Supporting Information

Multiplexed photo-activation of mRNA with single-cell resolution

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The Supporting Information contains: supplemental methods and general materials; chemical synthesis of compound (3), compound (4) and compound (8); NMR and HRMS spectrums; HPLC trace of purified compound (8); in vitro transcription and maturation of mRNA; in vitro TGT labeling and purification of 17-nt RNA oligo and mRNA transcripts; live cell fluorescence imaging and photo-uncaging experiments.

General Materials

Reagents and instruments

Commercially available methanesulfonyl chloride, sodium azide, tetra-*n*-butylammonium fluoride in THF (1M), *N*-succinimidyl carbonate, 4-dimethylaminopyridine, *N*,*N*-diisopropylethylamine, copper(I) bromide and common organic solvents were obtained from Sigma-Aldrich. Deuterated chloroform ($CDCl_3$) was obtained from Cambridge Isotope Laboratories. All reagents obtained from commercial suppliers were used without further purification. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F_{254} plates. Silica gel flash chromatography was performed using E. Merck silica gel (type 60SDS, 230-400 mesh). Solvent mixtures for chromatography are reported as v/v ratios. HPLC analysis was carried out on an Eclipse Plus C8 analytical column with *Phase A*/*Phase B* gradients [*Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. HPLC purification was carried out on Zorbax SB-C18 semipreparative column with *Phase A*/*Phase B* gradients [*Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a VarianVX-500 MHz spectrometer, and were referenced relative to residual proton resonances in $CDCl_3$ (at 7.24 ppm). Chemical shifts were reported in parts per million (ppm, δ) relative to tetramethylsilane (at 0.00 ppm). ^{1}H NMR splitting patterns are assigned as singlet (s), doublet (d), triplet (t), quartet (q) or pentuplet (p). All first-order splitting patterns were designated on the basis of the appearance of the multiplet. Splitting patterns that could not be readily interpreted are designated as multiplet (m) or broad (br). Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian VX-500 MHz spectrometer, and were referenced relative to residual proton resonances in $CDCl_3$ (at 77.23 ppm). Electrospray Ionization-Time of Flight (ESI-TOF) spectra were obtained on an Agilent 6230 Accurate-Mass TOF mass spectrometer.

DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and Eton Bioscience (California, CA). Molecular biology reagents such as restriction digestion enzymes, Q5 DNA polymerase, T7 RNA polymerase, Vaccinia Capping System, *E. coli* Poly(A) Polymerase, nucleotide stains and competent bacterial strains were purchased from New England Biolabs (Ipswitch, MA), Promega (Madison, WI), or Life Technologies (Carlsbad, CA). Dynabeads™ M-280 Streptavidin was purchased from Thermo Fisher Scientific (Waltham, MA). All images, unless otherwise indicated, were acquired on a Yokagawa spinning disk system (Yokagawa, Japan) built around an Axio Observer Z1 motorized inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 20x 1.42 NA or 63x (bottom) objective to an Evolve 512x512 EMCCD camera (Photometrics, Canada) using ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany). Green fluorescent protein (GFP) was excited with a 488 nm OPSL laser. Red fluorescent protein (RFP) mCherry was excited with a 561 nm Diode (SHG) laser. Images were acquired using Zen Blue software (Carl Zeiss) and processed using Image J (Fiji).

Reaction Buffers

TGT Storage Buffer: 25 mM HEPES, pH 7.3, 2 mM DTT, 1 mM EDTA, and 100 µM PMSF.
TGT Reaction Buffer: 100 mM HEPES, pH 7.3, 5 mM DTT, and 20 mM $MgCl_2$.
T7 Reaction Buffer: 40 mM Tris pH 7.5, 5 mM DTT, 25 mM MgCl2, 2 mM spermidine.
Dynabeads™ streptavidin binding and washing (B&W) buffer 2X: 10 mM Tris-HCl (pH 7.5), 1mM EDTA, 2 M NaCl.
Dynabeads™ streptavidin binding and washing buffer with Tween (BW&T) 2X: 10 mM Tris-HCl (pH 7.5), 1mM EDTA, 2 M NaCl, 0.1% Tween 20.

Dynabeads™ streptavidin solution A: DEPC-treated 0.1 M NaOH, DEPC-treated 0.05 M NaCl.

Dynabeads™ streptavidin solution B: DEPC-treated 0.1 M NaCl.

Dynabeads™ streptavidin elution buffer: 950 µL formamide, 20 µL 500 mM EDTA, 30 µL RNase free water.

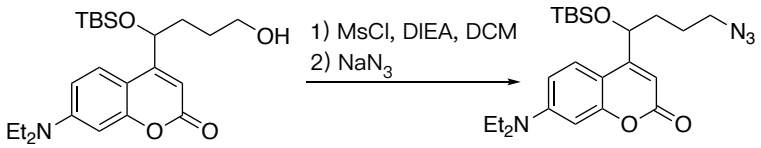
In vitro uncaging light source and irradiation period

456 nm LED (50W max) light source was purchased from KESSIL. 30 seconds of irradiation completely removes biotin-DEACM-preQ1 from RNA in vitro.

365 nm (4W) light source was purchased from Analytik Jena US. 60 seconds of irradiation completely removes biotin-Bac-preQ1 from RNA in vitro.

Chemical Synthesis

Synthesis of compound **(3)**



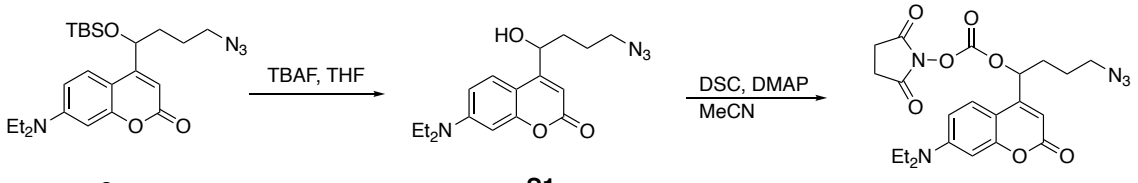
2 **3**

Scheme S1. Synthesis of compound **(3)**

4-(4-azido-1-((tert-butyldimethylsilyl)oxy)butyl)-7-(diethylamino)-2H-chromen-2-one **(3)**

A solution of the previously reported 4-(1-((*tert*-butyldimethylsilyl)oxy)-4-hydroxybutyl)-7-(diethylamino)-2*H*-chromen-2-one **(2)** (50.0 mg, 0.12 mmol) in DCM (5 mL) was treated with DIEA (29.8 mg, 0.24 mmol) and MsCl (20.6 mg, 0.18 mmol). [1, 2] Then the reaction mixture was stirred for 12 hours at room temperature. Afterwards, the reaction solution was quenched with water in ice bath and extracted with 5 mL DCM for 3 times. The combined organic solution was washed with brine and dried with Na_2SO_4. All organic solvent was removed *in vacuo*. The resulting product was dissolved in DMF (3 mL). Next, NaN_3 (15 mg, 0.24 mmol) was added to the solution. The reaction solution was heated at 80°C for 5 hours. After cooling the reaction to room temperature, water (10 mL) was added to quench the reaction, followed by EtOAc (5mL*6) extraction. Then the organic solution was dried with Na_2SO_4 and removed *in vacuo*. The resulting crude material was then purified by flash chromatography (0-10% EtOAc in hexanes) to afford compound **(3)** as a yellow solid (43.2 mg, 81%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.45 (d, *J* = 9.0 Hz, 1H), 6.57 (d, *J* = 9.1 Hz, 1H), 6.52 (d, *J* = 2.5 Hz, 1H), 6.18 (s, 1H), 4.93-4.88 (m, 1H), 3.45-3.37 (m, 4H), 3.37-3.23 (m, 2H), 1.92-1.74 (m, 2H), 1.74-1.66 (m, 2H), 1.21 (t, *J* = 7.1 Hz, 6H), 0.92 (s, 9H), 0.09 (s, 3H), –0.03 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 162.47, 158.10, 156.56, 150.27, 125.04, 108.35, 105.87, 105.87, 97.90, 70.50, 51.27, 44.68, 44.68, 35.17, 25.77, 25.77, 25.77, 24.69, 18.15, 12.44, 12.44, – 4.70, –5.15. HRMS ($M+H^+$) calcd for $[C_{23}H_{37}N_4O_3Si]^+$ 445.2629, found 445.2633.

