nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-020-00439-4

Check for updates

Characterizing proteins in a native bacterial environment using solid-state NMR spectroscopy

Siddarth Narasimhan[1,2], Cecilia Pinto[1,3], Alessandra Lucini Paioni[1], Johan van der Zwan[1], Gert E. Folkers[1] and Marc Baldus[1]✉

For a long time, solid-state nuclear magnetic resonance (ssNMR) has been employed to study complex biomolecular systems at the detailed chemical, structural, or dynamic level. Recent progress in high-resolution and high-sensitivity ssNMR, in combination with innovative sample preparation and labeling schemes, offers novel opportunities to study proteins in their native setting irrespective of the molecular tumbling rate. This protocol describes biochemical preparation schemes to obtain cellular samples of both soluble as well as insoluble or membrane-associated proteins in bacteria. To this end, the protocol is suitable for studying a protein of interest in both whole cells and in cell envelope or isolated membrane preparations. In the first stage of the procedure, an appropriate strain of *Escherichia coli* (DE3) is transformed with a plasmid of interest harboring the protein of interest under the control of an inducible T7 promoter. Next, the cells are adapted to grow in minimal (M9) medium. Before the growth enters stationary phase, protein expression is induced, and shortly thereafter, the native *E. coli* RNA polymerase is inhibited using rifampicin for targeted labeling of the protein of interest. The cells are harvested after expression and prepared for ssNMR rotor filling. In addition to conventional $^{13}C/^{15}N$-detected ssNMR, we also outline how these preparations can be readily subjected to multidimensional ssNMR experiments using dynamic nuclear polarization (DNP) or proton (^{1}H) detection schemes. We estimate that the entire preparative procedure until NMR experiments can be started takes 3–5 days.

Introduction

Solid-state nuclear magnetic resonance (ssNMR) spectroscopy offers atomic-level insight into the structure and dynamics of complex biomolecules. Such studies have, for example, vitally contributed to our view of the structural and dynamic nature of membrane bilayers[1,2], as well as provided novel insight into the functional profile of membrane proteins[3–5] including membrane-embedded protein pumps[6], channels[7–9], and receptors[10,11]. In addition, ssNMR has offered valuable insights into the structural and dynamical properties of protein assemblies[12] and amyloid proteins[13], and general preparation protocols for in vitro ssNMR studies for such systems are in place[14,15].

Especially in recent years, advancements in high-sensitivity ssNMR techniques, such as dynamic nuclear polarization (DNP[16]) and ^{1}H-detected ssNMR[15,17,18], have further expanded the potential of ssNMR for in situ studies. Such studies are motivated by growing evidence that a full description of cellular processes requires structural biology approaches in native cellular environments, where ssNMR is jointly applied together with other modalities including cryogenic electron microscopy/tomography and light microscopy (see, e.g., refs. [19–22]).

To date, natural-abundance ssNMR has been sufficient to obtain valuable insights into the molecular and structural properties of bacterial samples[23]. Uniform isotope labeling in combination with dedicated spectral filtering and separation via multidimensional ssNMR allows the examination of cellular components such as DNA or peptidoglycans[24,25]. In addition, bacterial cell lysates can be elegantly studied by both solution (see, e.g., refs. [26,27]) and solid-state NMR[28]. However, more advanced sample preparation methods are often needed to conduct in-depth studies inside bacterial cells. To achieve this, molecules of interest must be selectively labeled with NMR-active isotopes while ensuring minimal background labeling of other cellular components. NMR studies in situ are further

[1]NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, the Netherlands. [2]Present address: Structural and Computational Biology Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. [3]Present address: Department of Bionanoscience, Kavli Institute of Nanoscience, Delft University of Technology, Delft, the Netherlands. ✉e-mail: m.baldus@uu.nl

complicated by the decrease in the desired signal, simply due to studying the molecule in a complex cellular environment. Over the last two decades, elegant approaches have been developed to employ solution-state NMR to study proteins directly inside cells[29–32]. However, such in-cell NMR experiments are limited to molecules that undergo fast tumbling, which complicates the study of insoluble and membrane proteins, as well as proteins engaged in complexes in cells. In addition, macromolecular crowding causes the protein of interest to engage in transient and nonspecific interactions. This leads to a higher 'apparent' viscosity of intracellular environments, which hinders solution-state NMR studies[33]. Previous work has shown that molecular crowding is significantly more pronounced in bacterial cells such as *E. coli* cells, when compared with human cells. As a result, even the study of moderately sized globular bacterial proteins using in-cell solution-state NMR can be precluded[34].

In this protocol, we describe procedures that can be used to produce bacterial (*E. coli*) samples for the study of both soluble (as well as potentially insoluble) and membrane proteins. The successful implementation of the protocol results in molecule-specific isotope labeling with a minimal background contribution. The traditional labeling strategy used in bacterial in-cell solution-state NMR involves overexpression of the protein of interest to the extent that NMR signatures of the background signals (especially those stemming from components such as lipids or labeled amino acids) can be neglected in the spectra[29,35]. However, such an approach cannot be directly applied to ssNMR, as the signals from the entire cell can contribute to the ssNMR spectrum, particularly when recorded under DNP conditions where molecular motion is absent. Our approach combines several complementary strategies to address these challenges. In the case of membrane proteins, we describe how to isolate the cell envelope or the specific membrane of interest. Such ssNMR preparations that preserve the native membrane setting have already been used to examine β-barrel proteins[25,36], ion and proton channels[37,38], and chaperones[20,36,39]. In addition, retinal proteins[40,41], electron transport proteins[42], as well as globular proteins[43,44], peptides[45,46], and entire protein complexes[47] have been studied using this strategy.

In addition to the existing methods, we employ an approach to perform targeted isotope labeling by suppressing native *E. coli* RNA-polymerase-dependent protein expression[48,49]. This method has been previously applied to cellular ssNMR of membrane proteins[20,36,50]. In the current context, we show how to adapt this approach to make it widely applicable to soluble proteins[51]. This approach has already been applied to study an artificial metalloenzyme that assembles in the bacterial cytoplasm[52] and may in the future enable noninvasive tracking of physiological processes including molecular synthesis[53] and enzymatic conversion[54] as well as to probe the bacterial response to environmental changes such as osmotic stress or toxicitiy[55]. In addition, such in situ studies are of general interest in the field of antibiotics and functional amyloids[56,57] as well as for the study of the bacterial cytoskeleton[58].

Development of the protocol

The approach described here relies on the use of the antibiotic rifampicin to minimize/eliminate the isotope labeling of the cellular background while allowing labeling of the protein of interest. This strategy was initially applied to in-cell solution-state NMR[35] and more recently in studies of membrane proteins in their native lipid environment using magic-angle spinning ssNMR (MAS-ssNMR)[20,36,50]. Here we describe the latter approach in detail and expand this method to suit the detection of diverse classes of proteins, including cytoplasmic and periplasmic proteins, whether soluble or insoluble, in whole bacterial cells. This expansion is particularly useful to study slowly tumbling proteins that are elusive in in-cell solution-state NMR studies.

Studies on cytoplasmic or periplasmic proteins in particular require us to use whole cells. Using whole cells results in 'dilution' of the protein of interest due to the presence of other cellular components, thereby increasing the need to employ sensitivity-enhanced ssNMR methods such as DNP[21,59] and ^{1}H-detected ssNMR[15,17,18]. In this case, we do not apply ^{1}H-detected ssNMR as used in membrane preparations to boost the sensitivity and resolution, since this would demand operation at high MAS rates (>40 kHz), a condition that can potentially promote cell lysis due to the high *g*-forces generated within the rotor. Instead, we employ low-temperature (~100 K) DNP to increase the low sensitivity and ensure that the cells are unaffected by the *g*-forces exerted by the MAS, an approach applied recently to mammalian cells[21,22,59]. This also allows measurements over longer periods at very low protein concentrations but without compromising the sample integrity during spectral acquisition. As we showed recently[22], ^{1}H-detected ssNMR may in the future become suitable to study

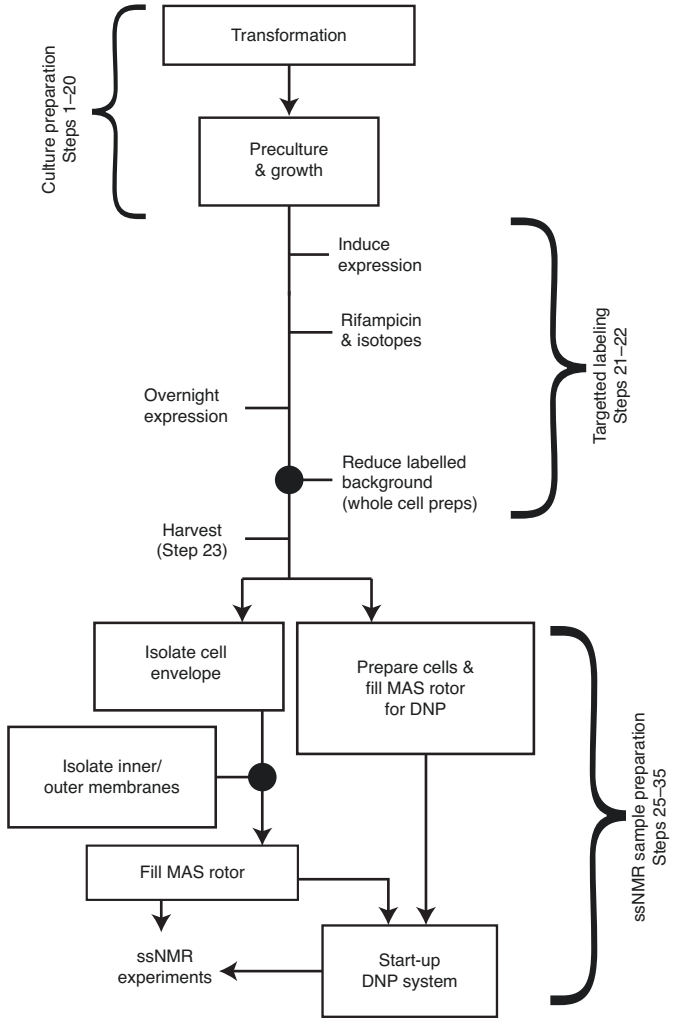

Fig. 1 | Overview of the protocol divided into three stages. Boxes indicate the different subsections of the procedure. Circular nodes indicate optional steps/procedures. Note that Steps 1–23 need to be performed in BSL-1 containment only.

three-dimensional (3D) cell cultures that are more robust to sample degradation as a result of temperature increase or cylindrical forces under MAS.

Analogous to recent DNP-ssNMR approaches to study proteins inside mammalian cells[21,59], three major challenges had to be overcome to carry out this work in bacteria. Firstly, and unlike in mammalian cells, proteins cannot be delivered into bacterial cells by electroporation. As a result, targeted labeling of the protein of interest must be achieved within the cells during overexpression. Secondly, sufficiently large DNP enhancements must be obtained to facilitate the acquisition of two-dimensional (2D) and 3D ssNMR experiments. Finally, it is vital to ensure that unwanted NMR signals stemming from the isotope-labeled cellular background, in particular those which originate from the nonprotein components, are minimized by using dedicated labeling strategies. Unlike with solution-state NMR, signals from the entire cellular background, in particular from labeled lipids, are spectroscopically enhanced in DNP-ssNMR, possibly due to their proximity to the radicals. Therefore, we prescribe measures to reduce the background labeling from these components.

Overview of the procedure

The procedure consists of three major stages, as summarized in Fig. 1. The first stage involves bacterial culture preparation, beginning with the introduction of the T7 regulated gene of interest into a bacterial strain engineered for expression (Steps 1–11 and Fig. 2, top panel). Bacteria are then

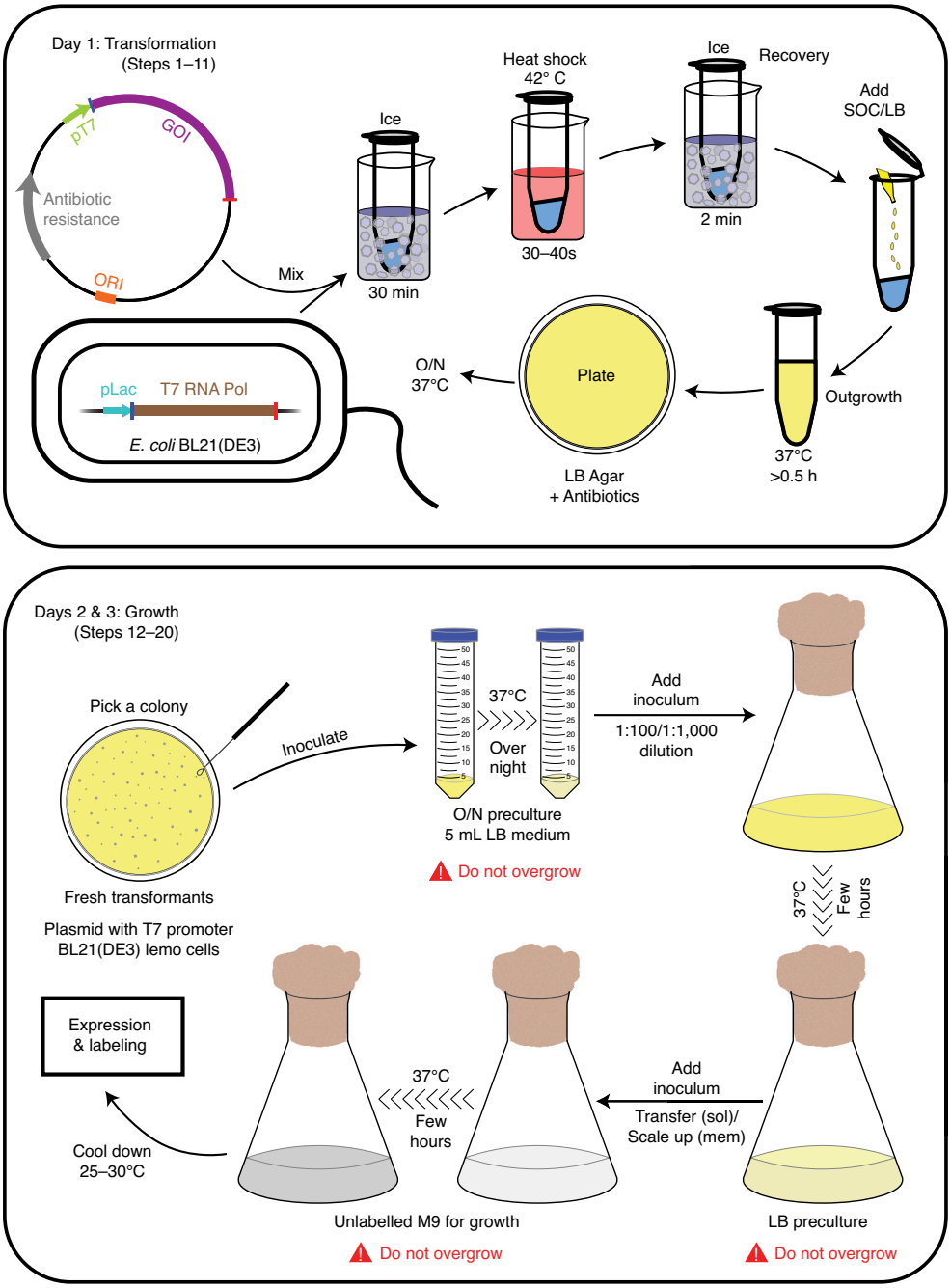

Fig. 2 | Overview of bacterial transformation and growth steps preceding labeled protein expression.

grown, and the expression of the T7 RNA polymerase is induced (Steps 12–20 and Fig. 2, bottom panel). In the second stage (Steps 21 and 22 and Fig. 3), targeted labeling and expression of the protein of interest is carried out. Rifampicin is used to block the native polymerase and to incorporate the isotopes into the nascent protein of interest. This stage is followed by harvesting the cells (Step 23) and an optional SDS-PAGE analysis to check expression (Step 24). In the final stage, the harvested cells are prepared for ssNMR experiments (Steps 25–35 and Fig. 3). Post cell harvest (Step 23), further procedures differ based on the type of sample, that is, whole cell or cell envelope/specific membrane preparations.

