In situ observation of conformational dynamics and protein ligand–substrate interactions in outer-membrane proteins with DEER/PELDOR spectroscopy

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Observation of structure and conformational dynamics of membrane proteins at high resolution in their native environments is challenging because of the lack of suitable techniques. We have developed an approach for high-precision distance measurements in the nanometer range for outer-membrane proteins (OMPs) in intact Escherichia coli and native membranes. OMPs in Gram-negative bacteria rarely have reactive cysteines. This enables in situ labeling of engineered cysteines with a methanethiosulfonate spin label (MTSL) with minimal background signals. Following overexpression of the target protein, spin labeling is performed with E. coli or isolated outer membranes (OMs) under selective conditions. The interspin distances are measured in situ, using pulsed electron–electron double resonance (PELDOR or DEER) spectroscopy. The residual background signals, which are problematic for in situ structural biology, contribute specifically to the intermolecular part of the signal and can be selectively removed to extract the desired interspin distance distribution. The initial cloning stage can take 5–7 d, and the subsequent protein expression, OM isolation, spin labeling, PELDOR experiment, and data analysis typically take 4–5 d. The described protocol provides a general strategy for observing protein ligand–substrate interactions, oligomerization, and conformational dynamics of OMPs in their native OM and intact E. coli.

Introduction

Membrane proteins often sample a broad conformational landscape and the activity of channels, transporters, or receptors often involves large-scale domain movements1. Thus, a mechanistic description of their function necessitates an understanding of conformational changes and equilibrium dynamics. Biomolecular structures of membrane proteins have primarily been determined with cryo-electron microscopy (cryoEM), solution NMR, and X-ray crystallography. All these techniques require the isolation of the target molecules from the native environment, which masks the effects of cellular conditions such as the lipid environment, interaction with other molecules/ions, molecular crowding, pH or ionic gradients, and the specific localization. These factors may critically influence protein structure, function, and dynamics. For example, there is increasing evidence for the vital role of the native lipid environment in membrane protein folding, structure, and activity2,3. In this protocol, we describe how to use PELDOR or DEER to observe OMP structure and dynamics in their native environment, in either E. coli or purified OMs.

EPR spectroscopy of intrinsically diamagnetic biomolecules

Continuous-wave (CW) electron paramagnetic resonance (EPR) spectroscopy is a powerful technique used for conformational studies of membrane protein, as it can provide information on water accessibility, polarity of the surrounding environment, dynamics, and intra- or intermolecular distances between sites in the range of 1–2 nm4. However, most biomolecules are not paramagnetic, and for EPR spectroscopy they must be modified with an appropriate spin label. For proteins, labels are normally attached by covalently linking a functionalized spin label to cysteines engineered using

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site-directed mutagenesis (SDM)\textsuperscript{5}. This requires the removal of native reactive cysteines in the target protein, and this usually does not impair protein function. Alternatively, spin probes can be incorporated using genetic encoding in response to a nonsense codon\textsuperscript{6-8}. The development of these site-directed spin-labeling approaches led to a rapid growth of EPR spectroscopy as a powerful tool for structural biology. The nitroxide-based MTSL (which forms the side chain denoted as R1) is the preferred spin label for proteins (Table 1). Numerous studies have been carried out with MTSL, and rotamer libraries have been created to describe the internal motion of R1 (ref.\textsuperscript{4}). It carries an unpaired electron (spin 1/2) localized along the N–O bond. The small size, high specificity, and reactivity make MTSL an ideal spin label for structural studies. It is also used in paramagnetic relaxation enhancement NMR experiments to determine long-range distance constraints (up to ~35 Å).

However, interspin distances beyond 2 nm cannot be determined using CW-EPR, because the line broadening due to dipolar interaction becomes too small to be extracted within the linewidth of the CW-EPR spectrum. To resolve the weak dipolar couplings resulting from longer distances, several pulsed dipolar EPR spectroscopy (PDS) techniques have been developed. PELDOR or DEER is currently the most widely utilized tool among a growing number of PDS techniques\textsuperscript{9,10}. It is a powerful technique for structural investigation of biomolecules in solution, membrane, or cellular environments\textsuperscript{11-15}. PELDOR can resolve distance distributions between spin pairs in the range of 1.5–16.0 nm\textsuperscript{16}. Such interspin distances have been used to explore conformational changes, equilibrium dynamics, and structural heterogeneity of several membrane protein complexes\textsuperscript{17-19}. Combined with simulations and modeling, such constraints can validate existing structures, provide novel structural information, and visualize alternative conformations that have not yet been observed in the crystal structure\textsuperscript{17,19,20}.

As in any probe-based technique, PELDOR provides sparse distance restraints. The distances are determined between the unpaired electrons, which are connected to the protein backbone through flexible linkers. The degrees of freedom for the rotation of the dihedral angles in the linker lead to a rotameric distribution of the spin label (σ = ~3 Å in the absence of protein backbone motion)\textsuperscript{1}. Changes in the interspin distance provide direct information on the extent and the nature of the conformational changes. However, to extrapolate those changes to the backbone or to use the distance distribution for structural modeling, the rotameric states of the spin label need to be described. This has been rather well established for MTSL\textsuperscript{21}, which permits the comparison of an available rotamer library with experimental data or allows modeling of a new functional or an oligomeric state from an existing structure\textsuperscript{19}.

**Theoretical rationale behind DEER/PELDOR spectroscopy**

PELDOR uses a refocused Hahn echo with the pulse sequence \(\pi/2-t_1-\pi-t_3-\text{echo}_1-\pi-t_2-\text{echo}_2\) applied at the observer frequency \(\nu_B\) on the A spin (Fig. 1, in gray). A second inversion pulse at the pump frequency \(\nu_p\) is applied on the B spin at a variable time \(t\) with respect to the observer echo. The observer sequence refocuses the inhomogeneous broadening of the A spin arising from \(g\) value (Landé \(g\)-factor) dispersion, hyperfine interaction, and coupling with other electron spins. Inversion of the B spin by the pump pulse changes the resonance frequency of the A spin by the dipolar coupling frequency \(\omega_{\text{dip}}\). Thus, varying the timing of the pump pulse leads to a phase gain of the \(\text{A spin by } \phi_{t} = \omega_{\text{dip}}t\) and oscillation of the echo amplitude \(V\). The resulting PELDOR is a product of two contributions\textsuperscript{8}:

\[
V(t) = B(t) \times F(t)
\]

\(B(t)\), the intermolecular contribution, commonly known as the background function, arises from the interaction between spins in the neighboring biomolecules. \(F(t)\), which is the wanted intramolecular interaction, arises from the interaction of the spins within the same biomolecular unit being observed. For a single isolated spin pair, \(F(t)\) can be expressed as\textsuperscript{8}:

\[
F(t) = \left\{ 1 - \lambda_B \left[ 1 - \cos(\omega_{\text{dip}}t) \right] \right\}
\]

For a macroscopically disordered sample, such as that of a biomolecule, \(F(t)\) can be calculated from an ensemble average according to the following equation:

\[
F(t) = \prod_i \left\{ 1 - \lambda_i \left[ 1 - \cos(\omega_{\text{dip}}t) \right] \right\}_{r,\theta}
\]

The indexing \(i\) refers to the B spins. The averaging runs for all the distances \(r\) over all the orientations \(\theta\) of the interspin vectors with respect to the external magnetic field \(B_0\). The parameter \(\lambda\) is the fraction of the coupled B spins that are excited by the pump pulse (also known as the
### Table 1 | Spin labels for in situ PELDOR experiment with proteins/peptides.

| Label                  | Linkage                  | Advantages/disadvantages                                                                                                                                                                                                 | Refs.          |
|------------------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| **Nitroxide labels**   |                          |                                                                                                                                                                                                                         |                |
| MTSL (RI)              | -S-S- (disulfide) bond via cysteines | Small size, high specificity and reactivity, and very well-studied label reduction of nitroxide label and the -S-S- bond under in situ conditions                                                                                     | 35,36,46,47    |
| 3-Maleimido-PROXYL     | -C-S- (thioether) bond via cysteine | Stable covalent attachment to the protein Less specific and irreversible linkage, may react with Arg or Lys, bulkier than MTSL, and limited literature data                                                                 | 12             |
| M-TETPO                | -C-S- (thioether) bond via cysteine | Very stable under reducing conditions and covalent attachment to the protein Disadvantages similar to those of 3-maleimido-PROXYL                                                                                                                                                        | 38             |
| *Gadolinium(Gd$^{3+}$)* labels |                          |                                                                                                                                                                                                                         |                |
| Gd(III)-DOTA-M         | -C-S- (thioether) bond via cysteine | Stable under reducing conditions Bulky and less specific, endogenous Mn$^{2+}$ may interfere with in situ experiments, requires higher frequency (94 GHz) for optimal sensitivity, thiol exchange with glutathione or hydrolysis of the succinimide ring may take place in situ | 14,40          |
| Gd-PyMTA               | -C-S- (thioether) bond via cysteine | Smaller linker and higher cysteine specificity, lower affinity than DOTA Limited literature data                                                                                                                      | 39             |
| GdL                    | -C-S- (thioether) bond via cysteine | Smaller linker and higher affinity (for Gd$^{3+}$) and reactivity to cysteines Limited literature data                                                                                                                  | 41             |

Table continued
inversion efficiency), which leads to a decay of $F(t)$ to $1 - \lambda$. For nitroxide spin labels separated by distances >1 nm, the unpaired electron can be approximated to be localized at the center of the $N$–$O$ bond with the quantization axis parallel to the direction of $B_0$. Thus, the dipole–dipole tensor can be described by the point–dipole interaction. The exchange coupling $J$ through bonds is negligible for distances >1.5 nm, especially when determined with biomolecules in frozen buffers or membranes. Typically, the difference between the pump and observer frequency ($\Delta \nu$) is much larger than $\omega_{\text{dip}}$ and therefore the pseudosecular term in the Hamiltonian for electron–electron interaction can be neglected\(^{22}\). Now $\omega_{\text{dip}}$ under the approximation of weak coupling ($\Delta \nu > \omega_{\text{dip}}$) is given by:

$$\omega_{\text{dip}} = \frac{1}{r_{AB}} \frac{\mu_0}{4\pi\hbar} g_A g_B \mu_B^2 (3\cos^2 \theta - 1)$$