Synthesis of compound **(4)**



3 **S1** **4**

Scheme S2. Synthesis of compound **(4)**

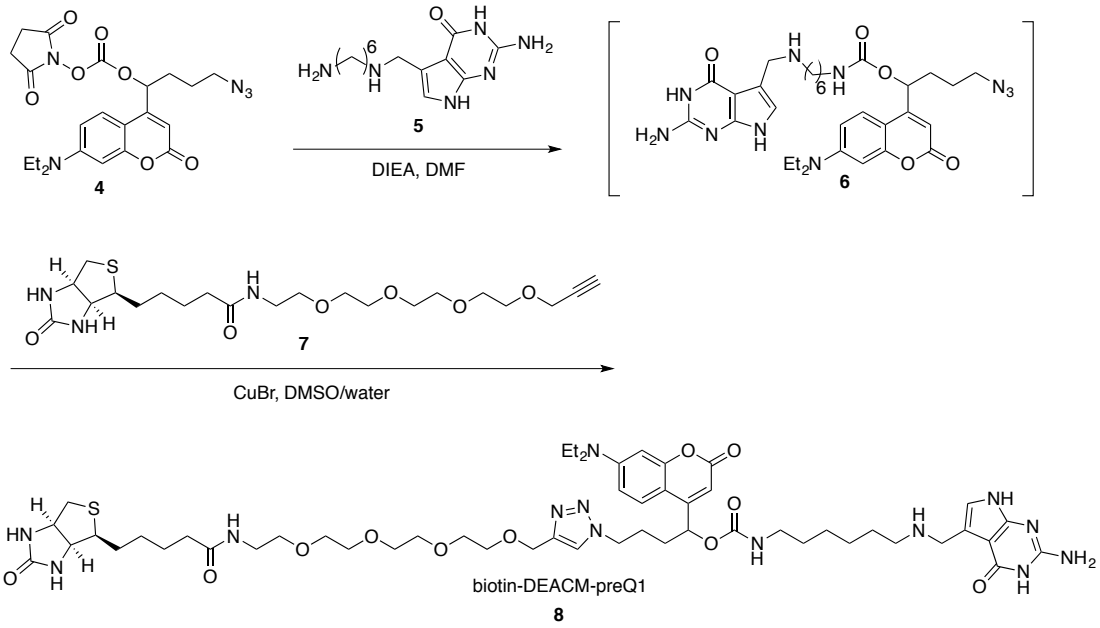
4-(1-((*tert*-butyldimethylsilyl)oxy)-4-hydroxybutyl)-7-(diethylamino)-2*H*-chromen-2-one **(S1)**

A solution of compound **(3)** (20 mg, 0.045 mmol) in THF (5 mL) was treated with TBAF solution (1 M, 1.35 mmol). The resulting solution was stirred at room temperature for 6 hours. After the deprotection, the solution was directly subjected to semipreparative HPLC purification, using a C18 column [gradient of H_2O with 0.1% formic acid and MeOH with 0.1% formic acid 95:5 (0 min) to 5:95 (10 min to 18min)]. Note: We tried to purify the compound by silica flash chromatography, which leads to complete decomposition of compound **(S1)**. The fractions containing product compound **(S1)** from semipreparative HPLC purification were dried *in vacuo* at room temperature on rotary evaporator. The resulting product was further dried *in vacuo* with mechanical pump for 1 hour at room temperature. Compound **(S1)** was obtained as yellow oil (18.1 mg, 96%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.34 (d, *J* = 8.9 Hz, 1H), 6.54 (d, *J* = 8.8 Hz, 1H), 6.43 (s, 1H), 6.20 (s, 1H), 4.97-4.91 (m, 1H), 3.41 (s, 1H), 3.36-3.27 (m, 4H), 1.88 (m, 1H), 1.82-1.64 (m, 3H), 1.13 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 162.88, 158.64, 156.39, 150.26, 125.25, 109.17, 105.91, 105.26, 98.20, 77.35, 69.33, 51.24, 44.95, 33.84, 25.11, 12.51, 12.36. HRMS (M+Na+) calcd for [C17H22N4O3Na]+ 353.1384, found 353.1586.

4-azido-1-(7-(diethylamino)-2-oxo-2H-chromen-4-yl)butyl (2,5-dioxopyrrolidin-1-yl) carbonate **(4)**

Compound **(S1)** (18.1 mg, 0.043 mmol) was dissolved in MeCN, followed by addition of DSC (22.0 mg, 0.086 mmol) and DMAP (15.9 mg, 0.13 mmol). The reaction solution was stirred for 12 hours at room temperature, followed by semipreparative HPLC purification, using a C18 column [gradient of H_2O with 0.1% formic acid and MeOH with 0.1% formic acid 95:5 (0 min) to 5:95 (10 min to 18 min)]. The fractions containing product **(4)** from semipreparative HPLC purification were dried *in vacuo* on rotary evaporator without heat. The resulting product (9.6 mg, 50%) was further dried *in vacuo* with mechanical pump for 1 hour at room temperature. Note: After dried in *in vacuo* at room temperature, there were small amount of unknown by-product generated. Thus, the NMR spectrums of compound **(4)** were not obtained. However, compound **(4)** is confirmed by low resolution mass spectrometry, using fresh fraction from semipreparative HPLC. LRMS ($M+H^+$) calcd for $[C_{22}H_{26}N_5O_7]^+$ 472.2, found 472.1.

Synthesis of compound **(8)**



Scheme S3. Synthesis of compound **(8)**

1-(7-(diethylamino)-2-oxo-2*H*-chromen-4-yl)-4-(4-(15-oxo-19-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-2,5,8,11-tetraoxa-14-azanonadecyl)-1*H*-1,2,3-triazol-1-yl)butyl (6-(((2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-d]pyrimidin-5-yl)methyl)amino)hexyl)carbamate **(8)**

Compound **(4)** (5.0 mg, 0.011 mmol) was dissolved in DMF (0.5 mL) followed by slow addition of the previously reported compound **(5)** [3] (8.8 mg, 0.032 mmol in 0.5 mL DMF) and DIEA (7.1 mg, 0.055 mmol). The reaction solution was stirred for 1 hour at room temperature. The crude substitution product was directly subjected to semipreparative HPLC purification, using a C18 column [gradient of H_2O with 0.1% formic acid and MeOH with 0.1% formic acid 95:5 (0 min) to 5:95 (10 min to 18min)]. Shielded from light, the semipreparative HPLC fractions containing product compound **(6)** (confirmed by low resolution mass spectrometry, LRMS ($M+H^+$) calcd for $[C_{31}H_{43}N_{10}O_5]^+$ 635.3, found 635.3) were dried *in vacuo* with ice bath. The resulting product was directly used for the next step of synthesis. Protected from light, CuBr (0.8 mg, 0.0055 mmol in DMSO:H_2O=0.3 ml:0.3 mL solution) was added to compound **(6)** followed by the addition of commercial available compound **(7)** (5.0 mg, 0.011 mmol in 0.4 mL DMSO; commercially available at BroadPharm, San Diego, USA; CAS# 1458576-00-5). The reaction mixture was stirred for 2 hours at room temperature, followed by semipreparative HPLC purification, using a C18 column [gradient of H_2O with 0.1% formic acid and MeOH with 0.1% formic acid 95:5 (0 min) to 5:95 (10 min to 18min)]. Protected from light, the fractions containing compound **(8)** from semipreparative HPLC were dried *in vacuo* at room temperature. After further vacuum drying in ice bath, there was a trace amount of water left in the vial due to the low temperature. To completely dry the final product compound **(8)**, a solvent mixture of H_2O:MeCN=0.3 mL:0.3 mL was added to the vial containing compound **(8)**, followed by lyophilization. Compound **(8)** was obtained as white residue (0.6 mg, 5% for 2 steps). HRMS ($M+H^+$) calcd for $[C_{52}H_{78}N_{13}O_{11}S]^+$ 1092.5659, found 1092.5664.

Transcription template

GFP-multiTag transcription template

pcDNA3-EGFP was a gift from Doug Golenbock (Addgene plasmid # 13031; http://n2t.net/addgene:13031 ; RRID:Addgene_13031). Ultramer DNA oligo containing three active tags was purchased from Integrated DNA Technologies (San Diego, CA). GFP-multiTag vector was obtained by inserting the ultramer DNA oligo into 5′-UTR of the GFP coding region through Gibson DNA assembly technique (NEB, Ipswitch, MA). DH5a competent cells (Life Technologies, Carlsbad, CA) were transformed with the ligation product and screened against ampicillin on agar plates overnight. Colonies were selected and overgrown for 16 hours. The overgrowth was subjected to DNA extraction with a QIAGEN Plasmid Maxi Kit (QIAGEN, Venlo, Limburg Netherlands). Sanger sequencing was performed to verify the inserted sequence.

GFP-multiTag sequence (from T7 promoter to 3' XbaI restriction enzyme cut site) is shown below. The 'Tag' sequences are underlined.