For membrane proteins, the cell envelope or individual membranes can be isolated to enhance the sensitivity and reduce spectral congestion (Step 25C(i–xv)). The membrane samples can then be used in either conventional ssNMR or DNP setups (Steps 26–35). For whole-cell measurements, which can

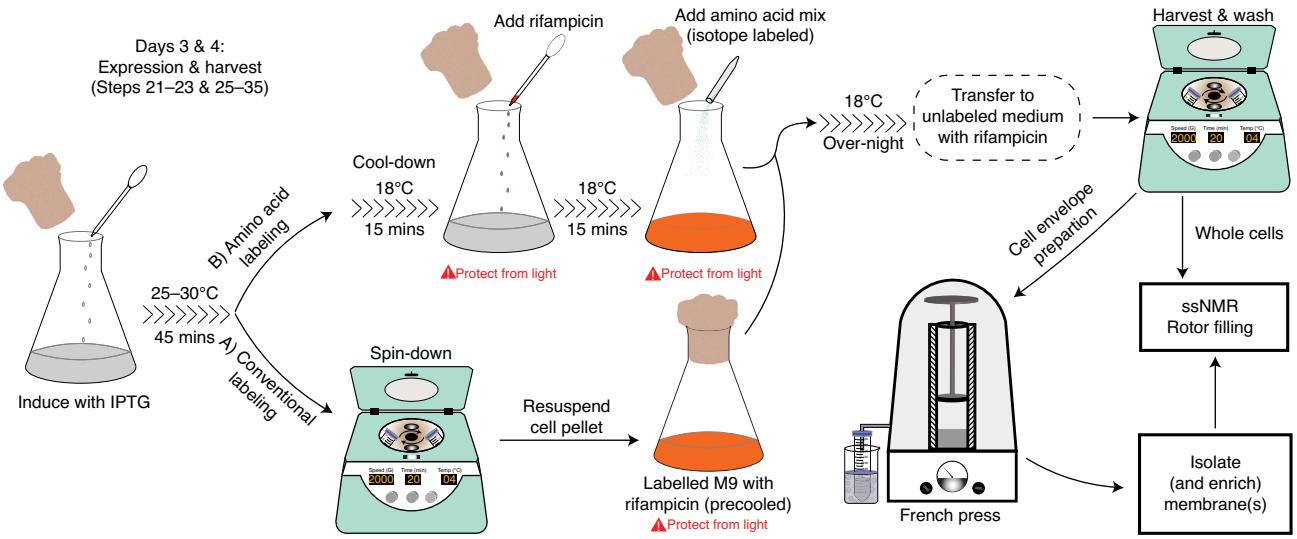

Fig. 3 | Overview of expression using either amino acid labeling or conventional labeling schemes followed by harvesting.

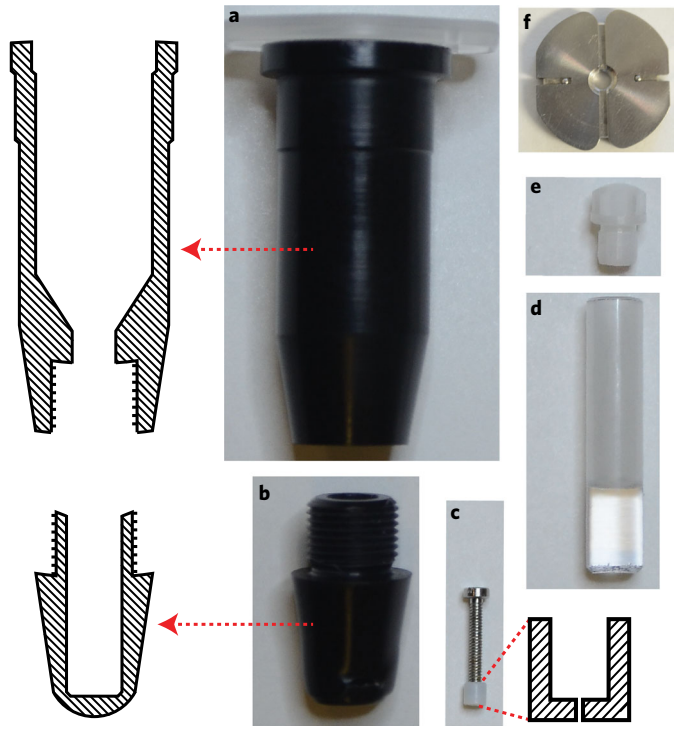

Fig. 4 | Toolkit required for filling ssNMR rotors. a–f, Top (**a**) and bottom (**b**) part of the rotor adaptor designed to mimic a 1.5-ml centrifuge tube similar to those manufactured by Beckman (ref. 357448) for use in a TLA-55 rotor, **c**, 3.2-mm rotor spacer made (in-house) using Teflon and a screw for easy handling, **d**, 3.2-mm DNP-ssNMR sapphire rotor and **e**, zirconia cap, and **f**, standard Bruker 3.2-mm cap removal tool. Panels **a**–**c** correspond to rotor filling tools made in an in-house machine shop. Note that similar designs of such tools exist, being routinely used for packing biological material into ssNMR rotors[75]. Cross-sectional views are shown.

in principle be applied to any type of protein, the cells are prepared for use with DNP, which is carried out at low temperatures. MAS rotors are filled using specialized tools (Step 25A(i–ix) and Fig. 4), (plunge-)frozen in liquid nitrogen (Step 25A(x) and Fig. 5), and carefully brought to the cold MAS probe (Step 25A(xi–xxiii) and Fig. 5). For an alternative DNP rotor filling method, see ref. [60]. Finally, the ssNMR experiments can be conducted.

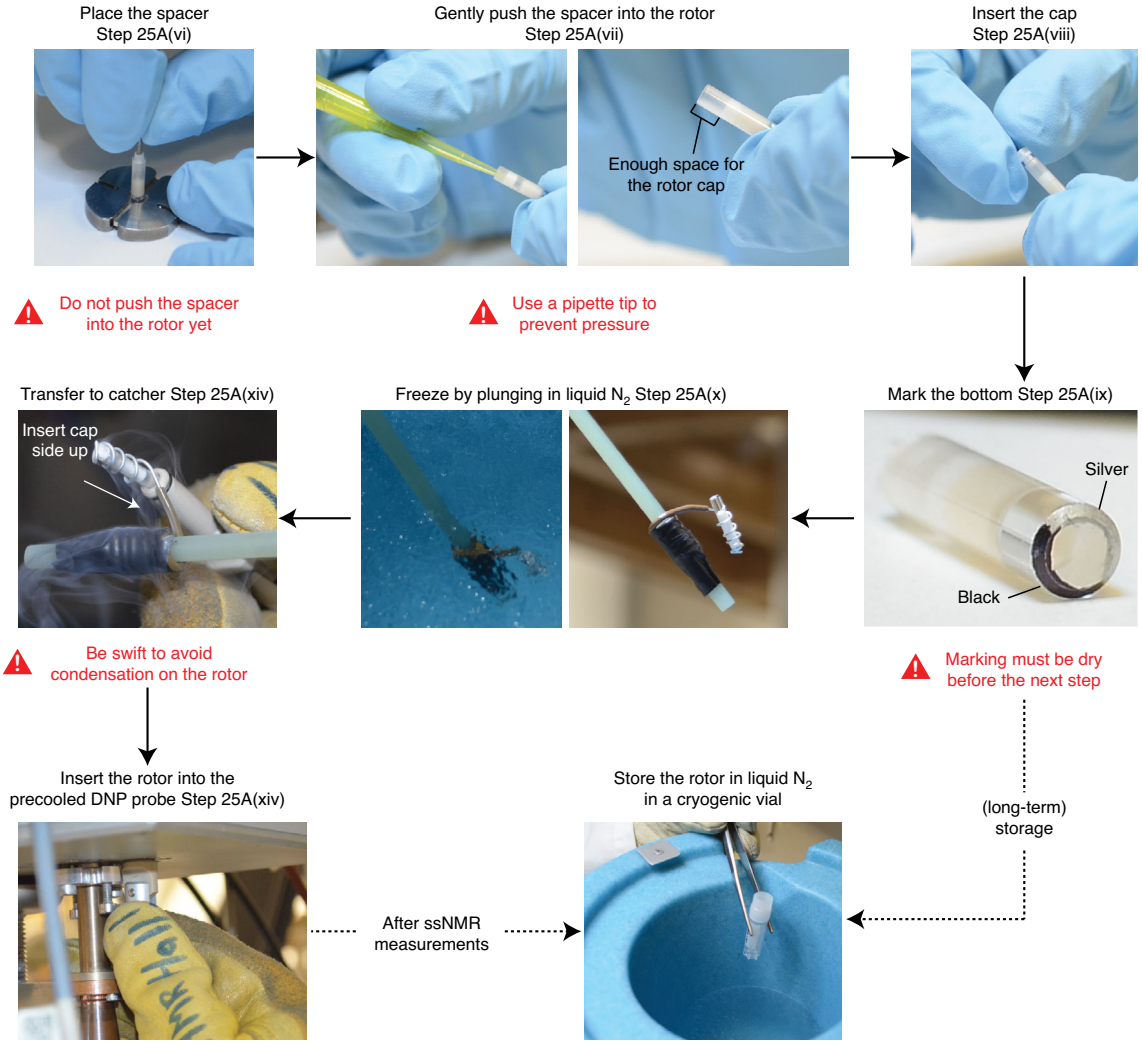

Fig. 5 | Preparing the filled rotor for DNP-ssNMR measurements.

Applications of the method

We have demonstrated the applicability of our method on a small soluble eukaryotic protein (ubiquitin, 8.5 kDa) and on two membrane proteins which are essential for the correct insertion and folding of other membrane proteins into their respective membranes: the pentameric β-barrel assembly machinery[36,61,62] (BAM, 200 kDa) located in the outer membrane and the inner membrane insertase YidC[20] (60 kDa). While ubiquitin is largely inert in bacteria, BAM and YidC are endogenous components of heterogeneous bacterial protein complexes that are involved in the assembly of membrane proteins, making them vital for survival. Note that we have also successfully tested our approach for soluble proteins on a globular metalloenzyme[52], and it may be suitable for proteins which so far have remained elusive to structural methods.

Furthermore, our approach can be fine-tuned with regards to protein expression times and levels if necessary. This extends the potential applications of our method to include studies on proteins at near-endogenous concentrations or those that suffer irreversible changes during traditional purification procedures (including disruption of membrane protein topology or increased proteolytic sensitivity). In addition, toxic proteins that can be damaging to the cells at high concentration can also be studied using this approach. Note that this is only possible due to the sensitivity enhancements offered by ^{1}H-detected and/or DNP-ssNMR. In particular, we explore here the possibility of employing 2D and 3D experiments to obtain detailed information when using DNP-ssNMR. In the future, further improvements in terms of the spectral resolution in the DNP-based experiments may be possible by recording spectra with higher dimensionality and/or conducting high-field DNP using improved DNP agents.

Limitations

Although we have seen that the approach is suitable for a variety of proteins, we cannot guarantee that it will be applicable to all proteins from all origins. For example, we have only tested the approach on Gram-negative bacteria, and in specific, strains of *E. coli* that have been engineered and optimized for high expression levels. Deviating from the strains and expression systems used in this protocol may require further optimization of the procedure. We estimated with ubiquitin that our 3.2-mm DNP rotors contained ~0.5 mg of isotope-labeled protein, with isotope incorporation levels comparable to conventional approaches for obtaining isotope-labeled purified ubiquitin (Supplementary Figs. 1 and 2).

As with more conventional ssNMR experiments, the applicability of this approach to large or very complex proteins may still be challenging, in particular if these proteins exhibit repetitive secondary structures or amino acid composition and/or represent multi-protein complexes that exhibit a greater degree of spectral crowding. Such limitations cannot simply be circumvented using the methods described in this protocol. As such, this method may prove more informative for proteins that are sufficiently small and abundant (such as Braun's lipoprotein, Lpp, ref. [25]) than for large or multimeric protein complexes. Likewise, the spectral resolution under low-temperature DNP conditions is generally lower compared with experiments carried out at ambient temperatures, in particular due to freezing out of protein motion, paramagnetic effects, and lower magnetic field strength (see, e.g., refs. [63–65]). We recently highlighted potential ways to surpass low spectral resolution[65], including the use of novel DNP agents that perform optimally at higher magnetic fields[66]. In addition, amino-acid-specific labeling to reduce spectral crowding can improve the spectral dispersion when dealing with large[47] or disordered proteins.

Expertise required

To prepare the cellular samples, familiarity with molecular biology and biochemistry techniques to the extent of expressing recombinant proteins is essential. In addition, one must possess basic microbiological skills to be able to conduct work in a sterile environment (BSL-1) with nonpathogenic strains of Gram-negative bacteria. To carry out ssNMR experiments, a basic (practical) understanding of MAS-ssNMR methods is required, in particular, the ability to set up and carry out multi-dimensional ssNMR experiments for biological applications, along with experience with very fast MAS-ssNMR ^{1}H-detected and/or DNP methods. For data analysis, we recommend a practical working knowledge of Topspin (Bruker BioSpin) and SPARKY[67] or equivalent NMR spectral data processing and visualization software. Since the execution of the whole approach requires a broad set of skills, we recommend working in a team comprising biochemists/molecular biologists and spectroscopists.

