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NAD tagSeq for transcriptome-wide identification and characterization of NAD^+-capped RNAs

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Several noncanonical initial nucleotides (NCINs) have been found to cap RNAs and possibly regulate RNA stability, transcription and translation. NAD^+ is one of the NCINs that has recently been discovered to cap RNAs in a wide range of species. Identification of the NAD^+-capped RNAs (NAD-RNAs) could help to unveil the cap-mediated regulation mechanisms. We previously reported a method termed NAD tagSeq for genome-wide analysis of NAD-RNAs. NAD tagSeq is based on the previously published NAD captureSeq protocol, which applies an enzymatic reaction and a click chemistry reaction to label NAD-RNAs with biotin followed by enrichment with streptavidin resin and identification by RNA sequencing. In the current NAD tagSeq method, NAD-RNAs are labeled with a synthetic RNA tag and identified by direct RNA sequencing based on Oxford Nanopore technology. Compared to NAD captureSeq, NAD tagSeq provides a simpler procedure for direct sequencing of NAD-RNAs and noncapped RNAs and affords information on the whole sequence organization of NAD-RNAs and the ratio of NAD-RNAs to total transcripts. Furthermore, NAD-RNAs can be enriched by hybridizing a complementary DNA probe to the RNA tag, thus increasing the sequencing coverage of NAD-RNAs. The strategy of tagging RNAs with a synthetic RNA tag and identifying them by direct RNA sequencing might be employed in analyzing other NCIN-capped RNAs. The experimental procedure of NAD tagSeq, including RNA extraction, RNA tagging and direct RNA sequencing, takes ~5 d, and initial data analysis can be completed within 2 d.

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Introduction

The 5′ end of eukaryotic mRNAs generally contains a methyl-7-guanosine (m^7G) cap, which is formed soon after transcriptional initiation[1,2]. The cap not only protects mRNAs from degradation by 5′–3′ exonucleases but also forms a cap-binding complex that mediates subsequent transcriptional and translational processes[3]. The recent findings that RNAs can be capped with numerous non-canonical nucleotide derivatives, such as NAD^+, FAD and dpCoA, indicate another layer of cap-mediated gene regulation[4–6]. Those regulations might involve alteration of RNA stability and translation initiation and interaction with proteins involved in mRNA transport[7,8].

NAD^+ is a central co-enzyme in cellular redox reactions and possibly regulates the NAD-RNA levels[9–11]. However, the identities of the NAD-RNAs, as well as the NAD^+ cap-mediated biological functions, are largely unknown. There is an urgent need to develop methods that can provide direct evidence and quantitative information about the presence of NAD^+ cap on individual RNA.

Existing methods for identifying NAD-RNAs

Several years ago, NAD captureSeq was developed for transcriptome-wide profiling of NAD-RNAs[12]. In NAD captureSeq, adenosine diphosphate-ribosyl cyclase (ADPRC) is used to replace the nicotinamide moiety of NAD^+ with an alkyne-containing molecule called 4-pentyn-1-ol. The alkynylated product is then linked with biotin-azide through a click chemistry reaction (copper-catalyzed azide-alkyne cycloaddition (CuAAC)). The biotin-labeled RNAs are enriched by streptavidin resin, followed by making a cDNA library sequenced by next-generation sequencing (NGS) technology. A negative

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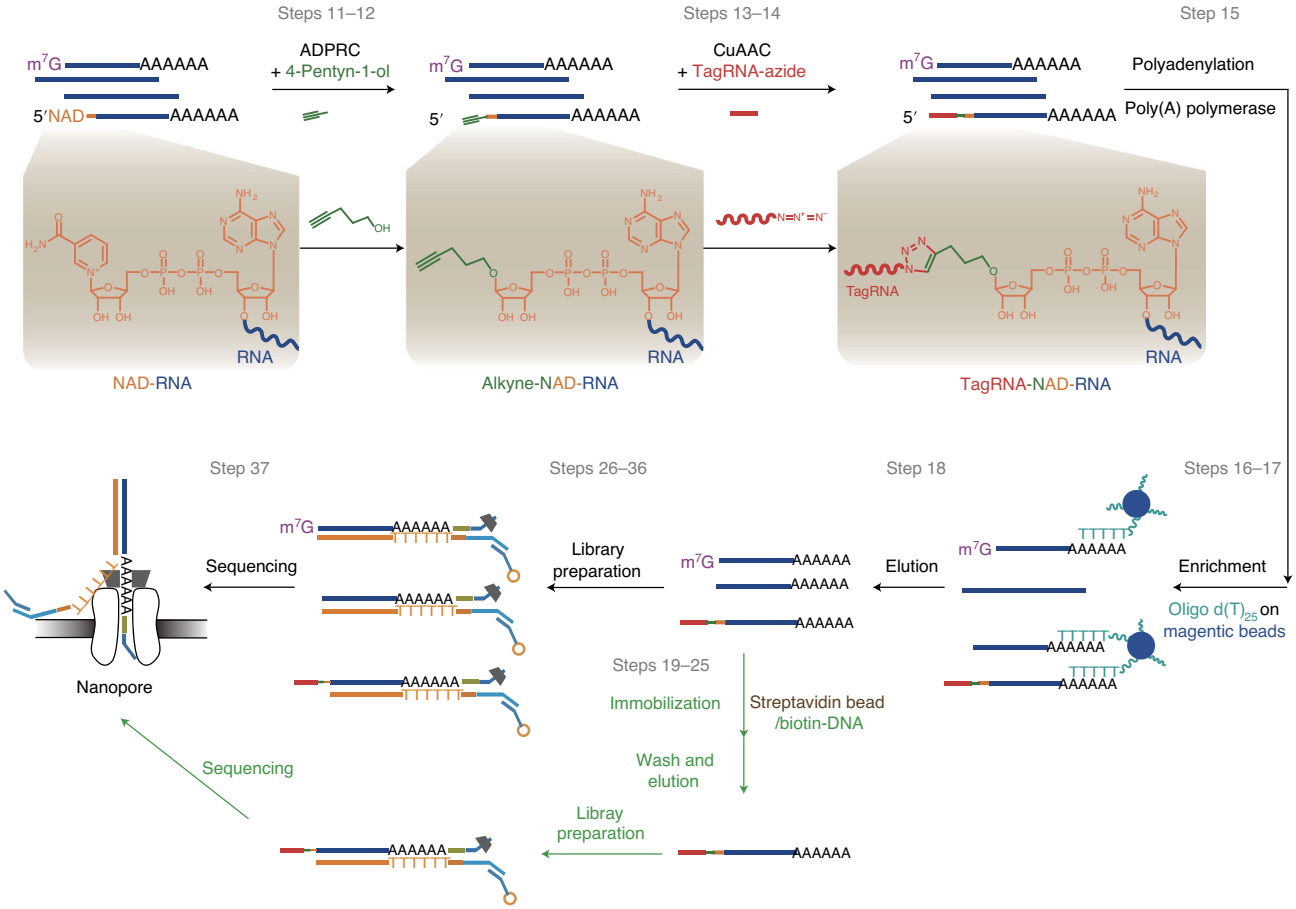

Fig. 1 | Schema of NAD tagSeq. First, the NAD^+ cap is modified with 4-pentyn-1-ol under ADPRC catalysis and then labeled with specific tagRNA-azide through CuAAC. Second, total RNAs are tailed with poly(A) with poly(A) polymerase and purified with oligo dT beads. Third, it is optional to enrich the tagged NAD-RNA by hybridization with a DNA probe (green arrows). The RNAs then undergo library preparation before Nanopore sequencing.

control RNA sample is treated in the same way but without ADPRC. If a specific RNA is enriched in the ADPRC-treated sample compared to the negative control, some of its transcripts are considered to be NAD^+ capped. Through NAD captureSeq, NAD-RNAs have been identified in bacteria[13], yeast[14], plants[5,15] and human cells[16].

In addition to NAD captureSeq, another method termed CapZyme-Seq was developed for the identification of RNAs that contain a NAD^+ cap or other noncanonical caps[17]. In this method, RNA decapping enzymes, such as NudC from *Escherichia coli*, are applied to hydrolyze the NCIN cap to form 5′-monophosphate RNAs. Decapped RNAs are then ligated with an RNA oligo, whereas m^7G-capped RNAs could not be ligated to the oligo. The RNAs ligated with the RNA oligo are then reverse transcribed to cDNAs for amplification and identification of the NCIN-capped RNAs by high-throughput sequencing.

Development of NAD tagSeq

One shortcoming of NAD captureSeq is the nonspecific binding of non-biotinylated RNAs to the streptavidin resin. Because NAD-RNA levels are likely low, the nonspecific binding could significantly reduce sensitivity and introduce false positives. In CapZyme-Seq, NudC not only hydrolyzes the NAD^+ cap but also cleaves other noncanonical caps, making it difficult to discriminate the authentic NAD-RNAs.

To overcome these issues, we developed the NAD tagSeq method for transcriptome-wide identification and characterization of NAD-RNAs (Fig. 1)[15]. Like NAD captureSeq, the NAD tagSeq method also uses the ADPRC-catalyzed reaction and the CuAAC reaction for labeling of NAD-RNAs. However, instead of tagging NAD-RNAs with biotin, NAD tagSeq uses azide conjugated with

a synthetic RNA tag in the click chemistry reaction, thus ligating the NAD-RNA with the RNA tag (Fig. 1). Then, the RNAs are directly sequenced by single-molecule sequencing technology such as the Oxford Nanopore sequencing platform. The RNA tag serves as an identifier for NAD-RNA, meaning that an RNA sequence read containing the tag sequence at its 5′ end is assumed as NAD^+ capped. The RNA tag can also be used to enrich the tagged RNAs through hybridization with a DNA oligo that has a complementary sequence and gets immobilized on beads. The enrichment step is optional. With enrichment, sequencing coverage of NAD-RNA can be increased. Without the enrichment, both tagged and nontagged transcripts are identified by Nanopore sequencing, thereby obtaining the ratios of NAD-RNAs to total transcripts. The NAD tagSeq method also enables quantification of relative abundance of NAD-RNAs and total transcripts from the same genes.