TAATACGACTCACTATAGGG***GCAGACTGTAAATCTGC***AGACCCAAGCTTGGTAGGTCAGTTGCAGTTACCG
AGCTCGGATCCACTAGTAACGGCCGC***GCAGACTGTAAATCTGC***CAGTGTGCTAGTCAGACAGATGGAATTC
TGCAGATATCCATCACACTGGCGGCCGCTCGA***GCAGACTGTAAATCTGC***GATGGTGAGCAAGGGCGAGGA
GCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTG
TCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGC
TGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGA
CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCT
TCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCAT
CGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAAC
AGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAA
CATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTG
CTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATC
ACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAATCT
AGA

RFP-multiTag transcription template

pcDNA3.3-mCherry was a gift from Derrick Rossi (Addgene plasmid # 26823 ; http://n2t.net/addgene:26823 ; RRID:Addgene_26823). Similarly, RFP-multiTag vector was prepared. RFP-multiTag mRNA vector sequence (from T7 promoter to 3' restriction enzyme cut site) is shown below. The 'Tag' sequences are underlined.

TAATACGACTCACTATAGGG***GCAGACTGTAAATCTGC***AGACCCAAGCTTGGTAGGTCAGTTGCAGTTACCG
AGCTCGGATCCACTAGTAACGGCCGC***GCAGACTGTAAATCTGC***CAGTGTGCTAGTCAGACAGATGGAATTC
TGCAGATATCCATCACACTGGCGGCCGCTCGA***GCAGACTGTAAATCTGC***GATGGTGAGCAAGGGCGAGGA
GGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACG
AGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGA
CCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTAC
GTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCG
TGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAAGACGGCGAGTTCAT
CTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACGATGGGC
TGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTG
AAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGC
TGCCCGGCGCCTACAACGTGAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAA

CAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAATATCTAACAC
AGACTCTCGGTACCATCATTTTCATATCCCCACCACCATCATTTAATGAATTCCATCAGGAATCCCTCACTTAA
AGCCCGCCGAAAGGCGGGCTTTTCTGTGTCTGCAGACTGGCCGTCGTTTTACACTCGAGCATGCATCTAGA

In vitro mRNA transcripts synthesis

In vitro transcription (IVT) reaction

GFP-multiTag vector was linearized using XbaI restriction digestion enzyme (NEB, Ipswitch, MA) to ensure uniform length transcription. For each IVT reaction, 10 µg of DNA plasmid was dissolved in cutsmart buffer (NEB, Ipswitch, MA) to a final concentration of 300 ng/µL. 40 units of XbaI restriction digestion enzyme was added into the reaction mixture. The reaction was carried out at 37 °C for 4 hours to ensure complete digestion. The reaction solution was allowed to cool down to room temperature. The DNA product was extracted with an equal volume of molecular biology grade phenol / chloroform / isoamyl alcohol (25:24:1) and vortexed for 2 minutes followed by a 5-minute centrifugation at 10,000 RCF. The top aqueous layer was transferred to a fresh tube and an equal volume of chloroform was added. The mixture was vortexed for 2 minutes followed by a 5 minute centrifugation at 10,000 RCF. The aqueous layer was transferred into a fresh tube for DNA precipitation. The linearized DNA was precipitated by the addition of 0.1x volumes of 3M sodium acetate (pH = 5.2) and 2.5x volumes of 95% ethanol. The sample was chilled to -20 °C overnight, centrifuged at 16,100 RCF for 20 minutes at 4 °C, followed by gently removing ethanol. The DNA pellet was air dried and resuspended in 200 µL of RNase free water. The obtained DNA solution was used directly for IVT reaction. Each IVT reaction was set up with 50 ng/µL of linearized DNA template, 5 mM of each NTPs (ATP, CTP, UTP), 9 mM of GTP (NEB, Ipswitch, MA), 0.004 unit/µL of thermostable inorganic pyrophosphatase (NEB, Ipswitch, MA), 0.25 µg/µL T7 RNA polymerase, 0.05% Triton X-100 (Sigma, St. Louis, MO) and 1 unit/µL RNase Inhibitors, Murine (NEB, Ipswitch, MA). The IVT reaction was carried out at 37 °C for 4 hours to allow for sufficient RNA synthesis. To remove DNA template, 2 µL of 100 mM $CaCl_2$ and 20 units of Turbo DNase (Life Technologies, Carlsbad, CA) were added to the mixture and incubated at 37 °C for 1 hour. The mixture was then centrifuged at 10,000 RCF for 5 minutes at room temperature to pellet any remaining magnesium pyrophosphate. The supernatant was resuspended in 200 µL RNase free water. To precipitate the *in vitro* transcribed mRNA (IVT-mRNA) product, the solution was added with 100 µL of 8 M LiCl and chilled to -20 °C for 4 hours, followed by 20 minutes of centrifugation at 16,000 RCF at 4 °C. The supernatant was removed gently. The remaining RNA pellet was resuspended in 200 µL RNase free water and quantified at 260 nm. The IVT-mRNA was confirmed as a single observable UV shadowing band by 4% denaturing PAGE (4% polyacrylamide in TBE with 8M urea) and kept frozen at -20 °C until used.

Maturation of *in vitro* transcribed mRNA (IVT-mRNA)

Vaccinia capping system (NEB, Ipswitch, MA) was used to add a 7-methylguanylate cap structure (Cap 0) to the 5′ end of IVT-mRNA to allow 5′-cap dependent translation *initiation.* IVT-mRNA was diluted in 29.2 µL RNase free water with a final concentration of 0.5 µg/µL. The RNA solution was heated to 65 °C and held for 5 minutes to denature RNA. The mixture was then chilled on ice and held for another 5 minutes, followed by the addition of 4 µL of 10x capping buffer, 2 µL of 10 mM GTP, 2 µL of 4 mM S-adenosylmethionine (SAM), 32 units of RNase inhibitor and 2 µL of Vaccinia Capping Enzyme. The reaction mixture was incubated at 37 °C for 45 minutes. The capped IVT-mRNA product was purified by LiCl precipitation and directly used for polyadenylation. *E. coli* Poly(A)

Polymerase (NEB, Ipswitch, MA) was used to polyadenylate IVT-mRNA. 30 μL of reaction mixture containing 15 μg of capped IVT-mRNA, 1 mM ATP, 30 units of RNase inhibitor was prepared in 1x *E. coli* Poly(A) polymerase reaction buffer and incubated at 37 °C for 1 hour. The maturated IVT-mRNA was purified by LiCl precipitation and directly used for *in vitro* TGT labeling.

TGT *in vitro* labeling and purification

TGT labeling of 17-nt RNA oligo

TGT labeling reaction was assembled with the following components in 1x TGT reaction buffer: 5 μM of 17-nt RNA oligo, 5 μM of TGT enzyme, 50 μM of small-molecule substrate (biotin-Bac-preQ1 or biotin-DEACM-preQ1), and 5 mM DTT. The reaction mixture was incubated at 37 °C for 4 hours. 1 μL of proteinase K was added into reaction mixture and incubated at 37°C for 30 minutes to terminate the labeling reaction. The crude labeled RNA was purified by EtOH precipitation. Crude labeling product was analyzed on 18% denaturing PAGE (Figure S1). By measuring RNA band intensity, the labeling efficiency of RNA oligo using biotin-Bac-preQ1 was about 93.6% and the labeling efficiency of RNA oligo using biotin-DEACM-preQ1 was about 96.7%. After biotin-streptavidin affinity purification, the unlabeled RNA oligo can be completely get rid of (Figure 2B). Note that sense biotin-Bac-preQ1 and biotin-DEACM-perQ1 are extremely photo-sensitive, all reaction and analysis show be performed in a dark room with minimal red lamp as ambient light. All materials should be handled carefully to protect from photo-degradation.

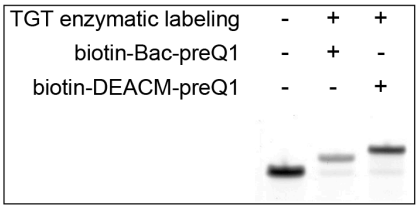


Figure S1. 18% denaturing PAGE analysis of in vitro TGT labeling of the 17-nt RNA oligo without affinity purification. Gel was stained with 1X GelRed.

TGT labeling of IVT mRNA transcript

TGT labeling conditions were adapted from our previous study. [3] Synthesized TGT substrate was dissolved in RNase free water. For *in vitro* TGT reaction, 1 μM of IVT-mRNA, 50 μM of small-molecule substrate (biotin-Bac-preQ1 or biotin-DEACM-preQ1), 2 unit/μL RNase inhibitor, 5 mM DTT and 1.5 μM of TGT enzyme was assembled in 1x TGT reaction buffer. The reaction mixture was incubated at 37 °C for 4 hours. 1 μL of proteinase K was added into reaction mixture and incubated at 37°C for 30 minutes to terminate the labeling reaction. The crude labeled mRNA was purified by EtOH precipitation.

