Combining Site-Directed Spin Labeling in Vivo and In-Cell EPR Distance Determination

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Structural studies on proteins directly in their native environment are required for a comprehensive understanding of their function. Electron paramagnetic resonance (EPR) spectroscopy and in particular double electron-electron resonance (DEER) distance determination are suited to investigate spin-labeled proteins directly in the cell. The combination of intracellular bioorthogonal labeling with in-cell DEER measurements does not require additional purification or delivery steps of spin-labeled protein to the cells. In this study, we express eGFP in E.coli and use copper-catalyzed azide-alkyne cycloaddition (CuAAC) for the site-directed spin labeling of the protein in vivo, followed by in-cell EPR distance determination. Inter-spin distance measurements of spin-labeled eGFP agree with in vitro measurements and calculations based on the rotamer library of the spin label.
Combining Site-Directed Spin Labeling in vivo and in-cell EPR Distance Determination

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Abstract

Structural studies on proteins directly in their native environment are required for a comprehensive understanding of their function. Electron paramagnetic resonance (EPR) spectroscopy and in particular double electron-electron resonance (DEER) distance determination are suited to investigate spin-labeled proteins directly in the cell. The combination of intracellular bioorthogonal labeling with in-cell DEER measurements does not require additional purification or delivery steps of spin-labeled protein to the cells. In this study, we express eGFP in E.coli and use copper-catalyzed azide-alkyne cycloaddition (CuAAC) for the site-directed spin labeling of the protein in vivo, followed by in-cell EPR distance determination. Inter-spin distance measurements of spin-labeled eGFP agree with in vitro measurements and calculations based on the rotamer library of the spin label.

Introduction

The understanding of protein function and structure is crucially linked to the ability to study proteins in their native environment. Effects of not only molecular crowding but also post-translational modifications, presence of a variety of specific or non-specific interaction partners or chaperones have a great impact on proteins, yet are only incompletely understood through in vitro studies.1

Facing the challenge of highly complex cellular compositions, in-cell electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL)2 has emerged as a valuable tool to provide structural information3 as many cellular components are diamagnetic and therefore EPR-silent. Pulsed techniques such as double electron-electron resonance (DEER) provide access to long-range distance restraints in the nanometer range by measuring the dipole-dipole interactions between paramagnetic spin labels.4, 5

So far, in vivo DEER studies on spin-labeled proteins have either been conducted on outer membrane proteins upon cysteine-based spin labeling on the cellular surface6-8 or relied on the delivery of spin-labeled protein into the cell, e.g. by microinjection into oocytes9-12 or permeabilization of the membrane via hypo-osmotic shock or electroporation13-18. Non-canonical amino acid (ncAA) incorporation and bioorthogonal in vivo spin labeling offer a more direct and elegant approach for in-cell EPR studies as they may combine expression, labeling and the EPR study of the protein of interest directly inside the same cell without additional delivery steps. While a number of studies on bioorthogonal spin labeling have been published in recent years,19-27 the corresponding DEER measurements were limited to in vitro measurements. Even in cases with confirmed in vivo labeling, purification and concentration of spin-labeled protein was required prior to a DEER measurement. The Steinhoff group has advanced nitroxide spin labeling via copper-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) and reported labeling of eGFP at one site between the ncAA N-ϵ-propargyl-L-lysine (PrK) and a nitroxide spin label in E. coli. However, for DEER distance determination, the protein was purified and conventional cysteine labeling with MTSSL was used to introduce the second spin label.20

In our previous work;26 we have identified the ncAA para-ethynyl-phenylalanine (pENF) as a suitable choice for bioorthogonal spin labeling with CuAAC. pENF was incorporated into E. coli thioredoxin with high labeling yields and exhibited favorable linker properties for DEER distance determination in in vitro measurements.26

Here, we extend this approach to bioorthogonal double spin labeling and DEER measurements directly in vivo (Figure 1). We report the incorporation of pENF at two sites of eGFP via amber stop codon suppression in E. coli, develop conditions for CuAAC with an azide-bearing nitroxide spin label and combine it with direct in-cell DEER distance
determination without any purification step. In-cell inter-spin distances of spin-labeled eGFP are comparable to \textit{in vitro} measurements and calculated distances based on the rotamer library of the label.