After the tagging step, a poly(A) tail is added to all RNAs by employing a polyadenylation enzyme, as only poly(A)-containing RNAs can be sequenced by Oxford Nanopore sequencing (Fig. 1)[18,19]. As a result, NAD-RNAs are labeled with an RNA tag at the 5′ end and polyadenylated at the 3′ end, whereas other RNAs only undergo polyadenylation. Then, library preparation is performed for all the RNAs, which includes ligation with reverse transcription adapter (RTA), reverse transcription and attaching RNA adapter. Finally, direct RNA sequencing by Nanopore is applied to detect the processed RNAs.

After obtaining the reads given by Guppy[20], we need to identify the NAD-RNAs. The base-caller is unable to recognize the sequences near the 1,2,3-triazole linkage; therefore, we use the tagged and untagged forms of RNA from each gene to represent the NAD-RNAs and noncapped RNA, respectively. Because the tag RNA is 40 nucleotides long, and the linkage can affect the base-calling, we sort out the reads with 12 consecutive tag RNA nucleotides in the first 40 nucleotides and classify them as the tagged RNAs. The reads are then aligned to the reference genome and transcriptome to find out genes that are enriched in the $ADPRC^+$ sample when compared to the $ADPRC^-$ sample. The structure of the tagged NAD-RNA can be visualized by Integrative Genomics Viewer[21].

Applications of the method

The NAD tagSeq protocol can be used for genome-wide identification and quantification of NAD-RNAs in different organisms, including eukaryotes and prokaryotes. It can be used to compare NAD-RNA profiles in different samples and analyze the overall levels of cellular NAD-RNAs and individual NAD-RNA levels. The method can yield information on abundance of NAD-RNAs and total transcripts from the same gene if the enrichment of the tagged RNAs is skipped. Furthermore, the whole sequence structure of NAD-RNAs can be revealed by the long-read sequencing technique[22]. This technique can be used to analyze NAD-RNA profiles under different physiological conditions and compare them with profiles of total transcriptomes and proteomes to reveal possible roles of NAD-RNAs in mediating gene expression.

Comparison with other methods

Numerous methods have been developed for the analysis of RNA caps. Some of the approaches are designed for quantifying the global level of RNA caps, whereas others are capable of mapping the NAD^+ cap to individual RNA.

The methods in the first category generally employ nucleases to digest RNA into single nucleotides before measurement. To separate the RNA caps, thin-layer chromatography (TLC) was developed based on the concept that nucleotides with distinct modifications differ from the unmodified counterparts by net charge, polarity and hydrophobicity[23]. ^{32}P radioactive labeling is used to enhance the detection sensitivity of TLC[24]. Liquid chromatography–mass spectrometry (LC–MS) has been used in numerous publications[25–28] to identify and quantify novel RNA caps, including NAD^+, FAD, dpCoA and m^7Gpppm^6AmGp. Moreover, NAD-capQ uses a colorimetric probe to quantify the nuclease-released NAD^+ cap[9]. These relatively simple methods are used mainly to determine the overall cap level in RNA[29].

The second category of methods primarily applies RNA-sequencing techniques to identify the NAD-RNAs[30]. These techniques include NAD captureSeq and CapZyme-Seq that rely on reverse transcription of RNAs and PCR amplification to make cDNA libraries that can be sequenced by NGS. Potential drawbacks of these methods include (1) the absence of direct evidence of the NAD^+ cap, (2) the fact that PCR amplification might cause bias[31], (3) the complexity and ambiguity during sequencing assembly[32], and (4) the loss of information about RNA post-transcription modifications, such as alternative splicing. In NAD captureSeq, nonspecific binding of RNA to streptavidin resin

could cause false-positive reads and reduce sensitivity. Fragmentation of RNAs during the biotin tagging process and subsequent cDNA library construction causes loss of the information on the overall sequence structures of NAD-RNAs. In the NAD tagSeq method, if a polyadenylation step is not included, fragmented NAD-RNAs will not be identified owing to their lack of a poly(A) tail. Measures to reduce RNA fragmentation, such as reducing the duration of the CuAAC reaction, can enhance the identification of full-length NAD-RNAs. In CapZyme-Seq, the sequencing results cannot discriminate different NCIN-capped RNAs by using nonspecific cleavage enzymes. Thus, it is necessary to develop novel enzymes for specific RNA caps.

Advantages of NAD tagSeq

NAD tagSeq has several advantages compared to other NAD-RNA sequencing methods, including NAD captureSeq[12] and CapZyme-Seq[17].

First, NAD tagSeq could use the RNA tag in the 5′ end as an effective tool to filter out some of the false positives. In NAD captureSeq, RNA molecules nonspecifically bound to streptavidin resin are converted to cDNA library together with biotin-labeled RNAs. The nonspecific binding issue also happens in NAD tagSeq; however, NAD tagSeq could use direct RNA sequencing to discriminate NAD-RNAs from other nonspecific binding ones by the presence of the RNA tag.

Second, NAD tagSeq does not need a PCR procedure as in NAD captureSeq, which could result in more accurate quantification of NAD-RNAs by avoiding bias caused by PCR[22]. NAD tagSeq also allows quantification of absolute abundance and relative abundance of NAD-RNAs in total transcripts.

Third, NAD tagSeq is simpler than NAD captureSeq, as RNAs can be directly identified after the tagging, therefore eliminating the whole process of making a cDNA library.

Fourth, NAD tagSeq can reveal the overall sequence of NAD-RNAs. Oxford Nanopore sequencing technology allows RNA molecules to be sequenced from the 3′ end to the 5′ end. Hence, the whole sequence of the RNA molecules can be revealed. It allows for analyzing how the caps regulate RNA function through post-transcriptional modifications like alternative splicing or 5′ untranslated regions.

Fifth, the idea of tagging NAD-RNA with a synthetic RNA tag followed by direct RNA sequencing might be applied in the future for developing similar methods to identify other NICN-capped RNAs if a specific enzyme and/or click chemistry reaction can be developed for tagging RNAs capped with other NCINs.

Limitations of NAD tagSeq

There are certain limitations for the NAD tagSeq method. Nanopore sequencing starts from the 3′ end to the 5′ end and sometimes results in truncated reads[33,34], meaning that the 5′ region of RNA might not be sequenced. Therefore, some sequence reads without the RNA tag might come from NAD-RNAs, which introduces some false negatives. Besides, fragmentation of RNAs, such as during the click chemistry reaction in the presence of copper ions, could lead to false negatives if a polyadenylation step is not included before tagging. Another disadvantage of NAD tagSeq is that it is more expensive than NAD captureSeq owing to the current cost of Oxford Nanopore flow cells (around US$800 per cell). In addition, the Oxford Nanopore sequencing platform has a limit in sequencing small RNAs. The shortest sequencing reads we obtained, not including the poly(A) tail and the RNA tag sequence, were ~80 nucleotides, raising a possibility that some small NAD-RNAs might be missed by this analysis. We speculate that Illumina sequencing might also be used for NAD tagSeq; however, it needs further testing of whether a reverse transcriptase can read through the joint between the RNA tag and the NAD cap to reach the RNA tag region.

Both NAD tagSeq and NAD captureSeq require a fairly high amount of input RNA (~100 μg of total RNA). There remains a possibility that the ADPRC-mediated reaction and/or the click chemistry reaction used in the NAD tagSeq and NAD captureSeq methods could also lead to tagging of other known or unknown NICN caps or RNA modifications, causing false positives. Despite the limitations, NAD tagSeq represents a very useful tool for identifying NAD-RNAs and understanding their molecular roles and biological functions.

Level of expertise needed to implement the protocol

Most steps of this protocol can be performed by researchers experienced in molecular biology techniques and bioinformatic analysis. The synthesis of model NAD-RNA by in vitro transcription and the labeling of NAD-RNAs with chemoenzymatic reactions are relatively easy to conduct, and the tagRNA-azide is custom designed and commercially available. Before NAD tagSeq, the

verification of the presence of NAD^+ in RNA requires experience in MS analysis. Alternatively, the colorimetric method based on NAD-capQ can be used. Finally, researchers need training for the Nanopore sequencing platform, which can be established in most labs.

Experimental design

Before performing the NAD tagSeq protocol, several preparations should be carried out. Besides preparing reagents (see 'Materials' section), several optional preparatory steps are described in this article.

Before the NAD tagSeq experiment, one might verify the NAD^+ presence or determine the NAD^+ levels in specific RNA samples by using LC–MS when working on a species for which NAD-RNAs have not been determined[26] (see Supplementary Methods: LC–MS analysis of NAD^+ presence in total RNA).

Validation of the tagging process using a model NAD-RNA

To analyze the labeling efficiency of the RNA tag, we synthesize a model NAD-RNA (38 nucleotides) by in vitro transcription using T7 RNA polymerase[35]. The transcription of model NAD-RNA is driven by T7 Class II promoter, and only the first transcribed nucleotide is adenosine so that NAD^+ can be incorporated in the transcript at the 5′ end (Box 1). The synthesized NAD-RNA is then purified using a PAGE gel (Box 1). The purity of model NAD-RNA is further monitored using PAGE gel (Steps 1 and 2). Then, the model NAD-RNA undergoes ADPRC-catalyzed chemoenzymatic reaction with 4-pentyn-1-ol and CuAAC reaction with tagRNA-azide (25 nucleotides; Steps 3–7). The products are monitored by PAGE gel, where a reaction lacking ADPRC ($ADPRC^-$) is used as a negative control (Steps 8 and 9).

