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A Method to Monitor the Introduction of Posttranscriptional Modifications in tRNAs with NMR Spectroscopy

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Abstract

During their biosynthesis, transfer RNAs (tRNAs) are decorated all along their sequence with a large number of posttranscriptional chemical modifications. Methods to directly detect the introduction of posttranscriptional modifications during tRNA maturation are rare and do not easily bring information on the temporality of modification events. Here, we report a methodology, using NMR as a tool to monitor tRNA maturation in a nondisruptive and continuous fashion in cellular extracts. This method requires the production for NMR spectroscopy of substrate tRNA transcripts devoid of modifications and of active cell extracts containing the cellular enzymatic activities. The present protocol describes these different aspects of our method and reports the time-resolved NMR monitoring of the yeast tRNA^{Phe} maturation as an example. The NMR-based methodology presented here could be adapted to investigate diverse features in tRNA maturation.

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transfer RNA, tRNA, posttranscriptional modifications, RNA modifications, yeast, RNA transcription, NMR spectroscopy, yeast extract, time-resolved NMR, in extract NMR

1. Introduction

Synthesis and maturation of transfer RNAs (tRNAs) involve posttranscriptional chemical modifications of their nucleotides. These modifications occur at specific sites in a tightly controlled manner, which ensures that the tRNA biogenesis process effectively leads to the formation of mature and functional tRNAs [1-4]. Currently, over 140 posttranscriptional modifications are reported in RNAs, the

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vast majority being found in tRNAs [5, 6]. This family of RNAs displays not only the largest variety of posttranscriptional decoration, but also the highest density of modification per RNA molecule, with ~8–25% of modified nucleotides in tRNAs depending on the organisms [7, 8]. Notably, all tRNA functions within cells are affected by modifications. In particular, modifications in and around the anticodon are implicated in the decoding process [1, 9, 10]. In addition, some modifications control cleavages producing tRNA fragments [11, 12]. And finally, cellular stress can result in changes in tRNA modifications, which can lead to translational reprogramming [13, 14].

Even though modifications are central in tRNA biology, methods to directly detect their introduction during tRNA biosynthesis are rare and do not easily provide information on the temporality of modification events. With the aim of filling this gap, we have developed a methodology, using NMR as a tool to monitor tRNA maturation in a nondisruptive and continuous manner [15]. Briefly, introducing isotope-labeled tRNAs into unlabeled cell extracts containing the cellular enzymatic activities combined with the use of isotope filters in NMR experiments, enables the detection of the tRNA of interest within the complex cell extract environment (Figure 1). RNA modification events are directly monitored in a time-resolved fashion, by measuring successive NMR experiments on a single sample directly incubated in the NMR spectrometer.

In this chapter, we describe the detailed procedure for monitoring tRNA maturation in cell extracts with NMR spectroscopy. We demonstrate our methodology with the maturation of the yeast tRNA^{Phe} in yeast extracts. Our methodology consists of three steps (Figure 1). In the first step, a ¹⁵N-labeled tRNA sample is produced by *in vitro* transcription with ¹⁵N-labeled nucleotides and purified by ion-exchange chromatography (Figure 1; step 1). After dialysis and concentration, the ¹⁵N-labeled tRNA sample is ready for further use. In the second step, an unlabeled cellular extract is produced from the lysis of a cell culture (Figure 1; step 2). To preserve as far as possible the cellular enzymatic activities in the extract, cell lysis is performed under gentle conditions and in the presence of anti-proteases. After ultracentrifugation of the cell debris, active unlabeled cellular extracts are obtained. In the third step, the NMR monitoring of tRNA maturation is performed (Figure 1; step 3). An *in extract* NMR sample is produced by mixing the ¹⁵N-labeled tRNA sample obtained in step 1 with the unlabeled cell extract obtained in step 2. This sample is incubated directly in the NMR spectrometer and series of NMR experiments are measured in order to monitor tRNA maturation events in a time-resolved fashion (Figure 1; step 3). In the NMR spectra, the progressive appearance of new signals and the correlated disappearance of signals from the unmodified tRNA sample are the signature of chemical modifications being introduced in the initial transcript. Since NMR spectra are measured as a time-course series, our methodology enables the identification of early and latter modifications events. Overall, NMR spectroscopy provides the means to observe sequential orders in the introduction of modifications along the tRNA maturation pathway.

