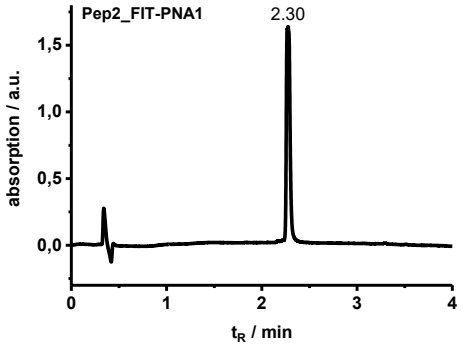
1000
1200
m / z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absorption / a.u.
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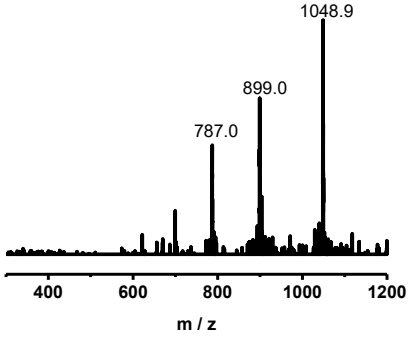
0
1
2
3
4
t_R / min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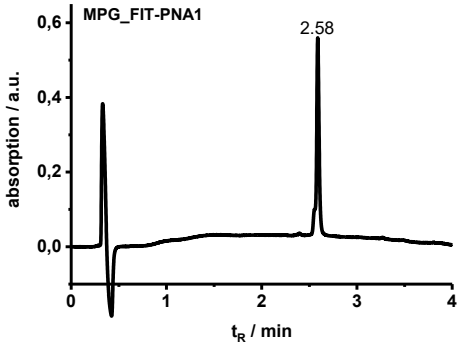
1000
1200
m / z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absorption / a.u.
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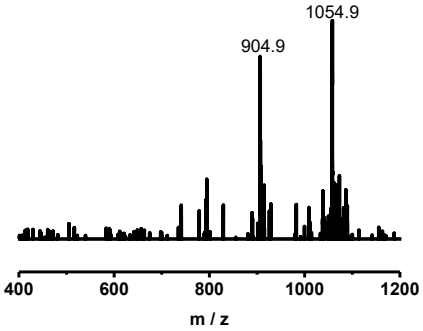
1
2
3
4
t_R / min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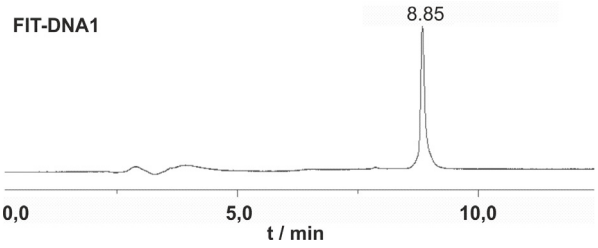
m / z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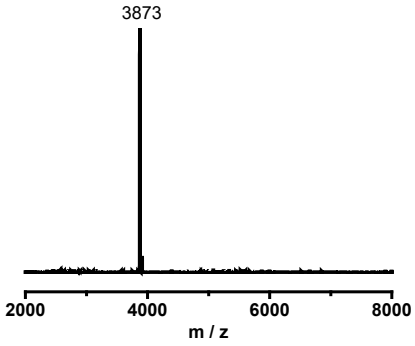
t / min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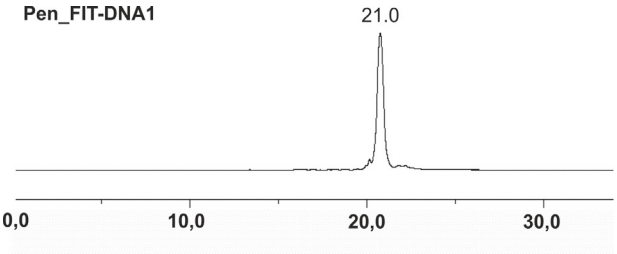
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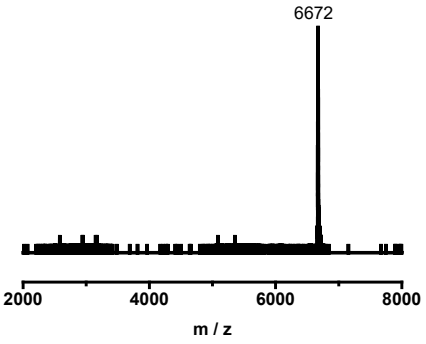
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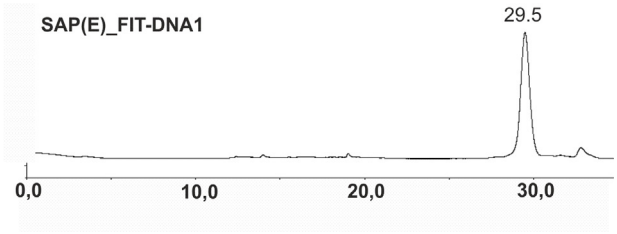
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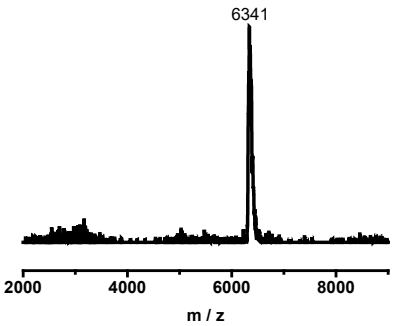
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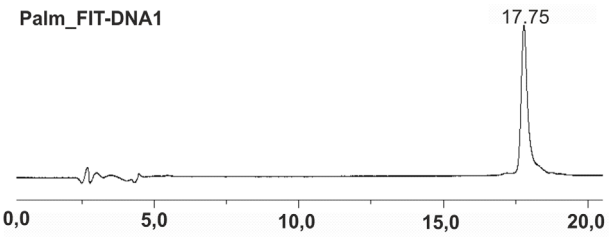
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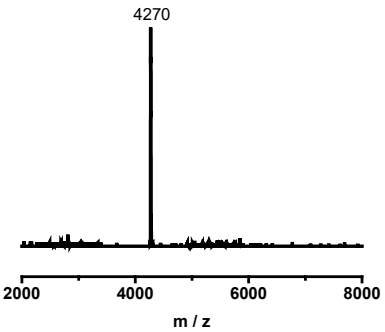
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m / z

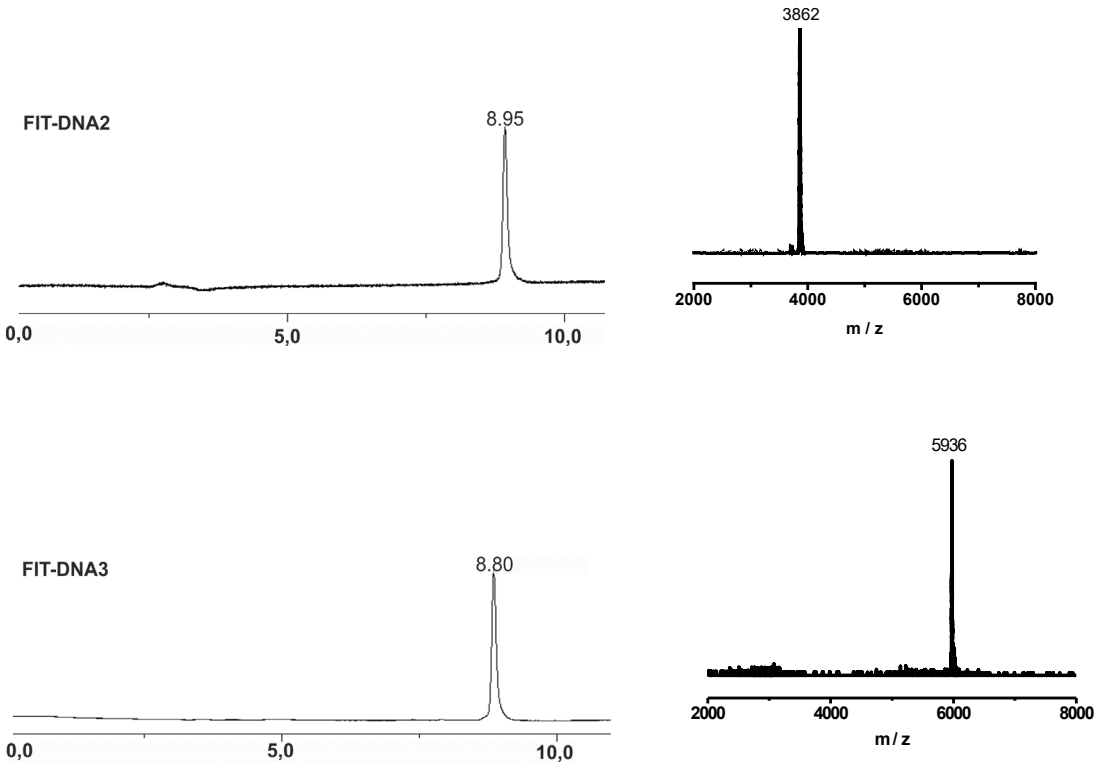


Figure S1. RP-HPLC-UV analysis (left) and MALDI-TOF-MS analysis (right) of purified FIT probes.

3. Optical spectroscopy data of FIT probes

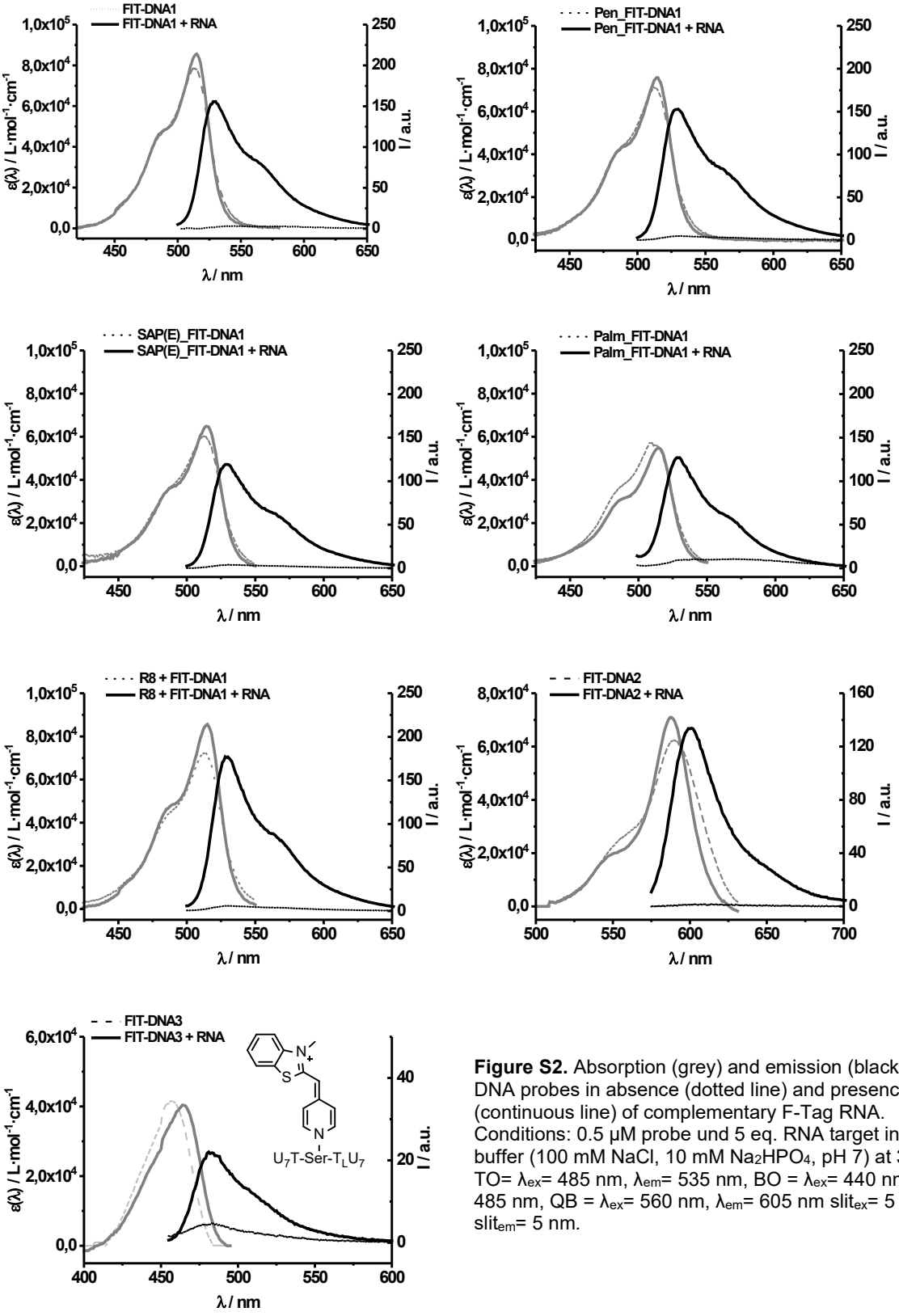


Figure S2. Absorption (grey) and emission (black) of FIT-DNA probes in absence (dotted line) and presence (continuous line) of complementary F-Tag RNA. Conditions: 0.5 µM probe und 5 eq. RNA target in PBS buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) at 37°C, TO= λ_{ex}= 485 nm, λ_{em}= 535 nm, BO = λ_{ex}= 440 nm, λ_{em}= 485 nm, QB = λ_{ex}= 560 nm, λ_{em}= 605 nm $slit_{ex}$= 5 nm, $slit_{em}$= 5 nm.

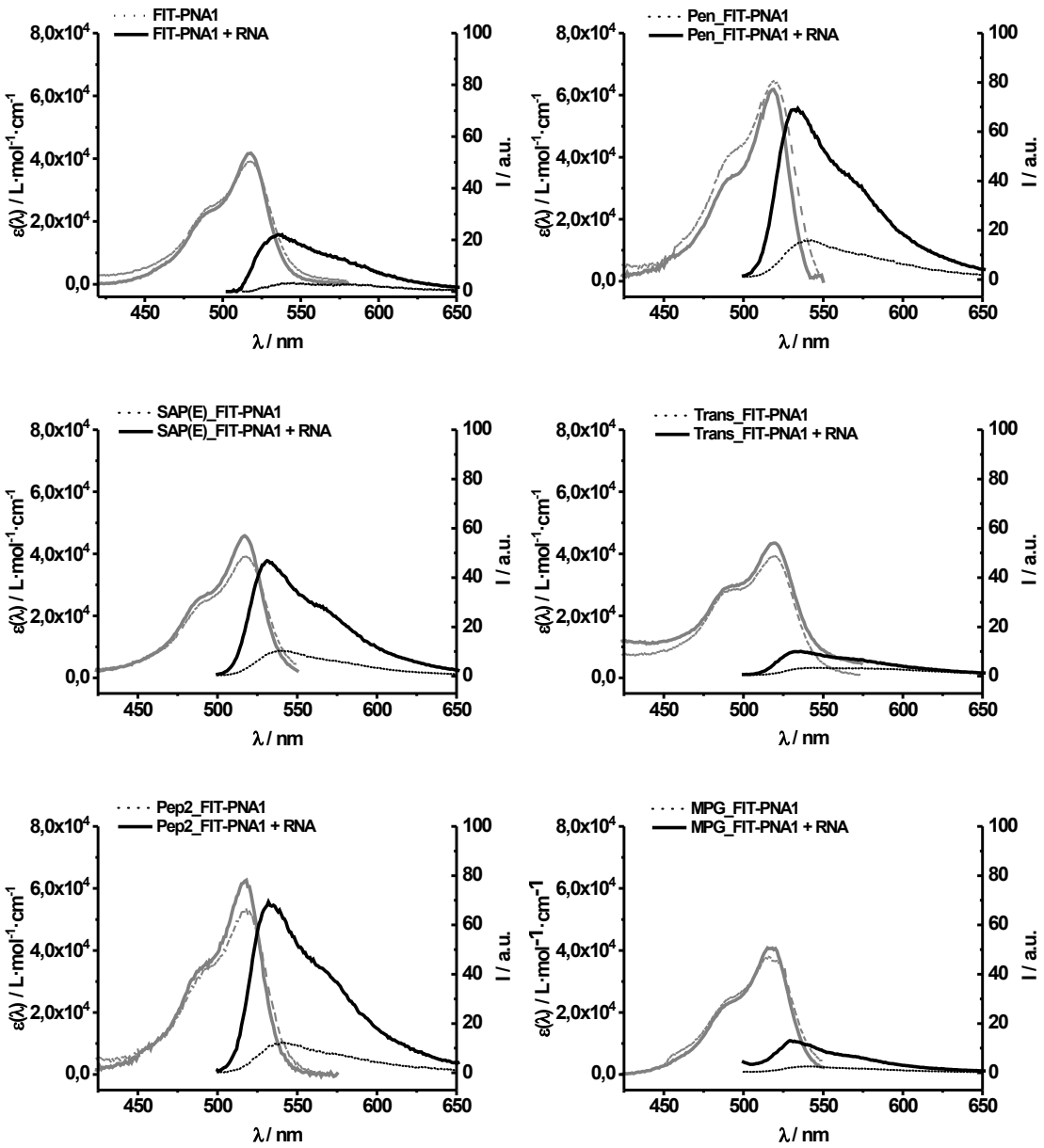


Figure S3. Absorption (grey) and emission (black) of FIT-PNA probes in absence (dotted line) and presence (continuous line) of complementary F-Tag RNA. Conditions: 0.5 µM probe und 5 eq. RNA target in PBS buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) at 37°C, λ_{ex}= 485 nm, λ_{em}= 535 nm, $slit_{ex}$= 5 nm, $slit_{em}$= 5 nm.

4. Melting analysis of the FIT probes and FIT probe conjugates

For melting analysis, the absorbance at 260 nm was monitored during a thermal cycle (3 times 20-90 °C in 0.5 °C/min) with 1 eq of RNA target. The probes were dissolved at 1 µM or 0.5 µM concentration using PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7).