(4)

where $\mu_0$ is the permeability of vacuum, $\hbar$ is the reduced Planck's constant, and $\theta$ is the angle between the interspin vector $r_{AB}$ and $B_0$. With an isotropic value of $g_A = g_B = 2.006$ approximated for nitroxide spins, $\omega_{\text{dip}}/(2\pi)$ has a value of 52.04 MHz/nm\(^3\). The intermolecular contribution $B(t)$, which arises from dipolar interaction between randomly distributed spins on neighboring objects, can also be expressed according to Eq. (3; ref. 9). Such intermolecular interactions lead to an exponential decay function as shown below:

$$B(t) = \exp\left(-\frac{C\lambda_{\text{d}}(kt)^{d/3}}{1 - \Delta}\right)$$

(5)

where, $C$ is the concentration of the $A$ spins that interacts through intermolecular interaction and $\lambda_{\text{d}}$ is the fraction of the $B$ spins excited by the pump pulse. The spins can have a homogeneous distribution in space (with $d = 3$) or could have a spatial distribution with fractional dimension, as in the case of membrane proteins reconstituted into proteoliposomes (with $d = 2.0$–2.5). Values >3 indicate contributions from exclusion volume effects due to large physical separation between the spins\(^{23}\). The constant $k$ is given as:

$$k = \frac{8\pi^2 \mu_0^2 g_A g_B}{9\sqrt{3} \hbar}$$

(6)

The modulation depth ($\Delta$) of $F(t)$ (Fig. 2) is the product of $\lambda_{\text{d}}$ (inversion efficiency in Eq. (3)) and the labeling efficiency (([spin]/[protein]) × 100%). Thus, depending on the spin-labeling efficiency, $F(t)$ decays to a final value of $1 - \Delta$ (when the labeling efficiency is <100%, $\lambda$ in Eqs. (2) and (3) should be substituted with $\Delta$). One of the critical steps in PELDOR data analysis is the separation of $F(t)$ from the original data. When the dipolar evolution ($t$) is observed for sufficiently long times, this can be achieved by dividing $V(t)$ by $B(t)$ (Eq. (1)). Reliable fit for $B(t)$ requires that the dipolar evolution $V(t)$ be observed for a much longer time ($t_{\text{max}}$) after the dipolar oscillations have fully decayed. Owing to the flexibility of the spin label and the protein backbone, the PELDOR data contain a distribution of frequencies/distances. The corresponding distance distribution is characterized by its mean, width, and shape. The width and the shape of the distance distribution are encoded in the decay rate and the shape of the decay envelope, respectively, of the dipolar oscillations. The observed $t_{\text{max}}$ puts an upper limit on the accurate determination of the mean distance by
$r_{\text{max}, r} \approx 5 \sqrt{t_{\text{max}}/(2 \mu s)} \text{nm}$ and on the width by $r_{\text{max}, \sigma} \approx 4 \sqrt{t_{\text{max}}/(2 \mu s)} \text{nm}^{13}$. To determine the shape of the distance distribution, $t_{\text{max}}$ should be even longer, with an extremely high signal-to-noise (S/N) ratio.

The molecules in the sample and hence the interspin vectors ($r_{AB}$) are randomly distributed. When all the orientations are excited, Fourier transformation of $F(t)$ yields a dipolar spectrum (or Pake pattern), and the interspin distance can be obtained directly from the frequencies at which the singularities ($\theta_\perp = 90^\circ$ and $\theta_\parallel = 0^\circ$) appear (Fig. 2d). Normally, the inherent flexibility of the protein backbone and the spin label cause broadening of the Pake pattern and the singularities are not defined anymore, which leads to inaccuracies in the probability distribution $P(r)$ of the interspin distances. Such a scenario leads to an inverse problem in which the $P(r)$ value needs to be computed from $F(t)$ or the dipolar spectrum. This is an ill-posed problem, as the noise in the time- or frequency-domain data can have an even larger effect on the computed distance distribution. To address this ill-posed problem, current approaches impose a variable smoothness to stabilize the solution against artifacts. Tikhonov regularization (TR) is one of the most common methods used to solve ill-posed problems. TR has also been used for PELDOR data analysis$^{24,25}$ and is implemented in the MATLAB-based software DeerAnalysis$^{26}$. During TR, the time-domain signal $S(t)$ for a given distance distribution $P(r)$ is simulated and fitted with the experimental form factor $F(t)$ with minimum deviation. Straight fitting of $S(t)$ to $F(t)$ would result in strong noise artifacts, and TR uses a regularization parameter $\alpha$, which stabilizes the solution against artifacts.
**Fig. 2 | In situ PELDOR in native OM.** a, Position 188 in the second extracellular loop and the TEMPO-labeled hydroxocobalamin (TEMPO-HOCbl, in green with the nitroxide-bearing moiety highlighted in red; 25 μM) are highlighted on the BtuB crystal structure (Protein Data Bank (PDB) 1NQH). The TEMPO-HOCbl was synthesized as described in Box 1. b, Original PELDOR data obtained in native OM as indicated. The data are slightly shifted along the vertical axis for clarity. For the 188R1 mutant, the data fit perfectly into a stretched exponential decay (μ = 2.2, in gray). Similarly, the intermolecular contribution for the 188R1-TEMPO-HOCbl spin pair also fits into a stretched exponential decay (μ = 2.5, in black). c, The dipolar evolution (orange) obtained for 188R1/TEMPO-HOCbl PELDOR after correction for the intermolecular contribution and the corresponding fit from Tikhonov regularization (TR) is overlaid (black). The modulation depth (Δ) is indicated. Overall, the data suggest a 2D distribution of the spins over the large cell surface and deviation of the value for d (from 2.0) might be for other reasons, including the membrane curvature and sample inhomogeneity. d, The dipolar spectrum obtained with Fourier transformation (orange) or TR (black) of c. Frequencies corresponding to the parallel (θ = 0°) and perpendicular (θ = 90°) orientations of the interspin vectors to the B0 are indicated. e, Interspin distance distributions obtained from TR of c, f, dipolar coupling frequency; $F(t)/F(0)$, normalized PELDOR data after correction for the intermolecular contribution; $P(r)$, probability for interspin distance; t, time; $V(t)/VO$, normalized original PELDOR data.

which is related to the smoothness of $P(r)$. A large α value means a broad distance distribution and, for well-defined narrow distances, a smaller α value is required. The optimum α value is calculated by the L curve criterion. This whole procedure works with $F(t)$, which requires a priori estimation of $B(t)$. For data with short dipolar evolution times, a priori estimation of $B(t)$ might be difficult or could lead to unrealistically large errors. In addition, when $P(r)$ consists of a mixture of narrow and broad components, this procedure may result in splitting of the broad component into multiple narrow peaks to fit the single value of the regularization parameter α used. To quantify the effect of the choice of α on the uncertainty in $P(r)$, a Bayesian statistical approach can be used with TR.27 Alternatively, the original PELDOR data can be simultaneously fitted for both $B(t)$ and $F(t)$ in a model-based approach, using simple analytical expressions of $P(r)$ such as Gaussian components.28 This does not require a priori estimation of $B(t)$ and also permits a rigorous statistical analysis on the fit parameters. However, none of these approaches can circumvent the length of the dipolar evolution time required for reliable determination of $B(t)$ and $P(r)$. Even when this condition is achieved, it is worthwhile to compare the shapes of $P(r)$ obtained with TR and model-based analysis.

The observable length of the dipolar evolution time is limited by the loss of phase memory due to transverse relaxation, and spectral-, spin-, and instantaneous diffusion, which together lead to an exponential decay with a time constant called the phase memory time ($T_M$). Under the typical conditions of PELDOR measurements on membrane proteins (50–100 μM spin and 50–80 K), $T_M$ is dominated by fluctuating hyperfine interaction with nearby protons, including those from the methyl groups and the higher local spin concentration. For membrane-reconstituted proteins, this reduces the $T_M$ to 1–2 μs (from typical values between 3 and 4 μs in detergent solution) and thereby limits the upper range of the accessible distances to ~5 nm. Sample deuteration and, recently, multiple refocusing pulses at the observer frequency have been implemented to prolong $T_M$ and increase the range of the accessible distances. This leads to the extension of the 4-pulse DEER toward 5-pulse and 7-pulse versions (known as the 5-pulse DEER and the 7-pulse CP-PELDOR, respectively)29,30, both of which can prolong the $T_M$ up to twofold for membrane-reconstituted proteins (Fig. 1). Each of these observer π pulses, which are applied under a Carr–Purcell (CP) condition, is accompanied by an inversion π pulse at the pump frequency. The non-ideal behavior of the successive pump pulses (N) leads to uneven excitation of the B spins and 2N dipolar signals. Therefore, additional data processing is required to remove the artifacts and extract the wanted dipolar pathway (in which the B spins are
excited by all the successive pump pulses). Although PELDOR is a double-frequency experiment, there exist single-frequency techniques for distance measurements, such as double-quantum coherence (DQC)\textsuperscript{31}, single-frequency technique for refocusing dipolar couplings (SIFTER)\textsuperscript{32}, and relaxation-induced dipolar modulation enhancement (RIDME)\textsuperscript{33}. DQC and SIFTER are used less frequently, mainly because of the requirement for nonselective broadband pulses, which can excite the whole spectrum (or both the coupled spins). RIDME has been shown to be a very sensitive technique for distance measurements involving fast-relaxing metal centers\textsuperscript{33,34}. However, a stronger background decay than in the PELDOR experiment makes the separation of $F(t)$ for longer distances more complicated.