2. Materials

All solutions are prepared using reagents of highest available purity and with ultrapure deionized water. Particular care should always be taken to avoid RNase contamination during preparation of reagents that are required for the RNA transcription reaction (see Note 1). The use of ultrapure deionized water is sufficient to assure RNase-free condition, provided that the water purification system is well maintained. All reagents required for RNA transcription and cellular extract preparation are stored at –20°C, unless indicated otherwise (see Note 2).

2.1. tRNA transcription and purification

1. Commercial or *in-house* produced (see Note 3) T7 RNA polymerase (stock at 1 mg/mL stored at –20°C in 20 mM Na-phosphate pH 7.7, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 50% (v/v) glycerol).

2. *In vitro* T7 transcription buffer concentrated 20 times (TB20x): 400 mM Tris-HCl pH 8.0, 10 mM spermidine, 50 mM DTT, 0.1% (v/v) Triton X-100 in water. Store at -20°C .
3. Stock solution of unlabeled ATP and CTP at 100 mM and pH 8.0 (*e.g.* Jena Bioscience, catalog numbers: NU-1010 and NU-1011). Store at -20°C .
4. Stock solution of ^{15}N -labeled UTP and GTP at 100 mM (Cambridge Isotope Laboratories, catalog numbers: NLM-4270-CA and NLM-4268-CA). Store at -20°C .
5. DNA oligonucleotide of high purity for use as template for the yeast tRNA^{Phe} transcription at 100 μM in water (see Note 4). For the yeast tRNA^{Phe}, use an oligonucleotide with the following sequence: 5' TGG TGC GAA TTC TGT GGA TCG AAC ACA GGA CCT CCA GAT CTT CAG TCT GGC GCT CTC CCA ACT GAG CTA AAT CCG CTA TAG TGA GTC GTA TTA 3'.
6. DNA oligonucleotide of high purity for use as T7 promotor primer at 100 μM in water (see Note 5). For example: 5' TAA TAC GAC TCA CTA TAG 3'.
7. Sterile syringe filters 0.22 μm .
8. MgCl_2 at 280 mM in water. Sterilize by filtration with 0.22 μm filter and store at -20°C .
9. GMP pH 7.5 at 80 mM in water. Sterilize by filtration with 0.22 μm filter and store at -20°C .
10. Water bath with temperature control.
11. Ethylenediaminetetraacetic acid (EDTA) prepared at 0.5 M pH 8.0 in water. Store at room temperature.
12. ÄKTA Purifier (GE Healthcare) or similar chromatographic system.
13. MonoQ 10/100 GL column (GE Healthcare).
14. Purification buffer A: 25 mM Na-phosphate pH 6.5, 50 mM NaCl, 5 mM MgSO_4 (filtrate with 0.22 μm filter).
15. Purification buffer B: 25 mM Na-phosphate pH 6.5, 1 M NaCl (filtrate with 0.22 μm filter).
16. NMR buffer: 10 mM Na-phosphate pH 6.5, 10 mM MgCl_2 .
17. NMR buffer stock solutions: 500 mM Na-phosphate pH 6.5, 1 M MgCl_2 .
18. Dialysis sacks with 3.5 kDa MWCO (*e.g.* Spectra/Por 3 Dialysis Tubing Spectra/Por).
19. Standard closures for dialysis tubing.
20. Concentrators Amicon Ultra-15 MWCO 10 kDa (Millipore/Merck).
21. Ultrapure deionized water.