RNA sample preparation for NAD tagSeq

Fragmentation and degradation of RNAs will reduce the efficiency of identifying NAD-RNAs by using NAD tagSeq. For RNA sample preparation, we recommend using fresh tissues for RNA extraction. Alternatively, tissues can be frozen in liquid nitrogen immediately after harvesting. RNase-free reagents, and consumables should be used for sample preparation. RNA can be extracted with Tri-reagents (such as TransZol) or RNeasy kits[36]. Then, DNA contamination is removed from the RNA sample with DNase. It is highly recommended to use a sufficient amount of RNA (10 μg or higher) for tagging and sequencing to reduce degradation or fragmentation by the copper ions in the CuAAC reaction. If a total RNA sample is used, we recommend 100 μg RNAs for the tagging experiment (see 'Limitations'). The RNA integrity is checked with agarose gel electrophoresis (Step 10). The RNA quality can also be monitored by NanoDrop, Qubit or Agilent BioAnalyzer 2100 systems. For long-term storage up to 2 months, the RNA samples should be aliquoted and stored at −80 °C.

Labeling NAD-RNA with RNA tag

To conduct the reaction, the model NAD-RNA or the total cellular RNAs is first modified with a clickable linker, 4-pentyn-1-ol, under the catalysis of ADPRC. Then, the alkyne-linked RNA undergoes click chemistry reaction with a specific tagRNA-azide (40 nucleotides; Steps 11–14). As a negative control, the sample is treated in the same way except that no ADPRC is added. Results from the negative control help to assess the noise level from nonspecific tagging of RNA molecules.

Polyadenylation of total RNA

The direct RNA sequencing by Nanopore requires the RNA to contain a poly(A) tail. If eukaryotic messenger RNA is used as an input sample, the polyadenylation step is not required. However, polyadenylation is required if the research is focused on other types of RNA without a poly(A) tail. This step is catalyzed by poly(A) polymerase to add a poly(A) tail to each RNA, and then the RNAs are purified by oligo dT beads (Steps 15–18).

Enrichment of NAD-RNA for in-depth sequencing

This step is optional, and it is conducted to increase the sequencing coverage of NAD-RNA. The tagged NAD-RNA is enriched by a DNA oligo that is immobilized on beads and has a sequence complementary to the RNA tag (Steps 19–25).

Box 1 | In vitro transcription and purification of model NAD-RNA ● Timing ~2 d

Procedure

1 Prepare dsDNA transcription template. Mix equal amounts of 20 μM of model NAD-RNA template-sense and 20 μM of model NAD-RNA template-antisense ssDNA in a PCR tube. Heat the sample to 90 °C for 5 min for annealing and cool down to 4 °C in a thermal cycler with a ramp rate of 0.2 °C/s.

■ PAUSE POINT The synthesized template dsDNA can be stored at −80 °C for several years.

2 Combine the following in vitro transcription components in a 1.5-ml tube and incubate at 37 °C for 4 h to synthesize model NAD-RNA. The detailed procedure for preparation of T7 RNA polymerase is included in Supplementary Methods: preparation of recombinant T7 RNA polymerase.

Reagent	Stock concentration	Volume (μl)	Final concentration
dsDNA template	10 μM	40	1 μM
T7 RNA polymerase	1 μg/μl	10	0.025 μg/μl
In vitro transcription buffer	10×	40	1×
DTT	100 mM	20	5 mM
DMSO	100%	20	5% (vol/vol)
NAD^+	50 mM	32	4 mM
GTP	50 mM	8	1 mM
CTP	50 mM	8	1 mM
UTP	50 mM	8	1 mM
Murine RNase inhibitor	40 U/μl	10	1 U/μl
Water		To 400	
Total		400	

▲ CRITICAL STEP The step is optimized from a published protocol[12] to increase the purity of model NAD-RNA (see 'Experimental design' section).

3 Add DNase I to 400 μl of the RNA sample in transcription buffer according to the table below to degrade the template DNA and incubate at 37 °C for 10 min.

Reagent	Stock concentration	Volume (μl)	Final concentration
Model RNA sample		400	
DNase I reaction buffer	10×	50	1×
DNase I (RNase-free), 50 U	2 U/μl	25	0.1 U/μl
Water		To 500	
Total		500	

▲ CRITICAL STEP This step is not used in NAD CaptureSeq. However, we highly recommend using DNase I to eliminate the DNA template before gel electrophoresis; otherwise, the band of DNA template might interfere with that of NAD-RNA.

4 Add 200 μl of acid phenol:chloroform to the reaction mixture and mix by vortex. Centrifuge at 12,000*g* at 4 °C for 5 min and transfer the supernatant to a new 1.5-ml tube. Add in 100 μl of chloroform and mix well. Centrifuge at 12,000*g* at 4 °C for 5 min and transfer the supernatant (~540 μl) to a new 2-ml tube.

5 Add in 60 μl of 3 M NaOAc (pH 5.5; final, 0.3 M) and then mix with 1.5 ml of EtOH and save at −20 °C for 2 h. Then, centrifuge at 12,000*g* at 4 °C for 30 min to precipitate RNA and collect the RNA pellets. Remove and discard the EtOH and air dry the RNA pellet no longer than 5 min.

6 Re-dissolve the RNA in 20 μl of water and then add in 20 μl of 2× RNA loading dye. Denature RNA at 95 °C for 2 min and load the sample into the wells of precast 10% (wt/vol) denaturing PAGE gel. Run the gel at 150 V until the bromophenol blue dye runs out of the gel.

7 Observe the products stained with RedSafe on a transilluminator. Excise the product by cutting the band with width of 2–3 mm into smaller pieces for RNA elution. Place the gel pieces in 2-ml tubes and grind the gel slice into a fine powder with a pipette tip.

8 Add in 1 ml of 0.3 M NaOAc (pH 5.5) to the gel powder. Put the tube on a shaker and incubate at 4 °C for 30 min with shaking speed of 600 r.p.m.

9 Pass the supernatant through a syringe capped with a sterile filter to remove gel pieces and add 8 μl of 5 mg/ml linear acrylamide and ice-cold EtOH to a final concentration of 70%. Cool the solution at −80 °C for 30 min and centrifuge in a pre-cooled microcentrifuge at 17,000*g* for 30 min at 4 °C. Remove and discard the supernatant and wash the pellet twice with 70% (vol/vol) EtOH. The dried RNA can be stored at −80 °C for up to 3 months for further uses.

Library preparation and sequencing with MinION

Typically, 200–500 ng of RNA is used for making a sequencing library. Following the direct RNA sequencing protocol of Oxford Nanopore Technologies, the RNA samples are ligated with RTA, reverse transcribed and linked with a sequencing adapter[19], which can all be accomplished with the

Nanopore Direct RNA Sequencing Kit. Then, the generated libraries can be sequenced on Nanopore sequencing devices. All these can be done following the manufacturer's instructions. Base-calling is conducted with the MinKNOW software and the embedded Guppy data processing toolkit (Steps 26–38).

Identification of NAD-RNA from sequencing reads

Although base-callers are unable to recognize the sequences near the 1,2,3-triazole linkage, the reads given by Guppy can still be used to determine the NAD-RNAs through the tag RNA (Steps 39–43). In the original application of NAD tagSeq[15], we developed Python scripts, combined with available tools such as Guppy and Minimap2 (ref. [37]), to implement data analysis. To facilitate the performance of this protocol, we established a computational pipeline of these tools termed TagSeqTools[38]. Here we present a set of detailed procedures for identification and quantification of NAD-RNA, which uses TagSeqTools and existing software such as FastQC[39]. We differentiate NAD-RNA and non-NAD-RNA using the TagSeek module in TagSeqTools. The tagged and non-tagged reads generated from the TagSeek step can be subjected to TagSeqQuant, which uses default alignment parameters of Minimap2. In TagSeqQuant, the reads also undergo quality check by FastQC[39], and an html file is generated to show the sequencing quality. After the aforementioned analysis steps, gene coverage files for NAD-RNAs and non-NAD-RNAs are generated for IGV visualization[21], and counts tables of genes and isoforms are also produced for further analysis.