**Spin labels for in situ PELDOR**

Routinely, spin labeling and PELDOR are performed following the extraction and purification of the protein from the native environment. With recent developments in spin-label chemistry, PELDOR experiments have been performed in a few cases under in situ conditions (Table 1). For soluble proteins, in situ PELDOR has been demonstrated following exogenous addition of the protein to *E. coli*, HeLa cells, or oocytes\textsuperscript{12,14,35,36}. However, with MTSL, the disulfide bond that connects the spin label to the cysteine in the protein of interest becomes less stable under the reducing cellular environment. The maleimido-proxy label (3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy), which is attached to the protein via a C–S bond, is more stable in situ\textsuperscript{2}, although the nitroxide moiety may still be reduced into a hydroxylamine. In addition, the maleimido-proxy may produce undesirable side reactions with amines. Sterically shielding the nitroxide moiety by substituting the gem-dimethyl with gem-diethyl groups has been shown to provide kinetic stability against reduction\textsuperscript{35}. Such a protected label when functionalized with the maleimide group (called M-TEMPO) is shown to be more stable in the cellular environment\textsuperscript{36}. The limited stability of the nitroxide spin labels stimulated the application of redox-stable Gd\textsuperscript{3+} and trityl (trialkylmethyl) spin labels for in situ EPR. Gd\textsuperscript{3+} was found to be stable inside several cell types and enabled PELDOR on exogenously introduced molecules with high sensitivity\textsuperscript{14,39–41}. With the ongoing efforts to further reduce the size of the chelator and the linker, Gd\textsuperscript{3+} could become an ideal spin label for in situ EPR\textsuperscript{42}. Trityl is also stable under reducing conditions and provides higher sensitivity due to its narrow EPR spectrum\textsuperscript{43}. Trityl-nitroxide PELDOR has been shown to be a versatile tool for observing protein–ligand interactions in native *E. coli* membranes\textsuperscript{44}. Trityl-Fe\textsuperscript{3+} distances have been determined using RIDME experiments on cytochrome P450 inside *Xenopus laevis* oocytes\textsuperscript{45}. The major drawbacks of trityl labels are their large size and the tendency to aggregate when exposed to membranes or whole cells\textsuperscript{46,44}. Genetic encoding has been tested for direct incorporation of an unnatural amino acid, which can be spin-labeled\textsuperscript{14} or carry a nitroxide label\textsuperscript{47}, but reduction of nitroxide in the cellular environment has prevented the in situ application of this approach. Despite this progress, in situ EPR is limited to only those molecules that can be purified and spin-labeled in vitro. For membrane proteins, this approach is not feasible, as the protein needs to be spin-labeled directly in the complex native environment. As a result, the labeling of membrane proteins in native environments for spectroscopic investigation presents a major challenge for structural biologists.

**In situ PELDOR of bacterial OMPs**

We have developed a general strategy to make accurate distance measurements within OMPs in intact *E. coli* and native OMs using PELDOR. The cell envelope of Gram-negative bacteria consists of an inner membrane (IM) surrounding the cytoplasm and an OM that protects the cells from harsh conditions, including antibiotics (Fig. 3). The periplasm separates these two membranes. The OM is an asymmetric bilayer made up of phospholipids (PLs) and lipopolysaccharides (LPSs), and it harbors numerous β-barrel proteins (OMPs). OMPs range in size between 8 and 26 β-strands, and many of them are being explored as targets for novel drugs. These proteins rarely have reactive cysteines, which decreases non-specific signals that might result from in situ spin labeling\textsuperscript{46,47}. We exploited this natural cysteine exclusion for selective MTSL labeling and in situ PELDOR measurements of OMPs\textsuperscript{46,47–49}. In *E. coli*, spin labeling can be performed at solvent-exposed sites on the extracellular side under ambient conditions. Alternatively, the use of isolated OM preparations provides access to both membrane surfaces for spin labeling and ligand binding. In this protocol, we provide a detailed procedure for SDM, protein expression, OM isolation, spin labeling in *E. coli* and OM, sample preparation, PELDOR setup, and data analysis.
Applications of the method

OMPs consist of autotransporters, channels for specific substrates, general porins, protein-folding machinery, transporters, and proteins involved in adhesion, invasion, or evasion of host cells. They are involved in diverse essential physiological processes, such as membrane biogenesis, motility, infection, immune response, transport, resistance to toxic compounds, and signaling, and many of them are highly sought-after targets for novel drugs. Currently, there is a need to understand the structure, function, and dynamics of OMPs in their native environments. Over the past few years, evidence that the native environment might critically influence folding, function, oligomerization, and structure of OMPs has accrued. For example, it has been observed that the detergent extraction required for high-resolution methods can alter OMP conformation. Some of the OMP complexes, such as the β-barrel assembly machinery (BAM) or the LPS transport system (Lpt) themselves, are responsible for the OM biogenesis. Unlike other membrane transport complexes, which transport substrates across the membrane, BAM and Lpt insert their substrates directly into the membrane. Thus, the asymmetric OM (which is too complicated to be reconstructed in vitro) forms an integral part of the overall structure of these complex machines. Our protocol describes an approach to validate OMP structures in the native OM and in intact E. coli. A comparison of in silico PELDOR simulations with experimental data can reveal the similarity or differences in structure between in vitro and in situ conditions.

OMPs show variations in the oligomeric structure from monomers to trimers. It remains unknown whether such oligomerization persists in situ. Recent biochemical and biophysical data suggest that the membrane environment can induce homo-oligomerization as well as heterologous protein–protein interaction for proteins, which were observed as monomers in the crystal structures. Such interactions may govern their local and global dynamics, as well as spatial clustering into micro-domains called OMP islands. Our protocol can be used to address such homo- or heteromolecular interactions at high resolution in native environments.

OMPs can undergo large conformational changes or sometimes a shift in the overall conformational equilibrium during function. Such changes might be mediated through interactions with other molecules such as lipids in the OM, substrates, ligands, toxins, transducers, or a subunit of the molecular complex. As we demonstrated earlier, our protocol enables the observation of such long-range conformational changes directly in E. coli and native OM (Fig. 4 and Supplementary Fig. 1). In this example, the extracellular ligands or the interacting molecules could be added from outside. Alternatively, such investigations can be performed in the absence of an interacting protein (e.g., through the knockout of a gene, which encodes an interacting protein located in the OM or the

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**Fig. 3 | Schematic view of the cell envelope of Gram-negative bacteria.** The cell envelope of Gram-negative bacteria consists of an IM and an OM, which are separated by the periplasm. The IM is a PL bilayer, whereas the OM is an asymmetric bilayer consisting of PL and LPS. The IM contains α-helical proteins and the OM harbors numerous β-barrel proteins (or OMPs), including the porins, which are essential to bacterial growth or pathogenicity. The OM also contains peripherally attached lipoproteins (LPs). The OMPs rarely have reactive thiols, and their cysteine mutants can be labeled with MTSL in E. coli or isolated OM with minimal background labeling.
**Comparison with other methods**

Our protocol offers several advantages for in situ structural biology of membrane proteins over existing structural biology tools. Although dynamic nuclear polarization (DNP)-enhanced solid-state NMR is emerging as a promising tool for structural investigation of membrane proteins under native conditions, difficulties with selective labeling, lower sensitivity and spectral resolution, insensitivity to longer distances (>0.2 nm), and difficulty in maintaining cell integrity during the measurement period are major challenges. Cryo-electron tomography with subtomogram averaging allows structural investigation in the native environment. However, reconstruction of a membrane protein structure in situ with sufficient resolution has not yet been achieved. PELDOR spectroscopy and single-molecule fluorescence resonance energy transfer (smFRET) are unique tools for addressing conformational changes and equilibrium dynamics of large membrane protein complexes. Both PELDOR and smFRET can be used to determine distances between two labeled sites. In particular, smFRET is useful for observing conformational dynamics with spatio-temporal resolution and superior sensitivity. However, the requirement for site-specific orthogonal labeling on two distinct sites, limited range of the distances for a given FRET pair, and the large size, as well as the uncertainty in the mutual orientation of the fluorophores, pose major challenges. Fluorescent proteins have been widely used for in situ fluorescence studies, but their large size limits the scope for smFRET. Current approaches for in situ smFRET use exogenous introduction of biomolecules labeled with organic fluorophores, which is limited to soluble molecules. By contrast, EPR spectroscopy uses identical spin labels at both sites that are relatively small in size and provide structural information about the

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**Fig. 4 | In situ PELDOR in E. coli.** a, The extracellular loops carrying the positions 188 and 399 are highlighted on the BtuB crystal structure (PDB 1NQH). b, Original PELDOR data in E. coli as indicated. For WT BtuB (which is naturally Cys-less), the data fit into a stretched exponential decay \(d = 2.2\), which could not be measured for longer time window because of the weak signal. The intermolecular contribution for the 188R1-399R1 spin pair fits into a stretched exponential decay \(d = 2.5\), in black). The data are slightly shifted along the vertical axis for clarity. c, The dipolar evolution (orange) obtained for the 188R1-399R1 PELDOR after correction for the intermolecular contribution \(d = 2.5\) and the corresponding fit from TR (in black). The modulation depth \(\Delta\) value is indicated. Overall, the data suggest a 2D distribution of the spins over the large cell surface and deviation of the value for \(d\) (from 2.0) might be for other reasons, including the membrane curvature and sample inhomogeneity. d, The dipolar spectrum obtained with Fourier transformation (orange) or TR black) of c, e, Interspin distance distributions obtained from TR of c. The corresponding simulation on the BtuB crystal structure (PDB 1NQH) using the MMM software is overlaid (in violet), which suggests a very good agreement between the conformations observed in the crystal structure and E. coli. f, xxxxxxxx; F, xxxxxxxx; P, xxxxxx; r, xxxxxxx; t, time; V, xxxxxx.
characterization. Spin labeling of cobalamin did not substantially alter its affinity for BtuB further reduces the background labeling and thereby enriches the signals for the cysteine mutants in the isolated OM (Supplementary Fig. 2a,b). Compared to E. coli, OM provides several advantages. Overall, sample handling and spin labeling are easier as there are no concerns about cell viability or spin-label reduction (see ‘Limitations’ section). Also, OM can be concentrated to a higher degree, thereby providing a higher S/N ratio while keeping the protein in the native membrane environment.

