# Chapter 2

# In-Cell NMR of Intrinsically Disordered Proteins in Prokaryotic Cells

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## Abstract

In-cell NMR, i.e., the acquisition of heteronuclear multidimensional NMR of biomacromolecules inside living cells, is, to our knowledge, the only method for investigating the three-dimensional structure and dynamics of proteins at atomic detail in the intracellular environment. Since the inception of the method, intrinsically disordered proteins have been regarded as particular targets for in-cell NMR, due to their expected sensitivity to the molecular crowding in the intracellular environment. While both prokaryotic and eukaryotic cells can be used as host cells for in-cell NMR, prokaryotic in-cell NMR, particularly employing commonly used protein overexpression systems in *Escherichia coli* cells, is the most accessible approach. In this chapter we describe general procedures for obtaining in-cell NMR spectra in *E. coli* cells.

**Key words:** In-cell NMR, *Escherichia coli*, Heteronuclear multidimensional NMR, Rapid NMR measurement, Protein structure, Protein dynamics

## 1. Introduction

Recent improvements in the measurement sensitivity of NMR spectra as the result of developments in hardware, pulse sequences, and stable isotope labeling techniques allow the observation of high-resolution heteronuclear multidimensional NMR spectra of proteins inside living cells (in-cell NMR) (1–8). Due to the noninvasive character of NMR spectroscopy and its ability to provide data at atomic resolution, in-cell NMR is an excellent tool for investigating protein behavior in detail inside living cells under macromolecular crowding (9).

In order to observe in-cell NMR spectra, the proteins of interest must be abundant and labeled selectively with NMR active stable isotopes such as $^{13}C$ and $^{15}N$. In-cell NMR experiments can be performed both in prokaryotic and eukaryotic cells, although different labeling procedures are required. For prokaryotic in-cell NMR

studies (10–12) the proteins of interest are labeled by utilizing protein expression systems in the host cells. The basic principle relies on the fact that recombinant protein production driven by strong promoters leads to the rapid intracellular accumulation of large amounts of the expression system-encoded proteins to the almost complete exclusion of new endogenous protein synthesis. Thus, switching from unlabeled to isotope-labeled growth medium shortly before recombinant protein induction results in the selective labeling of the protein of interest. For eukaryotic in-cell NMR, labeled proteins are first purified, and then incorporated into cells by microinjection (13–15), by combining with a cell-penetrating peptide (16), or through resealable pores (17).

Prokaryotic, particularly *Escherichia coli*, in-cell NMR is the easiest system, and thus has been used for the investigation of various protein systems and biological events, such as protein–protein interactions (4, 18), protein–DNA interactions (19), intracellular protein dynamics (20), protein stability (21), and the screening of small molecule interactor libraries (22). We have recently demonstrated three-dimensional protein structure determination exclusively on the basis of information obtained by in-cell NMR (23).

The behavior of intrinsically disordered proteins (IDPs) in cells is also an important target for in-cell NMR. Recently, in-cell NMR using *Xenopus* oocytes has been applied to the human amyloid protein Tau, one of the largest known IDPs (15). Prokaryotic in-cell NMR has also been used for the investigation of IDPs since the inception of this method, and it has been demonstrated that the bacterial protein FlgM is completely unfolded in vitro, but appears partially folded when analyzed in the context of live bacteria (24). With interest in the structure–function relationship of IDPs, particularly inside cells, growing and with the technique's relative experimental convenience, we anticipate the application of prokaryotic in-cell NMR to various IDPs in the near future.

Thus, we describe here a general procedure for *E. coli*-based in-cell NMR experiments, including sample preparation, rapid NMR measurement, data processing, and analysis. Being critical for the validation of in-cell NMR, procedures for checking the sample condition and the viability of the cells are also described.

## 2. Materials

In this section, we list the materials required in our typical procedure for the preparation of $^{15}$N-labeled *E. coli* cell samples and in-cell NMR measurement. The modifications required for other stable isotope labeling patterns are described in Note 1. The details of apparatus required for standard molecular biological and biochemical experiments, e.g., devices for aseptic techniques, incubators, centrifuges, etc., are omitted.

**2.1. Large Scale E. coli Culture and Stable Isotope Labeling**

1. Expression plasmid encoding the protein of interest (see Note 2).

2. *E. coli* strains: e.g., JM109 (DE3) *E. coli*, BL21 (DE3) *E. coli* (see Note 3).

