# Investigating macromolecules inside cultured and injected cells by in-cell NMR spectroscopy

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**The noninvasive character of NMR spectroscopy, combined with the sensitivity of the chemical shift, makes it ideally suited to investigate the conformation, binding events and dynamics of macromolecules inside living cells. These 'in-cell NMR' experiments involve labeling the macromolecule of interest with a nonradioactive but NMR-active isotope ($^{15}$N or $^{13}$C). Cellular samples are prepared either by selectively overexpressing the protein in suitable cells (e.g., bacterial cells grown on isotopically labeled media), or by injecting isotopically labeled proteins directly into either cells or cell extracts. Here we provide detailed protocols for in-cell NMR experiments in the prokaryotic organism *Escherichia coli*, as well as eukaryotic cells and extracts employing *Xenopus laevis* oocytes or egg extracts. In-cell NMR samples with proteins overexpressed in *E. coli* can be produced within 13–14 h. Preparing *Xenopus* oocyte samples for in-cell NMR experiments takes 6–14 h depending on the oocyte preparation scheme and the injection method used.**

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## INTRODUCTION

Three features of NMR spectroscopy make it unique among biophysical methods for the investigation of biological macromolecules *in vivo*. The first is its ability to provide information about molecules under physiological conditions. This trait has enabled spectroscopists to study metabolites and ions in systems ranging from cellular suspensions to entire perfused organs, and is the cornerstone of magnetic resonance imaging (MRI), which provides spatially resolved information about the composition of entire organisms. The second is the selectivity of NMR for certain, relatively rare atomic nuclei, especially $^{13}$C and $^{15}$N, which have natural abundances of 1.11% and 0.37%, respectively. Thus, most molecules in cells contain almost exclusively $^{12}$C and $^{14}$N, and act as invisible players in heteronuclear NMR experiments, allowing for the discrimination of a single isotopically-labeled macromolecular species in the context of a crowded macromolecular environment. The third and final distinctive feature is the sensitivity of the chemical shift of an NMR-active nucleus to changes in its chemical environment. This characteristic has made NMR spectroscopy an excellent tool for studying the interaction of biological macromolecules with binding partners, including other macromolecules, small ligands and medically relevant drugs. In-cell NMR capitalizes on these attributes not necessarily to solve structures directly in the cellular environment, but to gather information about changes in the state of a macromolecule in its natural surroundings. Post-translational modifications, localization to different organelles, conformational changes and binding events all result in changes in the resonance frequencies of the affected nuclei, and can thus be measured quantitatively via in-cell NMR experiments.

So far, most *in vivo* examinations of biological macromolecules have relied on fluorescence techniques. Although these methods can identify the location of a particular macromolecule within the cell, only limited information about the conformation of a protein can be obtained. NMR-based *in vivo* techniques can close this gap, and make the investigation of conformational changes, dynamics and binding events possible.

A prerequisite for the observation of macromolecules by liquid-state NMR spectroscopy, however, is that they can tumble freely in solution with a rotational correlation time that is not longer than a couple of tens of nanoseconds. The slower the tumbling rate, the broader the resonance lines become until they disappear completely. Although the size and shape of a macromolecule are well-known parameters affecting the correlation time, the recent introduction of transverse relaxation-optimized spectroscopy (TROSY) and similar techniques has raised the so-called 'size limit' of NMR, thereby enabling the study of, for example, the bacterial chaperonin GroEL with a mass of $\sim$900 kDa[1]. Other less-commonly considered parameters that are especially relevant to in-cell NMR experiments are the viscosity of the intracellular environment, and the likelihood that the protein of interest might join large and relatively immobile macromolecular complexes. Fortunately, the cellular viscosity is, at most, twice the viscosity of water[2]. Based on the linear relationships between the viscosity, rotational correlation time and molecular mass of a protein, this twofold increase in viscosity leads to a twofold increase in the apparent molecular mass of a macromolecule; this modest increase can be surmounted, if necessary, by applying the same approaches used to study larger proteins *in vitro*. However, recruitment of the protein to cellular components, such as membranes or the cytoskeleton, can dramatically increase the rotational correlation time, and might lead to the disappearance of its resonances despite TROSY. A lack of signal might itself be informative, especially when coupled with perturbations, such as the addition of mutations or small molecules designed to disrupt the relevant interaction, thereby making the tumbling properties of the protein, as measured by in-cell NMR, a quantitative readout. In addition, the detection of side-chain methyl groups within the almost immobilized protein might still be possible due to their fast internal rotation[3].

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In-cell NMR samples are prepared either by overexpressing a protein in cell culture, or by purifying the protein and injecting it into cells. These cultured cells might be bacteria, yeast or insect cells, or any other type capable of expressing the target protein in sufficient quantities. Intracellular protein deposition by microinjection is restricted to a few eukaryotic cell types that can be manipulated in this way. Additionally, as most cell-injection procedures are time consuming and elaborate, the number of cells that must be manipulated and are required to fill a Shigemi NMR tube to a volume of 250 μl should be as small as possible. *Xenopus laevis* oocytes are ideal in this regard as they are large (1 μl in volume) and only ∼200 are required for an in-cell NMR sample. Moreover, *Xenopus* oocytes are widely used in many research institutions, and protocols for their injection are well established and easily performed. Mature (stage VI) *Xenopus* oocytes have the additional advantage of being naturally synchronized and arrested in prophase at the G2/M boundary of the first meiotic division. During oocyte-to-egg maturation, a hormonal trigger activates synchronized cell-cycle progression into metaphase of meiosis II. For isolated oocytes in an *in vitro* setting, these events can be executed by the external addition of progesteron, which renders this system an important laboratory tool for the study of eukaryotic cell-cycle progression. Cellular extracts from *Xenopus* eggs are easily obtained in a largely undiluted form, and similarly recapitulate most of the biological activities of intact cells. They are frequently used as alternative cell-free systems for *ex vivo* analyses of cellular processes in many 'biological' applications and are equally well suited for in-extract NMR analyses.