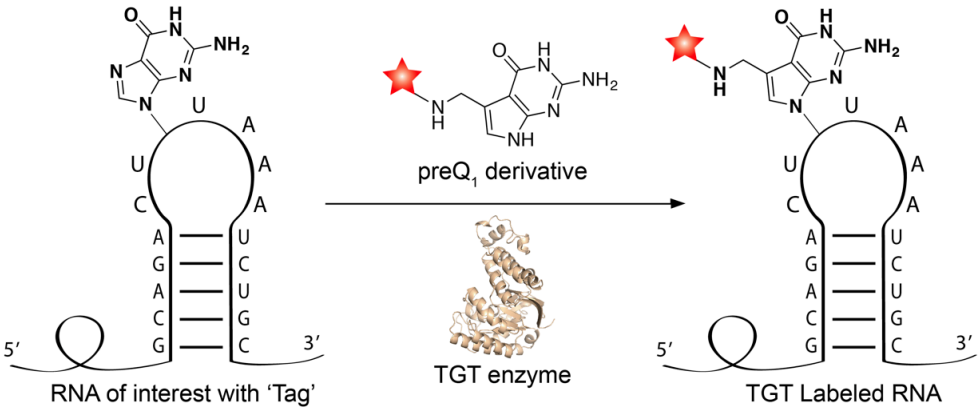


Figure S2. RNA labeling through 'RNA-TAG'. The bacterial tRNA guanine transglycosylase (TGT) exchanges a guanine nucleobase within the 17-nucleotide RNA stem-loop structure ('Tag') with a synthetic substrate (preQ1 derivative) carrying a small-molecule functioning group (shown as red star).

Purification of TGT labeled mRNA transcript

The crude TGT labeling product was further purified using Dynabeads™ M-280 Streptavidin. Dynabeads™ M-280 Streptavidin beads stock solution (Thermo Fisher Scientific, Waltham, MA) was vortexed for 30 seconds. 150 µL of the well-mixed stock solution was transferred to a 2 mL Eppendorf tube. Following manufacture's protocal, the magnetic beads were washed with 300 µL of 1x BW&T buffer for 3 times, 300 µL of solution A for 2 times, 300 µL of solution B for 2 times and 300 µL of 1x BW&T buffer for 2 times After washing, the beads were incubated with labeled IVT-mRNA in 1x BW&T buffer for 25 minutes to allow for the binding of IVT-mRNA to the magnetic beads. The solution was then rotated at room temperature for 25 minutes to allow for the binding of IVT-mRNA and the magnetic beads. The RNA bound beads were washed with 300 µL of 1x BW&T buffer 2 times and 300 µL of 1x B&W buffer 1 time. The beads were then resuspended in 100 µL elution buffer and incubated at 65 °C for 3 minutes, followed by centrifugation at 7,000 RCF for 1 minute. The resulting supernatant was then subjected to ethanol precipitation with the addition of 0.1x volumes of 3M sodium acetate and 4x volumes of ethanol. The purified labeled IVT-mRNA was stored in - 20 °C until used. The purification yield was around 60%.

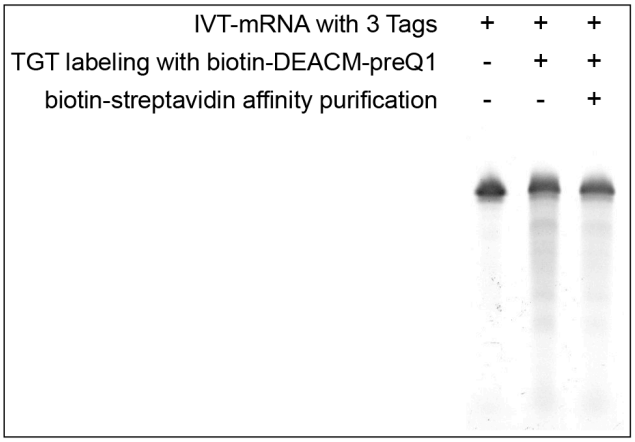


Figure S3. 4% denaturing PAGE analysis of IVT-mRNA labeling with biotin-DEACM-preQ1. Unlike labeling short RNA oligos, the molecular weight difference of labeled mRNA and unlabeled mRNA is too small to be visualized on 4% denaturing PAGE. Thus, in order to determine the labeling efficiency of biotin-DEACM-preQ1 onto mRNA transcript, we performed fluorescence biotin quantitation assay (Figure S4, Figure S5).

Agarose gel analysis of biotin-labeled mRNA

An mRNA with ***one*** active Tag sequence located at the 5′-UTR of the mRNA was labeled with biotin through TGT enzymatic labeling. The labeled mRNA was further purified by biotin-streptavidin affinity purification. 100 ng biotin-labeled and purified mRNA or control mRNA without TGT labeling was incubated with 1 μM streptavidin in 10 μL solution at room temperature for 15 minutes. The samples were then analyzed on 1% GelRed pre-stained agarose gel under 90 V for 45 minutes (Figure S4).

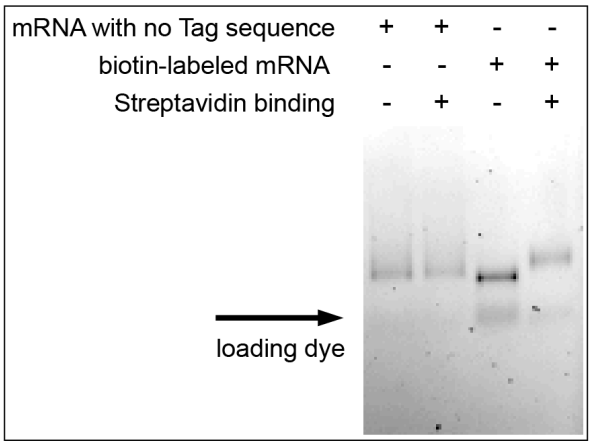


Figure S4. Biotinylation of mRNA by TGT labeling. 1% agarose Gel-shift analysis was performed to demonstrate successful incorporation of biotin at the 5′-UTR of the mRNA (as shown in line 4).

Degree of labeling of biotin-DEACM-preQ1 on IVT-mRNA transcript

In our previous paper [3], we performed mRNA labeling efficiency test using the biotin-Bac-preQ1 analog. We performed the following experiment to determine the labeling efficiency of mRNA using biotin-DEACM-preQ1 analog. To quantify the labeling efficiency of biotin-DEACM-preQ1 onto mRNA, we performed a fluorescence biotin quantitation assay (Thermo Scientific, Waltham, MA). This commercially available microplate-based assay measures the fluorescence of the Thermo Scientific DyLight Reporter (fluorescent avidin and HABA premix) upon binding with a biotinylated sample, in this case, the TGT labeled biotinylated RNA. The avidin fluoresces when the weakly interacting HABA (4′-hydroxyazobenzene-2-carboxylic acid) is displaced by the biotinylated RNA. The amount of biotin for each sample can be determined by comparing the fluorescence intensity of the sample with a purified and signally biotinylated mRNA reference (one labeled biotin per mRNA molecule, Figure S4).
As shown in Figure S5, there is an average number of 2.12 biotins per mRNA transcript for the labeled but unpurified mRNA with 3 Tags inserted at the 5′-UTR. After Dynabeads™ streptavidin purification, there is an average number of 2.32 biotins per mRNA transcript. After photo-irradiation with 456 nm light for 30 seconds, there is an average number of 0.04 biotins per mRNA transcript which is similar to unmodified mRNA control (0.037), demonstrating complete photo-cleavage of the biotin-DEACM-preQ1 analog from mRNA transcript using 456 nm LED light.