Materials

Reagents

- 4-Pentyn-1-ol (Sigma-Aldrich, cat. no. 302481) **! CAUTION** 4-Pentyn-1-ol is an irritant to skin, eyes and the respiratory tract. Wear gloves or operate in a fume hood.
- Acid phenol:chloroform (with IAA, 125:24:1, pH 4.5; Ambion, cat. no. AM9722) **! CAUTION** Phenol and chloroform are toxic. Wear protective gloves and clothing to prevent contact.
- Acrylamide/bis solution (40% (wt/vol) 29:1; Bio-Rad, cat. no. 1610146) **! CAUTION** Acrylamide is a neurotoxin and potential carcinogen. Wear masks, gloves and protective clothing when using it.
- Adenosine 5′-triphosphate disodium salt (Na_2ATP; Sigma-Aldrich, cat. no. A2383)
- ADPRC (Sigma-Aldrich, cat. no. A9106)
- Agarose (Affymetrix USB, cat. no. AAJ10906Q1)
- Ammonium persulfate solution(APS; Bio-Rad, cat. no. 1610700) **! CAUTION** APS is an irritant. Wear gloves and protective clothing.
- Boric acid (Sigma-Aldrich, cat. no. B7901) **! CAUTION** Boric acid is an irritant. Wear gloves and protective clothing.
- Chloroform (VWR International, cat. no. 22711.324) **! CAUTION** Chloroform is a suspected carcinogen. It should be protected from light, and it is best to operate in a fume hood.
- Concentrated hydrochloric acid (37% (wt/wt) HCl; VWR International, cat. no. 20252.42) **! CAUTION** 37% (wt/wt) HCl is highly volatile and corrosive. Operate in a fume hood and wear protective gloves and clothing.
- Copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$; Sigma-Aldrich, cat. no. C3036) **! CAUTION** $CuSO_4$ is an irritant to skin and eyes. Wear protective gloves and clothing when operating.
- Cytidine 5′-triphosphate disodium salt (Na_2CTP; Sigma-Aldrich, cat. no. C1506)
- Diethyl pyrocarbonate (DEPC; Sigma-Aldrich, cat. no. D5758) **! CAUTION** DEPC is toxic and an irritant to skin and eyes. Wear protective gloves and clothing to prevent direct contact.
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D8418)
- Dithiothreitol (DTT; Sigma-Aldrich, cat. no. 43815) **! CAUTION** DTT is toxic and causes skin and eye irritations. Wear gloves and protective clothing to avoid direct contact.
- DNase I (New England Biolabs, cat. no. M0303), supplied with DNase I reaction buffer (10×)
- *E. coli* poly(A) polymerase (New England Biolabs, cat. no. M0276), supplied with poly(A) polymerase reaction buffer (10×) and ATP solution (10 mM)
- Ethanol (EtOH; VWR International, cat. no. 20821.321DP) **! CAUTION** EtOH is inflammable. Keep it from sources of ignition.
- EDTA solution (0.5 M, pH 8.0; Thermo Fisher Scientific, cat. no. 15575020)
- GTP (Sigma-Aldrich, cat. no. G8877)
- Isopropanol (VWR International, cat. no. 20842.330) **! CAUTION** Isopropanol is inflammable. Keep it away from sources of ignition.

- Linear acrylamide (5 mg/ml; Invitrogen, cat. no. AM9520)
- Lithium chloride (LiCl; Sigma-Aldrich, cat. no. L4408)
- Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$; Sigma-Aldrich, cat. no. M2670)
- Murine RNase inhibitor (New England Biolabs, cat. no. M0314)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Nuclease-free water (Ambion, cat. no. AM9930)
- Oligo $d(T)_{25}$ magnetic beads (New England Biolabs, cat. no. S1419S) **! CAUTION** Store at 4 °C for 2–3 years, or until the expiration date on the bottle, and avoid freezing.
- Potassium hydroxide hydrate (KOH; Supelco, cat. no. 1.05002) **! CAUTION** KOH is a severe irritant. Wear gloves and protective gloves when handling.
- RedSafe nucleic acid staining solution (20,000×; iNtRON, cat. no. 21141) **! CAUTION** RedSafe is toxic. Wear gloves and protective clothing and operate in fume hood to avoid direct contact.
- RNA loading dye (2×; New England Biolabs; cat. no. B0363S) **! CAUTION** Bromophenol blue and formamide in the loading buffer are toxic. Wear protective gloves and clothing when using.
- Sodium acetate (NaOAc, 3 M, pH 5.5; Ambion, cat. no. AM9740)
- Sodium ascorbate (Sigma-Aldrich, cat. no. A7631)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5886)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no.S8045) **! CAUTION** NaOH is a severe irritant to skin and other human tissues. Wear gloves to avoid direct contact.
- Spermidine (Sigma-Aldrich, cat. no. S2626) **! CAUTION** Spermidine can cause skin and eye damage. Wear gloves and goggles when handling and use fume hood to prevent exposure.
- Streptavidin magnetic resins (New England Biolabs, cat. no. S1421S) **! CAUTION** Store at 4 °C for 2–3 years, or until the expiration date on the bottle, and avoid freezing.
- T7 RNA polymerase (New England Biolabs, cat. no. M0251; Promega, cat. no. P2077)
- Tetramethylethylenediamine (TEMED; Bio-Rad, cat. no. 1610800) **! CAUTION** TEMED is toxic and volatile. Wear gloves, protective clothing and goggles. Operate in a fume hood.
- TransZol reagent (TransGenBiotech, cat. no. ET101-01) **! CAUTION** TransZol exposure can cause serious chemical burns. Operate in a fume hood and wear protective gloves and clothing.
- TBE buffer (10×; Pierce, cat. no. 28355; Invitrogen, cat. no. 15581044)
- Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA; Click Chemistry Tools, cat. no. 1010)
- Tris (Thermo Fisher Scientific, cat. no. 17926)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Urea (Thermo Fisher Scientific, cat. no. 15505027)
- Uridine 5′-triphosphate trisodium salt hydrate (Na_3UTP; Sigma-Aldrich, cat. no. U6625)
- β-NAD^+ hydrate (Sigma-Aldrich, cat. no. N1636)

Oligonucleotides

▲ CRITICAL All DNA oligos purified by PAGE and azide RNA oligos purified by HPLC are custom tailored and commercially available. They are delivered as dry powder. The oligos are dissolved with nuclease-free water to a final concentration of 100 μM, and aliquots can be stored at −20 °C for up to 6 months. Repeated free-thaw cycles should be avoided.

- DNA-biotin molecule (5′-TTCAGGTTCAGGTTCAGGTTCAGG-biotin-3′; Beijing Genomics Institute), synthesis scale of 2 OD is available.
- Model-NAD-RNA template-antisense (5′-GCCCGCGAGAGAGAGACAGCAGACGACGACGACACAGTAATAGTGAGTCGTATTAGTGATC-3′; Beijing Genomics Institute), synthesis scale of 4 OD is available.
- Model-NAD-RNA template-sense (5′-GATCAC**TAATACGACTCACTATT**ACTGTGTCGTCGTCGTCTGCTGTCTCTCTCTCGCGGGC-3′ (boldface letters denote T7 class II promoter (ϕ2.5); Beijing Genomics Institute), synthesis scale of 4 OD is available.
- TagRNA-azide, 25 nt tag (5′-GCCAUUGCCAUUGCCAUUGCCAUUG/3AzideN/-3′; Integrated DNA Technologies), synthesis scale of 200 nmol and final yield of ~10 nmol are available.
- TagRNA-azide, 40 nt tag (5′-CCUGAACCUGAACCUGAACCUGAACCUGAACCUGAACCUG/3AzideN/-3′; Integrated DNA Technologies), synthesis scale of 500 nmol and final yield of ~10 nmol are available.

Reagents for library preparation for Nanopore direct RNA sequencing

- Agencourt RNAClean XP beads (Beckman Coulter, cat. no. A63987)
- Direct RNA Sequencing Kit (Oxford Nanopore Technologies, SQK-RNA001), containing RTA, RMX (RNA adapter mix), RCS (RNA CS), WSB (wash buffer), ELB (elution buffer) and RRB (RNA running buffer), supplied with Flow Cell Priming Expansion Pack, which includes FB (flush buffer) and FLT (flush tether). Store RTA, RCS, ELB, WSB on ice, RRB and RMX in a freezer until needed. Now the kit has been updated to Direct RNA Sequencing Kit (SQK-RNA002) that is provided together with Flow Cell Priming Kit (EXP-FLP002)
- Flow Cell Wash Kit (Oxford Nanopore Technologies, EXP-WSH003)
- dNTP mix (10 mM each nucleotide, dATP, dCTP, dGTP, dTTP; Invitrogen, cat. no. 18427013)
- NEBNext Quick Ligation Reaction Buffer (5×; New England Biolabs, cat. no. B6058S)
- Qubit dsDNA HS Assay Kit (Invitrogen, cat. no. Q32851)
- Qubit RNA HS Assay Kit (Invitrogen, cat. no. Q32855), containing Qubit RNA HS Reagent, Qubit RNA HS Buffer, Qubit RNA HS Standard#1 and Qubit RNA HS Standard#2
- SuperScript III Reverse Transcriptase (Invitrogen, cat. no. 18080085), supplied with 5× first-strand buffer and 0.1 M DTT
- T4 DNA ligase (New England Biolabs, cat. no. M0202T)

Equipment

- Agarose gel electrophoresis power supply (Major Science, Minis-150) and agarose gel precast system (Major Science, MT-108)
- Autoclave (Hirayama, HV-85)
- Balance (Shimadzu, ATX224)
- ChemiDoc XRS+ (Bio-Rad)
- Freezer (−80 °C; Panasonic, Ultra-Low Freezer, ALT-501 Model)
- Fume hood (Flores Valles, FL 5331 Model)
- High-speed centrifuge (Beckman Coulter, Avanti JXN-26)
- Magnetic rack (New England Biolabs, cat. no. S1509S)
- Micropipette tips (RNase-free, 2 µl, 20 µl, 200 µl, 1 ml and 10 ml)
- Micropipettes (Eppendorf, P2.5, P10, P20, P200 and P1000)
- Milli-Q reference water purification system (Merck, C79625)
- NanoDrop spectrophotometer (Hangzhou Allsheng Instruments, Nano-100)
- PAGE-running apparatus for 10 × 8-cm gels, including electrophoresis chamber, power supply, glass plates, spacers and combs (Bio-Rad, Mini-PROTEAN Tetra Cell, cat. no. 1660828EDU)
- PCR tubes (0.2 ml, with flat cap; Axygen Scientific, cat. no. PCR-02-C)
- pH meter (Thermo Fisher Scientific, cat. no. STARA2110)
- Qubit 3.0 fluorometer (Invitrogen, cat. no. Q33216)
- RNA Clean Kit 1 (RNA Clean & Concentrator-5; Zymo Research, cat. no. R1015), including RNA binding buffer, RNA prep buffer, RNA wash buffer, Zymo-Spin IC columns, collection tubes and nuclease-free water
- RNA Clean Kit 2 (RNA Clean & Concentrator-25; Zymo Research, cat. no. R1017), containing RNA binding buffer, RNA prep buffer, RNA wash buffer, Zymo-Spin IC columns, collection tubes and RNase-free water
- Rotator mixer (Hula mixer; Thermo Fisher Scientific, cat. no. 15920D)
- Sterile filter (0.2-µm pore size; PALL, cat. no. PN4612)
- Tabletop centrifuge (Eppendorf, Model 5417R)
- Thermal cycler (Bio-Rad, T100)
- Thermal shaker and heater (Eppendorf, ThermoMixer C)
- Transilluminator (UltraBright-LED Blue Light; Syngene, SBLT-2)
- Vertical autoclave (Hirayama, HV-85)
- Vortex mixer (Scientific Industries, Vortex Genie 2)

