

Video Article

Nuclear Magnetic Resonance Spectroscopy for the Identification of Multiple Phosphorylations of Intrinsically Disordered Proteins

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Abstract

Aggregates of the neuronal Tau protein are found inside neurons of Alzheimer's disease patients. Development of the disease is accompanied by increased, abnormal phosphorylation of Tau. In the course of the molecular investigation of Tau functions and dysfunctions in the disease, nuclear magnetic resonance (NMR) spectroscopy is used to identify the multiple phosphorylations of Tau. We present here detailed protocols of recombinant production of Tau in bacteria, with isotopic enrichment for NMR studies. Purification steps that take advantage of Tau's heat stability and high isoelectric point are described. The protocol for *in vitro* phosphorylation of Tau by recombinant activated ERK2 allows for generating multiple phosphorylations. The protein sample is ready for data acquisition at the issue of these steps. The parameter setup to start recording on the spectrometer is considered next. Finally, the strategy to identify phosphorylation sites of modified Tau, based on NMR data, is explained. The benefit of this methodology compared to other techniques used to identify phosphorylation sites, such as immuno-detection or mass spectrometry (MS), is discussed.

Video Link

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Introduction

One of the main challenges of healthcare in the 21st century are neurodegenerative diseases such as Alzheimer's disease (AD). Tau is a microtubule-associated protein that stimulates microtubule (MT) formation. Tau is equally involved in several neurodegenerative disorders, so-called tauopathies, of which the best known is AD. In these disorders, Tau self-aggregates in paired helical filaments (PHFs) and is found modified on many residues by posttranslational modifications (PTMs) such as phosphorylation¹. Phosphorylation of Tau protein is implicated in both regulation of its physiological function of MT stabilization and pathological loss of function that characterizes AD neurons.

Furthermore, Tau protein, when integrated in PHFs in diseased neurons, is invariably hyperphosphorylated². Unlike normal Tau that contains 2-3 phosphate groups, the hyperphosphorylated Tau in PHFs contains 5 to 9 phosphate groups³. Hyperphosphorylation of Tau corresponds both to an increase of stoichiometry at some sites and to phosphorylation of additional sites that are called pathological sites of phosphorylation. However, overlap exists between AD and normal adult patterns of phosphorylation, despite quantitative differences in the level⁴. How specific phosphorylation events influence function and dysfunction of Tau remains largely unknown. We aim to decipher Tau regulation by PTMs at the molecular level.

To deepen the understanding of the molecular aspects of Tau, we have to address technical challenges. Firstly, Tau is an intrinsically disordered protein (IDP) when isolated in solution. Such proteins lack well-defined three-dimensional structure under physiological conditions and require particular biophysical methods to study their function(s) and structural properties. Tau is a paradigm for the growing class of IDPs, often found associated with pathologies such as neurodegenerative diseases, hence increasing the interest to understand the molecular parameters underlying their functions. Secondly, characterization of Tau phosphorylation is an analytical challenge, with 80 potential phosphorylation sites along the sequence of the longest 441 amino-acid Tau isoform. A number of antibodies have been developed against phosphorylated epitopes of Tau and are used for detection of pathological Tau in neurons or brain tissue. Phosphorylation events can take place on at least 20 sites targeted by proline-directed kinases, most of them in close proximity within the Proline-rich region. The qualitative (which sites?) and quantitative (what stoichiometry?) characterization is difficult even by the most recent MS techniques⁵.

NMR spectroscopy can be used to investigate disordered proteins that are highly dynamic systems constituted of ensembles of conformers. High-resolution NMR spectroscopy was applied to investigate both structure and function of the Tau protein. In addition, the complexity of Tau's phosphorylation profile led to the development of molecular tools and new analytical methods using NMR for the identification of phosphorylation sites⁶⁻⁸. NMR as an analytical method allows for the identification of Tau phosphorylation sites in a global manner, visualization of all the single-site modifications in a single experiment, and quantification of the extent of phosphate incorporation. This point is essential since although phosphorylation studies on Tau abound in the literature, most of them have been performed with antibodies, leaving a large degree of uncertainty over the complete profile of phosphorylation and thus the true impact of individual phosphorylation events. Recombinant kinases including PKA, Glycogen-synthase kinase 3 β (Gsk3 β), Cyclin-dependent kinase 2/cyclin A (CDK2/CycA), Cyclin-dependent kinase 5 (CDK5)/p25 activator protein, extracellular-signal-regulated kinase 2 (ERK2) and microtubule-affinity-regulating kinase (MARK), which show phosphorylation activity towards Tau, can be prepared in an active form. In addition, Tau mutants that allow for generating specific Tau protein isoforms with well-characterized phosphorylation patterns are used to decipher the phosphorylation code of Tau. NMR spectroscopy is then used to characterize enzymatically modified Tau samples⁶⁻⁸. Although *in vitro* phosphorylation of Tau is more challenging than pseudo-phosphorylation such as by mutation of selected Ser/Thr into glutamic acid (Glu) residues, this approach has its merits. Indeed, neither the structural impact nor interaction parameters of phosphorylation can always be mimicked by glutamic acids. An example is the turn motif observed around phosphoserine 202 (pSer202)/phosphothreonine 205 (pThr205), which is not reproduced with Glu mutations⁹.

Here, the preparation of isotopically labeled Tau for NMR investigations will be described first. Tau protein phosphorylated by ERK2 is modified on numerous sites described as pathological sites of phosphorylation, and thus represents an interesting model of hyperphosphorylated Tau. A detailed protocol of Tau *in vitro* phosphorylation by recombinant ERK2 kinase is presented. ERK2 is activated by phosphorylation by mitogen activated protein kinase/ERK kinase (MEK)¹⁰⁻¹². In addition to the preparation of modified, isotopically-labeled Tau protein, the NMR strategy used for identification of the PTMs is described.