2.2. Cellular extract preparation

1. Ampicillin stock solution at 100 mg/mL in water. Store at -20°C .
2. YEPD-Ampicillin agar plates: Dissolve 10 g of yeast extract, 20 g of peptone, and 20 g of agar in 950 mL of water and sterilize by autoclaving. Prepare a solution of 40% (w/v) of glucose in water and sterilize by filtration with 0.22 μm filter. Add 50 mL of the sterile-filtered glucose solution in the autoclaved medium to obtain 1 L of YEPD agar medium containing 2% (w/v) of glucose. Allow the solution to cool to $\sim 50^{\circ}\text{C}$ and add ampicillin at a final concentration of 100 $\mu\text{g/mL}$. Mix well and pour 25 mL of the solution per Petri dish. Plates can be stored upside down at 4°C for several weeks.
3. YEPD medium: Dissolve 10 g of yeast extract and 20 g of peptone in 950 mL of water and sterilize by autoclaving. Prepare a solution of 40% (w/v) of glucose in water and sterilize by filtration with 0.22 μm filter. Solutions can be stored for months at room temperature. Add 50 mL of the sterile-filtered glucose solution in the autoclaved medium to obtain 1 L of YEPD medium containing 2% (w/v) of glucose.
4. Glycerol stock of c13-ABYS-86 *S. cerevisiae* strain stored at -80°C (see Note 6).
5. Static and shaking incubators at 30°C . Sterile 100 mL, and 2 L Erlenmeyer flasks.
6. Spectrophotometer to measure the absorbance at 600 nm (OD600).
7. Centrifugation bottles for cell harvesting and S30 cell extract preparation and corresponding rotors (*e.g.* Beckman Coulter JA-10 and JA-20). Ultracentrifugation bottles for S100 cell extract preparation and corresponding rotor (*e.g.* Beckman Coulter Type 70.1 Ti).
8. Centrifuge and Ultracentrifuge with temperature control that allows cooling to 4°C .
9. Mechanical device to disrupt yeast cells such as an Eaton press or a French press (see Note 7).
10. Lysis buffer: 25 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 7.0, 10 mM MgCl_2 , 0.1 mM EDTA. Sterilize by filtration with 0.22 μm filter and divide into aliquots of 10 mL. Store at -20°C .
11. DTT stock solution at 1 M: Dissolve 3.085 g of 1,4-dithio-DL-threitol into 20 mL of 10 mM sodium acetate pH 5.2. Sterilize by filtration with 0.22 μm filter and divide into aliquots of 1 mL. Store at -20°C .
12. Phenylmethanesulfonyl fluoride (PMSF) solution at 100 mM in isopropanol. Store at 4°C .
13. Benzamidine stock solution at 500 mM: Dissolve 437 mg of benzamidine hydrochloride into 5 mL of water. Sterilize by filtration with 0.22 μm filter and divide into aliquots of 500 μL . Store at -20°C .
14. Antiprotease stock solutions concentrated 5000x: On one hand, dissolve 5 mg of leupeptin and 5 mg of antipain into 1 mL of water and divide into aliquots of 200 μL . On the other hand, dissolve 5 mg of pepstatin A and 5 mg of chymostatin into 1 mL of DMSO and divide into aliquots of 200 μL . Store both antiprotease stock solutions at -20°C .
15. Liquid nitrogen (see Note 8).

2.3. NMR monitoring of tRNA maturation

1. Maturation buffer concentrated ten times (MB10x): 1.5 M NaH₂PO₄/K₂HPO₄ pH 7.5, 50 mM NH₄Cl, 20 mM DTT, 1 mM EDTA in water (see Note 9). Sterilize by filtration with 0.22 μ m filter and divide into aliquots of 500 μ L. Store at -20°C .
2. MgCl₂ at 1 M in water. Sterilize by filtration with 0.22 μ m filter and divide into aliquots of 500 μ L. Store at -20°C .
3. Stock solution of S-adenosyl-L-methionine (SAM) at 100 mM (see Note 10) in a 5 mM H₂SO₄ solution (see Note 11). Divide into aliquots of 25 μ L. Store at -20°C for up to 4–6 months.
4. Stock solution of ATP at 100 mM and pH 8.0 (*e.g.* Jena Bioscience, catalog number: NU-1010). Store at -20°C .
5. Stock solution of β -Nicotinamide adenine dinucleotide 2'-phosphate reduced (NADPH) at 100 mM (see Note 12) and pH 8 in water (see Note 13). Divide into aliquots of 25 μ L. Store at -20°C for up to 1–2 months.
6. Deuterium oxide 99.96% (D₂O) (*e.g.* Eurisotop, catalog number: D215B).
7. 5 mm Shigemi NMR tubes (see Note 14).
8. Manual centrifuge equipped with a swing-out rotor and 15 mL conical tube adaptors for the centrifugation of NMR tubes (*e.g.* Hettich, catalog numbers: HET-1011 and HET-1014).
9. Solution-state NMR spectrometer operating at 600 MHz or higher, equipped with a cryogenically cooled triple-resonance probe with z-axis gradients.
10. RNase-free water

3. Methods

3.1. tRNA transcription and purification

The optimal T7 *in vitro* transcription conditions depend greatly on the MgCl₂ and NTP concentrations. The [MgCl₂]/[NTP] ratio should be adjusted to optimize the yield of the RNA of interest. We recommend testing small-scale reactions (*e.g.* 40 μ L) before large-scale production of RNAs (5–20 mL) (see Note 15). For large scale *in vitro* transcription, up-scale the best conditions determined with small-scale reactions. In our hands, large amount of unmodified yeast tRNA^{Phe} ¹⁵N-labeled on Us and Gs (see Note 16) sufficient for monitoring its maturation with NMR, can be obtained from a 10 mL transcription reaction as described here.