There are intrinsic advantages and disadvantages to both approaches. Performing in-cell NMR experiments on the same cells expressing the protein of interest involves far less sample manipulation, and allows for the study of proteins that are difficult to purify or are unstable outside the cellular context. Injecting eukaryotic cells allows in-cell NMR analyses of biological processes that are characteristic of higher organisms, ensures that the NMR-active isotope label is enriched only in the intended molecules, works for proteins that do not overexpress to high intracellular concentrations and facilitates the precise control of the intracellular concentration of the labeled protein. In addition, injecting a protein expressed and purified from *Escherichia coli* facilitates the investigation of post-translational modification processes, as the labeled protein encounters the eukaryotic cellular environment in an initially unmodified 'naked' form. Upon extract suspension or intracellular injection, the protein of interest undergoes covalent modifications, which are determined by the signaling state of the cell and executed by endogenous enzymes. Determining whether a particular protein is amenable to study by in-cell NMR, and which approach to use, depends on the protein and the biology in question.

**Considerations for in-cell NMR in cultured cells**

As discussed above, measuring the in-cell NMR spectra of biological macromolecules relies on distinguishing the resonance frequencies of the macromolecules of interest from those of all other cellular components. This discrimination is achieved by selectively labeling the proteins with NMR active isotopes, mainly $^{15}$N or $^{13}$C. In some cases, $^{19}$F has also been used, although it requires the chemical modification of amino acids (e.g., 5-fluorotryptophan), which can change the chemical properties and behavior of the investigated macromolecules[4].

Overexpressing, labeling and performing measurements on macromolecules inside cells require the latter to be cultured in an isotopically labeled medium. In principle, this procedure bears the risk of creating high levels of background, as all components of the cell might become isotopically labeled. Detailed investigations, however, have shown that in the case of $^{15}$N labeling only a small number of background peaks appear, even if the cells are kept and grown from the beginning in labeled medium[5]. Comparisons of different schemes with bacterial cells have shown that they can be grown on an unlabeled medium to the desired optical density, harvested and then resuspended in labeled media just before induction. The best in-cell NMR spectra are obtained with $^{15}$N-labeled and deuterated rich media. The higher expression level combined with the narrower resonance line width due to deuteration result in high sensitivity[5].

By contrast, full $^{13}$C labeling produces strong background signals that make the unambiguous identification of resonances of the macromolecule of interest impossible, with the exception of characteristic resonances such as high field-shifted methyl groups or anomeric protons of sugars. The significantly higher background levels in the case of $^{13}$C labeling as compared with $^{15}$N labeling are due to the greater number of C–H groups than N–H groups in proteins and non-proteinaceous molecules. In addition, many solvent-exposed amide protons exchange quickly with the bulk water, broadening their resonance lines beyond the detection limit[3]. Unambiguous identification of the carbon resonances of the overexpressed macromolecule can, however, be achieved by selective labeling procedures. Addition of $^{13}$C methyl group-labeled methionine, for example, leads to almost background-free in-cell NMR spectra in which the methyl groups of the overexpressed macromolecule can be identified. Similarly, the δ-methyl groups of isoleucines are excellent probes for in-cell NMR investigations[3].

**Considerations for in-cell NMR in injected cells**

The major concern when incorporating the isotopic label selectively into the target protein is alleviated when the latter is initially purified and then added to egg extracts or injected into *Xenopus* oocytes[6]. However, additional issues arise here. The study of proteins in egg extracts or oocyte cells necessitates the preparation of highly concentrated stock solutions. The injection volume per oocyte is restricted to a maximum of ∼50 nl (1 μl cell volume), which results in a 20-fold cellular dilution. In analogy, resuspending labeled proteins in egg extracts results in sample dilutions, which are ideally kept to a minimum to ensure cell-free conditions that most closely resemble the native situation. Thus, if the final intracellular concentration of labeled protein in the NMR tube is to be 50 μM, an extract or an oocyte experiment will require a starting protein concentration of at least 250 μM or 1 mM, respectively. Note that in order to reach an overall protein concentration of 50 μM in an oocyte sample, the intracellular concentration should be higher (by a factor of 1.3–1.5), as the spherical oocytes will not occupy the entire space of the NMR tube.

Protein chemists are accustomed to finding conditions that keep the protein of interest soluble and active; however, the need to introduce the highly concentrated protein into a living system entails additional considerations. Ideally, protein buffers will be similar in composition and pH to buffers used to make egg extracts.

In addition, although many proteins are stable at high concentrations only at low temperatures (usually 4 °C), *Xenopus* oocytes are most viable at 18 °C and will not tolerate prolonged exposure to 4 °C, and even a small amount of protein precipitation can quickly clog the injection needle. We have successfully performed oocyte injections in a 4 °C room by placing the plate containing oocytes on an improvised microscope stage that comprises a heat block set to 22 °C and by working quickly. Fortunately, it appears that once the protein is injected into the oocyte, it is usually stable at room temperature (22–26 °C). Whether this is due to dilution, molecular crowding, the presence of chaperones in the oocyte or some combination of these factors is not yet clear. These particular considerations will not apply for all protein samples; however, they illustrate the need to find ways to reconcile the requirements of the protein with those of the oocytes.