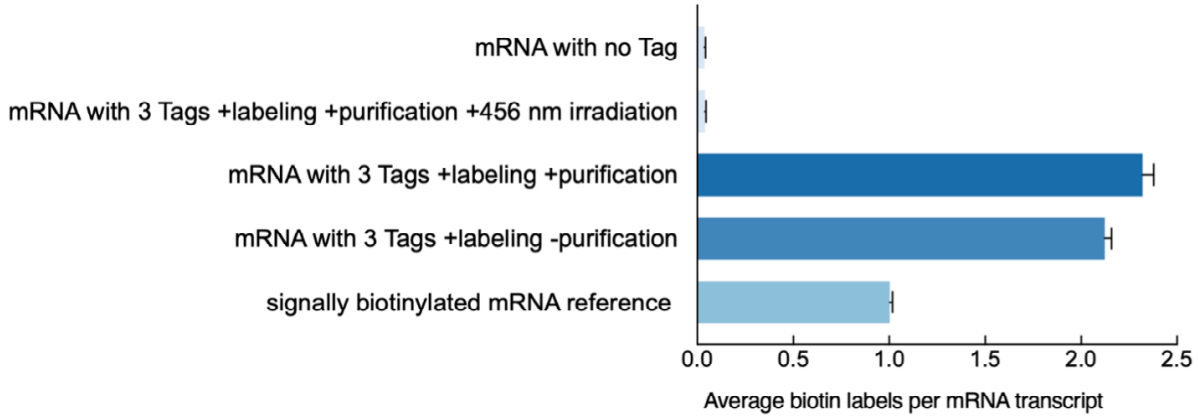


Figure S5. The average number of TGT labeled biotins per mRNA transcript obtained from the fluorescence biotin quantitation assay. The signally biotinylated mRNA was used as normalization reference.

Live cell imaging and photo-uncaging

IVT mRNA transfection

HEK-293 cells (ATCC, Manassas, VA) were cultured in DMEM media (Life Technologies, Carlsbad, CA) with 10% FBS and P/S. HEK-293 cells were plated at an initial density of around 70,000 cells per well in a Nunc Lab-Tek 8 well chamber slide (Thermo Scientific, Waltham, MA) pre-coated with 0.5% (w/v) poly-lysine in H_2O in order to limit cell movement during imaging. Cells were allowed to adhere overnight, washed with Opti-MEM media (Life Technologies, Carlsbad, CA) and subsequently transfected in Opti-MEM media with an addition of 400-600 ng of IVT-mRNA and 0.8-1.1 µL of Lipofectamine® RNAiMAX (Life Technologies, Carlsbad, CA). (*Note:* mRNA transfect protocal has to be regularly optimized to obtain highest transfection efficiency. IVT-mRNA and Lipofectamine® RNAiMAX amount and ratio have to be optimized for different cell passage numbers. Usually, 400-600 ng of mRNA and 0.8-1.1 µL of Lipofectamine® RNAiMAX should be used for transfecting HEK-293 cells. Optimization was performed following Lipofectamine® RNAiMAX manufacture's protocol.) Cells were transfected for 2 hours before washed with DMEM to remove any transfection reagents. Cells were allowed to grow in DMEM at 37 °C with 0.5% CO_2 to allow for protein expression.

mRNA stability of unlabeled mRNA and caged mRNA

Same amount of unlabeled GFP-mRNA, biotin-Bac-preQ1 caged GFP-mRNA and biotin-DEACM-preQ1 caged GFP-mRNA were transfected into HEK-293 cells. 2 hours post transfection, transfection medium was removed and change to full growth medium (DMEM, 10% FBS). Total cellular mRNA was extracted 4 hours and 24 hours after transfection, following by reverse transcription and qPCR analysis of the GFP-mRNA (primers were shown below). Zymo Direct-zol RNA Purification Kits was used to extra total RNA from cell samples. Oligo-dT(18)-VN (purchased from IDT) was used as primer for reverse transcription (Maxima Reverse Transcriptase, EP0742, Thermo Scientific, TM). NEB-luna qPCR 2X master mix was used for qPCR analysis. qPCR primers were listed below. GFP-mRNA level was plotted as relative mRNA levels normalized to actin-mRNA. As a result, we didn't observe significant mRNA stability difference among these 3 mRNAs at 4 hours post transfection. After 24 hours, we observed slightly lower (about 20%) relative mRNA level of the caged mRNA compared to unlabeled mRNA. This difference might due to the fact that the caged mRNA went through extra in vitro labeling and purification

steps, which may cause mRNA degradation, not necessarily due to the incorporation of the photo-cages. The degradation of mRNA after in vitro TGT labeling and biotin-streptavidin purification can be visualized on 4% denaturing PAGE (Figure S3).

GFP-mRNA forward primer:

ACGTAAACGGCCACAAGTTC

GFP-mRNA reverse primer:

AAGTCGTGCTGCTTCATGTG

Actin-mRNA forward primer:

agagctacgagctgcctgac

Actin-mRNA reverse primer:

ctccatgcccaggaaggaagg

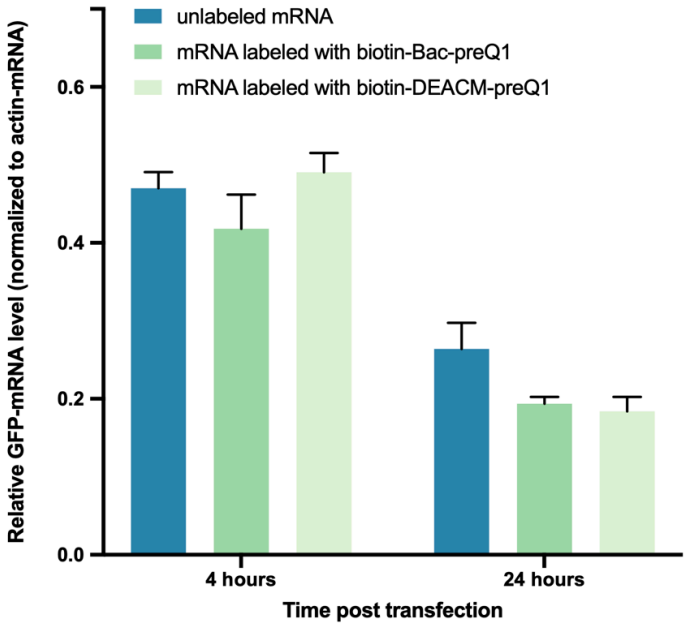


Figure S6. qPCR analysis of mRNA stability of unlabeled and caged mRNAs. GFP-mRNA level was normalized to actin-mRNA shown as relative GFP-mRNA level in Figure S6.

Translation activity estimation of the biotin-DEACM-preQ1 caged GFP-mRNA

In order to estimate mRNA translation activity of biotin-DEACM-preQ1 caged GFP-mRNA and how much protein expression could be restored after light irradiation, we performed the following cell imaging and photo-uncaging experiments. Following mRNA transfection protocol described in previous section, we transfected the same amount (500 ng mRNA, 0.9 μL Lipofectamine® RNAiMAX) of unlabeled mature GFP-mRNA, biotin-DEACM-preQ1 caged GFP-mRNA, biotin-DEACM-preQ1 caged and then in vitro 456 nm LED uncaged GFP-mRNA into cultured HEK-293 cells. Two hours post transfection, transfection medium was removed from cultured cells and the plate of cells that were transfected with biotin-DEACM-preQ1 caged mRNA were irradiated with 456 nm LED light for 30 seconds. Cells were imaged 8 hours after the light irradiation event (Figure S7A). For fluorescent protein expression quantification, average fluorescence level (relative fluorescence units, RFU) was measured from more than 80 cells (Figure S7B). As shown in Figure S7, the installment of biotin-DEACM-preQ1 reduced mRNA translation activity to about 12% relevant to the unlabeled mRNA. The in vitro photo-uncaged mRNA

showed about 96% translation activity relevant to the unlabeled mRNA. The in vivo photo-uncaged mRNA showed about 76% translation activity relevant to the unlabeled mRNA.