1. Prepare the large-scale transcription reaction of 10 mL in a 50 mL conical tube as follow: 40 μ L of DNA template pre-mixed with the T7 promotor primer at a 1:1 ratio (stock at 50 μ M), 500 μ L of TB20x transcription buffer, 500 μ L of non-labeled ATP (stock at 100 mM), 500 μ L of non-labeled CTP (stock at 100 mM), 500 μ L of ¹⁵N-labeled UTP (stock at 100 mM), 500 μ L of ¹⁵N-labeled GTP (stock at 100 mM), 1300 μ L of MgCl₂ (stock at 280 mM), 500 μ L of GMP (stock at 80 mM), 250 μ L of T7 RNA polymerase (stock at 1 mg/mL), and complete with ultrapure deionized water to 10 mL.

2. Incubate in a water bath for ~3–4 h at 37°C (see Note 17).
3. Stop the reaction by adding 1.0–1.5 mL of 0.5 M EDTA pH 8.0 (see Note 18). The transcription reaction can be stored few days at -20°C until its purification or purified directly as described below.
4. Wash the purification system (ÄKTA Purifier or equivalent FPLC purification system) and the column (MonoQ 10/100 GL) with 0.5 M NaOH and rinse them extensively with water (see Note 19). Then, equilibrate the MonoQ column in purification buffer A.
5. Filtrate the large-scale transcription sample with 0.22 μm filter and load up to 5–6 mL of the transcription sample (see Note 20) on the MonoQ column previously equilibrated with purification buffer A at a flow rate of 2 mL/min.
6. Elute the tRNA transcript from the MonoQ column with a 20%–80% gradient of purification buffer B over 75 mL (~10 column volumes) at a flow rate of 3 mL/min. Collect 1 mL fractions. Transcribed yeast tRNA^{Phe} is usually eluted between 50% and 55% of buffer B (see Note 21).
7. Repeat steps 5 and 6 with the remaining of the 10 mL large-scale transcription, and pool the fractions containing the tRNA of interest from both purification runs.
8. Dialyze extensively the pooled fractions of yeast tRNA^{Phe} in a dialysis sack against 1 mM Na-phosphate pH 6.5 (see Note 22).
9. Recover the desalted tRNA^{Phe} in a 50 mL conical plastic tube and refold it by heating the sample at 95°C for 5 min and letting it cool down slowly at room temperature. Add NMR buffer stock solutions (*e.g.* 500 mM Na-phosphate pH 6.5 and 1 M MgCl₂) to place the refolded sample in the final NMR buffer, namely 10 mM Na-phosphate pH 6.5 and 10 mM MgCl₂ (see Note 23).
10. Concentrate the yeast tRNA^{Phe} sample in the NMR buffer up to 1.5–2.0 mM using Amicon concentrators of 10 kDa. Typically, a 10 mL transcription with ¹⁵N-labeled Us and Gs provides a final NMR sample of 300 μL at ~1.8 mM, corresponding to 0.5–0.6 μmol of purified tRNA. Store the concentrated yeast tRNA^{Phe} NMR sample at -20°C until needed (see Note 24).

3.2. Cellular extract preparation

All steps after cell harvest are performed on ice, unless indicated otherwise. Pre-cool centrifugation bottles, rotors and centrifuges before usage.

1. Streak yeast c13-ABYS-86 cells from glycerol stock on YEPD-Ampicillin agar plate and incubate the plate in a static incubator at 30°C for ~2 days until the colonies appear.
2. Start a preculture by transferring an individual colony with a sterile inoculation loop into a 100 mL Erlenmeyer containing 25 mL of YEPD medium supplemented with 100 $\mu\text{g/mL}$ of ampicillin. Incubate the preculture in a shaking incubator at 30°C and 200 rpm for 20 h.
3. Measure the OD₆₀₀ of the preculture. Start a culture with an OD₆₀₀ of 0.15 by transferring the appropriate volume of preculture into a 2 L Erlenmeyer containing 500 mL of YEPD medium. Incubate the culture in a shaking incubator at 30°C and 200 rpm for 24 h.
4. Harvest the cells by a 20 min centrifugation at $3500 \times g$ at room temperature in 500 mL centri-

fugation bottles. Resuspend the cell pellet with 40 mL of cold water and transfer the suspension into a 50 mL conical tube. Centrifuge cells at $3500 \times g$ for 20 min at 4°C. Weight the cell pellet (see Note 25). Store the cells at -80°C until needed for cellular extract preparation.