Protocol

1. Production of ¹⁵N, ¹³C-Tau (Figure 1)

- Transform pET15b-Tau recombinant T7 expression plasmid^{13,14} into BL21(DE3) competent *Escherichia coli* bacterial cells¹⁵.
NOTE: the cDNA coding for the longest (441 amino acid residues) Tau isoform is cloned between *NcoI* and *XhoI* restriction sites in the pET15b plasmid.
 - Mix gently 50 μ l of competent BL21(DE3) cells, forming 1-5 $\times 10^7$ colonies per μ g of plasmid DNA, with 100 ng of plasmid DNA in a 1.5 ml plastic tube.
NOTE: Codon-usage optimized bacterial strains for eukaryotic cDNA expression are not essential to produce human Tau.
 - Place the cell mixture on ice for 30 min and then heat shock for 10 sec at 42 °C. Place the tube back on ice for 5 min and add 1 ml of room temperature LB (Luria-Bertani) medium. Incubate the bacterial suspension at 37 °C for 30 min under gentle agitation.
- Spread using an inoculation loop 100 μ l of cell suspension evenly onto an agar plate of LB medium containing 100 μ g/ml of ampicillin antibiotic.
- Incubate the selection plate for 15 hr at 37 °C.
- Keep the selection plate at 4 °C until proceeding to the culture step, for a maximum of 2 weeks approximately.
NOTE: a glycerol stock of bacterial culture (50% glycerol), stored at -80 °C, can be prepared to start the culture at a later stage.
- Add 1 ml 1 M MgSO₄, 1 ml 100 mM CaCl₂, 10 ml 100x MEM vitamin complement, 1 ml 100 mg/ml ampicillin to 1 L of autoclaved M9 salts (6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl).
NOTE: A white precipitate will form upon addition of the CaCl₂ solution to the M9 salts that quickly dissipates.
- Solubilize 300 mg of ¹⁵N, ¹³C-complete medium, 1 g of ¹⁵NH₄Cl and 2 g of ¹³C₆-glucose in 10 ml of M9 medium. Filter-sterilize the isotope solution using a 0.2 μ m filter, directly into the M9 medium.
- Suspend using an inoculation loop one colony of pET15b-Tau transformed bacteria from the selection plate in 20 ml of LB medium supplemented with 100 μ g/ml of ampicillin.
- Incubate the inoculated medium at 37 °C for about 6 hr.
- Measure optical density at 600 nm (OD₆₀₀) on 1 ml of a ten-fold dilution of bacterial culture in a plastic spectrometer cuvette.
NOTE: Turbidity of the bacterial culture corresponding to OD₆₀₀ of 3.0-4.0 indicates that saturation growth phase is reached.
- Add 20 ml of the saturated LB culture to 1 L of M9 growth medium supplemented with ampicillin (100 μ g/ml final concentration), in 2 L Erlenmeyer plastic baffled culture flask.
- Place the culture flask in a programmable incubator set to 10 °C and 50 rpm. Program the incubator to switch to 200 rpm and 37 °C early in the morning of the next day.
- Measure OD₆₀₀ on 1 ml of bacterial culture in a plastic spectrometer cuvette. Add 400 μ l of 1 M IPTG (isopropyl β -D-1-thiogalactopyranoside) stock solution (kept at -20 °C) when OD₆₀₀ reaches a value of about 1.0 to induce the expression of recombinant Tau protein.
- Continue the incubation at 37 °C for a further 3 hr. Collect the bacterial cells by centrifugation at 5,000 $\times$ g for 20 min.
- Freeze the bacterial pellet at -20 °C. Keep frozen until the purification step, for an extended period if needed.

2. Purification of ¹⁵N, ¹³C-Tau (Figure 2)

- Autoclave cation-exchange (CEX) purification buffers at 121 °C under 15 psi for 20 min. Store buffers at 4 °C.
- Thaw the bacterial cell pellet and resuspend thoroughly in 45 ml of extraction CEX A buffer (50 mM NaPi buffer pH 6.5, 1 mM EDTA) freshly supplemented with protease inhibitor cocktail 1x (1 tablet) and DNaseI (2,000 units).
- Disrupt the bacterial cells using a high-pressure homogenizer at 20,000 psi. 3-4 passes are necessary. Centrifuge at 20,000 $\times$ g for 40 min to remove insoluble material.
- Heat the bacterial cell extract for 15 min at 75 °C using a water bath.
NOTE: a white precipitate is observed after a few minutes.

5. Centrifuge at 15,000 x g for 20 min and keep the supernatant containing the heat-stable Tau protein.
6. Store at -20 °C until the following purification step, if needed.
7. Perform a cation-exchange chromatography on a strong CEX resin packed as a 5 ml bed column using a fast protein liquid chromatography (FPLC) system (**Figure 3 A**).
 1. Set flow rate to 2.5 ml/min.
 2. Equilibrate the column in CEX A buffer
 3. Load the 60-70 ml heated-extract containing Tau using a sample pump, or alternatively pump A, depending on the system. Collect the flow-through for analysis to verify that Tau protein is efficiently binding to the resin (see 2.8).
 4. Wash the resin with CEX A buffer until absorbance at 280 nm is back to baseline value.
 5. Elute Tau from the column using a three-step NaCl gradient obtained by gradual increase of CEX B buffer (CEX A buffer with 1 M NaCl). Program the FPLC as follows: first step of the gradient to 25% CEX B buffer in 10 column volumes (CV) to reach 250 mM NaCl, second step to 50% CEX B buffer in 5 CV to reach 500 mM NaCl, and third step to 100% CEX B buffer in 2 CV to reach 1 M NaCl. Collect 1.5 ml fractions during the elution steps.
8. Analyze 10 µl of the fractions collected during the elution step by SDS-PAGE (12% SDS-acrylamide gel) and Coomassie staining (**Figure 3 A**)¹⁶. Check the loading step on the column as well by analyzing 10 µl of the flow-through.
9. Choose the fractions containing Tau and pool these fractions for the next step.
10. Perform a buffer exchange on Tau-containing pooled fractions (**Figure 3 B**).
 1. Equilibrate a desalting column of 53 ml G25 resin packed bed (26 x 10 cm) in 50 mM ammonium bicarbonate (volatile buffer) using a FPLC system.
 2. Set flow rate to 5 ml/min. Inject the Tau sample on the column via a 5 ml injection loop. Collect fractions corresponding to the absorption peak at 280 nm.
 3. Repeat the injection 3-4 times, depending on the volume of the initial CEX pool.
11. Calculate the amount of purified Tau protein by using the peak area of the chromatogram at 280 nm (1 mg of Tau corresponds to 140 mAU*ml).
NOTE: The extinction coefficient of Tau protein at 280 nm is 7,550 M⁻¹cm⁻¹. Tau does not contain any Trp residues.
12. Pool all Tau fractions.
13. Aliquot the sample into tubes containing the equivalent of 1 to 5 mg of Tau. Choose these tubes so that the volume of solution is small compared to the volume of the tube (for example 5 ml of solution in a 50 ml tube).
14. Punch holes in the tube caps using a needle. Freeze Tau samples at -80 °C.
15. Lyophilize Tau samples. Lyophilized Tau protein can be kept at -20 °C for long periods of time.