3. LB medium: 10 g/l Bacto tryptone, 5 g/l Bacto Yeast Extract, 10 g/l NaCl. Sterilize by autoclaving.

4. $MgSO_4$ stock solution: 1 M $MgSO_4$. Store at room temperature.

5. $CaCl_2$ stock solution: 50 mM $CaCl_2$. Store at room temperature.

6. $FeCl_3$ stock solution: 5 mM $FeCl_3 \cdot 6H_2O$. Store at 4°C.

7. Metal mixture stock solution: 4 mM $ZnSO_4 \cdot 7H_2O$, 1 mM $MnSO_4 \cdot 5H_2O$, 4.7 mM $H_3BO_3$, 0.7 mM $CuSO_4 \cdot 5H_2O$. Store at 4°C.

8. Thiamine stock solution: 0.3 M Thiamine hydrochloride. Store at −20°C.

9. M9 minimal medium (unlabeled, 100 ml): 1.2 g $Na_2HPO_4$, 0.6 g $KH_2PO_4$, 0.1 g $NH_4Cl$, 0.1 g NaCl, 0.2 g D-glucose, 200 μl $MgSO_4$ stock solution, 200 μl $CaCl_2$ stock solution, 40 μl $FeCl_3$ stock solution, 40 μl thiamine stock solution, 100 μl Metal mixture stock solution. Prepare just before use. Filter-sterilized.

10. M9 minimal medium ($^{15}N$-uniformly labeled, 100 ml): Same as the composition of unlabeled M9 minimal medium with the exception that unlabeled $NH_4Cl$ was replaced by the same concentration of $^{15}NH_4Cl$. Prepare just before use. Filter-sterilized (see Note 1).

11. Reagent for induced expression of the target protein. For example, isopropyl thio-β-D-thiogalactoside.

12. Antibiotics stock solution. For example, ampicillin stock solution: 50 mg/ml Ampicillin sodium. Store at −20°C.

**2.2. NMR Measurement**

1. NMR spectrometer (for specifications and pulse sequences see Notes 4 and 5, respectively).

2. Linux computer system for data processing and analysis of spectra (for specifications and installed software see Notes 6 and 7, respectively).

## 3. Methods

In this section, we focus on the methods required for typical in-cell NMR experiments of proteins overexpressed in *E. coli* cells; thus, standard procedures and troubleshooting for the construction of protein expression systems in *E. coli* are omitted.

*3.1. Preparation of Proteins Inside Living E. coli Cells*

1. Transform *E. coli* cells with the overexpression plasmid.

2. Grow the *E. coli* cells in 2 ml LB media at 37 °C with shaking to a high $OD_{600}$ of ~2.0 (see Note 2).

3. Subculture the *E. coli* cells (100 μl) in 100 ml unlabeled M9 media, and incubate the culture at 37 °C until the $OD_{600}$ reaches 0.5–0.6.

4. Centrifuge the culture at ~800 × *g* for 20 min at room temperature.

5. Decant the supernatant. Centrifuge the pellet again at ~800 × *g* for 5 min at room temperature.

6. Remove the supernatant by pipetting and resuspend the cells in 100 ml stable isotope-labeled M9 media (see Note 8).

7. Incubate the cells at 37 °C without shaking for 1 h.

8. Induce the production of the target protein (for example, by adding isopropyl thio-β-D-thiogalactoside to a final concentration of 0.5 mM).

9. Continue protein expression with shaking at optimal temperature (for example, 3 h at 37 °C in our previous in-cell NMR study of *T. thermophilus* HB8 TTHA1718 (23)).

10. Harvest the cells by centrifugation at ~400 × *g* for 30 min at room temperature.

11. Remove the supernatant by aspiration and resuspend the cells by adding small amounts of unlabeled M9 media (140–160 μl) and carefully pipetting the solution up and down until the entire cell pellet has been suspended. The cells are harvested by gentle centrifugation and placed as a ~60 % slurry with M9 medium.

12. Add $D_2O$ (10 % of the final sample volume) to the bacterial slurry, and transfer the sample into an NMR tube (see Note 9).