5. Add one equivalent volume of lysis buffer (*i.e.* 1 mL of lysis buffer per g of yeast cells) to the frozen cells on ice and re-suspend cells gently. Add DTT at a final concentration of 2 mM, PMSF and benzamidine both at a final concentration of 1 mM, and leupeptin, antipain, pepstatin A and chymostatin each at a final concentration of 1 $\mu\text{g}/\text{mL}$.
6. Lyse the cells by two sequential passages in a pre-cooled Eaton pressure chamber at -80°C operated via a hydraulic press with a working pressure of 20,000-30,000 psi. Alternatively, cells may be lysed by two sequential passages in a pre-cooled French press at 4°C operating at a pressure of 10,000-20,000 psi (see Note 7).
7. Evaluate the volume of lysed cell suspension recovered after lysis and add again DTT, PMSF, benzamidine, leupeptin, antipain, pepstatin A and chymostatin to double the previously mentioned final concentrations (see Note 26).
8. Centrifuge the disrupted cells at $30,000 \times g$ for 1 h at 8°C. Carefully recover the supernatant and transfer it into a clean ultra-centrifugation bottle. Ultra-centrifuge the yeast S30 cell extract at $100,000 \times g$ for 1 h at 8°C. Transfer 250 μL aliquots of the supernatant into 1.5 mL safe-lock tubes. Snap freeze the S100 yeast extract aliquots into liquid nitrogen (see Note 8) and store the cellular extracts at -80°C until needed (see Note 27).

3.3. NMR monitoring of tRNA maturation

The monitoring of tRNA maturation can simply be monitored by adding the unmodified ^{15}N -labeled tRNA prepared in step 1 into the unlabeled cellular extract prepared in step 2 (Figure 1). In our hands, with the yeast tRNA^{Phe}, sufficient signal-to-noise ratio in NMR measurements can be achieved with a concentration of ^{15}N -labeled tRNA of 40 μM in the cellular extract (see Note 28). Additionally, cofactors of modification enzymes should be added to the reaction to ensure efficient activity of the cell extract. In practice, we add S-adenosyl-L-methionine (SAM), the almost universal methyl donor used by RNA methyltransferases, and reduce nicotinamide adenine dinucleotide phosphate (NADPH), a hydride donor implicated in the formation of dihydrouridines. All components of the maturation reaction should be prepared and mixed together on ice. The ^{15}N -labeled tRNA must be added last and the delay from its mixing to the starting of the NMR experiments must be as quick as possible and kept shorter than ~5–10 min (see Note 29). Incubation is done directly in the NMR spectrometer, and a series of NMR experiments are measured in a time-resolved fashion in order to visualize the sequential order of tRNA modification events (Figure 2). Although the observation of NMR chemical shift changes that correspond to modification events is easily performed at this step, the interpretation of these changes in terms of posttranscriptional modifications is not necessarily straightforward and might necessitate in-depth NMR studies (see Note 30).

1. Prepare the tRNA maturation reaction of 280 μL in a 1.5 mL tube on ice as follow: 185 μL of yeast cellular extract prepared in Subheading 3.2, 28 μL of MB10x maturation buffer, 1.4 μL of MgCl_2 (stock at 1 M; 5 mM final), 11.2 μL of SAM (stock at 100 mM; 4 mM final), 11.2 μL of ATP (stock at 100 mM; 4 mM final), 11.2 μL of NADPH (stock at 100 mM; 4 mM final), 14 μL of D_2O (5% (v/v) final), 6.3 μL of ^{15}N -labeled yeast tRNA^{Phe} prepared in Subheading 3.1 (stock at 1.8 mM; 40 μM final), and complete with RNase-free water to 280 μL (see Note 31).

