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Supplementary Information for

Enhanced incorporation of sub-nanometer tags into cellular proteins for fluorescence nanoscopy via optimized genetic code expansion

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Abbreviations and definitions

AF647 = Alexa Fluor 647
AF647$_{Az+}$ = azide-plus Alexa Fluor 647
ATP = adenosine triphosphate
BCN = Nε-Bicyclononyne-L-Lysine
BTTAA = 2-(4-((Bis((1-(*tert*-butyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid
ch = chimeric
CpK = Nε-cyclopropene-L-Lysine
CuAAC = copper-catalyzed azide-alkyne cycloaddition
DMSO = dimethyl sulfoxide
DNA = deoxyribonucleic acid
EF-Tu = elongation factor thermo unstable
eEF = eukaryotic elongation factor
EGTA = ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
GCE = genetic code expansion
GPCR = G-protein coupled receptor
HCl = hydrochloric acid
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
iEDDA = inverse electron-demand Diels-Alder reaction
IPTG = isopropylthio-β-galactoside
iRFP = infrared fluorescent protein
iRFP$_{713}$ = infrared fluorescent protein with maximum emission wavelength 713 nanometers
LB = Luria broth
M$_2$R = M2 muscarinic acetylcholine receptor
Mb = *Methanosarcina barkeri*
MES = 2-(*N*-morpholino)ethanesulfonic acid
Mm = *Methanosarcina mazei*
mRNA = messenger ribonucleic acid
ncAA = noncanonical amino acid
OD$_{XYZ}$ = optical density at wavelength XYZ nanometers
OmpC = outer membrane protein C
PACE = phage-assisted continuous evolution
PALM = photoactivated localization microscopy
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PFA = paraformaldehyde
PrK = Nε-propargyl-L-Lysine
Pyl = pyrrolysine
RIPA = radioimmunoprecipitation assay buffer
RS = aminoacyl-tRNA synthetase
RS$_{2020}$ = aminoacyl-tRNA synthetase with “2020” set of mutations (see **Fig. 3** or **Fig. S4.1**)
RS$_{IPYE}$ = aminoacyl-tRNA synthetase with “IPYE” mutations described in Bryson et al. (2017)
RS^{F} = aminoacyl-tRNA synthetase with “F” mutation described in Yanagisawa et al. (2008)
sfGFP = superfolder-green fluorescent protein
SiR = silicon rhodamine
STED = stimulated emission depletion microscopy
STORM = stochastic optical reconstruction microscopy
TBS = Tris-buffered saline
TBTA = Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TCEP = tris(2-carboxyethyl)phosphine
TCO = Nε-trans-Cyclooct-2-en-L-Lysine
TCP = targeted coordinate pattern
TEMPOL = 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
tRNA = transfer RNA

Technical replicate = separate coverslip from the same experiment performed on the same day
Biological replicate = distinct repetition of an experiment performed with a different cell passage on a different day

1 General materials & methods

Cell lines. For all cloning and DNA amplification, electrocompetent DH5α and electrocompetent Top10 *E. coli* were used. BL21(DE3) competent *E. coli* (Novagen, Merck KGaA, Darmstadt, Germany) were used for all bacterial protein expression experiments in this study (**Fig. 2E, Fig. 3D, Fig. 4C, SI Fig. S6.1-6.2**). All quantitative ncAA incorporation experiments in mammalian cells (**Fig. 2F, Fig. 3B-C, Fig. S3.1, Fig. S4.1-4.3**) and M_2R GPCR imaging experiments (**Fig. 4B & Fig. S5.1-5.4**) were done in HEK293T cells. β-actin imaging experiments (**Fig. 5, Fig. 6, Fig. 7, Fig. S7.1, Fig. S12.1, Fig. S13.2**) were done in U2OS cells (ATCC, Manassas, VA, USA). Mammalian cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose (ThermoFisher 31966047) and GlutaMAX (ThermoFisher, Waltham, MA, USA) supplemented with 10% [v/v] fetal bovine serum (Bio&SELL S 0615) and 100 U/ml penicillin + 100 µg/ml streptomycin (Sigma P0781), and 1 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO, USA).

Primers. All primers were ordered from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA) at 0.2 µmol scale at 100 µM in TE buffer with desalt purification. Primers were subsequently stored at -20°C.

Geneblocks. All geneblocks were ordered dry from Integrated DNA Technologies (Integrated DNA Technologies BVBA, Leuven, Belgium) and were resuspended in diH2O upon receipt at a concentration of 10 ng/µL and were then stored at -20°C.

DNA concentration and purity. All DNA concentrations were determined using a Nanodrop 1000 (PEQlab). For mammalian transfection experiments, all plasmids had an OD_{260}/OD_{280} ratio between 1.8 and 1.9.

Preparing electrocompetent *E. coli*. Fresh overnight culture of bacteria was inoculated at 1:100 dilution in LB medium and grown to an OD_{600} = 0.6-1.0. Then, they were incubated on ice for 15-30 minutes, pelleted, washed with H_2O (twice), washed with 10% glycerin (v/v, resuspended in 2% of the original volume of LB), and then resuspended in 10% glycerin (0.3% of the original volume of LB). Then, they were aliquoted into 50 µl portions and flash-frozen with liquid nitrogen. Cells were subsequently stored at -80°C until use.

Bacterial electroporation. All bacteria were transformed via electroporation. 50 µL aliquots of frozen bacteria in 10% glycerin were thawed from -80°C on ice for 15-20 minutes. DNA and 200µL of diH2O were added and tubes were flicked to mix. For amplification of Gibson Assembly products, 5 µL of the reaction product was added. For amplification of plasmids for cellular transfection, 0.5 ng of DNA were added. After electroporation, 1 mL of LB medium was immediately added to the electroporation product and the mixture was transferred to a shaker at 37°C for 1 hour. Finally, electroporation product was plated onto pre-warmed LB/Agar plates with the relevant antibiotic(s) for selection (for pULTRA, spectinomycin was added to a final concentration of 50 µg/mL; for pBAD or pET101, ampicillin was added to a final concentration of 100 µg/mL).

Transfection for GCE in mammalian cells. Cells on 12-well plates were transfected with 1 µg of protein-of-interest plasmid and 1 µg of the relevant GCE plasmid (encoding RS and tRNA). Cells on 24-well plates were transfected with 500 ng of protein-of-interest plasmid and 500 ng of the relevant GCE plasmid. Lipofectamine3000 (ThermoFisher Scientific, Waltham, MA, USA) was used at 1.5 µL of reagent per 1 µg of total DNA according to the manufacturer's protocol, except with a doubled volume of Opti-MEM medium (11058021, ThermoFisher Scientific, Waltham, MA, USA), i.e., final delivery volume of 200 µL per well for 12-well plates and 100 µL per well for 24-well plates. The transfection solution was then added into each well on top of the existing medium and plates were very gently shaken back and forth to mix. Cells were then incubated at 37°C, 5% CO_2.

DNA Sanger sequencing. All plasmids were sequenced by a commercial Sanger Sequencing service (Microsynth Seqlab, Göttingen, Germany).

DNA amplification/purification. After Gibson Assembly, products were transformed into DH5α *E. coli* via electroporation and single colonies were amplified using the QIAGEN Plasmid Miniprep Kit (QIAGEN Inc., Germantown, MD, USA). Sequences were confirmed via Sanger sequencing. Those which were to be used for bacterial transformation were further amplified via the QIAGEN Plasmid Plus Midi Kit (QIAGEN Inc., Germantown, MD, USA), while those for mammalian transfection were amplified via the GenElute Endotoxin-free Plasmid Midiprep Kit (Sigma Aldrich, St. Louis, MO, USA).

Gibson assembly. First, the vector for Gibson Assembly was amplified from 10 ng of starting plasmid using the Q5 High-Fidelity 2X Master-Mix (New England Biolabs GmbH, Frankfurt am Main, Germany), with forward and reverse primer concentrations of 500 nM in a final volume brought up to 50 µL with diH_2O. A touchdown PCR protocol was utilized, consisting of an initial 3-minute denaturing step at 98°C followed by 19 cycles of: 1) 10 second denaturing at 98°C, 2) 30 second annealing, 3) 8-minute extension at 72°C. During the first cycle, annealing was performed at 65°C. With each cycle, the annealing temperature was lowered by 0.5°C until a final annealing temperature of 55°C was reached. Finally, 14 extra cycles were done with a 55°C annealing temperature. After amplification, the PCR product was run on a 0.8% agarose gel stained with GelGreen Nucleic Acid Gel Stain (Biotium Inc., Fremont, CA, USA). The desired band was cut from the gel and DNA was extracted using the NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel). Gibson Assembly was then performed using the NEBuilder HiFi DNA Assembly Kit (New England Biolabs GmbH, Frankfurt am Main, Germany). 75 ng of vector DNA purified from the gel was combined in a 2:1 ratio of Geneblock insert to vector (computed via NEBioCalculator DNA Ligation Calculator). The final volume was brought up to 20 µL and the mixture was incubated at 37°C for 1 hour before transformation. All unused product was stored at -20°C.

Bacterial ncAA incorporation. Reporter and GCE plasmids were electroporated into BL21-DE3 *E. coli*. Single colonies were picked, and primary cultures were grown in 5 mL of LB supplemented with relevant antibiotics for 16 hours at 37°C. Subsequently, overnight colonies were diluted 1:100 in 250 µL of LB medium supplemented with antibiotics in wells of a 24-well plate. Cells were then incubated in a shaker at 37C for 3.5 hours. During this incubation, additional LB medium was prepared that was supplemented with antibiotics, inducers, and ncAA. For expressions of pULTRA and pET101 vectors, IPTG was added at a final concentration of 2 mM from a 1M aqueous stock. For expression of pBAD vectors, arabinose was added at a final concentration of 3 mM from a 1.33 M aqueous stock. To add ncAA to LB, a 250 mM stock solution of ncAA was freshly prepared in 1N NaOH. This solution was diluted in LB to achieve the desired final concentration of ncAA. An equal volume of 1N HCl was added to neutralize the pH (0.75x the volume of 1N HCl was added if the ncAA was PrK, which was purchased as an HCl salt). After the 3.5-hour incubation, 250 µL of the LB medium supplemented with inducers and ncAA was added to the corresponding wells of the 24-well plate and cells were incubated overnight at 37°C in a shaker at 37°C for 24 hours.

Mammalian ncAA incorporation. 100 mM stocks of ncAA were freshly prepared in 15% DMSO/85% 0.2N NaOH (v/v) and were added to growth medium along with HEPES buffer, such that the HEPES:ncAA stock ratio (v:v) was 3:1 and such that the concentration of the ncAA was 4x the desired final concentration. For cells on 24-well plates 250 µL of this solution was gently added to each well, on top of the existing medium, while for cells on 12-well plates 500 µL of this solution was added. Cells were incubated at 37°C, 5% CO_2 for 60 minutes.

Stock solutions for labeling.
0.5% casein blocking buffer (w/v in PBS). Prepared as described in cited reference.[1]
1.7 mM TBTA (ABCR GmbH, Karlsruhe, Germany) dissolved in 20% (v/v) DMSO in *t*-butanol*

85 mM BTTAA (Click Chemistry Tools, Scottsdale, AZ, USA) aqueous stock**
5 mM copper sulfate (Merck) aqueous stock***
20 mM copper sulfate aqueous stock***
100 mM hydroxylamine-HCl (Acros organics, Belgium) aqueous stock†
100 mM sodium ascorbate (Fluka, Buchs, Switzerland) stock†
100 mM TEMPOL (Sigma Aldrich, St. Louis, MO, USA) stock†
5 mM DMSO stock of picolyl-azide SiR* (provided by Alexey Butkevich, Max Planck Institute for Medical Research, Heidelberg, Germany)
5 mM DMSO stock of azide plus AlexaFluor 647* (Click Chemistry Tools, Scottsdale, AZ, USA)
5 mM DMSO stock of tetrazine-silicon rhodamine* (Spirochrome AG, Stein am Rhein, Switzerland)

†prepared fresh
*stored at -20°C for <6 months
**stored at -20°C for <3 months
***stored at 4°C for <3 months

PrK labeling (mammalian, fixed). Based on a previously described protocol,[1] per 1 mL of labeling solution: 50 µL of 20 mM $CuSO_4$ stock was mixed with 58.82 µL of 1.7 mM TBTA stock. 25 µL of hydroxylamine stock was added and the tube was capped, wrapped with parafilm, and flicked to mix. The mixture was incubated at room temperature for 10 minutes. Subsequently, 866.18 µL of casein blocking buffer and 1 µL of azide dye stock (either picolyl-azide silicon rhodamine or azide-plus AlexaFluor647 specified for each experiment) were added and the tube was vortexed to mix. Labeling solution was added to cells for 120 minutes and vessels were lidded, wrapped with parafilm, and covered with aluminum foil to minimize oxygen and light exposure. Finally, cells were rinsed in casein blocking buffer with gentle rocking for 5 minutes (repeated 3 times).

PrK labeling (mammalian, live). Based on a previously described protocol,[1] per 1 mL of labeling solution: 20 µL of 5 mM $CuSO_4$ stock was mixed with 5.88 µL of BTTAA stock. 50 µL of sodium ascorbate stock and 2 µL of TEMPOL stock were added to the mixture. The tube was capped, wrapped with parafilm, vortexed to mix, and then incubated at room temperature for 10 minutes. 922.12 µL of PBS pre-warmed to 37°C and 2 µL of azide-dye stock were added to the labeling tube. The labeling solution was vortexed to mix. Cells were rinsed two times with PBS pre-warmed to 37°C after which labeling solution was distributed to cells. The plate was covered with aluminum foil and incubated at room temperature for 10 minutes. After 10 minutes, labeling solution was removed and 500 µM bathocuproine sulfonate (Alfa Aesar, Tewksbury, MA, USA) in growth medium (pre-warmed to 37°C) was added to each well. Cells were incubated at room temperature for 1 minute, after which they were rinsed with the pre-warmed growth medium three times. Finally, cells were incubated at 37°C, 5% CO_2 for 60 minutes before fixation.

BCN/CpK/TCO labeling (mammalian, live). Based on a previously described protocol,[2] per 1 mL of labeling solution: 1 µL of tetrazine dye stock was added to 900 µL of PBS pre-warmed to 37°C and the solution was mixed by vortex. Cells were rinsed two times with PBS pre-warmed to 37°C after which labeling solution was added and plates were covered with aluminum foil. After a 20-minute incubation at room temperature, cells were rinsed with growth medium pre-warmed to 37°C three times and were then incubated at 37°C, 5% CO_2 for 60 minutes before fixation.

M_2R expression and labeling. 18mm round 1.5H coverslips in a 12 well-plate were coated with 100 mg/L poly-$_D$-Lysine mol wt 70,000-150,000 (Sigma Aldrich, St. Louis, MO, USA) dissolved in diH_2O for 2 hours at 37°C. Coverslips were then rinsed with PBS three times and with growth medium two times, such that each well contained 800 µL of growth medium. 70-80% confluent HEK293T cells were resuspended and diluted in fresh growth medium to $5x10^5$ cells/mL. 500 µL of cell suspension were deposited to each well and cells were incubated for 2 days at 37°C, 5% CO_2. ncAA was incorporated

as described above in the “mammalian ncAA incorporation” protocol. Subsequently, cells were transfected and were incubated for 16-24 hours. Next, cells were rinsed with fresh growth medium (3x) and incubated for 1 hour at 37°C, 5% CO_2 (repeat twice). Cells were then labeled via either the PrK labeling (mammalian, live) protocol with azide-plus AlexaFluor647 or the BCN labeling (mammalian, live) protocol with tetrazine-silicon rhodamine, after which they were fixed with 4% PFA (prepared from formaldehyde, K51726405 104, Sigma, Darmstadt, Germany) for 20 minutes at room temperature. Cells were then rinsed with PBS, washed with 50 mM NH_4Cl for 5 minutes, rinsed again with PBS (three times), and imaged.

OmpC expression and labeling. Single colonies of transformed E. coli were picked, and primary cultures were grown in 5 mL LB medium supplemented with relevant antibiotics at 37°C overnight. Subsequently, a secondary culture was prepared in 5 mL LB medium supplemented with the relevant antibiotics. The primary culture was diluted 1:100 in this fresh medium and was incubated for 2.5 hours at 37°C. During incubation, additional LB medium was prepared that was supplemented with antibiotics, IPTG inducer, ncAA, and HCl to neutralize PrK. The ncAA was added from a freshly prepared 250 mM stock solution in 1 N NaOH. The supplemented LB medium had a final PrK concentration of 5 mM, 15 mM HCl, and 200 mM IPTG. 5 mL of this medium was added to the secondary culture and was incubated overnight. On the following day, to wash out excess PrK, the cells were pelleted and washed once per hour over 5 hours with fresh LB medium. After washing, the cells were re-suspended in M9/0.8% glycerol medium supplemented with the relevant antibiotics and stored at 4°C on a gentle shaker overnight. The next day, OD_{600} was measured, cells were pelleted for 10 minutes and were resuspended in 0.5% casein buffer so that the OD_{600} for each condition = 3. While the bacteria were being spun down, 50 µL of 20 mM copper sulfate stock was combined with 5.88 µL of 85 mM BTTAA stock in a 1.7-mL tube. 25 µL of 100 mM hydroxylamine was added and the tube was capped, wrapped in parafilm, flicked to mix, and incubated for 10 minutes. Subsequently, 866.16 µL of the re-suspended bacteria and 2 µL of 5 mM azide plus AlexaFluor 647 were added. The tube was briefly vortexed and was covered in aluminum foil. It was then incubated while gently shaking for 20 minutes. The bacteria were then washed with M9/0.8% glycerol every hour over the next 5 hours. 80 µL of bacterial solution was transferred to a coverslip in a Petri dish with wet KimWipes and was left to incubate for 30 minutes at room temperature. The coverslips were then rinsed with PBS three times. Cells were fixed with 4% PFA for 20 minutes. Subsequently, the cells were rinsed with PBS three times and were mounted onto microscope slides with PBS.