**Applications**

The unique aptitude of in-cell NMR measurements can best be described by a brief survey of experiments already conducted to investigate (i) the conformation of proteins in the cellular environment, (ii) protein–protein interactions, (iii) protein–drug interactions, (iv) protein–metal ion interactions and (v) the dynamics of proteins. Although all these studies have been conducted in bacteria, the recent development of in-cell NMR in *Xenopus* oocytes and egg extracts will facilitate similar measurements in eukaryotic environments[6]. Methodologies for implementing in-cell NMR for all of the above are provided.

Although the overall conformation of small well-folded single-domain proteins rarely depends on the environment, many proteins involved in signal transduction are only partially folded, and their conformation can be readily modulated by the presence of the interacting partners or by the crowded conditions of the intracellular space. For example, Gary Pielak's group has demonstrated that the bacterial protein FlgM is completely unfolded *in vitro*, but appears partially folded when analyzed in the context of live bacteria. Moreover, the folded *in vivo* conformation of FlgM was similarly displayed *in vitro* in solutions that contain high concentrations of BSA, ovalbumin or glucose, suggesting that this cellular state is induced by macromolecular crowding[7]. For other proteins, molecular crowding might stabilize the unfolded state. α-synuclein, which is a natively disordered protein in its pure form and in dilute solutions, exhibits a conformational transition at 35 °C that includes the formation of secondary structure elements within the amino (N)-terminal 100 amino acids. In the crowded periplasm of *E. coli* cells, however, this transition cannot be observed at elevated temperatures[8,9].

Investigation of potential interactions between proteins can, in principle, be studied by in-cell NMR experiments. A disadvantage of NMR spectroscopy is its inherently low sensitivity, which requires intracellular concentrations in the tens of μM range. These quantities are necessary for sufficient signal-to-noise ratios in NMR experiments and for in-cell NMR measurements within a reasonable amount of time. Unfortunately, most endogenous proteins are not found at these concentrations, which excludes the detection of binding events that involve endogenous protein components. Nevertheless, if the specific interaction between two different proteins should be studied by in-cell NMR experiments, one approach suggests that the sequential overexpression of both binding partners should be studied *in vivo*. To distinguish these proteins, the research group of Alexander Shekhtman has designed a labeling scheme that allows the expression of the first protein in an isotopically labeled medium under the control of an L-arabinose-inducible promoter. After harvesting the cells and resuspending them in a non-labeled medium, IPTG-induction can be used to initiate overexpression of the (non-labeled) interaction partner. Binding can thus be detected from changes of the chemical shifts of the first protein after induction of the overexpression of the second. The Shekhtman laboratory has used this system to study the interaction between ubiquitin and two interaction partners, the signal-transducing adaptor molecule STAM2 and a peptide derived from the ataxin 3 protein in bacterial cells[10].

In addition to specific interactions between different proteins, in-cell NMR experiments can also be used to investigate nonspecific interactions. One such example is FKBP, which is a proline isomerase; although it cannot be observed by amide proton based in-cell NMR experiments, labeling of the methyl groups of methionines with $^{13}$C enables the detection of their resonances. These data suggest that FKBP is interacting with other proteins in the cellular environment, creating complexes that tumble so slowly that the amide proton signals are line-broadened beyond the detection limit. The inherently higher sensitivity of methyl groups, due to the greater number of protons coupled to the heteronucleus and their high internal rotation rates, allows their detection and makes them an ideal probe for studying the behavior of large complexes in the cellular environment[3].

The sensitivity of the chemical shift towards changes in the chemical environment of a given spin has made NMR spectroscopy a standard tool in the pharmaceutical industry for studying the interactions between proteins and potential drugs[11,12]. One disadvantage of these *in vitro* assays, however, is that they do not encompass the complexity of potential cellular encounters and modifications that the drug might experience *in vivo*. For example, a drug might not be able to pass the cellular membrane, might get pumped out of the cell quickly, might be metabolized or might interact more strongly with other cellular components. These adverse effects can, potentially, be detected and interpreted by in-cell NMR experiments. Hubbard and colleagues compared the NMR spectra of the $^{15}$N-labeled bacterial signal transduction protein CheY *in vitro* and *in vivo*, and showed that addition of the drug BRL-16492PA to either created the same chemical-shift differences. From these data, they concluded that this drug is capable of passing the bacterial membrane and interacting with the protein inside the cellular environment[13].

In addition to protein–drug interactions, the metal-binding state of a protein can also be investigated by this technique. Comparisons of the in-cell NMR spectra of CheY with the corresponding *in vitro* spectra of the protein bound to different metal ions have revealed that it preferentially binds to $Mg^{2+}$ ions in the bacterial cytoplasm[13]. Other investigations have shown that calmodulin is found predominantly in the apo (metal ion-free) form in bacteria, which is not surprising given that the intracellular calcium concentration is insufficient to promote the calcium-bound form. Additional peaks that do not belong to the apo form of calmodulin suggest that other more abundant ions than calcium might be bound in the bacterial cytoplasm[5].

NMR spectroscopy is capable of providing information not only about the structure and conformation of a protein, but also about its dynamics[14]. These investigations are based on measuring NMR

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relaxation parameters, mainly longitudinal and transverse relaxation rates of the $^{15}$N nucleus, as well as the heteronuclear nuclear Overhauser effect (NOE) between the amide proton and the $^{15}$N nucleus. These experiments can be performed using the same pulse sequences that have been established for *in vitro* investigations, with the added concern that the living samples cannot last for more than several hours in the NMR tube. Longer experiments can be performed in a number of different ways. *E. coli* can be embedded in an agarose matrix[15] and supplied with oxygen and nutrients without compromising the quality of the in-cell NMR spectra of the samples. In addition, for bacteria or *Xenopus* oocytes or egg extracts, multiple equivalent samples can be made and measured separately with reference scans conducted to normalize any differences in protein concentration or homogeneity.

