Supporting information for:

A Genetically Encoded Fluorescent Probe in Mammalian Cells

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Materials and methods

General:

All chemicals were obtained from commercial sources and used without further purification. PCR reactions were carried out with Platinum-pfx polymerase (Invitrogen), following manufacturer's protocol. DNA oligomers for PCR reactions was purchased from IDT. Restriction enzymes and T4 DNA ligase from NEB were used. Plasmid DNA was purified using Qiagen spin miniprep kits. Macherey-Nagel Nucleospin columns were used to purify DNA following digestion or gel electrophoresis. The synthesis of Anap follows the procedure that was previously reported.[19] Protein mass spectra were acquired at the Scripps Center for Mass Spectrometry (La Jolla, CA). *E. coli* DH10B cells were used for routine cloning and DNA propagation. Site-directed mutagenesis was performed using the Agilent QuikChange mutagenesis kit.

Construction of pAnap and the reporter plamids

The amber suppressor $tRNA_{CUA}^{Leu}$ was derived from *E. coli* $tRNA_{UAA}^{Leu}$ in which U35 and A36 are mutated to C and U, respectively. The gene lacking 3′-CCA but with a 3′-TTTTTT sequence, driven by the human H1 promoter was synthesized by IDT. This was PCR amplified, digested with BglII and BamHI and was inserted into the BglII site of pCDNA3.1. Additional copies of the tRNA expression cassette were introduced in the resulting plasmid by successive rounds of BglII digestion, ligation of the BglII/BamHI digested insert, and verification of the insert orientation in the resulting plasmid by DNA sequencing and digestion analysis. AnapRS was PCR amplified and was inserted into pCDNA3.1-8x H1-$tRNA_{CUA}^{EcLeu}$ between HindIII and XhoI restriction sites to generate pAnap. Similarly, Histone H3 (Thr59TAG) and Grp94 (Leu7TAG and Ser127TAG) were PCR amplified and inserted into pmCherry-N1 (Clontech) to afford expression plasmids for these reporter proteins. GalT1-pmCherry-N1 was a gift from William Balch group (TSRI), to which the amber mutation was introduced by site-directed mutagenesis.

Mammalian cell culture:

CHO cells were grown in a medium containing F-12, 10% FBS, 1% Pen-Strep, and 2 mM L-glutamine at 37 ºC in a humidified atmosphere of 5% CO_2. HEK293 and HeLa

cells were cultured in DMEM media supplemented with 10% FBS and was maintained at 37 ºC in a humidified atmosphere of 5% CO_2.

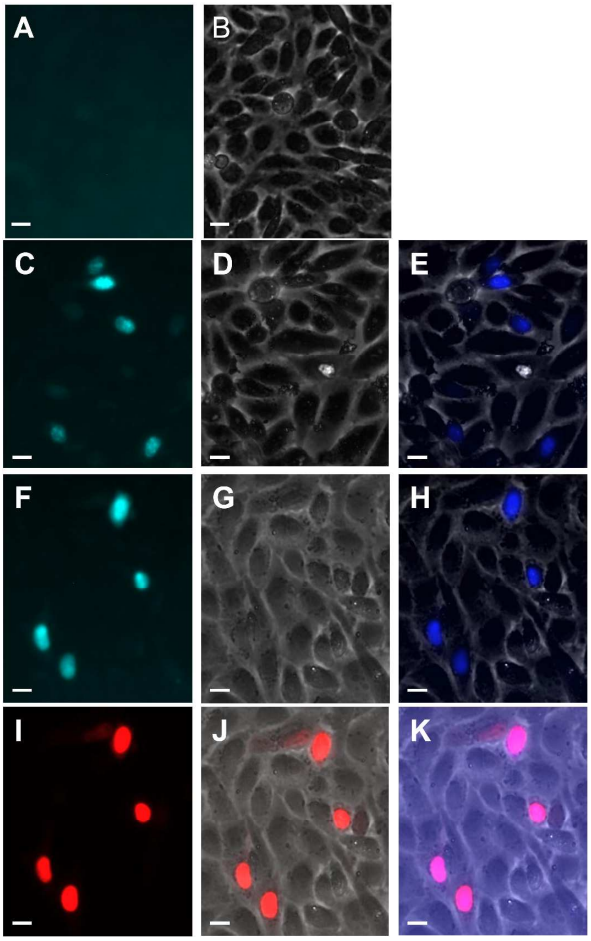
Expression and purification of the EGFP-40-Anap mutant