β-actin expression and labeling. 800 µL of growth medium was added to 18mm 1.5H coverslips (high precision, 0117580 Marienfeld, Lauda-Königshofen, Germany) in a 12 well-plate. 70-80% confluent U2OS cells were resuspended and diluted in fresh growth medium to $5x10^5$ cells/mL. 500 µL of cell suspension were deposited to each well and cells were incubated for 2 days at 37°C, 5% CO_2. 100 mM stocks of ncAA were freshly prepared in 15% DMSO (Acros Organics)/85% 0.2N NaOH (v/v) and were added to growth medium along with HEPES (A3724, PanReac AppliChem, Darmstadt, Germany), such that the HEPES:ncAA stock ratio (v:v) was 3:1 and such that the concentration of the amino acid was 4x the desired final concentration. 500 µL of this solution was then gently added to each well, on top of the existing medium, and cells were incubated at 37°C, 5% CO_2 for 60 minutes. Subsequently, cells were transfected and were incubated for 16-24 hours. Next, cells were rinsed with fresh growth medium (3x) and incubated for 1 hour at 37°C, 5% CO_2 (repeat twice). Cells were rinsed two times with PBS pre-warmed to 37°C. Cells were then extracted and fixed as described in a previously published protocol.[3] PBS was removed and replaced with an extraction solution: 5 mM glucose, 0.25% Triton X-100 (w/v, K42092903 350, Merck, Darmstadt, Germany), 0.3% glutaraldehyde (R1020, Agar Scientific, Essex, UK) dissolved in cytoskeleton buffer (10 mM MES pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$). After 10 minutes at room temperature, extraction solution was very slowly removed from each well and fixation solution (5 mM glucose, 2% glutaraldehyde dissolved in cytoskeletal buffer) was added for 10 minutes. Wells were then rinsed with PBS three times and with TBS. Cells were then incubated

with 0.5% TCEP (Sigma Aldrich, St. Louis, MO, USA) in TBS for 3 hours after which they were rinsed with PBS three times. The PBS was then replaced with 1% $NaBH_4$ (Fluka, Buchs, Switzerland) in PBS for 30 minutes. During this time, coverslips were manually kept down in the solution with a pipette tip (bubbles occasionally push them up to the surface). Coverslips were then rinsed with PBS, after which they were transferred to a new plate and rinsed again twice with PBS. Coverslips were gently rocked in the second PBS wash for 15 minutes, after which they were again rinsed with PBS three times. Finally, coverslips were blocked with 0.5% casein blocking buffer for 1 hour, after which they were labeled via PrK labeling (PrK labeling (mammalian, fixed) protocol) with picolyl-azide silicon rhodamine for STED or azide-plus AlexaFluor647 for MINFLUX. Coverslips were then rinsed with 1% Triton X-100 three times and with PBS three times before they were mounted and imaged.

Confocal and STED imaging. Confocal and STED images were captured with an Abberior Instruments Expert Line STED Microscope. A 640 nm excitation laser was used together with a 775 nm 40 MHz STED laser. When relevant, DAPI was excited using a 405 nm light-emitting diode. Confocal images were acquired with a 50 nm pixel size and a 10 µsec dwell time per pixel. STED images were acquired with a 15 or 20 nm pixel size with 20 µsec dwell time per pixel.

3D MINFLUX sample stabilization. To stabilize the samples during MINFLUX imaging, all coverslips were treated with gold nanorods (A12-25-980-CTAB-DIH-1-25, Nanopartz Inc., Loveland, CO, USA) as fiducial markers. The commercial nanorod suspension was diluted 1:3 in phosphate buffered saline (P4417, Sigma-Aldrich, St. Louis, MO, USA) and was then sonicated for ~10 minutes. 300 µl of this solution was then added to biological samples for 10-40 minutes at room temperature. Afterwards, the samples were washed three to four times with PBS.

3D MINFLUX sample mounting. A standard STORM blinking buffer was used, containing 0.4 mg/ml glucose oxidase (G2133, Sigma-Aldrich, St. Louis, MO, USA), 64 µl/ml catalase (C100-50MG, Sigma-Aldrich, St. Louis, MO, USA), 50 mM Tris-HCl pH 8.0, 10 mM NaCl, 50 mM MEA (cysteamine hydrochloride, M6500, Sigma-Aldrich, St. Louis, MO, USA) and 10% (w/v) glucose. The coverslips were mounted on single-welled microscope slides together with the imaging buffer and sealed with picotwinsil speed 22 (picodent Dental-Produktions- und Vertribes-GmbH, Wipperfürth, Germany).

3D MINFLUX imaging. For 3D-MINFLUX imaging, a previously described instrument[4] was used and the performance of the microscope was validated daily. The photon count time traces were processed as previously described[4], resulting in several localizations per emission event. The online detection of fluorophore emission was realized with a static photon count threshold. We chose a low threshold to avoid missing valid localizations, hence we had to remove background contributions from the resulting localizations. We used previously described metrics[4] to judge whether a localization was dominated by the emission of a single molecule or by background. Background emission usually leads to localizations far from the center of the MINFLUX targeted coordinate pattern (TCP) and to similar emission probabilities in all exposures (emission probability in exposure K when having in total 7 exposures: $p_K = \frac{1}{7}$). Thus, we discarded localizations at a distance r_{rel} > 35 nm from the TCP center as well as localizations with emission probabilities in the central exposure of p_0 > 0.125. To guarantee a certain localization precision, localizations with photon number < 1000 in the last iteration were omitted. To estimate the localizations of the fluorophores we employed a grid-search based maximum-likelihood estimator. Localizations that coincide with at least one border pixel in the searching grids were discarded, as convergence of the maximum-likelihood estimator cannot be ensured in these cases.

3D MINFLUX precision estimation. In the majority of recorded single molecule blinking events, multiple independent MINFLUX iterations could be performed during the event. That allows direct measurement of the localization precision by estimating the standard deviation in 3D of MINFLUX localizations corresponding to a single blinking event. The given precisions in the main text are the

median values for these standard deviations over all recorded blinking events where each event showed more than three MINFLUX localizations.

3D MINFLUX localization combination. In the analysis of the raw data stream, positions were estimated within certain time intervals corresponding to equal numbers of detected photons. The information about the molecule (uninterrupted photon traces) from which these localizations originated was preserved as the trace-id number. For the combined localizations, the average value of all localization positions with the same trace id was taken.

MINFLUX plot-profile analysis. To generate plot profiles of MINFLUX localizations (**Fig. 6A, Fig. 7B, Fig. S12.1**), localizations were rotated in *xy* to position them parallel to the axis being integrated for plot profile generation. They were then binned into pixels, as specified for each experiment, and analyzed in ImageJ using the rectangular selection tool. Plot profiles were then generated without interpolation and data were exported. Absolute localization counts were obtained by multiplying profile values with the length of the rectangular selection in pixels (Fig. 6A: 6 px, otherwise: 66 px).

3D MINFLUX rendering. MINFLUX localizations were drift-corrected (see **SI §11**) and were sorted into $(2\ \text{nm})^3$ voxels. The voxels intensities were blurred in *yz* and *xy* with a Gaussian (2.0 nm standard deviation). Voxel colors were then assigned by their z-position or y-position. RGB values were then normalized by the square-root of the relative voxel intensity (where maximum intensity = 1) and resulting images were rendered with ImarisViewer 9.8.0. Given the high magnification, voxels in the expanded field of **Fig. 6B** were normalized by the relative voxel intensity, not the square-root of the relative voxel intensity.

2 Measuring relative noncanonical amino acid incorporation efficiencies into sfGFP in *E. coli*

2.1 Assay overview

Incorporation of ncAAs in *E. coli* was assessed via a previously described sfGFP assay (**Fig. S2.1**).[5] A UAG stop codon is cloned into sfGFP, replacing asparagine at position 150. Since this position is upstream of the fluorochrome in the protein, unsuccessful ncAA incorporation will result in translation termination, leading to a truncated, non-fluorescent product. On the other hand, incorporation of ncAA at position 150 allows for full-length expression of fluorescent sfGFP. In this way, more efficient tRNA/RS pairs allow for more fluorescent signal per cell. All fluorescence was normalized by OD_{600} to account for inter-condition variation in the number of *E. coli* cells expressing protein.

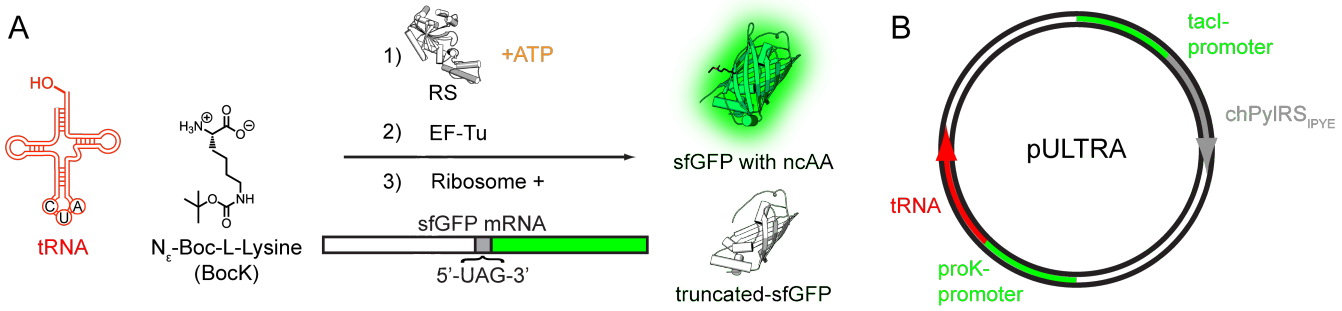

Fig. S2.1 | Assay and GCE plasmid used to test synthetase/tRNA pairs in *E. coli*. NB- this assay was used in main text Fig. 2 and Fig. 3. **A)** First, the aminoacyl-tRNA synthetase uses ATP to generate an aminoacyl-tRNA molecule. Second, the aminoacyl-tRNA enters the A-site of the ribosome with the help of EF-Tu and the ncAA is incorporated when the UAG codon of the mRNA is reached in the ribosome. **B)** Plasmid overview of the pULTRA vector used to encode the RS/tRNA pairs for bacterial genetic code expansion.

2.2 Plasmids

tRNA/RS plasmids:

pULTRA-chPylRS$_{IPYE}$/tRNA^{Pyl*} (Plasmids section, Plasmid E1)
pULTRA-chPylRS$_{IPYE}$/tRNA^{M15} (Plasmids section, Plasmid E2)

sfGFP reporter plasmids:

pBAD-sfGFP$_{WT}$ (Plasmids section, Plasmid A1)
pBAD-sfGFP$_{N150*}$ (Plasmids section, Plasmid A2)

2.3 Preparation and measurement protocol

Spectroscopic measurement parameters. sfGFP fluorescence intensity and OD_{600} were measured on a Tecan Spark 20M plate reader. To measure sfGFP intensity, an excitation wavelength of 483 nm was used with 5 nm bandwidth, and an emission wavelength of 508 nm was used with 5 nm bandwidth. Per measurement, 30 flashes were performed with an integration time of 40 µsec and a manual gain of 100. A fixed z position was used across all measurements. Absorbance at 600 nm was measured with 10 flashes and a 300 msec settle time.

Sample preparation and measurement protocol. Samples were prepared according to the "bacterial ncAA incorporation" protocol (see materials & methods section). Bacteria were transformed with both a tRNA/RS plasmid and a sfGFP reporter plasmid. All medium was supplemented with spectinomycin

to a final concentration of 50 µg/mL and ampicillin to a final concentration of 100 µg/mL. After 24-hour overnight incubation, 150 µL of LB medium were added to wells of a 96-well plate and measured on the plate reader as described above, serving as a “blank”. Subsequently, 75 µL of LB medium was removed and 75 µL of desired overnight bacterial suspension was added to the corresponding wells. The solution was mixed by pipetting up and down several times, and then measured on the plate reader as described above. The measurements for the blanks were then subtracted from those of the samples. Each plate featured a sample of wild-type pBAD-sfGFP as an internal control.

3 Measuring relative noncanonical amino acid incorporation efficiencies into iRFP$_{713}$-sfGFP in mammalian cells

3.1 Assay overview

Incorporation of ncAAs in HEK cells was assessed using a modified version of a previously described iRFP$_{713}$-sfGFP assay (**Fig. S3.1A**).[6] Briefly, in an iRFP$_{713}$-sfGFP fusion protein, a UAG stop codon is cloned into the sfGFP, replacing Tyr at position 151 of sfGFP. Unsuccessful ncAA incorporation results in translation termination, leading to expression of iRFP$_{713}$ with a C-terminally truncated, non-fluorescent sfGFP fragment. On the other hand, incorporation of ncAA at position 151 allows for full-length expression of fluorescent iRFP$_{713}$-sfGFP. Since iRFP$_{713}$ and sfGFP fluorescence are found to scale linearly with concentration in the experimental protein concentration ranges (**Fig. S3.1C**), the sfGFP fluorescence is then normalized to the iRFP$_{713}$ fluorescence to determine the proportion of protein molecules that have had ncAA incorporated.

3.2 Plasmids

tRNA/RS plasmids:

pNEU-*Mb*PylRS^{F}/tRNA^{M15} (Plasmids section, Plasmid M01)
pNEU-chPylRS$^{F}{}_{IPYE}$/tRNA^{M15} (Plasmids section, Plasmid M03)
pNEU-chPylRS$^{F}{}_{IPYE}$/tRNA^{Pyl*} (Plasmids section, Plasmid M04)

iRFP$_{713}$-sfGFP reporter plasmids:

pNEU-iRFP$_{713}$-sfGFP$_{WT}$ (Plasmids section, Plasmid M26)
pNEU-iRFP$_{713}$-sfGFP$_{Y151*}$ (Plasmids section, Plasmid M27)

3.3 Preparation and measurement protocol

Spectroscopic measurement parameters. sfGFP and iRFP$_{713}$ fluorescence intensity were measured on a Tecan Spark 20M plate reader. To measure sfGFP intensity, an excitation wavelength of 473 nm was used with 20 nm bandwidth, and an emission wavelength of 518 nm was used with 20 nm bandwidth. Per measurement, 30 flashes were performed with an integration time of 40 µsec and a manual gain of 75. To measure iRFP$_{713}$ intensity, an excitation wavelength of 688 nm was used with 20 nm bandwidth, and an emission wavelength of 743 nm was used with 30 nm bandwidth. Per measurement, 30 flashes were performed with an integration time of 40 µsec and a manual gain of 150. All conditions for each biological replicate were measured at the same time on the same 96-well plate. A fixed z position was used across all measurements. For each replicate, the z-position of the measurement was optimized from the condition treated with the *M. barkeri* synthetase and the maximal amount of ncAA. The same z-position was used to measure each well of the 96-well plate.

Sample preparation and measurement protocol. Wells of 24 well-plates were coated with 100 mg/L poly-$_{D}$-Lysine mol wt 70,000-150,000 (Sigma Aldrich, St. Louis, MO, USA) dissolved in diH_2O for 2 hours at 37°C. Coverslips were then rinsed with PBS three times and with growth medium two times, such that each well contained 400 µL of growth medium. 70-80% confluent HEK293T cells were resuspended and diluted in fresh growth medium to 5x10^5 cells/mL. 250 µL of cell suspension were deposited to each well and cells were incubated for 2 days at 37°C, 5% CO_2. ncAA was incorporated as described above in the “mammalian ncAA incorporation” protocol for 24-well plates (see Materials & Methods). Subsequently, cells were transfected as described in the “transfection for GCE in mammalian cells” protocol (see Materials & Methods) and were incubated for 40-44 hours. Two hours prior to the end of this incubation, PBS and RIPA-lysis buffer with protease inhibitor pill were stored on ice. After the end of the incubation, cells were rinsed with ice-cold PBS (2x) and in each well the PBS

was replaced with 230 µL of the RIPA buffer solution. The plate was kept on ice and gently shaken for 15 minutes. Afterwards, the lysate from each well was transferred to a 1.7-mL tube and centrifuged at 14,000xg for 15 minutes. Finally, 50 µL of each supernatant was transferred to a 96-well plate and measured on the plate reader as described above.