Materials

Biological materials

- BL21 competent cells with isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase expression (DE3), available from many vendors (e.g, Thermo Fischer Scientific, Promega, and New England Biolabs). pLysS or Lemo21 systems prevent leaky expression of proteins, which is essential to express certain protein types (such as membrane proteins or toxic proteins). The applicability of the targeted labeling approach described here has been tested on BL21 DE3 star (using BAM[36]) and Lemo21 strains (using YidC[20] and ubiquitin)
- Plasmid with a T7/lac repressor/promotor harboring the (preferably codon-optimized) gene of interest, e.g., pET expression vector series (Merck). The plasmids of the proteins described in this protocol are available to academic researchers for reference/control experiments from the corresponding author upon request

Reagents

- Yeast extract (granulated, Merck, cat. no. 1037530500)
- Tryptone (Merck, cat. no. 1072131000)
- (Optional) SOC medium (Thermo Fisher Scientific, cat. no. 15544034); SOC medium can be substituted by LB (see 'Reagent setup')
- Bacto agar (Sigma-Aldrich, cat. no. A5306)
- Antibiotic salt: ampicillin sodium salt (Sigma-Aldrich, cat. no. A9518), chloramphenicol (Carl Roth, art. no. 3886), or streptomycin sulfate salt ($C_{21}H_{39}N_7O_2{\cdot}1.5H_2SO_4$, Sigma-Aldrich, cat. no. S9137),

depending on the antibiotic resistance conferred by the plasmid used **! CAUTION** Harmful when inhaled or ingested. Use gloves and handle in a fume hood.

- Sodium phosphate dibasic (Na_2HPO_4, Sigma-Aldrich, cat. no. 1.06580)
- Potassium phosphate monobasic (KH_2PO_4, Honeywell, cat. no. 04243)
- Sodium chloride (NaCl, Sigma-Aldrich, cat. no. 1.06404)
- (Optional) sodium hydroxide (NaOH, Sigma-Aldrich, cat. no. 221465). This reagent is required only for cell envelope/membrane preparations (Step 25C) **! CAUTION** NaOH causes irritation to skin and eyes. Use gloves and handle in a fume hood.
- Hydrochloric acid (fuming HCl 37%, Merck, cat. no. 100317) **! CAUTION** HCl is corrosive. Fuming HCl (37%) produces highly irritating vapors. Use diluted solutions (e.g., 30%), gloves and handle in a fume hood.
- Magnesium sulfate ($MgSO_4$, Sigma-Aldrich, cat. no. M5921)
- Calcium chloride ($CaCl_2$, Interchema, cat. no. i-V6.202.49A)
- Ferrous ammonium sulfate (also known as Mohr's salt, $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, Sigma-Aldrich, cat. no. 203505) **! CAUTION** Harmful when inhaled or ingested. Use gloves and handle in a fume hood.
- Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS{\cdot}HCl$, Sigma-Aldrich, cat. no. T4625)
- Ammonium molybdate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, Fluka, cat. no. 09878)
- Boric acid (H_3BO_3, Sigma-Aldrich, cat. no. B6768)
- Cobalt chloride ($CoCl_2$, J.T. Baker, cat. no. 1670)
- Copper sulfate ($CuSO_4$, J.T. Baker, cat. no. 1843)
- Manganese chloride ($MnCl_2$, Merck, cat. no. 105927)
- Zinc chloride ($ZnCl_2$, Merck, cat. no. 108816)
- D-Biotin ($C_{10}H_{16}N_2O_3S$, Sigma-Aldrich, cat. no. B4501)
- Choline chloride ($C_5H_{14}ClNO$, Sigma-Aldrich, cat. no. C1879)
- Folic acid ($C_{19}H_{19}N_7O_6$, Sigma-Aldrich, cat. no. F7876)
- Myo-inositol ($C_6H_{12}O_6$, Sigma-Aldrich, cat. no. I5125)
- Nicotinamide ($C_6H_6N_2O$, Sigma-Aldrich, cat. no. N3376)
- Pantothenic acid ($C_9H_{17}NO_5$, Sigma-Aldrich, cat. no. 21210)
- Pyridoxal hydrochloride ($C_8H_9NO_3{\cdot}HCl$, Sigma-Aldrich, cat. no. 271748)
- Riboflavin ($C_{17}H_{20}N_4O_6$, Sigma-Aldrich, cat. no. R4500)
- IPTG ($C_9H_{18}O_5S$, Sigma-Aldrich, cat. no. I6758)
- Rifampicin (Serva, cat. no. 34514) **! CAUTION** Harmful when inhaled or ingested. Use gloves and handle in a fume hood.
- (Optional) D-glucose ($C_6H_{12}O_6$, Sigma-Aldrich, cat. no. G7021). This reagent is not required if cellular background deuteration is needed (see Steps 15–17 in Procedure).
- (Optional) deuterated D-glucose (d_7) (for cellular background deuteration). (Cambridge Isotope Labs, item no. DLM-2062-10). This reagent is required only when cellular background deuteration is needed (see Steps 15–17 in Procedure)
- Ammonium chloride (NH_4Cl, Sigma-Aldrich, cat. no. 1.01145)
- (Optional) ^{13}C-enriched (≥99%) D-glucose (Cambridge Isotope Laboratories, item no. CLM-1396-10). This reagent is required only when conventional isotope labeling schemes are used (see Step 21A in Procedure)
- (Optional) ^{15}N-enriched (≥98%) ammonium chloride (Cambridge Isotope Laboratories, item no. NLM-467-10). This reagent is required only when conventional isotope labeling schemes are used (see Step 21A in Procedure)
- (Optional) $^{13}C/^{15}N$-enriched (≥98%) algal amino acid mixture (Cortecnet, item no. CCN070P1). This reagent is required only when isotope-enriched amino acids are used for labeling (see Step 21B in Procedure)
- (Optional) deuterium oxide (D_2O ≥98%, Cortecnet, cat. no. CD5251P1000 or Eurisotop, ref. DLM-4-99-PK). This reagent is required only when cellular background deuteration is needed (see Steps 15–17 in Procedure) or if DNP is performed (Steps 25A & 26)
- Dimethyl sulfoxide (DMSO, $(CH_3)_2SO$, Sigma-Aldrich, cat. no. D8418)
- (Optional) phenylmethanesulfonylfluoride (PMSF, $C_7H_7FO_2S$, Sigma-Aldrich, cat. no. P7626). This reagent is required only for cell envelope/membrane preparations (Step 25C) **! CAUTION** PMSF is cytotoxic; use in well-ventilated area, such as a fume hood.
- Glycerol ($C_3H_8O_3$, Sigma-Aldrich, cat. no. G5516)
- Bromophenol Blue ($C_{19}H_{10}Br_4O_5S$, Sigma-Aldrich, cat. no. B0126)
- Sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$, Thermo Fisher, cat. no. 28312)

- β-Mercaptoethanol (BME, C_2H_6OS, Sigma-Aldrich, cat. no. M6250). **! CAUTION** BME is highly toxic and a skin irritant. Do not ingest or inhale. Use it only in a fume hood and wear gloves.
- Tris-HCl (Merck, cat. no. 648317)
- (Optional) ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$, Sigma-Aldrich, cat. no. 03609). This reagent is required only for cell envelope/membrane preparations (Step 25C)
- (Optional) ^{2}H- (≥98%)/^{12}C (≥99.5%)-enriched glycerol ($C_3D_8O_3$, Cortecnet, item no. CCD1060P1, or Cambridge Isotope Laboratories, item no. CDLM-8660-PK). This item is required only when DNP is used (Steps 25A and 26)
- (Optional) DNP agent: AMUPol (Cortecnet, cat. no. C010P005). Alternatively, equivalent water-soluble biradicals may also be used. This item is required only when DNP is used (Steps 25A and 26)

Equipment

- Sterile working environment to grow the bacteria (equivalent to BSL-1 containment level)
- Centrifuge microtubes (1.5 ml) (e.g., Sarstedt AG & Co., ref. no. 72.690.001)
- Centrifuge tubes (50 and 15 ml) (e.g., Grenier Bio-one cellstar tubes, cat. nos. 188 271 (15 ml) and 227 261 (50 ml))
- Table-top centrifuge to spin 1.5-ml tubes (e.g., Thermo Sorvall Legend Micro 17) operating at room temperature (20–25 °C) and at 4 °C or an equivalent device with variable temperature
- (Optional) French pressure cell press (e.g., Stansted SPCH-10). This item is required only for cell envelope/membrane preparations (Step 25C)
- (Optional) ultracentrifuge (e.g., Beckman Optima L-90K) and swinging bucket rotor (e.g., Beckman SW 32Ti). This item is required only for cell envelope/membrane preparations (Step 25C)
- (Optional) table-top ultracentrifuge (e.g., Beckman Optima MAX-XP), rotor (e.g., Beckman TLA-55), and compatible 1.5-ml tubes (e.g., Beckman ref. 357448). This item is required only for cell envelope/membrane preparations (Step 25C)
- Shaking incubator with variable temperature (e.g., New Brunswick Scientific Innova 44)
- Incubator for Petri dishes (e.g., Thermo Heratherm incubator)
- Autoclave (e.g., Varioklav steam sterilizer)
- Vortex mixer (e.g., Scientific Industries Vortex Genie 2)
- (Optional) sonicator for cell lysis (e.g., Qsonica). This item is required only for cell lysate preparations (Step 25B)
- Ice machine (e.g., Hoshizaki FM-120DE-50)
- (Optional) 5-mm NMR tubes (e.g., Wilmad cat. no. Z271993-1PAK or Shigemi cat. no. BMS-005V). This item is required only for cell lysate preparations (Step 25B)
- (Optional) 3.2-mm MAS-ssNMR zirconia rotors with caps (Bruker Biospin, cat. no. H12135). This item is required only for cell envelope/membrane protein preparations (Step 25C) or insoluble fractions of cell lysates (Step 25B)
- (Optional) 3.2-mm MAS-ssNMR sapphire rotors with zirconia caps (Bruker Biospin, cat. no. H13861). This item is required only when DNP is used (Steps 25A and 26)
- (Optional) 1.3-mm MAS-ssNMR rotors with caps (Bruker Biospin, cat. no. HZ14752). This item is required only for cell envelope/membrane protein preparations (Step 25C) or insoluble fractions of cell lysates (Step 25B)
- Centrifuge rotor adaptors for 3.2- and/or 1.3-mm rotors (designed in-house, see Fig. 4)
- (Optional) Cryogenic rotor plunger (designed in-house, see Fig. 5, Step 5). This item is required only for whole-cell DNP preparations (Step 25A).
- Cap removal tools (Bruker Biospin, for 3.2-mm rotors cat. no. HZ16913, for 1.3-mm rotors, cat. no. HZ14706)
- Sharpie permanent marker, black and silver (silver is required only for DNP-ssNMR) **▲ CRITICAL** We highly recommend using Sharpie brand markers to mark the rotor for the MAS frequency optical sensor. The optical sensor does not accurately perceive marks made using many other inks.
- (Optional) A 9.4-T wide-bore ssNMR magnet equipped with a gyrotron for DNP (Bruker Biospin) was used here. Other commercial or custom-made setups are also suitable. This item is required only when DNP is used (Steps 25A and 26)
- (Optional) A 3.2-mm triple-resonance (^{1}H, ^{13}C, ^{15}N) DNP-ssNMR probe compatible with the 9.4-T magnet (Bruker) was used here. Other commercial or custom-made setups are also suitable. This item is required only when DNP is used (Steps 25A and 26)

• (Optional) A 21.1-T solution-state NMR magnet 5-mm triple-resonance (^{1}H, ^{13}C, ^{15}N) cryogenically cooled probe (Bruker Biospin) is used here. This item is required only for lysate analysis (Step 25B)

Reagent setup

▲ **CRITICAL** Do not autoclave D_2O or any solutions made with D_2O as this would lead to the exchange of hydrogen and deuterium (H/D exchange). Autoclaving can be substituted by filter sterilization. To prevent H/D exchange during long-term storage, ensure that the stocks are well sealed (e.g., using parafilm around the lids of the containers) or prepare fresh.

LB medium and LB agar plates

For 1 l of LB medium, add 10 g tryptone, 10 g NaCl, and 5 g yeast extract to demineralized water. Dissolve completely prior to sterilization by autoclaving. Prepare LB agar by adding 15 g Bacto agar to the LB medium just before autoclaving. Add antibiotic(s) only once the solution temperature is below 45 °C, and immediately pour into 10-cm Petri dishes to prepare the LB agar plates with antibiotics (see below, 'Antibiotics for resistance selection'). When sealed using parafilm or cling film, LB agar plates can be stored for no longer than a month at 4 °C. ▲ **CRITICAL** The Bacto agar will not dissolve readily at room temperature, therefore it is essential to add this just prior to autoclaving. Also, the LB agar solution becomes solid at room temperature. Either store at 55 °C or microwave for short periods to restore the ability to pour into Petri dishes. ▲ **CRITICAL** Do not add antibiotics when the LB agar is hot, as the antibiotics are sensitive to heat.

M9 salts

Prepare 10× concentrated M9 salts by combining 60 g l^{-1} Na_2HPO_4, 30 g l^{-1} KH_2PO_4, and 5 g l^{-1} NaCl in H_2O or D_2O. Autoclave or filter sterilize, and store at room temperature for up to 6 months. Ensure that the solution is clear when this is stored for an extended amount of time.

$MgSO_4$ and $CaCl_2$ stocks

Prepare 1 M $MgSO_4$ and $CaCl_2$ stocks by dissolving the appropriate amount of $MgSO_4$ (500× concentrated) and $CaCl_2$ (10^5× concentrated) into a desired final volume of H_2O or D_2O. Autoclave or filter sterilize, and store at room temperature for up to a year.

0.01 M Mohr's salt stock

Prepare 0.01 M Mohr's salt stock by dissolving an appropriate amount of Mohr's salt (1,000× concentrated) into the desired final volume of H_2O or D_2O. Filter sterilize, and store the Mohr's salt stock solution at room temperature for 2–3 days, or at 2–8 °C for longer storage. Protect from exposure to light. ! **CAUTION** Harmful when inhaled or ingested. Use gloves and handle in a fume hood. ▲ **CRITICAL** Do not autoclave. Discard and prepare the stock anew when a brownish deposit (oxidation) occurs on the walls of the container over time.