**Box 1 | TEMPO-HOCbl synthesis, purification, and characterization**  ● **Timing 30 h**

**TEMPO-HOCbl synthesis**

▲ **CRITICAL** TEMPO-HOCbl is synthesized according to a protocol that we previously described for the synthesis of TEMPO-cyanocobalamin (TEMPO-CNCbl)\(^ {64}\). A short description of the procedure is provided below.

1. Activate the ribose-5'-hydroxyl of TEMPO-HOCbl with CDT and then react with 4-amino-TEMPO (steps 2–4).

▲ **CRITICAL STEP** The synthesis should be performed using dry solvents under argon atmosphere.

2. Dissolve 0.1 g of HOCbl in 50 mL of DMSO and add 0.036 g of CDT.

3. Stir the reaction mixture for 30 min at RT.

4. Add 0.13 g of 4-amino-TEMPO and stir the reaction mixture at RT for 12 h.

5. Precipitate TEMPO-HOCbl by adding 200 mL of a 1:1 (vol/vol) mixture of acetone and diethyl ether.

6. Separate the precipitate by centrifugation at 4 °C for 15 min at 4,000 g.

7. Repeat the centrifugation once more with the supernatant.

8. Wash the pooled precipitate (TEMPO-HOCbl) with acetone and centrifuge as described in Step 6.

9. Dry the precipitate overnight under air, and then freeze-dry. The yield of the raw product is typically 0.091 g (0.059 mol), which corresponds to 80% of the theoretical yield.

■ **PAUSE POINT** The raw product can be stored at −20 °C for several years.

**TEMPO-HOCbl purification and characterization**

10. The crude product from the above reaction is analyzed using HPLC on a BDS-C18 column with detection at both 254 and 316 nm (Supplementary Fig. 3), using the following parameters:

- Inject 8 µL of 1 mM TEMPO-HOCbl and elute at a 1 mL/min flow rate with 400-bar pressure at RT
- Use the following solvent system for elution: solvent A: water; solvent B: methanol.
- Perform elution at a linear gradient of 15–60% solvent B over 40 min.

11. We perform MALDI-ToF–MS analysis on a Voyager STR Workstation DE PRO with 100-kW laser peak power (337 nm) in a matrix made of 2,5 dihydroxybenzoic acid and 6-aza-thiothymine (Supplementary Fig. 4). The LC-ESI–MS measurements are performed with a Shimadzu LCMS-2020 system (Supplementary Fig. 5). A water–acetonitrile solvent system (both containing 0.1% (vol/vol) formic acid) is used for column elution with a capillary voltage of 3–4 kV, 5-bar N2 pressure, and a mass range of 80–2,000 Da, with single-quad detection.

The labeling procedure is rather easy and, as PELDOR uses identical spin labels at both sites, site-specific labeling with orthogonal labels as in FRET is not required. Labeling is achieved by adding MTSL to the cell exterior, and unreacted spin labels can be removed by a few washing steps. As we previously demonstrated with a trityl label\(^ {44}\), this protocol can be adopted to attach other spin labels, which are functionalized to react with cysteines. In practice, any solvent-exposed sites can be spin-labeled with cysteine mutants in the isolated OM (Supplementary Fig. 2a,b). Compared to E. coli, OM provides several advantages. Overall, sample handling and spin labeling are easier as there are no concerns about cell viability or spin-label reduction (see ‘Limitations’ section). Also, OM can be concentrated to a higher degree, thereby providing a higher S/N ratio while keeping the protein in the native membrane environment.
concentration after labeling for different time intervals. MTSL labeling was performed at OD600
MTSL (overall, which took an additional 6
MTSL for different times as indicated. For the zero time point, cells were pelleted immediately after mixing with
concentration after labeling at different OD600 values. Labeling was performed with 500
depth, we are able to measure a high-quality 4-
into micro domains with other proteins. Despite such a background decay and a lower modulation
OM. This indicates inhomogeneous distribution of BtuB (spin labels) and possibly spatial clustering
(biology. Here, the signals from background labeling contribute exclusively to the intermolecular part
Fig. 5 | In situ MTSL labeling of BtuB in E. coli. a, RT CW-EPR spectra of BtuB obtained in live E. coli after labeling
with 500 μM MTSL at OD_{600} = 25 for 10 min at 25 °C. b-d, MTSL labeling of BtuB 188C–399C in E. coli at 25 °C.
Spin concentrations of the E. coli (normalized to unit OD_{600}) are given on the y axis. b, Spin concentration after labeling with different MTSL concentrations. Labeling was performed at OD_{600} = 25 for 10 min. c, Spin concentration after labeling for different time intervals. MTSL labeling was performed at OD_{600} = 25 with 500 μM
MTSL for different times as indicated. For the zero time point, cells were pelleted immediately after mixing with
MTSL (overall, which took an additional 6–7 min, including centrifugation and EPR measurement). d, Spin concentration after labeling at different OD_{600} values. Labeling was performed with 500 μM MTSL for 10 min at different OD_{600} values as indicated. The inset shows a contour plot summarizing the experiments in c and d. The shaded area indicates a small window for the incubation time and the cell density, at which maximal labeling can be achieved. Error bars indicate a 15% error, which is typical for spin quantification using RT CW EPR spectroscopy. Similar trends were observed between independent experiments.

Limitations
The protocol described here can be used for studying OMPs in E. coli and native OM. In E. coli,
labeling is limited to solvent-exposed residues on the extracellular side. We have shown that positions
located on the extracellular loops and the barrel lumen can be labeled in E. coli47,48. Spin labeling can,
however, not be achieved in the periplasm under the conditions described here using MTSL or the
maleimido-proxyl label, which is attached to the protein through a C–S covalent bond (Table 1).
Although our optimized protocol provides good labeling efficiency, labeling has a limited time
window due to reduction of the MTSL molecules. After addition to the cell suspension, MTSL is
rapidly reduced, with a half-life of ~10 min (Supplementary Fig. 6), and most of the labeling happens
within the first few minutes (Fig. 5c). The mechanism for nitroxide reduction by E. coli remains
unknown as of now. Currently, we are working on alternative strategies to introduce more stable spin
labels (such as the protected nitroxide, Gd^{3+}, or Cu^{2+}), which would also allow us to extend spin
labeling into the periplasm. Notably, spin-label reduction is not an issue with the native OM
preparation.

Although the OMPs are devoid of reactive cysteines, we always observed background labeling with
E. coli and OM (see the wild-type (WT) in Figs. 4b and 5a). It is possible that the labels bind to the
membranes through physical adsorption. In agreement, previously we observed that a more
hydrophobic trityl label shows a higher binding (and aggregation) in the OM as compared to MTSL.44
Thus, adhesion to the cell membrane might be the major contribution, if not the only one, for
nonspecific labeling. In general, background labeling is one of the major hurdles for in situ structural
biology. Here, the signals from background labeling contribute exclusively to the intermolecular part
(B(t) in Eq. (1)) of the PELDOR signal. In effect, this reduces the modulation depth to 8–10% (λ_{P} in
Eq. (2)), but, most importantly, background signals do not interfere with distance measurements on
the target proteins (Figs. 2b and 4b). Even though the bulk spin concentration is in the range of
50–100 μM, the background B(t) of the PELDOR data shows a rather fast decay in both E. coli and
OM. This indicates inhomogeneous distribution of BtuB (spin labels) and possibly spatial clustering
into micro domains with other proteins. Despite such a background decay and a lower modulation
depth, we are able to measure a high-quality 4-μs trace within ~12 h. The decreased modulation
Experimental design
In this protocol, we describe a detailed protocol for spin labeling and PELDOR measurements on OMPs in E. coli and native OM. The protocol consists of the following stages: SDM to introduce cysteines (Steps 1 and 2), protein expression in E. coli (Steps 3 and 4), spin labeling in E. coli (Steps 5–7 and 8A(i–vii)) or OM (Steps 5, 6, and 8B(i–xii), PELDOR measurements (Steps 9–20), and data analysis (Step 21). Our procedure enables the observation of protein–protein or protein–ligand interactions as well as the characterization of long-range conformational changes of OMPs in their native environments.

Introducing cysteines for spin labeling
SDM is done using standard protocols to introduce cysteines at the desired positions in the WT protein. OMPs rarely have reactive cysteines; when present, they are either buried or cross-linked and often there is no need to create the Cys-less background. When performing PELDOR measurements in E. coli, positions located on the extracellular loops and the plug domain inside the β-barrel can be mutated to cysteines for labeling. By contrast, when performing PELDOR measurements on native OM, mutation and spin labeling can be performed at positions on either side of the membrane. The accessibility of the target site for the spin label is critical for successful labeling, and buried sites should therefore be avoided. This also precludes any possible structural distortion of the protein. In addition, residues with functional or structural roles, such as Gly, His, and Pro, and charged or aromatic amino acids should be avoided when possible. Residues such as Ala, Ile, Leu, Met, Ser, Thr, or Val would be more favored for SDM and labeling. Whenever possible, the functional integrity of the mutants should be verified (using in situ or in vitro functional assays) after spin labeling. We recommend that an in silico spin labeling and PELDOR experiment be performed for the selected sites (when a structure is available), e.g., by using one of the available programs such as MMM, MtsslWizard, or PRONOX before proceeding with SDM.