3.4 Calculating relative fluorescent protein concentration from measured fluorescence intensity

At low protein concentrations, fluorescence intensity should scale linearly with protein concentration. To determine if the preparation protocol described above yields samples that fall within this linear range, a full length, "wild-type," iRFP$_{713}$-sfGFP construct was transfected into mammalian cells as described above. At the end of the sample preparation protocol, the supernatant was serially diluted (1:2) to generate a range of samples ranging in concentration from the original tube to a 1:512 dilution of the same tube. A linear relationship was expected between the relative protein concentration and the fluorescence intensity of that sample. Indeed, this was observed over three replicates **(Fig S 3.1C)**. An average regression for sfGFP and iRFP$_{713}$ was then used to calculate relative concentrations of the two proteins in each sample **(eqn 1, Fig. S 3.1E).** Given that a perfectly efficient enzyme would result only in expression of full-length iRFP$_{713}$-sfGFP (i.e., without any truncation product featuring fluorescent iRFP and non-fluorescent sfGFP), a perfectly efficient GCE pair would result in [sfGFP]/[iRFP$_{713}$] = 1. Conversely, a totally ineffective GCE pair would lead to expression of only truncation product and would

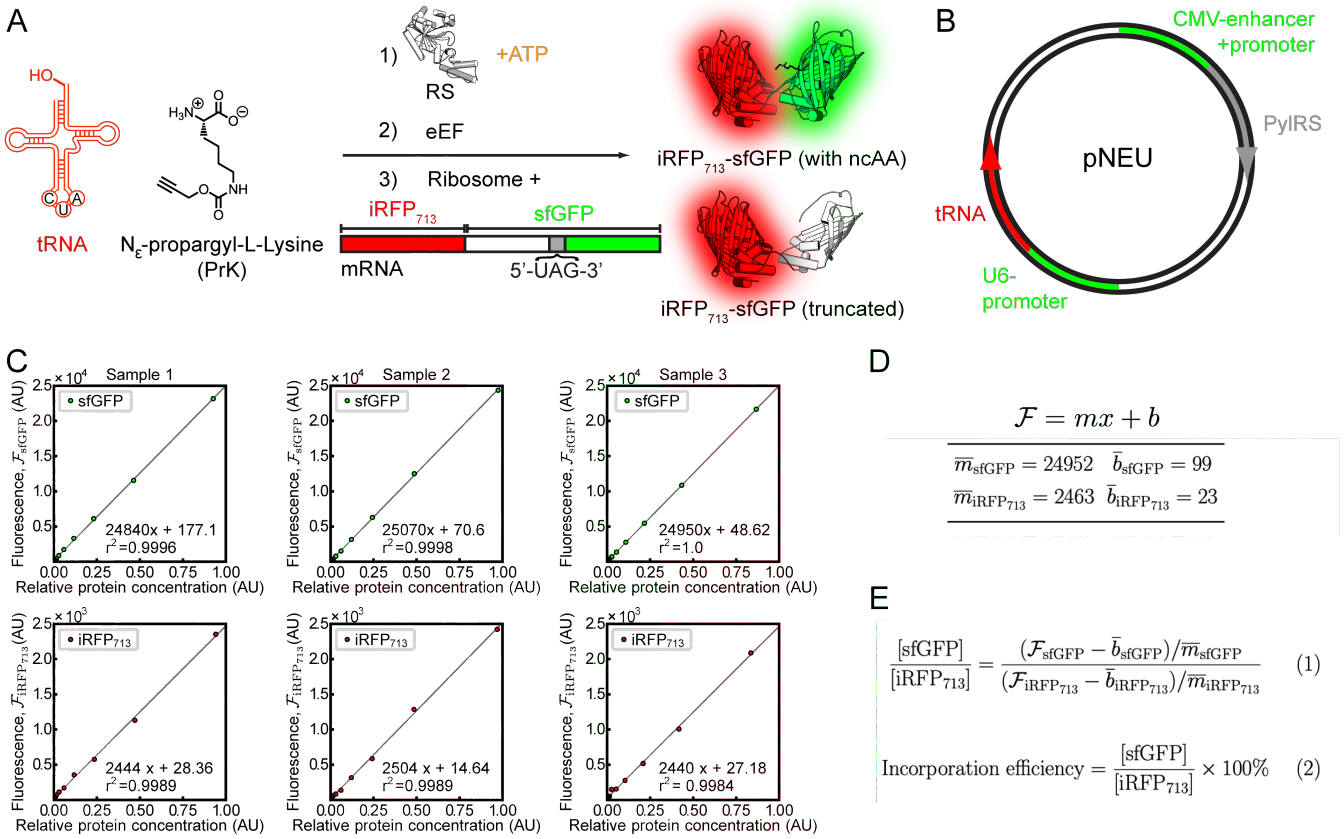

Fig. S3.1 | Assessing noncanonical amino acid incorporation efficiency in mammalian cells. (A) Incorporation efficiency was tested with an iRFP$_{713}$-sfGFP$_{Y151*}$ fusion protein. Successful ncAA incorporation would result in expression of iRFP$_{713}$ with fluorescent sfGFP, while unsuccessful incorporation would lead to expression of iRFP$_{713}$ with truncated, non-fluorescent sfGFP. **(B)** Overview of the pNEU vector used to express GCE machinery for mammalian incorporation. **(C)** To relate fluorescent protein concentration with measured fluorescence intensity, three serial dilutions of full-length, wild-type iRFP$_{713}$-sfGFP were performed. Fluorescence was found to scale linearly with concentration in all cases. **(D)** Average regressions relating fluorescence intensity with fluorescent protein concentrations. **(E)** Measured fluorescent intensity and average regressions are used to calculate incorporation efficiency.

thus result in [sfGFP]/[iRFP$_{713}$] = 0. Thus, incorporation efficiency was defined as the ratio of [sfGFP]/[iRFP$_{713}$] **(eqn 2, Fig. S 3.1E)**.

4 Mutation analysis and generation of chPylRS variants

4.1 Plasmids

pNEU-*Mb*PylRS^{F} + tRNA^{M15} (Plasmids section, Plasmid M01)
pNEU-*Mm*PylRS^{F} + tRNA^{M15} (Plasmids section, Plasmid M02)
pNEU-chPylRS$^{F}_{2020}$ + tRNA^{M15} (Plasmids section, Plasmid M14)
pNEU-*Mb*BCNRS + tRNA^{M15} (Plasmids section, Plasmid M19)
pNEU-*Mm*BCNRS + tRNA^{M15} (Plasmids section, Plasmid M20)
pNEU-chBCNRS$_{2020}$ + tRNA^{M15} (Plasmids section, Plasmid M21)
pNEU-*Mb*PylRS^{AF} + tRNA^{M15} (Plasmids section, Plasmid M22)
pNEU-*Mm*PylRS^{AF} + tRNA^{M15} (Plasmids section, Plasmid M23)
pNEU-chPylRS$^{AF}_{2020}$ + tRNA^{M15} (Plasmids section, Plasmid M24)
pNEU-chPylRS$^{F}_{IPYE}$ + tRNA^{M15} (Plasmids section, Plasmid M03)
pNEU-chPylRS$^{F}_{IPYE}$ (+D2N) + tRNA^{M15} (Plasmids section, Plasmid M05)
pNEU-chPylRS$^{F}_{IPYE}$ (+K3N) + tRNA^{M15} (Plasmids section, Plasmid M06)
pNEU-chPylRS$^{F}_{IPYE}$ (+E119K) + tRNA^{M15} (Plasmids section, Plasmid M07)
pNEU-chPylRS$^{F}_{IPYE}$ (+S158N) + tRNA^{M15} (Plasmids section, Plasmid M08)
pNEU-chPylRS$^{F}_{IPYE}$ (+D161N) + tRNA^{M15} (Plasmids section, Plasmid M09)
pNEU-chPylRS$^{F}_{IPYE}$ (+K258Q) + tRNA^{M15} (Plasmids section, Plasmid M10)
pNEU-chPylRS$^{F}_{IPYE}$ (+G343D) + tRNA^{M15} (Plasmids section, Plasmid M11)
pNEU-chPylRS$^{F}_{IPYE}$ (+S158N+D161N) + tRNA^{M15} (Plasmids section, Plasmid M12)
pNEU-chPylRS$^{F}_{IPYE}$ (+S158N+G343D) + tRNA^{M15} (Plasmids section, Plasmid M13)
pNEU-chPylRS$^{F}_{IPYE}$ (+S158N+D161N+G343D) + tRNA^{M15} (Plasmids section, Plasmid M15)
pNEU-iRFP$_{713}$-sfGFP(Y159*) (Plasmids section, Plasmid M27)

4.2 Mutational analysis of chPylRS variants

chPylRS$_{IPYE}$ was identified through phage-assisted continuous evolution (PACE) by Bryson et al[7] and features four mutations: V31I, T56P, H62Y, A100E.

In a study by Suzuki et al.,[8] three additional variants of chPylRS were identified: chPylRS$_{32A}$, chPylRS$_{24B}$, chPylRS$_{25C}$. All featured the H62Y mutation. chPylRS$_{32A}$ & chPylRS$_{24B}$ featured the T56P mutation from PACE (chPylRS$_{25C}$ featured a T56A variant). chPylRS$_{25C}$ also featured a frameshift with a R100N mutation, of which two nucleotide mutations fell in the same codon as that encoding A100E in chPylRS$_{IPYE}$. chPylRS$_{32A}$ also featured the previously described activity enhancing Y349F mutation.[9] In addition, the three variants featured the mutations shown in **Fig. S4.1A.**

We sought to determine whether any of the mutations from these three enzyme variants could be added onto chPylRS$^{F}_{IPYE}$ to further enhance its incorporation of the clickable amino acid, PrK. Each of the identified individual mutations were added into the plasmid encoding chPylRS$^{F}_{IPYE}$ + tRNA^{M15} (see Plasmids section, Plasmids M05-M11) and were screened via the assay described in SI Section 3. Addition of S158N, D161N, and G343D substantially enhanced incorporation efficiency (**Fig. 3B**).

To see if these mutations could be combined, all combinations of these three variants were generated (see Plasmids section, Plasmids M15-M18). Furthermore, variants of chPylRS$^{F}_{IPYE}$ featuring the full sets of additional mutations from chPylRS$_{32A,}$ chPylRS$_{24B,}$ and chPylRS$_{25C}$ were generated (see Plasmids section, Plasmids M12-M14) and compared. Out of all the enzymes, chPylRS$^{F}_{IPYE}$ with the D161N and G343D mutations performed most efficiently (**Fig. S4.1B-D**). This enzyme was chosen for all subsequent experiments and was renamed chPylRS$^{F}_{2020}$.

A

chPylRS$_{32A}$	chPylRS$_{32A\text{-}Nter}$	chPylRS$_{24B}$	chPylRS$_{25C}$
D2N	D2N		
K3N	K3N	K3N	K3N
E119K		S158N	D161N
K258Q		G343D	G343D

Additional mutations (excluding those at residues 56, 62, 100, 349.

B

chPylRS$_{2020}$ final mutation list

V31I
T46P
H62Y
A100E
D161N
G343D

chPylRS$^{F}_{2020}$= chPylRS$_{2020}$ with Y349F

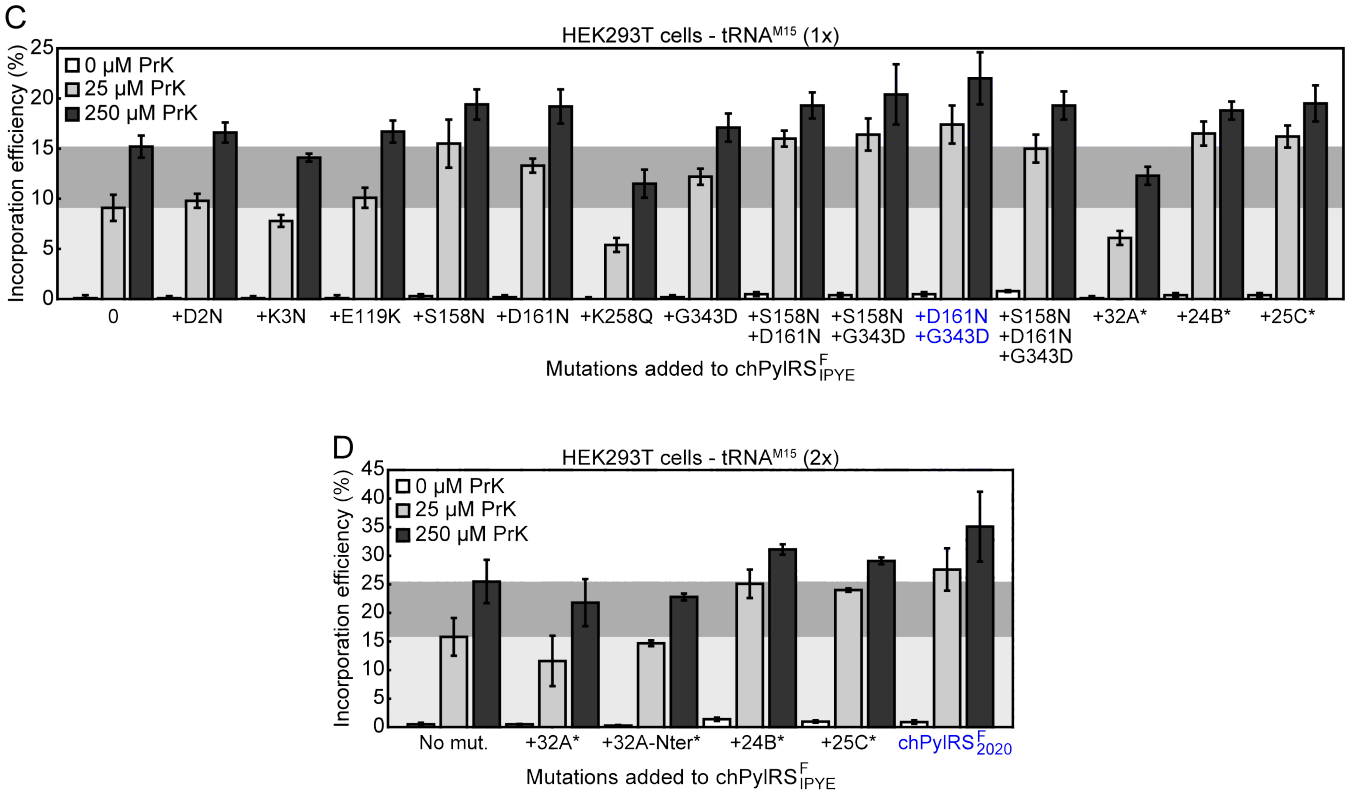

Fig. S4.1 | Mutation screening and generation of chPylRS$^{F}_{2020}$. A) Mutations in chPylRS from Suzuki et al.[8], excluding those at positions mutated in chPylRS$_{IPYE}$ and the activity-enhancing mutation Y349F. **B)** Mutations in the final chPylRS$^{F}_{2020}$ enzyme described in the main text. **C)** Combinatorial screening of mutations added into chPylRS$^{F}_{IPYE}$ with one copy of tRNA^{M15}. +32A* refers to the addition of mutations from chPylRS$_{32A}$ (the analogous mutation sets are denoted in the same way). **D)** Addition of mutation sets from panel A and chPylRS$^{F}_{2020}$ with two copies of tRNA^{M15} compared against chPylRS$^{F}_{IPYE}$. Note, the plasmids for experiment in panel D were amplified and purified using the QIAGEN Plasmid Plus Midi Kit (QIAGEN Inc., Germantown, MD, USA).

To test whether the "2020" set of mutations is generalizable to other amino acids and synthetases, we tested the incorporation of four clickable amino acids (**Fig S4.2A**). PrK and CpK were introduced via chPylRS$^{F}_{2020}$. BCN was incorporated after previously described binding pocket mutations for an *M. barkeri* enzyme[10] were ported into chPylRS$_{2020}$ (Plasmids section, Plasmids M19-M21). Finally, TCO was incorporated after introduction of an additional reported Y271A mutation into the binding pocket of the enzyme (chPylRS$^{AF}_{2020}$, see Plasmids section, Plasmids M22-M24). In addition, for all these experiments, we also tested the "wild-type" variant of the enzyme from *Methanosarcina mazei* featuring the Tyr → Phe mutation, which has not previously been paired with tRNA^{M15}. For PrK and BCN, chPylRS$^{F}_{2020}$ and chBCNRS$_{2020}$ (respectively) outperformed the *M. barkeri* and the *M. mazei* enzymes (**Fig S4.2B**). On the other hand, for CpK and TCO all three enzymes performed similarly well, suggesting that the relative positioning of the tRNA and ncAA might already be close to optimal for these two amino acids. Interestingly, for BCN it was found in all cases that the high concentrations of ncAA (250 μM) reduced the total levels of protein expressed (**Fig S4.3**), so 25 μM was used for all experiments with BCN going forward.

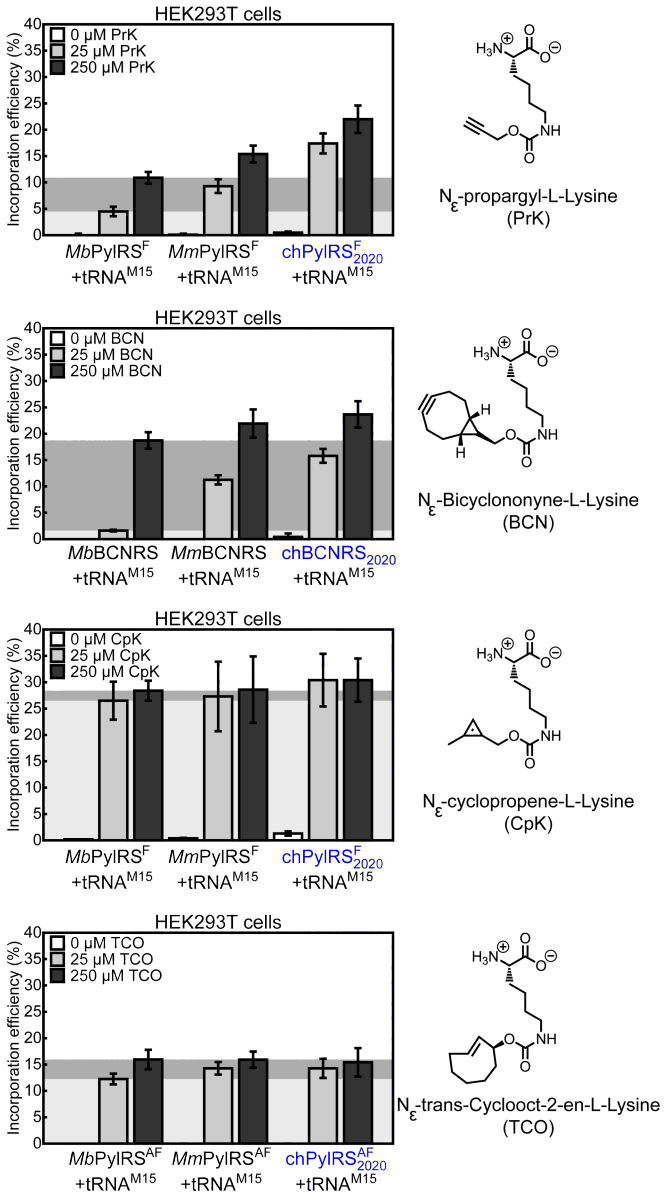

Fig. S4.2 | Incorporation of four clickable amino acids with the corresponding M. barkeri, M. mazei, and "2020" synthetases. Incorporation efficiencies for the different RS/tRNA pairs and clickable ncAAs. The 2020 synthetase performed better than, or as well as, the other variants in all cases. All error bars represent standard deviation of biological replicates. Chemical structures of the four tested ncAAs shown on the right.

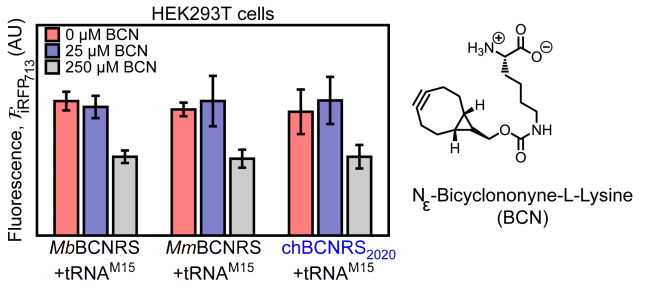

Fig. S4.3 | High concentrations (250 μM) of BCN reduce total protein expression. Error bars represent standard deviation of biological replicates.