Melting curves at 1 µM concentration

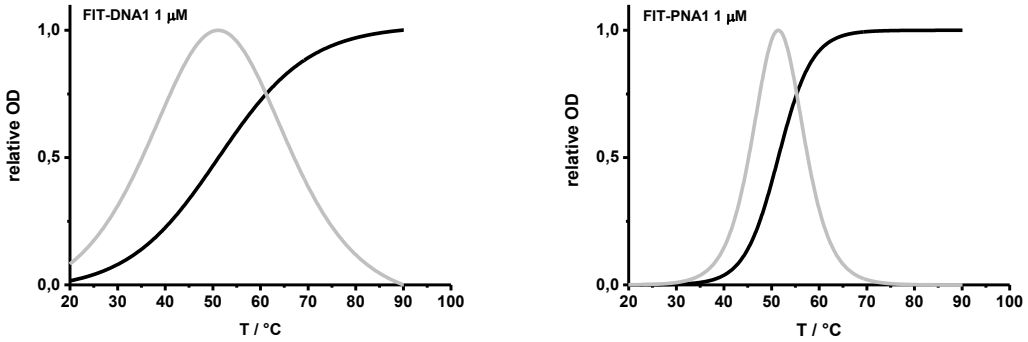
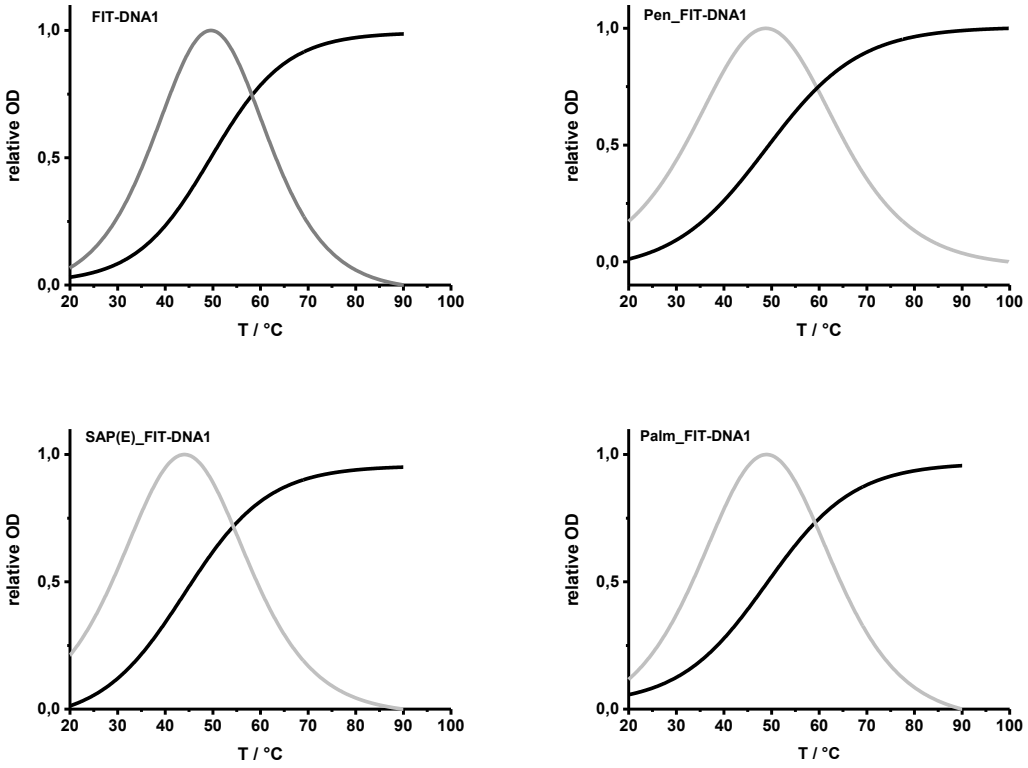


Figure S4. Normalized melting curve (black) and derivation (grey). Conditions: 1 µM probe and 1 eq. RNA target in PBS (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) 20-90 °C. λ_{em}= 260 nm.

Melting curves of 0.5 µM DNA FIT-Probes and DNA FIT-Probe conjugates



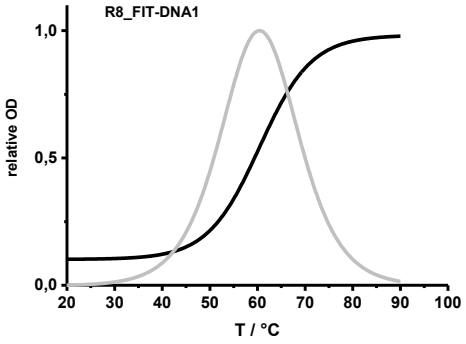
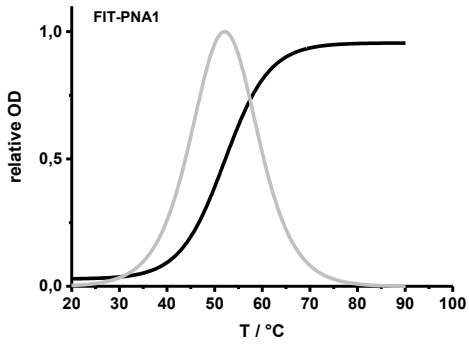
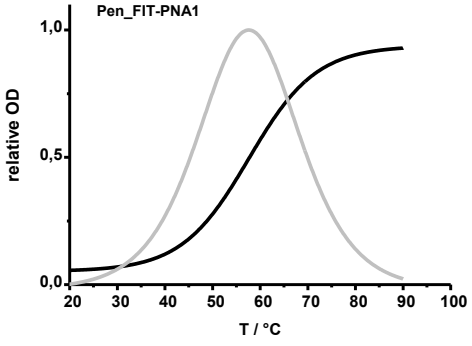
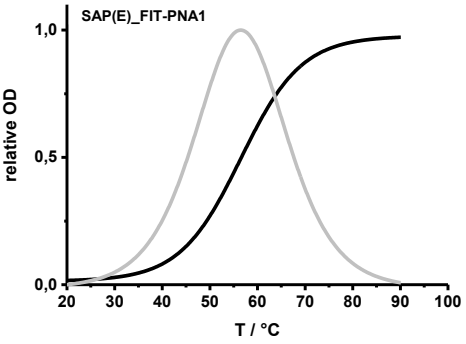


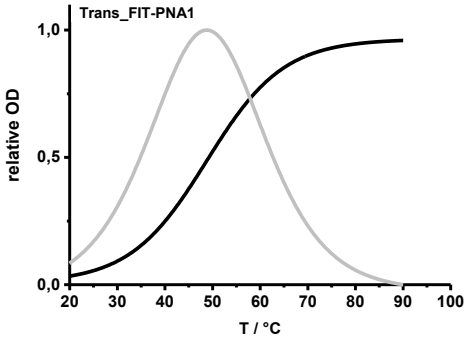
Figure S5. Melting curve (black) and derivation (grey). Conditions: 0.5 µM probe and 1 eq. RNA target in PBS buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) 20-90 °C. λ_{em}= 260 nm.

Melting curves of 0.5 µM PNA FIT-Probes and PNA FIT-Probe conjugates









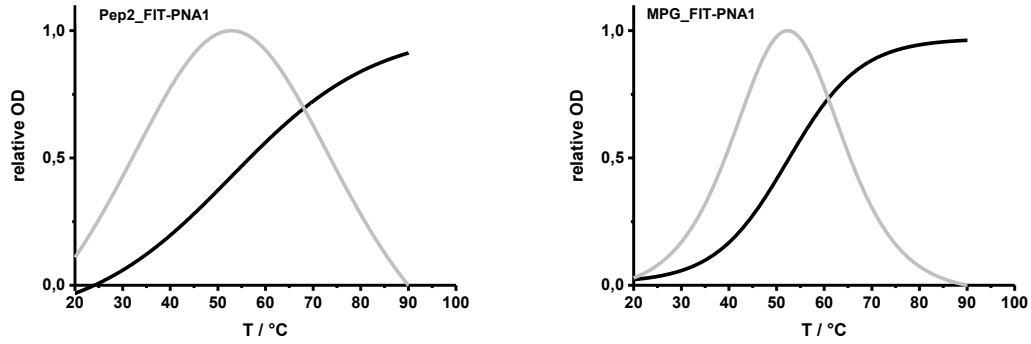


Figure S6. Melting curve (black) and derivation (grey). Conditions: 0.5 µM probe und 1 eq. RNA target in PBS buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7) 20-90 °C. λ_{em}= 260 nm.

5. Permanent transfection of Flp-In™ 293 T-Rex cells™ with F-tagged mCherry

To express a repeat sequence of mRNA together with an mCherry open reading frame under control of an inducible promoter, we employed oligo condensation and MultiSite-Gateway™ recombination cloning from *Invitrogen* (Carlsbad, USA). 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) from *Invitrogen* (Carlsbad, USA) using all three donor plasmids and a custom destination vector containing R4 and R3 recombination sites for the Multiside-Gateway system as well as an FRT site for *Flp-In™* recombination. Stable cell lines were generated using *Flp-In™ 293 T-Rex cells™* 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. Inducible F-RNA expression was measured by quantitative real-time PCR using primers specific for the mCherry ORF. Protein translation from induced F-RNA was validated by Western Blot as well as time-lapse microscopy imaging of mCherry fluorescent protein.

5'- GAT CCT AAT CAA CGG CCG GAC GTG CAT CTA ATC AAC GGC CGG ACG TGC ATA -3'

5'- TAT GCA CGT CCG GCC GTT GAT TAG ATG CAC GTC CGG CCG TTG ATT AGG ATC -3'

Figure S7. 51 nt long sequence oft he synthesized oligonucleotides for the repeat sequence.

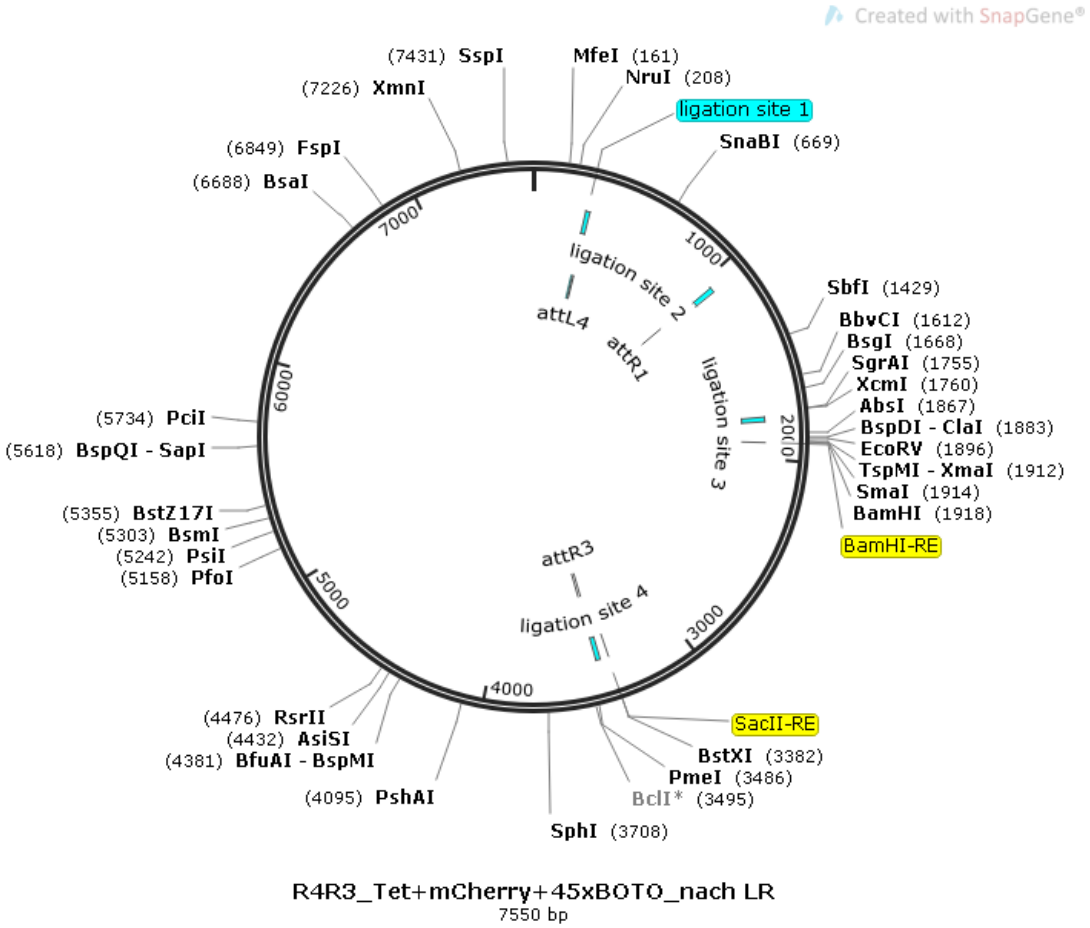


Figure S8. Plasmid map used for transfection of the Flp-In™ 293 T-Rex cells™

6. PCR (Polymerase Chain Reaction) of mcherry mRNA

Permanently transfected Flp-In™ 293 T-Rex cells™ were trypsinated after different times of doxycycline incubation (15 min, 30 min, 45 min and 60 min) in phenol red free DMEM. For purification of total RNA, the RNeasy Mini Kit (Qiagen) was used following the manufacturer's instructions. Total RNA was eluted in 30 µL RNase free H_2O. RNA concentration was determined by an optical density (OD) measurement. For in vitro cDNA synthesis a *High Capacity cDNA reverse Transcription Kit* from *Applied Biosystems* and a *TGradient* from *Biometra* was used. Real-time PCR was performed in a

volume of 20 µL in 96-wellplates by using an iQ-Thermocycler (*BioRad*). Specific primers (forward: 5´-cagctttcttgtacaaagtggca-3´; reverse: 5´-attaggatcccccgggctg-3´) for the amplification of a 139-bp sequence between the mCherry and the F-tag region (Seq1) were selected with Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers and synthetic templates were purchased from Biomers (Ulm, Germany). As negative control (*No Template Control, NTC*) the cDNA was replaced by RNase free water. All measurements were performed in triplicates. The mastermix for 20 µL consisted of 9.8 µL H_2O, 2.0 µL 10 x reaction buffer S, 4.0 µL 5x enhancer solution P (both *VWR*, #01-8120), 1.0 µL 25mM $MgCl_2$, 0.4 µL dNTP´s (mix, 10 mM each), 0.8 µL of each primer (10 µM), 1.0 µL SYBRGold (10x), 1.0 µL cDNA solution (10ng/µL), 1unit HotStart Taq polymerase from *VWR* (#01-8120*)*. The solutions were transferred into 96-wellplates and cDNA was amplified under the following cycle-conditions: initial denaturation at 95°C for 4 min followed by 30 to 40 cycles of denaturation (95°C, 10 sec), annealing (59°C, 20 sec) and elongation (72°C, 20sec).

For quantification a calibration curve was generated with different concentrations (0.1 – 10^{-6} ng/µl) of a synthetic 139-bp DNA template (5´-CAGCTTTCTTGTACAAAGTGGCATAATGATGAATTG TAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATA AGCTTGATATCGAATTCCTGCAGCCCGGGGGATCC-3´) analogous to the PCR product Seq1 from cDNA. The cycle of threshold (Ct) values correspond to a certain number of copies in 1 µL of the starting material (Fig. S9).

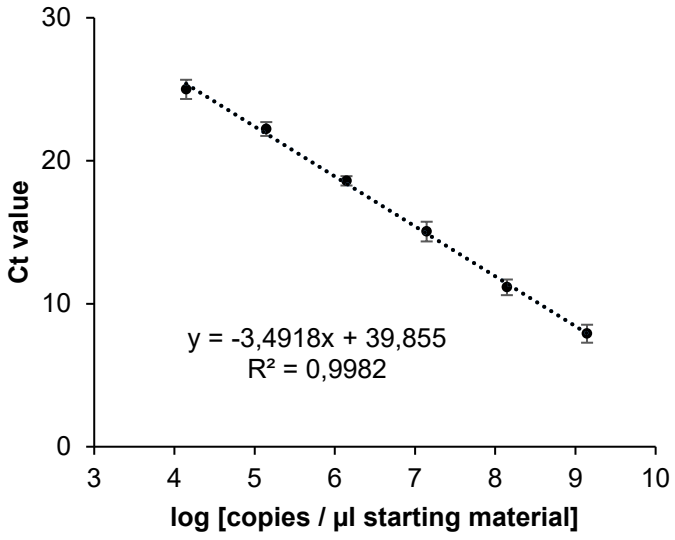


Figure S9. Logarithmic presentation of the calibration curve after amplification of a 139-bp DNA target encoding for Seq1, a sequence between the mCherry and the F-tag region in a 10-fold dilution series (0.1 – 10^{-6} ng/µL). The equation describes the correlation between the Ct value and the number of copies in 1 µl starting material.

The equation shown in Fig. S9 allows the conversion of any Ct value determined by PCR of cDNA into a copy number of Seq1 per ng starting material (copies/ng = 10 exp. (Ct value – 39.855) / -3,4918). Considering the number of counted cells per sample we calculated the number of target copies per cell (Table S3, Fig. S10).

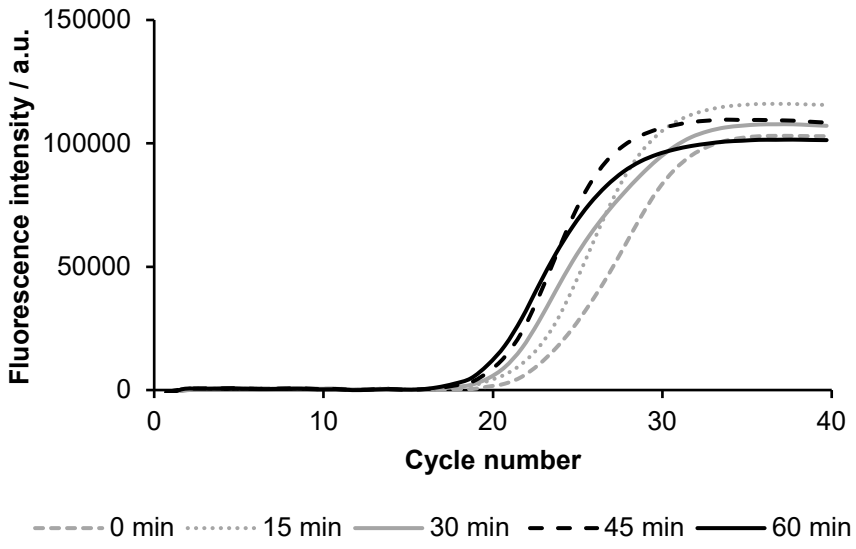


Figure S10. Amplification curves for quantitative PCR analysis of mRNA extract obtained after different incubation times of *Flp-In™ 293 T-Rex cells™* with 2 µg / mL doxycycline (SYBRGold as fluorescence marker). The curves represent the means of three experiments with 3 replicates each.

Table S3: Ct values (mean of at least three experiments with 3 replicates each) and calculated numbers of Seq1 copies per *Flp-In™ 293 T-Rex cell™* after different times of incubation with doxycyclin.

Sample-Nr.	Incubation time (min)	Ct value	Copies / ng starting material	starting material / sample (ng)	copies / sample	cells / sample	copies / cell
1	0	20.4	38628	1140	4.404E+07	200000	220
	15	20.8	28383	2130	6.046E+07	200000	302
	30	19.8	57187	1260	7.206E+07	200000	360
	45	20.2	41855	1500	6.278E+07	200000	314
	60	19.1	85788	1860	1.596E+08	200000	798
2	0	24.0	3382	1170	3.957E+06	120000	33
	15	20.9	66459	240	1.595E+07	120000	133
	30	18.9	254022	240	6.097E+07	120000	508
	45	18.3	163689	540	8.839E+07	120000	737
	60	20.0	49628	870	4.318E+07	120000	360
3	0	23.1	6398	600	3.839E+06	75000	51
	15	22.8	14059	330	4.639E+06	75000	62
	30	24.0	3275	630	2.063E+06	75000	28
	45	19.9	50508	1020	5.152E+07	75000	687
	60	17.7	166606	810	1.350E+08	75000	1799
4	60	18.3	18578	4681	8.695E+07	140000	621
5	60	16.8	12891	5640	2.467E+08	140000	670

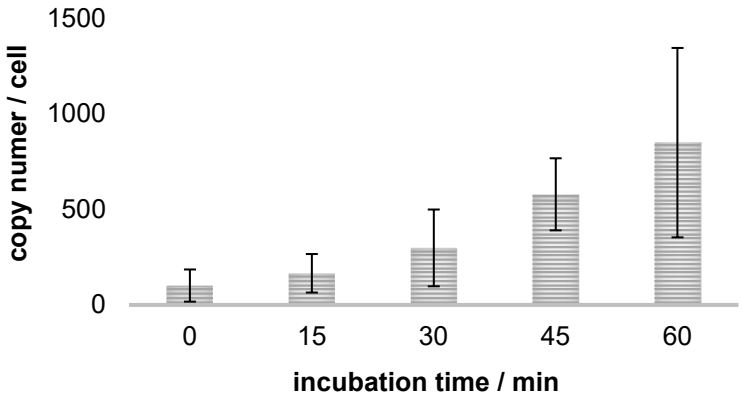


Figure S11. Average copy number of the Seq1 template per cell after different incubation periods with 2 µg/mL doxycycline. Mean of three independent experiments (Table S3) is shown with SD.

