Supporting Information: Combining Site-Directed Spin Labeling *in vivo* and in-cell EPR Distance Determination

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Chemicals

All chemicals were obtained from Carl Roth or Sigma Aldrich unless stated otherwise. 2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl) acetic acid (BTTAA) was purchased from Jena Bioscience and 4-ethynyl-L-phenylalanine hydrochloride (pENF) from Achemblock. 3-(Azidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (az-proxyl) was synthesized according to published procedure.[1]

Protein Expression

Cloning and transformation of *E. coli*

Amber mutations were introduced into pBAD_Y39TAG-GFP_His6[2] or pBAD_GFP-WT_His6 at position 221 by site-directed mutagenesis (QuikChange II Site-directed mutagenesis Kit, Agilent) using the primer pairs GCG ATC ACA TGG TCC TGT AGG AGT TCG TGA CCG CCG CCG / CGG CGG TCA CGA ACT CCT ACA GGA CCA TGT GAT CGC GC.

Chemically competent BL21-Gold (DE3) *E. coli* were co-transformed with the plasmids pEVOL_pCNPhe as provided by the Schultz lab and either pBAD_GFP-WT_His6[3] or the gene construct containing the respective mutation for amber stop codon suppression, pBAD_Y39TAG_GFP_His6[2], pBAD_L221TAG_GFP_His6 or pBAD_Y39/L221TAG_GFP_His6. 100 µl competent bacteria were thawed on ice, mixed with an appropriate amount of the respective plasmids and incubated on ice for 30 min. The cells were then subjected to a heat-shock at 42 °C for 1 min and incubated for another 2 min on ice, before being added to 1 mL pre-warmed (37°C) Super Optimal Broth with carbolite repression (SOC-medium). Afterward, cells were incubated for 1 h at 37°C and 1400 rpm and grown on an LB-Agar plate (Lennox) containing 34 µg/mL chloramphenicol and 50 µg/mL carbenicillin overnight. Carbenicillin was used as a substitute for ampicillin due to its improved stability when used in growth media. For bacterial glycerol stock creation, LB-medium containing 34 µg/mL chloramphenicol and 50 µg/mL carbenicillin was inoculated with a single colony from the agar plate, incubated overnight at 37°C and 180 rpm, and afterward, 500 µL overnight culture were mixed with 500 µL 50 % (v/v) glycerol in a 2 mL cryovial and stored at -80°C.

Protein expression

An overnight culture from the glycerol stocks was grown in LB-medium containing 34 µg/mL chloramphenicol and 50 µg/mL carbenicillin at 37°C and 180 rpm. For protein expression, this culture was diluted 1:100 into fresh LB medium supplemented with the same antibiotic concentrations and incubated at 37°C and 180 rpm until an OD_{600} of 0.3 to 0.4 was reached. Cell cultures were then induced with 0.2 % L-arabinose from a 20 % w/v stock solution (sterile-filtrated in MilliQ-water) and supplemented with 0.5 mM pENF by directly adding the respective amount of the solid ncAA to the medium. The protein expression took place under the same incubation conditions for 12 h.

Protein purification

After overnight protein expression, cells were harvested by centrifugation (4°C, 4000 rpm, 15 min) and the supernatant was discarded. Protein samples were kept on ice the whole time. For protein purification by Ni-NTA affinity chromatography, cell pellets were resuspended in bacterial protein extraction reagent (B-PER, Thermo Fisher Scientific) supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF) and lyzed via sonification (Q700 QSONICA, cycles of 1 s pulse and 1 s pause for 60 s). Cell lysates were centrifuged (10 min, 15 000 rpm and 4°C), the supernatant was added to HisPur Ni-NTA resin and incubated for 1 h at 4°C. The beads were then washed several times with washing buffer (50 mM NaH_2PO_4, 300 MM NaCl, pH 8,0) containing up to 30 mM imidazole. eGFP was eluted from the beads in the same buffer supplemented with 500 mM imidazole. Protein concentrations were determined photometrically via absorption at 280 nm. The proteins were dialyzed against PBS buffer pH 7.5 for the CuAAC labeling reaction or MilliQ-water for ESI-MS.

Copper toxicity

Growth curves and plate sensitivity assay

A culture of BL21-Gold (DE3) *E.coli* cotransformed with pBAD_Y39/L221TAG_GFP_His6 and pEVOL_pCNF were grown overnight in 50 ml LB medium containing 34 µg/mL chloramphenicol and 50 µg/mL carbenicillin at 37°C and 180 rpm. Cells were harvested by centrifugation (25°C, 4000 rpm, 15 min) and washed 3 x with 25 mL PBS buffer pH 7.5 and the optical density (O.D.600) was adjusted to 160 prior to treatment with CuAAC labeling reagents. Cells were added to the indicated labeling reagents and incubated for 40 minutes at 25 °C and 400 rpm.

For the E.coli growth monitoring, treated cells were diluted with LB medium and the appropriate antibiotics by a factor of 10^5. Absorption at 600 nm was measured with a microplate reader (SPARK®, Tecan) for 14 h at 37°C. Results were confirmed in 2 independent experiments, each averaged over measurements from 2 wells with 200 µl bacterial solution.