OMP expression
The OMP can be expressed in either a constitutive or an induced manner and in a minimal or rich medium, and the best conditions should be tested in a case-by-case manner. Although we did not encounter any issues with expression levels in different strains, different vectors, strains, media, and
protocols can be tested when the expression level is not sufficient. The RK5016 strain (MC4100, metE70, argH, btuB, and recA mutations) we used for BtuB expression is derived from the E. coli K-12/MC4100 strain, which has been extensively used for genetic experiments; in addition, its genome is well characterized. This strain allowed us to perform in situ PELDOR in both whole cells and native OM. Another E. coli K-12 strain (KDF541) was used for in situ EPR of the ferric enterobactin transporter (FepA) in whole cells. In both cases, the protein was expressed in a constitutive manner (BtuB from the lac promoter and FepA from the native promoter) in minimal medium. E. coli BL21(DE3) cells, one of the most popular and commercially available strains can also be used, as we demonstrated for in situ PELDOR of the ferrichrome transporter FhuA in native OMs. We also achieved similar results for the ferric enterobactin transporter FecA. For spin labeling in native OMs, an identical protocol works with both K-12 and BL21(DE3) strains, suggesting that our protocol can be used for studying any of the OMPs in their native membranes. In our experience, the K-12 strains appear to be better suited to investigations in whole cells. However, we anticipate that BL21(DE3) or other commercial strains will also be useful for whole-cell investigations with further optimization for protein expression and spin labeling.

**Obtaining efficient spin labeling**

Calculating the labeling efficiency in situ is a difficult task. If a spin-labeled ligand (or an interacting protein fragment) is available, binding analysis using room temperature (RT (22–26 °C)) CW-EPR can be used to quantify the expression level and the labeling efficiency of the protein of interest. Signals for the WT background (under identical conditions) should be subtracted to account for nonspecific labeling and ligand binding. For surface-exposed positions, a high labeling efficiency of >80% can usually be achieved. Most often, overexpression of the OMP does not cause protein aggregation in the membrane, and it can be checked by measuring interspin distances between singly labeled variants (Fig. 2b). When there is evidence for nonspecific interaction or aggregation, the expression vector, E. coli strain, or the growth conditions can be further optimized. Spin labeling in the complex cellular environment requires more selective and stringent conditions as compared to the labeling of purified proteins. Labeling is fraught with background labeling and the fast reduction of MTSL. Thus, the labeling conditions, as well as the properties of the spin label itself, become critical to successful labeling. We tested labeling at different cell densities, MTSL concentrations, and incubation times. In our experience, maximal labeling is achieved with 50 μM MTSL, in the first few minutes of incubation. Higher MTSL concentrations have no effect, and prolonging the incubation time reduces the overall signal intensity (Fig. 5b,c). Overall, labeling appears to be more efficient at lower OD600 (≤10) values, and MTSL is reduced faster at higher cell densities (Fig. 5d). By prolonging the incubation time at higher spin concentrations (e.g., 1 h with 150 μM MTSL), it is possible to reduce signals from nonspecific labeling, although the labeling of the cysteine is decreased as well. Following incubation, free spin labels are easily removed by two to three rounds of washing and resuspension. Free spin label reduces the modulation depth and hence the overall sensitivity; therefore, the number of wash steps can be adjusted as necessary to reduce unbound label to undetectable levels.

**Isolation of the OM**

Removal of the IM (from the cell envelope), which carries several α-helical proteins, is necessary for efficient spin labeling in the native OM (Supplementary Fig. 2a,b). When labeled on the intact envelope, cysteine mutants and the WT sample revealed similar spectra. Selective solubilization of IM using sarkosyl reduces nonspecific labeling and yields much larger signals for cysteine mutants as compared to WT samples. Cysteines located on either the extracellular or the periplasmic side can be labeled with isolated OM (Fig. 2 and Supplementary Fig. 2c,d), and excess MTSL is removed after a few rounds of washing. Spin-labeled ligands or proteins (tested up to 10 kDa) can be added to observe their interaction with the target OMP from either side of the membrane without performing any extrusion or freeze–thaw cycles, meaning that the native OMs are also accessible to larger substrates. Although our current labeling protocol provides samples with sufficient quality for the PELDOR experiment, it may be possible to reduce the background labeling through further optimization of the labeling conditions. In addition, alternative methods for OM isolation, such as density-gradient centrifugation, can be tested.

**Sample handling and PELDOR measurements**

We have seen that the MTSL signals are gradually lost even if the cells are kept on ice. Therefore, the cells should be transferred to the PELDOR tubes and frozen immediately after spin labeling.
Care should be taken to ensure that the cells are not too concentrated, as they may lyse during transfer to the EPR tubes. Although much smaller than the signals for the double cysteine mutants, WT cells also give (nonspecific) signals under the optimized labeling conditions (Figs. 4b and 5a). The more apolar the spin label, the higher the nonspecific labeling becomes\(^7\), suggesting a physical adsorption of the labels to the cells. Therefore, it is strongly recommended to always measure the WT sample to rule out the contribution from background labeling. Owing to the stochastic nature of the nonspecific labeling, the WT sample does not yield any particular distance and contributes only to the intermolecular component \((B(t))\) of the PELDOR signal. The PELDOR experiments described in this protocol are performed following standard procedures\(^{11,71}\) (Steps 9–20). Addition of 20% (vol/vol) glycerol-\(d_8\) prolongs \(T_M\) in both \(E.\) coli and OM\(^{77}\). This enables the observation of the dipolar evolution for up to 4 \(\mu\)s within ~12 h. By combining advanced sequences such as the 5-pulse DEER or the 7-pulse CP-PELDOR with complete deuteration (of proteins and lipids by growing in deuterated medium), it might be possible to further reduce the measurement time and prolong the observation window of the dipolar evolution.

### Materials

#### Biological materials
- \(E.\) coli K-12/RK5016 (ref. \(^{68}\)) (MC4100, metE70, argH, \(btuB\), and \(recA\)) or BL21(DE3) (ThermoFisher Scientific) cells. Another K12 strain (KDF541) has been used for FepA\(^{46}\). \(E.\) coli RK5016 cells are available upon request from the corresponding author.

#### Reagents
- Expression vectors for the protein of interest (POI); in the example described in this protocol, we use a pUC8-based plasmid (pAG1) carrying \(btuB\) under the control of the lac promoter for constitutive expression. Inducible expression from the T7 promoter under the control of the lac operator (pHK763) also worked for FhuA\(^{63}\). These plasmids are available upon request from the corresponding author.
- QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, cat. no. 210519)
- Plasmid purification kit (Qiagen, cat. no. 27104)
- \(K_2\)HPO\(_4\) (Sigma-Aldrich, cat. no. P8281)
- \(KH_2\)PO\(_4\) (Sigma-Aldrich, cat. no. P5655)
- \((NH_4)_2\)SO\(_4\) (Sigma-Aldrich, cat. no. A4418)
- Sodium citrate (Sigma-Aldrich, cat. no. W302600)
- \(l\)-Methionine (Sigma-Aldrich, cat. no. M9625)
- \(l\)-Arginine (Sigma-Aldrich, cat. no. A5006)
- \(d\)-\((+)-Glucose (Sigma-Aldrich, cat. no. G5767)
- MgSO\(_4\) (Sigma-Aldrich, cat. no. M2643)
- CaCl\(_2\) (Sigma-Aldrich, cat. no. C5670)
- Thiamine (Sigma-Aldrich, cat. no. T4625)
- Ampicillin (100 mg/mL; Sigma-Aldrich, cat. no. A5354)
- MOPS (4-morpholinepropanesulfonic acid sodium salt; Sigma-Aldrich, cat. no. M9024)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- PMSF (phenylmethanesulfonyl fluoride; Sigma-Aldrich, cat. no. 78830) CAUTION: PMSF has acute toxicity and can cause damage to skin or eyes. Wear appropriate eye and skin protection.
- N-Laurylelsarcosine (or sarcosyl; Sigma-Aldrich, cat. no. 61743) CAUTION: Sarkosyl is very toxic and can cause serious damage to eyes and skin. Wear appropriate eye and skin protection.
- MTS\(_L\) (1-Oxyl-2,2,5,5-tetramethyl-\(\Delta_3\)-pyrroline-3-methyl; methanethiosulfonate spin label; TRC, cat. no. O87500)
- TEMPO (Sigma-Aldrich, cat. no. 214000)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650) CAUTION: DMSO is an irritant to eyes and skin. Wear appropriate eye and skin protection.
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S2770) CAUTION: NaOH is corrosive to the skin and can cause serious eye damage. Wear appropriate eye and skin protection.
- Glycerol-\(d_8\) (Sigma-Aldrich, cat. no. 447498)
- CDT (1,1’-carbonyl-di-(1,2,4-triazole); Sigma-Aldrich, cat. no. 21861)
- Acetone (Sigma-Aldrich, cat. no. 650501)
- Diethyl ether (Sigma-Aldrich, cat. no. 296082)
- 2,5-Dihydroxybenzoic acid (Sigma-Aldrich, cat. no. 149357)
- 6-aza-thiothymine (Alfa Aesar, cat. no. A1416706)
- Formic acid (0.1% (vol/vol); Merck, cat. no. 159013)
- Acetonitrile (Sigma-Aldrich, cat. no. 271004)
- Isopropanol (Sigma-Aldrich, cat. no. 650447)
- LB medium (MP Biomedicals, cat. no. 3002-011)
- 4-amino-TEMPO (Sigma-Aldrich, cat. no. 163945)
- Hydroxocobalamin (HOCbl; Sigma-Aldrich, cat. no. H1428000)
- Liquefied N2 (Linde) **CAUTION** Contact of liquid N2 with skin can cause frostbite.
- Liquefied He (AirLiquide) **CAUTION** Inhalation of helium leads to oxygen-deficient symptoms.