2. Quickly transfer the tRNA maturation mix to a 5 mm Shigemi tube. Spin down the reaction mix using a manual centrifuge adapted for NMR tube centrifugation. Put the Shigemi plunger in place and maintain it in position with sealing plastic film (*e.g.* Parafilm). Insert the NMR tube into the NMR spectrometer probe previously equilibrated at 30°C.
3. Perform a rapid adjustment of the shims and quickly start the series of 2D (^1H , ^{15}N) correlation spectra with NMR parameters determined on a reference sample of similar composition (see Note 29). In our hands, with the yeast tRNA^{Phe}, high-quality NMR spectra were measured with 2D (^1H , ^{15}N)-BEST-TROSY experiments [16] with 210 transients and interscan delays of 200 ms. Sweep widths were set to 24.0 and 26.0 ppm and 3072×96 complex points were measured for the ^1H and ^{15}N dimensions, respectively. With these settings, a 2D NMR spectra is recorded in ~120 min. Record a series of 12 identical spectra in a continuous fashion, for monitoring tRNA maturation during 24 hours.
4. Process the series of spectra with cosine-bell apodization functions and zero filling to 4096 and 512 data points in the ^1H and ^{15}N dimensions, respectively. Other more complicated apodization schemes may also be used to achieve better S/N ratios or resolution.
5. Analyze the series of NMR spectra and identify the NMR chemical shift changes that correspond to modification events. For accurate interpretations of the changes appearing upon incubation, an in-depth NMR study may be needed (see Note 30). The acquisition of NMR spectra in a continuous and time-resolved fashion enables the identification of a potential sequential order in the introduction of posttranscriptional modifications. In the case of the yeast tRNA^{Phe}, some modifications appear early in the modification process, such as $\Psi 55$, m⁷G46, and T54, and some appear late, such as m¹A58 (Figure 2).

4. Notes

1. The introduction of undesired RNase activity into the transcription reaction can significantly reduce the efficiency of transcription and thereby the overall yield of tRNA production by T7 *in vitro* transcription.
2. Storage at -20°C significantly prolongs the lifetimes of hydrolysis-sensitive reagents. We usually do not observe any significant deterioration of stored reagents within 1 year at -20°C, unless indicated otherwise. Yeast cellular extracts are more sensitive and should be stored at -80°C.
3. Protocol for the expression and purification of T7 RNA polymerase in *E. coli* is not detailed here, but has been described comprehensively elsewhere, and active T7 RNA polymerase can be easily prepared following published protocols [17, 18].
4. We usually order the oligonucleotide at 1 μmol of synthesis scale with a PAGE purification. The above sequence can simply be changed to produce other tRNAs of interest for studying their maturation with NMR.
5. Efficient transcription are typically obtained from an 18-nucleotide-long T7 promotor primer: 5' TAA TAC GAC TCA CTA TAG 3' [19]. We usually order the oligonucleotide at 0.2 μmol of synthesis scale purified with a simple desalting step.
6. The multiple protease deficient strain c13-ABYS-86 (genotype *MAT α ura3 Δ 5 leu2-3,112 his3 pral-1 prb1-1 prc1-1 cps1-3*) [20] lacks the four major non-specific protease, namely the two

endoproteinases A and B and the two carboxypeptidases Y and S. Cell extracts prepared from this strain have a much reduced ability to degrade unspecific protein substrates, namely ~1% of the remaining protease activity as compared to a wild type strain harboring these enzymes [21].