Figure 1 Schematic overview of \textit{in vivo} spin labeling approach via copper-catalyzed [3+2] azide-alkyne cycloaddition followed by \textit{in-cell} EPR distance determination. The ncAA pENF is incorporated site-specifically into eGFP using amber stop codon suppression. The eGFP-expressing E. coli cells are subjected to CuAAC-based spin labeling and subsequent, DEER distance determination is performed directly inside the cell.

Results and Discussion

We decided on eGFP as a model protein for our study since \textit{in vivo} CuAAC-based spin labeling on this protein has previously been shown\textsuperscript{20} and the fluorescence properties of eGFP facilitate \textit{in-cell} monitoring of the protein. We chose position Y39TAG in the β2-β3 loop region and position L221TAG in the β11-strand of the barrel as labeling sites.\textsuperscript{28} We co-transformed plasmids pBAD_GFP_Y39/L221-TAG and pEVOL_pCNF (encoding a polyspecific \textit{Methanocaldococcus jannaschii} tRNA\textsubscript{Tyr} (CUA)/tyrosyl-tRNA-synthetase (YRS) pair evolved for the genetic encoding of para-cyano-L-phenylalanine, pCNF)\textsuperscript{29, 30} into BL21-Gold (DE3) \textit{E. coli}, induced the culture and purified Y39/L221pENF eGFP by Ni-NTA chromatography via its C-terminal His6-tag. ESI-MS data confirmed the correct incorporation of pENF at two positions (Figure S1). \textit{In vitro} labeling reactions were performed as previously described\textsuperscript{26} with 1 mM CuSO\textsubscript{4}, 3 mM 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), 1 mM sodium ascorbate (NaAsc) and 1 mM 3-(azidomethyl)-2,2,5,5-tetramethyl-1-pyrroolidinylloxyl (az-proxy) and yielded Y39/L221pENF-L eGFP with a labeling efficiency of approximately 70 % based on the ratio of spin concentration to protein concentration. Incorporation of pENF and labeling did not significantly affect the function of eGFP as \textit{in vitro} excitation and emission fluorescence spectra of Y39/L221pENF before and after labeling did not differ from eGFP wild-type spectra (Figure S2). \textit{In vitro} DEER measurements of spin-labeled Y39/L221pENF-L eGFP resulted in a narrow distance distribution (HWHM: 0.5 nm) with a mean peak at 2.3 nm (Figure S3) and agree well with the calculated distances based on the crystal structure of eGFP (PDB structure 4EUL) and our previously published rotamer library\textsuperscript{26} of the pENF-L spin label.
Figure 2 Toxicity of CuAAC labeling reagents for E. coli. (A) E. coli growth after treatment with CuAAC 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) (n = 2) (B) Plate sensitivity assay. Diluted E. coli suspensions of cells treated with Cu(II)SO₄, BTTAA, NaAsc (1/3/1 mM) (blue), and untreated control sample (gray) were plated, incubated overnight and the number of colony-forming units (CFU) was counted (n = 3). (C) Dead cell/ alive cell identification with flow cytometry. E. coli cells expressing Y39/L221pENF eGFP were either treated with ethanol (EtOH), subjected to CuSO₄, BTTAA, NaAsc (1/3/1 mM, labeling mix) or treated with 30 mM Cu(II)SO₄. Untreated cells (control) served as alive control sample. Dead cell detection was facilitated by PI staining and the indicated numbers represent the percentage of dead cells.