7. Live cell imaging

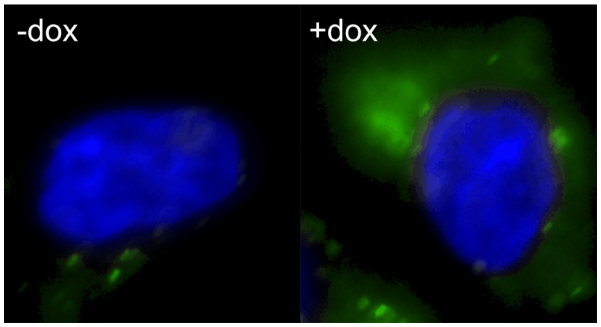


Figure S12. Zoomed in fluorescence microscopy images of living HEK293 cells after 30 min incubation with 1 µM Pen_FIT-PNA1 in PBS at 37 °C and 5% CO_2. The left image and the right image show cells without (-dox) and with (+dox) induction of F-tagged mCherry mRNA. Green colour shows the signal from TO emission with 500/24 nm filter ; -dox, +dox = without and with addition of doxycycline (2 µg/ mL) 1 h before incubation with probes. Scale bar is 20 µm. Blue colour shows the signals from nucleus.

Complexation of FIT-DNA1 with octaarginine

FIT-DNA1 (12 pmol) and different amounts of octaarginine were mixed in PBS and filled into the gel pockets (10 µL) of a 1% agarose gel. The electrophoresis was performed for 20 min at 100 V in 1 x TAE buffer using a *PowerPac HC* from *Bio-Rad*. Afterwards the FIT probe was stained for 5 min with *SYBR*Gold™ and fluorescence imaged by using a *Kodak Image Station 4000 MM Pro* (λ_{ex}= 495 nm and λ_{em}= 535 nm). The lack of DNA staining by *SYBR*Gold™ was taken as an indicator for quantitative DNA complexation, which appeared complete when at a positive/negative charge ratio P/N=16.

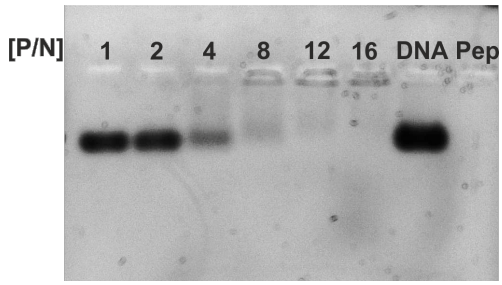
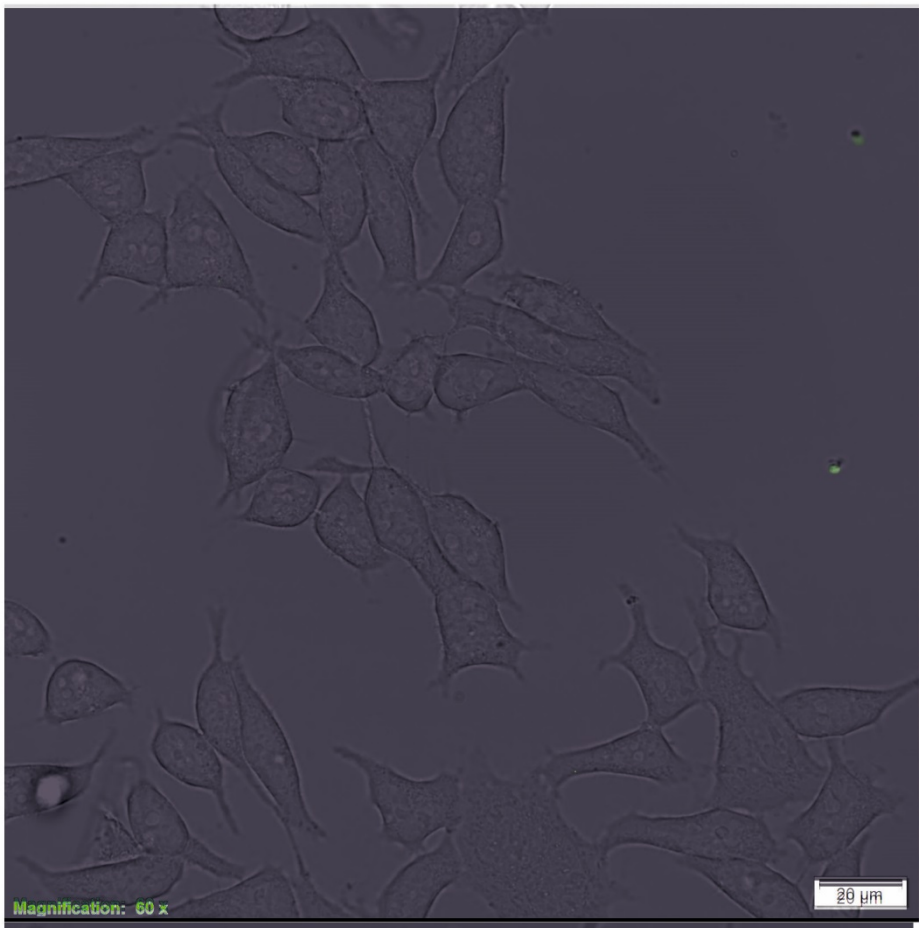


Figure S13. 1%-agarose gel analysis of SYBRGold™-stained mixtures of FIT-DNA1 and octaarginine at various positive/negative charge ratios P/N.

FIT-DNA1

-DOX



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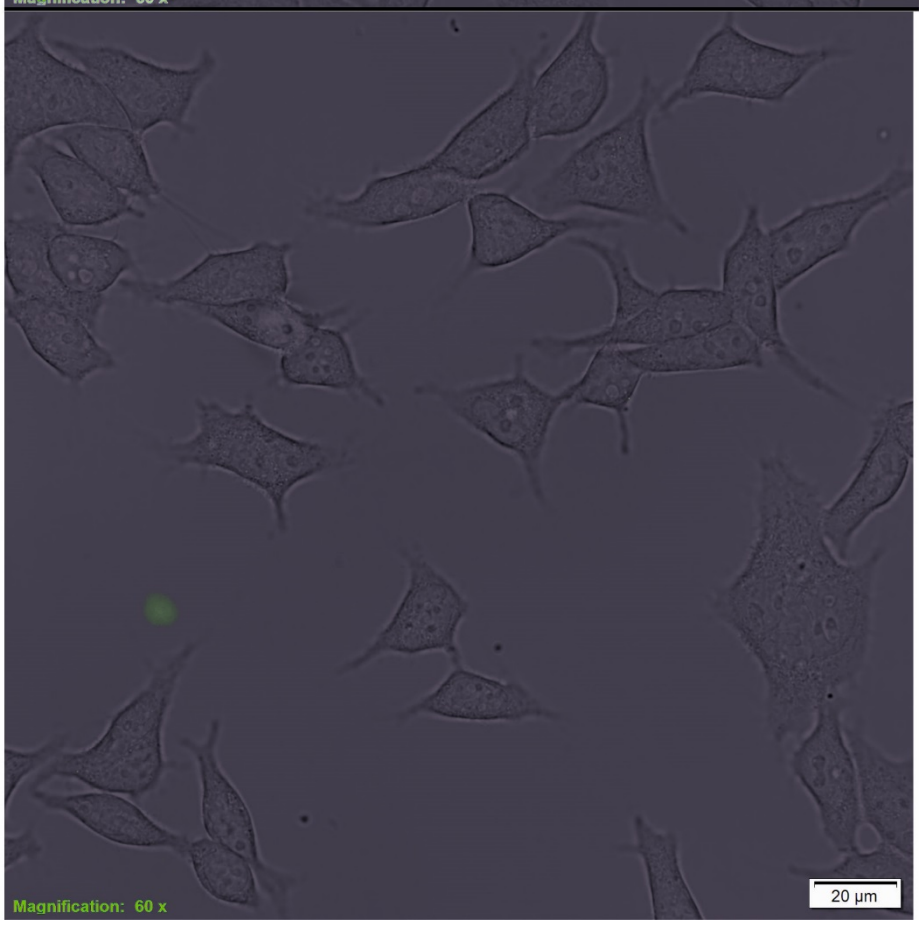

Pen_FIT-DNA1

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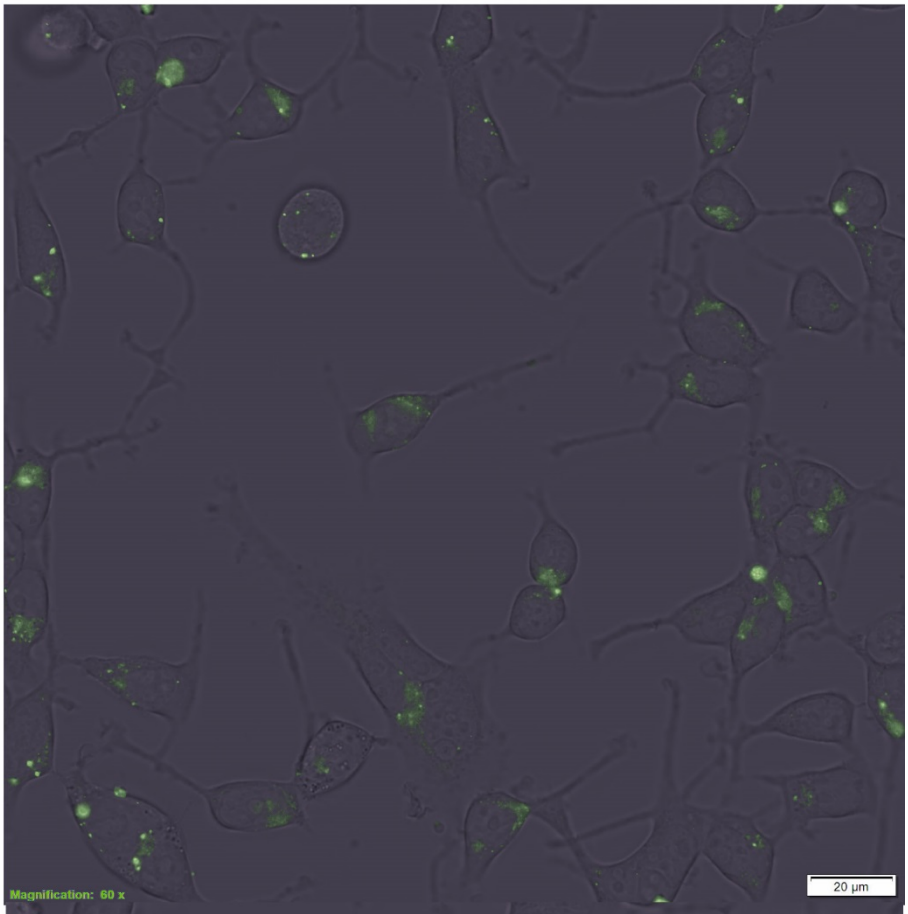

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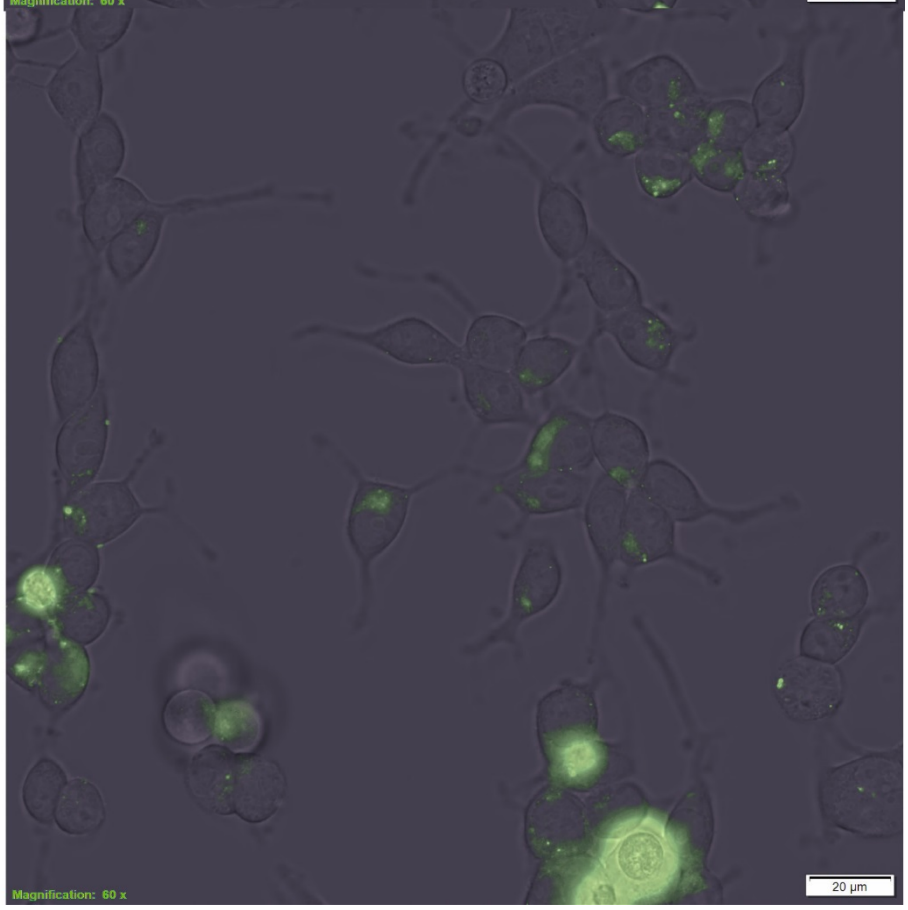

SAP(E)_FIT-DNA1

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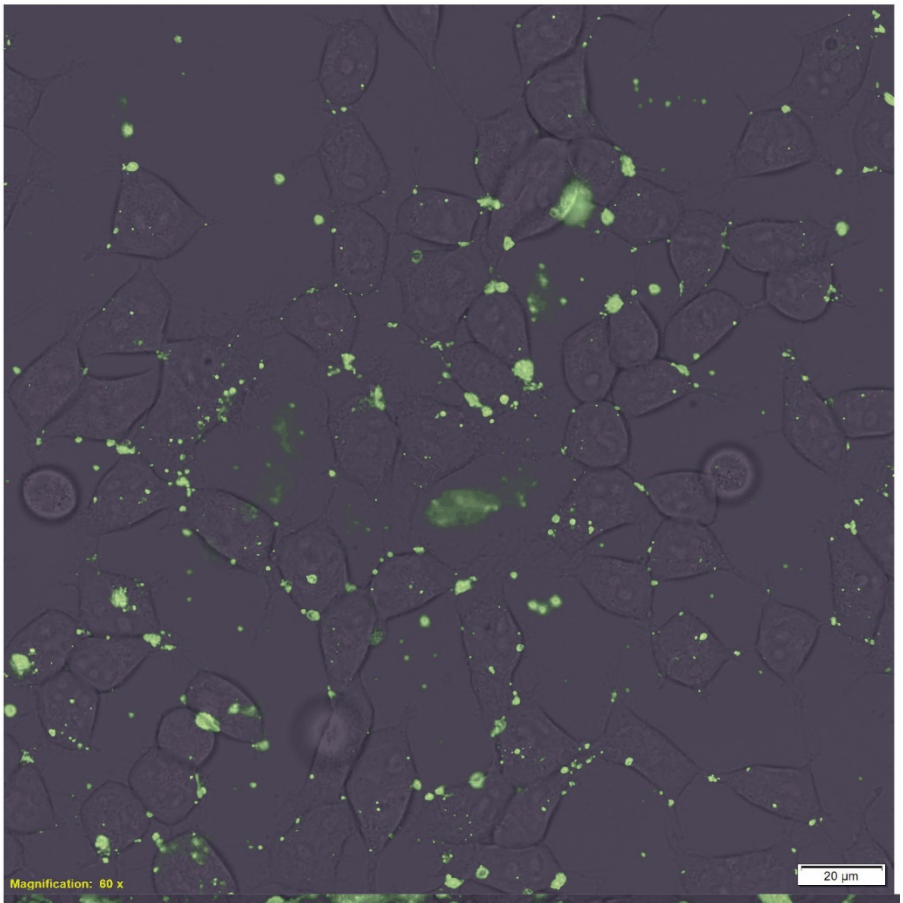

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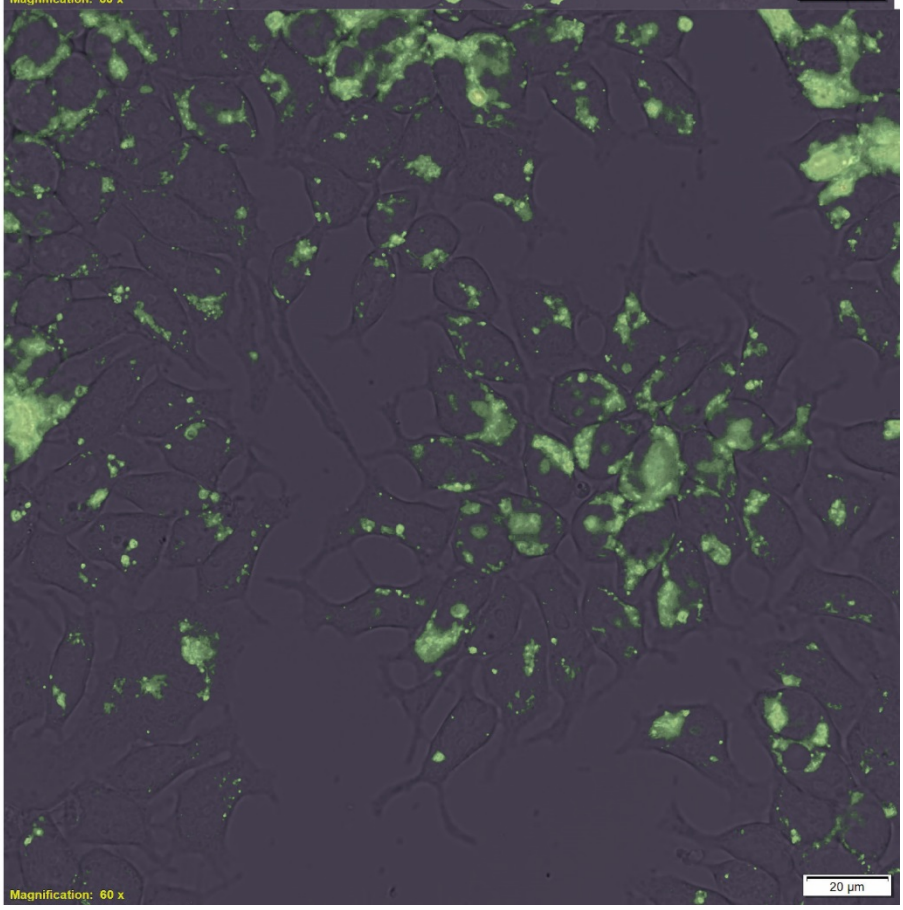