5 M_2R GPCR labeling and imaging

5.1 Plasmids

pNEU-*Mb*PylRS^{F}+tRNA^{M15} (Plasmids section, Plasmid M01)
pNEU-chPylRS$^{F}_{2020}$+tRNA^{M15} (Plasmids section, Plasmid M17)
pNEU-*Mb*BCNRS+tRNA^{M15} (Plasmids section, Plasmid M19)
pNEU-chBCNRS$_{2020}$+tRNA^{M15} (Plasmids section, Plasmid M21)
pNEU-M_2R_{V186*}+tRNA^{M15} (Plasmids section, Plasmid M28)

5.2 Biological replicates for M_2R labeling in HEK293T cells treated with 250 µM PrK

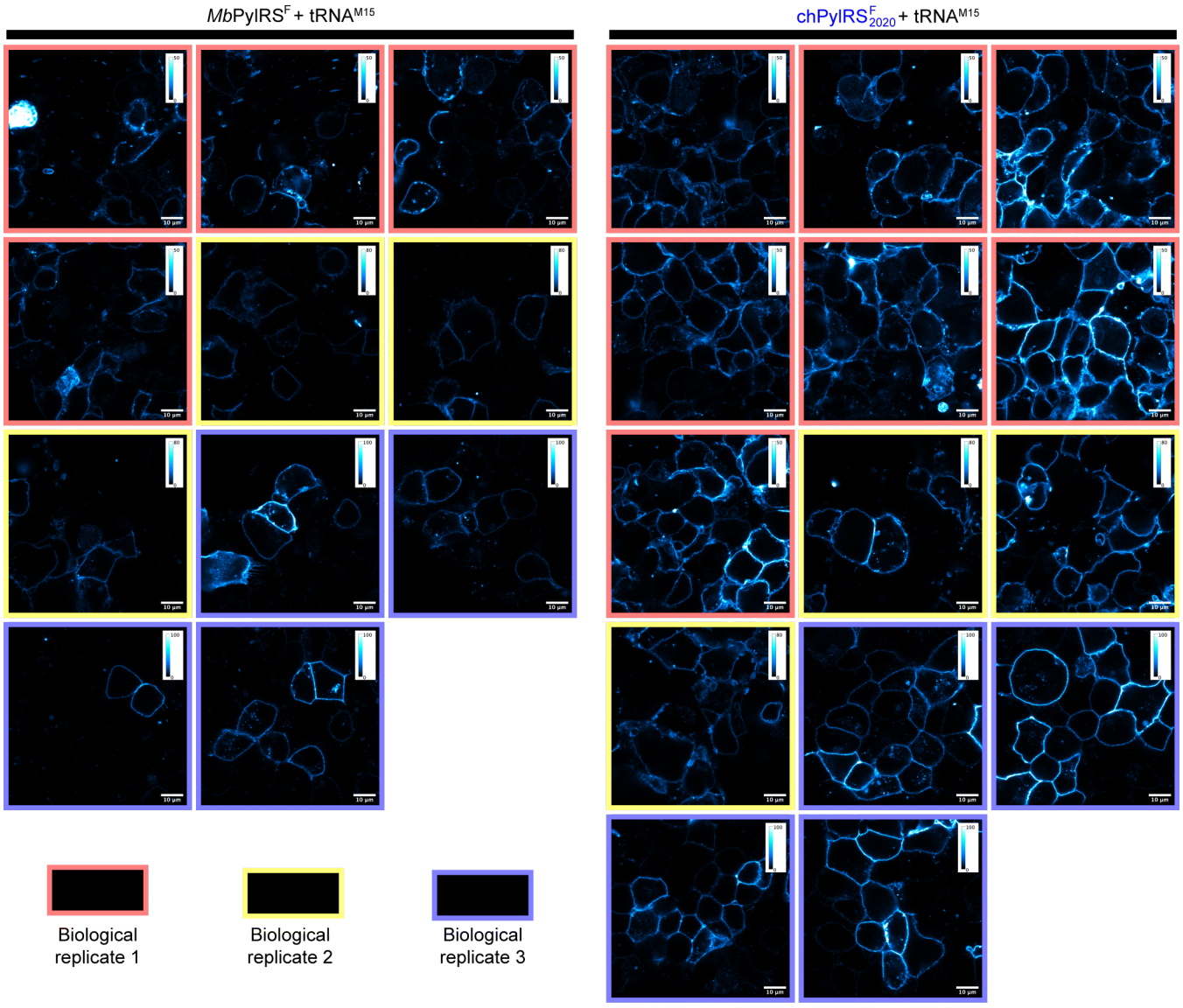

Fig. S5.1 | Acquired images of M_2R_{PrK186} labeled with a rapidly reacting azide-plus variant of the AlexaFluor-647 fluorescent dye. 250 µM PrK was used for ncAA incorporation in HEK293T cells. The images are from three biological replicates.

5.3 Biological replicates for M_2R labeling in HEK293T cells treated with 25 µM PrK

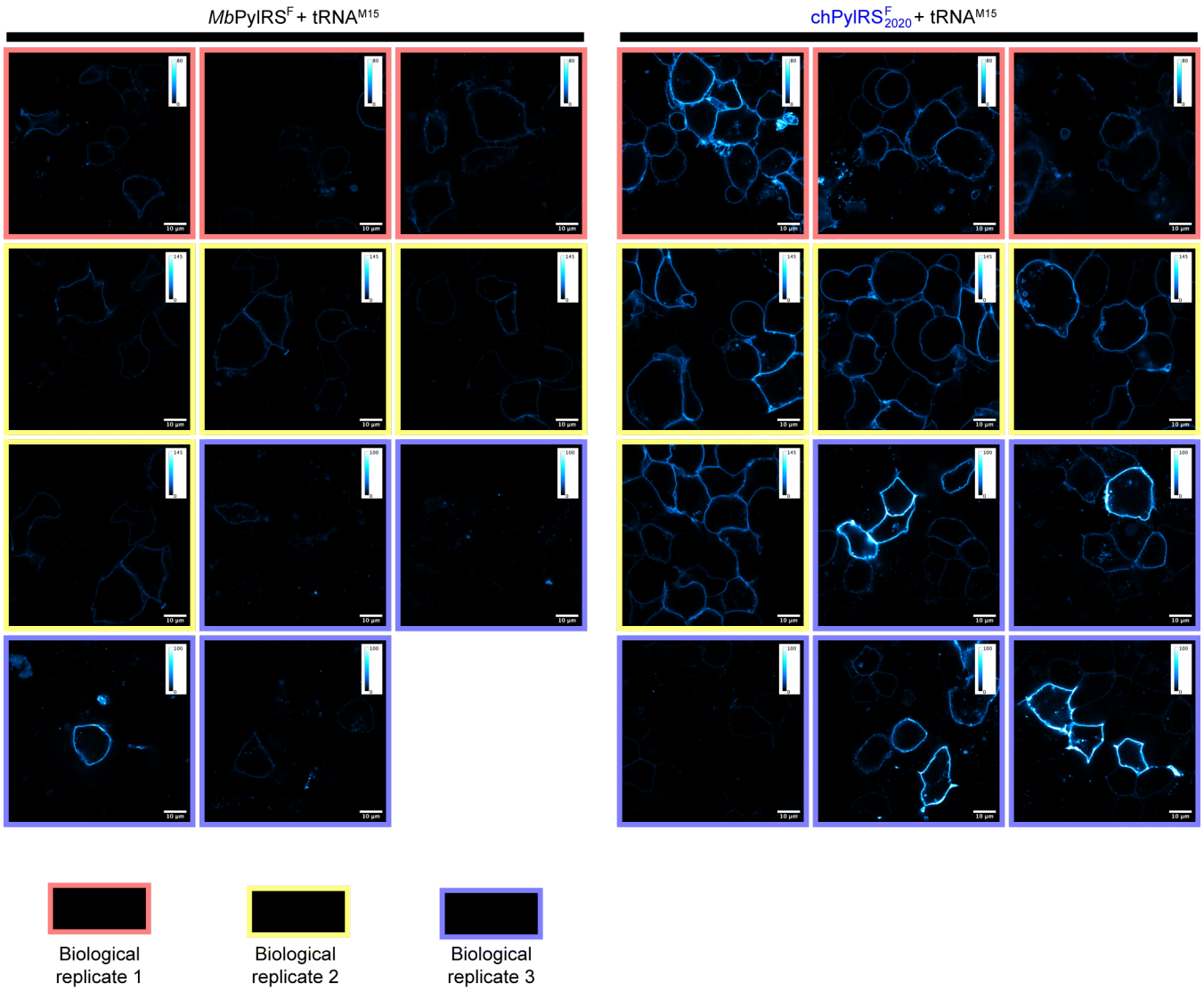

Fig. S5.2 | Acquired images of M_2R_{PrK186} labeled with a rapidly reacting azide-plus variant of the AlexaFluor-647 fluorescent dye. 25 µM PrK was used for ncAA incorporation in HEK293T cells. The images are from three biological replicates.

5.4 Biological replicates for M_2R labeling in HEK293T cells treated with 25 µM BCN

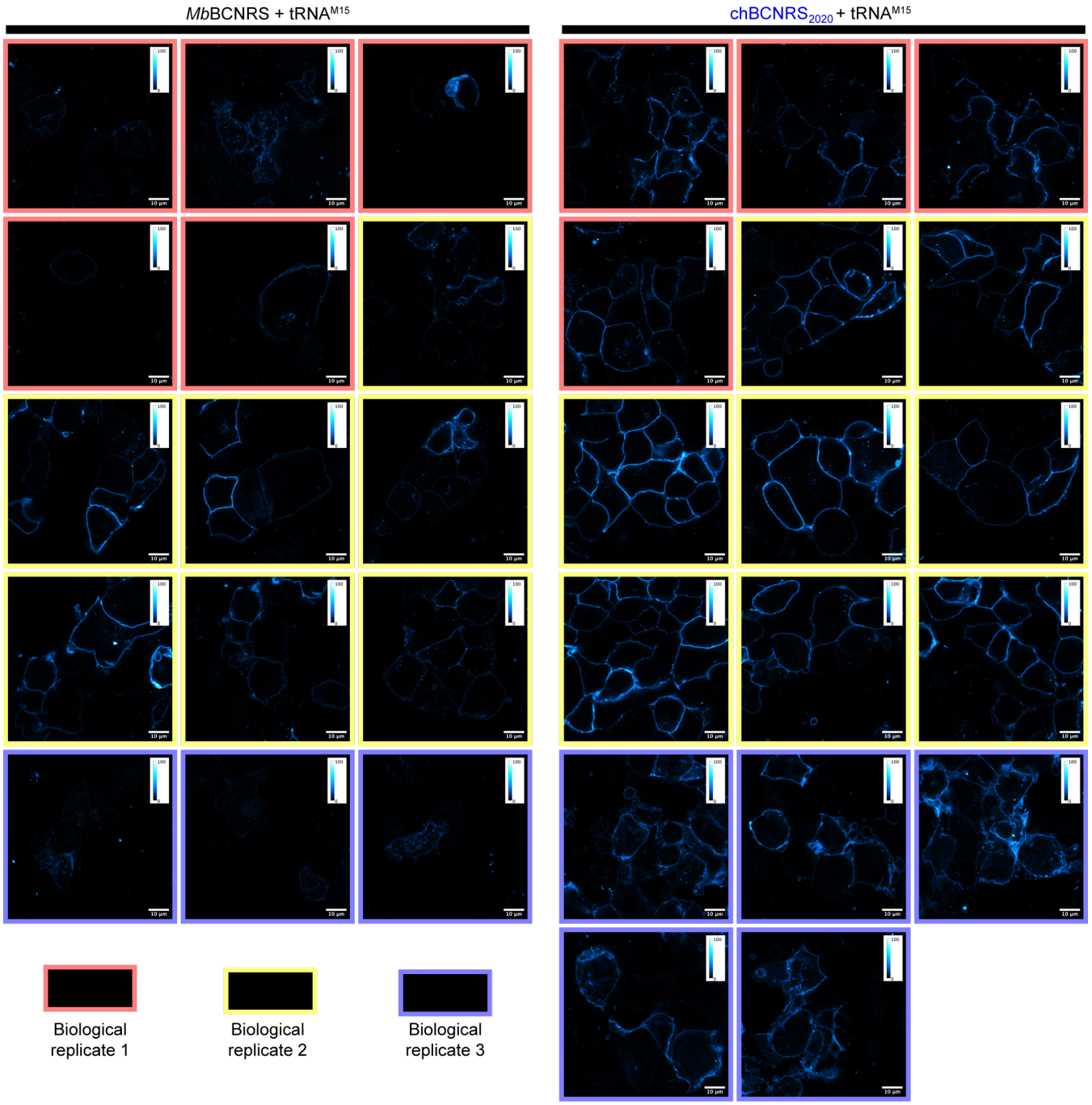

Fig. S5.3 | Acquired images of M_2R_{BCN186} labeled with a rapidly reacting azide-plus variant of the AlexaFluor-647 fluorescent dye. 25 µM BCN was used for ncAA incorporation in HEK293T cells. The images are from three biological replicates.

5.5 Quantitative analysis of M_2R labeling in HEK293T cells

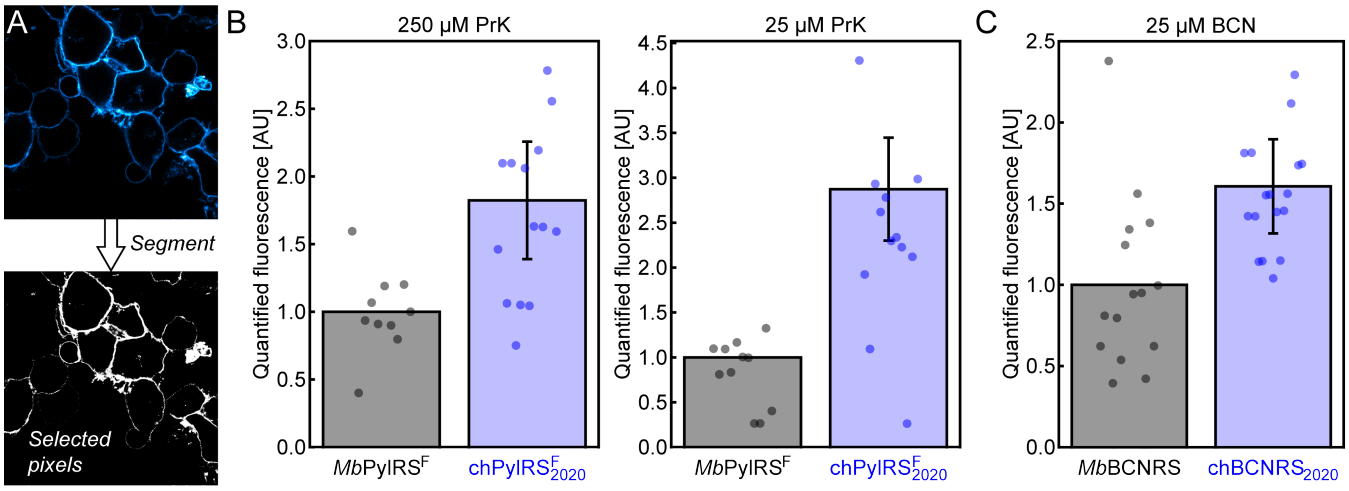

Fig. S5.4 | Quantification of M2R labeling in HEK293T cells. A) Images were converted to an 8-bit format and then segmented in ImageJ by converting them to a binary mask using automatic thresholding (Process→Binary→Convert to Mask). Mean gray values were then extracted from the non-zero pixels in the mask by referencing intensity values from the original image. **B)** Comparison of mean gray values of *M. barkeri* and "2020" PylRS enzymes paired with $tRNA^{M15}$ (internally normalized to *M. barkeri*). Bars & error-markers represent the mean and standard deviation (respectively) of the three biological replicates. Dots show the mean gray value of the segmented pixels for each analyzed image.

6 OmpC imaging

6.1 Plasmids

pULTRA-chPylRS$_{IPYE}$+tRNA^{Pyl*} (Plasmids section, Plasmid E1)
pULTRA-chPylRS$^{F}_{2020}$+tRNA^{M15} (Plasmids section, Plasmid E4)
pET101-OmpC$_{D311*}$ (Plasmids section, Plasmid E5)

6.2 Biological replicates for OmpC labeling in *E. coli* (BL21-DE3) treated with 2.5 mM PrK

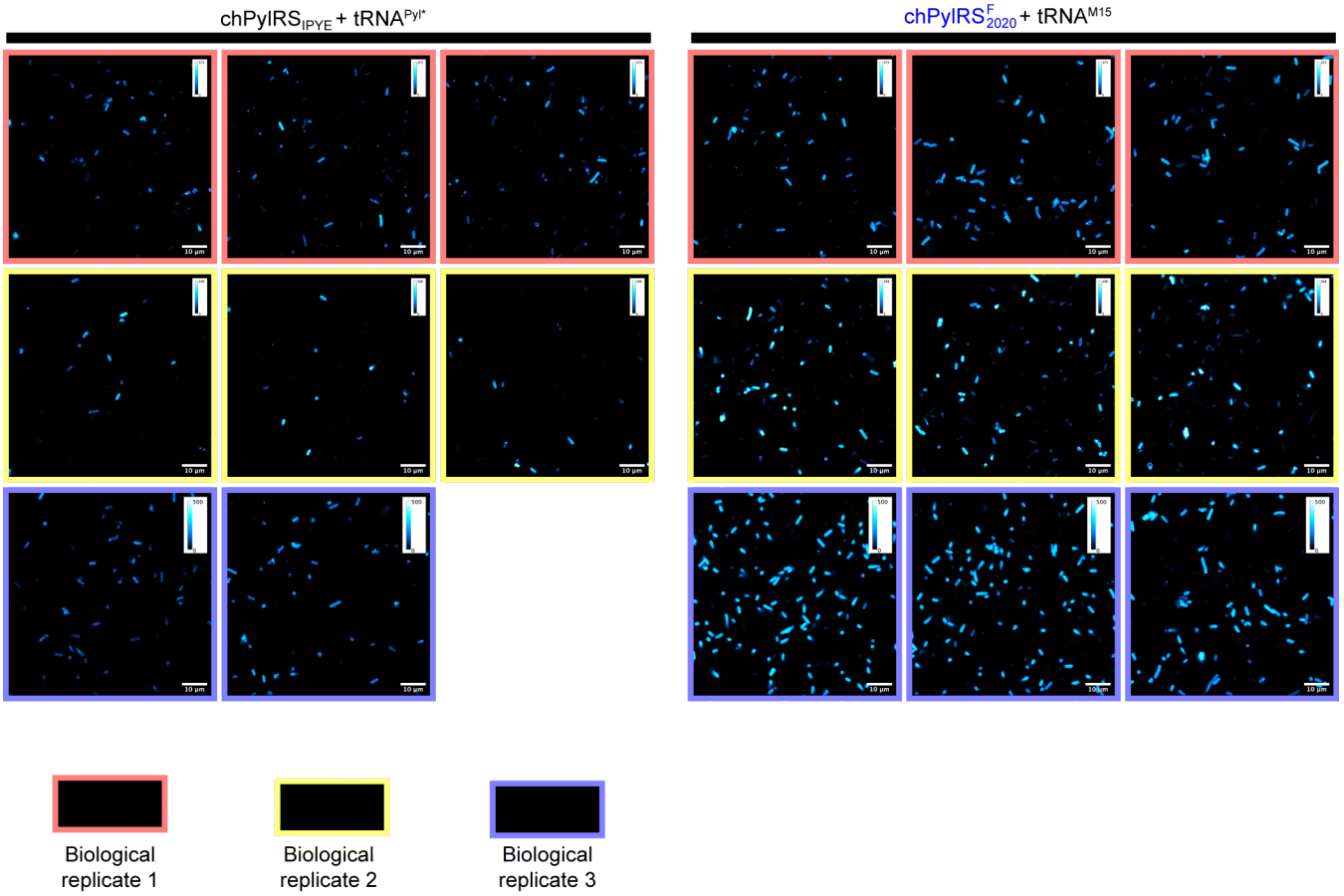

Fig. S6.1 | Acquired images of OmpC$_{PrK311}$ labeled with a rapidly reacting azide-plus variant of the AlexaFluor-647 fluorescent dye. 2.5 mM PrK was used for ncAA incorporation in *E. coli* (BL21-DE3) cells. The images are from three biological replicates.