*3.2. Checking the Sample Conditions for Sample Stability and Viability of the Cells During In-Cell NMR Experiments (see Note 10)*

1. Insert the in-cell NMR sample into the magnet and tune the probehead (see Note 11).

2. Perform a series of short, diagnostic NMR experiments (e.g., 2D $^1H$-$^{15}N$ HSQC experiment with 20 min duration). The appearance of significant changes in the spectra indicates the limit of sample stability.

3. Between each NMR experiment, transfer 10 μl from the NMR sample to a microfuge tube and centrifuge at high speed to pellet the bacteria. Store the pellet and supernatant separately for SDS-PAGE (see Note 12) to check the stability of the expressed protein.

4. Check the viability of the cells during NMR experiments can be investigated by a plating colony test. Spread a small volume of the NMR sample (~10 μl) taken before and after the experiments on LB plates containing the appropriate antibiotic.

Incubate the plates overnight at 37 °C and count the colonies. In our laboratories the acceptable percentage of viability limits is set to be 80–85 %.

**3.3. NMR Measurements, Data Processing, and Analysis**

1. Insert the in-cell NMR sample into the magnet and tune the probehead.

2. Check the sample's condition by measuring 1D $^1$H-NMR spectra and 1D (or 2D) $^1$H-$^{15}$N HSQC spectra.

3. Collect 2D/3D NMR spectra (see Notes 13 and 14). Figure 1a shows the 2D $^1$H-$^{15}$N HSQC spectrum of an IDP, the C-terminal region of *S. cerevisiae* Mre11, in living *E. coli* cells, in comparison with the spectrum from the purified sample (Fig. 1b). Due to the higher viscosity inside cells (25), which increases the rotational correlation time and apparent molecular mass of proteins, and the inhomogeneity of in-cell NMR samples, the line shape for both $^1$H and $^{15}$N dimensions of cross peaks are much broadened when compared to the in vitro sample. The duration of the NMR experiments for an in-cell
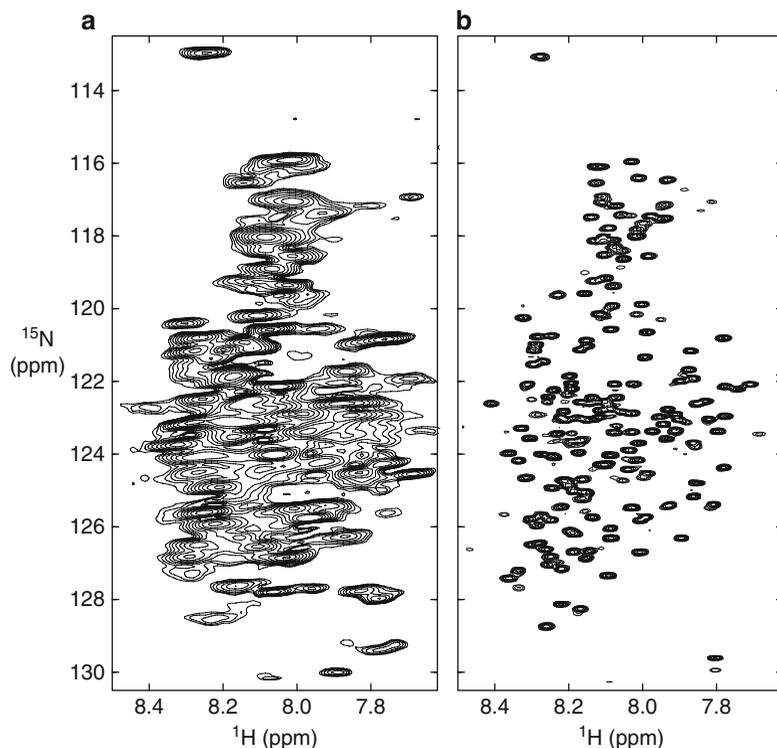


Fig. 1. 2D $^1$H-$^{15}$N HSQC spectra of the C-terminal region of *S. cerevisiae* Mre11 in *E. coli* cells (**a**) and in vitro (**b**). The spectra were acquired with eight transients and a total of 1,024 ($t_2$, $^1$H$^N$) × 64 ($t_1$, $^{15}$N) complex points at a probe temperature of 37 °C. For in-cell NMR measurement, *E. coli* strain BL21 (DE3) was used. Resolution enhancement for the $t_1$ dimension was achieved by applying linear prediction prior to Fourier transform on Azara v2.8 software.