The long-term growth of E.coli was assessed in a plate sensitivity assay. A dilution series of treated cells ranging from 10^1 to 10^8 was spotted onto LB agar plates supplemented with chloramphenicol and carbenicillin to find a suitable concentration to count individual colony forming units (CFU). Treated (1 mM CuSO4, 3 mM BTTAA, 1 mM NaAsc) and untreated (PBS control) cells were diluted by a factor of 10^7 after incubation with the labeling reagents, plated and incubated overnight at 37°C. The number of CFU per plate was counted for 3 independent experiments.

Flow cytometry

After overnight protein expression, Y39/L221pENF expressing cells were harvested by centrifugation (25°C, 4000 rpm, 15 min), washed 3x with 25 mL PBS buffer, pH 7.5 and the optical density was adjusted to 160 for treatment with CuAAC labeling reagents. Cells were incubated with 1 mM CuSO4, 3 mM BTTAA and 1 mM NaAsc, 30 mM CuSO4 or PBS (untreated control) for 40 minutes at 25°C, then washed 4 x with 1 mL PBS buffer pH 7.5. For flow cytometry analysis, cells were diluted to O.D.600 = 0.01 and stained with propidium iodide solution (Thermo Fisher) for 5 minutes. The dead samples of cells were prepared by fixing in 75% ethanol and stained in the same way. Cells were analyzed on a BD LSRFortessa ™ (BD Bioscience). GFP and PI were excited at 488 nm or 561 nm and detected with a 505 LP and 530/50BP or 600LP and 610/20BP filters. 50,000 cells were counted. Data analysis was performed with the FlowJo Software 10.6.1.

CuAAC labeling protocols

In vitro CuAAC labeling reaction

The labeling reaction was performed according to the previously published protocol[1] with small alterations. Briefly, copper(II)-sulfate ($CuSO_4$) and the ligand BTTAA[4] were mixed in water in a 1:3 ratio. Ascorbic acid was added in a 1:1 ratio to copper to reduce Cu(II) ions into the catalytically active Cu(I) species. The labeling reagents were then diluted with PBS buffer pH 7.5 and protein, as well as 100 mM 3-(azidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (az-proxyl) label in dimethyl sulfoxide (DMSO), was added, resulting in a final concentration of 1 mM copper(II)-sulfate, 3 mM BTTAA, 1 mM sodium ascorbate, 50 µM protein, and 1 mM az-proxyl spin-label. The CuAAC reaction took place at 25°C for 90 min and 800 rpm in an Eppendorf ThermoMixer C. Afterward, excess reagents were removed by size-exclusion chromatography *via* spin desalting columns (Zeba™ Spin Desalting column, 7 K MWCO, 2 mL column material, Thermo Fisher Scientific). Additional washing steps were performed *via* ultrafiltration in centrifugal filter units (Amicon Ultra-0.5 mL Centrifugal Filters, 3.5 K MWCO, Merck; 15 min, 12 000 rpm, 4°C) to remove excess reagents and concentrate the protein sample in the process: Protein samples were washed 4x with 400 µL PBS buffer containing 1

mM EDTA to remove remaining copper ions, followed by either 6x 400 µL MilliQ-water (samples prepared for fluorescence spectroscopy) or 6x 400 µL 1.25x PBS (samples prepared for cw EPR and DEER measurements). DEER samples were prepared in PBS buffer containing 20% deuterated glycerol. The protein concentration was adjusted to 18 µM which resulted in an overall spin concentration of 25 µM. A volume of 60 µL was filled into 3 mm outer diameter quartz tubes (Fused quartz tubing, Technical Glass Products) and shock-frozen in liquid nitrogen.

In vivo CuAAC labeling reaction

After overnight protein expression, cells were harvested by centrifugation (25°C, 4000 rpm, 15 min) and the supernatant was discarded. The cell pellet was dissolved in 25 mL pre-warmed LB medium containing 34 µg/mL chloramphenicol, 50 µg/mL carbenicillin and 0.2 % L-arabinose and incubated at 37°C and 180 rpm for 1.5 h. Cells were then washed 3x by centrifugation (4°C, 4000 rpm, 10 min) and resuspension of the pellet in 20 mL cold PBS buffer pH 7.5. After the last washing step, cells were resuspended in 1 mL PBS buffer and the optical density (OD_{600}) of the suspension was adjusted to OD_{600} = 160 for the labeling reaction. *In vivo* labeling took place for 40 min and 25 °C in a reaction volume of 500 µl and with the same reagent concentrations as for *in vitro* labeling. The excess spin-label was then washed away by centrifugation and resuspension of the pellet in PBS buffer. (2000 g, 2 min 4°C, 4x 1 mL washing solution). After discarding the supernatant, cell pellets were either subjected to room temperature cw EPR spectroscopy or shock-frozen in liquid nitrogen for DEER distance determination. On average, 60 minutes passed between exposure of the nitroxide labeling reagent to the cells and shock-freezing of the sample or start of the cw EPR experiment, respectively.