R8+FIT-DNA1

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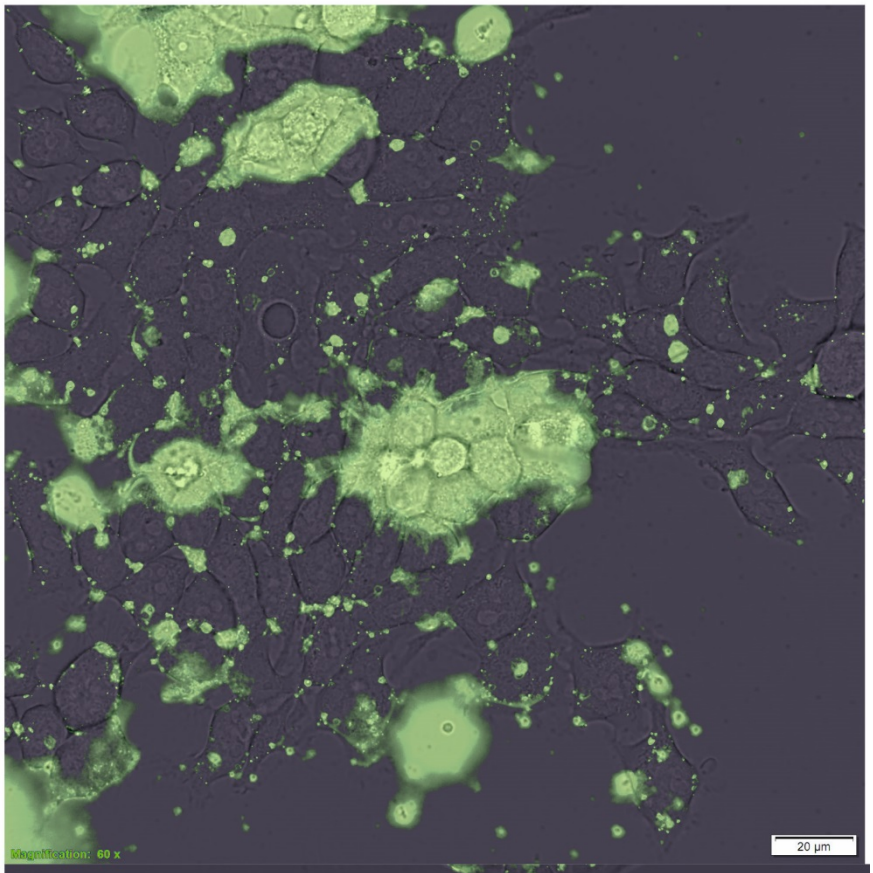

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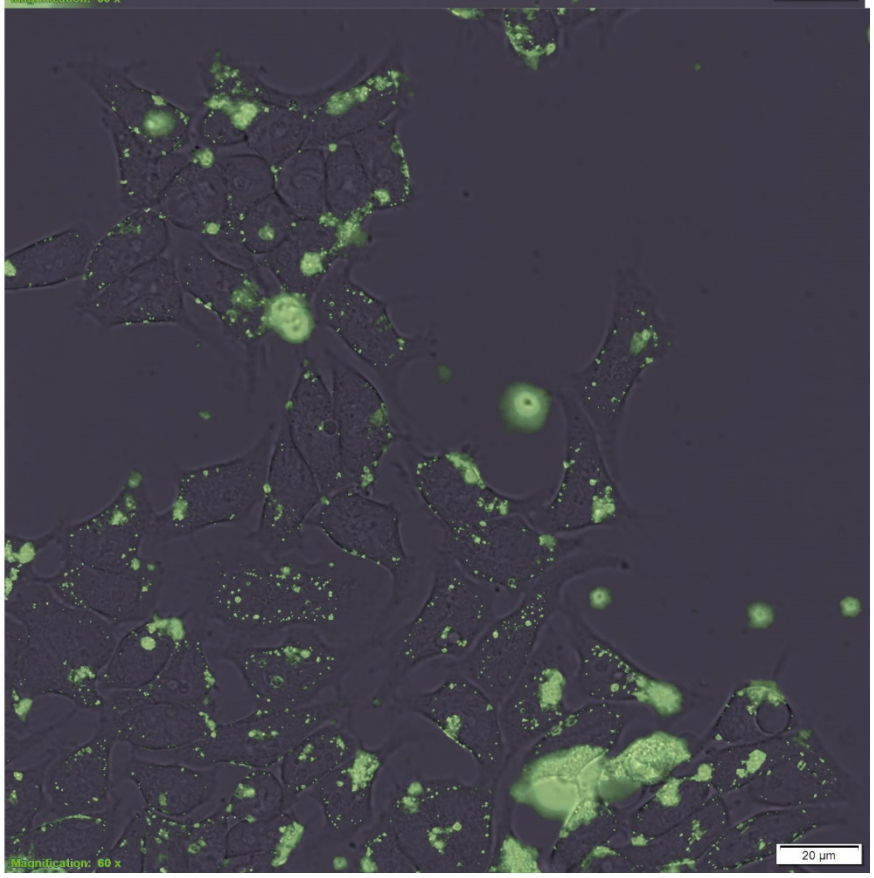

Palm_FIT-DNA1

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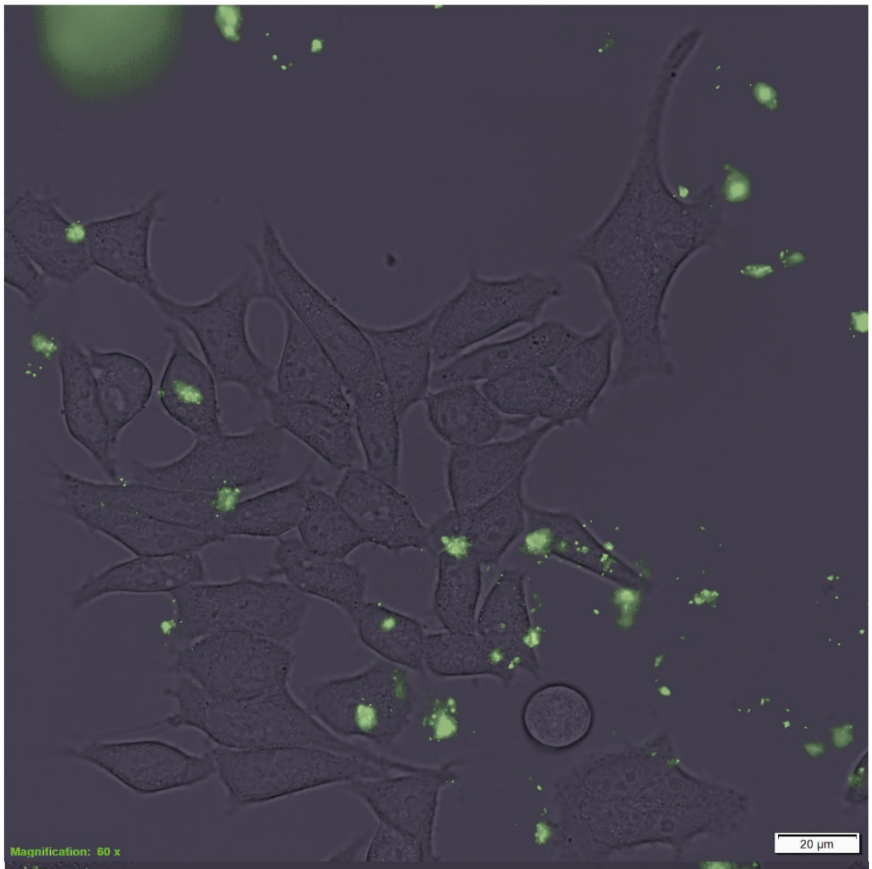

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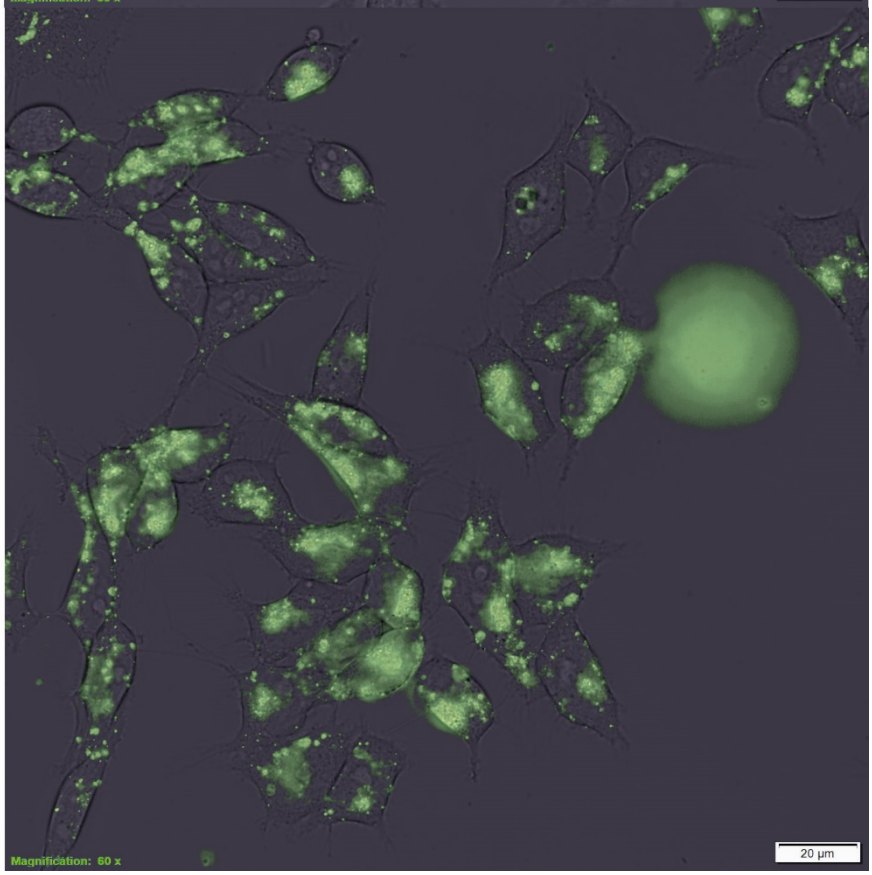

Palm_FIT-DNA1 + CQ

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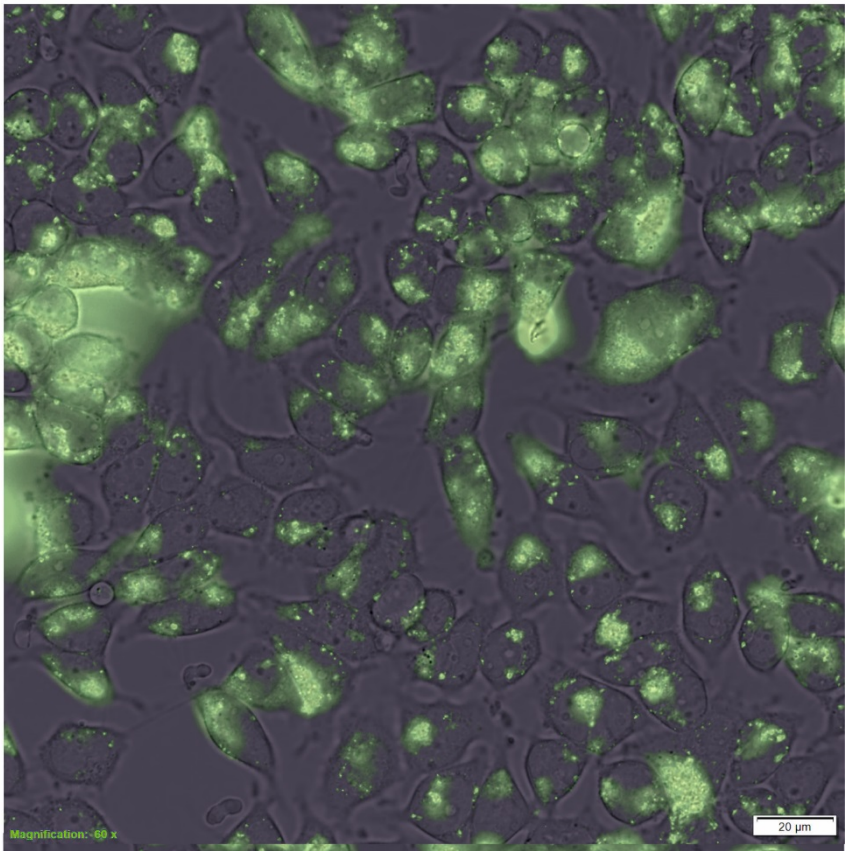

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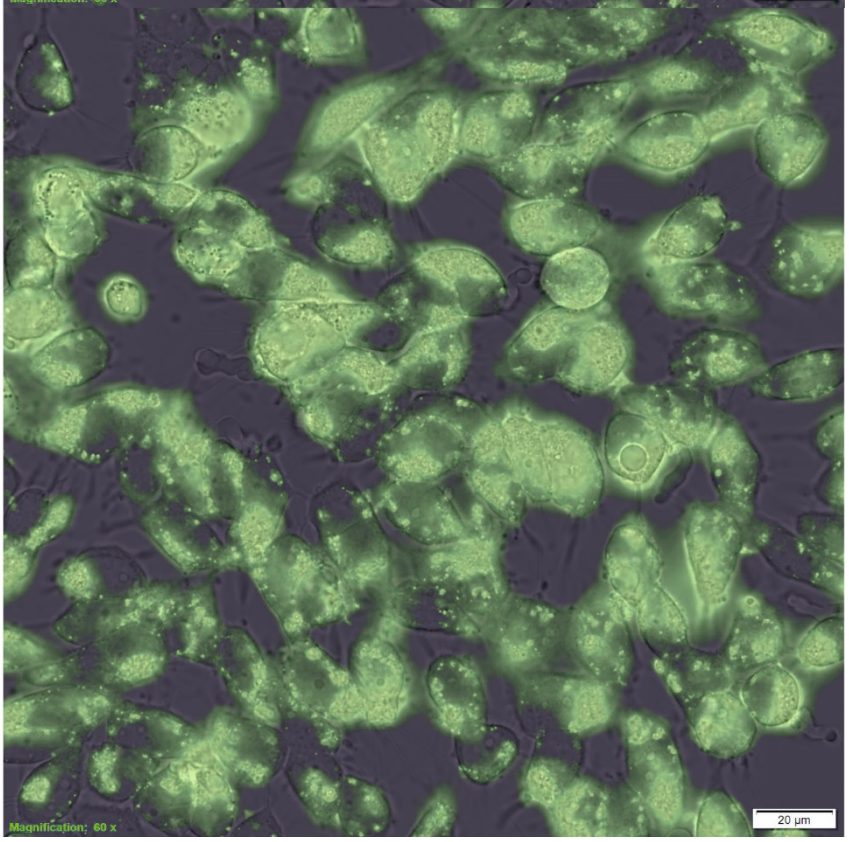

FIT-DNA1

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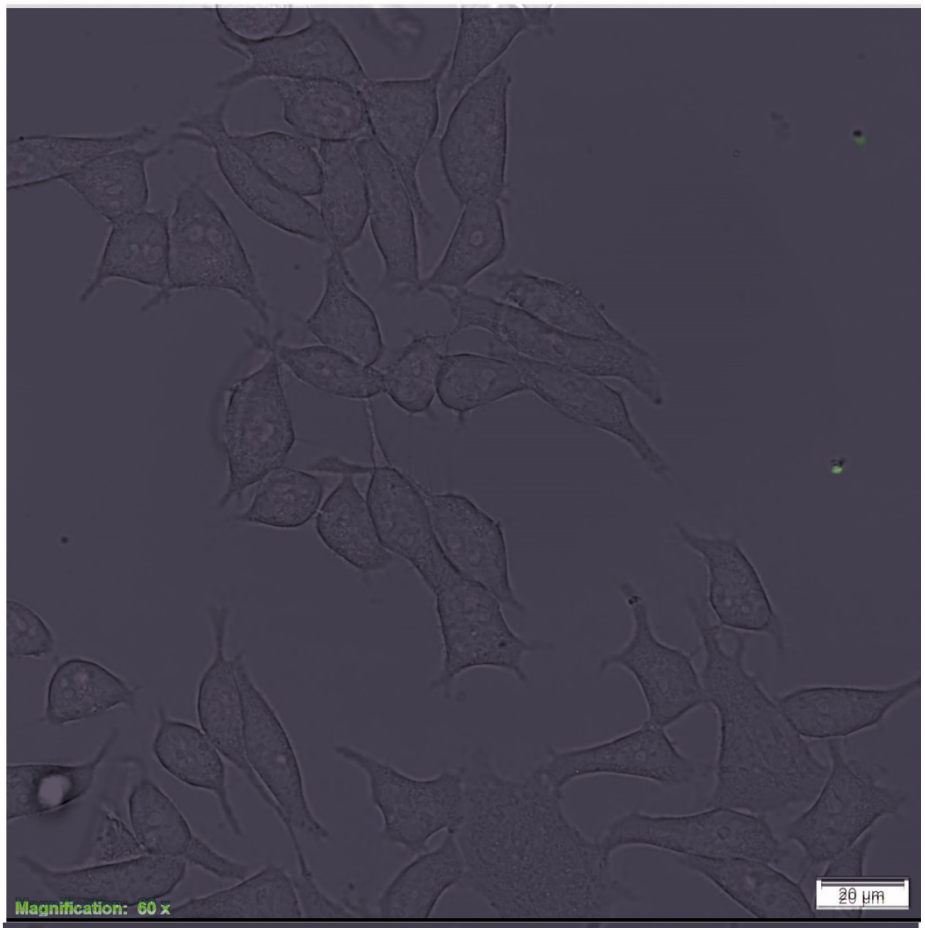

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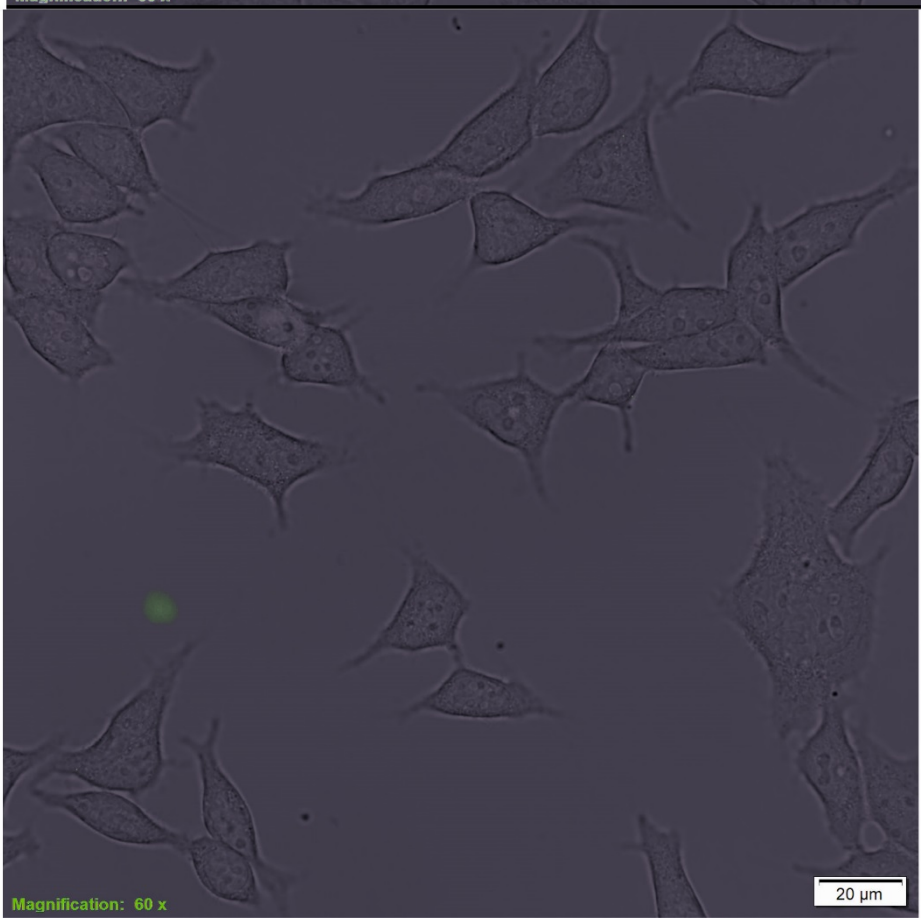