Equipment for library preparation for direct RNA sequencing by Nanopore

- DNA LoBind tubes (1.5-ml and 2.0-ml Eppendorf, cat. nos. 0030108051 and 0030108078)
- Flow cell Mk I SpotON (Oxford Nanopore, cat. no. FLO-MIN106 R9 version)
- MinION Mk1B (Oxford Nanopore, cat. no. MIN-101B)

Software

- Image Lab Software 6.0.1 for ChemiDoc XRS+
- ImageJ 1.50i for image adjustment (https://imagej.nih.gov/ij/)
- MinKNOW 19.6.8, with base-caller of Guppy embedded, from Oxford Nanopore Technologies
- Ubuntu 18.04.3 LTS, Linux-based operating system or UNIX (https://ubuntu.com/download)
- Python 2.7 (https://www.python.org/downloads/)
- R 3.2.1 (https://cran.r-project.org/)
- Biopython 1.16.6 (https://biopython.org/wiki/Download)
- Regex 2020.2.20 (https://pypi.org/project/regex/)
- FastQC 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
- Minimap2 (v2.12; https://github.com/lh3/minimap2)
- Samtools 1.7 (http://samtools.sourceforge.net/)
- A homemade Python script called TagSeqTools, which contains TagSeek.py and TagSeqQuant.py, is available on GitHub (https://github.com/dorothyzh/TagSeqTools) and as Supplementary Software
- Integrative Genomics Viewer 2.7.2 (IGV; https://software.broadinstitute.org/software/igv/)

Reagent setup

0.1% (vol/vol) DEPC water

For 1 L, add 1 ml of DEPC to 1 L of Milli-Q water and mix well. Then, incubate for 12 h at 37 °C to inactivate RNases in glassware, autoclave at 120 °C for 20 min and cool down before use. The DEPC-treated water can be stored at room temperature (RT) or 4 °C for 1 month.

1% (wt/vol) agarose gel

Mix 0.3 g of agarose, 3 ml of 10× TBE and 27 ml of water and microwave for 5 min to dissolve the agarose. After cooling down to ~60 °C, mix the gel with 1 µl of RedSafe. Then, pour the mixture to gel precast set and let it stand for 15 min to solidify.

10% (wt/vol) APS

Add in 1 g of APS, raise the volume to 10 ml with Milli-Q water and vortex until dissolved. Aliquot to 100 µl and store at −20 °C for 3 months.

10% (wt/vol) denaturing PAGE solution

Prepare freshly before use. For 10 ml, combine 4.8 g of urea, 2.5 ml 40% (wt/vol) of acrylamide/bis (29:1), 100 µl 10% (wt/vol) of APS, 4 µl of TEMED and 1 ml of 10× TBE and then raise the volume to 10 ml with water. ▲ **CRITICAL** Avoid low temperatures to prevent precipitation of urea.

TBE (10×)

For 1 L, dissolve 108 g of Tris and 55 g of boric acid in 800 ml of water. Then, add in 40 ml of 0.5 M EDTA solution (final, 20 mM). Filter the solution with a 0.2-µm filter and store at 25 °C. The buffer can be stored for up to 1 week unless white precipitates form. 10× TBE buffer is also commercially available.

ADPRC reaction buffer (5×)

For 10 ml, add in 2.5 ml of HEPES-KOH stock solution (final, 250 mM) and 250 µl of 1 M $MgCl_2$ stock solution (final, 25 mM), add 6 ml of water and then adjust the pH to 7.0, raising the volume to 10 ml. Store at 4 °C in the dark for 1 year.

ADPRC stock solution (125 µg/ml, ~4.25 µM)

Dissolve 100 µg of ADPRC in 400 µl of 1× APDRC reaction buffer and add 400 µl of glycerol. Store as aliquots of 100 µl at −80 °C for several months. ▲ **CRITICAL** Avoid repeated freeze-thaw cycles.

ATP stock solution (50 mM)

Dissolve 27.56 mg of Na_2ATP in 100 µl of water. Pass the solution through a 0.2-µm filter. Store at −20 °C for 1 year.

CTP stock solution (50 mM)

Dissolve 26.36 mg of Na_2CTP in 1 ml of water. Filter the solution with a 0.2-µm filter. Store at −20 °C for 1 year.

CuAAC reaction buffer (5×, pH 7.0)
For 50 ml, add 2.98 g of HEPES (final, 250 mM) in 35 ml of water and adjust the pH to 7.0. Add 1.25 ml of 1 M $MgCl_2$ (final, 25 mM) and fill up to 50 ml with water. Store at 4 °C in the dark, and the buffer can be saved for at least 3 months.

$CuSO_4$ stock solution (50 mM)
Dissolve 12.48 mg of $CuSO_4 \cdot 5H_2O$ in 1 ml of water. ▲ **CRITICAL** Freshly prepare before use.

DTT stock solution (100 mM)
Dissolve 154.2 mg of DTT in 1 ml of 0.1% (vol/vol) DEPC water. Pass the solution through a 0.2-μm filter. Dispense into 100-μl aliquots. Store at −20 °C for up to 3 months.

GTP stock solution (50 mM)
Dissolve 26.16 mg of GTP in 1 ml of water. Pass the solution through a 0.2-μm filter. Store at −20 °C and use within 1 year.

HEPES-KOH stock solution (pH7.6, 1 M)
For 10 ml, add 2.383 g of HEPES to 7 ml of ddH_2O and then add ~77 mg of KOH pellets to adjust the pH to 7.6, raise the volume to 10 ml with ddH_2O and sterilize by filtration through a 0.2-μm filter. Store at 4 °C and protect HEPES-containing solutions from light for 3 months. Equilibrate to RT to re-dissolve some salts before use.

In vitro transcription buffer (10×)
For 15 ml of in vitro transcription buffer (10×): 6 ml of 1 M Tris-HCl (pH 8.0; final, 400 mM), 1.5 ml of 100 mM spermidine (final, 10 mM), 3.3 ml of 1 M $MgCl_2$ (final, 220 mM), 15 μl of Triton X-100 and 4.185 ml of water. Filter-sterilize the solution. Store at 4 °C and protect Triton X-containing buffer from light. The buffer is stable for 6 months.

LiCl stock solution (5 M)
Dissolve 10.60 g of LiCl in 50 ml of water. Filter the solution through a 0.2-μm filter into a sterile tube. The solution can be stored at 4 °C for 1 year.

$MgCl_2$ stock solution (1 M)
Add 203.3 mg of $MgCl_2 \cdot 6H_2O$ to 1 ml of water. Pass the solution through a 0.2-μm filter. The solution can be stored at 4 °C for 1 year.

NaCl stock solution (5 M)
Dissolve 292.2 mg of NaCl in 1 ml of water. Filter the solution and store at RT for up to 1 month.

NAD^+ stock solution (50 mM)
Dissolve 33.17 mg of NAD^+ in 1 ml of water. Filter through a 0.2-μm filter and aliquot into 100 μl. Store at −80 °C for 1 month.

NaOH stock solution (10 M)
Dissolve 20.0 g of NaOH in 50 ml of water. Store in a plastic bottle at RT for 1 year.

Oligo $d(T)_{25}$ 2× binding buffer
For 50 ml, add 5 ml of 1 M Tris-HCl stock solution (pH 7.5; final, 100 mM), 10 ml of 5 M LiCl stock solution (final, 1 M) and 200 μl of 0.5 M EDTA solution (final, 2 mM) and raise the volume to 50 ml with water. Store at 4 °C for several months.

Oligo $d(T)_{25}$ elution buffer
For 50 ml, add 1 ml of 1 M Tris-HCl stock solution (pH 7.5; final, 20 mM) and 100 μl of 0.5 M EDTA solution (final, 1 mM) and raise the volume to 50 ml with water. Store at 4 °C for several months.

Oligo d(T)$_{25}$ equilibration buffer
For 50 ml, add 1 ml of 1 M Tris-HCl stock solution (pH 7.5; final, 20 mM), 5 ml of 5 M LiCl stock solution (final, 500 mM) and 100 µl of 0.5 M EDTA solution (final, 1 mM) and raise the volume to 50 ml with water. Store at 4 °C for several months.

Oligo d(T)$_{25}$ low-salt buffer
For 50 ml, add 2.5 ml of 1 M Tris-HCl stock solution (pH 7.5; final, 50 mM), 1.5 ml of 5 M LiCl stock solution (final, 150 mM) and 100 µl of 0.5 M EDTA solution (final, 1 mM) and raise the volume to 50 ml with water. Store at 4 °C for several months.