**Equipment**
- Microcentrifuge (up to 20,000g; Heraeus; Thermo Scientific, cat. no. 75002410)
- Falcon tubes (15 and 50 mL; Fisher Scientific, cat. nos. 12-565-269 and 12-565-271)
- Petri dishes (Fisher Scientific, cat. no. FB0875713)
- Thermal cycler (Eppendorf, cat. no. 6311000010)
- ThermoMixer (Eppendorf, cat. no. 538200015)
- Syringe filters for sterilization (polyvinylidene fluoride (PVDF), 0.22 μM; Millipore, cat. no. SLGV033RS)
- Centrifuges for pelleting cells (~5,000g; Sorvall, cat. no. 70900171)
- Centrifuges for pelleting membranes (~200,000g; Beckman Coulter, cat. no. A94471 and MTX 150 Micro-Ultracentrifuge; Sorvall, cat. no. 46962)
- French pressure cells and press (SLM Instruments, cat. nos. FA-073 and FA-078E1) and nylon balls (Diversified Equipment, cat. no. P/N FA-925)
- Ultrasonic water bath (Branson, cat. no. CPX-952-539R)
- Micropipettes (20 μL; BRAND, cat. no. 708718)
- Micropipette controller (BRAND, cat. no. 25800)
- Disposable syringe needles (120-mm length, 0.8-mm diameter; B Braun, cat. no. 466564/3)
- Disposable syringes (1 mL; B Braun, cat. no. 9166017V)
- Spectrometer (X-band (9.4 GHz)) equipped with a SHQE cavity (Bruker, model no. E500 CW)
- EPR spectrometer (with pulsed Q-band (34 GHz); Bruker, model no. ELEXYS E80) equipped with a PELDOR unit (Bruker, model no. E580–400U)
- Continuous-flow helium cryostat (Oxford Instruments, model no. CF935)
- Temperature control system (Oxford Instruments, model no. ITC 502)
- Accessory unit (ELEXYS SuperQ-FT, 150-W traveling wave tube (TWT) amplifier; Applied Systems Engineering)
- Resonator (cavity; Bruker, model no. EN5107D2)
- Suprasil quartz EPR tubes (1.6-mm outer diameter; Wilmad-LabGlass, cat. no. WG-222T-RB)
- Rotor (Sorvall, model no. SLA-3000)
- Rotor (Sorvall, model no. SS-34)
- Rotor (Thermo Fisher Scientific, model no. T-647.5)
- Rotor (Thermo Fisher Scientific, model no. S100-AT4)
- Column (5 μM, 2 × 250 mm, BDS C18; Agilent Technologies, cat. no. 79926BD-582)
- Workstation (Voyager STR Workstation DE PRO; Applied Biosystems)
- LCMS system (Shimadzu, model no. 2020)
- HPLC system (Agilent, 1200 series)
- Milli-Q Reference Water Purification System (Merck, cat. no. Z00QSV0WW)

**Software**
- Linux (for EPR data acquisition; [https://www.linux.org/pages/download/](https://www.linux.org/pages/download/)) and Windows or Mac environments (for data processing and analysis)
- MATLAB from MathWorks ([https://www.mathworks.com/products/matlab.html](https://www.mathworks.com/products/matlab.html))
- MATLAB-based DeerAnalysis software ([http://www.epr.ethz.ch/software.html](http://www.epr.ethz.ch/software.html)) or MtsslWizard ([https://pymolwiki.org/index.php/MtsslWizard](https://pymolwiki.org/index.php/MtsslWizard))
Reagent setup

10× Minimal medium
Dissolve 105 g of KH₂PO₄, 45 g of KH₂PO₄, 10 g of (NH₄)₂SO₄, and 5 g of sodium citrate in 1 L of Milli-Q water and autoclave. This solution can be stored at RT for several weeks. Alternatively, a rich medium such as LB (Luria–Bertani) or TB (Terrific Broth) can be used.

2% (wt/vol) Methionine and arginine
Dissolve 2 g each of methionine and arginine in 100 mL of Milli-Q water and filter-sterilize. The sterile solution can be stored at RT for several weeks when properly handled to prevent microbial contamination.

20% (wt/vol) Glucose + thiamine
Dissolve 20 g of glucose and 50 mg of thiamine in 100 mL of Milli-Q water and filter-sterilize. The sterile solution can be stored at RT for several weeks when properly handled to prevent microbial contamination.

1 M MgSO₄
Dissolve 12.04 g of MgSO₄ in 100 mL of Milli-Q water and filter-sterilize. The sterile solution can be stored at RT for several weeks when properly handled to prevent microbial contamination.

1 M CaCl₂
Dissolve 1.11 g of CaCl₂ in 10 mL of Milli-Q water and filter-sterilize. The sterile solution can be stored at RT for several weeks when properly handled to prevent microbial contamination.

1× Minimal medium
Mix 100 mL of 10× minimal medium with 5 mL each of 2% (wt/vol) arginine and methionine, 10 mL of 20% (wt/vol) glucose + thiamine, 3 mL of 1 M MgSO₄, and 300 μL of 1 M CaCl₂ and make up the final volume to 1 L. ▲ CRITICAL Prepare fresh each time; mixing should be done on the clean bench to keep the medium sterile.

Plasmids for the expression of BtuB
T188C or T188C-G399C mutations are introduced into BtuB (which is cloned into a pAG1 vector) using a QuikChange Lightning site-directed mutagenesis kit.

100 mM PMSF
Dissolve 174.2 mg of PMSF in 10 mL of isopropanol and store at −20 °C for up to several months.

Spin-labeling buffer
Dissolve 5.2 g of MOPS and 1.6 g of NaCl in Milli-Q water and adjust the pH to 7.5 in a final volume of 500 mL. Prepare the buffer fresh before the experiment.

100 mM MTSL
Dissolve 10 mg of MTSL in 377 μL of DMSO and store at −20 °C for up to a year.

TEMPO standard (calibration standard for estimating spin concentration)
Dissolve 1.57 mg of TEMPO in 10 mL of Milli-Q water for a 1 mM stock. Perform a serial dilution to prepare four to five samples in the range of 50–300 μM. The solutions can be stored at −20 °C for up to a year.
Plasmid construction and mutagenesis ● **Timing 5–7 d**

1. Clone the POI into a suitable bacterial expression vector. Please see ‘Reagents’ section for the expression vectors that worked in our hands. In the example described here, we used the pUC-8-based pAG1 plasmid, which constitutively expresses \textit{btuB} (see ‘Reagents’ section). The plasmid is available upon request from the corresponding author.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** The POI can be expressed in either a constitutive or induced manner and should be tested in a case-by-case manner (see ‘Experimental design’ section).

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Pause Point}}}}** Once the plasmid has been constructed, it can be stored in $-20 \, ^\circ \mathrm{C}$ until proceeding with the next step.

2. Introduce the cysteines at the positions that will be labeled with MTSL, using SDM and following the manufacturer’s instructions.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** If the POI has any native cysteines, they should first be mutated to generate a Cys-less protein. If the cysteines are buried, with low accessibility for MTSL, they need not be mutated. Native cysteines can be mutated to alanine, valine, serine, or to a combination of those amino acids. With regard to hydrophobicity, alanine or valine is a better match for cysteine; however, difference in the overall size may lead to structural or functional distortions in some cases. Serine is a good match in terms of size, but its increased polarity may have an adverse effect on the protein. Therefore, it is recommended to test the function of the Cys-less construct with purified protein when feasible.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** It may be necessary to generate several cysteine mutants for optimal levels of expression and spin labeling (see ‘Experimental design’ section). With the double-cysteine mutations for PELDOR measurements, we recommend measuring one or both of the corresponding single-cysteine mutants to rule out aggregation or to identify oligomerization, if any. When feasible, we advise testing spin-labeling efficiency and PELDOR with the purified protein as well.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Troubleshooting}}}}** The mutated plasmids can be stored at $-20 \, ^\circ \mathrm{C}$ for several years until proceeding with the next step.

Transformation ● **Timing 1.5 h**

3. Transform the plasmid into \textit{E. coli} as follows: mix 1 \textmu L of the plasmid with 50 \textmu L of \textit{E. coli} cells in a sterile 1.5-mL microcentrifuge tube. Incubate at 42 \, ^\circ \mathrm{C} for 45 s and place the tube in ice for 2 min. Add 250 \textmu L of sterile LB medium and incubate at 37 \, ^\circ \mathrm{C} for 1 h. Spread 100 \textmu L of the culture on a sterile LB agar plate containing 100 \mu g/mL ampicillin and incubate at 37 \, ^\circ \mathrm{C} overnight.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** Alternatively, the BL21(DE) strain can be used for \textbeta-D-1-thiogalactopyranoside (IPTG)-induced expression and spin labeling. Both K-12 and BL21(DE3) strains are useful for investigations in native membranes, whereas K-12 strains appear to be better suited to studies in whole cells (see ‘Experimental design’ section for a detailed discussion).

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** Perform transformation with the WT (or with the Cys-less background for proteins having native cysteines) plasmid as well to characterize the signals from background labeling.

Cell culture and protein expression ● **Timing 24 h**

4. The next day, transfer a single colony from the agar plates to 25 mL of 1× minimal medium (see ‘Reagent setup’ section) and incubate at 33 \, ^\circ \mathrm{C} for 6–8 h until the culture turns cloudy. Transfer 5 mL of culture to 1 L of sterile 1× minimal medium in a 3-L Erlenmeyer flask and incubate at 33 \, ^\circ \mathrm{C} for 12–16 h.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** The incubation time and temperature should be thoroughly optimized to avoid nonspecific protein–protein interaction or aggregation. Overgrowth may lead to cell lysis, which can result in MTSL reduction and poor spin-labeling efficiency in \textit{E. coli}. Expression can be performed in a rich medium such as LB as well.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Critical Step}}}}** Typically, 1-L culture (on both minimal and rich media) yields far more cells than required for preparing several CW and PELDOR samples. A smaller culture volume can be used if the protein expression level is not negatively affected.

   **\textbf{\textit{\textbf{\textcolor{red}{\textbf{Troubleshooting}}}}**
Preparation for in situ spin labeling of the protein in *E. coli* and in the OM ● **Timing 30 min**

5 Following protein expression, pellet the cells by centrifugation at 4 °C in 500-mL tubes at 5,000 g for 10 min using a Sorvall SLA-3000 rotor.