Pen_FIT-PNA1

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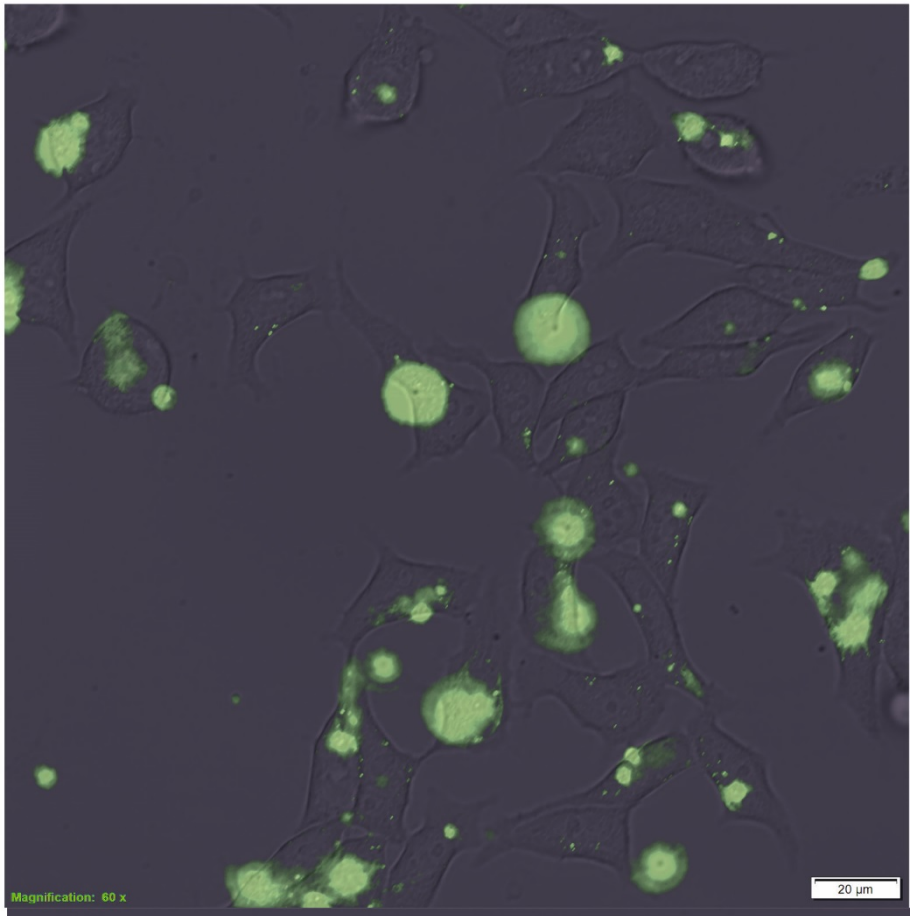

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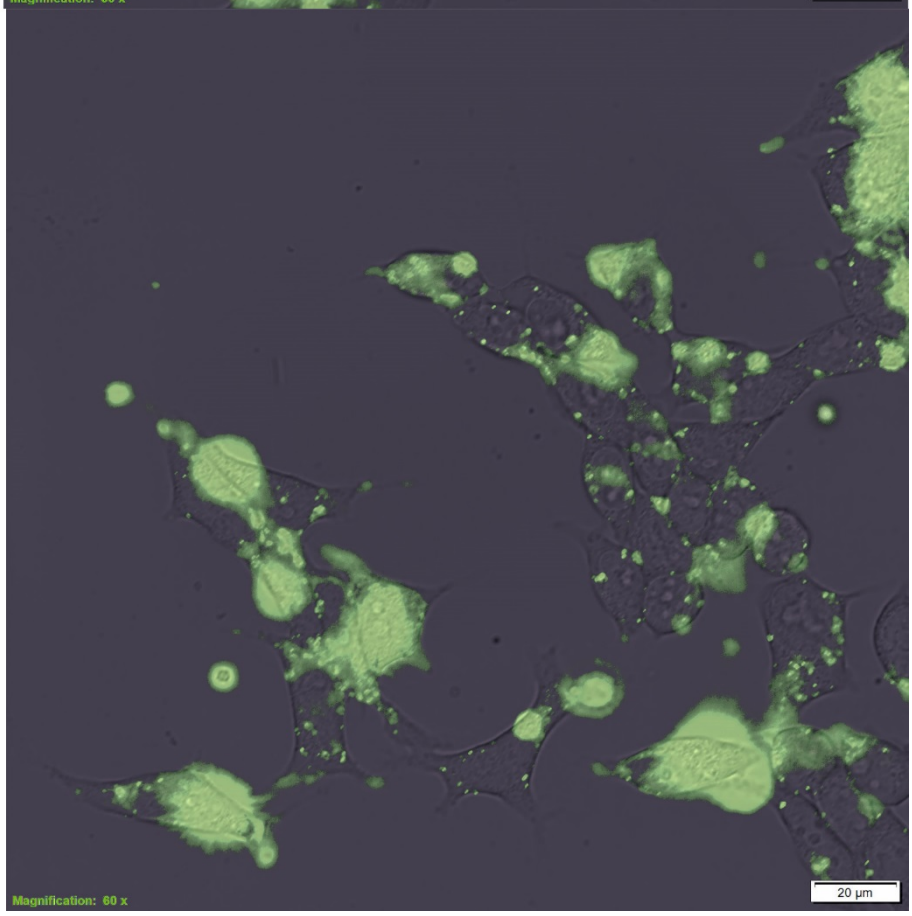

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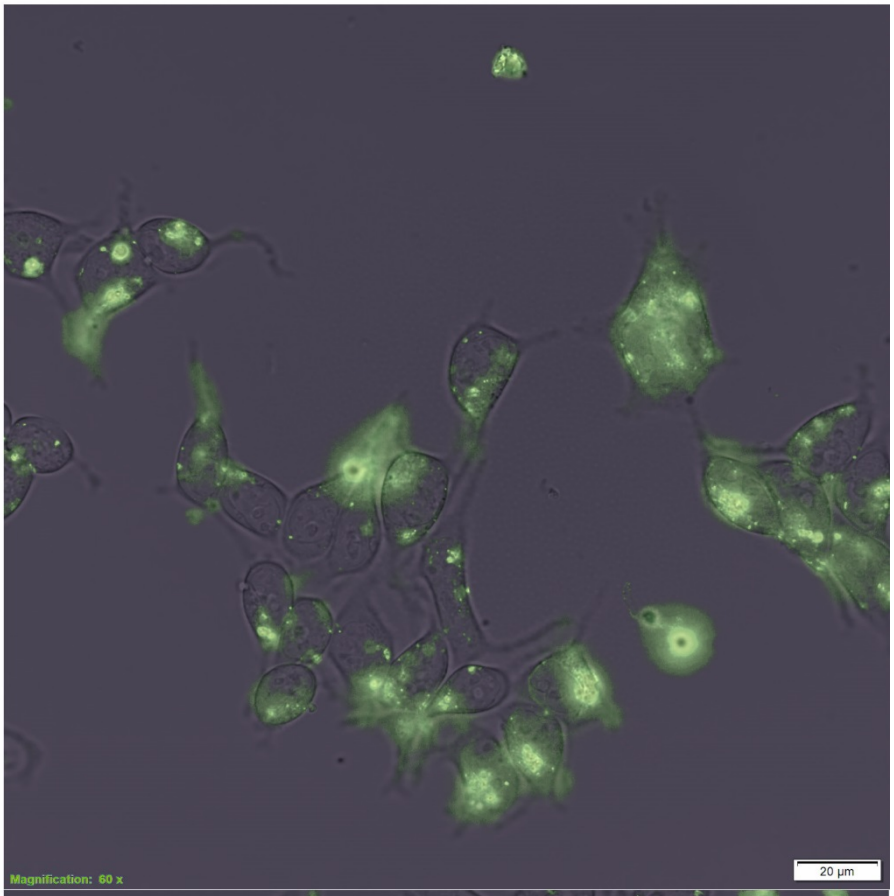

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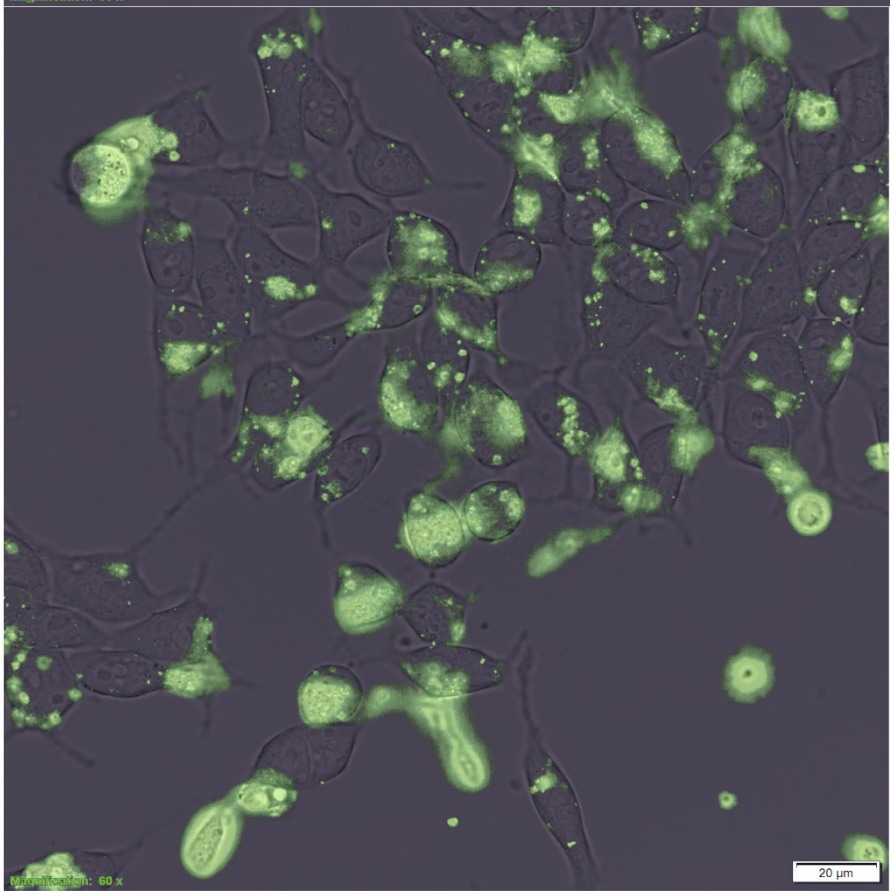

Pep2_FIT-PNA1

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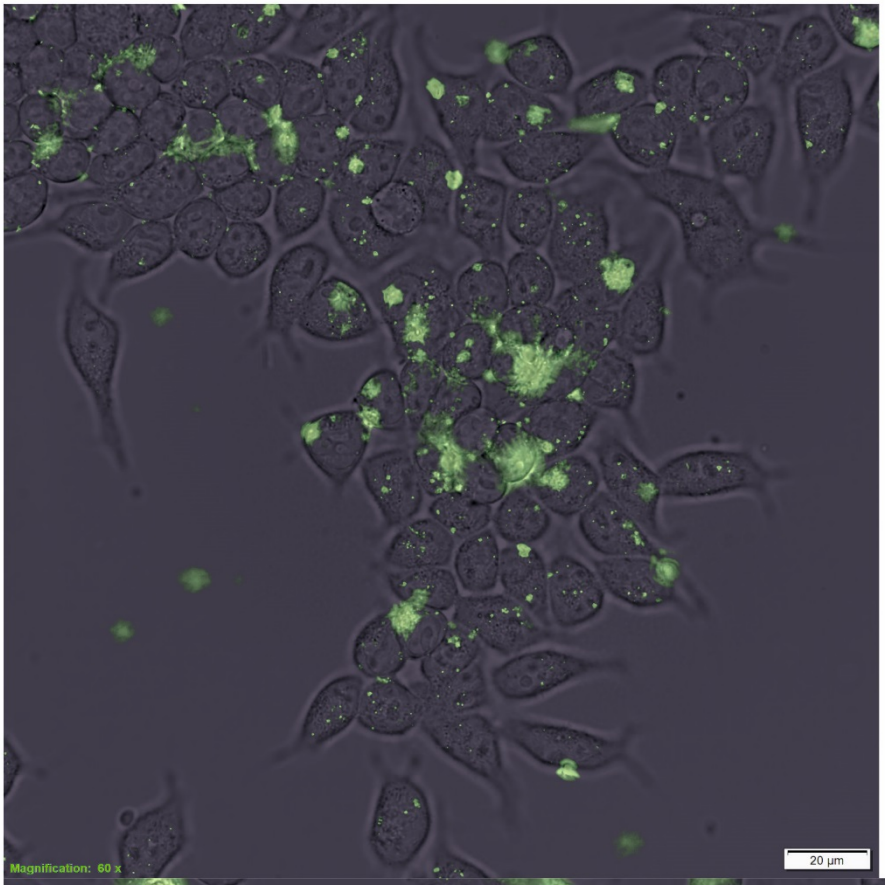

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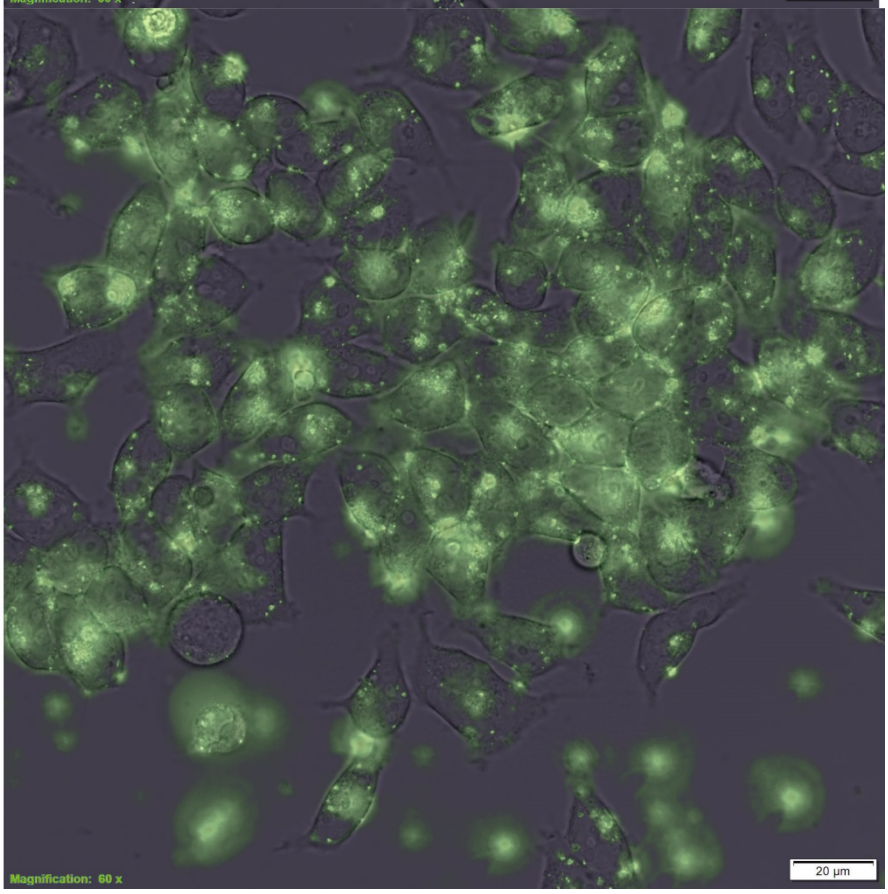

Trans_FIT-PNA1

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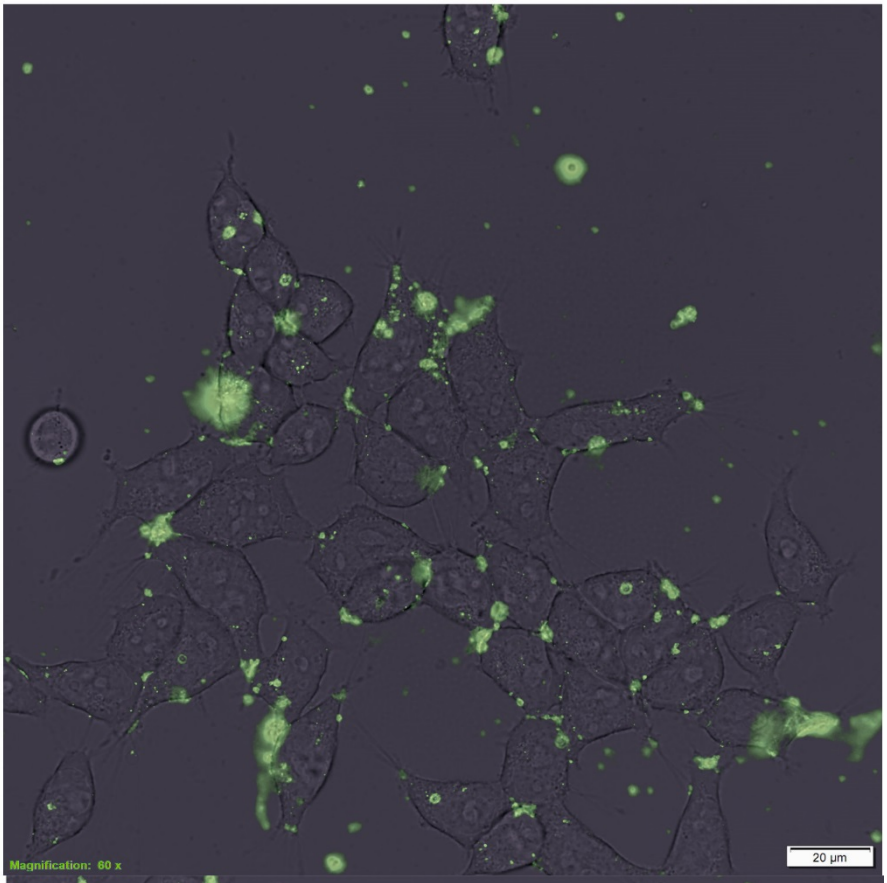