Oligo d(T)$_{25}$ wash buffer
For 50 ml, add 2.5 ml of 1 M Tris-HCl stock solution (pH 7.5; final, 50 mM, 5 ml of 5 M LiCl stock solution (final, 500 mM) and 100 µl of 0.5 M EDTA solution (final, 1 mM) and raise the volume to 50 ml with water. Store at 4 °C for several months.

NaOAc solution (0.3 M, pH 5.5)
For 1 ml, dissolve 100 µl of 3 M NaOAc solution in 1 ml of water. Store at 4 °C for several months.

Sodium ascorbate stock solution (500 mM)
For 1 ml, dissolve 99.06 mg of sodium ascorbate in 1 ml of water. ▲CRITICAL Freshly prepare before use.

Spermidine stock solution (100 mM)
For 10 ml, dissolve 145.2 mg in 10 ml of water. Aliquot into 2 ml and store at −20 °C for several months.

Streptavidin 2× binding buffer
For 50 ml, mix 2 ml of 1 M Tris-HCl (pH 7.5; final, 40 mM), 10 ml of 5 M NaCl stock solution (final, 1 M) and 200 µl of 0.5 M EDTA solution (final, 2 mM) and raise the volume up to 50 ml. The buffer can be stored at 4 °C for 2 months.

Streptavidin wash buffer
For 50 ml, mix 25 ml of streptavidin 2× binding buffer with 25 ml of water. The buffer can be stored at 4 °C for 2 months.

Streptavidin elution buffer
For 50 ml, mix 0.5 ml of 1 M Tris-HCl (pH 7.5; final, 10 mM) and 100 µl of 0.5 M EDTA solution (final, 1 mM) and then raise the volume up to 50 ml. Store the buffer at 4 °C for 2 months.

Streptavidin low-salt buffer
For 50 ml, add in 1 ml of 1 M Tris-HCl (pH 7.5; final, 20 mM), 0.483 g of NaCl (final, 0.15 M) and 100 µl of 0.5 M EDTA stock solution (final, 1 mM). Store the buffer at 4 °C and use within 2 months.

THPTA stock solution (50 mM)
Dissolve 26.53 mg of THPTA in 1 ml of water. Store aliquots of 100 µl at −20 °C for 1 year. ▲CRITICAL Aliquots can be frozen and thawed several times.

Tris-HCl stock solutions (1 M, pH 7.5 and pH 8.0)
For 50 ml Tris-HCl, add 6.06 g of Tris to 40 ml of water and add an appropriate volume of 37% (wt/wt) HCl stock solution to adjust the pH to 7.5 and 8.0, respectively. Then, raise the volume to 50 ml. Store at 4 °C for up to 3 months. ▲CRITICAL Adjust the pH using either 10 M HCl or 10 M NaOH.

UTP stock solution (50 mM)
Dissolve 27.50 mg of Na_3UTP in 1 ml of water. Pass the solution through a 0.2-µm filter. Store at −20 °C for 1 year.

Equipment setup

Sterilize pipettes, tips and tube racks

Use RNase-free tubes and pipette tips. Keep pipettes and racks in paper towels soaked in 70% (vol/vol) EtOH overnight. Then, rinse the racks with DEPC-treated water. ▲ **CRITICAL** Use gloves at all times to avoid RNase contamination.

Procedure

▲ **CRITICAL** Use nuclease-free water or autoclaved Milli-Q water with 0.1% (vol/vol) DEPC whenever water is needed. Use latex gloves or equivalent at all times to avoid RNase contamination. Use RNase-free tubes whenever needed.

Ligation of model NAD-RNA with RNA tag before gel electrophoresis ● Timing ~1 d

1 Synthesize model NAD-RNA by in vitro transcription as described in Box 1. Re-dissolve model NAD-RNA in 30 µl of nuclease-free water and measure RNA concentration (~150 ng/µl) with a Qubit fluorometer. Dilute the RNA to 100 ng/µl and take 10 µl to mix with 10 µl of 2× RNA loading dye. Denature RNA at 95 °C for 2 min. Load the sample into a well of a 10% (wt/vol) denaturing PAGE gel and run the gel at 150 V for ~20 min.

▲ **CRITICAL STEP** The synthesis of model NAD-RNA through in vitro transcription refers to that of NAD captureSeq[12] with minor adjustment. The synthesized model NAD-RNA can be aliquoted for several uses and stored at −80 °C.

2 Stain the gel with RedSafe and visualize the purified NAD-RNA using ChemiDoc XRS+. See 'Anticipated results' section and Fig. 2 for an example.

? TROUBLESHOOTING

3 Combine the following reagents in a 1.5-ml tube and incubate at 37 °C for 30 min. A reaction without ADPRC (ADPRC$^-$) is used as a negative control.

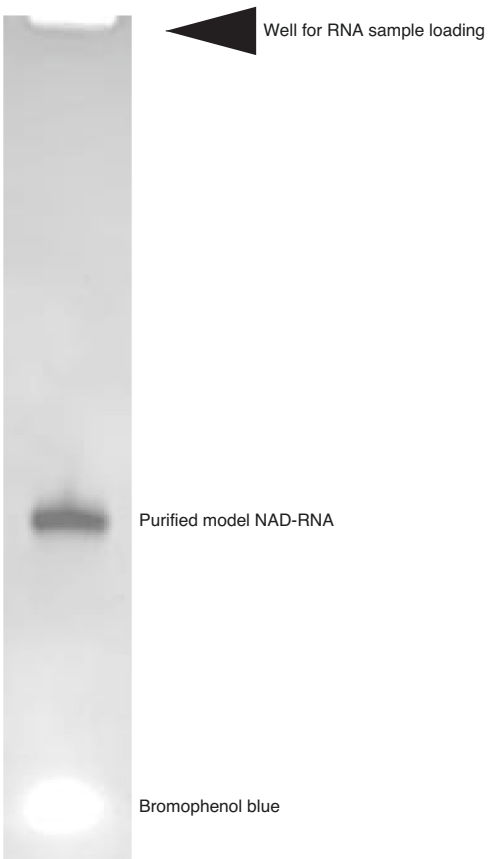

Fig. 2 | Gel electrophoresis of the purified model NAD-RNA. The band in the gel is model NAD-RNA, and the band sharpness is an indicator of purity and integrity of NAD-RNA. Bromophenol blue is used as a tracking dye.

Reagent	Stock concentration	Volume (µl)	Final concentration
ADPRC	4.25 µM	20 (0 for ADPRC$^-$)	0.85 µM
4-Pentyn-1-ol	100%	10	10% (vol/vol)
Model NAD-RNA (38 nt)	100 ng/µl	4.5	4.5 ng/µl
ADPRC reaction buffer	5×	20	1×
Water		To 100	
Total		100	

▲ **CRITICAL STEP** This step refers to the protocol of NAD captureSeq[12] and achieves alkynylation of the model NAD-RNA through the ADPRC-catalyzed reaction.

? TROUBLESHOOTING

4 Purify the RNA sample with RNA Clean Kit 1. Elute the RNA sample with 15 µl of water. Determine the model NAD-RNA concentration using a Qubit fluorometer and dilute to 20 ng/µl.

? TROUBLESHOOTING

5 Before the CuAAC reaction, prepare the click mix by thoroughly mixing the following components.

Reagent	Stock concentration	Volume (µl; one sample)	Final concentration
$CuSO_4$	50 mM	2	1 mM
THPTA	50 mM	1	0.5 mM
Sodium ascorbate	500 mM	0.4	2 mM
Total (click mix)		3.4	

6 Combine the following components in a 1.5-ml tube and incubate at 25 °C for 30 min, with gentle vortex mixing, to ligate the alkynylated model NAD-RNA with tagRNA-azide.

Reagent	Stock concentration	Volume (µl)	Final concentration
CuAAC reaction buffer	5×	20	1×
Click mix		3.4	
Model NAD-RNA (alkynylated; 38 nt; Step 4)	20 ng/µl	15	3 ng/µl
TagRNA-azide (25 nt)	2 mM	12.5	250 µM
H_2O		To 100	
Total		100	

▲ **CRITICAL STEP** Do not exceed 25 °C and 30 min, to avoid RNA degradation.

7 Purify the RNA sample with RNA Clean Kit 1 and elute RNA sample into 15 µl of RNase-free water.

■ **PAUSE POINT** The RNA sample can be stored at −80 °C for several days.

8 Mix the 15 µl of RNA sample with 15 µl of 2× RNA loading dye and then denature the RNA samples by heating at 95 °C for 2 min. Load the samples (ADPRC$^+$ and ADPRC$^-$) onto a precast 10% (wt/vol) denaturing PAGE gel for electrophoresis. The gel running apparatus is set as 40 mA and runs until the bromophenol blue reaches 2 cm from the bottom of the gel.

9 Stain the gel with RedSafe and visualize the product band using ChemiDoc XRS+. See 'Anticipated results' section and Fig. 3 for an expected result.

? TROUBLESHOOTING

Labeling of NAD-RNAs in a total RNA sample with RNA tag ● Timing ~4 h

▲ **CRITICAL** A minimum of ~100 µg of starting RNA per reaction is needed for the identification of NAD-RNA in total RNA (see 'Limitations').

10 Extract and purify the RNA sample. After gel electrophoresis, observe the integrity of the extracted RNA using ChemiDoc XRS+. The detailed procedure for *Arabidopsis thaliana* RNA extraction is included in Supplementary Methods: *A. thaliana* growth and RNA extraction. See 'Anticipated results' and Fig. 4 for an example.