NMR sample must be set considering the life time of the *E. coli* cells under the measurement conditions (see Note 15).

4. Proteins may leak from the cells, invalidating the measured data, so to make sure that the measured NMR data are definitely from the protein molecules inside cells, remove the cells from the sample after measurements by centrifugation (e.g., $\sim 16{,}000 \times g$ for 5 min). Retain the supernatant of the measured sample, make it up with unlabeled M9 medium to the initial sample volume, and measure a $^1$H-$^{15}$N HSQC experiment of it. Check that no or only very weak cross peaks due to the target protein is observed in the spectrum (see Note 16).

5. To make sure that the target protein is inside the cells, lyse the harvested cells, fill up with unlabeled M9 medium to the initial sample volume, and measure $^1$H-$^{15}$N HSQC experiment of it. Check that much sharper cross peaks are observed in the spectrum (see Note 16).

6. Process NMR spectra with appropriate procedure (e.g., maximum entropy reconstruction for nonlinearly sampled data) (see Notes 5 and 7).

7. Analyze NMR spectra with interactive NMR spectrum analysis software (see Note 7).

## 4. Notes

1. The choice of stable isotope labeling pattern depends on the purpose of the experiments. For simple observation of or monitoring the behavior of proteins of interest inside *E. coli* cells by 2D $^1$H-$^{15}$N correlation experiments, uniform $^{15}$N-labeling is sufficient. On the other hand, when resonance assignments are required from spectra acquired from in-cell NMR samples, uniform $^{13}$C/$^{15}$N-labeling is required and $^{13}$C/$^{15}$N-labeled M9 minimal medium is used for sample preparation, in which unlabeled D-glucose in the $^{15}$N-labeled M9 medium is replaced by the same concentration of uniformly $^{13}$C-labeled glucose, e.g., U-$^{13}$C-glucose.

   Since IDPs tend to provide $^1$H-$^{15}$N correlation spectra with poorer peak separation than proteins with defined structures (see Fig. 1a), selective labeling procedures may be very useful. For selective $^{15}$N-labeling of amino acids which are end products of biosynthetic pathways in *E. coli* (e.g., lysine), addition of a supply of the $^{15}$N-labeled amino acid(s) to the unlabeled M9 minimal medium is sufficient (11, 26). Though we have not tested them in our groups, auxotrophic *E. coli* strains lacking some transaminase activity may be required for the cases where isotope scrambling is expected during biosynthesis (27).
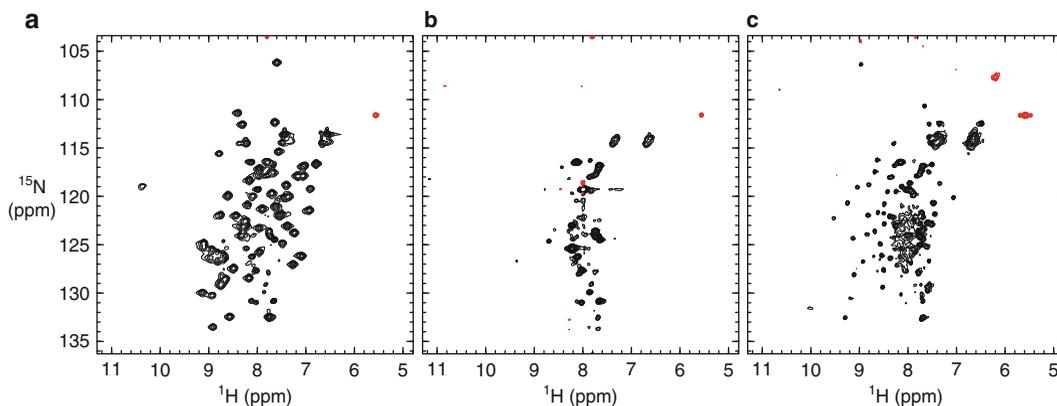
Fig. 2. 2D in-cell $^1$H-$^{15}$N HSQC spectra of *T. thermophilus* HB8 TTHA1718 (**a**), TTHA1431 (**b**) and TTHA1912 (**c**) in *E. coli* strain JM109 (DE3). The expression plasmids encoding TTHA1431, TTHA1718 and TTHA1912 (51) are purchased from RIKEN BioResource Center (http://www.brc.riken.jp/lab/dna/en/thermus_en.html). The spectra were acquired with eight transients and a total of 1,024 ($t_2$, $^1$H$^N$) × 64 ($t_1$, $^{15}$N) complex points at a probe temperature of 37 °C.