HEK293 or CHO cells were transiently transfected with pAnap and pEGFP at 50-70% confluency using Fugene HD (54 µl Fugene HD + 3 µg of pAnap + 9 µg of pEGFP for 12 ml cell culture in 75 cm^2 tissue culture flasks). After 6 h, fresh media containing 0.5 mM Anap was introduced and the cells were grown for an additional 24 h before being detached and lysed in RIPA buffer with protease inhibitor cocktail (Roche). After dialysis with native purification buffer (50 mM NaH_2PO_4, pH 8.0, 200 mM NaCl, and 10 mM imidazole), the protein expressed was purified by Ni-NTA affinity chromatography under native conditions according to the manufacturer's protocol (QIAGEN), and was analyzed by SDS-PAGE and ESI-MS.

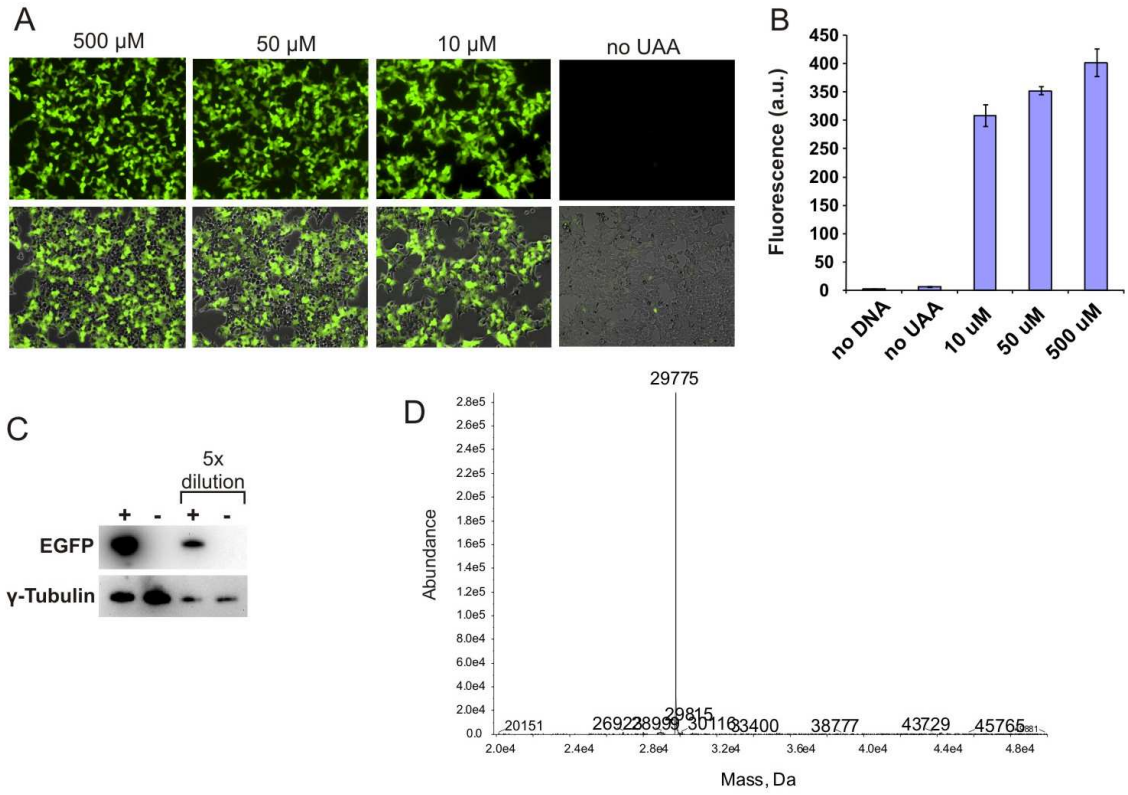
Expression of reporter proteins and subsequent imaging