? TROUBLESHOOTING

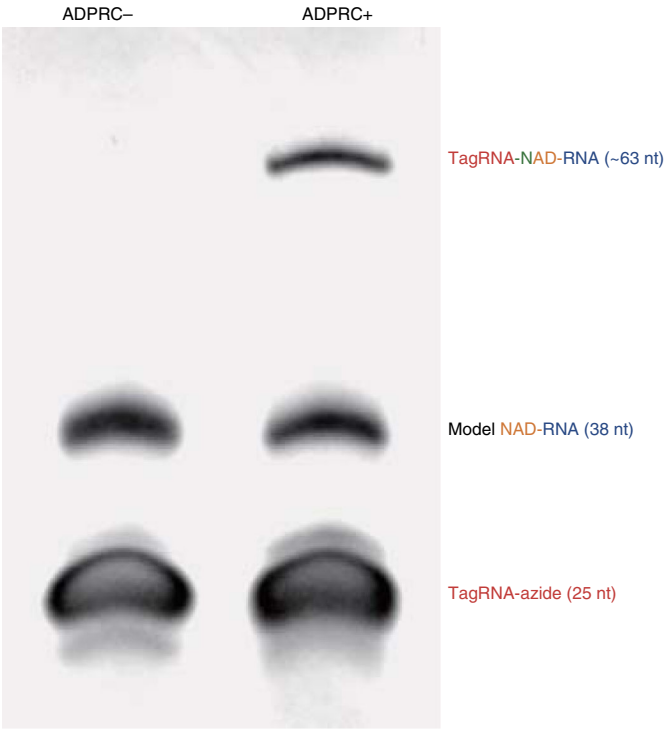

Fig. 3 | Gel analysis of ligation of model NAD-RNA with tagRNA-azide to obtain tagRNA-NAD-RNA using 10% (wt/vol) denaturing PAGE gel. 'ADPRC^{-}' sample processed without ADPRC was used as a negative control. A similar result was published in Zhang et al.[15].

11 Dissolve the extracted RNA sample and dilute it to 2.5 μg/μl. Conduct ADPRC-catalyzed reaction to modify NAD-RNA with 4-pentyl-1-ol. The ADPRC^{-} reaction is used as a negative control.

Reagent	Stock concentration	Volume (μl)	Final concentration
ADPRC	4.25 μM	20 (0 for ADPRC^{-})	0.85 μM
4-Pentyl-1-ol	100%	10	10% (vol/vol)
Total RNA sample, 100 μg	2.5 μg/μl	40	1 μg/μl
ADPRC reaction buffer	5×	20	1×
Water		To 100	
Total		100	

12 Purify the RNA sample with RNA Clean Kit 2. Add 71.6 μl of water directly and centrifuge at 16,000*g* at 4 °C for 30 s to elute RNA. Determine RNA concentration using a Qubit fluorometer. **PAUSE POINT** The eluted RNA can be used immediately or stored at −80 °C for up to 3 days.

13 Prepare fresh click mix as shown in Step 5 and then combine the following reagents in a 1.5-ml tube and incubate at 25 °C for 30 min with gentle vortex on a ThermoMixer with a speed of 700 r.p.m.

Reagent	Stock concentration	Volume (μl)	Final concentration
CuAAC reaction buffer	5×	20	1×
Click mix		3.4	
Alkynylated RNA, ~80 μg		71.6	~1.1 μg/μl
TagRNA-azide (40 nt)	100 μM	5	5 μM
Total		100	

▲CRITICAL STEP Do not exceed 25 °C and 30 min to avoid RNA degradation.

14 Purify the tagged RNA using RNA Clean Kit 2 and elute with 50 μl of nuclease-free water.

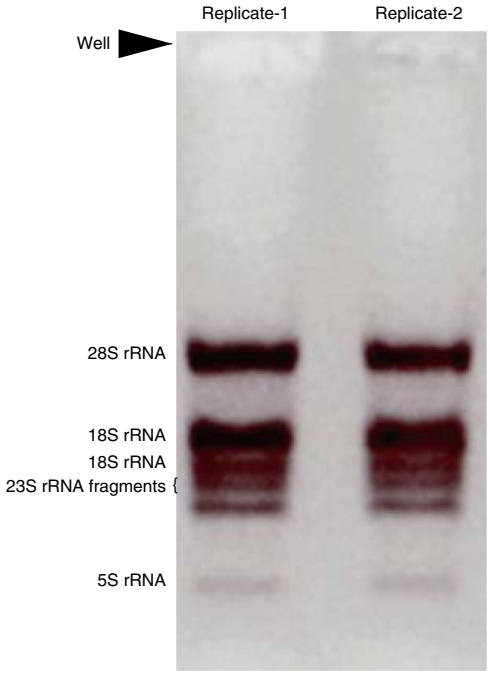

Fig. 4 | RNA integrity analysis using agarose gel electrophoresis, showing 28S rRNA, 18S rRNA and 5S rRNA from *A. thaliana* seedling. The two lanes are from one sample and loaded in the gel as technical replicates.

Polyadenylation of total RNA and purification with oligo d(T)$_{25}$ beads ● Timing ~6 h

▲ **CRITICAL** Direct RNA sequencing by Nanopore requires RNA molecules to have a poly(A) tail for library preparation. In total RNA, only mRNA and some noncoding RNAs are known to have a poly(A) tail, so it is necessary to add a poly(A) tail to other RNA for total RNA sample sequencing.

15 Put the RNA sample in 1 ml of reaction with the following components and incubate at 37 °C for 30 min.

Reagent	Stock concentration	Volume (µl)	Final concentration
RNA after tagging, ~70 µg	~1.4 µg/µl	50	~0.07 µg/µl
Poly(A) polymerase reaction buffer	10×	100	1×
ATP solution	10 mM	200	2 mM
E. coli poly(A) polymerase, 500 U	5 U/µl	100	0.5 U/µl
Water		To 1,000	
Total		1,000	

16 Equilibrate oligo d(T)$_{25}$ beads. Transfer 1 ml of oligo d(T)$_{25}$ beads to a 2-ml LoBind tube, which can be used for both ADPRC^{+} and ADPRC^{-} groups. Gently agitate for 2 min at RT. Put the tube on a magnetic rack for 3 min to settle the beads. Remove and discard the supernatant and resuspend the beads in 400 µl of equilibration buffer. Put the tube on a magnetic rack for 3 min and discard the supernatant. Repeat the wash using the equilibration buffer. Then, resuspend the beads with 200 µl of 2× binding buffer.

17 Purify the polyadenylated RNA (ADPRC^{+} or ADPRC^{-}; ~70 µg) with RNA Clean Kit 1 and then elute with 100 µl of water. Next, purify the polyadenylated RNA with half of the oligo d(T)$_{25}$ magnetic beads. Add an equal volume (100 µl, ~70 µg) of total RNA to the beads. Gently vortex at RT for 10 min to allow poly(A)-containing RNA to hybridize with oligo d(T)$_{25}$ beads. Put the tube onto a magnetic rack and keep it there for 3 min. Transfer the supernatant to a new 1.5-ml tube and retain it. Wash the beads separately with 400 µl of wash buffer and 400 µl of low-salt buffer. Gently vortex for 2 min at RT and then remove and discard the buffer with the help of the magnetic rack.

18 Resuspend the beads in 200 µl of elution buffer and then elute the bound RNA by incubating the tube on a thermal shaker set at 50 °C and 800 r.p.m. for 2 min. Place the tube on a magnetic rack and transfer the eluate to a new 1.5-ml tube. Reuse the beads and transfer the retained supernatant

from Step 17 to the beads. Follow the binding, wash and elution steps and then combine two parts of the eluate.

PAUSE POINT The eluted RNA can be used immediately or stored at −80 °C for use within 1 month.

Enrichment of tagged NAD-RNA with DNA probe (optional) ● Timing ~3 h

CRITICAL This step is optional. It aims to increase the sequencing depth of NAD-RNA.

19 Prepare the streptavidin magnetic resin. Gently agitate and aliquot 150 µl of streptavidin magnetic resin into a 1.5-ml tube. Remove and discard the supernatant with the help of a magnetic rack. Add 200 µl of streptavidin wash buffer and briefly vortex to suspend the beads. Remove and discard the supernatant.

20 Add 75 µl of 2× streptavidin binding buffer, 70 µl of water and 5 µl of 100 µM DNA-biotin to the streptavidin magnetic resins and incubate for 5 min with occasional agitation at RT to immobilize the DNA-biotin probe onto the beads. Then, discard the supernatant after putting the tube on a magnetic rack. Wash beads twice with 200 µl of wash buffer. Discard the supernatant.

21 Combine (~60 µg, 400 µl) poly(A)-tailed RNA sample (Step 18) with an equal volume of 2× binding buffer. Put the mixture on ice and add it to the prepared magnetic beads immobilized with DNA-biotin.

22 Heat at 80 °C for 5 min and then slowly cool to RT on a ThermoMixer with gentle shaking. Place the tube on a magnetic rack and remove and discard the supernatant.

23 Wash the beads twice, each time with 200 µl of streptavidin wash buffer on ice, followed by one wash with 100 µl of streptavidin low-salt buffer on ice.

24 Elute the enriched tagRNA-linked RNA by incubation with 100 µl of elution buffer at 85 °C for 2 min. Transfer the supernatant to a new 1.5-ml tube. Repeat the elution and collect the supernatant together.

25 Purify the eluant using RNA Clean Kit 2. Elute with 10 µl of RNase-free water. Determine RNA concentration using a Qubit fluorometer. The expected RNA concentration is 0.2–0.4 µg/µl.