3. In Vitro Phosphorylation of ¹⁵N-Tau

1. Dissolve 5 mg of lyophilized Tau in 500 µl phosphorylation buffer (50 mM Hepes-KOH, pH 8.0, 12.5 mM MgCl₂, 1 mM EDTA, 50 mM NaCl).
2. Add 2.5 mM ATP (25 µl of 100 mM stock solution kept at -20 °C), 1 mM DTT (1 µl of 1 M stock solution kept at -20 °C), 1 mM EGTA (2 µl of a 0.5 M stock solution), 1x protease inhibitor cocktail (25 µl of a 40x stock obtained by dissolving 1 tablet in 1 ml phosphorylation buffer) and 1 µM activated His-ERK2 (250 µl in conservation buffer 10 mM Hepes, pH 7.3, 1 mM DTT, 5 mM MgCl₂, 100 mM NaCl and 10% glycerol, stored at -80 °C) in a total sample volume of 1 ml.
NOTE: The activated His-ERK2 can be prepared in-house^{5,8} by phosphorylation with the MEK kinase.
3. Incubate 3 hr at 37 °C.
4. Heat the sample at 75 °C for 15 min to inactivate ERK kinase.
5. Centrifuge at 20,000 x g for 15 min. Collect and keep the supernatant.
6. Desalt the protein sample into 50 mM ammonium bicarbonate using a column of 3.45 ml G25 resin packed bed (1.3 x 2.6 cm), which is suitable for a 1 ml sample.
7. Run a 12% SDS-PAGE¹⁶ with 2.5 µl of the protein sample to check both its integrity and efficient phosphorylation (**Figure 4**).
8. Lyophilize the phosphorylated Tau sample. Store the powder at -20 °C.

4. Acquisition of NMR Spectra (Figure 5)

1. Solubilize 4 mg of lyophilized ¹⁵N, ¹³C ERK-phosphorylated-Tau in 400 µl NMR buffer (50 mM NaPi or 50 mM deuterated Tris-*d*¹¹.Cl, pH 6.5, 30 mM NaCl, 2.5 mM EDTA and 1 mM DTT).
2. Add 5% D₂O for field locking of the NMR spectrometer and 1 mM TMSP (3-(trimethylsilyl)propionic-2,2,3,3-*d*₄ acid sodium salt) as internal NMR signal reference. Add 10 µl of a 40x stock solution of complete protease inhibitor cocktail.
3. Transfer the sample in a 5 mm NMR tube using an electronic syringe with a long needle or Pasteur pipette. Close the NMR tube using the plunger. Remove any air bubble trapped between plunger and liquid by plunger movements.
4. Place the NMR tube in a spinner. Adjust its vertical position in the spinner with the appropriate gauge for the NMR probe head used, such that most of the sample solution will be inside the NMR coil.
5. Start the air flow: click *lift* in the magnet control system window. Carefully place the spinner with the tube in the airflow at the top of the magnet bore. Stop the air flow (click *lift*) and let the tube descend into place inside the probe head in the magnet.
6. Set temperature to 25 °C (298 K).
7. Perform semi-automatic tuning and matching of the probe head to optimize power transmission. Type *atmm* on the command line.
8. Lock the spectrometer frequency using the D₂O signal of the sample recorded on the deuterium channel. Click *lock* in the magnet control system window.
9. Start the shimming procedure to optimize homogeneity of the magnetic field at the position of the sample. Type *topshim gui* on the command line to open the shim window. Click *start* in the shim window. Check the residual B0 standard variation value to verify that shims are optimal (less than 2 Hz is good).

10. Calibrate the p1 parameter (length of a proton radiofrequency pulse in μsec), which is necessary to obtain a 90° rotation of proton spins. Aim for the 360° pulse using a 1D spectrum of water protons (**Figure 6**).
11. Adjust the frequency offset by setting the o1 parameter (in Hz) to the proton water frequency in the 1D spectrum (**Figure 6**).
12. Start acquisition of a 1D proton spectrum (pulse sequence with watergate sequence for water signal suppression, for example zgpgw5) to verify signals from the sample (**Figure 7**). Adapt the number of scans to the relative protein concentration. Type *zg* on the command line to start the acquisition.
13. Set up additional parameters for the acquisition of a 2D [^1H , ^{15}N] HSQC spectrum (pulse sequence hsqcetf3gpsi, **Figures 8-9**).
 1. For a ^{15}N , ^{13}C labeled sample, decouple ^{13}C during the ^{15}N indirect evolution.
 2. Set the number of points and the spectral width (ppm) in the ^1H (F2) and ^{15}N (F1) dimensions.
NOTE: Adapt the number of acquisition data points to the spectrometer field to keep a similar number of Hz per point and to limit decoupling times: use 3,072 points at 900 MHz and 2,048 points at 600 MHz, in the ^1H dimension.
 3. Optimize additional parameters in the pulse sequences, corresponding to delays, pulse lengths, offset frequencies, power levels. Type *ased* on the command line to display all parameters relevant for the experiment.
14. Set parameters for the acquisition of a 3D [^1H , ^{15}N , ^{13}C] HNCACB spectrum (pulse sequence hncacbgpwg3d, **Figure 10A**) at 600 MHz.
15. Set parameters for a 3D [^1H , ^{15}N , ^{15}N] HNCANH experiment (pulse sequence hncannhgw3d) at 600 MHz. Set the number of points to 2048 in the ^1H and, 64 and 128 points in the two ^{15}N dimensions. Define the spectral widths as 14, 25, 25 parts per million (ppm) centered on 4.7, 119, 119 ppm in the ^1H , ^{15}N and ^{15}N dimensions. Duration of acquisition with 16 scans is 1 day and 22 hr.