In case of in vivo CuAAC, a major concern is copper-mediated cytotoxicity. Copper ions are linked to the formation of reactive oxygen species (ROS) and are known to impair cytoplasmic proteins with Fe-S clusters due to their thiophilic tendency. However, it has been shown that this toxicity is greatly reduced by adding chelators to the copper species with BTTAA being a ligand that shows especially promising properties for in vivo CuAAC applications. To investigate the effects of in vivo CuAAC on E. coli, we monitored the bacterial growth immediately after CuAAC treatment as well as the long-term growth behavior with a plate sensitivity assay (Figure 2). Cells were treated with the labeling reagents copper(II)sulfate, BTTAA and sodium ascorbate (in a 1:3:1 ratio), diluted, and the optical density of the cells at 600 nm (O.D.600) was measured over time and compared to an untreated control (Figure 2 A, S4). The growth rate of E. coli cells treated with CuAAC labeling reagents under labeling conditions did not differ from the untreated controls (only under far higher copper concentrations of 10 mM a reduction of growth rate was observed). In addition, we investigated the long-term growth behavior of treated and untreated cells by plating out diluted E. coli suspensions onto LB agar plates and incubating overnight at 37 °C. After counting the number of colony-forming units, we concluded that CuAAC treatment did not impact the number of viable cells (Figure 2 B, S5). Finally, to evaluate the integrity of the bacterial membrane after CuAAC labeling, we performed dead cell/ alive cell discrimination via FACS analysis (Figure 2 C, S6). Events were pre-gated based on their forward (FSC-A) and sideward scattering (SSC-A) to exclude cell debris and buffer crystals. E. coli cells were then gated based on SSC-A and GFP fluorescence, and the fraction of dead cells was detected based on staining with propidium iodide (PI). Untreated cells and cells treated with 75 % ethanol were used as controls to determine the level of PI intensity characteristic for live and dead cells, respectively. We did not observe an increase in the dead cell fraction in the CuAAC-treated compared to the untreated cells. For higher copper(II)sulfate concentrations of 30 mM, FACS data suggested the onset of copper-mediated cell death. The FACS analysis was conducted approximately 60-65 minutes after the start of the labeling reaction, the time point at which samples intended
for in-cell EPR distance determination were frozen in liquid nitrogen as part of the DEER sample preparation. We therefore expect a similar level of integrity as seen during FACS measurements in the respective DEER samples.

Figure 3 In-cell nitroxide stability after CuAAC spin labeling. (A) CW EPR spectra of E. coli cells expressing the tRNA^{76}_T(CUA)/YRS pair for the incorporation of pENF as well as eGFP wild-type (black) or Y39/L221pENF eGFP (green) after in vivo CuAAC spin labeling. Spectra were averaged over 5 scans. Indicated time points refer to the passed time after addition of the nitroxide labeling reagent to the cells. T = 60 min marks the end of the labeling procedure and the start of the EPR measurement. (B) For a qualitative description of the nitroxide reduction, the amplitude of the centerline from averaged CW spectra was plotted against the time. The signal intensity at t = 60 min was set to 100%.

Next, we were interested in studying possible nitroxide reduction inside E. coli (Figure 3). Proxyl-based spin labels as employed in this study are prone to fast biological reduction and it has been shown that they can be rendered EPR-inactive on a minute timescale. However, Bleiken et al. have recently stressed the effective spin concentration as a critical parameter for cellular nitroxide reduction kinetics and demonstrated that the addition of spin label in the millimolar range practically overloads cellular reduction mechanisms and can thereby prolong the time scale on which nitroxide signal is still detectable. In case of CuAAC-based spin labeling, Kucher et al. have already reported on the successful in vivo nitroxide labeling at a single labeling site, while sufficient double-labeling in vivo has yet to be proven. We limited the labeling times for the in vivo labeling approach to 40 minutes at room temperature, followed by additional washing steps to remove unbound nitroxide spin labels. In total, approximately 60 minutes passed between the addition of the nitroxide spin labeling reagent to E. coli cells and the start of the EPR measurement. Time-dependent EPR spectra are shown in Figure 3 A. For a qualitative description of the signal reduction, we plotted the amplitude of the nitroxide center field peak against the time (Figure 3 B). Upon performing the CuAAC-based spin labeling procedure, E. coli cells expressing Y39/L221pENF eGFP show a typical nitroxide EPR spectrum that prevails up to 120 minutes after the addition of azido-proxyl spin labeling reagent.