Thiamine stock

Prepare 100× concentrated thiamine stock by dissolving 0.5 g l^{-1} thiamine-HCl in an appropriate final volume of H_2O or D_2O. Filter sterilize, and store at room temperature or at 2–8 °C for up to a year. ▲ **CRITICAL** Do not autoclave.

Micronutrients stock

Prepare 1,000× concentrated micronutrients stock with 3 μM ammonium molybdate (588.03 μg l^{-1}; first make a higher concentration stock, and dilute thereafter for ease), 0.4 mM boric acid (24.73 mg l^{-1}), 30 μM cobalt chloride (3.9 mg l^{-1}), 10 μM copper sulfate (1.6 mg l^{-1}), 80 μM manganese chloride (10.07 mg l^{-1}), and 10 μM zinc chloride (1.36 mg l^{-1}) in H_2O or D_2O. Store at room temperature, or between 2–8 °C for up to a year. Protect from exposure to light. ▲ **CRITICAL** Do not autoclave. Filter sterilize the stock before use.

Vitamins stock

Prepare 1,000× concentrated vitamins stock by dissolving 50 mg l^{-1} riboflavin, 1 g l^{-1} D-biotin, 1 g l^{-1} myo-inositol, and 0.5 g l^{-1} each of choline chloride, folic acid, nicotinamide, pantothenic acid, pyridoxal HCl, and thiamine HCl in H_2O or D_2O. Prepare small aliquots, and store at −20 °C for

several years. ▲CRITICAL Avoid repeated freeze–thaw cycles. Do not autoclave. Filter sterilize the stock before use.

Ammonium chloride

Prepare 1,000× concentrated stock of NH_4Cl by dissolving 0.5–1 g ml^{-1} in H_2O or D_2O. Use ^{15}N-enriched NH_4Cl when labeling is necessary. Filter sterilize, and store at room temperature for up to a year.

Glucose

Prepare 40× concentrated stock of D-glucose by dissolving 200 g l^{-1} in H_2O or D_2O. For deuteration of the cellular background, use ^2H-enriched D-glucose (see Procedure, Steps 15–17). Use ^{13}C-enriched D-glucose for ^{13}C labeling during protein expression (see Procedure, Step 21A). Note that a final concentration of 2 g l^{-1} of ^{13}C-D-glucose is sufficient for overnight expression. Glucose should be prepared fresh but can be stored at room temperature for up to 3 months. ▲CRITICAL Do not autoclave. Filter sterilize the stocks before use. Prepare ^2H-enriched D-glucose stocks in D_2O.

Antibiotics for resistance selection

Prepare 1,000× concentrated stocks of chloramphenicol (35 mg ml^{-1}) in ethanol and ampicillin (50 mg ml^{-1}) or streptomycin (50 mg ml^{-1}) in H_2O. Prepare small aliquots, and store at −20 °C for several years if freeze–thaw cycles are eliminated. !CAUTION Antibiotics are harmful when inhaled or ingested. Use gloves and handle in a fume hood. ▲CRITICAL Do not autoclave. Avoid repeated freeze–thaw cycles. Ensure that your bacterial strain/plasmid confers resistance to the antibiotics used.

IPTG

Prepare 1 M concentrated stock of IPTG (238 mg ml^{-1}) in H_2O. Mix thoroughly and filter sterilize. Prepare small aliquots, and store at −20 °C for several years. ▲CRITICAL Do not autoclave. Avoid repeated freeze–thaw cycles.

Rifampicin

Prepare 1,000× concentrated rifampicin stock by dissolving 100 mg ml^{-1} rifampicin in DMSO. Prepare small aliquots, and store at −20 °C for years. !CAUTION Harmful when inhaled or ingested. Use gloves and handle in a fume hood. ▲CRITICAL Do not autoclave. Avoid repeated freeze–thaw cycles. Protect from exposure to light.

M9 minimal medium

Freshly prepare M9 minimal medium by diluting stock solutions of M9 salts (10×), $MgSO_4$ (500×), $CaCl_2$ (10^5×), Mohr's salt (1,000×), thiamine (100×), micronutrients (1,000×), vitamins (1,000×), ammonium chloride (1,000×), and glucose (40×) before use (see above). Ensure that all stocks are prepared in demineralized H_2O or D_2O (for deuterated media). Inspect the stock solutions before each use (e.g., check for the presence of precipitates, contamination, etc.). Add antibiotics to the freshly prepared medium just before inoculation. ▲CRITICAL Add the components one at a time, preferably adding the $CaCl_2$ first, followed by gentle swirling and then addition of the remaining components. This diminishes the formation of calcium phosphate precipitation. Well-prepared M9 minimum medium should be clear and devoid of any turbidity or particles. Prepared medium or concentrated medium stocks should not be stored for long periods (maximum 1–2 days at 2–8 °C).

French press lysis buffer

Prepare stock solutions of 0.5 M Tris-HCl (adjust the pH to 8.0 using HCl) and 100 mM EDTA (note that EDTA does not dissolve at pH below 8, use NaOH to adjust pH). Dilute the stocks to obtain 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 25% (wt/vol) sucrose, and 1 mM PMSF. !CAUTION NaOH causes irritation to skin and eyes. PMSF is cytotoxic. HCl is harmful to skin and eyes. Use these reagents in well-ventilated area, such as in a fume hood, and wear gloves. ▲CRITICAL The half-life of PMSF in aqueous solutions is very short (up to ~2 h). Prepare the PMSF stock (100 mM) in anhydrous ethanol, DMSO, or isopropanol. Always add it just before use.

2× Laemmli buffer

Prepare stock solution of 1.5 M Tris-HCl (adjust the pH to 6.8 using HCl) and 10% (wt/vol) SDS. Dilute the stocks to obtain 50 mM Tris HCl pH 6.8, 1% (wt/vol) SDS, 25% (vol/vol) glycerol, 1% (vol/

vol) BME, and 0.05% (wt/vol) Bromophenol Blue. The buffer can be stored in aliquots at −20 °C for several years, or at room temperature for up to 6 months. **! CAUTION** BME is highly toxic and a skin irritant. HCl is harmful to skin and eyes. Do not ingest or inhale. Use these reagents in a well-ventilated area, such as a fume hood, and wear gloves.

Procedure

▲ CRITICAL All microbiological procedures must be carried out in BSL-1 environments, preferably around a flame or within a laminar flow cabinet to enable aseptic procedures.

Bacterial transformation ● Timing 1 day

▲ CRITICAL See Fig. 2 (top panel) for a quick overview of the following steps:

1 Retrieve the desired competent *E. coli* cells from the −80 °C freezer and thaw on ice (10–30 min).

2 Add plasmid(s) containing the gene(s) of interest directly to the competent cells (~1–10 ng for 20 µl of competent cells). You may gently flick the bottom of the tube to increase mixing (optional but recommended). Include a plasmid-free control.

3 Incubate on ice for 30 min.

4 Heat shock the cells at 42 °C for 30–60 s in a water bath or on a heating block.

5 Allow the cells to recover on ice for 2 min, then add SOC or LB medium equivalent to 5× the volume of competent cells.
▲ CRITICAL STEP Do not add antibiotics, to allow the expression of the antibiotic resistance gene (s) from the plasmid.

6 Grow the bacteria in a shaking incubator at 37 °C for at least 30 min when only ampicillin will be used in Step 8, and at least 1 h when other antibiotics are (also) used.

7 (Optional) To remove excess growth medium, spin down the cells for 5 min at 4,000*g* and 25 °C. Remove the supernatant, and resuspend the cell pellet in ~100 µl of the supernatant.

8 Plate ~100 µl of the outgrown transformation mixture on LB agar plates containing the appropriate antibiotic (see 'Reagent setup') using a sterile L-shaped glass rod or other equivalent means.

9 (Optional) place the plate without the lid into a sterile laminar airflow chamber for 10–15 min to dry. This step can be skipped if most of the supernatant was removed by following Step 7.

10 Incubate the plates covered with the lid overnight at 37 °C.

11 Check for colonies the following day. If a plasmid-free control was also included at Step 2, ensure that there are no colonies on it.
■ PAUSE POINT Well-sealed plates can be stored at 4 °C for up to 3 weeks, but note that we have observed a decrease in expression upon prolonged storage.
? TROUBLESHOOTING

Preculture and growth ● Timing 1–2 days

▲ CRITICAL See Fig. 2 (bottom panel) for a quick overview of the following steps:

12 Using a sterile inoculation loop or a pipette tip, pick an isolated colony from the plate.

13 Inoculate the colony in 1–5 ml of LB with the appropriate antibiotic(s).

14 Allow this preculture to grow in a shaking incubator until the end of the day at 37 °C or overnight at 25 °C and 220 r.p.m.
▲ CRITICAL STEP Larger quantity of preculture can be obtained by inoculating a colony in a larger volume of LB medium, thereby skipping the next step, but this increases the risk of very slow growth and having to delay the experiment.
▲ CRITICAL STEP Overgrowth (OD_{600} > 2.0) leads to induction of expression, leading to compromised samples.
? TROUBLESHOOTING

15 Inoculate the preculture into a larger volume (50–1,000 ml) of LB at 1:500 to 1:1,000 dilution, and grow in a shaking incubator at 37 °C and 220 r.p.m.
▲ CRITICAL STEP The volume of LB used in this step should be at least half of the final volume of the expression medium (M9). At least 50 ml of expression medium is required for whole-cell samples, and at least 1 l for cell envelope/membrane preparations. Unlabeled M9 medium may be used directly in this stage to skip Steps 16 and 17.
▲ CRITICAL STEP For cellular background deuteration, use deuterated M9 medium prepared using deuterated glucose (see 'Reagent setup'). Ensure that the starting OD_{600} in deuterated M9 is ~0.1 so that an adequate amount of the cells are deuterated. Some bacteria may however maladapt to M9,

leading to slow or no growth. This is especially true when deuterated M9 medium is used to achieve deuteration of cellular background. This can be resolved by adapting the cells in LB medium prepared with D_2O or minimal medium prior to transfer into the expression medium.

16 (Optional) when the OD_{600} is at least 0.1, spin-down the cells grown in LB for 10 min at 2,000*g* and 25 °C. This step can be skipped when M9 medium is used in Step 15.

17 (Optional) discard the supernatant, resuspend the cell pellet in 50–1,000 ml of freshly prepared unlabeled M9 medium (see 'Reagent setup') such that the final OD_{600} is 0.1–0.3, and grow in a shaking incubator at 37 °C and 220 r.p.m. This step can be skipped when M9 medium is used in Step 15.

▲ **CRITICAL STEP** For cellular background deuteration, use deuterated M9 medium prepared using deuterated glucose (see 'Reagent setup'). Ensure that the starting OD_{600} in deuterated M9 is ~0.1 so that an adequate amount of the cells are deuterated. Some bacteria may however maladapt to M9, leading to slow or no growth. This is especially true when deuterated M9 medium is used to achieve deuteration of cellular background. This can be resolved by adapting the cells in LB medium prepared with D_2O or minimal medium prior to transfer into the expression medium.

? TROUBLESHOOTING

18 Grow the cells at 37 °C until the OD_{600} approaches 0.6–1.0, and incubate in a shaking incubator for another 15 min at 25–30 °C and 220 r.p.m.

? TROUBLESHOOTING

19 Induce expression of T7 RNA polymerase by adding 0.5–1 mM IPTG.

▲ **CRITICAL STEP** We recommend collecting a small amount of the culture (~20 µl), mixing it with 20 µl of 2× Laemmli buffer, and boiling it for 5 min at 95 °C for SDS-PAGE analysis.

■ **PAUSE POINT** After boiling, the SDS-PAGE sample can be stored at −20 °C until use. To avoid artifacts, boil and homogenize the frozen samples, by gently pipetting up and down multiple times.

20 Incubate the bacterial culture in a shaking incubator at 25–30 °C and 220 r.p.m. for 30–45 min.

▲ **CRITICAL STEP** The duration and incubation temperature vary by protein. The parameters indicated are a good starting point. Note that short or prolonged incubation may lead to lower yield/lower overall amount of the labeled protein of interest, so small-scale optimization is recommended.

Expression, labeling, and harvesting ● Timing 22 h to 1 day

▲ **CRITICAL** See Fig. 3 for a quick overview of the following steps:

21 Express the protein of interest either in medium containing ^{13}C-glucose and ^{15}N-ammonium chloride (Option A) or in a ^{13}C-/^{15}N-enriched mix of (or specific) amino acids (Option B).

(A) **Expression and labeling using conventional isotope labeling schemes**

▲ **CRITICAL** This labeling scheme is intended mainly for studying membrane proteins by ^{1}H-detected ssNMR experiments. Note that using this labeling scheme may produce significant background labeling of lipids, which would dominate the ^{13}C-detected spectra, especially when combined with DNP.

(i) Freshly prepare isotope-labeled M9 medium (see 'Reagent setup') with 0.5–1 mM IPTG (optional) and 100 µg ml^{-1} rifampicin. The volume of this M9 medium should be the same as the unlabeled M9 medium used for growth and induction.

▲ **CRITICAL STEP** Precool the M9 medium with rifampicin to the expression temperature (18 °C). IPTG is optional because there will be effectively no expression of T7 RNA polymerase after the addition of rifampicin.

(ii) Spin down the cells from Step 20 for 10–20 min at 2,000*g* and 18–25 °C.

(iii) Resuspend the cell pellet in freshly prepared isotope-labeled M9 medium from Step i.

▲ **CRITICAL STEP** Gently resuspend the cell pellet with a 10-ml disposable sterile pipette, minimizing the risk of damaging the cells.

(iv) Culture the cells in a shaking incubator for 15–20 h at 18 °C and 220 r.p.m. For DNP-ssNMR studies on whole cells, reduce the expression time by 4–6 h.

▲ **CRITICAL STEP** The temperature must not exceed 20 °C when rifampicin is used. Cover the culture flask(s) with aluminum foil to protect from light. Temperatures and expression lengths vary by protein. They range from a couple of hours to a couple of days. The above conditions serve as a good starting point, but optimization is recommended.

(B) **Expression and labeling using isotope-labeled amino acids**

▲ **CRITICAL** See Fig. 3 for a quick overview of the following steps:

(i) Cool the culture from Step 20 in a shaking incubator at 18 °C and 220 r.p.m. for at least 15 min.

▲ **CRITICAL STEP** This step ensures that the medium is cool enough when rifampicin is added. As whole cells will be used at the end, failure to carry out this step may lead to increased cell death and hence compromise sample integrity.

(ii) Add rifampicin (from 1,000× DMSO stock) and incubate for another 15 min to stop the expression of T7 RNA polymerase.

(iii) Add 500 mg l^{-1} of ^{13}C-/^{15}N-algal amino acid mixture or 50 mg l^{-1} of individual amino acids to the culture and mix gently.