5. Identification of Phosphorylation Sites

1. Process spectra using acquisition and processing NMR software.
 1. Perform a Fourier transformation of the data (**Figure 7**). Type *ft* for a 1D spectrum, *xfb* for a 2D spectrum or *ft3d* for a 3D spectrum, on the command line.
 2. Phase and reference all spectra (**Figure 7C**) using the interactive windows.
2. Identify resonances of interest in the 2D HSQC potentially corresponding to phosphorylated Ser and Thr residues (**Figure 9**, red box).
3. Extract planes (*i.e.* 2D ^1H - ^{13}C spectra) from the 3D ^1H - ^{15}N - ^{13}C spectrum using the ^{15}N chemical shifts of resonances of interest in the 2D. Use the cursor of dimension 2 (w2) to choose the ^{15}N frequency corresponding to the plane (w1-w3) to be visualized (**Figure 10B**).
4. Pick the resonance frequencies of 'CA' and 'CB' ^{13}C nuclei of the 'i' and 'i-1' residues (weaker set of signals compared to those of the i residue) for each [^1H , ^{15}N] resonance of interest in the HNCACB 3D spectrum by clicking on the resonance, in pointer mode menu find/add peak, to add the chemical shift value in a peak list file.
 1. Identify the i residue type, pSer or pThr, by comparing the chemical shifts in the peak list to known values of CA and CB chemical shifts of pSer and pThr¹⁷.
 2. Identify the presence of a Pro residue at the i+1 position by a characteristic additional +2 ppm shift of the CA chemical shift value¹⁸.
 3. Compare the chemical shift values of the CA and CB resonances corresponding to the i-1 residue to a table of chemical shifts predicted for Tau amino acid residues¹⁹ to identify the nature of the residue at the i-1 position.
5. Pick the resonance frequencies of ^{15}N nuclei of the 'i' and 'i-1' residues for each [^1H , ^{15}N] resonance of interest in the HNCANH spectrum.
6. Compare the ^{15}N chemical shift values to the chemical shift assignment of the Tau protein²⁰⁻²³.
7. Compare the identified dipeptides with the Tau sequence to define the sequence specific assignment.

Representative Results

Figure 3A shows a major absorption peak at 280 nm observed during the elution gradient. This peak corresponds to purified Tau protein as seen on the acrylamide gel above the chromatogram. **Figure 3B** shows a well separated absorption peak at 280 nm and peak of conductivity, ensuring that desalting of the protein is efficient. **Figure 4** shows protein gel-shift observed by SDS-PAGE analysis¹⁶ characteristic of multiple protein phosphorylation (compare lanes 2 and 3). **Figure 6** shows a series of proton (^1H) 1D spectra with increasing pulse lengths (in μsec). To setup the length of the pulse that will rotate ^1H spin magnetization by 90° , a pulse corresponding to a 360° rotation is used in practice, as it is easier to calibrate by minimizing the signal. The signal of water protons is null when the 360° pulse length is adequately defined. The value corresponding to a 360° rotation is then divided by 4. p1 in this experiment is 10.5 μsec . Enlarged region: The frequency of the residual signal is used to define the o1p parameter (frequency offset for ^1H). **Figure 7A** shows a free induction decay (FID), visualized to ensure that an NMR signal is detected. **Figure 7B** shows a 1D ^1H spectrum with an incorrect phase, as seen by resonances appearing as asymmetric peaks. **Figure 7C** shows a 1D ^1H spectrum with good signal to noise ratio, indicating that the basic acquisition parameters were correctly set and a signal from the protein sample can be detected. **Figure 8** shows 2D ^1H , ^{15}N HSQC spectra of recombinant ^{15}N -Tau at 900 MHz; **Figure 8A** with good sensitivity and resolution and **Figure 8B** with detection of proteolysis in the sample, as seen by the appearance of additional peaks in the spectrum (blue box). **Figure 9** shows 2D ^1H , ^{15}N HSQC spectra of recombinant ^{15}N -Tau **Figure 9A** at 600 MHz, with good sensitivity but less resolution compared to **Figure 8A**. **Figure 9B** at 600 MHz, shows appearance of additional peaks in the spectrum corresponding to phosphorylated residues (red box). **Figure 9C** at 900 MHz, shows peaks in the spectrum corresponding to phosphorylated residues (red box). Resolution is better than in **Figure 9B**. **Figure 10A** shows projections from the 3D NMR spectrum used to evaluate a successful experiment. **Figure 10B** shows a ^1H - ^{13}C plane extracted from the ^1H - ^{15}N - ^{13}C 3D spectrum with good signal intensity allowing to detect ^{13}C signals from both i and i-1 residues.

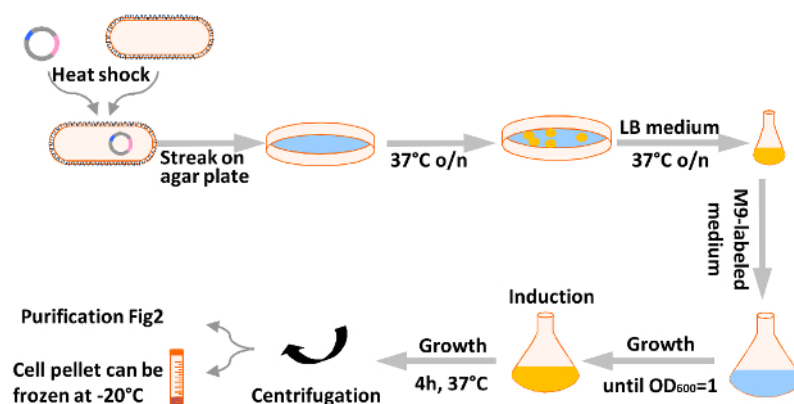


Figure 1: Scheme of the main steps of recombinant protein production and isotopic labeling. Steps from bacteria transformation to recombinant protein production are outlined as described in paragraph 1 of the protocol. [Please click here to view a larger version of this figure.](#)