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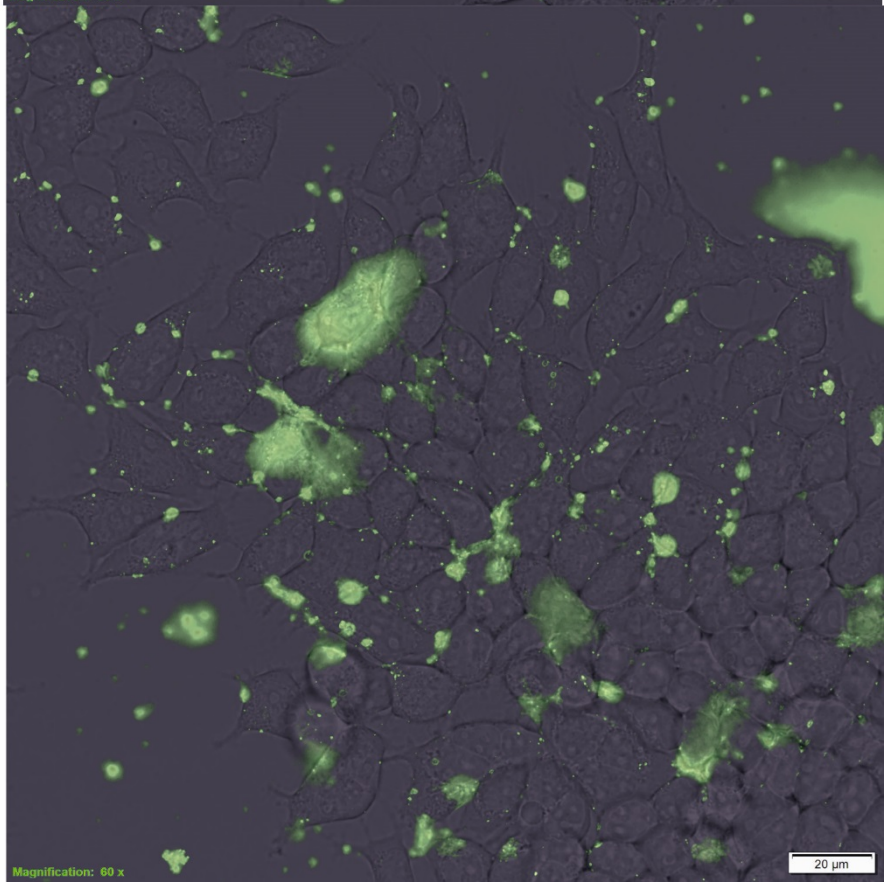

MPG_FIT-PNA1

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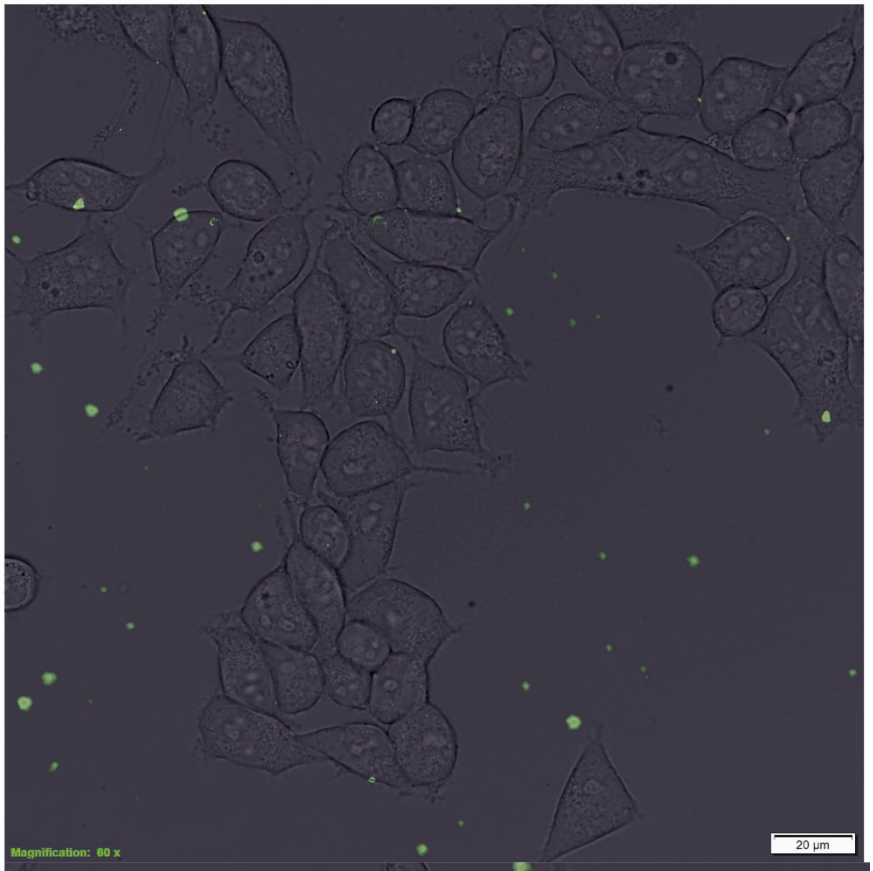

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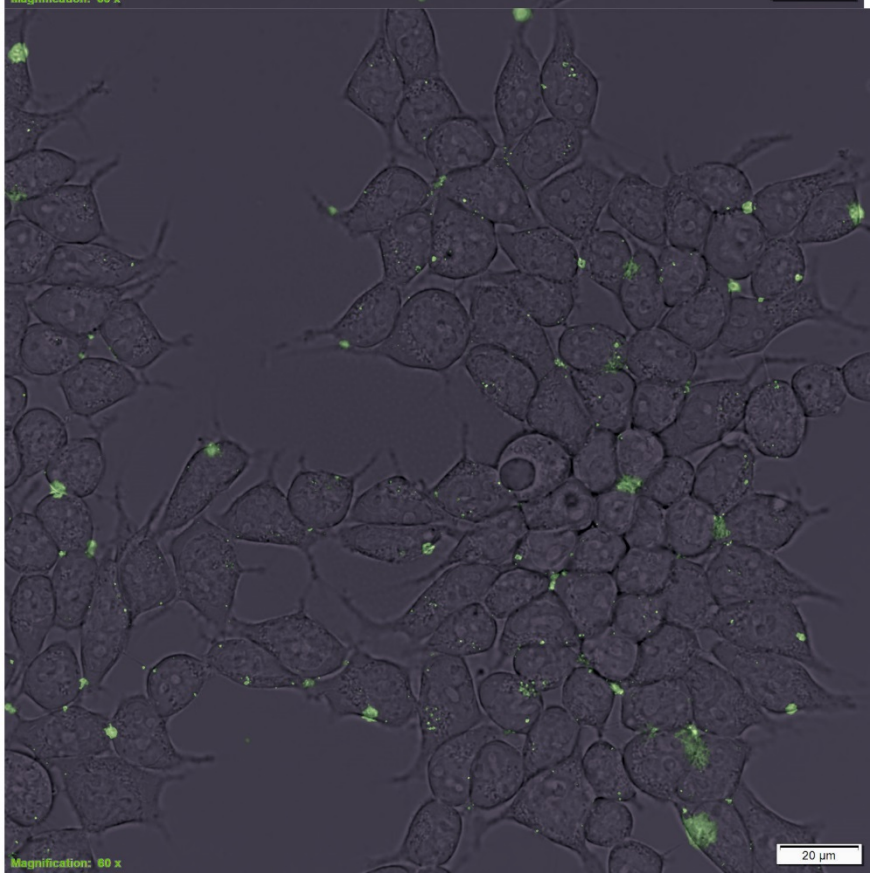


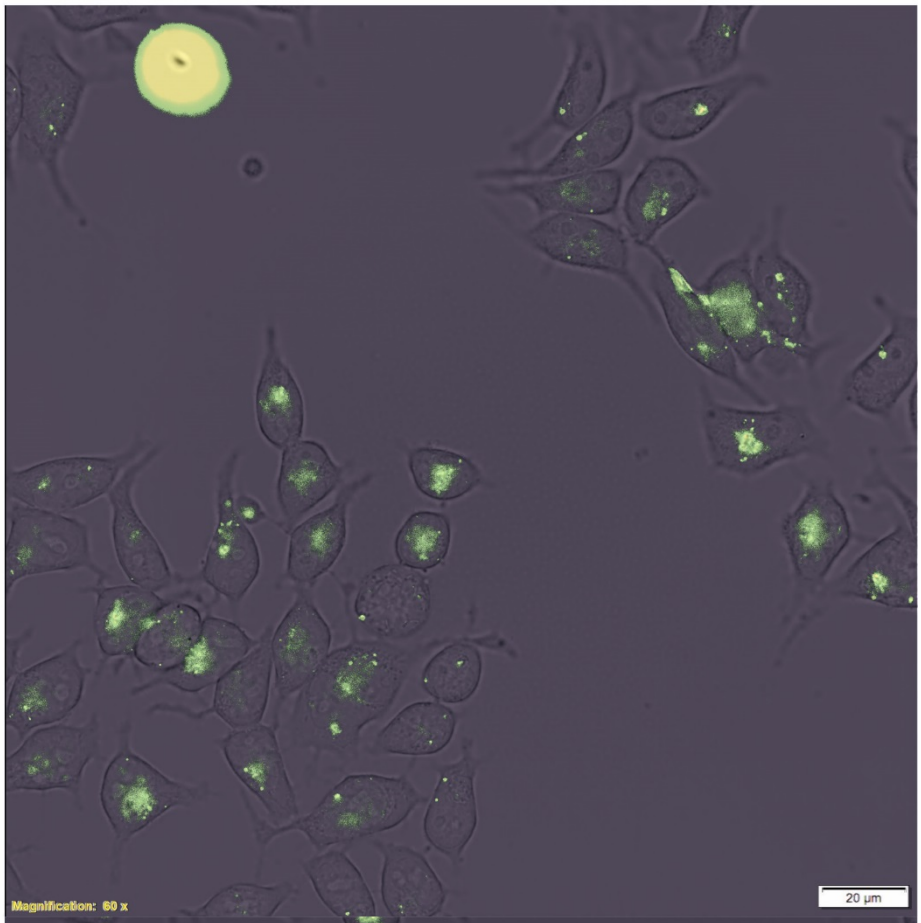
Figure S14. Magnified merged bright field and fluorescence microscopy images of permanently transfected Flp-In™ 293 T-REx™ cells without (-dox) and with (+dox) induction of F-tag-mCherry mRNA expression after incubation with DNA (2 µM) or PNA FIT probes (1 µM) for 30 min in PBS at 37 °C and 5% CO_2. -dox, +dox = without and with addition of doxycycline (2 µg/ mL) 1 h before incubation with probes. Filter sets: TO ex = 500/24 nm, TO em = 545/40 nm.

Transfection over night

The cells were incubated over night with probe (1 µM FIT-DNA1 or 250 nM FIT-PNA2) in antibiotic free DMEM at 37°C and 5 % CO_2. 2 µg/ mL doxycycline was added and after 1 h the cells were washed twice with DPBS + 1 mM $MgCl_2$, and imaged in DPBS or for longer time in phenol red free DMEM.

Pen_FIT-DNA1

-DOX



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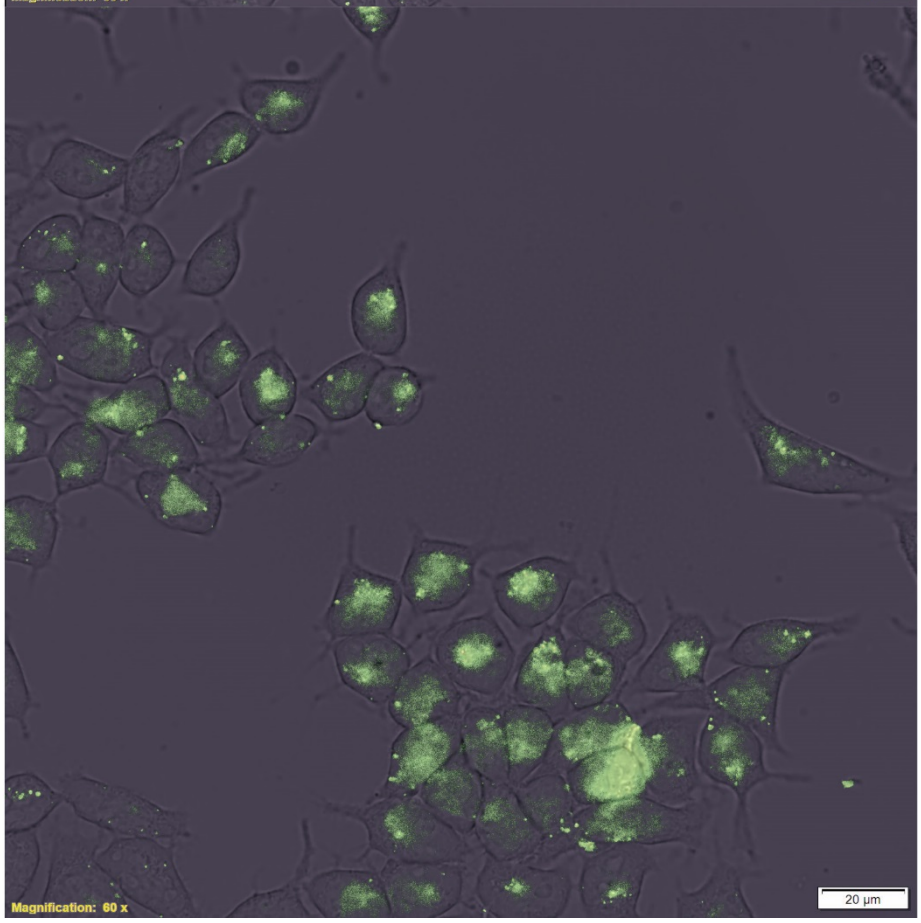

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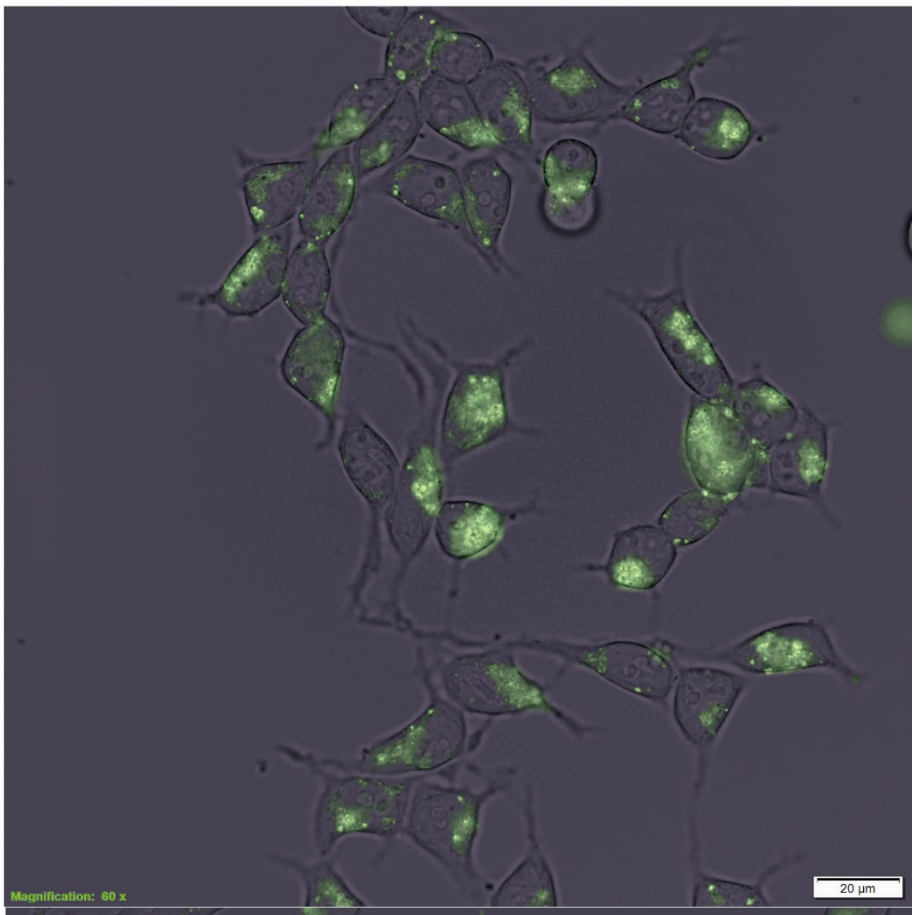

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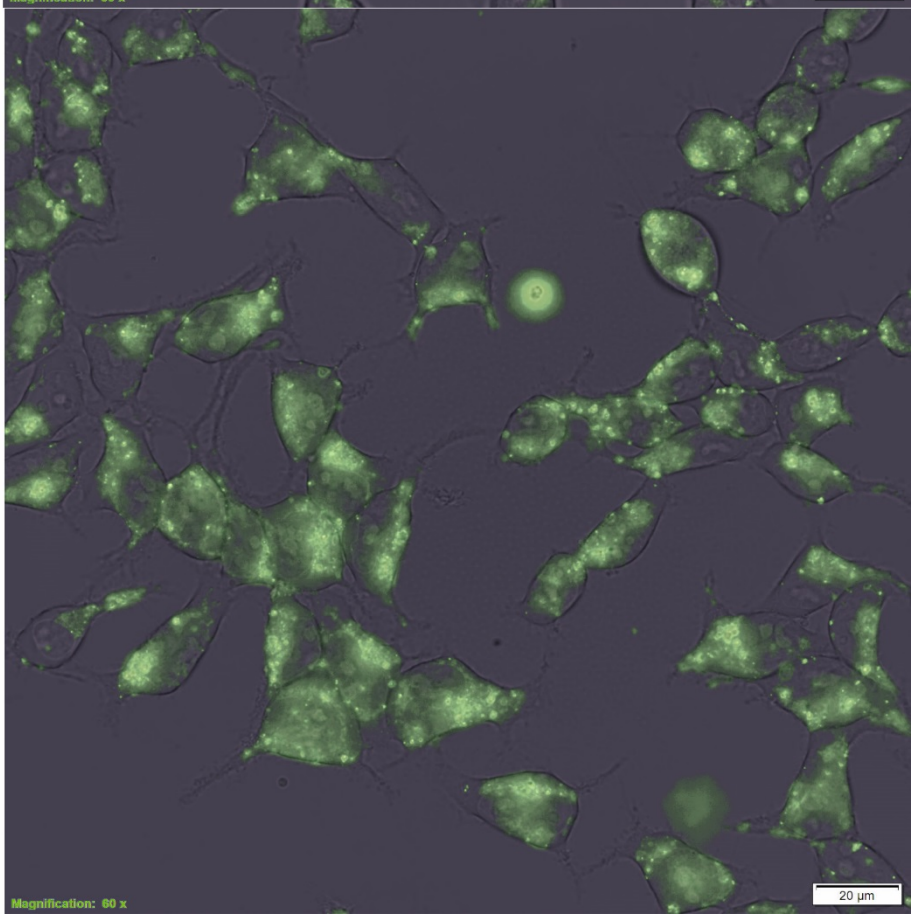