6.3 Quantification of OmpC labeling in *E. coli* (BL21-DE3)

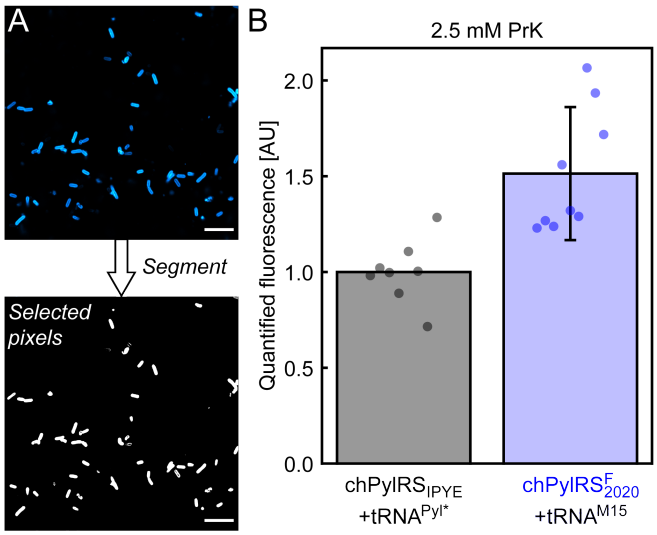

Fig. S6.2 | Quantification of OmpC labeling in *E. coli* (BL21-DE3) cells. A) Images were converted to an 8-bit format and then segmented in ImageJ by converting them to a binary mask using automatic thresholding (Process→Binary→Convert to Mask). Mean gray values were then extracted from the non-zero pixels in the mask by referencing intensity values from the original image. **B)** Comparison of mean gray values of published chPylRS$_{IPYE}$+tRNA^{Pyl*} pair and the new chPylRS$^{F}_{2020}$+tRNA^{M15} pair (internally normalized to chPylRS$_{IPYE}$+tRNA^{Pyl*}). Bars & error-markers represent the mean and standard deviation (respectively) of the three biological replicates. Dots show the mean gray value of the segmented pixels for each analyzed image.

7 β-actin labeling via PrK, BCN, TCO in U2OS cells

7.1 Plasmids

pNEU-chPylRS$^{F}_{2020}$+tRNA^{M15} (Plasmids section, Plasmid M14)
pNEU-chBCNRS$_{2020}$+tRNA^{M15} (Plasmids section, Plasmid M21)
pNEU-chPylRS$^{AF}_{2020}$+tRNA^{M15} (Plasmids section, Plasmid M24)
pNEU-β-actin$_{K118*}$ (Plasmids section, Plasmid M30)

7.2 Confocal images of β-actin labeled via PrK, BCN, and TCO

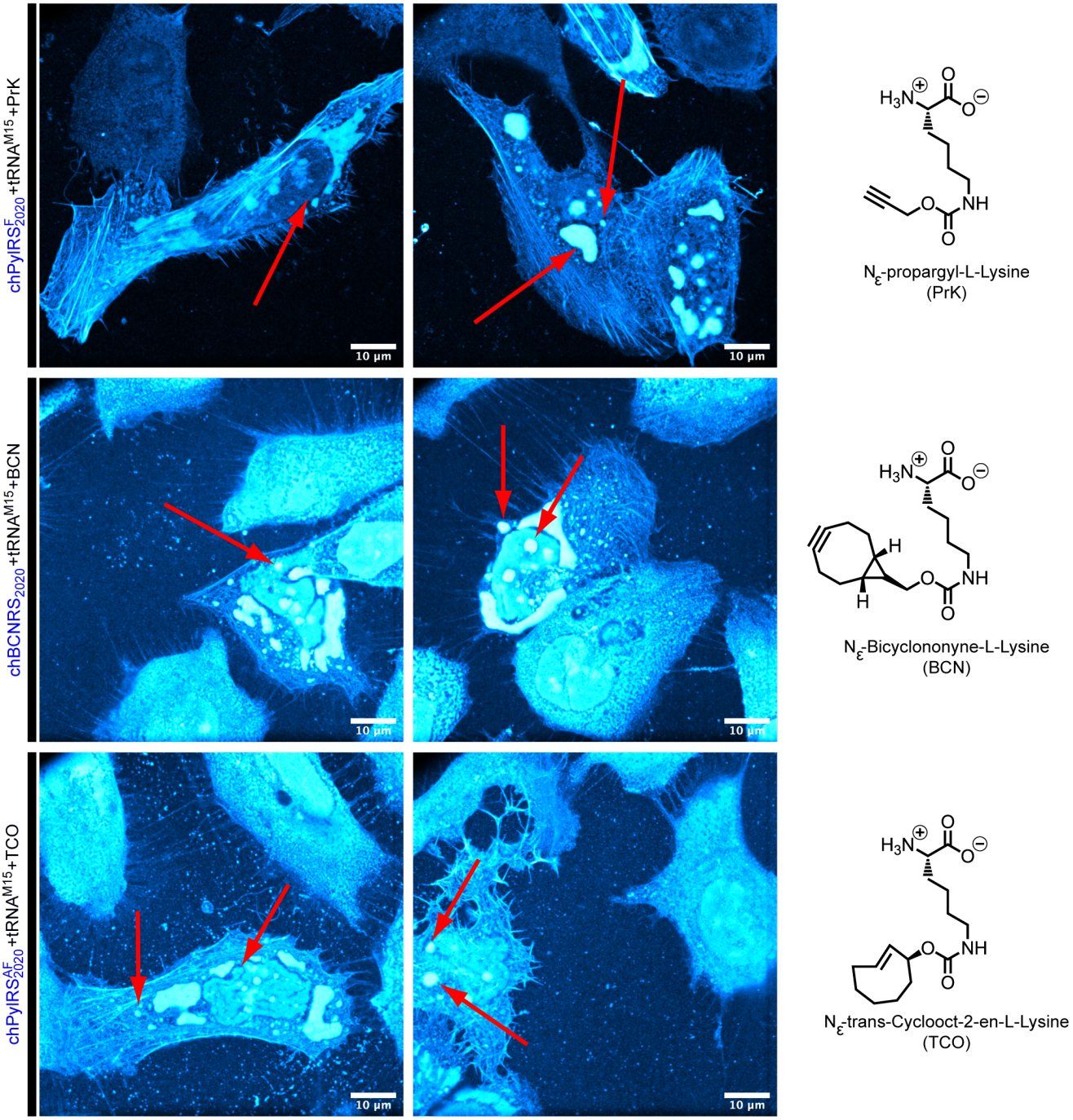

Fig. S7.1 | Comparison of ncAAs for labeling β-actin in U2OS cells. PrK was labeled with 250µM picolyl-azide silicon rhodamine. TCO and BCN were labeled with 250 µM tetrazine-silicon rhodamine. PrK labeling shows substantially lower background signal than TCO and BCN labeling. The red arrows point out non-specific inclusions which have been previously ascribed to labeled aminoacylated-tRNAs.[2,6,15]

8 Additional information for β-actin STED and MINFLUX imaging in U2OS cells

Example image from the STED experiment is shown in **main text Fig. 5**.

8.1 Plasmids

For STED
pNEU-chPylRS$^{F}{}_{2020}$+tRNA^{M15} (Plasmids section, Plasmid M14)
pNEU-β-actin$_{K118*}$ (Plasmids section, Plasmid M30)

For MINFLUX
pNEU-chPylRS$^{F}{}_{2020}$+tRNA^{M15} (2x) (Plasmids section, Plasmid M25)
pNEU-β-actin$_{K118*}$ (Plasmids section, Plasmid M30)

9 Simulated renderings of β-actin bundles

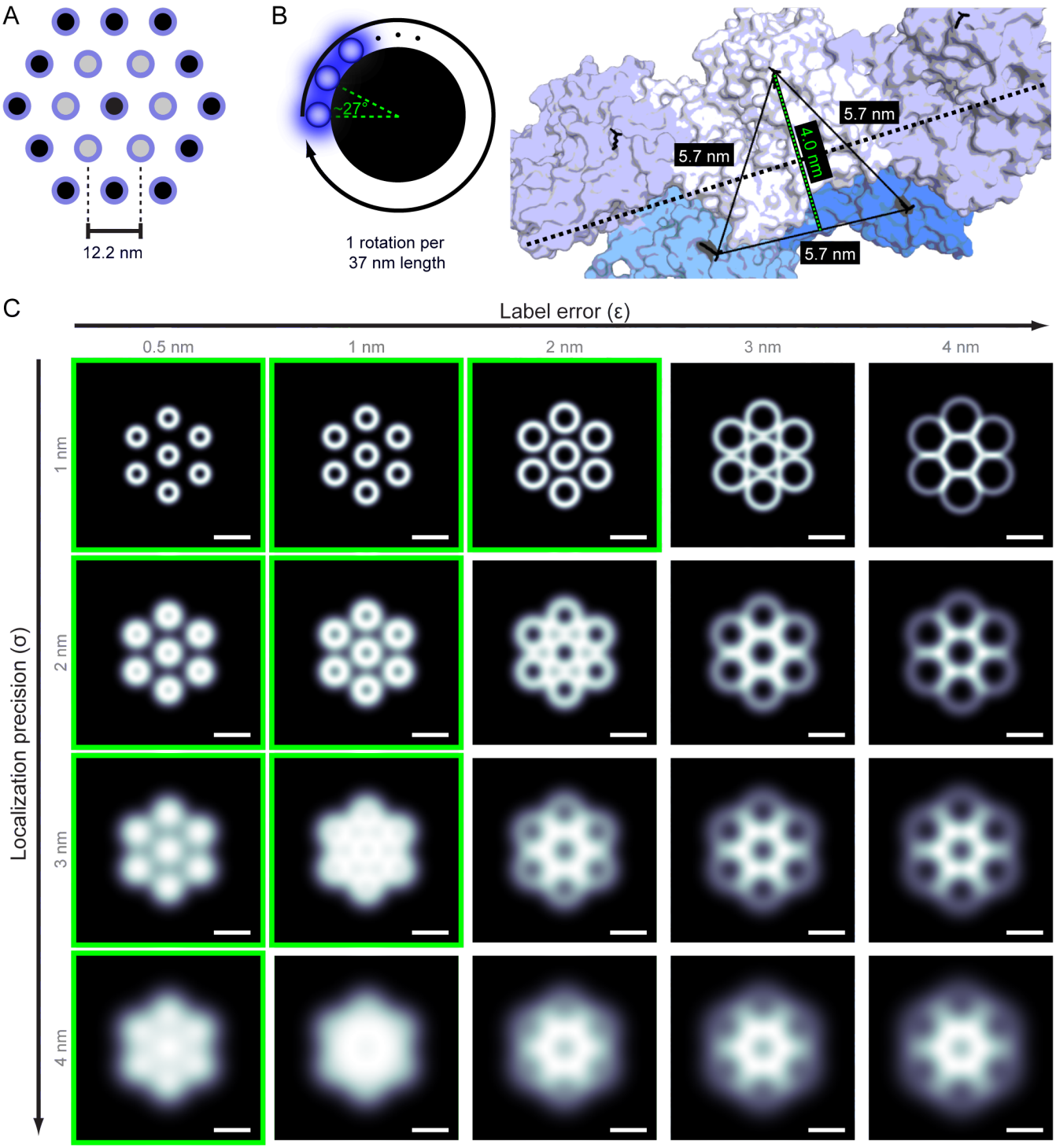

Fig. S9.1 | Simulated ideal cross-sectional projections of actin filaments in filopodia spanning a variety of localization precisions and tag offsets. A) Within filopodia, β-actin filaments are arranged on a triangular lattice featuring 12.2 nm ± 0.9 nm(σ) between adjacent filaments. **B)** *Left:* Since single filaments are double helices which undergo a full twist every 37 nm, when viewed in cross-section the labeling sites occupy positions on a circle separated by 27°. *Right:* The labeling sites (residues 118) are separated by 5.7 nm between adjacent single molecules, resulting in a cross-sectional diameter of 4.0 nm. **C)** The physical parameters highlighted in panel B were used to render filopodia (modeled as circles with 4 nm diameter) in cross section with a range of simulated tag offsets (distance between fluorophore and Cα of residue 118) and localization precisions. Conditions highlighted in green demonstrate signal maxima corresponding to single filaments rather than to combinations of adjacent filaments.

10 Estimated size of tag offset

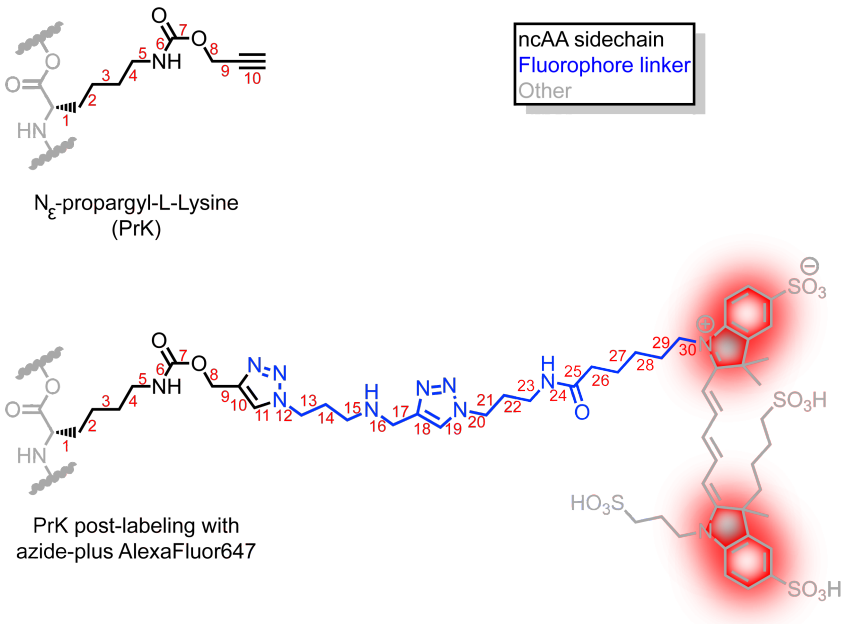

Fig. S10.1 | Length of labeled and unlabeled PrK sidechain. ***Top***: the unlabeled PrK sidechain features 10 bonds, most of which are fully saturated. ***Bottom:*** the labeled PrK sidechain features 30 bonds, most of which are fully saturated.