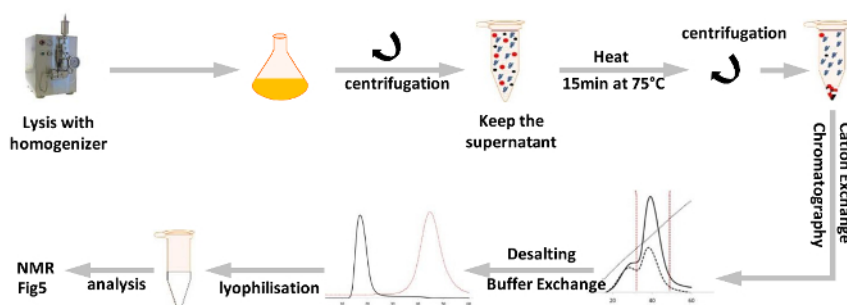


Figure 2: Scheme of the main steps of recombinant Tau protein purification. Steps from bacterial cells lysis to recombinant protein purification are outlined as described in paragraph 2 of the protocol. [Please click here to view a larger version of this figure.](#)

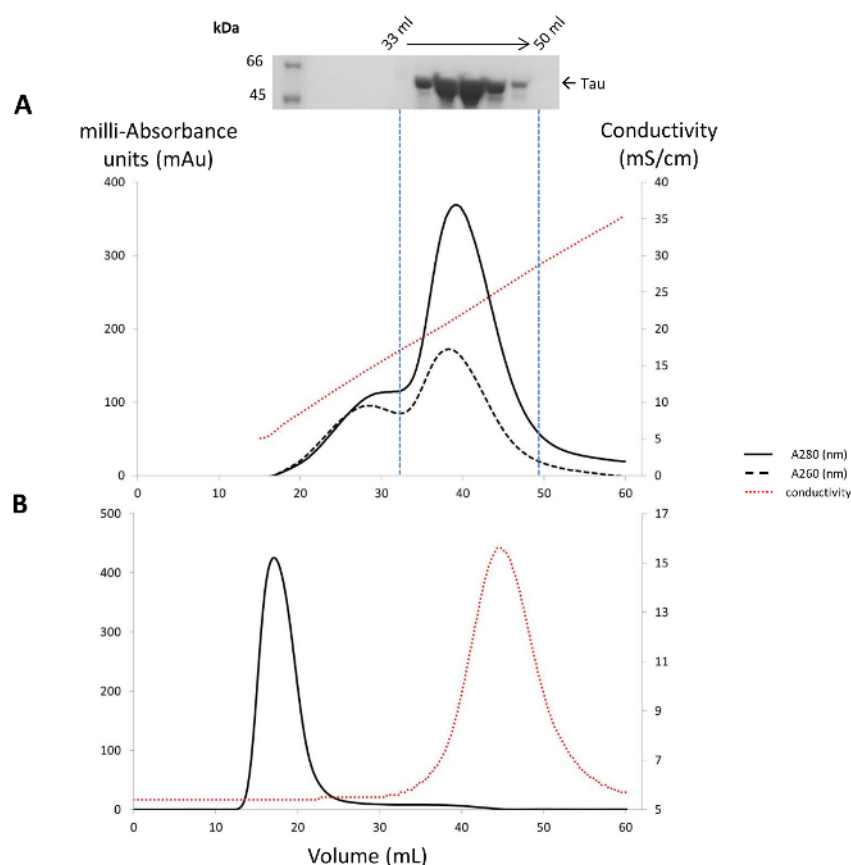


Figure 3: Liquid chromatography steps of protocol. (A) Cation exchange chromatography fractionation of the heated bacterial extract. The absorbance at 280 nm, 260 nm and the conductivity correspond respectively to solid and dashed black lines and dotted red line. 12% SDS-PAGE analysis of the collected fractions is shown above the chromatogram. (B) Desalting of the Tau protein into a buffer suitable for lyophilization. The amount of purified Tau protein estimated from the peak area (2,260 mAU*ml) is 16 mg of Tau. [Please click here to view a larger version of this figure.](#)

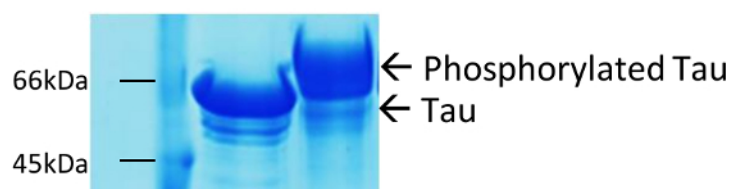


Figure 4: 12% SDS-PAGE analysis of Tau. Lane 1, molecular weight marker; lane 2, 10 µg of Tau; lane 3, 10 µg of ERK-phosphorylated Tau. Tau, as other IDPs, runs in an anomalous manner on SDS-PAGE, at an apparent molecular weight of about 60 kDa instead of the expected 46 kDa. [Please click here to view a larger version of this figure.](#)

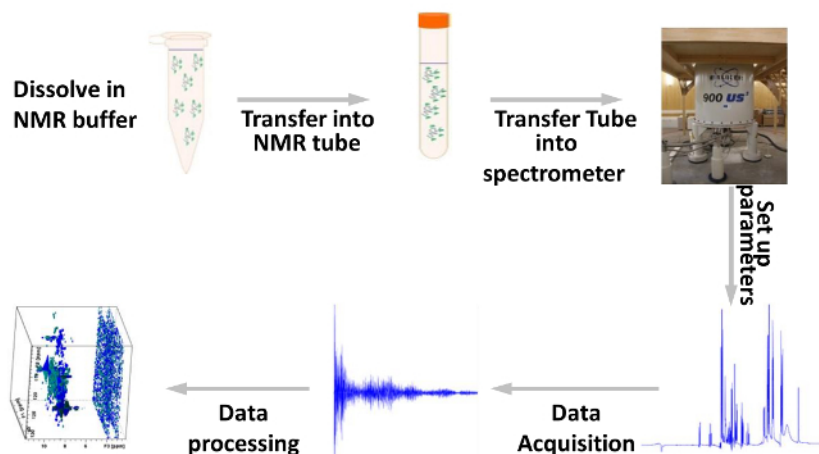


Figure 5: Scheme of the main steps for NMR sample preparation, NMR spectroscopy data acquisition and data processing. Steps from NMR sample preparation to data acquisition and processing are outlined as described in paragraph 4 of the protocol. [Please click here to view a larger version of this figure.](#)