▲ **CRITICAL STEP** When individual amino acids are used, supplement all other amino acids in the unlabeled form at 100 mg l^{-1} concentration each to prevent scrambling. (For example, for labeling of valine amino acid, add 50 mg l^{-1} labeled valine, and all other amino acids in unlabeled form at 100 mg l^{-1}.)

(iv) Incubate in a shaking incubator at 18 °C and 220 r.p.m. for 15–18 h.

▲ **CRITICAL STEP** The temperature must not exceed 20 °C when rifampicin is used. Cover the culture flask(s) with aluminum foil to protect from light. Temperatures and expression lengths vary by protein. They range from a couple of hours to even several days. The above conditions serve as a good starting point.

22 (Optional) to reduce the background labeling in whole-cell samples for DNP, spin down the cells for 10–20 min at 2,000*g* and 18 °C. Resuspend the pellet in precooled unlabeled M9 medium (optionally deuterated) with 100 μg l^{-1} rifampicin during the last 4–6 h of expression. The volume of this M9 medium should be the same as the M9 medium used for growth, induction, and expression.

▲ **CRITICAL STEP** This step ensures that the unincorporated ^{13}C/^{15}N-amino acids are removed. Failure to do so will lead to uninterpretable/complicated ^{13}C-based spectra. This step may be redundant for proteins with a high expression level when amino acid-specific labeling is performed, because the available amino acids are likely to be fully incorporated.

23 Harvest the cells by centrifuging at 4,000*g* and 4 °C for 10–20 min. Discard the supernatant.

▲ **CRITICAL STEP** If the protein of interest is soluble, it is recommended that the culture volume (50 ml as recommended in Step 15) be divided into two. Use one half for DNP-ssNMR (Step 25A), and freeze the other to run solution-state NMR experiments on lysates (Step 25B).

▲ **CRITICAL STEP** Post harvest, keep the cells <4 °C (on ice) as much as possible.

■ **PAUSE POINT** The cell pellet (or resuspended pellet in a buffer of choice with cryoprotectant, such as glycerol) can be stored for extended time periods in a −80 °C freezer. Do not freeze the pellet if you intend to use whole-cell samples for DNP (Step 25A).

▲ **CRITICAL STEP** We recommend collecting a small amount of the culture (~20 μl) before harvesting, mixing it with 20 μl of 2× Laemmli sample buffer, and boiling it for 5 min at 95 °C for SDS-PAGE analysis.

■ **PAUSE POINT** After boiling, the SDS-PAGE sample can be stored at −20 °C until use. To avoid artifacts, boil and homogenize the frozen samples, by gently pipetting up and down multiple times.

Final preparations for ssNMR

24 (Optional) run SDS-PAGE gels using the samples collected in Steps 19 and 23 to confirm expression of your protein of interest before proceeding to the ssNMR sample preparation.

? TROUBLESHOOTING

25 For DNP-ssNMR experiments on whole cells, follow Option A. For parallel solution-state NMR analysis of lysates, follow Option B. For membrane proteins, the cell envelope and the membrane of interest can be isolated by following Option C.

(A) **DNP-ssNMR of whole cells** ● **Timing 2–3 h**

▲ **CRITICAL** Note that the temperatures for the DNP-ssNMR steps are given in units of kelvin (K). The exact operation procedure varies by equipment. Refer to the user manual of the manufacturer. It is advisable to perform the steps in this option in a team of two scientists for safety reasons. See Fig. 5 for a step-by-step demonstration.

(i) Take one of the nonfrozen pellets from Step 23 and resuspend the pellet in 50 μl of deuterated DNP juice (60% (vol/vol) deuterated d_8- and ^{12}C-enriched glycerol, and 15 mM AMUPol in D_2O, with an appropriate buffer, e.g., 1× M9 salts). A volume of 50 μl of DNP juice is required per 25-ml culture with an OD_{600} of 1.0. This amount is sufficient to fill one 3.2-mm sapphire rotor.

▲ **CRITICAL STEP** It is recommended that the pellet be washed twice before this step by resuspending it in ice-cold 1× M9 salts and centrifuging at 4,000*g* and 4 °C for 10–20 min. Resuspend carefully and ensure there are no cell clumps. The pipette tip may be cut at the bottom to widen the opening. This allows easy pipetting of the viscous DNP juice.

(ii) Inspect for visible damage or scratches on the sapphire rotor and the cap using a magnifying glass.
▲ **CRITICAL STEP** Precool the centrifugal adaptor and the rotor components to < 4 °C to prevent the cells from warming up during the filling process. The following Steps iii–x need to be done as quickly as possible to prevent the DNP agents from getting reduced.
! **CAUTION** Using a compromised rotor or cap may lead to rotor crashes inside the probe while magic-angle spinning.

(iii) Place the rotor in the centrifuge adapter (see Fig. 4) and fill it with the suspension of cells from Step i.
▲ **CRITICAL STEP** Pipette the resuspended cells against the inner wall of the rotor carefully using a 200-µl pipette tip. Allow the cell suspension to slide down to the bottom of the rotor. Ensure that air bubbles are not formed.

(iv) Spin down the rotor for 5–10 min at 4,500*g* and 4 °C. Remove the supernatant containing excess DNP juice by pipetting it out carefully without disrupting the cell pellet.
▲ **CRITICAL STEP** Use a swingout rotor to ensure that the cells pack evenly. Uneven sample packing may lead to unstable magic-angle spinning.

(v) Repeat Steps iii–iv until the rotor is full.
▲ **CRITICAL STEP** Do not fill the sample to the full volume of the rotor; ensure there is enough space to add a protective Teflon spacer in the next step and the rotor cap (see Fig. 4).

(vi) Place the rotor on a flat surface. Use the cap removal tool for support as shown in Fig. 5. Carefully position the Teflon spacer using the spacer screw on top of the rotor.
▲ **CRITICAL STEP** Do not push the spacer all the way through into the rotor. This may cause cell lysis due to high pressure exerted by the spacer and the screw.

(vii) Remove the spacer screw slowly and use a pipette tip to push the spacer into the rotor, ensuring that the opening is not blocked to allow for pressure release.

(viii) Fix the cap by pushing it gently onto the rotor.

(ix) Mark the bottom of the rotor, one half in silver and the other half in black, using permanent markers (Sharpie).
▲ **CRITICAL STEP** Ensure that the marking is visible and dry before proceeding to the next step.

(x) Place the rotor, cap side down, in a rotor plunger (see Fig. 5) and plunge the rotor into liquid nitrogen. Allow at least 30–60 s for the rotor and the sample to freeze.
! **CAUTION** Wear appropriate hand and eye gear to protect yourself from cryogenic burns.
▲ **CRITICAL STEP** Ensure that the sample is frozen before proceeding to the next step.
■ **PAUSE POINT** As an alternative to freezing the rotor and measuring immediately, the rotor can be transferred to a cryovial and frozen by plunging the vial into liquid nitrogen. The frozen vial can be stored for very long periods (at least a few months) in a cryogenic freezer cooled using liquid nitrogen as commonly used to store mammalian cell lines.

(xi) Start up the heat exchanger unit and cool down the DNP probe to ~90 K.
! **CAUTION** MAS at cryogenic temperatures is prone to rotor crashes. Follow the instructions carefully and refer to your device's user manual.

(xii) Set the MAS unit to manual mode and set the variable temperature (VT) to 135 l h^{-1} and the bearing and drive gas flows to ~6 l h^{-1}.
▲ **CRITICAL STEP** The flow rates are the lowest nonzero flow rates at which the MAS unit operates.

(xiii) Engage 'eject' on the MAS console.

(xiv) Transfer the frozen sample from Step x from the liquid N_2 into the sample catcher and place it in the probe, as shown in Fig. 5. Membrane protein samples (from Step 35) can be placed in the catcher directly by hand, as these are not prior frozen.
▲ **CRITICAL STEP** Transfer the cold rotor to the probe quickly (in <5 s) to avoid ice condensation on the surface, which would prevent proper insertion of the sample into the probe. This does not apply to the membrane protein samples.

(xv) Manually increase the probe bearing pressure (Bp) to ~500 mbar before engaging the 'insert'. This is advisable for stable and safe spinning of the rotor.

(xvi) If the sample is inserted properly, the Bp should drop significantly. Wait for 5–10 s until the rotor equilibrates at a stable spinning rate of ~400 Hz (note that this value may vary by equipment). Gently apply drive pressure (Dp) of ~20 mbar, and allow the spinning to equilibrate.
? TROUBLESHOOTING

(xvii) Manually increase the drive pressure in 50-mbar increments. Wait for the MAS frequency to equilibrate at each step.
! CAUTION Ensure that the bearing pressure is high enough to maintain rotor stability; generally, a pressure of 1,000 mbar is regarded as safe.
? TROUBLESHOOTING

(xviii) When a MAS frequency of 3 kHz is reached, increase the bearing pressure to 2,000 mbar in steps of <200 mbar. Allow a 2- to 3-s gap between each stepwise increase.
? TROUBLESHOOTING

(xix) Gently increase the drive pressure to the desired MAS frequency in steps of 50 mbar. During this procedure, the probe temperature will rise moderately.
? TROUBLESHOOTING

(xx) Increase the VT gas flow to 1,470 l h^{-1} in small steps to lower the probe temperature.
? TROUBLESHOOTING

(xxi) Wait until a temperature of ~90 K is reached and the MAS frequency is stable. A stable MAS frequency indicates that temperature equilibration is complete.
▲ CRITICAL STEP Do not change the MAS spinning frequency and the VT gas flow simultaneously. Wait for the MAS rate to equilibrate after each stepwise increase in the VT gas flow rate.
? TROUBLESHOOTING

(xxii) After temperature equilibration, you may have to adjust the drive pressure slightly to bring it within the range (±50 Hz) of the desired MAS rate. Wait for the MAS rate and temperature to equilibrate again. Switch the MAS unit to auto mode to keep a constant MAS frequency throughout the measurements.
? TROUBLESHOOTING

(xxiii) Check for DNP enhancements with and without microwaves using standard one-dimensional (1D) ^{1}H–^{13}C or ^{1}H–^{15}N cross-polarization experiments (see, e.g., refs. [16,66,68] for further information) before proceeding with multidimensional experiments.
? TROUBLESHOOTING

(B) **Quick (detergent-free) cell lysis for solution-state NMR of lysates ● Timing 1–2 h**

(i) Thaw one of the two pellets on ice in case the cells were frozen after Step 23.
▲ CRITICAL STEP The other cell pellet should be used for parallel DNP-ssNMR of whole cells in Step 25A.

(ii) Resuspend the pellet in ice-cold buffer of 1× M9 salts (whose volume is at least a fifth of the corresponding culture volume of the pellet). Spin down again for 10–20 min at 4,000*g* and 4 °C. Repeat this step once more.
▲ CRITICAL STEP Include 10% D_2O in the final resuspension of 1× M9 salts to enable field locking for solution NMR.

(iii) Sonicate the cells ten times with 10- to 15-s 13-kHz pulses with a delay of at least 45 s between pulses to prevent sample heating. Ensure that the cells are on ice during the whole period.

(iv) Obtain the clear lysate by spinning down insoluble material for >10 min at >10,000*g* and 4 °C. Transfer the supernatant to the NMR tube.

(v) Carry out the solution NMR experiments as soon as possible[26,27,32].
▲ CRITICAL STEP Pre-shim the magnet and identify the range at which hard pulses need to be optimized using a purified protein in the same buffer. This will save time and prevent proteolysis of the protein of interest. Note that running the samples on an SDS-PAGE gel would confirm if proteolysis has occurred.
? TROUBLESHOOTING

(vi) (Optional) the insoluble pellet may be subjected to ssNMR experiments for sample integrity tests by following Steps 26–35.
? TROUBLESHOOTING

(C) **Membrane protein sample preparations ● Timing 4–8 h for cell envelope samples (Steps i–x), another 1 day for membrane fractions (Steps xi–xv).**
▲ CRITICAL See Fig. 3 for a quick overview of the steps in this Option.

(i) *Isolating the cell envelope (Steps i–ix):* Thaw the pellet on ice in case the cells were frozen after Step 23.

(ii) Resuspend the harvested cell pellet in 20 ml ice-cold French press lysis buffer (see 'Reagent setup') per liter of initial bacterial culture.

(iii) Freeze the (resuspended) pellet at −80 °C.

PAUSE POINT Cellular pellets can be stored at −80 °C for years.

CRITICAL STEP From this point forward, samples must be kept cold or on ice as much as possible. Precool the French press and thaw cells on ice to avoid excessive heating during lysis step. Wash French press with ice-cold H_2O and French press lysis buffer.

(iv) Thaw the cellular pellets on ice. Pass the cells through the French press at 35 kpsi, for five times.

(v) Spin down the lysate for 20 min at 4,000*g* and 4 °C.

(vi) Retain the supernatant and discard the pellet containing unbroken cells.

? TROUBLESHOOTING

(vii) Repeat Steps v and vi until you no longer obtain a pellet (or the pellet is negligible).

CRITICAL STEP If there is a large pellet, this indicates that whole cells are still present in the sample. It may be necessary to repeat this step multiple times to remove them.

(viii) Spin down the lysate in an ultracentrifuge for 30 min at 80,000*g* and 4 °C.

(ix) Resuspend the pelleted cell envelope in 1 ml 20 mM phosphate buffer pH 7.0 and ultracentrifuge again in a table-top appropriate Eppendorf for 1 h at 90,000*g* and 4 °C.

? TROUBLESHOOTING

(x) Measure the sample directly in ssNMR (Steps 26–35). To further separate the inner and outer membranes, proceed to Steps xi–xv below.

PAUSE POINT Cell envelopes can be stored at −80 °C in an appropriate buffer. Note that long-term storage is not recommended, but if necessary, include a cryoprotectant such as glycerol.

(xi) *(Optional) separating the inner and outer membranes (Steps xi–xv):* When using cell envelope samples stored with glycerol, first spin down the pellet at 90,000*g* and 4 °C for 30 min, dilute the pellet with 1 mL 20 mM Tris pH 8.0, 0.15 M NaCl, and pellet by spinning down. Repeat this wash twice, and finally resuspend in up to 3.3 ml of buffer. This will eliminate glycerol from the sample.