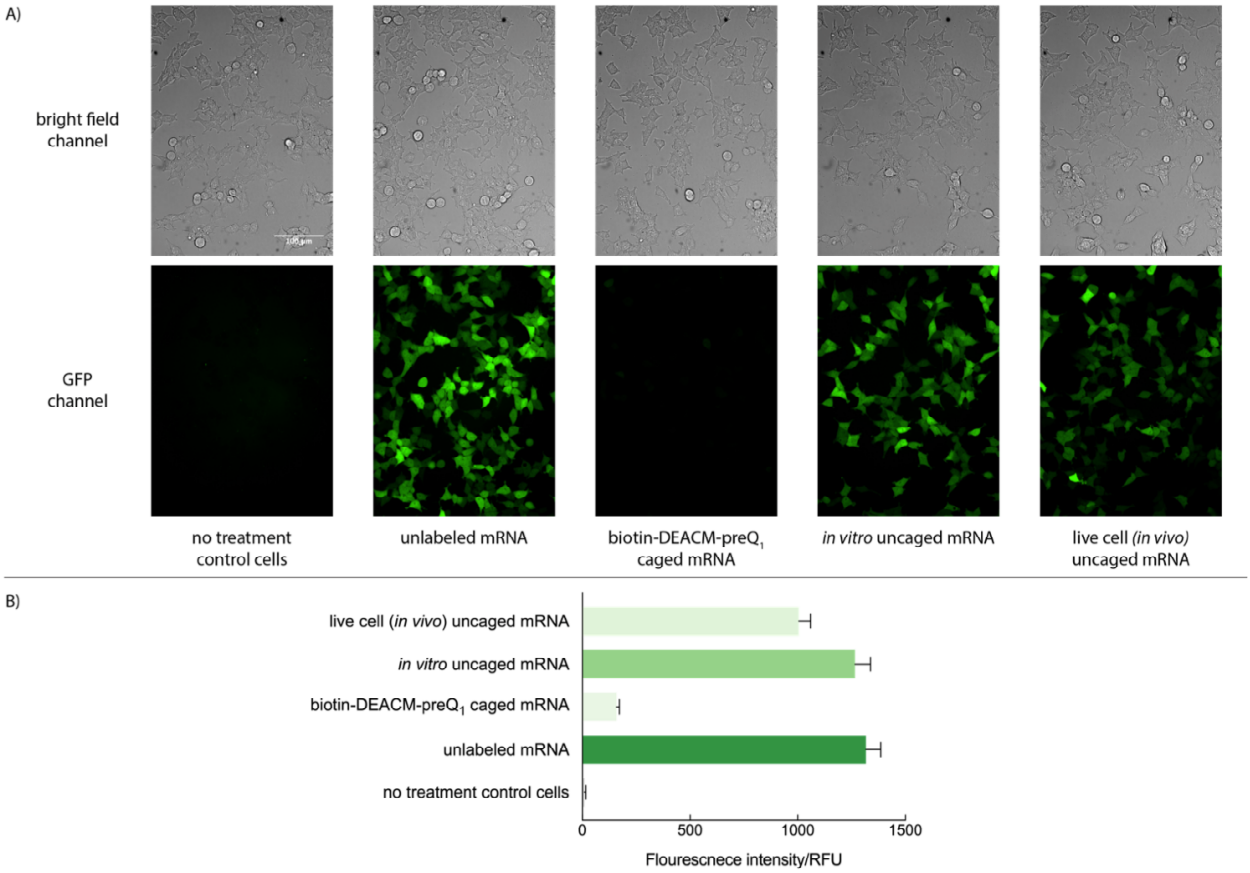


Figure S7. Cell imaging and protein expression estimation. A) Images of HEK-293 cells with different treatments. GFP fluorescence channels of all images were window leveled the same. Fluorescence intensity was relevant to GFP protein expression level. Scale bar = 100 μm. B) To obtain a quantitative estimation of GFP expression among differently treated cells, fluorescence intensity of individual cell in each cell image was measured using Fiji software. Fluorescence intensity of more than 80 cells were measured and averaged. The average relative fluorescence units (RFU) value with error bar (SEM) from each sample was plotted in Figure S7B.

Additional cell images

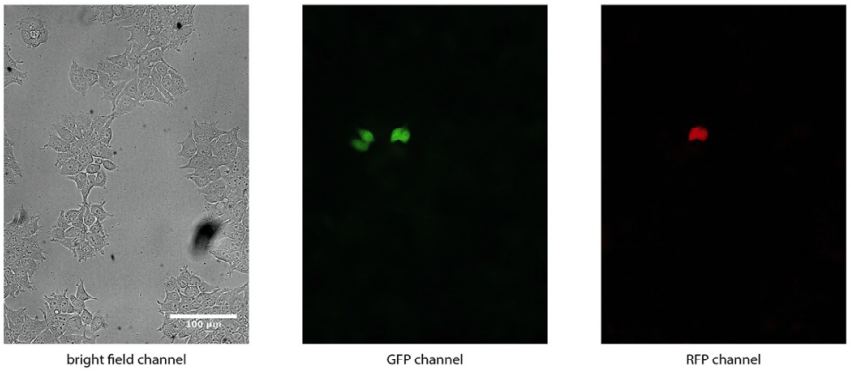


Figure S8. Additional view of Figure 3 in the main text showing a greater part of the slide. Cell images were taken using a 20x bottom objective. Scale bar = 100 μm.