The offset from the backbone is equivalent to the end-to-end distance of the sidechain and the linker. This assembly was assumed to be a freely rotating chain, of which the mean-squared end-to-end distance, $<D^2>$ equals:

$$< D^2 >= nd^2\left(\frac{1+\cos(\theta)}{1-\cos(\theta)}\right)$$

where *n*= the number of links, *d*= the link length, *θ*= azimuthal angle from the preceding link. Given that most bonds are fully saturated, and often carbon-carbon bonds, the molecular assemblies shown above (**Fig. S10.1**) were modeled as freely rotating chains with *d*=0.1535-nm and *θ*=68°. Computing root-mean-squared end-to-end distance for the unlabeled PrK and for PrK labeled with azide-plus AlexaFluor-647 gives:

$$< D^2 >^{1/2}_{\text{unlabeled}} = 0.72 \text{ nm}$$

$$< D^2 >^{1/2}_{\text{labeled}} = 1.25 \text{ nm}$$

11 Drift-correction of MINFLUX measurements

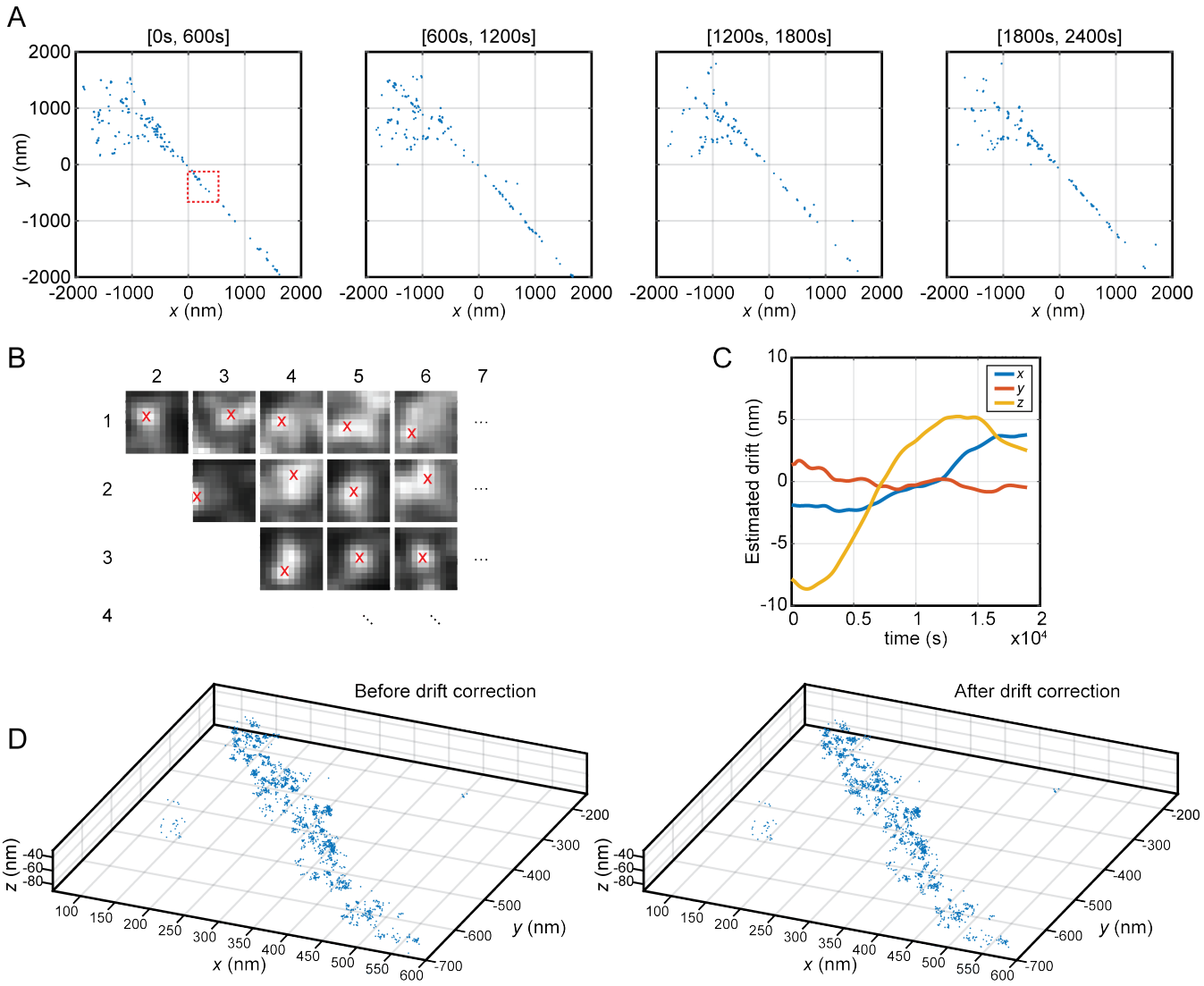

Fig. S11.1 | Drift correction of 3D-MINFLUX filopodia measurement from Fig. 7 (main text). (A) To correct drift in the measurements, localizations were divided into non-overlapping time windows of equal length (600 seconds, corresponding to the approximate time taken to fully scan the object one time). For each time window, a 3D-histogram of the localizations was generated with 5 nm pixel size and was Gaussian-blurred (10 nm full-width half-maximum). **(B)** Subsequently, each time window was then cross-correlated with each other (rows vs. columns) using fast Fourier transforms. The center of each cross-correlation stack was then estimated with sub-pixel accuracy by computing Gaussian-weighted averages of the cross-correlation peak amplitudes between all pairs of correlated time windows. **(C)** After computing the shift of each frame relative to the preceding frame, an initial drift curve was estimated by cumulatively summing these shifts. To refine the curve, a least squares estimator was applied to minimize the squared distances between all pairwise measured frame shifts and the predicted drift curve in *x*, *y*, and *z*, independently. Between the centers of each time window, a cubic spline interpolation was used to interpolate drift. Finally, each localization was corrected by correcting the computed drift in *x*, *y*, and *z* using the time at which it was measured. **(D)** Data from the red rectangle in A with and without drift correction. Only subtle changes are visible.

12 Slice analysis of drift-corrected β-actin filopodia MINFLUX measurements

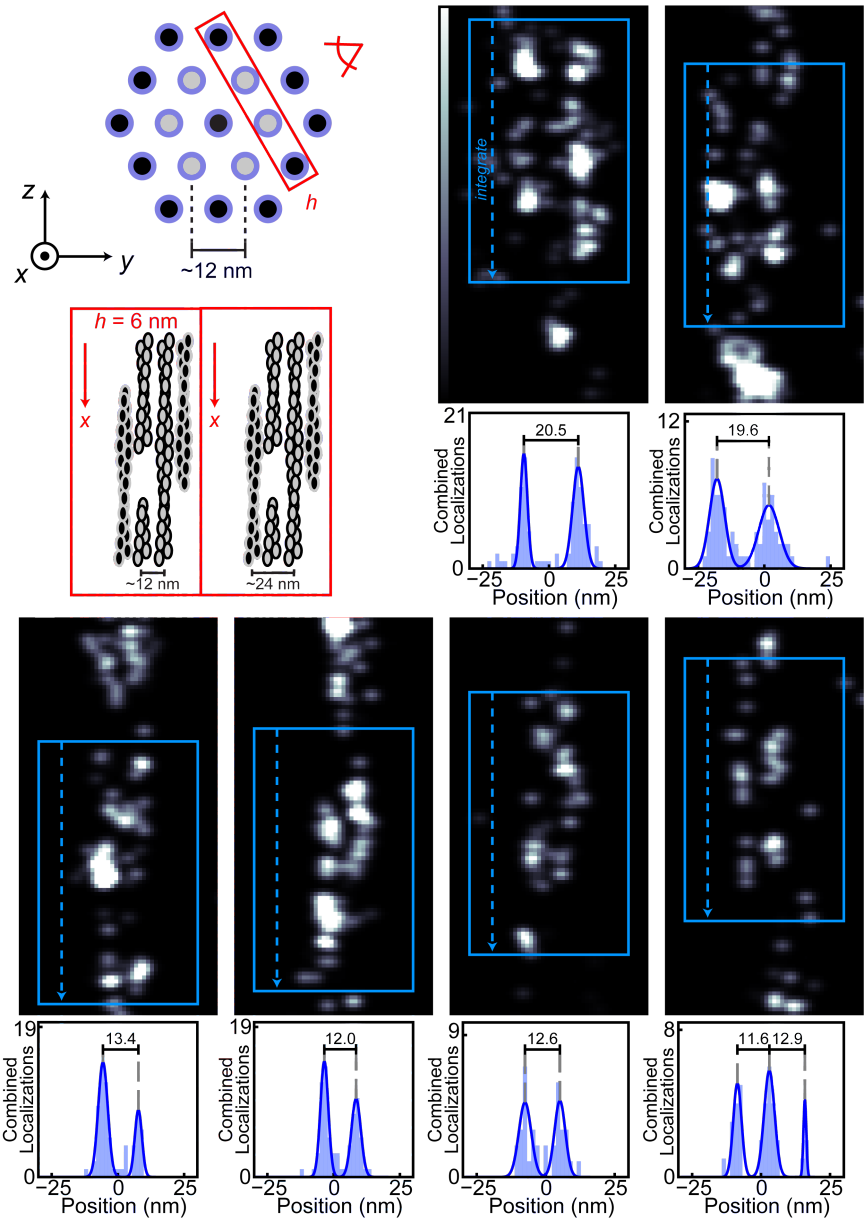

Fig. S12.1 | Analysis of adjacent filaments identified in 6 nm-thick slices of filopodia measured with MINFLUX. 3D-MINFLUX measurements of filopodia with combined localizations (SI §1, 3D-MINFLUX localization combination) were rotated such that the *x*-axis was a regression line down the center of the filopodia. The filopodia were then rolled (rotated around *x*) in 10-degree increments spanning from 0 degrees to 360 degrees. Localizations were binned into $(1.5\ \text{nm})^3$ voxels. The volume was then sliced to generate overlapping 6 nm-thick slice stacks (separated by 1.5 nm in *z*). The roll angle with the single brightest 6 nm slice was chosen for further analysis. The localizations were then analyzed as previously described in SI §1, MINFLUX plot-profile analysis, with binning into $(1.5\ \text{nm})^3$ voxels and generation of overlapping 6 nm-thick slice stacks (separated by 1.5 nm in *z*). Several examples of slices featuring adjacent filaments are shown. The integrated fluorescence signal was analyzed as a linear combination of two or three Gaussians (*right*). Blue boxes are 60-nm wide and 99-nm tall. Histograms are rendered from crude localization counts. To render the displayed images, each 1.5 nm-thick *z*-slice was blurred with a Gaussian (1.5-nm std. dev) in *xy* and in *yz*.

13 Cross-section analysis of MINFLUX filopodia measurements

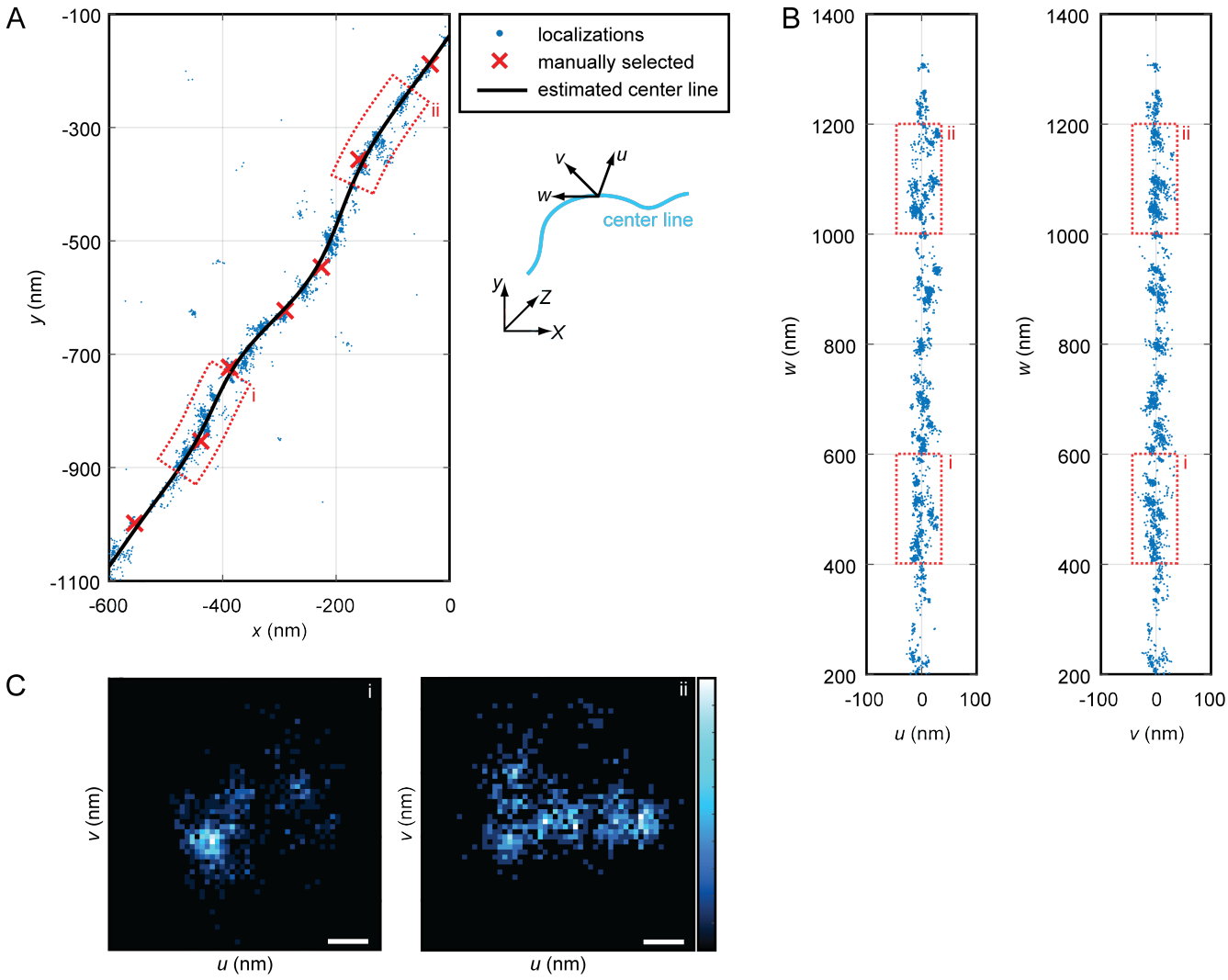

Fig. S13.1 | Modeling the central axis of filopodia and transforming localization coordinates into a coordinate system moving along the center line (A) MINFLUX localizations were shown in *xy* and positions along the length of the 2D-filopodium projection were manually selected (red crosses). The line connecting the manual selection was resampled and smoothed (Savitzky-Golay 2nd order filtering) and extended to *z* by estimating the local center of mass of the *z*-coordinates of the localizations (filopodia were mostly oriented within the *xy* plane).The center line of the filopodium was estimated by iteratively alternating between a smoothing step (Savitzky-Golay 2nd order filtering) and a center of mass estimation step with a Gaussian distance weight of localization contributions to the center of mass with a σ ~ 20 nm. The center line was finally resampled with a pixelation of 1 nm and a coordinate system was generated at each position with the first axis pointing along the line to the next point (*w*), the second axis maximally aligned with the *z* axis (*u*), and the final axis the cross product of the two (*v*). The *uvw* coordinate system moves along the estimated center line. **(B)** Same data as in A but in the moving *uvw* coordinate system. **(C)** Cross-section, i.e., a rendering (each localization is shown as a Gaussian of σ = 1.5 nm in the lateral *uv* directions over a distance of 200nm along the estimated center line (as shown Figure 6 (main text) and Figure S13.2.) for the regions marked i and ii in (A) and (B). Scale bar: 12nm.

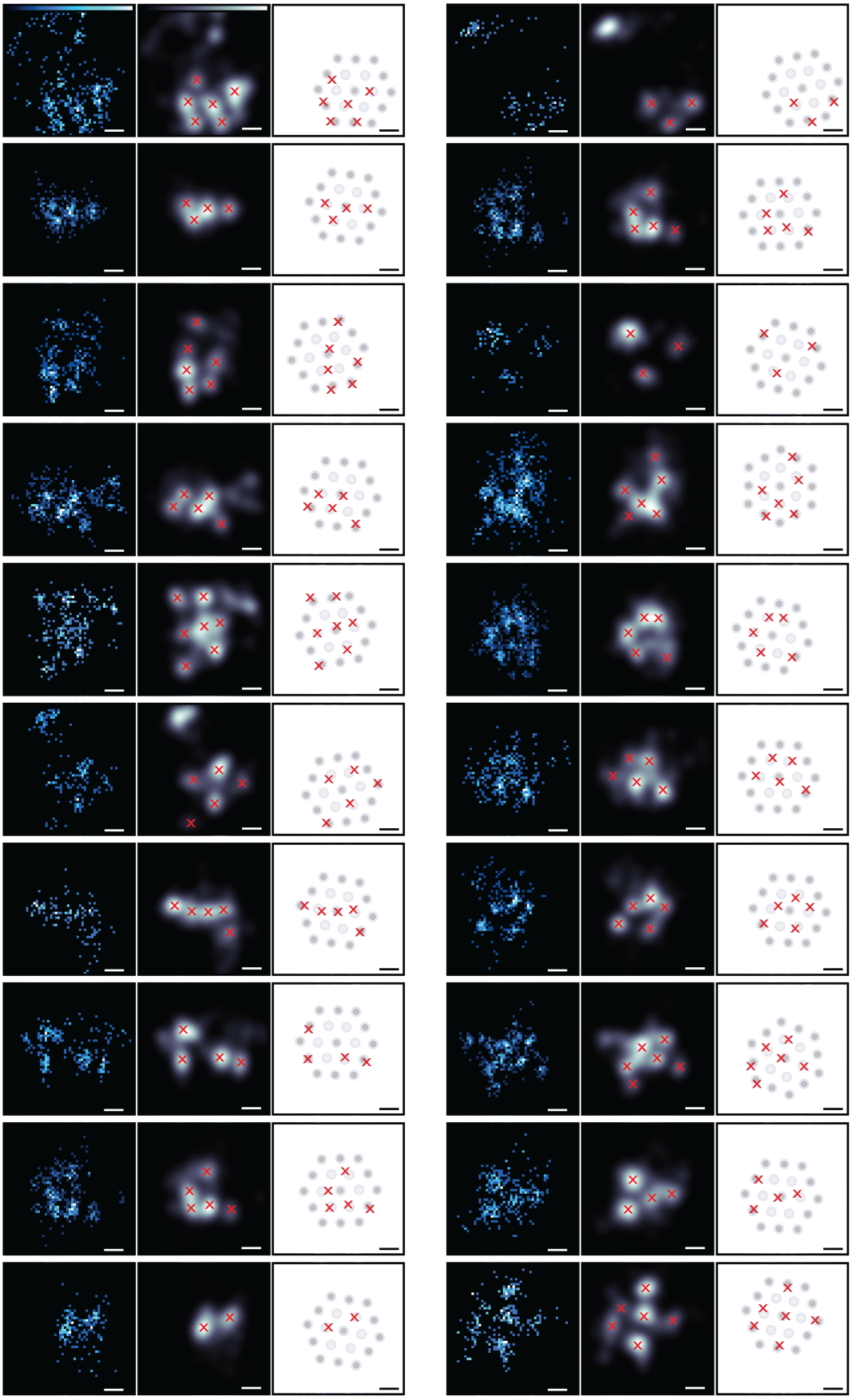

Fig. S13.2 | Additional examples of patterns observed in cross-sections of filopodia measured with 3D-MINFLUX. *Left*: intensity image. *Center*: blurred with Gaussian (3.5-nm standard deviation). Scale bar: 12nm *Right*: overlay of approximate peak centers on theoretical lattice.