Selective $^{13}$C-labeling at side-chain methyl groups is another useful option. Serber et al. employed methyl $^{13}$C-labeling at methionine residues as probes in in-cell NMR (28). In our previous in-cell NMR study (23), aimed at observing NOEs involving methyl groups, we employed selectively $^1$H/$^{13}$C-labeling at methyl groups of aliphatic amino acid residues by using amino acid precursors (29). Typically, 10 mg of $^{13}$C- or $^2$H/$^{13}$C-labeled amino acids or precursors, e.g., [3-$^{13}$C] alanine (ISOTEC), [u-$^{13}$C, 3-$^2$H] α-ketoisovalerate (Cambridge Isotope Laboratories) for leucine and valine residues, and [u-13C, 3,3-$^2$H] α-ketobutyrate (Cambridge Isotope Laboratories) for isoleucine residues, are supplemented in 100 ml of M9 minimal medium prepared using 100 % D$_2$O.

In addition, $^{19}$F-labeling utilizing fluoro amino acids (30), as well as site-specific incorporation of labeled nonnatural amino acids (31), has been reported recently.

2. Higher expression level does not always promise good in-cell NMR spectra. Despite high intracellular concentration, no, or very weak cross peaks are sometimes observed from the target protein in in-cell NMR spectra. Fig. 2a, b show 2D $^1$H-$^{15}$N HSQC spectra of *Thermus thermophilus* HB8 TTHA1718 and TTHA1431 in *E. coli* cells, respectively. While both of these proteins express very well inside *E. coli* cells, theTTHA1431 sample shows very poor spectra in which only background cross peaks are observed, in contrast to TTHA1718. The reasons for such results will be case-specific with motional restrictions due to nonspecific interactions with other cellular components inside *E. coli* cells being a possible explanation. Usually drastic improvement cannot be guaranteed, but it is worth exploring alternative

protein expression conditions, varying, e.g., *E. coli* host strains, timing of induction, temperature, and incubation times.

3. We usually try at least two *E. coli* strains, e.g., JM109 (a K-12 strain) and BL21 (a B strain) when starting in-cell NMR experiments with a new protein. In many cases, we obtain similar results from both strains, but we have also experienced the case that good $^1$H-$^{15}$N HSQC spectra were observed when using JM109, while broadened cross peaks were observed when using BL21.

4. NMR spectrometers and probeheads must be equipped for heteronuclear $^1$H-detection experiments or triple-resonance ($^1$H/$^{13}$C/$^{15}$N) experiments. Hardware enabling pulsed field gradients is highly recommended, since efficient water suppression in in-cell NMR experiments is very difficult to achieve without them. As sensitivity is one of the limiting factors of in-cell NMR experiments, a cryogenic probehead and a magnetic field strength corresponding to a $^1$H frequency of at least 500 MHz are recommended.

5. A major hurdle for in-cell NMR experiments is the limited lifetime of the cells inside the NMR sample tube. Standard 3D NMR experiments usually require 1–2 days of data collection, which is an unacceptably long time for live cells. NMR techniques for rapid measurement of multidimensional experiments are therefore very important. As was demonstrated in the in-cell NMR study using human cultured cells (16), application of SOFAST-HMQC and related techniques (32, 33) to 2D/3D experiments will be advantageous. In addition, nonlinear sampling (non uniform sampling) techniques for the indirectly acquired dimensions (34–36) are also very helpful. Spectrometers should be prepared so as to run these new techniques (they are starting to be usable as standard tools on very recent NMR spectrometers). For nonlinear sampling, the pulse sequences have to be prepared so as to control sampling points according to sampling schedule lists as opposed to conventional sampling of every point on a regularly spaced grid (26, 36).

6. Data processing and spectral analysis can in principle be run on a computer with a single processor. In case the project includes structure calculations, multicore PC systems or PC clusters are preferable.