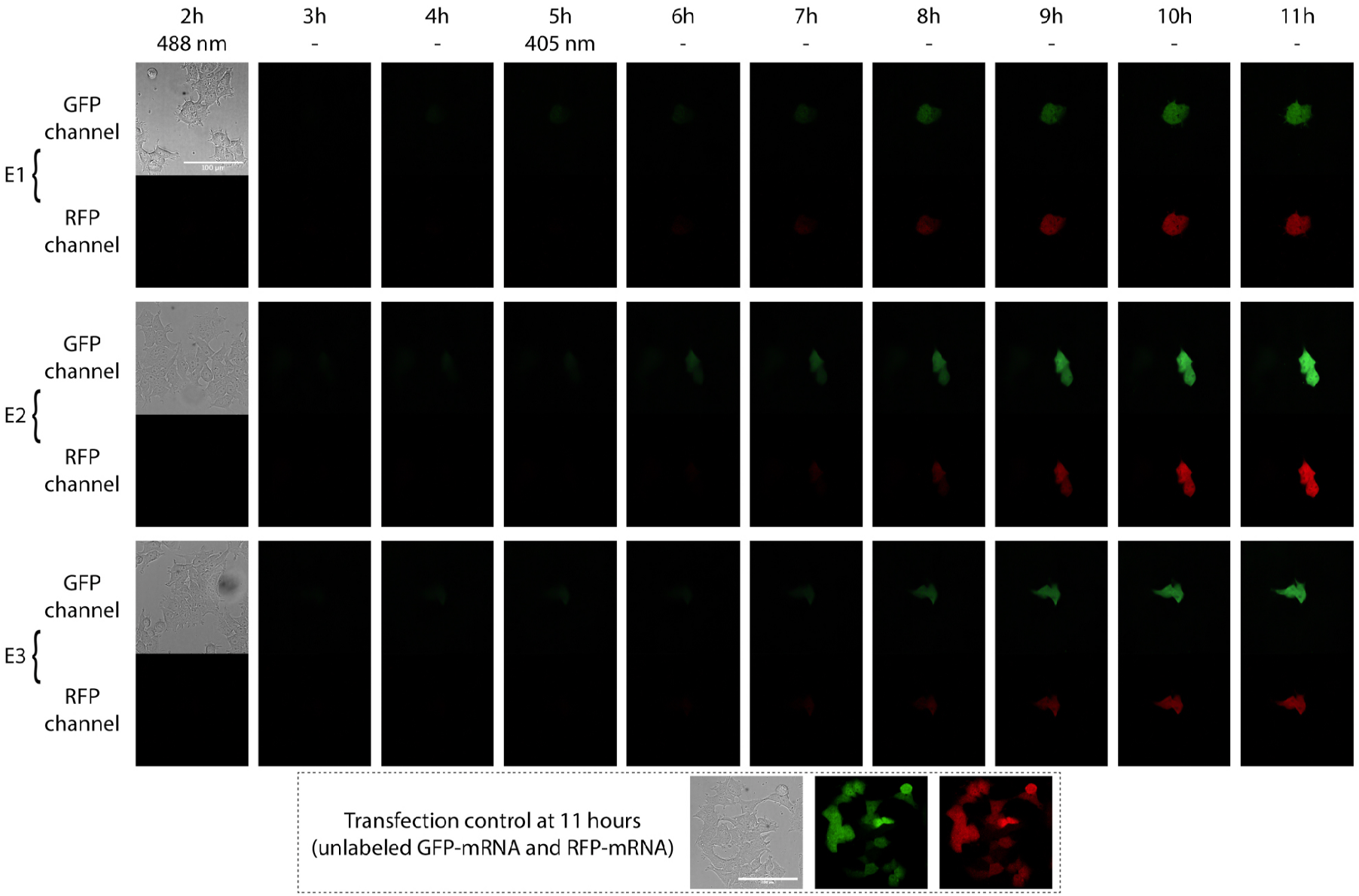
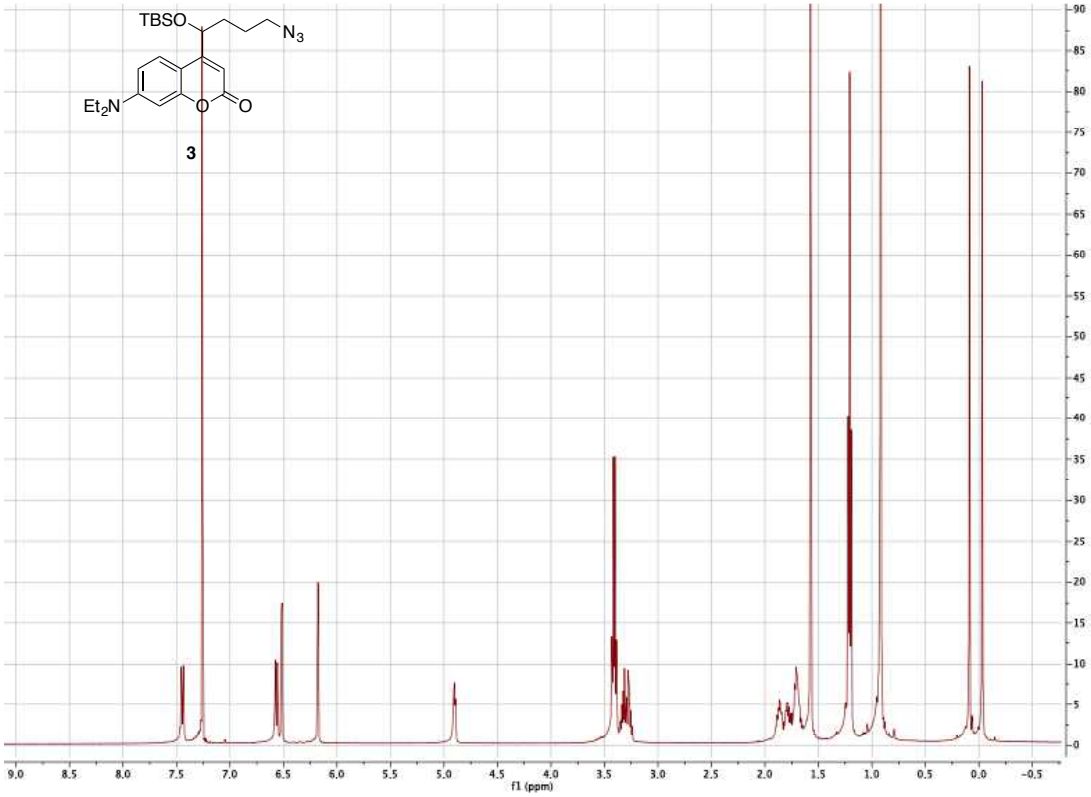
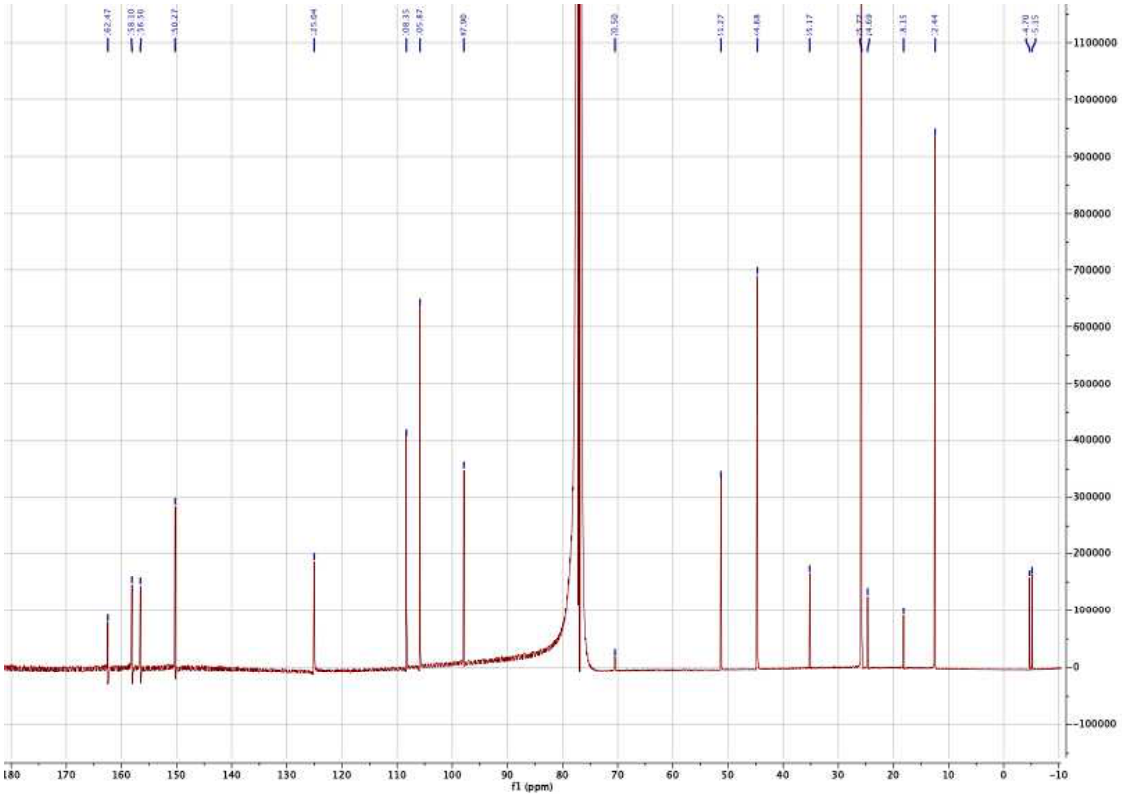

2h
488 nm
3h
-
4h
-
5h
405 nm
6h
-
7h
-
8h
-
9h
-
10h
-
11h
-
GFP channel
E1
RFP channel
GFP channel
E2
RFP channel
GFP channel
E3
RFP channel
Transfection control at 11 hours
(unlabeled GFP-mRNA and RFP-mRNA)

Figure S9. Replicates of sequential live cell uncaging experiments. Caged IVT-mRNAs coding for GFP and RFP were co-transfected into HEK-293 cells. Two hours post transfection, the selected cell was first irradiated with 488 nm laser light to trigger the release of biotin-DEACM-preQ1 from GFP-mRNA. Five hours post transfection, the same cell was then irradiated with 405 nm laser light to trigger the release of the biotin-Bac-preQ1 from RFP-mRNA. Cells were continuously imaged to quantify protein expression level. As control, cells transfected with unlabeled mRNA coding for GFP and RFP were images at the end time point (11 hours post transfection). Fluorescence intensity was measured and averaged. The average relative fluorescence intensity of GFP from laser irradiated cells was 78.2% comparing to cells transfected with unlabeled GFP-mRNA. The average relative fluorescence intensity of RFP from laser irradiated cells was 82.5% comparing to cells transfected with unlabeled RFP-mRNA.