X-band cw EPR

Room-temperature cw EPR spectra were recorded at 20°C and with an X-band spectrometer (EMX-Nano, Bruker with a cylindric cavity mode TM110). Typically, 40 µL sample volume was filled into a glass capillary (HIRSCHMANN® ringcaps®; inner diameter 1.02 mm). Spectra were recorded with a modulation amplitude of 1 G, a microwave attenuation of 15 dB corresponding to a power of 3.162 mW, a sweep width of 200 G, a sweep time of 30 s (*in vivo*) or 60 s (*in vitro*). *In vitro* measurements were averaged over 20 scans to increase the signal-to-noise ratio. Quantitative spin concentrations were directly obtained via the built-in EMXnano reference-free spin counting module (Xenon software, Bruker). The labeling efficiency was calculated as the ratio of the spin concentration to the protein concentration. For *in vivo* measurements, 2D-field sweeps experiments were performed to record spectra from individual scans time-dependently. Afterwards, the spectra either averaged over 5 scans for nitroxide reduction kinetics (shown in Figure 3 A) or consecutive scans were added up until the highest possible signal-to-noise ratio was achieved (Figure S8, 100 Scans).[5] All recorded spectra were processed using MatLab2018A (MatWorks, Inc.).

Q-band pulsed EPR

Pulsed EPR experiments were performed at Q-band (34 GHz) frequency at 50 K with a shot repetition time of 4 ms to avoid nitroxide saturation. Echo signals were detected with an integrator gate width corresponding to the respective π pulse length and a video-bandwidth of 20 MHz.

Echo-detected field sweep

Echo-detected field sweeps were recorded with a Hahn echo sequence (π/2 – 800 ns – π – 800 ns –echo), a sweep width of 4000 G and 50 shots per point. Pulse lengths were optimized with a nutation experiment for nitroxide species (B = 12096 G), yet similar pulse lengths were obtained in nutation experiments for copper species (B = 11800 G).

Phase memory relaxation

The phase memory time T_m was determined by increasing the interpulse delay of a Hahn echo sequence (starting with τ = 800 ns) and extracting the time point at which the signal intensity decreased to 1/e of the initial intensity at t = 0µs.

DEER measurement, data analysis, and evaluation

EPR inter-spin distance measurements were performed at Q-band (34 GHz) on a commercially available Bruker Elexsys E580 spectrometer operating with a SpinJet-AWG unit (Bruker Biospin) and a 150 W pulsed traveling-wave tube (TWT) amplifier (Applied Systems Engineering). Samples were held at cryogenic temperatures (50K) with the EPR Flexline helium recirculation system (CE-FLEX-4K-0110, Bruker Biospin, ColdEdge Technologies) comprising a cold head (expander, SRDK-408D2) and a F-70H compressor (SHI cryogenics), controlled by an Oxford Instruments Mercury ITC. The commercial Q-band resonator (ER5106QT-2, Bruker Biospin) was over-coupled for four-pulse DEER experiments[6, 7]. A sech/tanh pulse[8, 9] (t_p = 100 ns, $\beta = 6/t_p$, $\Delta\nu$ = 90 MHz) was programmed with EasySpin, adjusted to 34 GHz and used as pump pulse. A frequency offset of 80 MHz (to 33.92 GHz) was chosen for the rectangular observer pulses. Observer pulse lengths were optimized for every sample with π-pulses typically varying from 24 to 28 ns and $\pi/2$-pulses from 12 to 14 ns. Nuclear modulations were averaged by incrementing the first observer interpulse delay in 8 steps of 16 ns each from an initial value of 600 ns. For phase-cycling, the eight-step phase cycle $[x](x)x_px$ as proposed by Tait and Stoll[9] was employed. *In vivo* samples were recorded for 24 h, *in vitro* samples for 12 h.

Distance distributions were derived from DEER traces via DeerAnalysis2018[10]. Zero times were automatically determined, cut-offs excluded the last 150 ns of the DEER trace to avoid "2+1" end artifacts[11] and background starts were manually determined. The *in vitro* DEER measurement of Y39/L221pENF-L eGFPF was fitted with a homogeneous background model (dimension d = 3). In cell DEER traces of singly-labeled Y39pENF-L eGFP and L221pENF-L could be completely described with a homogeneous background model (d= 2.2-2.3, set background start to 0 ns), and analogously, *in vivo* DEER measurement of Y39L221pENF-L eGFP were fitted with d = 2.36.

Distance distributions were obtained by Tikhonov regularization using the L-curve criterion and assigned alpha parameters were chosen according to the L curve corner recognition as implemented in the DeerAnalysis software. Distance distributions of Y39/L221pENF-L were validated (prune level 1.15) by variation of the noise (1.5 / 5 steps), the starting time of the background fit (*in vitro*: 300-1600 / 14 steps, in vivo: 150-500 / 8 steps) and also the background dimension (in vitro: 3 – 3.5 / 6 steps, in vivo: 2.0-3.0 / 11 steps).

Mass Spectrometry

Samples were diluted to 50 µM and the buffer changed to MilliQ-water. Intact proteins were analyzed by direct infusion on an amaZon speed ETD mass spectrometer (Bruker) with a flow rate of 4 µL/min. The mass spectrometric data were acquired for about 3 minutes and the final mass spectrum was averaged over the whole acquisition time. Mass spectrometric data were evaluated using the Data Analysis Version 4.4 (Bruker) software.