R8 + FIT-DNA1

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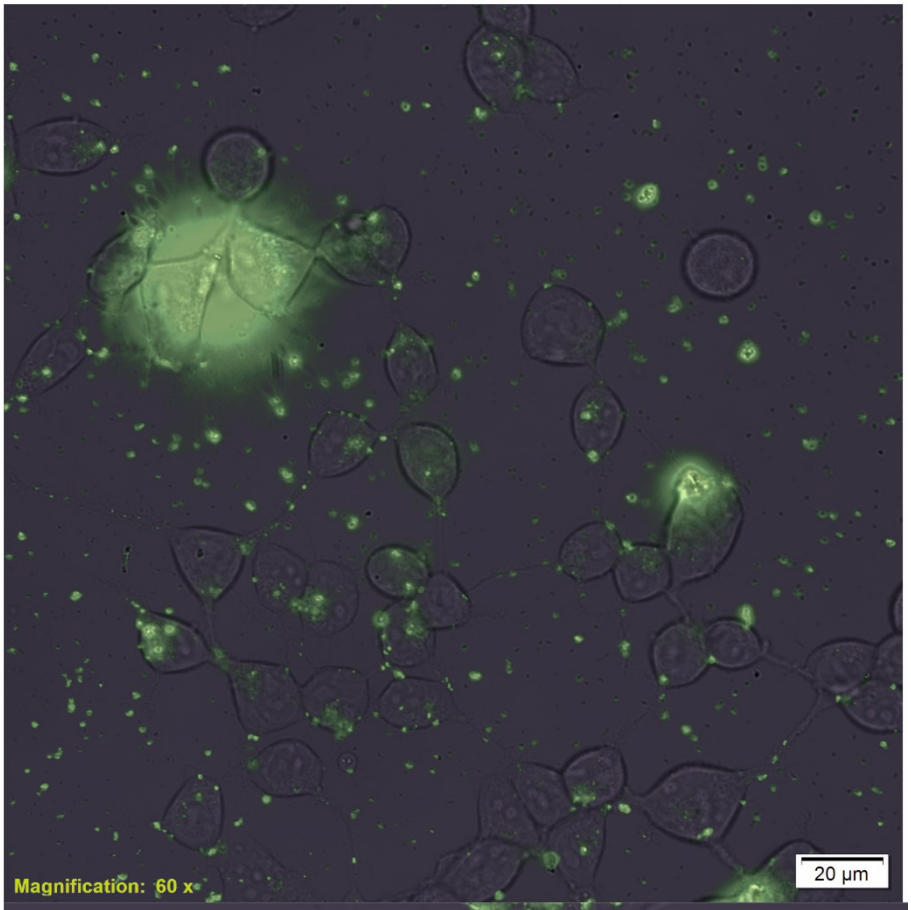

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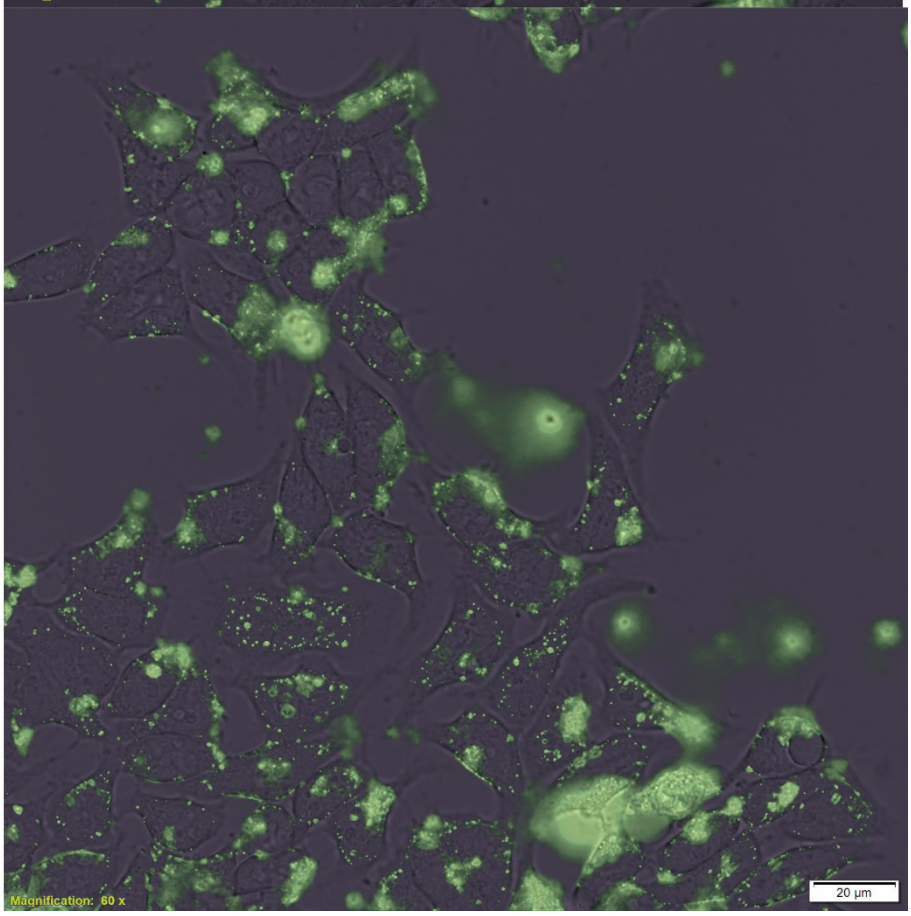
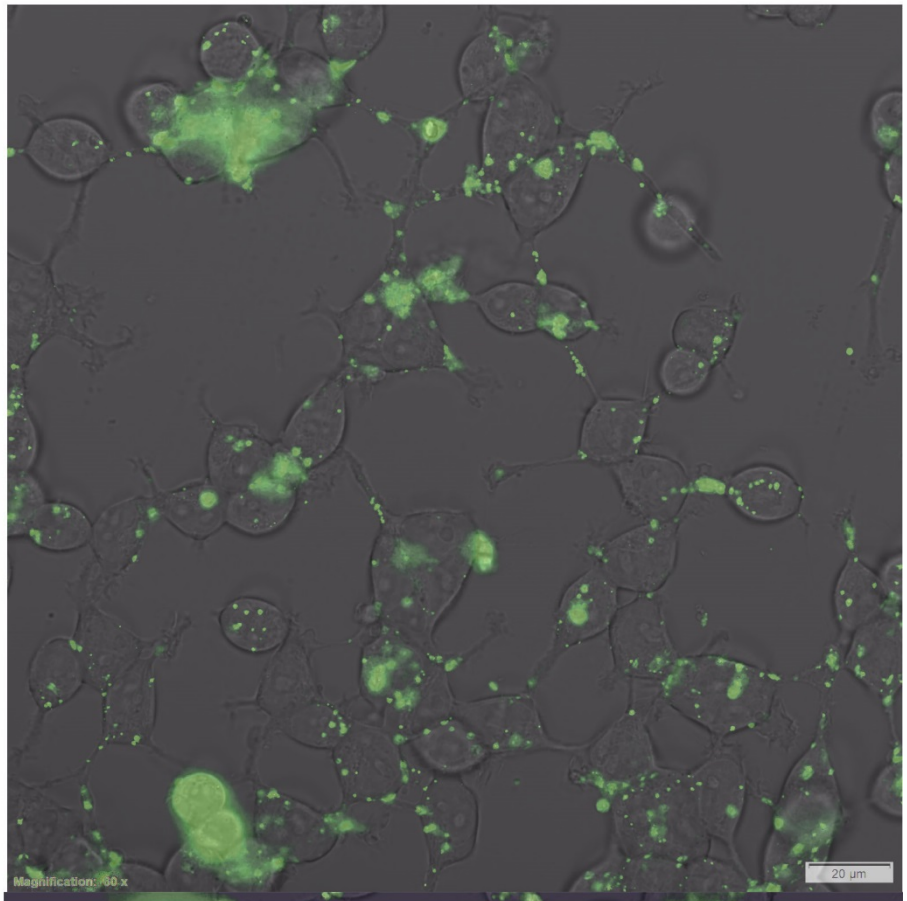


Pen_FIT-PNA1

-DOX



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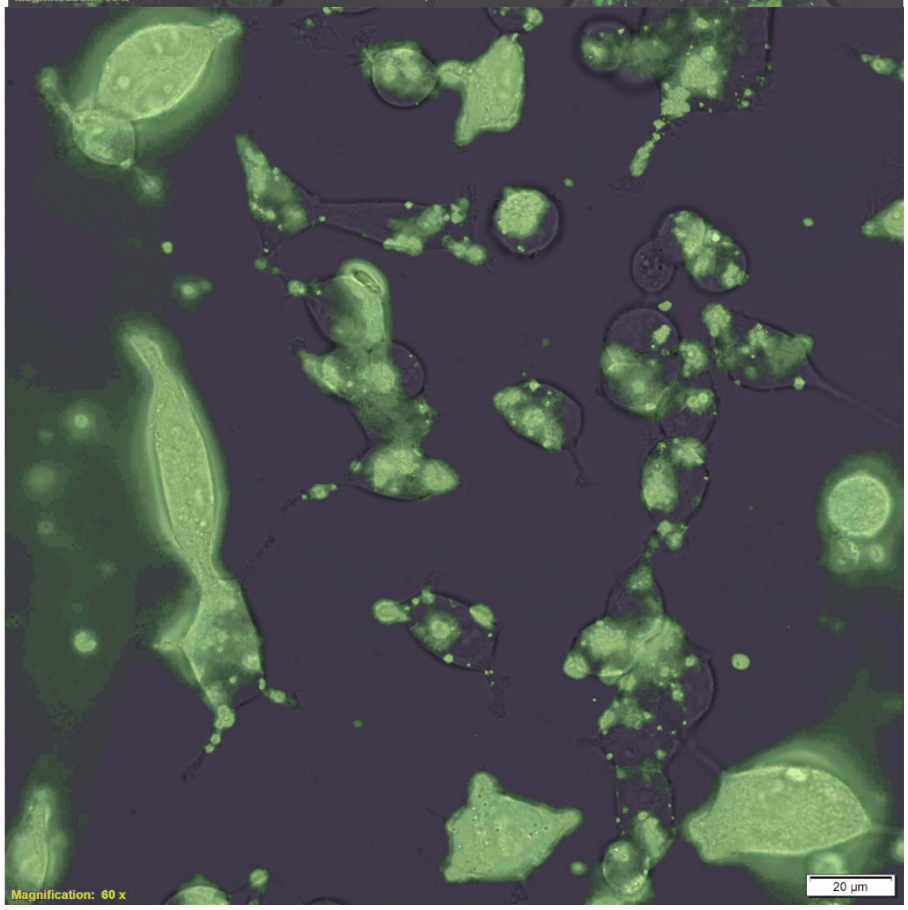
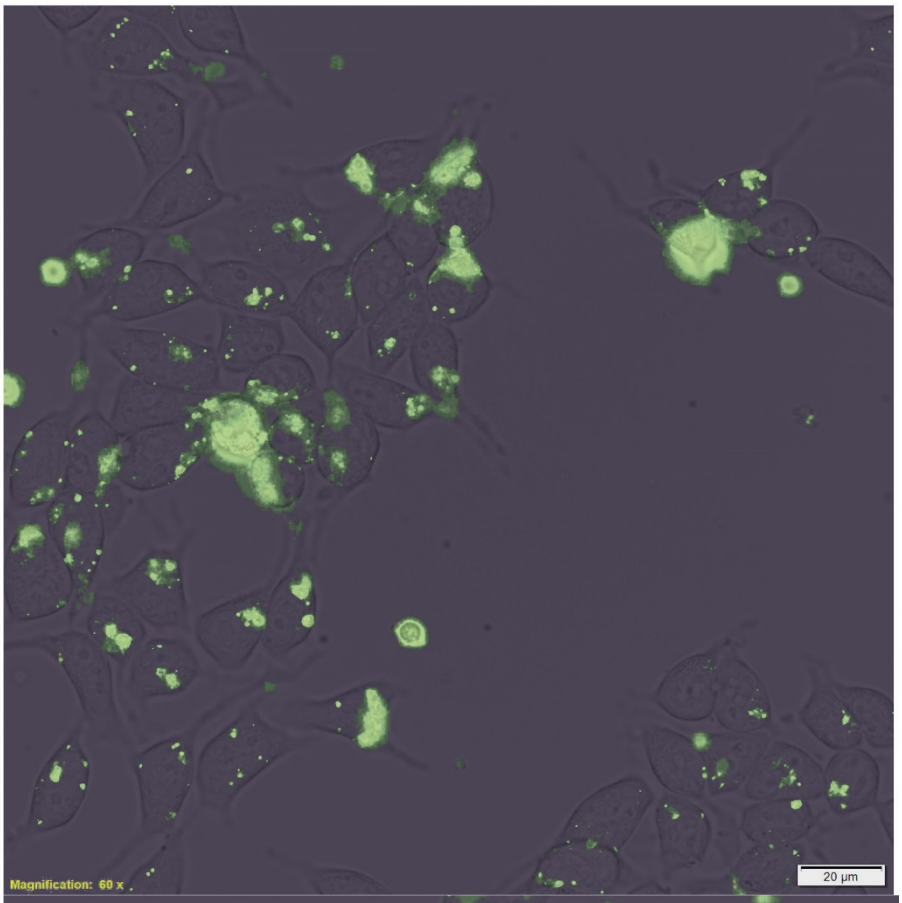


SAP(E)_FIT-PNA1

-DOX



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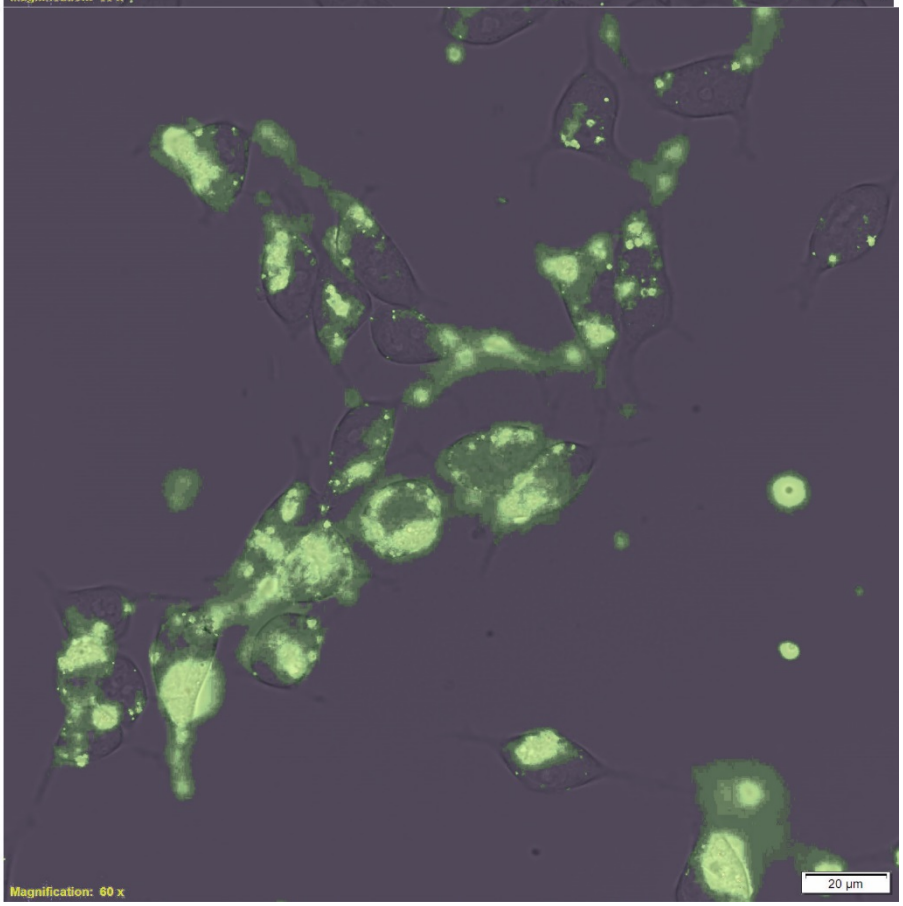
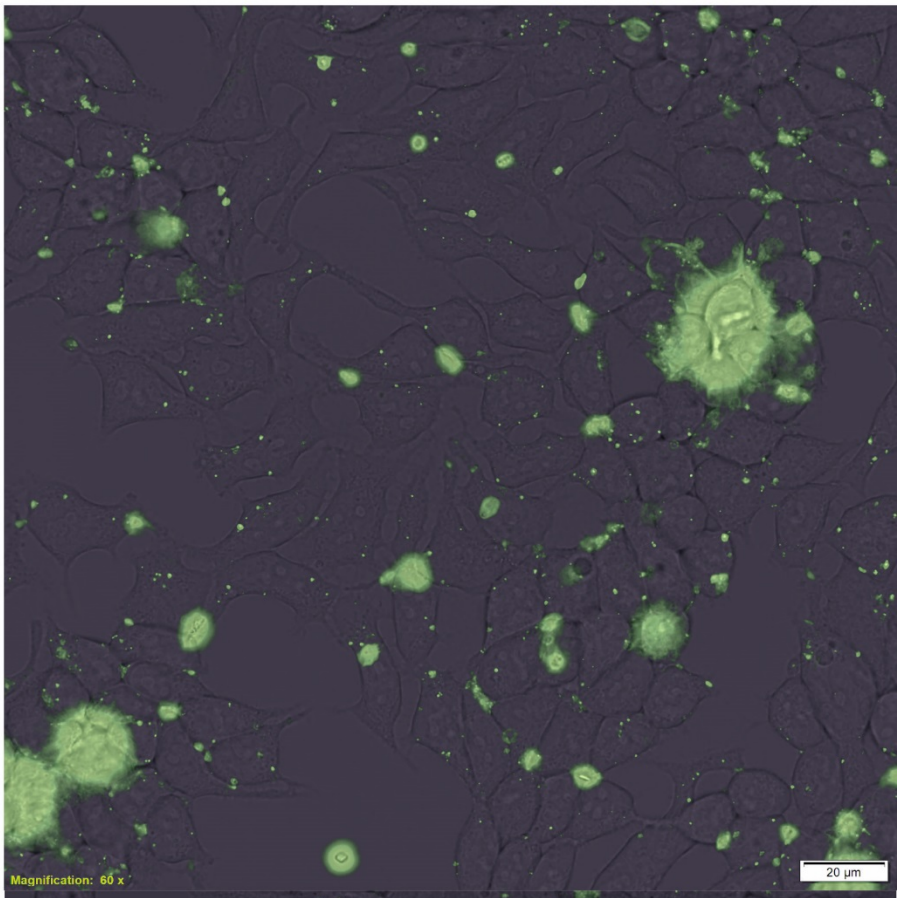