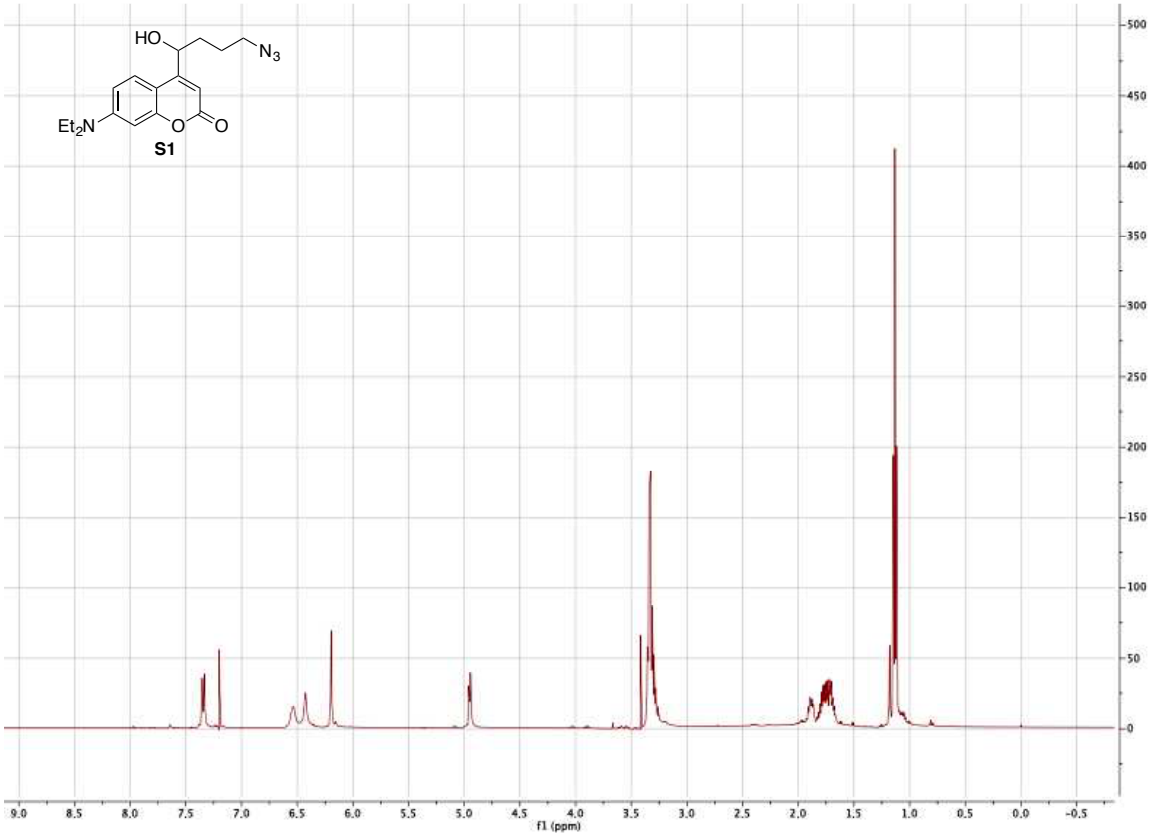
Spectrums

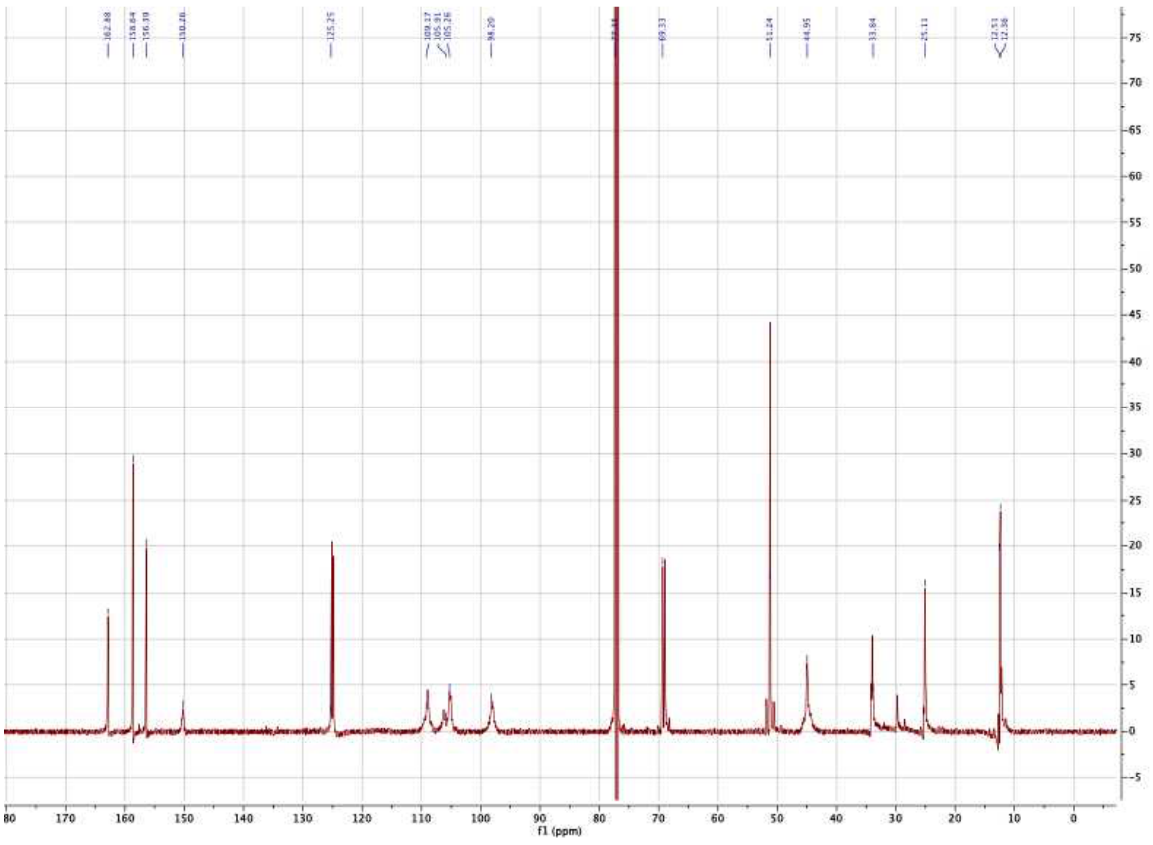
Compound **(3)**



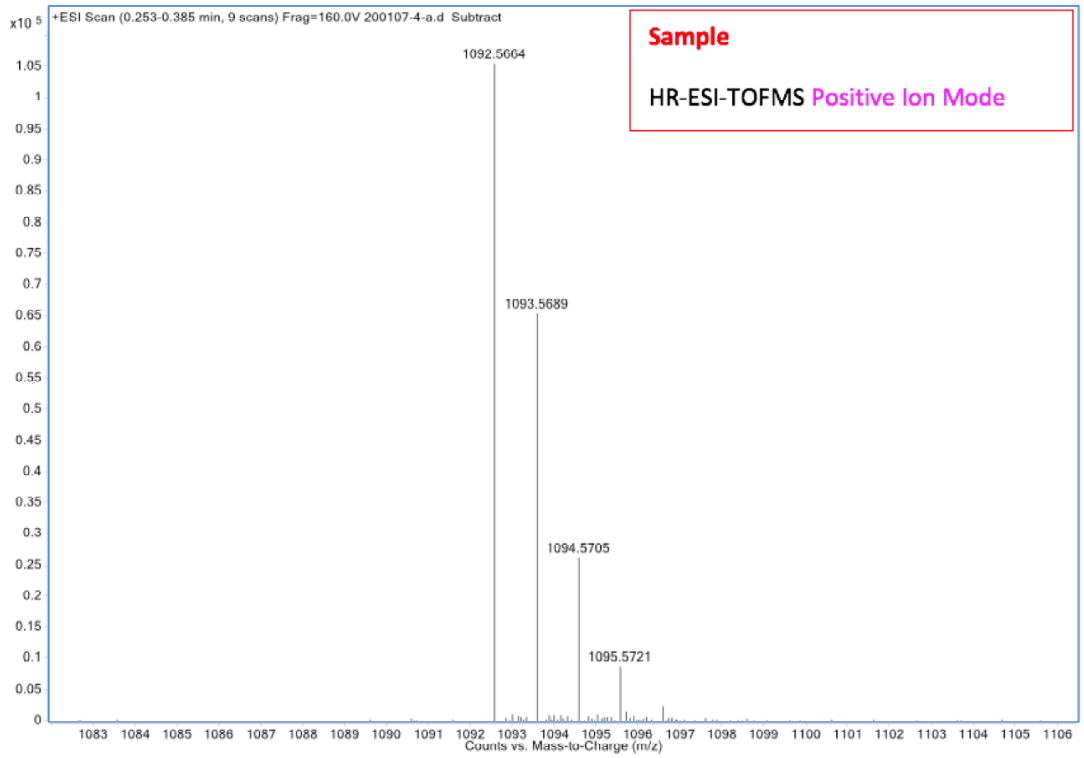


Compound **(S1)**


HO
N3
Et2N
O
O
S1
f1 (ppm)


162.88
158.64
156.39
150.26
125.25
109.17
105.91
105.26
94.29
77.16
69.33
51.24
44.95
33.84
25.11
12.51
12.36
f1 (ppm)

HRMS and HPLC trace of compound **(8)**



Mass Measured	Theo. Mass	Delta (ppm)	Composition
1092.5664	1092.5659	-0.5	$[C_{52}H_{78}N_{13}O_{11}S]^+$

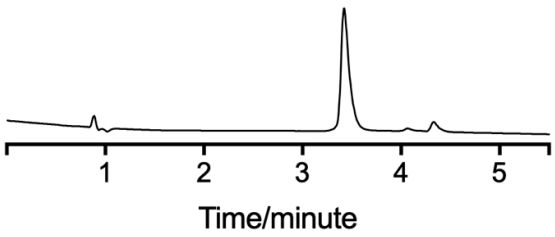


Figure S10. HPLC (405 nm absorbance) trace of compound **(8)**. Analytical HPLC: t_R = 3.48 min (Analytical HPLC condition 20 to 95% Phase B over 5 min, then 95% Phase B for 1 min, Eclipse Plus C8 analytical column. *Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid.)

References

[1] Goguen, B. N., Aemissegger, A., & Imperiali, B. (2011). Sequential activation and deactivation of protein function using spectrally differentiated caged phosphoamino acids. Journal of the American Chemical Society, 133(29), 11038-11041.

[2] Yamazoe, Sayumi, et al. "Sequential gene silencing using wavelength-selective caged morpholino oligonucleotides." Angewandte Chemie International Edition 53.38 (2014): 10114-10118.

[3] Zhang, Dongyang, et al. "Light-activated control of translation by enzymatic covalent mRNA Labeling." Angewandte Chemie International Edition 57.11 (2018): 2822-2826.