Fluorescence spectroscopy

200 nM protein solutions of WT eGFP, Y39/L221pENF eGFP, and spin-labeled Y39/L221pENF-L eGFP were filled in quartz cuvettes (High Precision Cell made of quartz SUPRASIL, Hellma Analytics) and fluorescence excitation and emission spectra of were recorded with a Cary Eclipse Fluorescence Spectrophotometer (Agilent) with a scanning rate of 600 nm/min and averaging time of 0.1 s and a recording range of 500-650 nm (scan mode emission, excitation at 488 m) or 300-500 nm (excitation scan mode, emission at 508 nm). Spectra were normalized to the maximum.

Supplementary figures

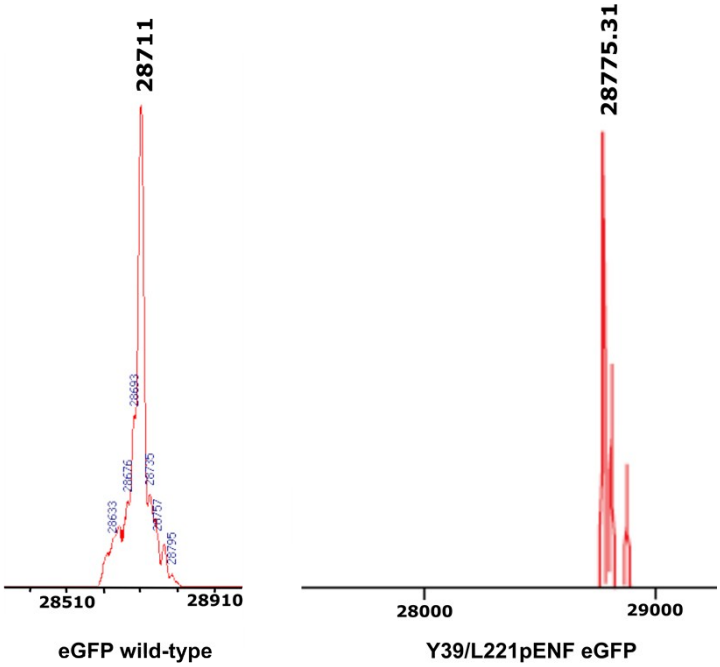

Figure S1 Full-length ESI-MS spectra of eGFP wild-type and Y39/L221pENF eGFP after expression and purification. The mass shift of 64.31 Da between both proteins corresponds to the successful exchange of Y and L with 2 pENF.

Sample preparation	Found m/z	Calculated m/z	Protein assignment
eGFP wild-type after expression	28711 Da		eGFP WT
Y39/L221pENF eGFP after expression	28775.31 Da	28776.88 Da	WT eGFP (28711 Da) – Y(181.07 Da) – L(131.09 Da) + 2 pENF (2*189.02 Da)

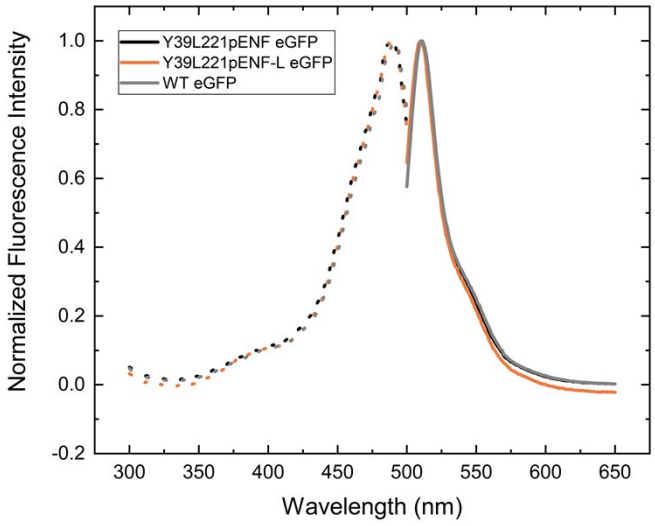

Figure S2 Fluorescence excitation (dashed lines, detection at 508 nm) and emission (solid line, excitation at 488 nm) spectra were recorded for eGFP wilde-type (gray), Y39/L221pENF eGFP (black) and spin-labled Y39/L221pENF-L eGFP (orange).