### Experimental design

Several general criteria apply for in-cell NMR experiments in cultured and injected cells, as described below.

**Cultured cells.** For preparing in-cell NMR samples by overexpression in bacteria, the total amount of cells should be in the range of 30 to 60% of the entire volume of typically 500 μl in a standard NMR tube (as determined by spinning down the cells at 20,000*g* in a tabletop centrifuge for 5 min at 4 °C). Higher concentrations of cells will result in worse homogeneity. The values of all volumes, time periods and centrifugal forces described in the detailed protocol below can and have been adjusted for individual experiments. The only critical requirements are that the over-expresison level must reach a minimum threshold (an intracellular concentration of ~200 μM for amide proton-detected experiments and 70 μM for methyl group-detected experiments), and that the bacterial slurry is homogeneous without clumps of tightly packed cells.

**Injected cells.** Studying proteins by in-cell NMR in the *Xenopus* model system requires the preparation of pure, concentrated and isotopically-labeled protein, as well as frog oocytes or egg extracts. Most scientists considering this protocol will probably have expertise in one or the other of these preparations, but not both. We strongly recommend referring to additional detailed texts to improve confidence with either aspect of the approach[16,17]. Oocytes are essentially prepared as described in ref. 16 and crude cytoplasmic extracts as described in ref. 18.

The preparation of sufficient amounts of isotopically labeled proteins is usually based on bacterial expression systems. Commonly, the gene encoding the protein of interest lies in a T7-inducible expression vector transformed into BL21 *E. coli*. In this case, grow the bacteria in labeled media to an $OD_{600}$ of 0.6 and induce expression with IPTG. The concentration of IPTG, the induction temperature and the duration of the induction can all be varied to find the optimal conditions for maximal soluble expression. Large affinity or fluorescent tags, such as glutathione *S*-transferase (GST), myelin basic protein (MBP) or GFP, which increase the solubility of the expressed proteins, are not recommended unless a specific protease cleavage site is introduced to enable the subsequent removal of the tag. Recombinant protein can also be expressed and isotopically labeled in other cell types, including yeast, insect cells or even mammalian cell culture.

Roughly 200 injected oocytes are required for every in-cell NMR experiment employing the *Xenopus* system. Oocytes are commonly injected with pulled glass needles mounted on a pneumatic or oil-driven injection device; each oocyte must be visually aligned with the tip of the needle and injected under a dissecting scope. In addition, we have also used a robotic device that automatically and reproducibly injects 96 oocytes in $<2$ min[19], enabling the preparation of dozens of NMR samples in a single day (see below). We have found that robotically injected oocytes are consistently more viable than their manually injected counterparts (at intracellular concentrations of exogenously added protein $<700$ μM) and show fewer signs of injection-inflicted incisions. The experimental read-out produced by in-cell NMR methods is comparable, although automated injected samples display a higher degree of quantitative reproducibility.

Proper animal care and safety protocols must be obeyed at all times. Check with your local animal care facility or appropriate institutional official to obtain the necessary training and licenses. Alternatively, at least one company in the US (Nasco Science) ships freshly harvested *Xenopus* ovaries by overnight courier. Animal training and licenses are usually not required for this service.

Standard reagents and equipment found in NMR spectroscopy and *Xenopus* laboratories are sufficient to perform all the experiments described here.

## MATERIALS

### REAGENTS

- OR2 buffer: 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES (pH 7.6)
- ND96 buffer: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2 \times 2H_2O$, 1 mM $MgCl_2 \times 6H_2O$ and 5 mM HEPES (pH 7.6)
- MBS buffer without calcium: 88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$ and 5 mM HEPES (pH 7.55)
- MMR buffer: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA and 5 mM HEPES (pH 7.8)
- XB buffer: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM potassium HEPES (pH 7.7) and 50 mM sucrose, supplemented with 2% (wt/vol) cysteine
- Cytostatic factor (CSF)-XB: XB buffer containing 2 mM $MgCl_2$ and 5 mM EGTA ▲ CRITICAL Can be made with 10% (vol/vol) $D_2O$ to avoid the need to add it to the NMR sample later
- Egg lysis buffer: 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM sucrose, 1 mM DTT, 50 μg $ml^{-1}$ cycloheximide and 10 mM HEPES (pH 7.7) ▲ CRITICAL Can be made with 10% (vol/vol) $D_2O$ to avoid the need to add it to the NMR sample later
- Optional: pregnant mare serum gonadotropin (PMSG; Calbiochem, cat. no. 367222)
- Optional: human chorionic gonadotropin (HCG; Sigma, cat. no. G1272)

### EQUIPMENT

- NMR spectrometer
- Optional: NMR tube (Shigemi) or conventional large volume tubes
- Optional: pre-pulled injection needles, nozzle aperture ~15 μm (Multi Channel Systems, cat. no. 38GC100TF-10)
- Optional: standard pneumatic oocyte injection device (Harvard Instruments)
- Optional: centrifugal filter device (Millipore) or stirred ultrafiltration cells (Millipore)
- Optional: 96-well cone-shaped plates (Greiner or Nunc)
- Optional: polypropylene centrifuge tubes (Beckmann)

### REAGENT SETUP

**MBS buffer** Can be used as a substitute for ND96 buffer at any stage of the oocyte preparation.

**Labeled media for preparation of in-cell NMR samples in bacterial cells** The labeled media can be either M9 minimal media with $^{15}$N ammonium chloride

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for full $^{15}N$-labeling or M9 media supplemented with $^{13}C$-labeled and/or $^{15}N$-labeled amino acids. For the highest expression level of fully $^{15}N$-labeled samples, commercially available labeled rich media can be used. Optimal sensitivity can be achieved by using $^{15}N$-labeled and deuterated media. If the amide proton-exchange rates of the protein of interest are slow at 37 °C, a deuterated extract or concentrate should be used dissolved in $H_2O$. This procedure will result in 50–80% deuteration, depending on the different chemical groups. If the exchange rate is fast, the expression can be carried out in 100% $D_2O$. During the final sample-preparation step, the sample will be resuspended in $H_2O$ and the amide proton will exchange back.