In principle, EPR signals in E. coli can stem from several possible species. Besides spin-labeled Y39/L221pENF-L eGFP, free spin label from the labeling reaction, as well as labeled pENF, both in free form and esterified to tRNA^{76}_T(CUA) might contribute to the signal. To limit off-target labeling, protein-expressing cells were transferred to fresh LB medium without pENF and protein expression was prolonged for 90 minutes prior to CuAAC labeling. In addition, approximately 9% of E. coli proteins are terminated with an amber stop codon (TAG) and pENF might also eventually be incorporated into these proteins. To investigate potential sources of signal, we co-transformed E. coli cells with plasmids for eGFP wild-type and the tRNA^{76}_T(CUA)/YRS pair and expressed eGFP wild-type in the presence of pENF in the medium. In this scenario, the EPR signal after CuAAC labeling procedure can only arise from free or tRNA-
esterified spin-labeled pENF or from labeled pENF incorporated at amber sites of off-target proteins. However, we did only observe neglectable signal intensities in this sample (Figure 3 A, black). In addition, we tested our washing protocol after CuAAC-based spin labeling with *E. coli* cells treated with CuAAC labeling reagents and azido-proxyl in the absence of pENF and found that free spin-label is effectively removed from the bacterial solution (Figure S7). We, therefore, assumed that the main contribution to the signal stems from spin-labeled Y39/L221pENF-L eGFP. Moreover, the spectral shape of the positive sample was similar to *in vitro* EPR spectra of *in vitro* labeled Y39/L221pENF-L eGFP (Figure S8).

Echo-detected field sweeps of *E. coli* samples after CuAAC spin labeling contained spectral contributions from Cu(II) species as a result of the incomplete removal of the catalyst after labeling (Figure S9). The phase memory time of the spins was reduced to 0.66 µs for cellular samples compared to 2.44 µs from the *in vitro* experiment in deuterated aqueous solution (Figure S10).

**Figure 4** In-cell DEER distance determination after bioorthogonal CuAAC spin labeling (A) Comparison of the form factors of Y39/L221pENF-L eGFP from DEER measurements conducted *in vitro* (orange) or *in vivo* (green). The modulation depth decreased from 38 % for *in vitro* DEER to approximately 2 % for *in vivo* DEER. (B) Derived distance distribution from form factors for DEER measurements *in vitro* (orange) and *in vivo* (green). The gray area indicates the expected distance distribution based on MMM calculations with the rotamer library of pENF-L and a crystal structure of eGFP (PDB entry 4EUL) (C) The crystal structure of eGFP (PDB entry 4EUL) with indicated labeling sites and expected label conformers based on the rotamer library of pENF-L.

For in-cell DEER measurements with the nitroxide spin label, we shock-froze *E. coli* cells expressing Y39/L221pENF eGFP in liquid nitrogen after the CuAAC-based spin labeling procedure. The dipolar evolution time was limited to 1.3 µs due to the reduced phase memory time in the cellular environment, but still long enough to provide reliable information about distance distributions in the range of the *in vitro* experiment. For background correction, we also recorded in-cell DEER traces with the two singly labeled eGFP variants Y39pENF-L and L221pENF-L eGFP which could be completely described by a homogeneous background model (Figure S11). A similar background model was then used for the background correction for doubly-labeled eGFP.

The resulting form factor upon background correction (Figure 4, S12) features a low modulation depth of roughly 2 %, compared to 38 % for the *in vitro* sample. We suspect that the large background contribution is a result of incomplete labeling of eGFP in the cellular environment as well as fast nitroxide reduction during the labeling procedure, while the presence of unbound spin label only plays a minor role. Form factor and the derived distance distribution were compared to the *in vitro* measurement of Y39/L221pENF-L eGFP and the expected distances based on the rotamer library. The shape of the in-cell form factor was strikingly similar to the *in vitro* measurement and the corresponding distance distribution contained distance constraints at 2.3 nm matching the distance distribution from the *in vitro* experiment.
In conclusion, we have advanced CuAAC-based spin labeling of alkyne-bearing proteins and direct DEER distance measurements to in vivo environments. Our labeling conditions do not exhibit copper-mediated cytotoxicity in E. coli. We observe moderate nitroxide reduction during labeling, allowing for double protein labeling with sufficient yields for subsequent DEER measurements. Moreover, we observe minimal background signal from nitroxides not attached to our target protein positions. This enables DEER studies that deliver valuable information on the structure of the labeled protein, and argue for a similar conformation that eGFP adopts in the E. coli cytoplasm and in vitro.