7. Several methods for lysing yeast cells are available, such as pressure chambers (*e.g.* Eaton press and French press), abrasives (glass bead vortexing), and enzymatic lysis (*e.g.* zymolyase). In our hands, best enzymatic activity is achieved with mechanical lysis on frozen cells in an Eaton pressure chamber [22]. If an Eaton press is not available in the laboratory, we recommend the use of a French press for cell lysis.
8. Cell extracts may also be snap freezed using an appropriate quantity of dry ice.
9. The composition of the maturation buffer aims at approaching cellular conditions, while at the same time remaining compatible with NMR spectroscopy [23].
10. Precise concentration of the SAM stock solution should be calculated from the millimolar extinction coefficient of SAM at 260 nm ($\epsilon_{260}(\text{SAM}) = 15.4 \text{ mM}^{-1}.\text{cm}^{-1}$) and the measurement of the absorbance at 260 nm of a suitable dilution of the solution.
11. We recommend preparing SAM stock solutions from SAM p-toluenesulfonate salt (*e.g.* Sigma-Aldrich, catalog number A2408). In addition, SAM is not stable in the presence of nucleophiles, and is therefore not stable in basic conditions. Sulfuric acid H_2SO_4 is a strong acid with a conjugate base SO_4^{2-} being a poor nucleophile. SAM solutions are quite stable in these conditions, if stored at -20°C . Note that the pH of a 5 mM H_2SO_4 solution is ~2, which may be checked by putting a drop of the SAM stock solution on a pH indicator paper.
12. Precise concentration of the NADPH stock solution should be calculated from the millimolar extinction coefficient of NADPH at 340 nm ($\epsilon_{340}(\text{NADPH}) = 6.3 \text{ mM}^{-1}.\text{cm}^{-1}$) and the measurement of the absorbance at 340 nm of a suitable dilution of the solution.
13. We recommend preparing NADPH stock solution from NADPH tetrasodium salt (*e.g.* Sigma-Aldrich, catalog number N1630). In addition, NADPH is not stable at acidic pH, therefore adjust pH of the stock solution to pH ~8 by adding a moderately concentrated base (*e.g.* NaOH 3–5 M) drop by drop with a micro-pipette. pH should be checked by putting a drop of the NADPH stock solution on a pH indicator paper.
14. Other types of NMR tubes, such as standard 5 mm or 3 mm tubes may also be used, but volumes of tRNA maturation reactions should be adjusted accordingly.
15. We recommend testing small-scale reactions of 40 μL before large-scale production of tRNAs. Transcriptions are performed with each of the four NTPs at 5 mM (total concentration of 20 mM). Concentration of MgCl_2 is varied in small steps in order to evaluate the efficiency of transcription with the $[\text{MgCl}_2]/[\text{NTPs}]$ ratio ranging from 0.3 to 3.0. Concentration of T7 RNA polymerase and DNA template, and the addition of GMP at 2–6 mM need also to be tested in a second step after determination of the best MgCl_2 concentration. The set-up and the analysis of small-scale transcriptions with denaturing urea-PAGE can be performed as described in reference [24].
16. Our methodology to monitor posttranscriptional modifications in tRNAs with NMR relies on the fact that imino signals are very sensitive to their chemical environment. Imino groups are carried by uridines and guanosines, and can be observed in (^1H , ^{15}N) correlation spectra on condition that the imino proton is protected from exchange with the solvent by hydrogen bonding. Since adenosines and cytosines do not carry imino groups, it is not necessary to produce a sample that

is ^{15}N -labeled on As and Cs. This has the advantage of drastically reducing the cost of sample production.

17. After ~ 1 h of incubation, a white precipitate of magnesium pyrophosphate becomes most of the time visible and is indicative of an efficient transcription.
18. After addition of EDTA and few minutes of incubation, the solution should turn clear. Magnesium ions are chelated by EDTA, which displaces the equilibrium of magnesium pyrophosphate precipitation. In case of remaining turbidity, add EDTA 0.5 M progressively, by steps of 0.1 mL until the solution becomes clear.
19. Transcribed RNAs are sensitive to RNases, one should therefore be careful to operate the purification in RNase free conditions. Washing the purification system and the column with NaOH 0.5 M is sufficient to remove most sources of RNase contamination. In addition, we recommend to run the purification at 4°C in a cold room or refrigerated cabinet, but purification may also be performed at room temperature.
20. It is not recommended to load more than 5–6 mL of transcription onto the column. With larger quantities of tRNAs, the purification becomes indeed difficult due to a broadening of the elution peaks. Repeat steps 5 and 6 as many times as needed for large-scale transcriptions of more than 5 mL.
21. Elution fractions can be analyzed with denaturing urea-PAGE. Yeast tRNA^{Phe} usually elutes between 50 and 55% of buffer B in five fractions of 1 mL each. Do not add fractions of tRNA^{Phe} eluted after 55% of buffer B, since they usually contain longer RNA transcripts originating from non-templated nucleotide addition at the 3'-end of the transcript.
22. For an efficient and extensive dialysis, we usually dialyze the sample against a 2 L dialysis solution for at least 1–2 hours and change it with a fresh solution three times.
23. We recommend performing the refolding step on diluted tRNAs at a low salt concentration. In the case of the yeast tRNA^{Phe}, we have observed that refolding at high salt and/or in the presence of MgCl_2 was less suitable for obtaining tRNAs of high-quality NMR spectra. Refolding with high-salt most probably leads to increased misfolding. However, the situation might be different for the refolding of other tRNAs and should be tested.
24. The yeast tRNA^{Phe} NMR sample can be stored at -20°C for at least 12–18 months without observable degradation and alteration of its NMR spectral properties.
25. The weight of the cell pellets can be evaluated by subtracting the weight of an empty conical tube.
26. Final concentrations in the yeast cell extract are as follow: 4 mM DTT, 2 mM PMSF, 2 mM benzamidine, and leupeptin, antipain, pepstatin A, and chymostatin each at $2\text{ }\mu\text{g/mL}$.
27. Yeast cellular extracts can be stored at -80°C for at least 12 months without a significant reduction of activity as seen in NMR monitoring of post-transcriptional tRNA modifications.
28. For the maturation studies, we selected a ^{15}N -labeled tRNA concentration of $40\text{ }\mu\text{M}$ as a compromise to achieve sufficient signal-to-noise ratio in NMR measurements while seeking to approach cellular tRNA concentrations. As a comparison, the concentration of total tRNAs has been estimated to be 100–200 μM in yeast and 200–350 μM in *E. coli*, with typical concentrations of individual tRNAs of 2–15 μM [25, 26].