**Protein buffer for injecting purified proteins into oocytes** Choose the final protein buffer while simultaneously considering the physiology of the oocytes or eggs extracts and the need to keep the protein soluble. A good starting point is buffer conditions similar to those used to make some egg extracts; for example, 50 mM sucrose, 50 mM NaCl, 25 mM HEPES (pH 7.5) and 1 mM DTT.

**EQUIPMENT SETUP**

**NMR spectrometer** Should be equipped with an inverse-detection probehead and at least two channels: one for protons and one for heteronuclei (either $^{15}N$ or $^{13}C$, depending on the specific application). The availability of pulsed field gradients is useful, but not a prerequisite for these measurements. As sensitivity is a limiting factor, cryogenic probes are recommended but are not absolutely necessary. Similarly, higher magnetic fields ($\geq$500 MHz) are recommended.

**Preparing the needle for injection** The concentrated protein is typically considerably more viscous than the RNA most often injected into *Xenopus* oocytes. Therefore, the aperture of the needle needs to be wide enough to allow the protein solution to pass through, but small enough to minimize the trauma inflicted upon the oocytes. The aperture diameter should be determined empirically for each protein. We suggest pulling needles with apertures of $\geq$15 μm. Alternatively, pre-pulled needles obtained from commercial sources have been found to consistently yield highly reproducible results, especially when used in conjunction with automated injection procedures (see below). Calibrate the needle by dispensing individual drops of sample in mineral oil, and measure the drop diameter under a microscope at the given injection-time and injection-pressure settings. Most dissecting microscopes contain built in micrometer scales that are visible through the ocular, which are then used to determine the drop volume by employing the arithmetic diameter/volume relation assuming a perfect sphere shape. We advise injecting volumes $\leq$50 nl.

**Automated injection device** Oocyte manipulations can be carried out using a fully automated injection device known as the Robocyte[19]. This system was originally devised to perform robotic injections and patch-clamp readout routines for use in automated high-throughput drug-screening trials of ion-transporting channels. We adapted the injection procedure of this system to perform large-scale manipulation routines within a minimal time period (<2 min per 96-well plate). Mount pre-pulled injection needles on a 'standard' pneumatic oocyte-injection device for sample loading through the needle tip and conventional drop-volume calibration in mineral oil. We have found that this approach is superior to 'back-loading' via the larger needle opening at the mounting end, as the formation of trapped air bubbles is consistently avoided by this procedure. Injections are typically carried out with settings of 0.1 bar holding pressure, 0.7 bar injection pressure, 200 ms injection time and 500 μm injection depth, which correspond to calibrated sample volumes of 50 nl per oocyte/injection (s.d. ±10% or ±5 nl).

## PROCEDURE

**1|** Prepare in-cell NMR samples either by overexpression in bacteria (A) or using egg extracts or oocytes (B).

**(A) Preparing in-cell NMR samples by overexpresison in bacteria**

(i) Grow *E. coli* cells harboring the overexpression plasmid in 70 ml Luria broth (LB; or other rich) media to a high $OD_{600}$ (1.4–1.6). ● TIMING **6–8 h**

(ii) Harvest the cells by centrifugation at ~1,800*g* for 15 min at 4 °C.

(iii) Pour off the supernatant and resuspend the bacteria in 50 ml labeled media for 10 min.

(iv) After 5–10 min recovery time at 37 °C, induce overexpression (for example with IPTG).

(v) Depending on the protein and cells used, express for ~4 h (pET11a vector in BL21 bacteria). ● TIMING **4 h**

(vi) Close to the end of the overexpression period, take 500 μl bacteria solution, add 50 μl $D_2O$ and use this sample for shimming the magnet. Use for in-cell NMR experiments with bacteria temperatures of 37 °C (20 min).

(vii) Harvest the cells by careful centrifugation at ~1,200*g* for 20 min to create a relatively soft pellet.

(viii) Pour off the supernatant and resuspend the cells by adding small amounts of supernatant (~150–200 μl) and carefully pipetting the liquid phase up and down until the entire cell pellet has been removed (10 min).

▲ CRITICAL STEP Avoid creating bubbles. Make sure that the cell suspension is homogeneous and does not contain clumps of cells, as a nonhomogeneous distribution will further degrade the magnetic homogeneity of the sample. For in-cell NMR experiments using samples labeled specifically with certain amino acids, the spectral quality can be improved by washing the pellet to remove the unincorporated label. Resuspending the cells in 50 ml label-free minimal media and harvesting them again immediately reduces the concentration of free amino acids by ~90%.

(ix) Add 50 μl $D_2O$ to the same, but emptied, NMR tube that was used for shimming. Add on top the homogeneous bacterial slurry to the exact level used for shimming. Use a long pipette with a wide opening.