(xii) Prepare the following sucrose gradient in 20 mM Tris-HCl pH 8, 0.15 M NaCl (bottom to top): 1.6 ml at 55%; 4.0 ml at 50%, 45%, 40%, and 35%; 1.6 ml at 30% (all wt/wt). Transfer 3.3 ml of the sample on top of the gradient. Spin the gradient for ~15 h at 175,000*g* and 4 °C using a swingout rotor (e.g., Beckman SW 32 TI)

CRITICAL STEP The sucrose gradients are very fragile. The distinct layers can easily break and mix, rendering the process useless. For application of the discrete layers of sucrose, we employ an empty syringe with a large-gauge needle (gauge 16 or larger), the tip of which rests against the wall of the ultracentrifuge tube. This allows slow and even distribution of the sucrose layers atop each other. The sucrose gradient can be prepared in advance. Note that diffusion will occur over time, so pre-prepared gradients should be used within 1 day. It is highly recommended to utilize the slowest acceleration and deacceleration options for the ultracentrifuge to minimize the possibility of disrupting the layers.

(xiii) Collect the inner and outer membrane fractions from the top of the 40% and 50% sucrose layers, respectively.

? TROUBLESHOOTING

(xiv) (Optional) analyze the inner and outer membrane fractions on an SDS-PAGE gel to check whether the protein of interest is in the expected layer and/or if the separation was successful.

(xv) Remove sucrose by spinning down the isolated membrane fractions for 30 mins at ≥90,000*g* at 4 °C. Resuspend in 20 ml sucrose-free buffer. Repeat this step multiple times (at least 3×) to ensure there are no traces of sucrose.

PAUSE POINT Membrane fractions can be stored at −80 °C in an appropriate buffer. Note that long-term storage is not recommended, but if necessary, include a cryoprotectant such as glycerol.

CRITICAL STEP When using membrane fractions stored with glycerol, first spin down the pellet at 90,000*g* and 4 °C for 30 min, dilute the pellet with 1 ml 20 mM Tris pH 8.0, 0.15 M NaCl, and pellet by spinning down. Repeat this wash twice, and finally resuspend in up to 3.3 ml of buffer. This will eliminate glycerol from the sample.

(Optional) filling ssNMR rotors with insoluble fractions and membrane protein preparations ● Timing 1–3 h

▲ CRITICAL The rotor filling procedure is compatible with the insoluble fraction collected in Step 25 Option B(vi) and with cell envelope preparations (Step 25C(ix)) and inner and outer membrane fractions (Step 25C(xv)).

26 (Optional) the insoluble fractions/membrane protein preparations can be made compatible for DNP-ssNMR by resuspending the pellet in 50 µl DNP juice (60% (vol/vol) deuterated d_8- and ^{12}C-enriched glycerol, and 15 mM AMUPol in D_2O, with an appropriate buffer, for example, 20 mM Tris pH 8, 0.15 M NaCl) and washing by spinning down the sample for 30–60 min at ≥90,000*g* and 4 °C.
▲ CRITICAL STEP Spin down the insoluble fraction/membrane protein preparations if it is present in a buffer, for 30 min at 90,000*g* and 4 °C, before resuspension in DNP juice.

27 Inspect for visible damage or scratches on the rotor and the cap using a magnifying glass.
! CAUTION Using partially damaged rotors or caps may lead to rotor crashes inside the probe while magic-angle spinning.

28 Use a centrifuge adapter for the rotor of choice (1.3 or 3.2 mm, see Fig. 4). Insert the rotor into the adapter.

29 Remove any excess liquid from the top of the pelleted sample from Step 26. Using a small spatula, apply a small amount of the pelleted sample to the upper part of the filling tool.

30 Spin down the sample into the rotor at speeds ≥20,000*g* for at least 20 min at 4 °C. Higher speeds lead to higher degree of packing in the rotor.
! CAUTION Ensure that the centrifuge adapter for the rotor of your choice is made of a durable material which can withstand such high *g*-forces.

31 Remove any excess buffer and check if the rotor is fully packed while ensuring there is enough room for the cap and, optionally, a protective spacer (applicable for 3.2-mm rotors).

32 Insert the protective spacer using the spacer screw (optional) and insert the cap gently.

33 If the rotor does not contain a mark for the MAS rate optical sensor, make a mark using a black Sharpie marker on the bottom side of the rotor, along the wall, such that it covers at least a third of the rotor's outer circumference. For DNP rotors, mark half the rotor with silver and the other half with black to increase the optical contrast and hence detectability by the MAS optical sensor.
▲ CRITICAL STEP This is a standard procedure; see Fig. 5 for guidelines. Ensure that the marking is clear, dry, and does not wipe off easily.

34 Inspect the rotor and the cap once again using a magnifying glass for visible damage and scratches that may have occurred while executing the above procedures.
■ PAUSE POINT Filled rotors can be stored in a refrigerator/freezer for a few days. The duration and optimal temperature vary by sample; sample integrity may be compromised in some cases.

35 Insert the rotor into the probe by conventional means. For DNP-ssNMR experiments, follow Step 25A(xi–xxiii). Note that the sample does not need to be frozen prior to DNP experiments.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
11	Too many colonies	Too much DNA was used for transformation	Reduce amount of DNA (1 ng DNA/20–100 µl cells)
	Few or no colonies	Improper heat shock	Ensure that temperature and duration are correct
		Poor-quality competent cells	Prepare new competent cells or purchase from a vendor
	Colonies in the control plate (without DNA)	Contamination occurred during transformation or competent cell preparation	Check competent cell stock for contamination
			Ensure you use fresh antibiotics and work in a sterile environment
	Satellite colonies	Antibiotic depletion around positive transformant colonies	Increase beta-lactam antibiotic concentration to 100 µg ml^{-1} and prevent long incubation times (<16 h)

Table continued

Table 1 (continued)

Step	Problem	Possible reason	Solution
14	No bacterial growth	No colony was picked from plate	Pick another colony or prepare multiple pre-cultures
17	Slow or no growth in deuterated M9	Cells are not adapted to deuterated medium	Prepare a secondary pre-culture in 90% deuterated M9, 10% LB
			Adapt the culture first in LB-D_2O and subsequently transfer to minimal medium
18	OD_{600} exceeds 1.0	Overgrowth of culture	If overgrowth reduces expression, restart culture and ensure it does not exceed OD_{600} 1.0
24	SDS-PAGE gel analysis reveals no expression of target protein	Protein is not expressed, or expression is too low under the employed conditions	Confirm expression with Western blots if an antibody is available for the protein of interest. Perform small-scale expression tests if necessary
25A(xvi)	The rotor does not insert/spin properly	Mark is not correctly made	Eject the rotor, warm it up to room temperature, dry the rotor surface, and make the mark as instructed in Step 25A(ix). If cellular samples are used, it is recommended that you make fresh samples if you thaw the rotor
		Condensation on the rotor	Transfer rotor to the probe quickly to avoid formation of condensation. If cellular samples are used, it is recommended that you make fresh samples if you thaw the rotor
25A (xvii–xxii)	The rotor does not spin properly or not stably	Sample packing is suboptimal	Stop spinning immediately. Ensure proper sample packing is done only in a swingout centrifuge rotor and the sample sinks to the bottom of the rotor. You may empty the rotor and recentrifuge properly for membrane protein preparations. If cellular samples are used, it is recommended that you make fresh samples if you thaw the rotor
25A(xxiii)	No DNP enhancement can be observed	DNP radicals are compromised	Test whether the stock solution is correct with a simple system such as a single amino acid
			Ensure the samples are kept on ice during rotor filling and the filling is done at cold temperatures (<4 °C). If cellular samples are used, it is recommended that you make fresh samples if you thaw the rotor
25B(v/vi)	No NMR signals from the clear lysate	Low de novo expression	Check expression using SDS-PAGE and Western blotting or other suitable biochemical means
		Protein precipitation	Check whether the insoluble fraction contains the precipitated protein. If yes, re-evaluate expression protocol using small-scale expression and solubility screening methods
25C(vi)	Large cell pellet	Cell lysis was incomplete	Ensure that pressure is correct on the French press
			Additionally, increase the number of freeze–thaw cycles (Step 25C(iii–iv)) or add lysozyme to weaken the membrane
25C(ix)	Large white/gray pellet at the bottom of the tube	Large portion of protein has precipitated	Make sure you work on ice and that your protein is compatible with the buffer used
		Protein expressed into inclusion bodies	Perform small-scale expression and solubility screening tests
25C(xiii)	Sucrose gradient fails to separate membranes	Sucrose gradient is broken	Prepare gradient in the cold (limits diffusion between layers) and handle gently. Additionally, reduce acceleration and deceleration speeds in the centrifuge

Timing

Reagent setup: 3–4 h
Steps 1–11, bacterial transformation: 1 day
Steps 12–20, preculture and growth: 1–2 days
Steps 21–24, expression, labeling, and harvesting: 22 h to 1 day

Table 2 | The protocol has been tested on three diverse proteins/protein complexes, using different ssNMR techniques

Protein	Labeling source	Sample type	ssNMR technique	Refs.
BAM complex	Isotope-labeled nutrients	Outer membrane	Conventional, ^{13}C-detected	36
YidC	Isotope-labeled nutrients	Cell envelope	55-kHz MAS, ^{1}H-detected	20
Ubiquitin	Isotope-labeled amino acid mix	Whole cells	DNP-enhanced, ^{13}C-detected	

Step 25A, DNP-ssNMR of whole cells: 2–3 h
Step 25B, quick (detergent-free) cell lysis for solution-state NMR of lysates: 1–2 h
Step 25C, membrane protein sample preparations: 4–8 h for cell envelope samples, another 1 day for membrane fractions
Steps 26–35, filling ssNMR rotors with insoluble fractions and membrane protein preparations: 1–3 h

Anticipated results

The procedure described in this protocol has been applied to three different protein samples, each using a slightly different ssNMR technique (Table 2). With these anticipated results, we demonstrate the versatility of the described protocol and its compatibility with different NMR methods .

Outer membrane preparations: ^{13}C-detected ssNMR

Despite its size and complexity, the β-barrel membrane protein insertase machinery (BAM, ref. [61]) is the most well-studied system that uses the described protocol for membrane proteins. For complex membrane protein samples, one can reasonably expect to discern alterations incurred in protein fold/assembly when compared with samples in other lipid environments (e.g., reconstituted in liposomes of varying composition[62] versus native membrane[36]). For example, Fig. 6 shows a 2D PARIS[69] spin diffusion spectrum (30 ms) of uniformly ^{13}C/^{15}N-labeled BamCDE in the outer membrane of *E. coli*. Protein signals are clearly visible despite the presence of lipid-derived correlations (indicated by arrows in the same figure). Such lipid correlations could potentially dominate the spectrum of proteins with lower expression levels. This is not the case with our approach, as these are drastically suppressed.

In addition, when combined with available assignments for soluble domains and predictions using the NMR analysis software FANDAS[70,71], these spectra prove even more useful. They allow the comparison of the complex in various conditions, which can highlight changes in dynamics/assembly. For example, the in-cell BamCDE spectrum displays fewer cross-peak correlations in spectral regions characteristic of CA–CB spectral correlations of serine and threonine (Fig. 6b) and leucine, aspartic acid, and asparagine (Fig. 6c) than expected from predictions of folded BamC (red crosses) and BamE (orange crosses) domains. These domains are, for example, present in free solution and can also be found in crystallographic and electron microscopy data of detergent-solubilized BAM complexes[61]. Since the employed 2D ssNMR experiment selects for rigid protein signals and mobile protein region should be absent, these findings suggest that at least segments of both BamC and BamE domains exhibit additional mobility in the natural cell envelope when expressed in the CDE subcomplex[36]. Additional high-resolution three-dimensional ^{1}H ssNMR experiments on such preparations confirm these findings.

Cell envelope preparations: ^{1}H-detected ssNMR

As described in the Introduction, proteins behave differently, depending on their environment. This is particularly highlighted by the ^{1}H-detected ssNMR correlation spectra of YidC, an inner membrane protein that helps fold and insert other inner membrane proteins[20], in cell envelopes (green) and purified and reconstituted in *E. coli* phospholipids (gray) at 55-kHz MAS (Fig. 7). While both preparations are characterized by similar spectral resolution, chemical shift differences between the case of native membranes as opposed to the reconstituted liposomes become apparent. The resolution offered by the high-MAS ^{1}H detection enables one to track these changes with ease. Note that ^{15}N-correlated, ^{1}H-detected spectra would only give rise to the protein peaks and the chemical shifts

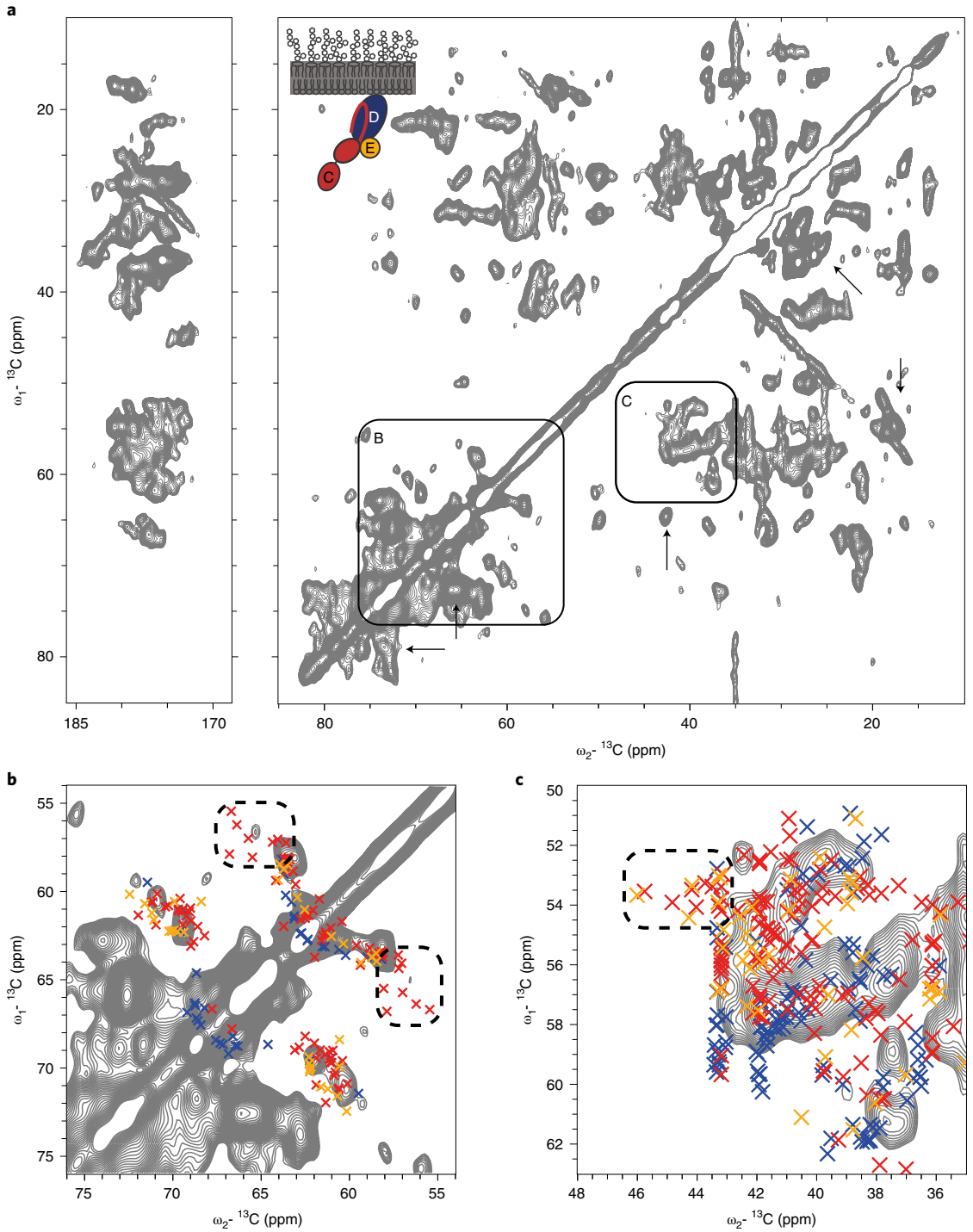

Fig. 6 | The (^{13}C, ^{13}C) correlated PARIS spin diffusion (30 ms) spectrum of uniformly [^{13}C, ^{15}N] labeled BamCDE in BL21 (DE3) star cell outer membranes. a–c, Spin diffusion spectrum (**a**), where arrows indicate regions of correlations stemming from nonproteinaceous components present in the outer membrane, showing (**b**,**c**) regions where predictions for correlations stemming from the protein components do not agree with the recorded spectra, possibly due to increased flexibility of these protein regions in membranes. Crosses in panels **b** and **c** reflect FANDAS predictions obtained from solution NMR assignments for BamC (red) and BamE (orange) (BMRB IDs 16035 (ref. [76]) and 16926 (ref. [77])) and predicted backbone correlations for BamD (blue). Figure reproduced from ref. [36] with permission from Elsevier.