14 Statistical analysis

Figure	Comparison y vs. x	t − test (statistic)	t − test (p − value)	DoF	Levene (statistic)	Levene (p − value)	Shapiro − Wilk (x, statistic)	Shapiro − Wilk (x, p − value)	Shapiro − Wilk (y, statistic)	Shapiro − Wilk (y, p − value)
2E	($chPylRS^{F}_{IPYE} + tRNA^{M15}$) vs. ($MbPylRS^{F} + tRNA^{M15}$)	−4.813	0.009	4	0.560	0.496	0.999	0.956	0.999	0.950
2F	($chPylRS^{F}_{IPYE} + tRNA^{M15}$) vs. ($MbPylRS^{F} + tRNA^{M15}$)	−4.594	0.010	4	0.004	0.954	0.999	0.952	0.989	0.800
3C (PrK, 25 μM)	($chPylRS^{F}_{2020} + tRNA^{M15}$) vs. ($MbPylRS^{F} + tRNA^{M15}$)	−10.664	4.380E − 4	4	0.472	0.530	0.955	0.593	0.871	0.298
3C (PrK, 250 μM)	($chPylRS^{F}_{2020} + tRNA^{M15}$) vs. ($MbPylRS^{F} + tRNA^{M15}$)	−6.655	0.003	4	0.626	0.473	0.999	0.952	0.926	0.473
3C (BCN, 25 μM)	($chBCNRS_{2020} + tRNA^{M15}$) vs. ($MbBCNRS + tRNA^{M15}$)	−18.495	5.029E − 5	4	0.862	0.406	0.922	0.460	0.787	0.085
3C (BCN, 250 μM)	($chBCNRS_{2020} + tRNA^{M15}$) vs. ($MbBCNRS + tRNA^{M15}$)	−2.894	0.044	4	0.399	0.562	0.939	0.522	1.000	0.987
3D (PrK, 1 mM)	($chPylRS^{F}_{2020} + tRNA^{M15}$) vs. ($chPylRS^{F}_{IPYE} + tRNA^{M15}$)	−3.250	0.031	4	0.878	0.402	0.949	0.566	1.000	1.000
3D (PrK, 2.5 mM)	($chPylRS^{F}_{2020} + tRNA^{M15}$) vs. ($chPylRS_{IPYE} + tRNA^{Pyl*}$)	−5.955	0.004	4	7.843E − 32	1.000	0.902	0.391	0.855	0.253
3D (PrK, 2.5 mM)	($chPylRS^{F}_{2020} + tRNA^{M15}$) vs. ($chPylRS^{F}_{IPYE} + tRNA^{Pyl*}$)	−1.753	0.154	4	0.154	0.715	0.815	0.150	0.855	0.253

Fig. S14.1 | Independent t-test analysis for comparisons between orthogonal pairs presented in main-text figures. Levene and Shapiro-Wilk tests were run to determine whether the homogeneity of variance and normality conditions required for valid t-testing are met. The comparison between $chPylRS^{F}_{2020}$+$tRNA^{M15}$ vs. $chPylRS_{IPYE}$+$tRNA^{Pyl*}$ in bacterial cells with 1 mM PrK could not be analyzed in this way because the data from the latter condition failed the Shapiro-Wilk normality test.

15 Plasmids

Nomenclature.
Plasmid A# ⇒ plasmid from Addgene
Plasmid I# ⇒ plasmid is a cloning intermediate that was not used for any experiments
Plasmid E# ⇒ plasmid for *E. coli*
Plasmid M# ⇒ plasmid for mammalian cells

15.1 Addgene Plasmids

Plasmid	Addgene name	
Plasmid A1	pULTRA-CNF	pULTRA-CNF was a gift from Peter Schultz (Addgene plasmid #48215; http://n2t.net/addgene:48215; RRID:Addgene_48215).[11]
Plasmid A2	pBAD-sfGFP	pBAD-sfGFP (Plasmids section, plasmid E3) was a gift from Ryan Mehl (Addgene plasmid #85482; http://n2t.net/addgene:85482; RRID:Addgene_85482)[12]
Plasmid A3	pBAD-sfGFP 150 TAG	pBAD-sfGFP 150 TAG (Plasmids section, plasmid E4) was a gift from Ryan Mehl (Addgene plasmid # 85483; http://n2t.net/addgene:85483; RRID:Addgene_85483)[12]
Plasmid A4	pET-G2K	pET-G2K was a gift from George Reed & Paul Sims (Addgene plasmid # 70169; http://n2t.net/addgene:70169; RRID:Addgene_70169)[13]
Plasmid A5	pNEU-hMbPylRS-4xU6M15	pNEU-hMbPylRS-4xU6M15 was a gift from Irene Coin (Addgene plasmid # 105830 ; http://n2t.net/addgene:105830 ; RRID:Addgene_105830)[14]

15.2 *E. coli* plasmids

Green boxes represent enzymes with "2020" mutations
Plasmid IXX ⇒ cloning intermediate not used in experiments
Plasmid EXX ⇒ plasmid used in *E. coli* experiments

		Cloning reagents			
Plasmid	Description	Starting plasmid	Forward primer	Reverse primer	Geneblock
Plasmid I1	**pULTRA *Mj*CnfRS*** + $tRNA^{Pyl*}$	Plasmid A1	Primer 01	Primer 02	Geneblock 01
Plasmid E1	**pULTRA chPylRS$_{IPYE}$** + $tRNA^{Pyl*}$	Plasmid I1	Primer 03	Primer 04	Geneblock 02
Plasmid E2	**pULTRA chPylRS$_{IPYE}$** + $tRNA^{M15}$	Plasmid E1	Primer 01	Primer 02	Geneblock 03
Plasmid E3	**pULTRA chPylRS$_{IPYE}$** (Y349F) + $tRNA^{M15}$	Plasmid E2	Primer 05	Primer 06	Geneblock 04
Plasmid E4	**pULTRA chPylRS$_{IPYE}$** (D161N+G343D+Y349F) + $tRNA^{M15}$	Plasmid E2	Primer 05	Primer 06	Geneblock 05
Plasmid E5	**pET101 OmpC(D311*)**	Plasmid A4	Primer 07	Primer 08	Geneblock 06

**Mj = Methanococcus jannaschii*

Plasmid I1 – pULTRA *Mj*CnfRS + tRNA^{M15}

_Mj_CnfRS tRNA^{Pyl*}

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Plasmid E3 – pULTRA chPylRS$_{IPYE}$ (Y349F) + tRNA^{M15}

also referred to as pULTRA chPylRS$^{F}_{IPYE}$+tRNA^{M15}

chPylRS$^{F}_{IPYE}$ tRNA^{M15}

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Plasmid E4 – pULTRA chPylRS$_{IPYE}$ (D161N+G343D+Y349F) + tRNA^{M15}

also referred to as pULTRA chPylRS$^{F}_{2020}$+tRNA^{M15}

chPylRS$_{IPYE}$(D161N+G343D+Y349F) tRNA^{M15}

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Plasmid E5 – pET101 OmpC(D311*)

OmpC(D311*)

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15.3 Mammalian plasmids

Green boxes represent enzymes with "2020" mutations
Plasmid IXX ⇒ cloning intermediate not used in experiments
Plasmid MXX ⇒ plasmid used in mammalian experiments

		Cloning reagents			
Plasmid	Description	Starting plasm.	Forward primer	Reverse primer	Geneblock
Plasmid I02	**pNEU *Mb*PylRS** (N-NES+Y349F+ C-FLAG) + $tRNA^{M15}$ (3x)*	Plasmid A5	Primer 09	Primer 10	Geneblock 07
Plasmid I03	**pNEU chPylRS$_{IPYE}$** (N-NES+ C-FLAG) + $tRNA^{M15}$ (3x)	Plasmid A5	Primer 11	Primer 10	Geneblock 08
Plasmid I04	**pNEU *Mb*PylRS** (N-NES+Y349F) + $tRNA^{M15}$ (3x)	Plasmid I02	Primer 09	Primer 12	Geneblock 09
Plasmid I05	**pNEU chPylRS$_{IPYE}$** (N-NES) + $tRNA^{M15}$ (3x)	Plasmid I03	Primer 11	Primer 13	Geneblock 10
Plasmid I06	**pNEU chPylRS$_{IPYE}$** (N-NES+Y349F) + $tRNA^{M15}$ (3x)	Plasmid I05	Primer 16	Primer 17	Geneblock 33
Plasmid M01	**pNEU *Mb*PylRS** (N-NES+Y349F) + $tRNA^{M15}$	Plasmid I04	Primer 14	Primer 15	Geneblock 11
Plasmid M02	**pNEU *Mm*PylRS** (N-NES+Y384F) + $tRNA^{M15}$	Plasmid M01	Primer 11	Primer 10	Geneblock 12
Plasmid M03	**pNEU chPylRS$_{IPYE}$** (N-NES+Y349F) + $tRNA^{M15}$	Plasmid I06	Primer 14	Primer 15	Geneblock 11
Plasmid M04	**pNEU chPylRS$_{IPYE}$** (N-NES+Y349F) + $tRNA^{Pyl*}$	Plasmid I06	Primer 14	Primer 15	Geneblock 12
Plasmid I07	**pNEU chPylRS$_{IPYE}$** (N-NES+Y349F) + $tRNA^{M15}$ *codons re-optimized for other mutations*	Plasmid M03	Primer 16	Primer 17	Geneblock 13
Plasmid M05	**pNEU chPylRS$_{IPYE}$** (N-NES+D2N+Y349F) + $tRNA^{M15}$	Plasmid I07	Primer 18	Primer 10	Geneblock 14
Plasmid M06	**pNEU chPylRS$_{IPYE}$** (N-NES+K3N+Y349F) + $tRNA^{M15}$	Plasmid I07	Primer 18	Primer 10	Geneblock 15
Plasmid M07	**pNEU chPylRS$_{IPYE}$** (N-NES+E119K+Y349F) + $tRNA^{M15}$	Plasmid M03	Primer 19	Primer 20	Geneblock 16
Plasmid M08	**pNEU chPylRS$_{IPYE}$** (N-NES+S158N+Y349F) + $tRNA^{M15}$	Plasmid M03	Primer 19	Primer 20	Geneblock 17
Plasmid M09	**pNEU chPylRS$_{IPYE}$** (N-NES+D161N+Y349F) + $tRNA^{M15}$	Plasmid M03	Primer 19	Primer 20	Geneblock 18
Plasmid M10	**pNEU chPylRS$_{IPYE}$** (N-NES+K258Q+Y349F) + $tRNA^{M15}$	Plasmid M03	Primer 16	Primer 17	Geneblock 19
Plasmid M11	**pNEU chPylRS$_{IPYE}$** (N-NES+G343D+Y349F) + $tRNA^{M15}$	Plasmid M03	Primer 16	Primer 17	Geneblock 20
Plasmid M12	**pNEU chPylRS$_{IPYE}$** (N-NES+S158N+D161N+Y349F) + $tRNA^{M15}$	Plasmid M03	Primer 19	Primer 20	Geneblock 21
Plasmid M13	**pNEU chPylRS$_{IPYE}$** (N-NES+S158N+G343DN+Y349F) + $tRNA^{M15}$	Plasmid M11	Primer 19	Primer 20	Geneblock 17

Plasmid M14	**pNEU chPylRS$_{IPYE}$** (N-NES+D161N+G343D+Y349F) + tRNA^{M15}	Plasmid M11	Primer 19	Primer 20	Geneblock 18
Plasmid M15	**pNEU chPylRS$_{IPYE}$** (N-NES+S158N+D161N+G343D+Y349F) + tRNA^{M15}	Plasmid M11	Primer 19	Primer 20	Geneblock 21
Plasmid I08	**pNEU chPylRS$_{IPYE}$** (N-NES+D2N+K3N+Y349F) + tRNA^{M15}	Plasmid I07	Primer 18	Primer 10	Geneblock 22
Plasmid I09	**pNEU chPylRS$_{IPYE}$** (N-NES+D2N+K3N+K258Q+Y349F) + tRNA^{M15}	Plasmid I08	Primer 16	Primer 17	Geneblock 19
Plasmid M16	**pNEU chPylRS$_{IPYE}$** (N-NES+D2N+K3N+E119K+K258Q+Y349F) + tRNA^{M15}	Plasmid I09	Primer 19	Primer 20	Geneblock 16
Plasmid I10	**pNEU chPylRS$_{IPYE}$** (N-NES+K3N+G343D+Y349F) + tRNA^{M15}	Plasmid M06	Primer 16	Primer 17	Geneblock 20
Plasmid M17	**pNEU chPylRS$_{IPYE}$** (N-NES+K3N+S158N+G343D+Y349F) + tRNA^{M15}	Plasmid I10	Primer 19	Primer 20	Geneblock 17
Plasmid M18	**pNEU chPylRS$_{IPYE}$** (N-NES+K3N+D161N+G343D+Y349F) + tRNA^{M15}	Plasmid I10	Primer 19	Primer 20	Geneblock 18
Plasmid M19	**pNEU *Mb*BCNRS** + tRNA^{M15}	Plasmid M01	Primer 21	Primer 17	Geneblock 23
Plasmid M20	**pNEU *Mm*BCNRS** + tRNA^{M15}	Plasmid M02	Primer 22	Primer 17	Geneblock 24
Plasmid M21	**pNEU chBCNRS$_{IPYE}$** (N-NES+D161N+G343D) + tRNA^{M15}	Plasmid M14	Primer 16	Primer 17	Geneblock 25
Plasmid M22	**pNEU *Mb*PylRS(Y271A+Y349F)** + tRNA^{M15}	Plasmid M01	Primer 23	Primer 17	Geneblock 26
Plasmid M23	**pNEU *Mm*PylRS(Y306A+Y384F)** + tRNA^{M15}	Plasmid M02	Primer 24	Primer 17	Geneblock 27
Plasmid M24	**pNEU chPylRS$_{IPYE}$** (N-NES+D161N+Y271A+G343D+Y384F) + tRNA^{M15}	Plasmid M14	Primer 16	Primer 17	Geneblock 28
Plasmid I11	**pNEU chPylRS** (N-NES+G343D+Y349F) + tRNA^{M15} (2x)**	Plasmid I05	Primer 16	Primer 17	Geneblock 20
Plasmid M25	**pNEU chPylRS$_{IPYE}$** (N-NES+D161N+G343D+Y349F) + tRNA^{M15} (2x)	Plasmid I11	Primer 19	Primer 20	Geneblock 18
Plasmid I12	**pNEU iRFP$_{713}$-sfGFP(Y150*)** + tRNA^{M15} (3x)	Plasmid A5	Primer 09	Primer 10	Geneblock 29
Plasmid M26	**pNEU iRFP$_{713}$-sfGFP** + tRNA^{M15} (3x)	Plasmid I12	Primer 09	Primer 25	Geneblock 30
Plasmid I12	**pNEU iRFP$_{713}$-sfGFP(Y150*)** + tRNA^{M15}	Plasmid I12	Primer 14	Primer 15	Geneblock 11
Plasmid M27	**pNEU iRFP$_{713}$-sfGFP(Y150*)**	Plasmid I12	Restriction digest using SalI-HF and PspXI.		
Plasmid M28	**pNEU M$_2$R(V186*) + tRNA^{M15}**	Plasmid M03	Primer 09	Primer 10	Geneblock 31
Plasmid M29	**pNEU β-actin(K118*) + tRNA^{M15}**	Plasmid M03	Primer 09	Primer 10	Geneblock 32

Plasmid M30	**pNEU β-actin(K118*)**	Plasmid M29	Restriction digest using SalI-HF and PspXI.

*Plasmid A5 found to have three copies of $tRNA^{Pyl*}$ after MiniPrep
**one copy of $tRNA^{M15}$ from was randomly lost during the cloning

Plasmid I02 – pNEU *Mb*PylRS (N-NES+Y349F+C-FLAG) + tRNA^{M15} (3x)

NES *Mb*PylRS(Y349F) FLAG tRNA^{M15}(3x)

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Plasmid I03 – pNEU chPylRS$_{IPYE}$ (N-NES+C-FLAG) + tRNA^{M15} (3x)

NES chPylRS$_{IPYE}$ FLAG tRNA^{M15}(3x)

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Plasmid I04 – pNEU *Mb*PylRS (N-NES+Y349F) + tRNA^{M15} (3x)

NES *Mb*PylRS(Y349F) tRNA^{M15}(3x)

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Plasmid I05 – pNEU chPylRS$_{IPYE}$ (N-NES) + tRNA^{M15} (3x)

NES chPylRS$_{IPYE}$ tRNA^{M15}(3x)

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Plasmid I06 – pNEU chPylRS$_{IPYE}$ (N-NES+Y349F) + tRNA^{M15} (3x)

NES chPylRS$_{IPYE}$(Y349F) tRNA^{M15}(3x)

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Plasmid M01 – pNEU *Mb*PylRS (N-NES+Y349F) + tRNA^{M15}

also referred to as pNEU *bm*PylRS^{F}+tRNA^{M15}

NES *Mb*PylRS(Y349F) tRNA^{M15}

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Plasmid M02 – pNEU *Mm*PylRS (N-NES+Y384F) + tRNA^{M15}

also referred to as pNEU *Mm*PylRS^{F}+tRNA^{M15}

NES *Mm*PylRS(Y384F) tRNA^{M15}

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Plasmid M03 – pNEU chPylRS$_{IPYE}$ (N-NES+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS^{F}$_{IPYE}$+tRNA^{M15}

NES chPylRS$_{IPYE}$(Y349F) tRNA^{M15}

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Plasmid M04 – pNEU chPylRS$_{IPYE}$ (N-NES+Y349F) + tRNA^{Pyl*}

also referred to as pNEU chPylRS$^{F}_{IPYE}$+tRNA^{Pyl*}

NES chPylRS$_{IPYE}$(Y349F) tRNA^{Pyl*}

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Plasmid I07 – pNEU chPylRS$_{IPYE}$ (N-NES+Y349F) + tRNA^{M15} *codons re-optimized for other mutations*

NES chPylRS$_{IPYE}$(Y349F) tRNA^{M15}

gacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacg
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Plasmid M05 – pNEU chPylRS$_{IPYE}$ (N-NES+D2N+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(D2N)+tRNA^{M15}

NES chPylRS$_{IPYE}$(D2N+Y349F) tRNA^{M15}

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Plasmid M06 – pNEU chPylRS$_{IPYE}$ (N-NES+K3N+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(K3N)+tRNA^{M15}

NES chPylRS$_{IPYE}$(K3N+Y349F) tRNA^{M15}

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Plasmid M07 – pNEU chPylRS$_{IPYE}$ (N-NES+E119K+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(E119K)+tRNA^{M15}