(x) Insert the sample into the magnet at a temperature of 37 °C. Shimming is insensitive, so only adjust the Z1 shim. If sedimentation of the cells over longer measurement periods becomes a problem, they can be encapsulated in low melting agarose as follows. Melt a solution of 2% (wt/vol) low melting agarose and slowly cool it to just below 40 °C. Add 500 μl of a concentrated bacterial slurry already containing 8% (vol/vol) $D_2O$ to an empty 1.5 ml tube. Add 100 μl of the melted, cooled agarose to the bacterial slurry, and mix well and rapidly. Use a long pipette to transfer the mixture immediately into the NMR tube and let the solution solidify.

▲ CRITICAL STEP Bacterial cells will be able to survive for several hours and will stay suspended for that time period. However, after only 30 min oxygen starvation will change the metabolism of the bacteria and result in a decrease in the cytoplasmic pH.

? TROUBLESHOOTING

(xi) To ensure that the observed NMR spectrum represents intracellular protein and that the signals are not caused by protein released from the bacteria due to cell lysis, move the sample to a centrifuge tube and centrifuge until all bacteria are collected in a pellet. Investigate the supernatant for any NMR signals.

(xii) The viability of the cells can be investigated by spreading the same small amount of sample before and after the NMR experiment (after dilution) on an LB plate containing the appropriate antibiotic. Counting the colonies reveals the survival rate of the bacteria.

**(B) Preparing in-cell NMR samples using egg extracts or oocytes**

(i) To prepare the protein of interest, express it recombinantly, incorporating the NMR-active isotope label or labels.

(ii) Purify the protein using standard protein chromatography approaches and the 'final protein buffer'.

(iii) Concentrate the pure protein with either a centrifugal filter device or stirred ultrafiltration cells. If using pre-harvested ovaries from a vendor, go to Step 1B(viii).

(iv) For microinjections, prime female frogs with 0.5 ml of 200 U $ml^{-1}$ PMSG between 2 and 5 d prior to harvesting the oocytes. Alternatively, oocytes can be obtained without hormone priming. This is especially suitable during northern hemisphere winter months (*X. laevis* is an amphibian from South Africa and its intrinsic 'seasonal' clock suggests a favorable summer climate when a cold climate prevails in the northern hemisphere).

(v) Anesthetize the frog by placing it in 1 l distilled water containing 1 g sodium bicarbonate and 1 g tricaine for 20 min.

(vi) Make a small incision through the abdominal tissue and remove the ovary lobe with forceps. ● TIMING **30 min**

(vii) Suture the incision closed and allow the frog to recover in isolation for 24 h before returning it to a communal tank. Alternatively, frogs can be sacrificed after oocyte removal, depending on the approved animal protocol. ● TIMING **30 min**

(viii) Place the freshly harvested ovaries on a plate of 2% (wt/vol) agarose made and covered with OR2 buffer. Vascular meninges surround the oocytes and must be removed prior to injections. There are two alternative approaches to removing these meninges: treatment with collagenase or manual peeling (2–6 h). Removal of the meninges with collagenase can be achieved as follows. Wash the ovaries in OR2 supplemented with 5% (wt/vol) collagenase, 1% (wt/vol) trypsin inhibitor and 1% (wt/vol) BSA for ~2 h at 18 °C on a rocking platform. Wash the collagenase-treated oocytes repeatedly with OR2 buffer and then with ND96 buffer.

▲ CRITICAL STEP Manually peeling the meninges off with a pair of forceps under a dissecting microscope requires considerable practice and is time consuming. The advantages over treating with collagenase are that the meninges removal is complete, the oocytes are typically healthier and components of the extracellular membrane have not undergone as harsh a treatment (which might not be critical for in-cell NMR analyses of intracellular protein samples).

(ix) Manually sort out healthy-looking stage VI oocytes. Stage VI cells are easily identified as the largest oocytes in the population, and are typically healthy when the darkly pigmented hemisphere (the animal pole) appears rich and uniformly colored.

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## BOX 1 | MANUAL AND AUTOMATED OOCYTE INJECTION PROCEDURES

**Manual injection**

1. Penetrate the immobilized oocyte at the equatorial plane separating the animal and the vegetal hemisphere of the cell with the injection needle.
▲ CRITICAL STEP Caution should be taken to insert the needle smoothly and with as little friction as possible. After sample deposition, withdraw the needle with equal precision and care.
● TIMING **2–4 h**
2. Upon completion of oocyte injections, inspect all manipulated cells for the degree of incision and sort out any obviously damaged cells.
3. Allow cells to recover for ≥3 h and again remove unhealthy discolored cells.

**Automated injection**

1. Transfer the injection needle containing the labeled protein sample onto the injection arm of the robotic injection device (Roboocyte).
2. Lock 96-well plates, containing one oocyte cell per well, onto the injection platform.
3. Align the injection needle and 96-well plate manually according to the manufacturer's instructions.
4. Perform the automated injection procedure.
5. Following injections, transfer the oocytes to large glass Petri dishes by collecting them from the 96-well plate with a standard pipette (cut tip).
6. Wash cells thoroughly and several times with excess volumes of ND96. We have found that automatically injected cells can be used immediately after washing as incisions are minimal. Hence, if kinetic studies of post-translational modifications are to be performed, for example, intracellular sample deposition is completed within 2 min, and collecting and washing cells can be achieved within 15 min. Equilibrating oocytes in ND96/$D_2O$ can be achieved within 20 min, and loading into NMR tubes is accomplished within <5 min. Hence, the in-cell NMR sample can be ready for NMR experiments within 45 min preparation time.

(x) Allow the cells to recover for 12 h in ND96 buffer at 18 °C prior to microinjection. When employing the robotic injection device, seed oocytes (one per well) into 96-well plates, and allow them to recover and adhere overnight before manipulations (see below).