6 Discard the supernatant and resuspend the cell pellet from each 500-mL tube in 30 mL of precooled (4 °C) spin-labeling buffer supplemented with 1% (wt/vol) glucose and keep on ice.

▲ **CRITICAL STEP** The cells should be handled as gently as possible to avoid lysis. Precool the buffer on ice before use. Immediately proceed to the next step.

7 Determine the OD_{600} value. The OD_{600} value is usually between 70 and 100 at this point. Dilute the sample to measure within the linear range OD_{600} = 0.01–0.4.

Spin labeling of the protein in *E. coli* or the OM

8 Spin-label the protein in situ in *E. coli* or in the OM by following option A or option B, respectively.

(A) In situ spin labeling of the protein in *E. coli* ● **Timing 2–3 h**

(i) Transfer an appropriate amount of the cell suspension to a sterile 1.5-mL Eppendorf tube in duplicate and dilute with spin-labeling buffer to 1 mL and a final OD_{600} value of 10. Store the remaining suspension on ice until used in Step 15.

▲ **CRITICAL STEP** Higher OD_{600} values lead to rapid reduction of MTSL and lower signals (Fig. 5d). Prepare the samples in duplicate for CW-EPR and PELDOR experiments. Multiple mutants can be prepared; however, the samples need to be rapidly processed in the subsequent steps, which may make it difficult to handle large number of samples simultaneously.

(ii) Add 1 μL of 100 mM MTSL stock solution (100 μM final concentration) and incubate on a thermal mixer at 25 °C for 1–5 min.

▲ **CRITICAL STEP** Longer incubation leads to rapid reduction of MTSL and lower signals. Our experience shows that shorter incubation times yield higher signals (Fig. 5c). Prolonged incubation (150 μM MTSL for 1 h at RT) can be used to reduce background signals \(^{47}\); however, this will reduce the overall signals for the cysteine mutants as well.

(iii) Pellet the cells by centrifugation at 5,000 g for 5 min at 4 °C.

(iv) Discard the supernatant, suspend the cells in 1 mL of precooled (4 °C) spin-labeling buffer and pellet as described in Step 8A(iii).

▲ **CRITICAL STEP** These washing steps should almost completely remove the free MTSL; otherwise the number of washing steps can be increased as required.

(v) Discard the supernatant and remove any residual buffer with a micropipette. Suspend the cells in 20 μL of precooled (4 °C) spin-labeling buffer and keep on ice. Immediately proceed with Step 8A(vi and vii).

▲ **CRITICAL STEP** The volume of spin-labeling buffer can be adjusted according to the amount of the cell pellet. Larger volumes will dilute the spin concentration and too-small volumes will make it difficult to transfer the cells to the EPR tubes. Keeping the cells too long may lead to loss of signals.

(vi) Add 20% (vol/vol) glycerol-\(d_8\) to the sample and mix gently. Use a syringe needle to transfer 10–15 μL of the sample to a Suprasil quartz EPR tube (diameter = 1.6 mm) and immediately freeze in liquid N\(_2\).

■ **PAUSE POINT** The EPR tubes with the sample can be stored at −80 °C (for several months) until performing the PELDOR measurements.

(vii) Transfer the duplicate sample to a 20-μL micropipette. Measure the signal with a Bruker E500 CW X-band spectrometer (or a similar instrument) and estimate the spin concentration from an external calibration curve of TEMPO. Measure the cells expressing WT protein to estimate background labeling.

▲ **CRITICAL STEP** 1 mL of an OD_{600} = 10 *E. coli* suspension contains \(\sim 8 \times 10^9\) cells. Depending on the final volume of the *E. coli* suspension (30–50 μL from Step 8A(vi)), the bulk spin (MTSL) concentrations varies between 30 and 100 μM, which is in a range obtained for purified membrane proteins. The active volume of the resonator is \(\sim 5\) μL, which means the DEER/PELDOR experiments are performed with \(\sim 10^9\) cells. A part of the signal may arise from background labeling depending on the labeling conditions.

? **TROUBLESHOOTING**

(B) In situ spin labeling of the protein in the OM ● **Timing 8 h**

(i) **Isolation of the OM (Step (i–vi)):** Add 300 μL of 100 mM PMSF stock solution (1 mM final concentration) to the cell suspension from Step 6.
(ii) Lyse the cells with a French press (press two to three times until the suspension is translucent) at ~10,000 p.s.i., using 1/8-inch nylon balls.

(iii) Remove cell debris by centrifugation at 17,000 g for 20 min at 4 °C in a Sorvall SS-34 rotor.

(iv) Collect the supernatant, which contains the cell envelope (OM + IM). Solubilize the IM with 0.5% (vol/vol) sarkosyl and mix it gently at RT for 2–3 min.

▲ CRITICAL STEP Mix gently, but thoroughly. Incomplete solubilization of the IM can result in larger background signals or even produce additional distances.

(v) Dilute the volume to 60 mL with spin-labeling buffer and pellet the OM by centrifugation at 220,000 g for 1.5 h at 4 °C in a T-647.5 rotor.

(vi) Remove the supernatant and suspend the pellet in 7 mL of spin-labeling buffer in a 15-mL Falcon tube.

▲ PAUSE POINT After suspension in the spin-labeling buffer, the pellet can be stored at −20 °C for several months.

(vii) In situ spin labeling of the protein in the OM (Step 8B(vii–xii)). Homogenize the OM suspension by gentle mixing in an ultrasonic water bath.

▲ CRITICAL STEP The membranes form small clusters during ultracentrifugation in Step 8B(v) and complete homogenization is necessary for efficient spin labeling.

(viii) Add 7 μL of 100 mM MTSL (100 μM final) and incubate at RT for 1 h.

(ix) Remove the free MTSL by pelleting the OM with centrifugation at 500,000 g for 45 min at 4 °C in a S100-AT4 rotor (a lower speed can be used with prolonged centrifugation, e.g., 1.5 h at 200,000 g).

(x) Discard the supernatant and collect the OM-containing pellet. Wash the OM by suspending in 7 mL of spin-labeling buffer and homogenize by repeatedly pipetting up and down or using an ultrasonic water bath. Centrifuge as in Step 8B(ix).

▲ CRITICAL STEP These washing steps should reduce the free MTSL concentration to below the level of detection; otherwise, the washing steps can be increased as required.

(xi) Remove the supernatant and collect the OM by suspending the pellet in a final volume of 250–500 μL of spin-labeling buffer. For preparing PELDOR samples, take 10–20 μL of the OM suspension and add 20% (vol/vol) glycerol-d8.

▲ PAUSE POINT The OM suspension can be stored at −80 °C (for several months) until performing the PELDOR measurements.

(xii) Take 20 μL of the sample and measure the spin concentration as detailed in Step 8A(vii). Measure the OM from cells expressing WT protein to estimate background labeling.

▲ CRITICAL STEP When suspended in 300–500 μL of buffer, OM isolated from 1 L of culture typically yields a 100–150 μM spin concentration after MTSL labeling. As with E. coli, some of those signals arise from background labeling.

? TROUBLESHOOTING

DEER/PELDOR measurement in E. coli or OMs  ● Timing 12–24 h

▲ CRITICAL DEER/PELDOR experiments are performed according to standard protocols11,71, with some modifications. The procedure below is described for a Q-band (34 GHz) Bruker E580 spectrometer having independent microwave (mw) pulse–forming units and equipped with a 150-W TWT amplifier, a continuous-flow helium cryostat, an intelligent temperature control system, an ELEXYS SuperQ-FT accessory unit, and an EN5107D2 resonator. Some modifications may be required, depending on the spectrometer configuration (e.g., for a spectrometer having an inbuilt arbitrary wave form generator (AWG)).

9 Cool the cryostat with the resonator to 50 K using liquid helium and wait until the temperature is stable. The cryostat and the transfer line must be evacuated to enable efficient cooling.

10 Turn on the spectrometer and the TWT amplifier. Press the tuning button in the ‘Microwave bridge tuning’ panel, switch to ‘Tune’ mode, and change the mw power from 60 to 20 dB, turn the reference arm off, and insert the frozen sample into the resonator. A frequency shift will be visible in the tune window. Change the frequency to find the resonance dip and adjust the position of the sample holder to overcouple the resonator. Readjust the frequency to the center of the resonator frequency.

? TROUBLESHOOTING

11 Turn the CW mw power off (attenuation of 60 dB), switch the spectrometer to ‘Operate’ mode and turn the ‘Reference arm’ on.
12 In the ‘New Experiment’ tab, create a new experiment by selecting the ‘Pulse’ tab with the option ‘Advanced’ and click on the ‘Activate’ button (indicated by the cursor as ‘parameter to hardware’). In the ‘FT Bridge’ panel, change the ‘Bridge Configuration’ from ‘CW’ to ‘Pulse’ mode.

13 Safety test (also see the Bruker E580 user manual): make sure that the TWT amplifier is in ‘standby’ mode and run a 2-pulse Hahn echo sequence $\pi/2-\tau-\pi$, in one of the pulse channels (e.g., in the $+x$ channel with a 16-ns ($\pi/2$)–500(\tau)–32 ns ($\pi$) sequence). Under ‘Patterns’ in the ‘FT EPR Parameters’ panel, create an acquisition trigger by entering an acquisition start point and an integration window and ensure that the protection switches (defense pulses) are visible in the ‘SpecJet’ window.

**Troubleshooting**

14 Switch the TWT amplifier to ‘operate’ mode and slowly decrease the attenuation to 0 dB (in ‘Receiver Unit’ under the ‘FT Bridge’ panel) to increase the mw power. Change the detection video bandwidth to 20 MHz. There should be no cavity ringing visible after the protection switches in the ‘SpecJet’ window.