7. Software for data processing must be installed. Particularly, when employing nonlinear sampling schemes for obtaining multidimensional NMR spectra, the software must be able to handle sparsely sampled data. In our laboratories, the two-dimensional maximum entropy method (2D MaxEnt) (37, 38) on AZARA 2.8 software (W. Boucher, http://www.bio.cam.ac.uk/azara) is used. The Rowland NMR Toolkit (http://webmac.rowland.

org/rnmrtk/) has also been used for this type of processing. Note that, in addition to MaxEnt, other processing procedures, such as multidimensional decomposition (39) and multidimensional Fourier transform (40, 41), can also be used.

An interactive NMR spectrum analysis software is required. In our laboratories, either an OpenGL version of ANSIG 3.3 (42, 43) or CcpNmr Analysis (44) is used. Alternatively, programs such as NMRView (One Moon Scientific, inc) (45, 46), Sparky (http://www.cgl.ucsf.edu/home/sparky/), XEASY (47) (http://www.mol.biol.ethz.ch/groups/wuthrich_group/software), and CARA (http://www.nmr.ch/) can be used.

When the project includes structural analysis, software for structure calculations has to be installed. In our laboratories, CYANA 3.0 (http://www.cyana.org) (48) and ARIA 2.3 (http://aria.pasteur.fr) (49) are used for automated NOESY assignment and structure calculation.

8. In order to produce a sample of *E. coli* cells in which only the target protein is labeled with $^{13}$C and $^{15}$N, the cells harboring the expression plasmid are first grown in unlabeled LB medium, and then transferred into M9 minimal medium containing stable isotopes where protein expression is induced.

9. The concentration of the expressed protein in *E. coli* cells can be estimated by comparing the density of the Coomassie-stained bands in SDS-PAGE gels with those of proteins with similar molecular size and known concentration.

10. To ensure that the observed in-cell NMR spectrum represents intracellular protein and that the signals are not caused by proteins released from the bacteria, the sample conditions and the viability of the cells during the experiments has to be checked before the "real" measurements.

11. Prior to the measurement of the in-cell NMR sample, shim the magnetic field with a separate NMR sample containing unlabeled M9 media (10 % D$_2$O), which is prepared with the same sample length as for the in-cell NMR sample.

12. Sometimes we experienced leakage of the target protein from cells during NMR experiments. Figure 2c shows a typical in-cell NMR spectrum when leakage has occurred. Sharp cross peaks emerge and increase their intensity during the timecourse of measurements. In another case, leakage was largely prevented by reducing the temperature for protein expression and NMR measurement.

Li et al. reported that encapsulation in alginate microcapsules stabilizes *E. coli* cells and prevents leakage (20).

13. It is highly recommended to estimate the allowable maximum experimental duration under the measurement conditions. In our previous in-cell NMR study of *T. thermophilus* HB8

TTHA1718, the virtual identity of $^1$H-$^{15}$N HSQC spectra recorded immediately after sample preparation and after 6 h in an NMR tube at 37 °C shows that TTHA1718 in-cell NMR samples are stable for at least 6 h, which was also confirmed by plating colony test (the viability of the bacteria in the in-cell samples after 6 h of NMR measurements was 85 ± 11 %) (23).

14. The present protocol does not require a specific set of NMR spectra. If duration of experiments and sensitivity permit, any of the common 2D and 3D experiments can be applied. In our previous study of in-cell structure determination of *T. thermophilus* HB8 TTHA1718 (23, 26), we measured three 3D NOESY-type experiments in addition to 2D $^1$H-$^{15}$N and $^1$H-$^{13}$C HSQC experiments. For application to IDPs, another 3D experiments which are widely used for backbone resonance assignment of IDPs, such as HNN and HN(C)N (50), can be considered, though we have not tested them on our in-cell NMR samples yet.

15. We recommend repeatedly monitoring the stability of the *E. coli* samples by 2D $^1$H-$^{15}$N HSQC spectra followed by plating colony tests, which allows for a comparison between the initial and current health of the cells.

    With the nonlinear sampling scheme the duration of each 3D experiment is reduced to 2–3 h. Repeat the measurement of each 3D experiment several times interleaved with a short 2D $^1$H-$^{15}$N HSQC experiment used to monitor the condition of the sample. Combine these 3D data to generate a new data set with improved signal-to-noise ratio up to the point that the 2D spectra exhibit significant changes.

16. These results were corroborated by SDS-PAGE, demonstrating that the contribution of extracellular protein to the observed signals is negligible.

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