Taken together, our approach combines natural translation, folding, and processing of a target protein with bio-orthogonal double labeling and direct DEER distance measurements directly in the natural environment of a bacterial cell. It overcomes the necessity for introducing spin-labeled proteins into cells, e.g. via electroporation or hypo-osmotic shock and thus represents a new access point to in-cell EPR studies of protein structure and function.

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References

[1] Kyne, C., and Crowley, P. B. (2016) Grasping the nature of the cell interior: from Physiological Chemistry to Chemical Biology, The FEBS journal 283, 3016-3028.
[2] Hubbell, W. L., Lopez, C. J., Allenbach, C., and Yang, Z. (2013) Technological advances in site-directed spin labeling of proteins, Current opinion in structural biology 23, 725-733.
[3] Jeschke, G. (2018) The contribution of modern EPR to structural biology, Emerging Topics in Life Sciences 2, 9-18.
[4] Jeschke, G. (2012) DEER distance measurements on proteins, Annual review of physical chemistry 63, 419-446.
[5] Jeschke, G. (2002) Distance measurements in the nanometer range by pulse EPR, Chemphyschem : a European journal of chemical physics and physical chemistry 3, 927-932.
[6] Joseph, B., Sikora, A., Bordignon, E., Jeschke, G., Cafiso, D. S., and Prisner, T. F. (2015) Distance Measurement on an Endogenous Membrane Transporter in E. coli Cells and Native Membranes Using EPR Spectroscopy, Angewandte Chemie (International ed. in English) 54, 6196-6199.
[7] Joseph, B., Sikora, A., and Cafiso, D. S. (2016) Ligand Induced Conformational Changes of a Membrane Transporter in E. coli Cells Observed with DEER/PEDOR, Journal of the American Chemical Society 138, 1844-1847.
[8] Dunkel, S., Pulagam, L. P., Steinhoff, H. J., and Klare, J. P. (2015) In vivo EPR on spin labeled colicin A reveals an oligomeric assembly of the pore-forming domain in E. coli membranes, Physical Chemistry Chemical Physics 17, 4875-4878.
[9] Singewald, K., Lawless, M. J., and Saxena, S. (2019) Increasing nitroxide lifetime in cells to enable in-cell protein structure and dynamics measurements by electron spin resonance spectroscopy, Journal of magnetic resonance (San Diego, Calif. : 1997) 299, 21-27.
[10] Lawless, M. J., Shimshi, A., Cunningham, T. F., Kinde, M. N., Tang, P., and Saxena, S. (2017) Analysis of Nitroxide-Based Distance Measurements in Cell Extracts and in Cells by Pulsed ESR Spectroscopy, Chemphyschem : a European journal of chemical physics and physical chemistry 18, 1653-1660.
[11] Qi, M., Groß, A., Jeschke, G., Gott, A., and Drescher, M. (2014) Gd(III)-PyMTA Label Is Suitable for In-Cell EPR, Journal of the American Chemical Society 136, 15366-15378.
[12] Igarashi, R., Sakai, T., Harra, H., Tenno, T., Tanaka, T., Tochio, H., and Shirakawa, M. (2010) Distance Determination in Proteins inside Xenopus laevis Oocytes by Double Electron–Electron Resonance Experiments, Journal of the American Chemical Society 132, 8228-8229.
[13] Yang, Y., Yang, F., Gong, Y.-J., Chen, J.-L., Goldfarb, D., and Su, X.-C. (2017) A Reactive, Rigid GdIII Labeling Tag for In-Cell EPR Distance Measurements in Proteins, Angewandte Chemie International Edition 56, 2914-2918.
[14] Yang, Y., Yang, F., Li, X.-Y., Xu, X.-C., and Goldfarb, D. (2019) In-Cell EPR Distance Measurements on Ubiquitin Labeled with a Rigid PyMTA-Gd(III) Tag, The Journal of Physical Chemistry B.
[15] Mascali, F. C., Ching, H. Y., Rasia, R. M., Un, S., and Tabares, L. C. (2016) Using Gene-Encodable Self-Assembling Gd(III) Spin Labels To Make In-Cell Nanometric Distance Measurements, Angewandte Chemie (International ed. in English) 55, 11041-11043.
[16] Martorana, A., Bellapadrona, G., Feintuch, A., Di Gregorio, E., Aime, S., and Goldfarb, D. (2014) Probing protein conformation in cells by EPR distance measurements using Gd³⁺ spin labeling, J Am Chem Soc 136, 13458-13465.
[17] Dalaloyan, A., Martorana, A., Barak, Y., Gataulin, D., Reuveny, E., Howe, A., Elbaum, M., Albeck, S., Unger, T., Frydman, V., Abdelkader, E. H., Otting, G., and Goldfarb, D. (2019) Tracking Conformational Changes in Calmodulin in vitro, in Cell Extract, and in Cells by Paramagnetic Resonance Distance Measurements, Chemphyschem : a European journal of chemical physics and physical chemistry 20, 1860-1868.
[18] Theillet, F. X., Binolfi, A., Bekei, B., Martorana, A., Rose, H. M., Stuiver, M., Verzini, S., Lorenz, D., van Rossum, M., Goldfarb, D., and Selenko, P. (2016) Structural disorder of monomeric alpha-synuclein persists in mammalian cells, Nature 530, 45-50.
[19] Fleissner, M. R., Brustad, E. M., Kalai, T., Altenbach, C., Cascio, D., Peters, F. B., Hideg, K., Peuker, S., Schultz, P. G., and Hubbell, W. L. (2009) Site-directed spin labeling of a genetically encoded unnatural amino acid, Proc. Natl. Acad. Sci. U S A 106, 21637.