29. To keep the delay between the mixing of the reaction components and start of the NMR experiments as short as possible, we recommend to set-up NMR experiments on a reference sample of similar composition. Specifically, shimming, tuning and matching of the probe and pulse calibrations are performed in advance on this reference sample. In these conditions, tRNA maturation experiments can be quickly started, since only a rapid adjustment of the shims on the real sample is needed.
30. One of the main challenge of NMR monitoring of tRNA maturation is to identify the NMR signature of individual modifications and thus to associate a particular chemical shift change on the NMR spectra with a specific tRNA modification event. For the yeast tRNA^{Phe}, we assigned its imino groups in three forms differing in their modification content [24, 27]. From the analysis of the differences between their (¹H, ¹⁵N) chemical shifts, we could identify the NMR signature of individual modifications [15, 27]. The requirements for such a detailed analysis might be system dependent, but we anticipate that for any system, the complete NMR analysis of at least two tRNA samples, with and without modifications, would be crucial.
31. In the situation where solution stocks of SAM, NADPH and ¹⁵N-labeled tRNA could not be prepared at the mentioned concentration, please adapt the volumes to reach a final concentration of SAM and NADPH of 4 mM and a final concentration of ¹⁵N-labeled tRNA of 40 μ M.

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Figure 1. Schematic description of the method. **(a)** Step 1: tRNA transcription with ^{15}N -labeled nucleotides. A ^{15}N -labeled tRNA (represented as red dots) is produced by T7 *in vitro* transcription. The transcription reaction is purified by ion-exchange chromatography (MonoQ column), and the purified ^{15}N -labeled tRNA is dialyzed and concentrated for use in step 3. **(b)** Step 2: unlabeled cell extract preparation. A cell culture is performed in an Erlenmeyer flask with unlabeled growth media. After cell lysis, cell debris are removed by ultracentrifugation, leading the unlabeled cellular extract subsequently used in step 3. **(c)** Step 3: NMR monitoring of tRNA maturation. The ^{15}N -labeled tRNA produced in step 1 is introduced in the unlabeled cellular extract produced in step 2. This mix is transferred to an NMR tube to yield an *in extract* NMR sample. The tRNA maturation sample is incubated directly in the NMR spectrometer. Successive NMR measurements are performed to provide time-resolved information on the tRNA maturation process. NMR chemical shift changes (indicated with arrows on the NMR spectra) are identified and linked to specific modification events. The acquisition of NMR spectra in a continuous and time-resolved fashion enables the identification of a sequential order in the introduction of posttranscriptional modifications.

Figure 2. Time-resolved NMR monitoring of RNA modifications in yeast tRNA^{Phe}. **(a)** Imino (¹H, ¹⁵N) correlation spectrum of a ¹⁵N-labeled yeast tRNA^{Phe} measured *in vitro* to provide a reference spectrum (first top left spectrum) and in a time-resolved fashion during a continuous incubation at 30°C in yeast cellular extract over 18 h (remaining five spectra). Each NMR spectrum measurement spreads over a 2 hours time period (incubation time indicated on each spectrum). Modifications occurring at the different steps are reported with arrows. **(b)** Schematic view of the sequential order of the introduction of modifications in yeast tRNA^{Phe} as observed with NMR.