Cells were seeded at $2x10^5$ in a 6-well plate. At 25-40% confluency, cells were transfected using Fugene HD (Roche; 8 µl Fugene 6 + 1.2 µg of pAnap + 1.2 µg of reporter plasmid for 2 ml cell culture in Costar 6-well cell-culture clusters). After 6 h of incubation, media was exchanged to fresh media containing 10-20 µM Anap, and cells were grown for an additional 12-24 h. For live cell-imaging (e.g., Supplementary Figures S1 and S2), cells were washed with DPBS (3x), incubated with fresh medium without Anap for 2 hours, washed (3x) and flooded with imaging buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 2 mg/mL glucose, pH 7.4), and visualized by fluorescence microscopy. Alternatively, for confocal microscopy, cells were grown on glass coverslips (#1, Fisher Scientific), fixed using cold (-20 °C) methanol for 5 min, washed with DPBS (3x), mounted on a glass slide using Vectashield hard set mounting medium and imaged subsequently (e.g., experiments in Figures 3 and 4). Low magnification fluorescent images were taken with Nikon, Ti-E inverted microscope. The cells were either excited at 360 nm to acquire Anap fluorescence images at 470–510 nm or excited at 540 nm to acquire mCherry fluorescence images at 580–610 nm. Higher resolution pictures were acquired using a Zeiss 710 laser scanning confocal

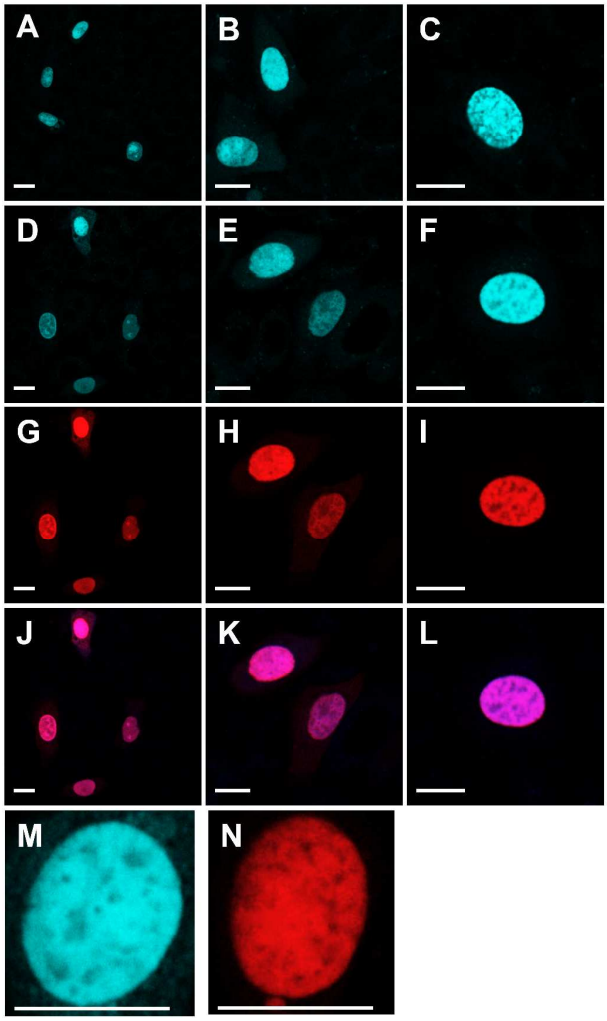
microscope (63x oil), equipped with a heated live cell imaging chamber. Fluorescent images using two-photon microscopy were acquired using a Leica SP5 confocal, two-photon microscope equipped with a Leica 63x / NA 0.9 water dipping objective. Illumination was provided by either a Spectra-Physics Mai-Tai Femtosecond IR Laser or a 543nm HeNe laser. The cells were either excited at 730 nm (two-photon) to acquire Anap fluorescence images at 420–500 nm or excited at 543 nm (one photon) to acquire mCherry fluorescence images at 600–700 nm. The imaging setup was controlled by Leica Application Suite-Advanced Fluorescence. Images were analyzed using ImageJ or Image Pro Plus software.