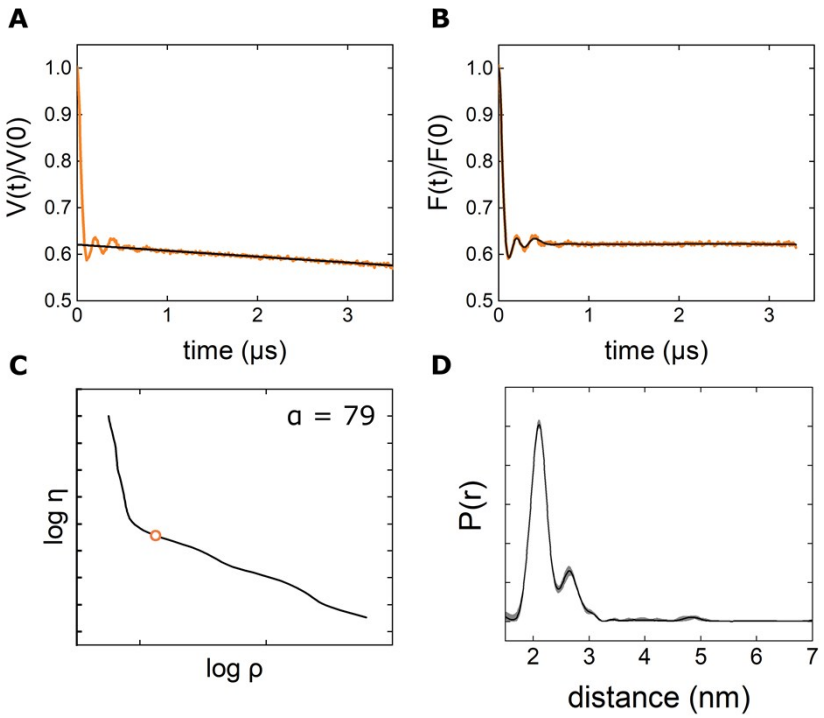

Figure S3 ***In vitro*** **DEER distance determination of purified Y39/L221pENF-L eGFP**. **(A)** Normalized DEER trace with homogeneous background fit **(B)** Background corrected form factor with fit by Tikhonov regularization **(C)** L-curve with the chosen alpha parameter **(D)** Validated distance distribution

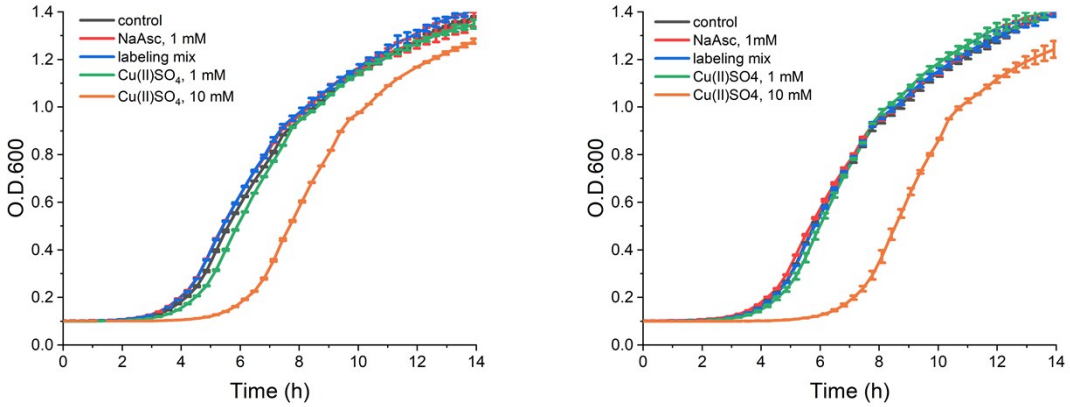

Figure S4 E. coli growth after treatment with CuAAC labeling reagents: 1 mM sodium ascorbate (red), labeling mix Cu(II)SO4, BTTAA, NaAsc (1/3/1 mM) (blue), 1 mM Cu(II)SO4 (green), 10 mM Cu(II)SO4 (orange), untreated control (gray) The growth curves are shown for 2 independent experiments, data points are averaged over 2 wells, error bars show standard deviation.

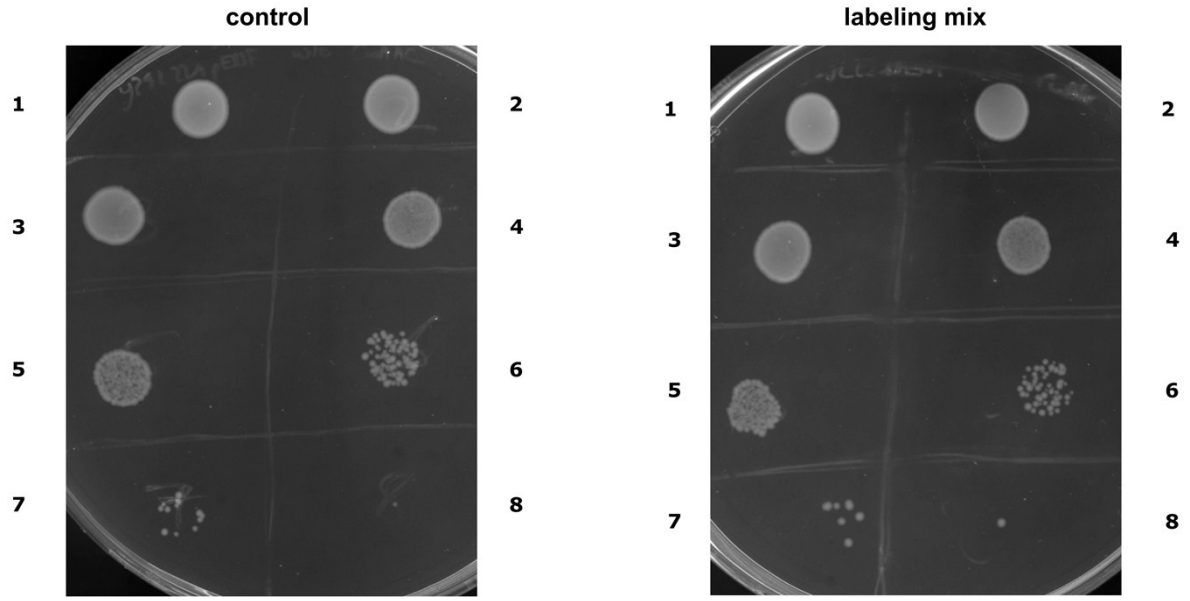

Figure S5 Plate sensitivity assay of *E.coli* cells. Bacterial cells were either treated with 1 mM CuSO4, 3 mM BTTAA, 1 mM NaAsc (labeling mix) or an equal volume of PBS (control) for 40 min at room temperature, then diluted with PBS buffer by a factor of 10^1-10^8 (numbers from 1-8) and spotted onto plates. The plates were incubated at 37°C for 14 h before read-out.