Pep2_FIT-PNA1

-DOX



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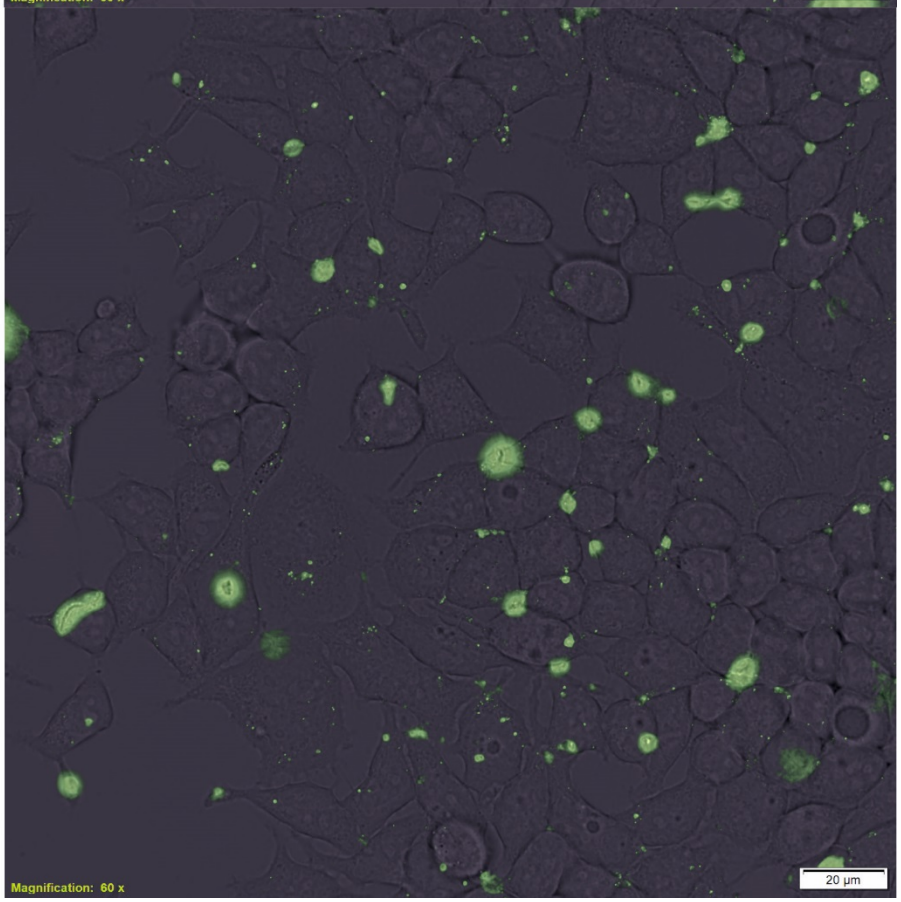
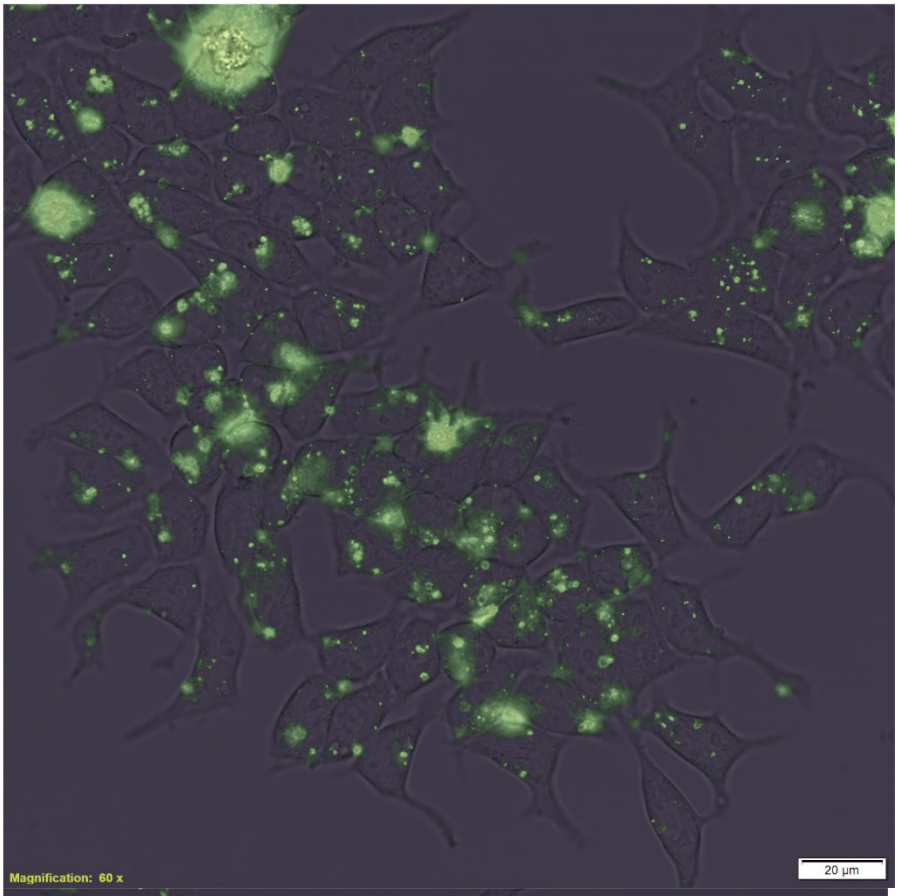
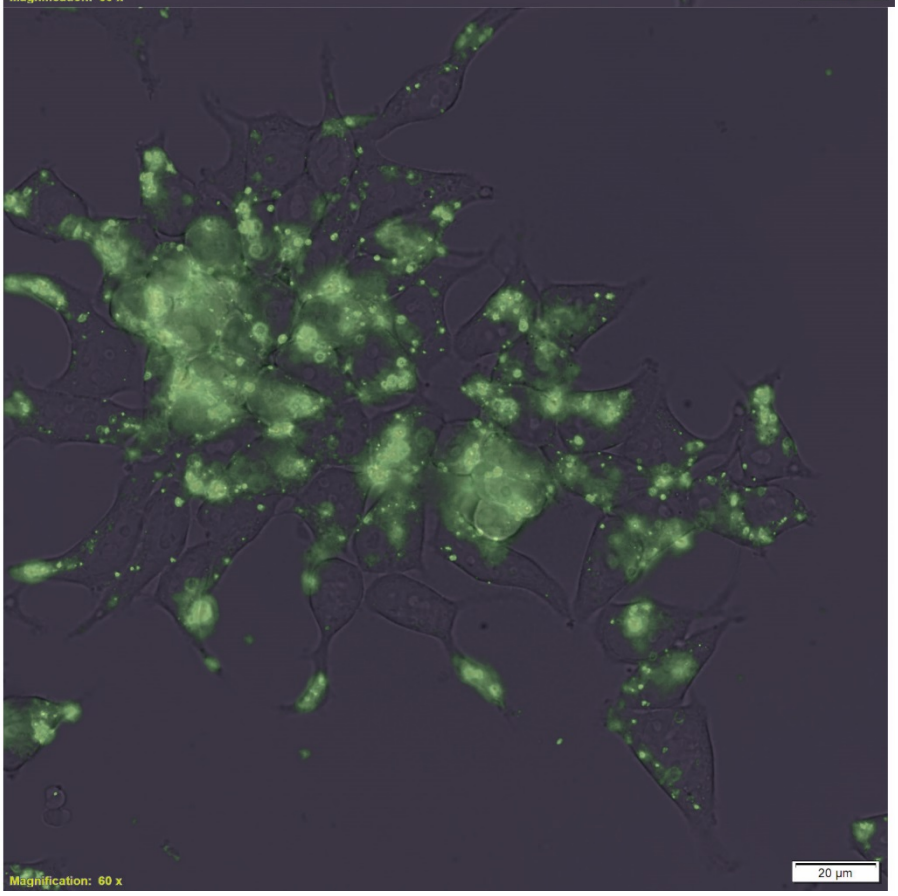


Trans_FIT-PNA1

-DOX

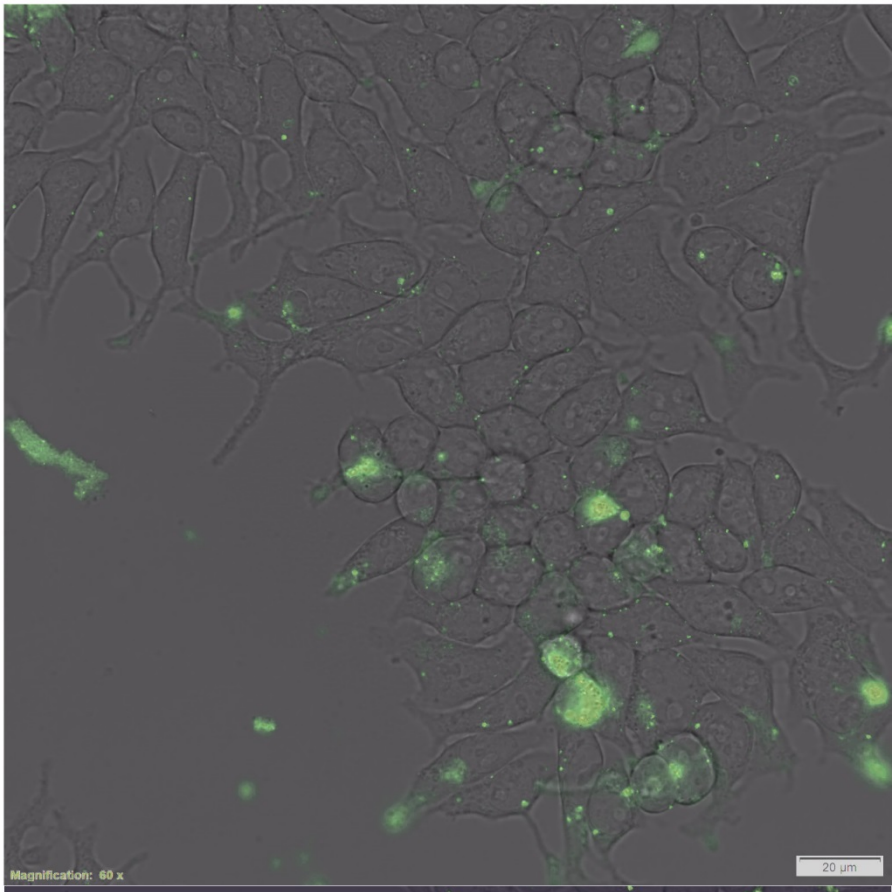


+DOX



MPG_FIT-PNA1

-DOX



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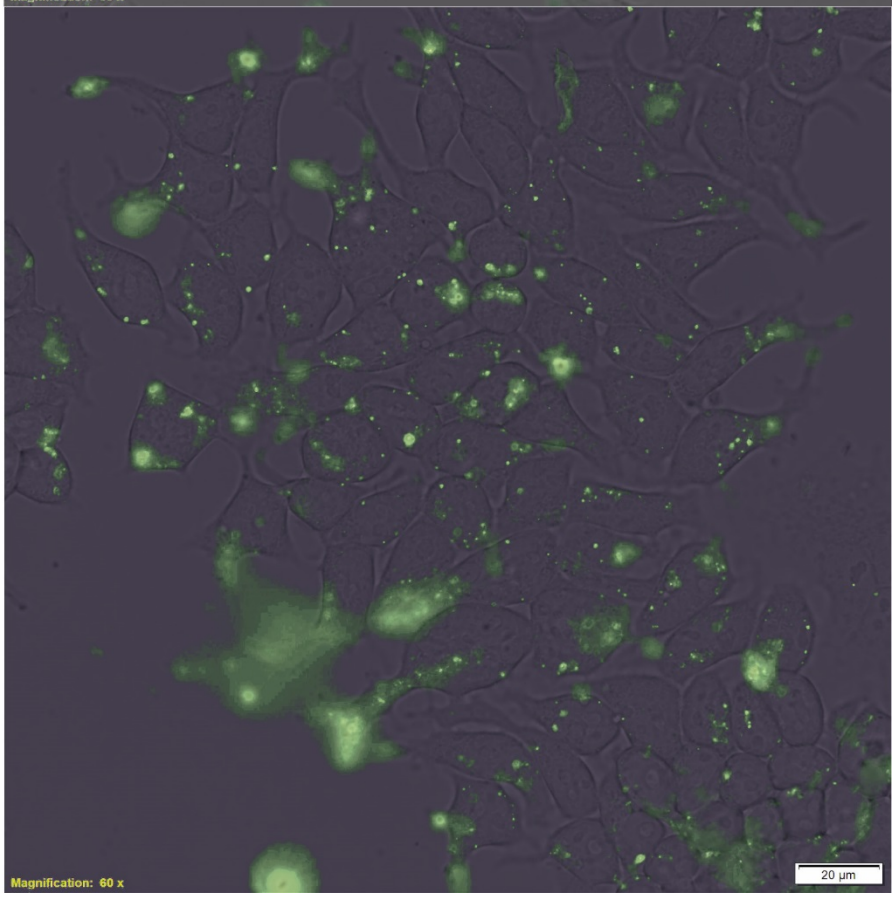

Figure S15. Bright field and fluorescence microscopy images of permanently transfected Flp-In™ 293 T-REx™ cells without (-dox) and with (+dox) induction of F-tag-mCherry mRNA expression over night after incubation with FIT DNA probes (1 µM) or FITPNA probes (250 nM). green = TO emission of FIT probes. Filter sets: TO ex = 500/24 nm, TO em = 545/40 nm. Scale bar is 20 µm.

Image analysis of the fluorescence images using ROI (Region Of Interest)

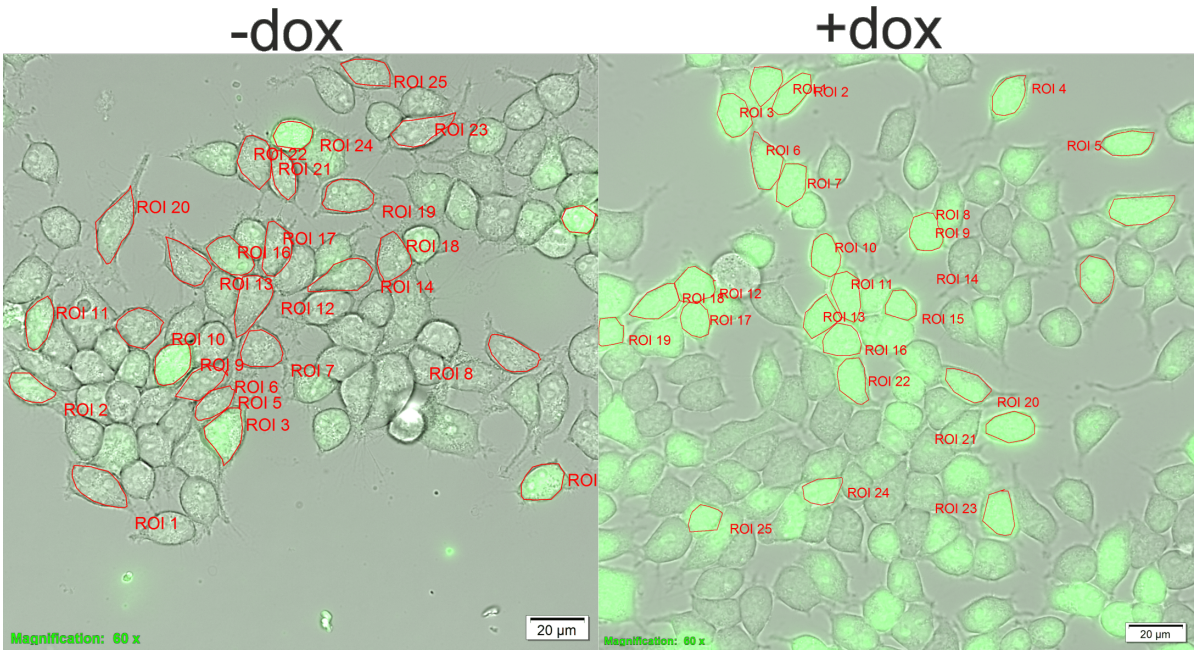

Figure S16. Representative analysis of the fluorescence microscopy images of permanently transfected HEK293 cells without (- dox) and with (+dox) induction of F-tagged mCherry mRNA obtained after SLO-mediated delivery of 2 µM FIT-DNA1. Cells showing high fluorescence intensity and healthy shape were surrounded by using the ROI tool of the Olympus IX83 software. Afterwards the mean intensity value of 50 cells was calculated.

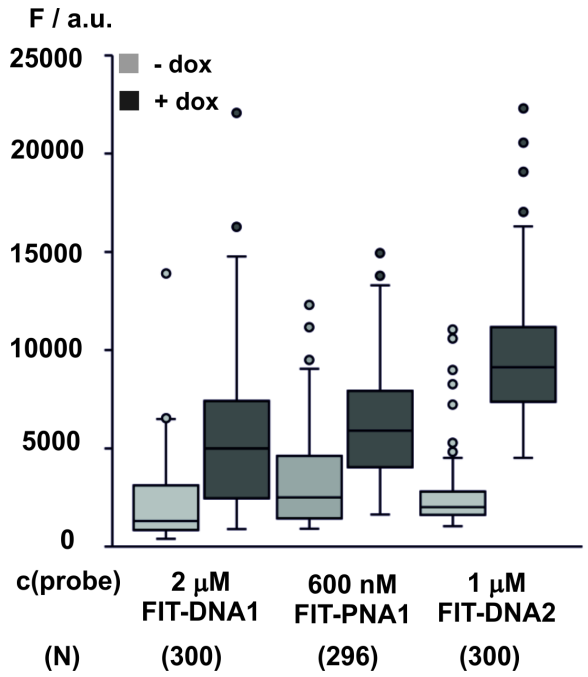

Figure S17. Mean fluorescence of Flp-In™ 293 T-REx™ cells after SLO induced delivery of FIT-DNA1, FIT-PNA1 and FIT-DNA2 without (– dox) and with (+dox) induction of F-tagged mCherry mRNA. The Box-Whisker plots (1.5 IQR) show the signal intensity of experiments with three different cell cultures. Fluorescence intensity was measured by image analysis (see Fig. S16). The number N of cells analysed is given in brackets. Conditions: 2 µg/ mL doxycycline (if added); after 1 h cells are permeabilized with 150 U/mL SLO for 10 min in PBS + 1mM $MgCl_2$ at 37°C and 5% CO_2 in presence of FIT probes. Filter sets: TO ex = 500/24 nm, TO em = 545/40 nm; QB ex = 575/25, QB em = 628/40 nm.

Cell Viability

Cells treated with probes or SLO were washed twice with DPBS + 2 mM $MgCl_2$ and then incubated with propidiumiodid in PBS (1µg/mL) purchased from *Thermo Fisher Scientific* (Rockford, USA) for 5 min followed by Hoechst 33342 staining (5 µg/mL) for 5 min. Afterwards cells with a propidiumiodid stain (dead cells) were counted and divided though all cells counted by the nucelus stain. 100-180 cells were included per measurement.

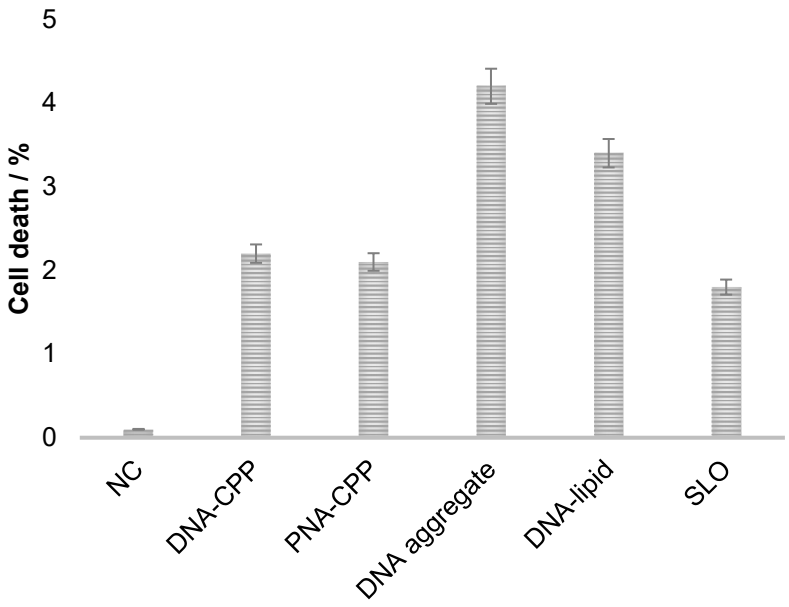

Figure S18. Bar chart of the cell death in % after different transfection treatments.

[1] I. G. Felix Hövelmann, Jasmine Chamiolo, Marc Kasper, Jonas Steffen, Anne Ephrussi, Oliver Seitz, *Chemical Science* **2016**, *7*, 128--135.