15 Hahn echo optimization: optimize the echo intensity observed in the SpecJet window by changing the magnetic field and adjusting the power and phase of the used pulse channel, video gain (from the ‘FT Bridge’ panel), and short repetition time (~2–3 ms for nitroxides, from the ‘FT EPR Parameters’ panel). Acquire a field-swept spectrum (sweep width ~150 G) and move the magnetic field to the maximum of this spectrum.

16 To get a first impression of the maximum possible time window for the 4-pulse PELDOR measurement, it is helpful to measure the phase memory time ($T_M$) by increasing the delay $\tau$ in the 2-pulse Hahn echo sequence and record a time-dependent decay curve. The acquisition trigger position displacement should be twice as large as the time increment for the $\pi$ pulse. Fit the curve with a mono- or stretched exponential decay to get the value for $T_M$. As a rule of thumb, the maximum feasible time window for the PELDOR experiment ($t_{max}$) is $\sim 2 \times T_M$.

17 **Pump (ELDOR) pulse optimization.** Insert a 12-ns-long pulse in the ELDOR channel 500 ns before the 2-pulse Hahn echo sequence optimized in Step 15 (therefore, shift the pulses of the Hahn echo and the acquisition trigger by 500 ns). Enter the spectrometer frequency as the ‘Current ELDOR frequency’ (in ‘Microwave’ in the ‘FT EPR Parameters’ panel). Increase the ELDOR mw power until the maximum inversion of the Hahn echo. Ideally, a mutation experiment should be performed by gradually increasing the ELDOR pulse length at a fixed mw power (of the ELDOR channel). Note the optimal ELDOR mw attenuation (for a 12-ns $\pi$ pulse).

**Critical step** With the TWT amplifier, shorter ELDOR pulses (<12 ns) can be used. However, with the used resonator and rectangular pulses, this can lead to a stronger overlap of the excitation bandwidths between the pump and observer pulses, resulting in decreased S/N ratio and artifacts. A large-bandwidth resonator\(^1\) or Gaussian pulses\(^2\) have been shown to eliminate the above problems.

18 **Set the observer pulses.** Decrease the mw power to 60 dB and change the frequency by ~70 MHz in the ‘Microwave Bridge Tuning’ panel. Carefully optimize the observer pulses to obtain a 16-ns$\pi/2$ pulse in the $+\xi\zeta$ and $-\xi\zeta$ channels with a phase difference of 180° and a 32-ns$\pi$ pulse in the $+\langle\xi\rangle$ channel as described in Step 5.

**Critical step** Longer pulses will yield smaller echo amplitude and shorter pulses can lead to an overlap of the excitation bandwidths between the pump and observer pulses. A larger frequency offset can be used when stronger pump or observer pulses are used; however, this will considerably reduce the echo amplitude and the S/N ratio. Note that for spectrometers generating the pulses with an AWG, additional phase-cycling steps are required to eliminate the echo crossing artifacts arising from coherence transfer pathways\(^3\).

19 **Setup of the 4-pulse PELDOR.** Press the ‘PulseSpel’ button in the ‘Acquisition’ tab in the ‘FT EPR Parameters’ panel and load the PulseSpel program with the corresponding variable definitions (available from Bruker). Load the observer pulse sequence $\pi/2-\tau_1-\pi-\tau_2-\pi$. Set the $\tau_1$ value between 100 and 200 ns, depending on the spectrometer configuration. To optimize the acquisition trigger and the echo integration window, set the delay $\tau_2$ initially to 1 $\mu$s. On the SpecJet, phase the echo and change the acquisition trigger to integrate symmetrically (e.g., for the length of the observer $\pi$ pulse) over the maximum of the echo.

20 Choose the delay $\tau_2$ according to the phase memory time of the sample. It has to be long enough to observe at least one full oscillation of the PELDOR signal (which depends on the expected distance) but short enough to get a reasonable S/N ratio within 12–24 h. Adjust the variables to increase $\tau_1$ by...
16 ns for eight steps to average out the deuterium modulation. In addition, move the pump pulse (by 8 or 16 ns) between the two observer \( \pi \) pulses with sufficient delays to avoid temporal overlap. The signals from the phase cycling of the first \( \pi/2 \) pulses are subtracted to eliminate the receiver offset. Accumulate as many scans as needed to get a reasonable S/N ratio.

**TROUBLESHOOTING**

**DEER/PELDOR data analysis**  
Timings: 10-20 min

Perform data analysis using the MATLAB-based DeerAnalysis software\(^{26}\), which can be downloaded from http://www.epr.ethz.ch/software.html (see the manual for details of data analysis and usage).

*CRITICAL STEP*  
Analysis of both WT (or the Cys-less background, for proteins having native cysteines) and the mutant is critical for reliable results. The signal from WT or single-cysteine mutants contributes to an exponential decay devoid of any particular distances (Figs. 2 and 4). The entire dipolar evolution for the WT or the single-cysteine mutant fits into a stretched exponential decay (with \( d \approx 2.0 \), which is in agreement with a 2D spatial distribution of the spins over the large membrane surface). The data for the doubly labeled protein provide the dipolar spectrum and the corresponding distance distribution. Fitting the data with \( d = 3.0 \) leads to the appearance of a small population of longer distances accompanied by distortions of the dipolar spectrum (Pake pattern, see Figs. 2 and 4). We strongly recommend checking for the shape of the Pake pattern after fitting, to ensure quality of the background \( B(t) \) correction. When data quality is good (with a sufficient S/N ratio and time window), background can automatically be determined with simultaneous fitting for \( d \) and the time window. The dipolar evolution should be acquired for a sufficiently long time window, as described earlier, in the ‘Theoretical rationale behind DEER/PELDOR spectroscopy’ section.

**TROUBLESHOOTING**

**Troubleshooting**

Troubleshooting advice can be found in Table 2

**Table 2 | Troubleshooting table**

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 2    | The cysteine mutant is expressed at very low levels | The mutated residue(s) is important for protein expression/stability | Choose another position for SDM. Test different vectors, strains, or modes of expression |
| 4    | No or poor cell growth after overnight culture | Only a few cells were seeded | Ensure that the pre-culture has sufficient cell density (\( \text{OD}_{600} \approx 0.3-0.5 \)) |
| 8A(vii) and 8B(xii) | Weak signals for the cysteine mutants after spin labeling | Labeling and sample handling under non-optimal conditions, resulting in cell lysis | For *E. coli*, process cells quickly after labeling |
|      | Large amounts of free MTSL (as evident from the narrow lines in the RT CW EPR spectrum) | Unsuccessful SDM | Increase the number of washing steps after labeling in Step 8A(iv) or 8B(x) |
|      | No difference in signal intensity between WT and the mutant (under identical conditions) | Limited accessibility of the target sites Insufficient washing of the cells or the OM | Optimize the labeling conditions or choose other positions for SDM |
|      | High background labeling Poor labeling of the target cysteines For OM preparations, incomplete solubilization of the IM | | Use a fresh stock of sarkosyl |

Table continued
Timing

Steps 1 and 2, plasmid construction and SDM: 5–7 d (additional time may be required to introduce the optimal mutations)
Step 3, transformation (for up to three samples): 1.5 h
Step 4, preculture preparation and protein expression overnight: 24 h
Steps 5–7, preparing the cells for in situ spin labeling: 30 min
Step 8A, in situ MTSL labeling of E. coli: 2–3 h
Step 8B(i–vi), isolation of native OM: 3 h
Step 8B(vii–xii), spin labeling of native OM: 5 h
Steps 9–20, PELDOR experiment: 12–24 h (for a 4-μs-long time trace)
Step 21, PELDOR data analysis: 10–20 min
Box 1, TEMPO-HOCl synthesis, purification, and characterization: 30 h

Anticipated results

With the conditions for protein expression and spin labeling described here, a 4-μs-long PELDOR trace can be obtained for double-cysteine mutants within 12–24 h of measurement in E. coli or in even less time with OM. When the background labeling is low, it will be difficult to observe a long time trace for the WT sample. Typical modulation depth (Δ) values for the double-cysteine mutants are ~8% (the maximum Δ under our experimental setup is ~25–30%). Although the background labeling in E. coli and OM does not give any particular distances, in effect it reduces the modulation depth. Such Δ values despite the background labeling suggest a good labeling efficiency, at least 60–70% or even more. As shown in Fig. 2, the interspin distances derived from the PELDOR experiments can provide structural information for ligand binding. In the absence of the ligand, the
PELDOR data fit into an exponential decay, and ligand binding leads to visible oscillations. The high data quality allows reliable determination of the Pake pattern and the interspin distance distribution. When compared with simulations on the corresponding crystal structure, such measurements can reveal the similarity or the difference for ligand binding between in vitro and in situ conditions. Also, the modulation depth ($\Delta$) can provide quantitative information for the amount of ligand binding. By performing such experiments using orthogonal labels (e.g., MTSL combined with trityl), greater sensitivity and selectivity can be obtained. A similar experiment performed with a doubly labeled protein enabled the observation of the extracellular loop conformation in BtuB (Fig. 4). A comparison between the experimental distance distribution and the simulation performed on the corresponding crystal structure revealed a rather good agreement. Thus, it is a powerful approach for observing or validating OMP conformation in native environments. Moreover, we observed that the second extracellular loop undergoes large conformational changes in the presence of the substrate (cyanocobalamin) or the ligand ($\text{Ca}^{2+}$). Thus, our protocol can be used to elucidate changes in conformation or conformational equilibrium of OMPs during function in their native environments.

**Reporting Summary**
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
B.J. conceived the idea and initiated the project on in situ DEER/FELDOR of OMPs in *E. coli* and isolated OMs and further developed it in collaboration with D.S.C. in the laboratory of T.F.P. A.S. participated in mutagenesis and spin labeling at the beginning of the project. B.J. and K.B. synthesized TEMPO-HOCbl. B.J. performed all the FELDOR experiments discussed in the main text. B.J and E.A.J. performed further optimizations for MTSL labeling and wrote the manuscript with input from other co-authors.

Competing interests
The authors declare no competing interests.
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