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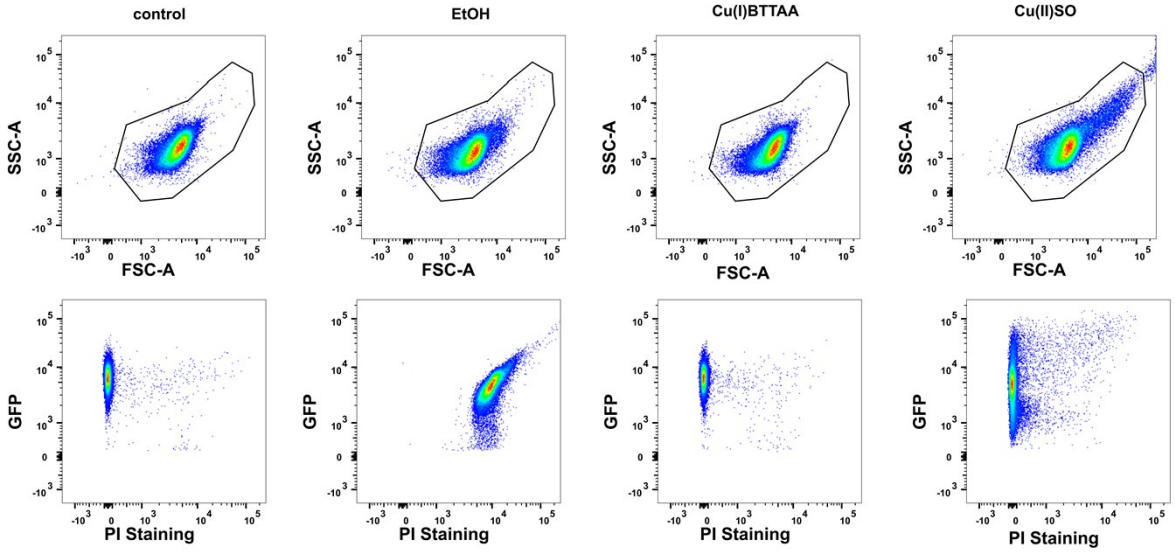

Figure S6 Flow cytometry analysis to determine the copper toxicity. Events were pre-gated based on the forward and sideward scattering (top row). Bottom row plots GFP fluorescence intensity against PI intensity.

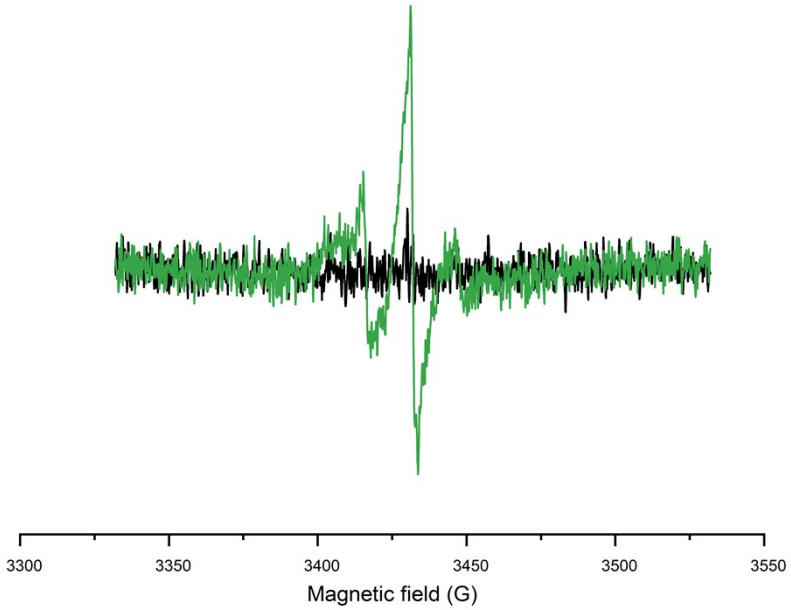

Figure S7 Removal of excess spin-label after CuAAC labeling reaction. *E. coli* cells expressing Y39/L221pENF eGFP (green) or WT eGFP in the absence of pENF (black) were subjected to the CuAAC labeling reaction and washing procedure. CW EPR spectra were recorded 60 minutes after nitroxide exposure to the cells and averaged over 5 scans to improve the signal-to-noise ratio. The black spectrum indicates that the unbound spin-label reagent az-proxyl is almost completely removed after the labeling and subsequent washing steps.