[20] Kucher, S., Korneev, S., Tyagi, S., Apfelbaum, R., Grohmann, D., Lemke, E. A., Klare, J. P., Steinhoff, H. J., and Klose, D. (2017) Orthogonal spin labeling using click chemistry for in vitro and in vivo applications, Journal of magnetic resonance (San Diego, Calif. : 1997) 275, 38-45.

[21] Loh, C. T., Ozawa, K., Tuck, K. L., Barlow, N., Huber, T., Otting, G., and Graham, B. (2013) Lanthanide Tags for Site-Specific Ligation to an Unnatural Amino Acid and Generation of Pseudocontact Shifts in Proteins, Bioconjugate Chemistry 24, 260-268.

[22] Kugele, A., Braun, T. S., Widder, P., Williams, L., Schmidt, M. J., Summerer, D., and Drescher, M. (2019) Site-directed spin labelling of proteins by Suzuki-Miyaura coupling via a genetically encoded aryliodide amino acid, Chemical communications (Cambridge, England) 55, 1923-1926.

[23] Abdelkader, E. H., Feintuch, A., Yao, X., Adams, L. A., Aurelio, L., Graham, B., Goldfarb, D., and Otting, G. (2015) Protein conformation by EPR spectroscopy using gadolinium tags clicked to genetically encoded p-azido-l-phenylalanine, Chemical Communications 51, 15898-15901.

[24] Garbuio, L., Bordignon, E., Brooks, E. K., Hubbell, W. L., Jeschke, G., and Yulikov, M. (2013) Orthogonal Spin Labeling and Gd(III)–Nitroxide Distance Measurements on Bacteriophage T4-Lysozyme, The Journal of Physical Chemistry B 117, 3145-3153.

[25] Evans, E. G., and Millhauser, G. L. (2015) Genetic Incorporation of the Unnatural Amino Acid p-Acetyl Phenylalanine into Proteins for Site-Directed Spin Labeling, Methods in enzymology 563, 503-527.

[26] Widder, P., Berner, F., Summerer, D., and Drescher, M. (2019) Double Nitroxide Labeling by Copper-Catalyzed Azide-Alkyne Cycloadditions with Noncanonical Amino Acids for Electron Paramagnetic Resonance Spectroscopy, ACS Chem Biol 14, 839-844.