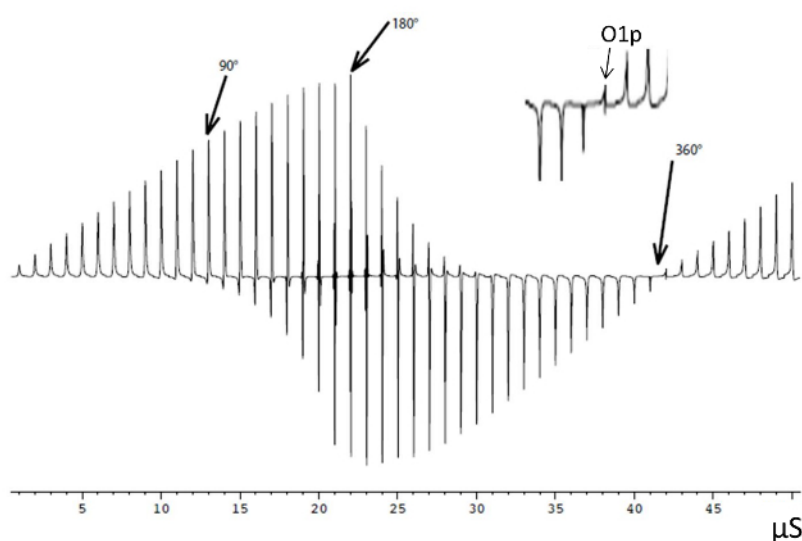


Figure 6: Set-up of the p1 parameter for NMR data acquisition. This parameter differs between samples and is mainly dependent on salt concentration. A standard ^1H nutation curve for 80% H_2O in D_2O is shown. Single-scan spectra with a recycle delay of 30 sec were collected and plotted horizontally. The pulse (p1) was varied from 1 μsec to 55 μsec in steps of 1 μsec . In theory, the signal should be maximal for a 90° pulse and equal to zero for a 180° pulse. However, in practice, radiation damping causes asymmetry and phase distortion problems which make it difficult to determine the 90° or 180° pulses directly. The second null point corresponds to a 360° pulse duration. The enlarged region shows a residual signal for a 360° pulse that is used to define the o1p frequency parameter. [Please click here to view a larger version of this figure.](#)

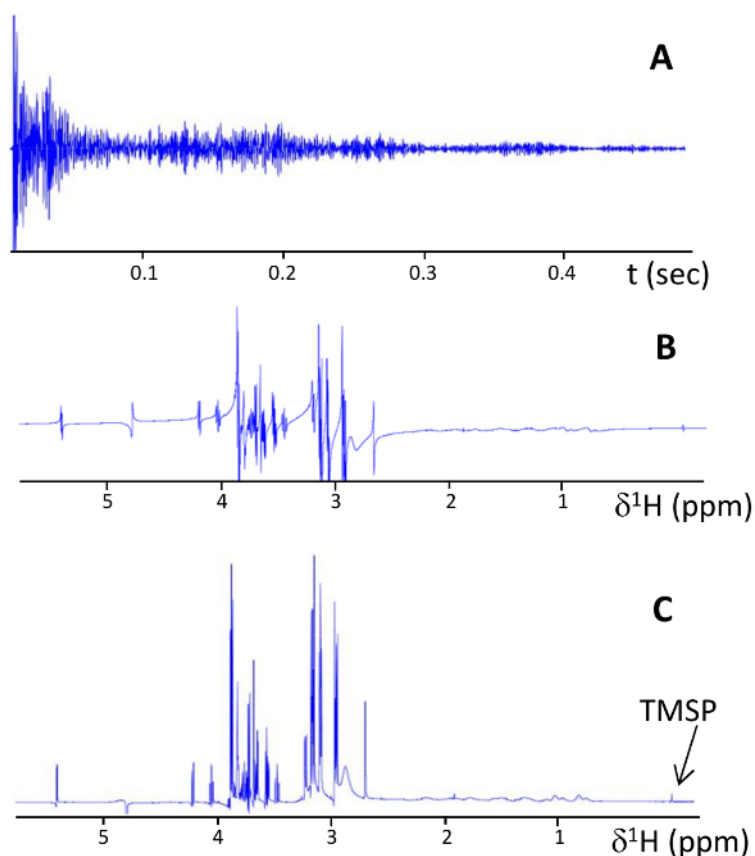


Figure 7: NMR data processing. (A) Free induction signal decay in the time domain. 1D proton spectra (B) resulting from Fourier transformation of the FID from panel A into the frequency domain but with incorrect phase (PHC0 -206°). (C) phased (PHC0 -113°) and referenced (TMSP signal at 0 ppm). [Please click here to view a larger version of this figure.](#)

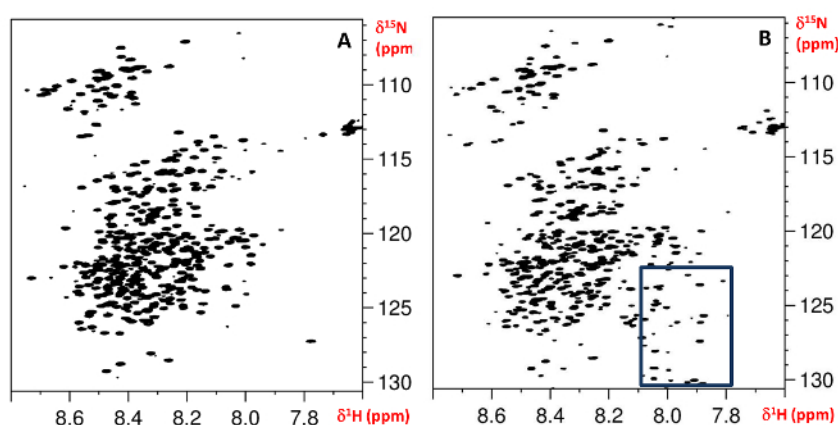


Figure 8: 2D ^1H , ^{15}N HSQC spectra of recombinant ^{15}N -Tau at 900 MHz. 3,072 and 416 acquisition data points were recorded using spectral widths of 14 and 25 ppm in the ^1H (F2) and ^{15}N (F1) dimensions, respectively. 16 scans were recorded per F1 increment, leading to a duration of the experiment of 4 hr 30 min. (A) good quality Tau sample (B) Tau sample showing degradation as revealed by the appearance of additional resonances in a particular region of the spectrum (high field ^1H , low field ^{15}N), here boxed in blue. This last sample was prepared without protease inhibitors. [Please click here to view a larger version of this figure.](#)