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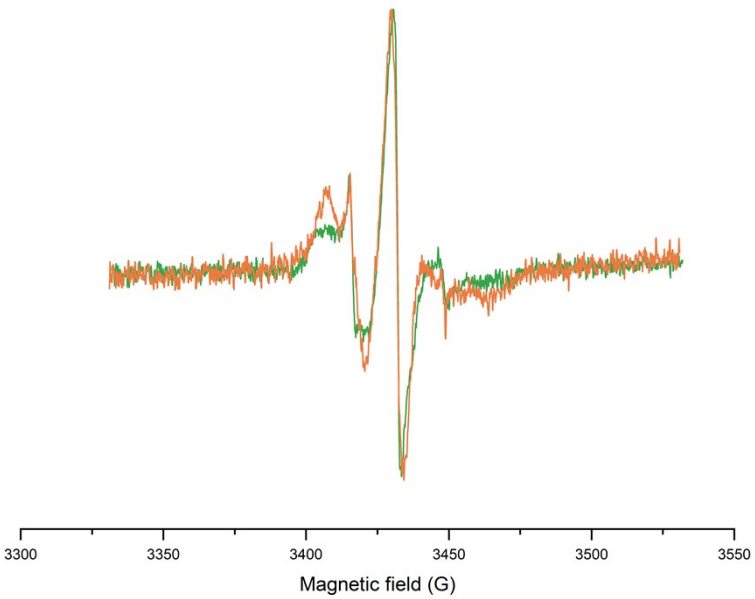

Figure S8 Comparison of Y39/L221pENF-L eGFP *in vitro* (orange) and *in vivo* (green). Cw EPR spectra were recorded in X-band at room temperature and accumulated over 10 minutes (*in vitro*) or 50 minutes (*in vivo*). The narrow component corresponds to a small fraction of unbound spin label.

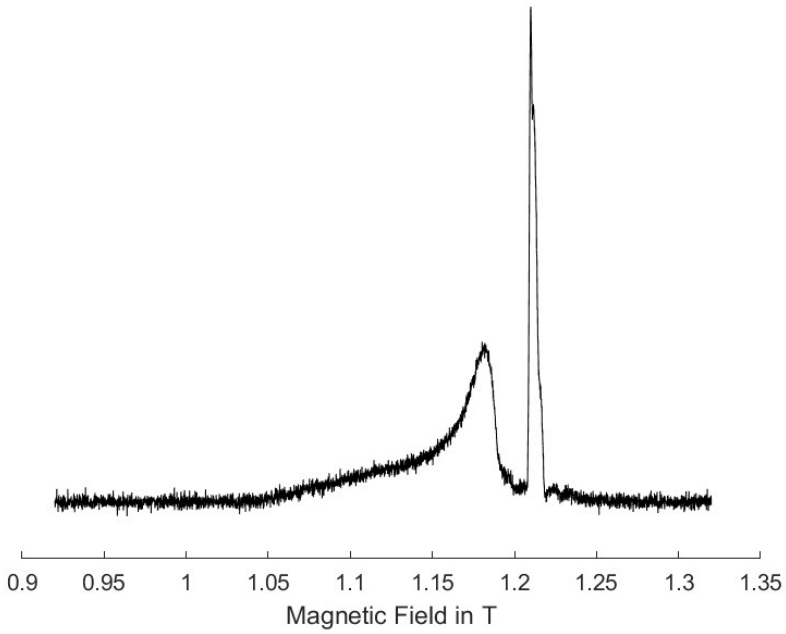

Figure S9 Q-band echo-detected field sweep of in-cell L221pENF-L eGFP at 50 K. The spectrum consists of contributions from remaining Cu(II) species after labeling (maximum at B = 1.18 T) and nitroxide species (maximum at B = 1.21 G). The Cu(II) contribution can be further decreased by additional washing of the cells, but at the cost of nitroxide signal intensity due to the rapid reduction in the cellular environment.