which correspond only to the protein of interest (YidC). Alterations in ssNMR signal intensities may be due to changes in backbone dynamics, as cross-polarization-based (dipolar) magnetization transfer is only effective for nuclei static on microsecond timescales or longer. Intensity variations may also result from alterations in hydrogen–deuterium exchange efficiencies, for example, due to the

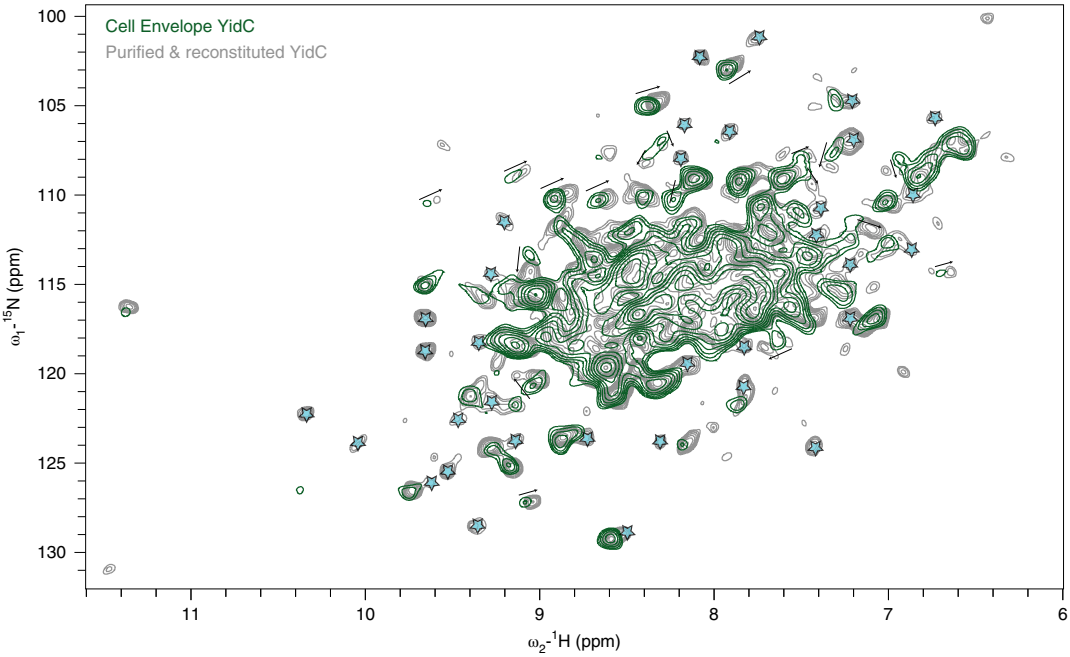

Fig. 7 | ^{1}H-detected ssNMR analysis of YidC in cell envelopes. ^{1}H-detected ^{15}N–^{1}H correlated ssNMR spectrum of YidC in the cell envelope (green) overlaid on the equivalent spectrum obtained using in vitro preparations of YidC in proteoliposomes (gray). Stars highlight peaks showing reduced intensities, and arrows indicate peaks that are perturbed. Both suggest that the structure and dynamics of YidC are different in native environments in comparison with in vitro conditions. Figure reproduced with permission from ref. [20].

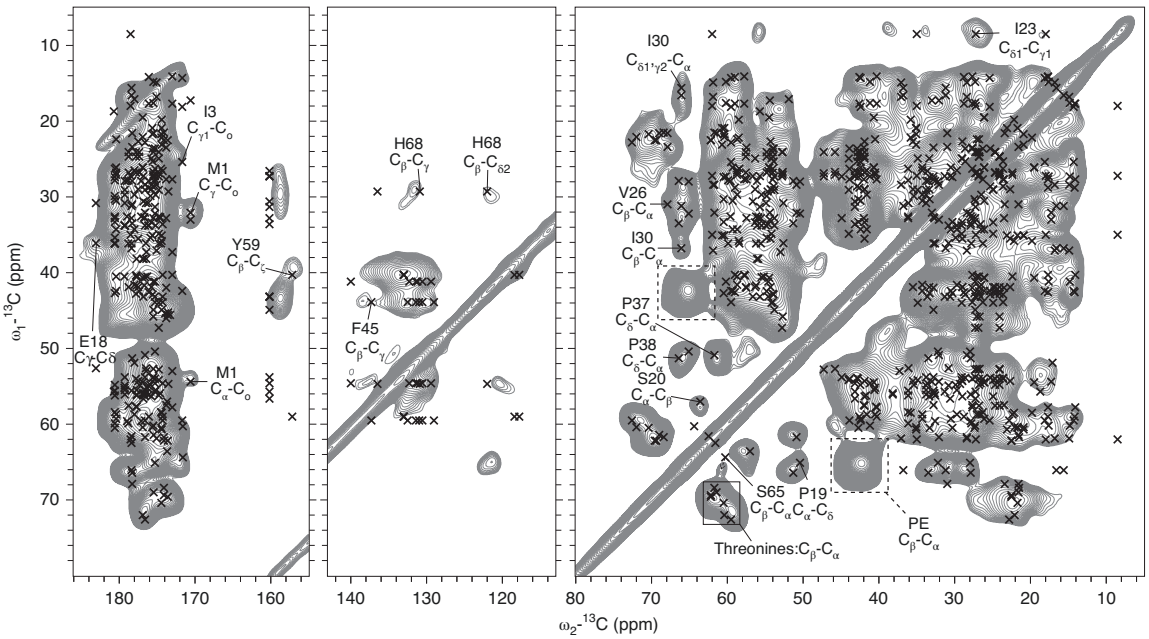

Fig. 8 | The (^{13}C, ^{13}C) correlated proton-driven spin diffusion (30 ms) of ubiquitin in deuterated Lemo21 cells. Resolved peaks are identified and indicated (at least one per residue identified). We attribute the only background peak to ^{13}C resonances of the head group of phosphatidylethanolamine (PE), which is indicated in a dashed box (see also Supplementary Fig. 6).Source data

differences in lipid composition, or they may reflect other aspects of the complexity of cellular envelope preparations. By contrast, the observed peak shifts most likely reflect changes in the structure of YidC.

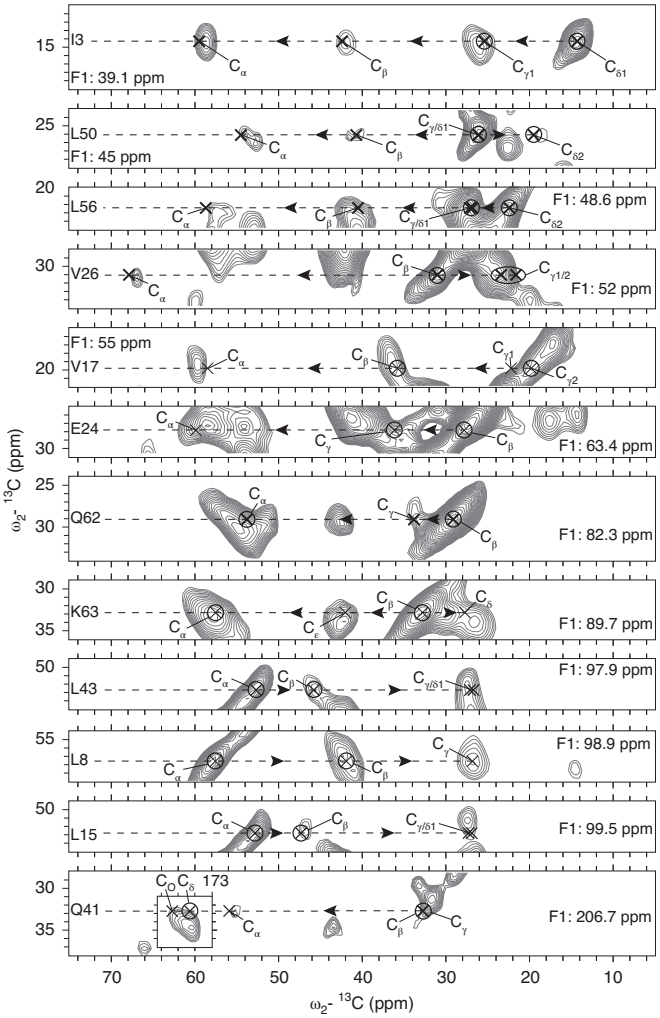

Fig. 9 | 3D 2Q-1Q-1Q correlated spectra of ubiquitin in deuterated Lemo21 cells. 2D (F_2, F_3) slices from the 3D (2Q-1Q-1Q) $^{13}C,^{13}C$ spectrum[73] corresponding to unambiguously identifiable peaks. The F_1 (2Q) frequency indicated in the slices corresponds to the sum of the chemical shifts of encircled peaks. The correlations in the F_1, F_2 slices are given in Supplementary Figs. 7–9.Source data

Whole-cell preparations

To demonstrate the application of the protocol to whole-cell preparations, ubiquitin was deliberately chosen for its robustness and because it is well characterized. Additionally, we expect ubiquitin to be relatively inert in bacterial environment, which makes it easy to detect using solution-state NMR for conducting reference experiments. Prior to DNP-ssNMR measurements of whole cells, it is important to test whether the protein of interest is expressed well and labeled in isolation (see Supplementary Notes 1–7 for all experimental parameters). In our case, ubiquitin expression was confirmed by Coomassie-stained Tris-tricine SDS-PAGE analysis[72] (Supplementary Fig. 1). Further, a combination of solution- and solid-state NMR on the soluble and insoluble fractions of the lysate from similar preparations confirmed targeted labeling (see Supplementary Figs. 3–5). The analysis of the lysate is crucial to eliminate signal ambiguities in DNP-ssNMR of whole cells where the samples are frozen at cryogenic temperatures, enabling the detection of all cellular components. Analyzing the lysate is also important to check for protein misfolding and intracellular degradation.

Finally, the DNP-ssNMR spectra show that the protein identified is indeed ubiquitin, as seen by the 2D and 3D ssNMR spectra (Figs. 8 and 9), showing good agreement with the chemical shift assignments. As also shown for the case of ubiquitin in human cells, 3D spectroscopy, for example, using 3D 2Q-1Q-1Q correlation methods[73] such as the one shown in Fig. 9, reduced the spectral crowding for correlations corresponding to residues I3, L8, L15, V17, E24, V26, Q41, L43, L50, L56, Q62, and K63.

Note that, despite trying to remove background labeling, one cannot fully eliminate signal contributions from phosphatidylethanolamine (PE), whose head group can be derived from serine. Scrambling between amino acids may occur when certain labeling schemes are used. See ref. [74] for options on using amino acids in combination.

Data availability

Source data are provided with this paper. All other data supporting the approach described in this protocol are available from the corresponding authors upon reasonable request.