[27] Kugele, A., Silkenath, B., Langer, J., Wittmann, V., and Drescher, M. (2019) Protein Spin Labeling with a Photocaged Nitroxide Using Diels-Alder Chemistry, ChemBiochem : a European journal of chemical biology.

[28] Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Crystal structure of the Aequorea victoria green fluorescent protein, Science (New York, N.Y.) 273, 1392-1395.

[29] Schultz, K. C., Supekova, L., Ryu, Y., Xie, J., Perera, R., and Schultz, P. G. (2006) A genetically encoded infrared probe, J Am Chem Soc 128, 13884-13895.

[30] Young, D. D., Young, T. S., Jahnz, M., Ahmad, I., Spraggon, G., and Schultz, P. G. (2011) An Evolved Aminoacyl-tRNA Synthetase with Atypical Polysubstrate Specificity, Biochemistry 50, 1894-1900.

[31] Kennedy, D. C., McKay, C. S., Legault, M. C., Danielson, D. C., Blake, J. A., Pegoraro, A. F., Stolow, A., Mester, Z., and Pezacki, J. P. (2011) Cellular consequences of copper complexes used to catalyze bioorthogonal click reactions, J Am Chem Soc 133, 17993-18001.

[32] Chillappagari, S., Seubert, A., Trip, H., Kuipers, O. P., Marahiel, M. A., and Miethke, M. (2010) Copper stress affects iron homeostasis by destabilizing iron-sulfur cluster formation in Bacillus subtilis, Journal of bacteriology 192, 2512-2524.

[33] Yang, M., Jalloh, A. S., Wei, W., Zhao, J., Wu, P., and Chen, P. R. (2014) Biocompatible click chemistry enabled compartment-specific pH measurement inside E. coli, Nature communications 5, 4981.

[34] Karthikeyan, G., Bonucci, A., Casano, G., Gerbaud, G., Abel, S., Thome, V., Kodjabachian, L., Magalon, A., Guigliarelli, B., Belle, V., Ouari, O., and Mileo, E. (2016) A Bioreistant Nitroxide Spin Label for In-Cell EPR Spectroscopy: In Vitro and In Oocytes Protein Structural Dynamics Studies, Angewandte Chemie (International ed. in English) 57, 1366-1370.

[35] Bleicken, S., Assafa, T. E., Zhang, H., Eisner, C., Ritsch, I., Pink, M., Rajca, S., Jeschke, G., Rajca, A., and Bordignon, E. (2019) gem-Diethyl Pyrroline Nitroxide Spin Labels: Synthesis, EPR Characterization, Rotamer Libraries and Biocompatibility, ChemistryOpen 0.

[36] Xie, J., and Schultz, P. G. (2005) An expanding genetic code, Methods (San Diego, Calif.) 36, 227-238.

[37] Jeschke, G. (2018) MMM: A toolbox for integrative structure modeling, Protein science : a publication of the Protein Society 27, 76-85.
Supporting Information for

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. BTTAA were purchased from Jena Bioscience and pENF from Achemblock. Azido-proxyl was synthesized according to published procedure.1

Protein Expression
Cloning and transformation of *E. coli*
Amber mutations were introduced into pBAD_Y39TAG-GFP_His62 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.

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_His63 or the gene construct containing the respective mutation for amber stop codon suppression, pBAD_Y39TAG_GFP_His62, 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 OD600 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 phenylmethanesulfonylfluoride (PMSF) and lysed 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 NaH2PO4, 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.

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 with small alterations. Briefly, copper(II)-sulfate (CuSO4) and the ligand 2-(((tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl acetic acid (BTTAA) 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 proxyl label in 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 D2O-PBS (samples prepared for cw EPR and DEER measurements). DEER samples were prepared in deuterated PBS buffer containing 20% deuterated glycerol. 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 (OD600) of the suspension was adjusted to OD600 = 160 for the labeling reaction. **In vivo** labeling took place for 40 min and 25 °C in a reaction volume of 500 µl 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 deuterated 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 TM1110). 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. **In vivo** measurements were either averaged over 5 scans for nitroxide reduction kinetics or consecutive scans were added up until the highest possible signal-to-noise ratio was achieved (Figure S8). All recorded spectra were processed using MatLab2018A (the 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 $\pi$ pulse length and a video-bandwidth of 20 MHz.