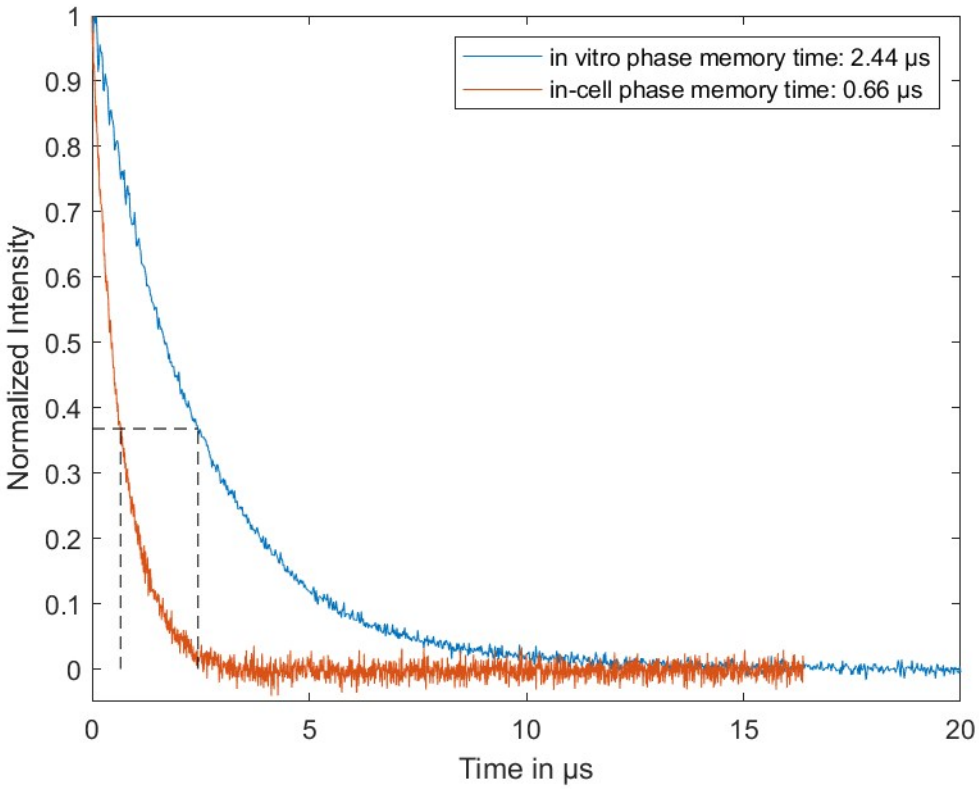

Figure S10 Echo relaxation measurement withY39/L221pENF-L eGFP *in vitro* and *in vivo.* Phase memory times were determined as the time point at which the signal intensity decayed to 1/e of the initial intensity (t = 0 µs). *In vivo* samples were prepared without deuterated solvents.

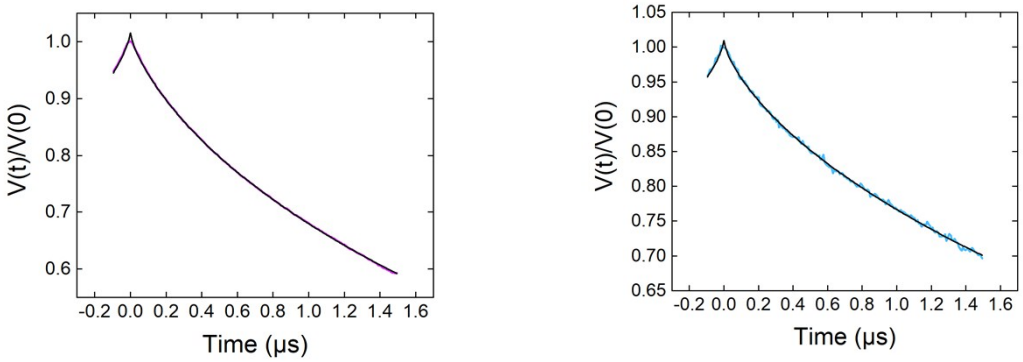

Figure S11 Normalized DEER traces with homogeneous background fit for in vivo DEER measurements of with singly-labeled eGFP mutants Y39pENF-L (purple) and L221pENF-L (blue) (background start set to 0 µs, background dimension is 2.2 or 2.3, respectively.)

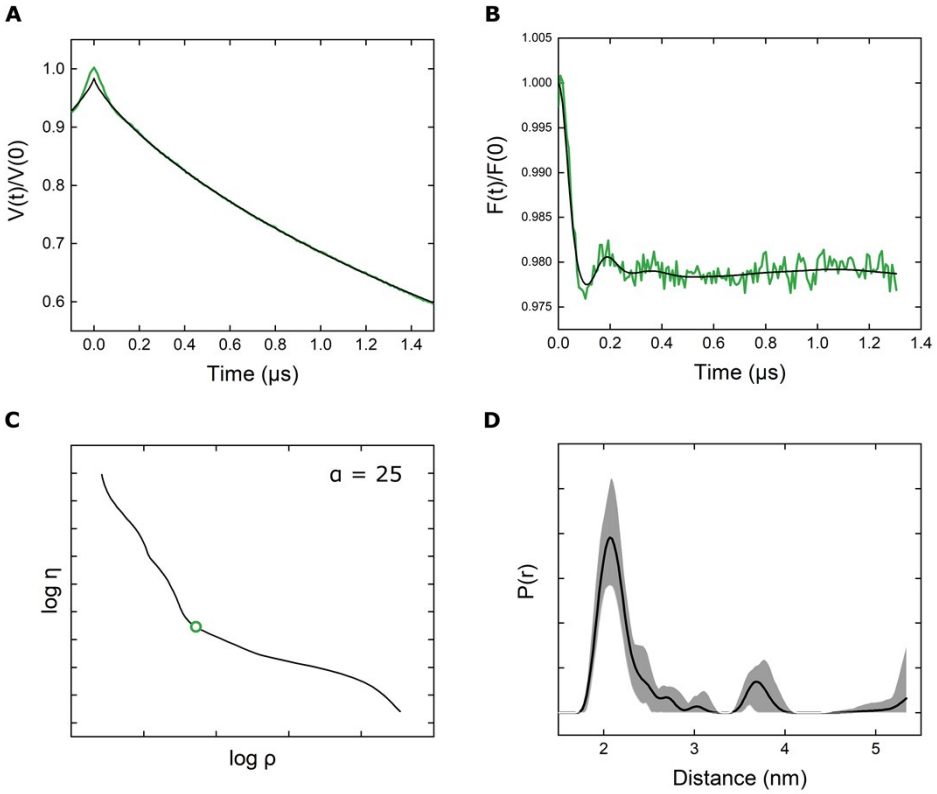

Figure S12 ***In vivo*** **DEER distance determination of Y39/L221pENF-L eGFP**. **(A)** Normalized DEER trace with homogeneous background fit (d = 2.36) **(B)** Background corrected form factor with fit by Tikhonov regularization. In triplicate measurements, we obtained varying modulation depths of 1 – 2% and comparable noise levels. **(C)** L-curve with the chosen alpha parameter **(D)** Validated distance distribution.

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