NES chPylRS$_{IPYE}$(E119K+Y349F) tRNA^{M15}

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Plasmid M08 – pNEU chPylRS$_{IPYE}$ (N-NES+S158N+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(S158N)+tRNA^{M15}

NES chPylRS$_{IPYE}$(S158N+Y349F) tRNA^{M15}

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Plasmid M09 – pNEU chPylRS$_{IPYE}$ (N-NES+D161N+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(D161N)+tRNA^{M15}

NES chPylRS$_{IPYE}$(D161N+Y349F) tRNA^{M15}

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Plasmid M10 – pNEU chPylRS$_{IPYE}$ (N-NES+K258Q+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(K258Q)+tRNA^{M15}

NES chPylRS$_{IPYE}$(K258Q+Y349F) tRNA^{M15}

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Plasmid M11 – pNEU chPylRS$_{IPYE}$ (N-NES+G343D+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(G343D)+tRNA^{M15}

NES chPylRS$_{IPYE}$(G343D+Y349F) tRNA^{M15}

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Plasmid M13 – pNEU chPylRS$_{IPYE}$ (N-NES+S158N+G343D+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(S158N+G343D)+tRNA^{M15}

NES chPylRS$_{IPYE}$(S158N+G343D+Y349F) tRNA^{M15}

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Plasmid M14 – pNEU chPylRS$_{IPYE}$ (N-NES+D161N+G343D+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{2020}$+tRNA^{M15}

NES chPylRS$_{IPYE}$(D161N+G343D+Y349F) tRNA^{M15}

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Plasmid M15 – pNEU chPylRS$_{IPYE}$ (N-NES+S158N+D161N+G343D+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(S158N+D161N+G343D)+tRNA^{M15}

NES chPylRS$_{IPYE}$(S158N+D161N+G343D+Y349F) tRNA^{M15}

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Plasmid I08 – pNEU chPylRS$_{IPYE}$ (N-NES+D2N+K3N+Y349F) + tRNA^{M15}

NES chPylRS$_{IPYE}$(D2N+K3N+Y349F) tRNA^{M15}

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Plasmid I09 – pNEU chPylRS$_{IPYE}$ (N-NES+D2N+K3N+K258Q+Y349F) + tRNA^{M15}

NES chPylRS$_{IPYE}$(D2N+K3N+K258Q+Y349F) tRNA^{M15}

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Plasmid M16 – pNEU chPylRS$_{IPYE}$ (N-NES+D2N+K3N+E119K+K258Q+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}_{IPYE}$(+32A*)+tRNA^{M15}

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Plasmid I10 – pNEU chPylRS$_{IPYE}$ (N-NES+K3N+G343D+Y349F) + tRNA^{M15}

NES chPylRS$_{IPYE}$(K3N+G343D+Y349F) tRNA^{M15}

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Plasmid M17 – pNEU chPylRS$_{IPYE}$ (N-NES+K3N+S158N+G343D+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}_{IPYE}$(+24B*)+tRNA^{M15}

NES chPylRS$_{IPYE}$(K3N+S158N+G343D+Y349F) tRNA^{M15}

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Plasmid M18 – pNEU chPylRS$_{IPYE}$ (N-NES+K3N+D161N+G343D+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{F}{}_{IPYE}$(+25C*)+tRNA^{M15}

NES chPylRS$_{IPYE}$(K3N+D161N+G343D+Y349F) tRNA^{M15}

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Plasmid M19 – pNEU *Mb*BCNRS(N-NES) + tRNA^{M15}

NES *Mb*BCNRS tRNA^{M15}

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NES *Mm*BCNRS tRNA^{M15}

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Plasmid M21 – pNEU chBCNRS$_{IPYE}$(N-NES+ D161N+G343D) + tRNA^{M15}

also referred to as pNEU chBCNRS$_{2020}$+tRNA^{M15}

NES chBCNRS$_{IPYE}$(D161N+G343D) tRNA^{M15}

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Plasmid M22 – pNEU *Mb*PylRS(N-NES+Y271A+Y349F) + tRNA^{M15}

also referred to as pNEU *Mb*PylRS^{AF}+tRNA^{M15}

NES *Mb*PylRS(Y271A+Y349F) tRNA^{M15}

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Plasmid M23 – pNEU *Mm*PylRS(N-NES+Y306A+Y384F) + tRNA^{M15}

also referred to as pNEU *Mm*PylRS^{AF}+tRNA^{M15}

NES *Mm*PylRS(Y306A+Y384F) tRNA^{M15}

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Plasmid M24 – pNEU chPylRS$_{IPYE}$(N-NES+Y271A+Y349F) + tRNA^{M15}

also referred to as pNEU chPylRS$^{AF}{}_{2020}$+tRNA^{M15}

NES chPylRS$_{IPYE}$(Y271A+Y349F) tRNA^{M15}

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Plasmid I11 – pNEU chPylRS$_{IPYE}$ (N-NES+G343D+Y349F) + tRNA^{M15} (2x)

NES chPylRS$_{IPYE}$(G343D+Y349F) tRNA^{M15} (2x)

gacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacg
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Plasmid M25 – pNEU chPylRS$_{IPYE}$ (N-NES+D161N+G343D+Y349F) + tRNA^{M15} (2x)

also referred to as pNEU chPylRS$^{F}{}_{2020}$+tRNA^{M15}(2x)

NES chPylRS$_{IPYE}$(G343D+Y349F) tRNA^{M15} (2x)

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Plasmid I12 – pNEU iRFP$_{713}$-sfGFP(Y150*) + tRNA^{M15} (3x)

iRFP$_{713}$ sfGFP(Y150*) tRNA^{M15} (3x)

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Plasmid M26 – pNEU iRFP$_{713}$-sfGFP + tRNA^{M15} (3x)

iRFP$_{713}$ sfGFP tRNA^{M15} (3x)

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Plasmid I13 – pNEU iRFP$_{713}$-sfGFP(Y150*) + tRNA^{M15}

iRFP$_{713}$ sfGFP(Y150*) tRNA^{M15}

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Plasmid M27 – pNEU iRFP$_{713}$-sfGFP(Y150*)

iRFP$_{713}$ sfGFP(Y150*)

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Plasmid M28 – pNEU M$_2$R(Y150*) + tRNA^{M15}

M$_2$R(V186*) tRNA^{M15}

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Plasmid M29 – pNEU β-actin(K118*) + tRNA^{M15}

β-actin(K118*) tRNA^{M15}

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Plasmid M30 – pNEU β-actin(K118*)

β-actin(K118*) tRNA^{M15}

gacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacg
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ggttttggcagtacatcaatgggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataag
cagagctctctggctaactagagaacccactgcttactggcttatcgaaattaatacgactcactatagggagacccaagctggctagcgccacc**ATGGACGACGATATCGCCGCTCTGGTGGTGGATAATGGCAGCGGAATGTGCAAGGCCGGAT**
TTGCCGGGGATGATGCCCCTAGAGCCGTGTTTCCTAGCATCGTGGGCAGACCTAGACACCAGGGCGTGATGGTTGGAATGGGCCAGAAAGACAGCTACGTGGGCGACGAGGCCCAGAGCAAGAG
AGGAATCCTGACACTGAAGTACCCCATCGAGCACGGCATCGTGACCAACTGGGACGACATGGAAAAGATCTGGCACCACACCTTCTACAACGAGCTGAGAGTGGCCCCTGAGGAACACCCTGTGC
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GGCAGAACAACCGGAATCGTGATGGATTCTGGCGACGGCGTGACCCACACCGTGCCTATCTATGAGGGATACGCCCTGCCTCACGCCATCCTGAGACTGGATCTGGCCGGCAGAGATCTGACCGA
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acgaacccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaga
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ttaccgctgtttgactccccgtcaagctctaaatcggggcatccct

16 Primers

All sequences written 5’ to 3’

Primer 01:	ACCAGCAATAGACATAAGC
Primer 02:	CGAGAAAGTCAGCAGG
Primer 03:	AACAGAATTTGCCTGGC
Primer 04:	CCACACATTATACGAGCC
Primer 05:	GATACCCTGGATGTTATGC
Primer 06:	GCTTTTGGTCAGTGCC
Primer 07:	TGAGCAATAACTAGCATAACC
Primer 08:	CCTATGCCTGTTACCAC
Primer 09:	CGACTGTGCCTTCTAG
Primer 10:	TCTCCCTATAGTGAGTCG
Primer 11:	CATCGCATTGTCTGAGT
Primer 12:	ACTCTCTATCCAGAGACAC
Primer 13:	CACTCTCTATCCAGAGGA
Primer 14:	CTCAGTACAATCTGCTCTG
Primer 15:	TCAGGTGGCACTTTTC
Primer 16:	ATTCCTCTGGATAGAGAGTG
Primer 17:	GAAGATCTGCTTGCTCAG
Primer 18:	ACAAGATCAAGCACTACG
Primer 19:	GATCTCCAGCAGATCTACG
Primer 20:	TTCTGCTGTTGTTCACG
Primer 21:	ACTGCTGAAAGTGATGC
Primer 22:	TTCAAGAACATCAAGAGGG
Primer 23:	GTGTCTCTGGATAGAGAGTG
Primer 24:	CCTGGAATCTATCATCACC
Primer 25:	CTGATGGTTCTCTCTTGC

17 Geneblocks

All sequences written 5’ to 3’

Geneblock 01

AAGCGGCCTGCTGACTTTCTCGCCGATCAAAAGGCATTTTGCTATTAAGGGATTGACGAGGGCGTATCTGCGCAGTAAGATGCGCCCCGCATTGGAAACCTGATCATGTAGATCGAATGGACTCTAAAT
CCGTTCAGCCGGGTTAGATTCCCGGGGTTTCCGCCAAATTCGAAAAGCCTGCTCAACGAGCAGGCTTTTTTGCATGCTCGAGCAGCTCAGGGTCGAATTTGCCATGGCGGCCACCAGGTACCACCGGC
GCCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGC

Geneblock 02

CATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACAAAGGAGGTGCGGCCGCATGGACAAAAAACCGCTGGATGTTCTGATTAGCGCAACCGGTCTGTGGATGAGCCGCACCGGTA
CACTGCATAAAATCAAACATTATGAAATCAGCCGCAGCAAAATCTATATTGAAATGGCATGTGGTGATCATCTGGTGGTGAATAATAGCCGTAGCTGTCGTCCGGCACGTGCATTTCGTTATCACAAATAT
CGTAAAACCTGCAAACGTTGCCGTGTGAGTGATGAAGATATTAACAATTTTCTGACCCGTAGCACCGAAGGTAAAACCAGCGTTAAAGTTAAAGTTGTGAGCGAGCCGAAAGTGAAAAAAGCAATGCCG
AAAAGCGTTAGCCGTGCACCGAAACCTCTGGAAAATCCGGTTAGCGCAAAAGCAAGCACCGATACCAGCCGTAGCGTTCCGAGTCCGGCAAAAAGCACCCCGAATAGTCCGGTTCCGACCAGCGCAA
GCGCACCGGCACTGACCAAAAGCCAGACCGATCGCCTGGAAGTTCTGCTGAATCCGAAAGATGAAATTAGCCTGAATAGCGGTAAACCGTTTCGTGAACTGGAAAGCGAACTGCTGAGCCGTCGTAAA
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CTGGAAAGCATTATTACCGATTTTCTGAACCATCTGGGCATCGATTTCAAAATTGTTGGTGATAGCTGCATGGTGTATGGTGATACCCTGGATGTTATGCATGGTGATCTGGAACTGAGTAGCGCAGTTG
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GCTATTATAACGGTATTAGCACCAATCTGTAAGCGGCCGCGTTTAAACGGTCTCCAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTG
ATAAAACAGAATTTGCCTGGCGGCAG

Geneblock 03

AAGCGGCCTGCTGACTTTCTCGCCGATCAAAAGGCATTTTGCTATTAAGGGATTGACGAGGGCGTATCTGCGCAGTAAGATGCGCCCCGCATTGGAAACCTGGTCAGGGAGACCGAACGGACTCTAAA
TCCGTTCAGCCGGGTTCGATTCCCGGGGTTTCCGCCAAATTCGAAAAGCCTGCTCAACGAGCAGGCTTTTTTGCATGCTCGAGCAGCTCAGGGTCGAATTTGCCATGGCGGCCACCAGGTACCACCGG
CGCCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGC

Geneblock 04

CGCACCGGCACTGACCAAAAGCCAGACCGATCGCCTGGAAGTTCTGCTGAATCCGAAAGATGAAATTAGCCTGAATAGCGGTAAACCGTTTCGTGAACTGGAAAGCGAACTGCTGAGCCGTCGTAAAA
AAGATCTGCAGCAGATTTATGCCGAAGAACGCGAAAACTATCTGGGTAAACTGGAACGTGAAATCACCCGTTTTTTTGTGGATCGTGGTTTCCTGGAAATCAAAAGCCCGATTCTGATTCCGCTGGAATA
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CTGCCGGATCCGATTAAAATCTTTGAAATTGGTCCGTGCTACCGCAAAGAAAGTGATGGTAAAGAACACCTGGAAGAATTCACCATGCTGAACTTTTGCCAGATGGGTAGCGGTTGTACCCGTGAAAATC
TGGAAAGCATTATTACCGATTTTCTGAACCATCTGGGCATCGATTTCAAAATTGTTGGTGATAGCTGCATGGTGTTTGGTGATACCCTGGATGTTATGCATG

Geneblock 05

CGCACCGGCACTGACCAAAAGCCAGACCAATCGTCTGGAAGTTCTGCTGAATCCGAAAGATGAAATTAGCCTGAATAGCGGTAAACCGTTTCGTGAACTGGAAAGCGAACTGCTGAGCCGTCGTAAAAA
AGATCTGCAGCAGATTTATGCCGAAGAACGCGAAAACTATCTGGGTAAACTGGAACGTGAAATCACCCGTTTTTTTGTGGATCGTGGTTTCCTGGAAATCAAAAGCCCGATTCTGATTCCGCTGGAATAT
ATTGAACGTATGGGCATTGATAACGATACCGAACTGAGCAAACAAATCTTTCGCGTGGATAAAAACTTTTGTCTGCGTCCGATGCTGGCACCGAATCTGTATAACTACCTGCGCAAACTGGATCGTGCAC
TGCCGGATCCGATTAAAATCTTTGAAATTGGTCCGTGCTACCGCAAAGAAAGTGATGGTAAAGAACACCTGGAAGAATTCACCATGCTGAACTTTTGCCAGATGGGTAGCGGTTGTACCCGTGAAAATCT
GGAAAGCATTATTACCGATTTTCTGAACCATCTGGGCATCGATTTCAAAATTGTTGATGATAGCTGCATGGTGTTTGGTGATACCCTGGATGTTATGCATG

Geneblock 06

GTCATGTGGTAACAGGCATAGGTATGAAAGTGAAAGTTCTGAGCCTGCTGGTTCCGGCACTGCTGGTTGCGGGTGCAGCAAATGCAGCCGAAGTTTATAACAAAGATGGCAACAAACTGGACCTGTAT
GGTAAAGTTGATGGCCTGCACTATTTCAGCGATAATAAAGATGTTGATGGCGACCAGACCTATATGCGTCTGGGTTTTAAAGGTGAAACCCAGGTTACCGATCAGCTGACCGGCTATGGTCAGTGGGAA
TATCAGATTCAGGGTAATAGCGCAGAAAACGAAAATAACAGCTGGACCCGTGTTGCATTTGCAGGTCTGAAATTCCAGGATGTTGGTAGCTTTGATTATGGTCGTAATTATGGCGTTGTGTATGATGTTA
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GCCTGGCATATCTGCAGAGCAAAGGTAAAAATCTTGGTCGCGGTTATTAGGATGAGGACATTCTGAAATACGTTGATGTTGGTGCCACCTACTACTTCAATAAAAACATGAGCACCTACGTGGACTACAA
AATTAACCTGCTGGATGATAATCAGTTTACCCGTGATGCAGGCATTAATACCGATAATATTGTTGCCCTGGGTCTTGTGTATCAGTTCTGAGTTTGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGA
GTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC

Geneblock 07

AATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGCCACCATGGCTTGTCCTGTGCCTCTGCAGCTGCCTCCTCTGGAAAGACTGACCCTGGACATGGACAAGAAACCCCTGGACGTGCTGATCT
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GATCAGCCTCGACTGTGCCTTCTAGTTGCCA

Geneblock 08

AATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGCCACCATGGCTTGTCCTGTGCCTCTGCAGCTGCCTCCTCTGGAAAGACTGACCCTGGACATGGACAAGAAACCCCTGGACGTGCTGATCT
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GTCTGAGTAGGTG

Geneblock 09

CCCGTGTCTCTGGATAGAGAGTGGGGCATCGACAAGCCCTGGATCGGAGCCGGATTTGGCCTCGAGAGACTGCTGAAAGTGATGCACGGCTTCAAGAACATCAAGCGGGCCAGCAGATCCGAGAGCT
ACTACAACGGCATCTCCACCAACCTGTGAGCGGCCGCTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCA

Geneblock 10

CCATTCCTCTGGATAGAGAGTGGGGCATCGATAAGCCCTGGATCGGAGCCGGCTTTGGCCTCGAGAGACTGCTGAAAGTCAAGCACGATTTCAAGAACATCAAGAGGGCCGCCAGAAGCGAGAGCTA
CTACAACGGCATCAGCACCAACCTGTGAGCGGCCGCTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGT
GCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTG

Geneblock 11

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TCTCAGTACAATCTGCTCTGATG

Geneblock 12

AATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGCCACCATGGCTTGTCCTGTGCCTCTGCAGCTGCCTCCTCTGGAAAGACTGACCCTGGACATGGACAAGAAGCCCCTGAACACCCTGATCA
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Geneblock 13

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Geneblock 14

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Geneblock 15

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Geneblock 16

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Geneblock 17

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Geneblock 18

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Geneblock 19

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Geneblock 20

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AGTGGG

Geneblock 21

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Geneblock 22

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Geneblock 23

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Geneblock 24

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Geneblock 25

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AGTGGG

Geneblock 26

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Geneblock 27

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Geneblock 28

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Geneblock 29

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Geneblock 30

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Geneblock 31

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Geneblock 32

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Geneblock 33

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AGTGGG

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