References

1. Griffin, R. G. Solid state nuclear magnetic resonance of lipid bilayers. *Methods Enzymol.* **72**, 108–174 (1981).
2. Seelig, J. Deuterium magnetic resonance: theory and application to lipid membranes. *Q. Rev. Biophys.* **10**, 353–418 (1977).
3. Brown, L. S. & Ladizhansky, V. Membrane proteins in their native habitat as seen by solid-state NMR spectroscopy. *Prot. Sci.* **24**, 1333–1346 (2015).
4. Hong, M., Zhang, Y. & Hu, F.H. Membrane protein structure and dynamics from NMR spectroscopy. *Annu. Rev. Phys. Chen*., 63, (eds. Johnson, M.A. & Martinez, T.J.) 1-24 (2012).
5. Kaplan, M., Pinto, C., Houben, K. & Baldus, M. Nuclear magnetic resonance (NMR) applied to membrane–protein complexes. *Q. Rev. Biophys.* **49**, e15 (2016).
6. Herzfeld, J. & Lansing, J. C. Magnetic resonance studies of the bacteriorhodopsin pump cycle. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 73–95 (2002).
7. Ketchem, R., Hu, W. & Cross, T. High-resolution conformation of gramicidin A in a lipid bilayer by solid-state NMR. *Science* **261**, 1457–1460 (1993).
8. Lange, A. et al. Toxin-induced conformational changes in a potassium channel revealed by solid-state NMR. *Nature* **440**, 959–962 (2006).
9. Cady, S. D. et al. Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. *Nature* **463**, 689–U127 (2010).
10. Luca, S. et al. The conformation of neurotensin bound to its G protein-coupled receptor. *Proc. Natl Acad. Sci. USA* **100**, 10706–10711 (2003).
11. Goncalves, J. A., Ahuja, S., Erfani, S., Eilers, M. & Smith, S. O. Structure and function of G protein-coupled receptors using NMR spectroscopy. *Prog. Nucl. Magn. Reson. Spectrosc.* **57**, 159–180 (2010).
12. Loquet, A., Habenstein, B. & Lange, A. Structural investigations of molecular machines by solid-state NMR. *Acc. Chem. Res.* **46**, 2070–2079 (2013).
13. Tycko, R. Solid-state NMR studies of amyloid fibril structure. *Annu. Rev. Phys. Chem*., 62 (eds. Leone, S.R., Cremer, P.S., Groves, J.T. & Johnson, M.A.) 279-299 (2011).
14. Das, N., Murray, D. T. & Cross, T. A. Lipid bilayer preparations of membrane proteins for oriented and magic-angle spinning solid-state NMR samples. *Nat. Protoc.* **8**, 2256 (2013).
15. Fricke, P. et al. Backbone assignment of perdeuterated proteins by solid-state NMR using proton detection and ultrafast magic-angle spinning. *Nat. Protoc.* **12**, 764–782 (2017).
16. Ni, Q. Z. et al. High frequency dynamic nuclear polarization. *Acc. Chem. Res.* **46**, 1933–1941 (2013).
17. Ishii, Y. & Tycko, R. Sensitivity enhancement in solid state N-15 NMR by indirect detection with high-speed magic angle spinning. *J. Magn. Reson.* **142**, 199–204 (2000).
18. Chevelkov, V., Rehbein, K., Diehl, A. & Reif, B. Ultrahigh resolution in proton solid-state NMR spectroscopy at high levels of deuteration. *Angew. Chem. Int. Ed.* **45**, 3878–3881 (2006).
19. Plitzko, J. M., Schuler, B. & Selenko, P. Structural biology outside the box—inside the cell. *Curr. Opin. Struct. Biol.* **46**, 110–121 (2017).
20. Baker, L. A. et al. Combined H-1-detected solid-state NMR spectroscopy and electron cryotomography to study membrane proteins across resolutions in native environments. *Structure* **26**, 161–170 (2018).
21. Narasimhan, S. et al. DNP-supported solid-state NMR spectroscopy of proteins inside mammalian cells. *Angew. Chem. Int. Ed.* **58**, 12969–12973 (2019).
22. Damman, R. et al. Development of in vitro-grown spheroids as a 3D tumor model system for solid-state NMR spectroscopy. *J. Biomol. NMR* **74**, 401–412 (2020).
23. Thongsomboon, W. et al. Phosphoethanolamine cellulose: a naturally produced chemically modified cellulose. *Science* **359**, 334–338 (2018).
24. Renault, M. et al. Solid-state NMR spectroscopy on cellular preparations enhanced by dynamic nuclear polarization. *Angew. Chem. Int. Ed.* **51**, 2998–3001 (2012).
25. Renault, M. et al. Cellular solid-state nuclear magnetic resonance spectroscopy. *Proc. Natl Acad. Sci. USA* **109**, 4863–4868 (2012).
26. Gronenborn, A. M. & Clore, G. M. Rapid screening for structural integrity of expressed proteins by heteronuclear NMR spectroscopy. *Prot. Sci.* **5**, 174–177 (1996).
27. Qing, G. et al. Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat. Biotechnol.* **22**, 877–882 (2004).
28. Frederick, K. K. et al. Sensitivity-enhanced NMR reveals alterations in protein Sstructure by cellular milieus. *Cell* **163**, 620–628 (2015).

29. Serber, Z. & Dötsch, V. In-cell NMR spectroscopy. *Biochemistry* **40**, 14317–14323 (2001).
30. Serber, Z. et al. Methyl groups as probes for proteins and complexes in in-cell NMR experiments. *J. Am. Chem. Soc.* **126**, 7119–7125 (2004).
31. Binolfi, A., Theillet, F.-X. & Selenko, P. Bacterial in-cell NMR of human α-synuclein: a disordered monomer by nature? *Biochem. Soc. Trans.* **40**, 950–954 (2012).
32. Barbieri, L., Luchinat, E. & Banci, L. Characterization of proteins by in-cell NMR spectroscopy in cultured mammalian cells. *Nat. Protoc.* **11**, 1101 (2016).
33. Tabaka, M., Sun, L., Kalwarczyk, T. & Hołyst, R. Implications of macromolecular crowding for protein–protein association kinetics in the cytoplasm of living cells. *Soft Matter* **9**, 4386–4386 (2013).
34. Siegal, G. & Selenko, P. Cells, drugs and NMR. *J. Magn. Reson.* **306**, 202–212 (2019).
35. Lee, K. M., Androphy, E. J. & Baleja, J. D. A novel method for selective isotope labeling of bacterially expressed proteins. *J. Biomol. NMR* **5**, 93–96 (1995).
36. Pinto, C. et al. Studying assembly of the BAM complex in native membranes by cellular solid-state NMR spectroscopy. *J. Struct. Biol.* **206**, 1–11 (2019).
37. Miao, Y. et al. M2 proton channel structural validation from full-length protein samples in synthetic bilayers and *E. coli* membranes. *Angew. Chem. Int. Ed.* **51**, 8383–8386 (2012).
38. Medeiros-Silva, J. et al. 1 H-detected solid-state NMR studies of water-inaccessible proteins in vitro and in situ. *Angew. Chem. Int. Ed.* **55**, 13606–13610 (2016).
39. Jacso, T. et al. Characterization of membrane proteins in isolated native cellular membranes by dynamic nuclear polarization solid-state NMR spectroscopy without purification and reconstitution. *Angew. Chem. Int. Ed.* **51**, 432–435 (2012).
40. Ward, M. E. et al. In situ structural studies of anabaena sensory rhodopsin in the *E. coli* membrane. *Biophys. J.* **108**, 1683–1696 (2015).
41. Etzkorn, M. et al. Complex formation and light activation in membrane-embedded sensory rhodopsin II as seen by solid-state NMR spectroscopy. *Structure* **18**, 293–300 (2010).
42. Yamamoto, K., Caporini, M. A., Im, S.-C., Waskell, L. & Ramamoorthy, A. Cellular solid-state NMR investigation of a membrane protein using dynamic nuclear polarization. *Biochim. Biophys. Acta* **1848**, 342–349 (2015).
43. Schanda, P. et al. Atomic model of a cell-wall cross-linking enzyme in complex with an intact bacterial peptidoglycan. *J. Am. Chem. Soc.* **136**, 17852–17860 (2014).
44. Reckel, S., Lopez, J. J., Loehr, F., Glaubitz, C. & Doetsch, V. In-cell solid-state NMR as a tool to study proteins in large complexes. *ChemBioChem* **13**, 534–537 (2012).
45. Medeiros-Silva, J. et al. High-resolution NMR studies of antibiotics in cellular membranes. *Nat. Commun.* **9**, 3963 (2018).
46. Shukla, R. et al. Mode of action of teixobactins in cellular membranes. *Nat. Commun.* **11**, 2848 (2020).
47. Kaplan, M. et al. Probing a cell-embedded megadalton protein complex by DNP-supported solid-state NMR. *Nat. Methods* **12**, 649–652 (2015).
48. Almeida, F. C. L. et al. Selectively labeling the heterologous protein in Escherichia coli for NMR studies: A strategy to speed up NMR spectroscopy. *J. Magn. Reson.* **148**, 142–146 (2001).
49. Galvão-Botton, L. M. P. et al. High-throughput screening of structural proteomics targets using NMR. *FEBS Lett.* **552**, 207–213 (2003).
50. Baker, L. A., Daniëls, M., van der Cruijsen, E. A. W., Folkers, G. E. & Baldus, M. Efficient cellular solid-state NMR of membrane proteins by targeted protein labeling. *J. Biomol. NMR* **62**, 199–208 (2015).
51. Serber, Z., Ledwidge, R., Miller, S. M. & Dötsch, V. Evaluation of parameters critical to observing proteins inside living Escherichia coli by in-cell NMR spectroscopy. *J. Am. Chem. Soc.* **123**, 8895–8901 (2001).
52. Chordia, S., Narasimhan, S., Lucini Paioni, A., Baldus, M. & Roelfes, G. In vivo assembly of artificial metalloenzymes and application in whole-cell biocatalysis (ChemRxiv, 2020).
53. White, S. W., Zheng, J., Zhang, Y. M. & Rock, C. O. The structural biology of type II fatty acid biosynthesis. *Annu. Rev. Biochem.* **74**,, 791–831 (2005).
54. Roelfes, G. LmrR: a privileged scaffold for artificial metalloenzymes. *Acc. Chem. Res.* **52**, 545–556 (2019).
55. Mitchell, A. M. & Silhavy, T. J. Envelope stress responses: balancing damage repair and toxicity. *Nat. Rev. Microbiol.* **17**, 417–428 (2019).
56. Fowler, D. M., Koulov, A. V., Balch, W. E. & Kelly, J. W. Functional amyloid – from bacteria to humans. *Trends Biochem. Sci.* **32**, 217–224 (2007).
57. Otzen, D. & Riek, R. Functional amyloids. *Cold Spring Harb. Perspect. Biol.* **11**, a:033860 (2019).
58. Kim, K. W. Prokaryotic cytoskeletons: in situ and ex situ structures and cellular locations. *Antonie van Leeuwenhoek* **112**, 145–157 (2019).
59. Albert, B. J. et al. Dynamic nuclear polarization nuclear magnetic resonance in human cells using fluorescent polarizing agents. *Biochemistry* **57**, 4741–4746 (2018).
60. Ghosh, R., Kragelj, J., Xiao, Y. & Frederick, K. K. Cryogenic sample loading into a magic angle spinning nuclear magnetic resonance spectrometer that preserves cellular viability. *JoVE* e61733 (2020).
61. Noinaj, N., Gumbart, J. C. & Buchanan, S. K. The β-barrel assembly machinery in motion. *Nat. Rev. Microbiol.* **15**, 197–204 (2017).
62. Pinto, C. et al. Formation of the β-barrel assembly machinery complex in lipid bilayers as seen by solid-state NMR. *Nat. Commun.* **9**, 4135–4145 (2018).

63. Koers, E. J. et al. NMR-based structural biology enhanced by dynamic nuclear polarization at high magnetic field. *J. Biomol. NMR* **60**, 157–168 (2014).
64. Corzilius, B., Andreas, L. B., Smith, A. A., Ni, Q. Z. & Griffin, R. G. Paramagnet induced signal quenching in MAS–DNP experiments in frozen homogeneous solutions. *J. Magn. Reson.* **240**, 113–123 (2014).
65. Narasimhan, S., Folkers, G. E. & Baldus, M. When small becomes too big: expanding the use of in-cell solid-state NMR spectroscopy. *ChemPlusChem* **85**, 760–768 (2020).
66. Zhai, W. et al. Postmodification via thiol-click chemistry yields hydrophilic trityl-nitroxide biradicals for biomolecular high-field dynamic nuclear polarization. *J. Phys. Chem. B* **124**, 9047–9060 (2020).
67. T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco.
68. Paioni, A. L., Renault, M. A. M. & Baldus, M. DNP and cellular solid-state NMR. *eMagRes* **7**, 51–61 (2018).
69. Weingarth, M., Bodenhausen, G. & Tekely, P. Broadband magnetization transfer using moderate radio-frequency fields for NMR with very high static fields and spinning speeds. *Chem. Phys. Lett.* **488**, 10–16 (2010).
70. Gradmann, S. et al. Rapid prediction of multi-dimensional NMR data sets. *J. Biomol. NMR* **54**, 377–387 (2012).
71. Narasimhan, S. et al. Rapid prediction of multi-dimensional NMR data sets using FANDAS. *Protein NMR Methods Protoc*. (ed. Ghose, R.) 111-132 (Springer, 2018).
72. Schägger, H. Tricine–SDS-PAGE. *Nat. Protoc.* **1**, 16–22 (2006).
73. Heise, H., Seidel, K., Etzkorn, M., Becker, S. & Baldus, M. 3D NMR spectroscopy for resonance assignment and structure elucidation of proteins under MAS: novel pulse schemes and sensitivity considerations. *J. Magn. Reson.* **173**, 64–74 (2005).
74. Lacabanne, D., Meier, B. H. & Böckmann, A. Selective labeling and unlabeling strategies in protein solid-state NMR spectroscopy. *J. Biomol. NMR* **71**, 141–150 (2018).
75. Mandal, A., Boatz, J. C., Wheeler, T. & van der Wel, P. C. A. On the use of ultracentrifugal devices for routine sample preparation in biomolecular magic-angle-spinning NMR. *J. Biomol. NMR* **67**, 165–178 (2017).
76. Knowles, T. J., McClelland, D. M., Rajesh, S., Henderson, I. R. & Overduin, M. Secondary structure and (1)H, (13)C and (15)N backbone resonance assignments of BamC, a component of the outer membrane protein assembly machinery in Escherichia coli. *Biomol. NMR Assign.* **3**, 203–206 (2009).
77. Kim, K. H. et al. Structural characterization of *Escherichia coli* BamE, a lipoprotein component of the β-barrel assembly machinery complex. *Biochemistry* **50**, 1081–1090 (2011).

Acknowledgements

The authors thank H. van Ingen for providing access to the solution-state NMR instrument, and D. Mance and Dr. Klaartje Houben for technical support and discussions. We are indebted to P. Tordo and O. Ouari (Aix-Marseille Université) for providing AMUPol for the DNP experiments S.N. was supported by the Netherlands' Magnetic Resonance Research School (NMARRS, project number 022.005.029). Furthermore, this work was supported by the Dutch Research Council (NWO, projects 700.26.121 and 700.10.443 to M.B.) and by iNEXT-Discovery (project number 871037), a project funded by the Horizon 2020 program of the European Commission.

Author contributions

S.N. and C.P. prepared samples and conducted ssNMR experiments. They were supervised by G.E.F. and M.B. In addition, the DNP experiments were supported by A.L.P. and J.v.d.Z. All authors contributed to writing the manuscript and approved the final version.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-020-00439-4.

Correspondence and requests for materials should be addressed to M.B.

Peer review information *Nature Protocols* thanks Lukas Trantirek and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 7 July 2020; Accepted: 9 October 2020;
Published online: 13 January 2021

Related links
Key references using this protocol:
Chordia, S. et al. Preprint at *ChemRxiv* (2020): https://doi.org/10.26434/chemrxiv.12485993.v1
Pinto, C. et al. *J Struct Biol* **206**, 1–11 (2019): https://doi.org/10.1016/j.jsb.2017.11.015.
Baker, L. A. et al. *Structure* **26**, 161–170 (2018): https://doi.org/10.1016/j.str.2017.11.011
Baker, L. A. et al. *J Biomol NMR* **62**, 199–208 (2015): https://doi.org/10.1007/s10858-015-9936-5