(xi) For microinjection, seed prepared oocytes onto injection grids if manual injections are to be performed, or into 96-well cone-shaped plates if robotic manipulations are envisaged. Align cells with identical orientations in order to allow for rapid injections, and comparable settings for needle penetration and sample deposition. In the case of automated injections, we allow cells to settle and partially adhere to the plate for ~12 h. These manipulations are typically carried out under a microscope.

(xii) Centrifuge the concentrated protein for 10 min at 20,000*g* in a tabletop centrifuge at 4 °C to pellet any particulate matter or precipitate that might otherwise clog the needle. Mount and load the needle with the concentrated protein.

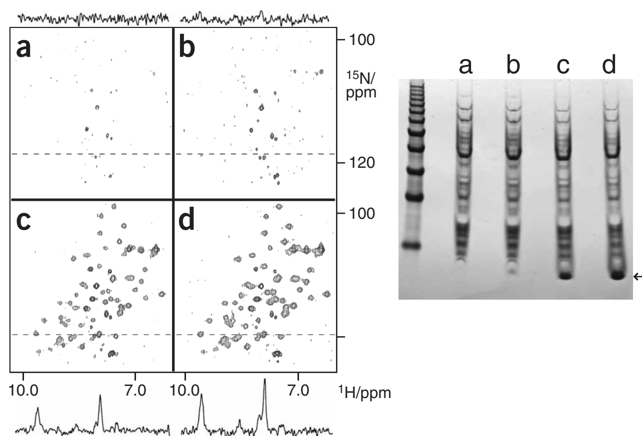


**Figure 1 |** Relationship between the level of protein expression and the quality of the in-cell NMR spectra. *E. coli* was grown in $^{15}$N-labeled M9 minimal medium for 10, 30, 60 or 120 min (**a–d**). In-cell NMR spectra (left) were acquired and SDS-PAGE with Coomassie staining (right) was performed for these samples. Reproduced from ref. 5 with permission.

(xiii) Inject the oocytes either manually or using an automated injection device (**Box 1**).

(xiv) To load the NMR tube, transfer the injected oocytes to ND96 buffer containing 10% (vol/vol) $D_2O$.

(xv) Fill a Shigemi NMR tube with ND96/$D_2O$ buffer.

(xvi) Carefully collect the oocytes with a pipette and add them individually to the top of the tube, allowing them to sediment by gravity. Occasionally swirl the tube to ensure optimal settling and packing.

(xvii) Count the number of oocytes per NMR sample and note the resulting volume of the specimen, as only this will allow you to accurately correlate the effective concentration of the sample to the intracellular molarity of your protein of interest.

▲ CRITICAL STEP Neither apply the Shigemi plunger nor remove the excess buffer from the tube for measurements.

(xviii) To produce *Xenopus* eggs, prime female frogs with 0.5 ml of 200 U ml$^{-1}$ PMSG and induce to lay 2 days later with 0.5 ml of 1,000 U ml$^{-1}$ HCG.

(xix) Collect and wash the eggs in MMR buffer. Remove the jelly surrounding the eggs by swirling them in XB buffer supplemented with 2% (wt/vol) cysteine (~20 min or until the surrounding jelly is no longer visible and the eggs 'align' more closely. Note that the presence of the jelly envelope functions to protect eggs in their natural environment and additionally serves as a spacer that prevents eggs from attaching to each other).

(xx) Rinse the eggs repeatedly with MMR buffer to remove the cysteine.

(xxi) For CSF extracts, wash the eggs in CSF-XB. For interphase extracts, wash the eggs in egg lysis buffer. CSF extracts mimic cells arrested in metaphase II of meiosis. Interphase extracts closely resemble the cytoplasm of eggs in interphase. These buffers can be made with 10% (vol/vol) $D_2O$ to avoid the need to add it to the NMR sample later.

(xxii) Transfer the eggs into polypropylene centrifuge tubes and remove the excess buffer by sucking off with a vacuum manifold.

(xxiii) Add cytochalasin B and protease inhibitors leupeptin, pepstatin A and chymostatin (LPC) at 10 μg ml$^{-1}$. Hereafter, keep the sample on ice or at 4 °C.

(xxiv) Pack the eggs by centrifuging for 1 min at 400*g* at 4 °C.

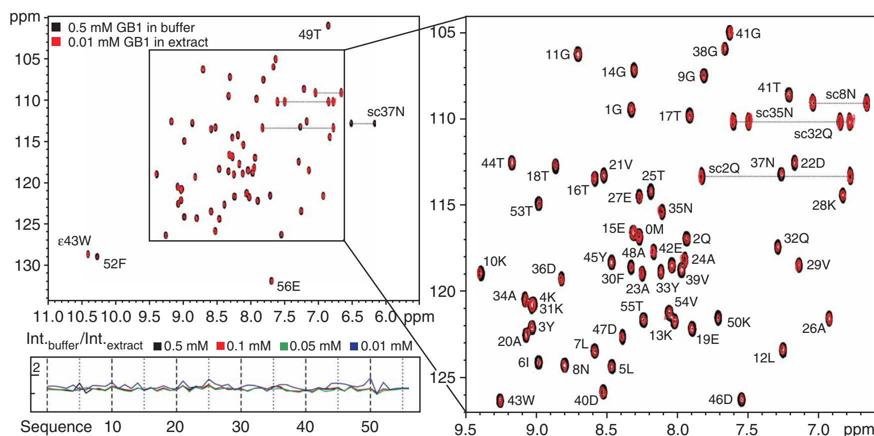


**Figure 2 |** Overlay of the [$^{15}$N, H]-HSQC spectra of purified GB1 (black) and GB1 in crude *Xenopus* egg extracts (red). Reproduced from ref. 6 with permission.