Supplementary Figure S1 Fluorescence images of labeled Histone H3 in CHO cells acquired with an inverted microscope**.** (A) CHO cells were grown in the presence of the $tRNA_{CUA}^{EcLeu}$/AnapRS pair and 0.5 mM Anap (excitation, 360 nm; emission 470-510 nm); (B) Bright field image for image A; (C) CHO cells with Thr59Anap-labeled histone H3; (D) Bright field image for image C; (E) Composite of images C and D; (F) CHO cells with Thr59Anap- and mCherry-labeled histone H3; (G) Bright field image for image F; (H) Composite of images F and G; (I) CHO cells with Thr59Anap- and mCherry-labeled histone H3 (excitation, 540 nm; emission 580-610 nm); (J) Composite of images G and I; (K) Composite of images F, G and I. Scale bars, 10 μm.



Supplementary Figure S2 AnapRS/$tRNA_{CUA}^{Leu}$ is able to maintain high fidelity and efficiency for amber suppression even at a low Anap concentration of 10 µM. (A) Fluorescence (top) and the composite of brightfield and fluorescence images (bottom) of HEK293 cells co-transfected with pAnap and pEGFP in the presence of different Anap concentrations in the media (indicated above). Robust EGFP expression is observed even at 10 µM Anap concentration, while little EGFP is expressed in its absence. (B) EGFP fluorescence (excitation 480 nm; emission 538 nm with a 530 nm cut-off filter) measured in cell free extracts prepared from 10^6 HEK293 cells for the experiment described above. The "no DNA" data point was obtained from an identical experiment, where HEK293 cells were not transfected with any plasmid. (C) Western-blot analysis for the expression of EGFP and γ-tubulin in cell free extracts prepared from 10^6 HEK293 cells co-transfected with pAnap and pEGFP and grown in the presence (+) or absence (-) of 10 µM Anap. (D) ESI-TOF MS analysis of EGFP-40-Anap, expressed in HEK293 cells in the presence of 10 µM Anap. Expected mass: 29773 Da, observed mass: 29775 Da



Supplementary Figure S3. Fluorescence images of labeled Histone H3 in CHO cells acquired with a confocal, two-photon microscope. (A) – (C) CHO cells with Thr59Anap-labeled histone H3 (excitation, 730 nm, two-photon; emission, 420–500 nm), without the mCherry label; (D) – (F) CHO cells with Thr59Anap- and mCherry-labeled histone H3 (excitation, 730 nm, two-photon; emission, 420–500 nm); (G) – (I) CHO cells with Thr59Anap- and mCherry-labeled histone H3 (excitation, 543 nm; emission, 600–700 nm); (J) Composite of images D and G; (K) Composite of images E and H; (L) Composite of images F and I; (M) CHO cells with Thr59Anap-labeled histone H3 (excitation, 730 nm, two-photon; emission, 420–500 nm); (N) CHO cells with Thr59Anap- and mCherry-labeled histone H3 (excitation, 543 nm; emission, 600–700 nm). Scale bars, 10 μm.

Supplementary Table 1. Viability of CHO cells grown under different conditions using tryptan blue assay.

	$tRNA_{CUA}^{Leu}$/ AnapRS	H3 (T59TAG)	mCherry	FugeneHD	Anap (0.5 mM)	Viable cells (%)
1	-	-	-	-	+	96
2	-	-	-	-	-	98
3	+	+	-	+	+	95
4	+	+	-	+	-	94
5	-	-	+	+	-	95
6	-	-	-	+	-	96

Sequence of pAnap derived from pCDNA3.1. H1 promoter regions are colored green; tRNACUAEcLeu encoding regions are colored orange; AnapRS gene is colored in orange and is shown in lowercase:

GACGGATCGGGAGATCTAATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAATCCCTATCAGTGA
TAGAGACTTATAAGTTCCCTATCAGTGATAGAGACACCGCCCGGATGGTGGAATCGGTAGACACAAGGGATTCTAAAT
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Sequence of pEGFP, derived from pCDNA3.1. EGFP (Tyr40TAG) gene is highlighted in green:

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTA
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