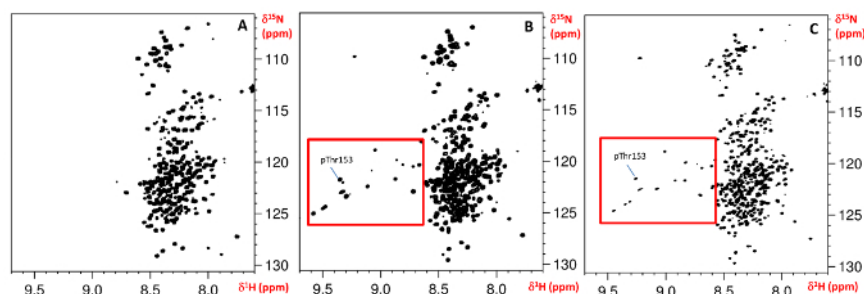


Figure 9: 2D ^1H , ^{15}N HSQC spectra of recombinant ^{15}N -Tau. (A) unphosphorylated Tau, 600 MHz spectrum (B) phosphorylated Tau, 600 MHz spectrum and (C) phosphorylated Tau, 900 MHz spectrum. Additional resonances in a particular region of the spectrum, here boxed in red, are observed in phosphorylated Tau spectra. These resonances, which correspond to proton amide (^1H - ^{15}N) correlations of pSer and pThr residues, are easily visualized in the region around 8.5 to 9.5 ppm for ^1H and 117 to 125 ppm for ^{15}N , outside the bulk of the ^1H , ^{15}N correlations of the unphosphorylated Tau spectrum. (A and B) correspond to spectra acquired at 600 MHz, 2,048 and 256 data points at spectral widths of 14 and 25 ppm were recorded in the ^1H (F2) and ^{15}N (F1) dimensions, respectively. 32 scans were used, and total duration of the acquisition was 2 hr 44 min and (C) at 900 MHz, 3,072 and 416 data points at spectral widths of 14 and 25 ppm were recorded in the ^1H (F2) and ^{15}N (F1) dimensions, respectively. 48 scans were used, and total duration of the acquisition was 6 hr 37 min. [Please click here to view a larger version of this figure.](#)

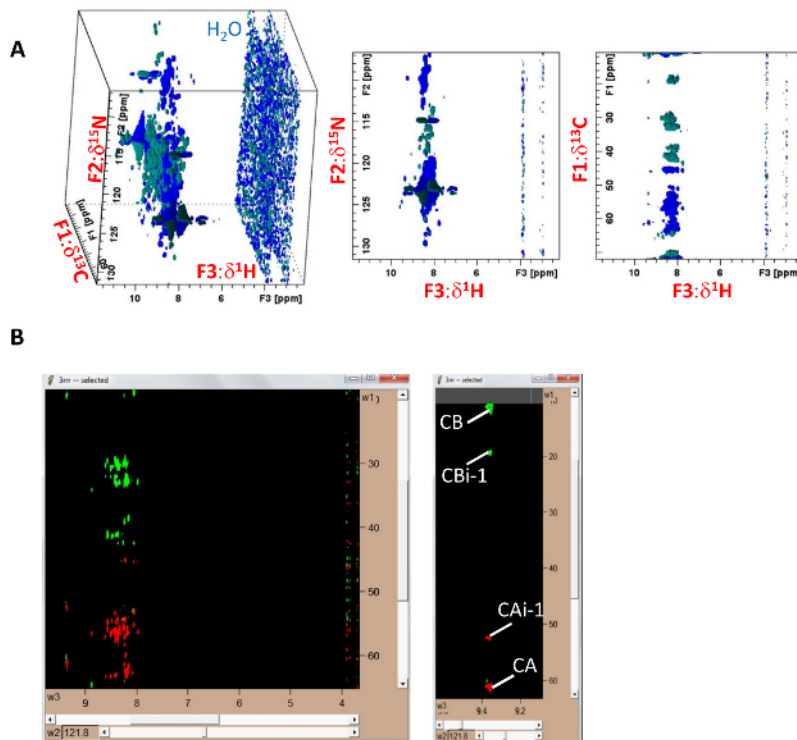


Figure 10: 3D ^1H , ^{15}N , ^{13}C NMR spectrum, projections and extracted 2D planes. 2,048, 72, and 256 data points were recorded in the ^1H (F3), ^{15}N (F2), and ^{13}C (F1) dimensions, respectively. Spectral widths are 14, 25, and 61 ppm, centered on 4.7, 119, and 41 ppm in the ^1H , ^{15}N and ^{13}C dimensions, respectively. Duration of the acquisition using 16 scans is 4 days and 6 hr. (A) Cube representation corresponding to the Fourier transformed 3D dataset of an HNCACB spectrum of ERK-phosphorylated Tau obtained at 600 MHz. The 2D ^1H , ^{15}N and the ^1H , ^{13}C planes are obtained by projection of the 3D data along the ^{13}C and ^{15}N dimension, respectively. Data processing and representation were done using NMR acquisition and processing software. (B) 2D ^1H , ^{13}C plane extracted from the 3D ^1H , ^{15}N , ^{13}C NMR dataset at a ^{15}N chemical shift of 121.8 ppm. A zoom (on the right) centered on the ^1H chemical shift of 9.38 ppm shows the ^{13}CA and ^{13}CB resonances of both residue i (pThr153) and i-1 (Ala152). The ^{13}CB resonance is aliased due to the width of the spectral window. Graphical representation and peak picking were performed using NMR analysis software. [Please click here to view a larger version of this figure.](#)