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(xxv) Remove the excess buffer and then crush the cells at 12,000*g* for 15 min at 4 °C. Note that crushing at 16,000*g* works equally well.

(xxvi) Remove the crude interphase extract by piercing the side of the tube with an 18-G needle attached to a 5-ml syringe.

■ **PAUSE POINT** CSF extracts can be used immediately or frozen in liquid nitrogen and stored at −80 °C for later use.

(xxvii) Add concentrated protein to the egg extracts so that the former comprises ≤20% of the final volume.

(xxviii) Pipette the mixture into a NMR tube.

## ● TIMING

Step 1A(i): 6–8 h
Step 1A(v): 4 h
Step 1B(vi): 30 min
Step 1B(vii): 30 min

## ? TROUBLESHOOTING

If measuring in-cell [$^{15}$N, $^{1}$H]-heteronuclear single-quantum coherence (HSQC) experiments of well-folded proteins in bacterial cells results in spectra that show only a limited number of signals, mainly between 8 and 8.5 ppm on the proton chemical shift axis, the protein of interest most likely interacts with cellular components that slow the rotational tumbling sufficiently to broaden its resonance lines beyond the detection limit. Use SDS–polyacrylamide gel electrophoresis (PAGE) to ensure that the protein is well expressed (visible as a strong band on the gel). If the expression level is not the problem, lyse the cells by adding lysozyme. Cell lysis will destroy weak complexes and make the NMR spectrum of the overexpressed protein visible.
If line-broadening due to weak nonspecific interactions with other cellular components is the reason for the failure to observe an in-cell NMR spectrum, try to label the protein with either $^{13}$C-methyl group-labeled methionine or δ-methyl group-labeled isoleucine. The higher sensitivity of methyl groups and, in particular, the faster internal rotation allows one to detect many proteins that are not visible by amide proton-based in-cell NMR experiments.

## ANTICIPATED RESULTS

**Figure 1** illustrates the relationship between protein expression levels and the quality of the in-cell NMR spectra.
On the left are a series of 10 min [$^{15}$N, $^{1}$H]-HSQC in-cell NMR experiments conducted on *E. coli* grown in $^{15}$N-labeled M9 minimal medium that have expressed a 7.5-kDa bacterial protein domain for 10 min, 30 min, 1 h and 2 h. On the right is a Coomassie-stained SDS-PAGE gel indicating the amount of overexpressed protein (arrow) found in each of the samples. The most prominent chemical shift resonances are just visible after 30 min when the overexpressed protein is barely detectable on the gel. Longer measurement times can compensate to some extent for lower expression levels, although increasing the number of scans by a factor *N* only improves the signal-to-noise ratio by $\sqrt{N}$.

**Figure 2** shows an overlay of an [$^{15}$N, H]-HSQC spectrum of purified GB1 (0.5 mM; black) and of GB1 in crude *Xenopus* egg extracts (10 μM; red). Both spectra were acquired with 16 transients within 43 min. In addition, intensity ratios of equivalent peaks in the 2D spectrum of purified protein and in the 2D spectrum of extracts are shown for GB1 concentrations ranging from 10 to 500 μM. For all concentrations analyzed, a similar peak reduction of ∼1.5 fold in the extract spectra relative to the spectra of the purified protein in buffer is observed.

**Figure 3** compares an [$^{15}$N, $^{1}$H]-HSQC spectrum of purified GB1 (0.5 mM; black) with a spectrum of GB1 in *Xenopus* oocytes (50 μM intracellular concentration; red). Both spectra were measured with 32 transients within 90 min. A calculation of the ratio of peak intensities in the buffer spectrum and the oocyte spectrum in the concentration range from 50 to 500 μM demonstrates an average fivefold intensity reduction in the oocyte spectra. The small insert in the spectrum shows a typical in-cell NMR sample of ∼200 sedimented oocytes in a Shigemi NMR tube.

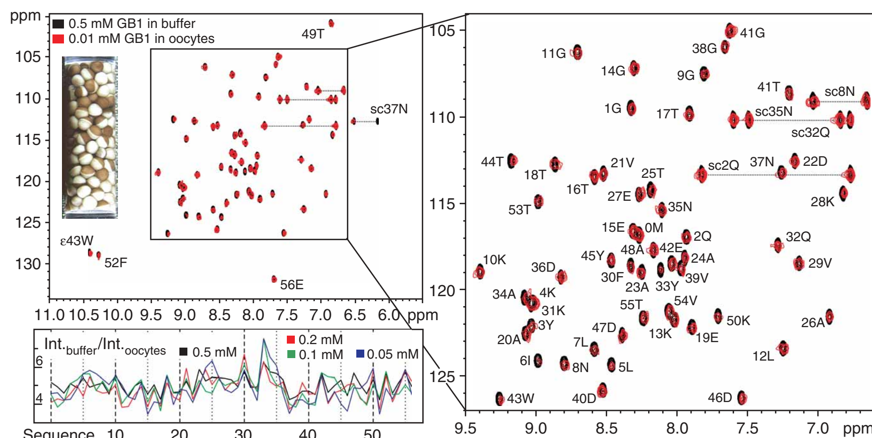


**Figure 3 |** Overlay of the [$^{15}$N, H]-HSQC spectra of purified GB1 (black) and GB1 in *Xenopus* oocytes (red). The insert is a photograph of a portion of an NMR tube containing *Xenopus* oocytes. Reproduced from ref. 6 with permission.

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**COMPETING INTERESTS STATEMENT** The authors declare that they have no competing financial interests.

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