**Echo-detected field sweep**

Echo-detected field sweeps were recorded with a Hahn echo sequence ($\pi/2$ – 800 ns – $\pi$ – 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 $\tau = 800$ ns) and extracting the time point at which the signal intensity decreased to 1/e of the initial intensity at $t = 0$ µs.

**DEER measurement, data analysis, and evaluation**

EPR inter-spin distance measurements were performed at Q-band (34 GHz) on a commercially available Bruker Elexys 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. A sech/tanh pulse ($t_p = 100$ ns, $\beta = 6/t_p$, $\Delta \nu = 90$ MHz) was used as pump pulse and adjusted to 34 GHz. 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 $\pi$-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 $[x](x)p(x)$ as proposed by Tait and Stoll 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. Zero times were automatically determined, cut-offs excluded the last 150 ns of the DEER trace to avoid “2+1” end artifacts and background starts were manually determined. The in vitro DEER measurement of Y39/L221pENF-L eGFP 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 Y39/L221pENF-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 wild-type (gray), Y39/L221pENF eGFP (black) and spin-labeled 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.

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.

**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*).
**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).

**Figure S10** Echo relaxation measurement with Y39/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 ($t = 0 \, \mu s$).
**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 (C) L-curve with the chosen alpha parameter (D) Validated distance distribution.
References

[1] Kucher, S., Korneev, S., Tyagi, S., Apfelbaum, R., Grohmann, D., Lemke, E. A., Klare, J. P., Steinhoff, H. J., and Klose, D. (2017) Orthogonal spin labeling using click chemistry for in vitro and in vivo applications, *Journal of magnetic resonance (San Diego, Calif. : 1997)* 275, 38-45.

[2] Plass, T., Milles, S., Koechler, C., Schultz, C., and Lemke, E. A. (2011) Genetically encoded copper-free click chemistry, *Angewandte Chemie (International ed. in English)* 50, 3878-3881.

[3] Schmidt, M. J., and Summerer, D. (2013) Red-light-controlled protein-RNA crosslinking with a genetically encoded furan, *Angewandte Chemie (International ed. in English)* 52, 4690-4693.

[4] Besanceney-Webler, C., Jiang, H., Zheng, T., Feng, L., Soriano del Amo, D., Wang, W., Klivansky, L. M., Marlow, F. L., Liu, Y., and Wu, P. (2011) Increasing the Efficacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study, *Angewandte Chemie International Edition* 50, 8051-8056.

[5] Pannier, M., Veit, S., Godt, A., Jeschke, G., and Spiess, H. W. (2000) Dead-time free measurement of dipole-dipole interactions between electron spins, *Journal of magnetic resonance (San Diego, Calif. : 1997)* 142, 331-340.

[6] Martin, R. E., Pannier, M., Diederich, F., Gramlich, V., Hubrich, M., and Spiess, H. W. (1998) Determination of End-to-End Distances in a Series of TEMPO Diradicals of up to 2.8 nm Length with a New Four-Pulse Double Electron Electron Resonance Experiment, *Angewandte Chemie (International ed. in English)* 37, 2833-2837.

[7] Stoll, S., and Schweiger, A. (2006) EasySpin, a comprehensive software package for spectral simulation and analysis in EPR, *Journal of Magnetic Resonance* 178, 42-55.

[8] Tait, C. E., and Stoll, S. (2016) Coherent pump pulses in Double Electron Electron Resonance spectroscopy, *Physical chemistry chemical physics : PCCP* 18, 18470-18485.

[9] Jeschke, G., Chechik, V., Ionita, P., Godt, A., Zimmermann, H., Banham, J., Timmel, C. R., Hilger, D., and Jung, H. (2006) DeerAnalysis2006—a comprehensive software package for analyzing pulsed ELDOR data, *Applied magnetic resonance* 30, 473-498.

[10] Jeschke, G. (2012) DEER distance measurements on proteins, *Annual review of physical chemistry* 63, 419-446.