Discussion

We have used NMR spectroscopy to characterize enzymatically modified Tau samples. The recombinant expression and purification described here for the full-length human Tau protein can similarly be used to produce mutant Tau or Tau domains. Isotopically enriched protein is needed for NMR spectroscopy, necessitating recombinant expression. Identification of phosphorylation sites requires resonance assignment and a ^{15}N ,

^{13}C doubly labeled protein. Given the cost of isotopes, good yield is required in the recombinant expression step. Glucose is the limiting factor for the bacterial growth in the M9 medium therefore the amount of $^{13}\text{C}_6$ -glucose can be increased to 4 g per liter of growth medium to improve yield. Addition of complete medium and MEM vitamins are not compulsory but help to stimulate growth and improve yield. Given the high cost of the complete labeled medium, this product is only used as a growth medium supplement. Bacterial growth is slow in M9 medium. Generally, bacterial cultures using M9 media supplemented with 3% complete medium reach time of induction after about 4 hr of incubation. An OD_{600} of 1.6-1.8 is usually obtained at the end of the culture. Expected yield of recombinant Tau protein is about 15 mg per liter of bacterial culture. The use of a programmable incubator allows to conveniently schedule protein production, collection of the bacterial pellet and analytical control of protein production during working day hours.

Sample concentration is important to obtain a good quality spectrum. A typical Tau sample sufficient for a 2D spectrum would contain 1 mg in 200 μl (i.e. 100 μM) Tau protein. For a 3D spectrum, at least 200 μM in 300 μl are required, provided that a cryogenic probe head is used. Access to a high-field spectrometer, such as the 900 MHz instrument used in this study will provide better signal-to-noise and reduce constraints on sample concentration (Figure 8). Given that Tau is a large disordered protein, its NMR spectra are characterized by considerable signal overlap, and a high-field NMR spectrometer will also be the best choice in terms of resolution (Figure 8). Nevertheless, the resonances corresponding to phosphorylation sites are in a distinct region of the spectrum and are easy to detect even with a 600 MHz spectrometer (Compare Figures 9B and 9C). In addition, due to its disordered nature, the Tau protein is sensitive to proteolysis (Figures 8B).

Sterilization of buffers is advised to limit Tau degradation. Addition of protease inhibitors to the NMR sample helps to protect Tau against degradation during data acquisition periods that can last from hours to days, depending on the pulse sequence. Low pH (i.e. pH below 7.0) is required to avoid too fast exchange between protein protons and water protons, which leads to signal broadening. Sample pH must also be well controlled to ensure reproducibility of the spectra. Indeed, since pK_a values of pSer and pThr are close to the optimal pH for NMR spectroscopy, chemical shifts of phosphorylated residues are very sensitive to pH variations. Adjustment of the pH of the NMR sample can be made directly using a pH-meter with a pH micro electrode, adapted to small volumes. Tau is a soluble protein and does not aggregate in standard conditions. Addition of polyanions, such as heparin sulfate, and incubation at 37 °C can initiate aggregation in Tau samples. In this case, given the solid nature of the aggregates, most of the resonances in the corresponding NMR spectrum broaden beyond detection. Even the phosphorylated Tau samples do not aggregate in the conditions described here for NMR data acquisition.

Compared to antibody analysis, NMR provides an overall view of PTMs. Mass spectrometry can also be used to identify the phosphorylation pattern of a protein sample. Isotopic labeling is not necessary for this technique, and required sample quantities are much smaller. However, characterization of a protein with multiple phosphorylations, such as Tau, remains challenging. Adjacent phosphorylations will produce isobaric peptides in bottom-up strategies of identification. Complete identification then needs MS/MS sequencing of the peptides obtained by sample proteolysis. An advantage of NMR consists in the intrinsic quantitative nature of the technique. The intensity of an NMR signal can be linked to the amount of the chemical group present at a specific site. We can thus define the proportion of chemical modification at each site. Most recent progress in MS applications have however shown that MS analysis of Tau multiple phosphorylation is feasible²⁴, even in a quantitative manner after proper normalization²⁵.

Here, we presented Tau phosphorylation by activated ERK2, but the methodology can be used for phosphorylation with other kinases as well^{6,7,26-28}. Kinetic experiments can be performed, which can help to define kinase specificity towards a protein substrate²⁸⁻³¹. Phosphorylation studies are not limited to recombinant kinases, and kinase activity of cell or tissue extracts can be analysed^{6,32,28}. An interesting development is the use of *in-cell* NMR to study *in situ* modifications^{33,34}. Conversely, NMR is also well-adapted to address phosphatase specificity, as was shown by studying the dephosphorylation of phosphorylated Tau by the PP2A phosphatase³⁵. NMR spectroscopy can be applied to the characterization of kinase inhibitors by comparing the phosphorylation profile of the protein substrate in a 2D spectrum in presence of the inhibitor compound with the spectrum issued from a control experiment⁶.

The interest of using NMR spectroscopy, compared to the more sensitive MS techniques, resides in the wide range of applications exploiting the protocol described here, rather than on its analytical capacity alone. It has proven crucial to identify phosphorylation sites to be able to link specific phosphorylations with structural or functional modifications that were mainly studied using NMR. NMR spectroscopy of phosphorylated Tau samples allows to explore the structural impact of phosphorylation on both transient local secondary structures and on global rearrangement of the dynamic ensemble of modified Tau^{36,37}. Functional aspects include the regulation of both interaction of Tau with protein partners^{7,38,39} and aggregation by Tau phosphorylation⁸. The phosphorylated Tau sample characterized by NMR can be further used to decipher phospho-dependent interactions, for example with 14-3-3 proteins⁴⁰, and engineered protein-protein interaction inhibitors^{41,42}. NMR allows definition of interaction site(s) at the residue level, and of the dependence of this interaction on phosphorylation. Additionally, NMR spectroscopy of phosphorylated Tau is a key methodology to characterize the proline *cis:trans* isomerase Pin1, an important phospho-dependent enzyme involved in Tau regulation⁴³⁻⁴⁵. In addition, phosphorylation analysis by NMR can be applied not only to IDPs but also to globular proteins⁴⁶. Finally, other types of Tau PTMs such as acetylations^{22,40,41} can be studied by NMR. The protocols described here have proven crucial to better understand the functional and structural regulation of Tau in physiological and pathological conditions.

Disclosures

The authors have nothing to disclose.

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