Library preparation and Nanopore sequencing ● Timing ~3 d

CRITICAL For each RNA sample, 500 ng of tagged RNA was used to prepare a library using the Nanopore Direct Sequencing Kit. Although direct nanopore RNA sequencing technology reads the RNA molecules directly, the reverse transcription reaction is necessary to guide the RNA strand to pass through the nanopore.

26 Mix the following reagents in a 0.2-ml PCR tube and incubate the reaction at RT for 10 min to ligate RTA to RNA samples.

Reagent	Stock concentration	Volume (µl)
NEBNext Quick Ligation Reaction Buffer		3
Tagged RNA, 500 ng (Step 25)		9
RCS	110 nM	0.5
RTA		1
T4 DNA ligase		1.5
Total		15

27 Prepare 23 µl of reverse transcription master mix using the following reagents:

Reagent	Stock concentration	Volume (µl)
Nuclease-free water		9
dNTPs	10 mM	2
First-strand buffer	5×	8
DTT	100 mM	4
Total		23

28 Add the master mix and then 2 µl of SuperScript III reverse transcriptase to the RNA sample in the PCR tube and then pipette to mix. Incubate the reaction in a thermal cycler according to the following table:

Temperature (°C)	Time (min)
50	50
70	10
4	Hold

29 Resuspend the stock of Agencourt RNAClean XP beads by vortexing. Add 72 µl of resuspended RNAClean XP beads to the reverse-transcribed RNA sample by pipetting. Incubate the tube on a Hula mixer for 5 min at RT.

30 Spin down the sample at 3,000*g* at 25 °C for 1 min. Pellet the beads on a magnet and pipette off the supernatant. Wash the beads with 150 µl of freshly prepared 70% (vol/vol) EtOH by rotating the tube by 180° to wait for the beads to migrate. Rotate again and discard the solution using a pipette.

31 Remove the tube from the magnetic rack. Resuspend the pellet using 20 µl of nuclease-free water and incubate at RT for 5 min. Pellet the beads on a magnetic rack until the eluate is clear. Transfer the eluate into a 1.5-ml DNA LoBind tube.

32 In the tube, mix the reagents according to the order in the following table and then incubate the reaction for 10 min at RT.

Reagent	Volume (µl)
Reverse-transcribed RNA (Step 31)	20.0
NEBNext Quick Ligation Reaction Buffer	8.0
RNA adapter (RMX)	6.0
Nuclease-free water	3.0
T4 DNA ligase	3.0
Total	40.0

33 Resuspend the stock of Agencourt RNAClean XP beads by vortexing. Add 40 µl of resuspended RNAClean XP beads to the adapter ligation reaction and mix by pipetting. Incubate on a Hula mixer for 5 min at RT.

34 Spin down the sample at 3,000*g* at 25 °C for 1 min, pellet the beads on a magnet and pipette off the supernatant. Wash the beads with 150 µl of the WSB twice, pellet the beads on a magnetic rack and pipette off the supernatant each time.

35 Add 21 µl of ELB to the beads. Incubate at 25 °C for 10 min. Pellet the beads on a magnetic rack until the eluate is clear and colorless. Transfer the 21 µl of eluate into a clean 1.5-ml DNA LoBind tube.

36 Pipette 1 µl of eluate to quantify RNA concertation using a Qubit dsDNA HS Assay Kit. The total amount of the recovered RNA should be ~200 ng.

37 Load the RNA library into a flow cell and sequence using MinION according to the manufacturer's protocol.
▲ **CRITICAL STEP** Refer to the official protocols. For the previous RNA Sequencing Kit (SQK-RNA001), the protocol is https://nanopore.yilimart.com/static/images/media/Direct%20RNA%20sequencing%20Control%20Experiment.pdf; for the updated RNA Sequencing Kit (SQK-RNA002) and Flow Cell Priming Kit (EXP-FLP002), the manual is https://store.nanoporetech.com/media/wysiwyg/pdfs/SQK-RNA002/Direct_RNA_sequencing_SQK-RNA002_-minion.pdf.

38 Run MinKNOW, which embeds Guppy to base-call all RNA reads. After running, the software will mainly generate fast5 files, which record the original signal obtained from nanopore, and fastq files, which are the base-called reads generated from Guppy base-caller.
▲ **CRITICAL STEP** MinKNOW can be run in Linux, Windows or MacOS systems. Refer to the manual for more information about the computer requirements for running MinKNOW: https://community.nanoporetech.com/requirements_documents/minion-it-reqs.pdf.
? TROUBLESHOOTING

Processing and analysis of sequencing reads ● Timing ~2 d

▲ CRITICAL All the following commands should be executed from the command prompt in Linux or UNIX shell and will be prefixed with '$'. Python, Minimap2, R and Samtools should be installed before running the following codes.

39 Download TagSeqTools with demo files:

```
$ wget https://github.com/dorothyzh/TagSeqTools/archive/TagSeqTools.v0.1.tar.gz
```

Uncompress TagSeqTools to a suitable location:

```
$ tar -zxvf TagSeqTools.v0.1.tar.gz
```

▲ CRITICAL STEP TagSeqTools encloses a set of demo files, including sequencing results in `demo.fastq` and the files for reference transcriptome `TAIR10.trans.fa.tar.gz` and reference genome `TAIR10.genome.fa.tar.gz`. This protocol will use these files for demonstration. Users should download the relevant reference genome when analyzing their own NAD tagSeq experiment.

40 Prepare a fastq file generated from the base-callers:

```
$ cd TagSeqTools-TagSeqTools.v0.1/demo
```

Prepare the fasta files for the reference genome:

```
$ tar -zxvf TAIR10.trans.fa.tar.gz
$ tar -zxvf TAIR10.genome.fa.tar.gz
```

▲ CRITICAL STEP It is also feasible to download sequencing data from the Gene Expression Omnibus repository. If the readers find that the fastq reads are in DNA format after opening the files, it is necessary to replace T with U using the command below:

```
$ sed 's/T/U/g' SRR8660547_1.fastq > SRR8660547.fastq.
```

41 Use the TagSeek module from TagSeqTools to sort out the RNAs with and without tag RNA sequence, with -s (similarity of tagRNA sequence and head of reads) 12—namely, at least 12 consecutive bases of tag sequence can be matched to the first 40 bases of a read:

```
$ python TagSeek.py -f demo -t 'CCUGAACCUGAACCUGAACCUGAACCUGAACCUGAACCUG' -s 12
```

This command will create `demo.tag.fastq`, `demo.nontag.fastq` and `Tag_statistics.txt` (a tagging quality statistics file).

42 Use the TagSeqQuant module from TagSeqTools for quantification and visualization of NAD-RNA. TagSeqQuant adopts Minimap2 to perform the alignment step, with the default mapping parameters.

```
$ python TagSeqQuant.py -n demo -tr TAIR10.trans.fa -g TAIR10.genome.fa
```

The output includes four folders, including `QC_results`, `Mapping_results`, `Mapping_statistics` and `Quantification_results`. The `QC_results` folder contains `demo_fastqc.html` to show the QC results. The reads quality assessment can be referred to the traditional assessment of Nanopore sequencing reads, meaning that median quality scores should be higher than 7, and the median read length should typically be larger than 400 nucleotides, but the read length distribution might vary slightly between different species. Moreover, each sample should result in around 1 million reads in a 48-h run. The `Mapping_statistics` folder contains `NAD_Map.html` and `nonNAD_Map.html` to show the statistics of mapping results, with the error rate included.

▲ CRITICAL STEP Executing TagSeqQuant requires that Minimap2 is available as an environment variable of the operating system (see Box 2 for the configuration of Minimap2).

43 Start IGV by the command below:

```
$ igv.sh
```

Box 2 | Installing the dependent software and configuring system environments ● Timing ~3 h

Data analysis with TagSeqTools requires an operating system capable of executing Python and R scripts. Also, running TagSeqTools needs the dependent Python libraries Biopython and Regex, as well as additional software programs including FastQC, Minimap2, Samtools and IGV. It is better to use a virtual environment to run these commands.

Procedure

1 Install the dependencies (Python libraries Biopython and Regex):

```
$ pip install biopython regex
```

2 Install FastQC on an Ubuntu system:

```
$ sudo apt install fastqc
```

Otherwise, the latest version of FastQC is available on its website (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Install FastQC by simply uncompressing the downloaded zip file into a suitable directory.
FastQC is a Java application. To configure the Java Runtime Environment, refer to the instructions of FastQC.

3 Install Minimap2 and add its path to an environment variable of the operation system ('user_name' should be replaced with the user's own name):

```
$ curl -L https://github.com/lh3/minimap2/releases/download/v2.17/minimap2-2.17_x64-linux.tar.bz2 | tar -jxvf -
$ export PATH=$PATH:/home/user_name/minimap2-2.17_x64-linux/
```

4 Install Samtools on an Ubuntu system:

```
$ sudo apt-get install -y samtools
```

Otherwise, install by the commands below:

```
$ git clone git://github.com/samtools/samtools.git
$ cd samtools/
$ make
```

5 Install IGV.

Download IGV from https://software.broadinstitute.org/software/igv/, unzip the downloaded file to a directory shown below and add it to the PATH environment variable, where the 'user_name' should be replaced by the user's own name, and ensure that the 'igv.sh' file exists in the directory.

```
$ export PATH=$PATH:/home/user_name/IGV
```

To visualize the mapping results, import the bam and bam.bai files in the `Mapping_results` folder generated by TagSeqQuant in Step 42 to IGV. For visualization of the alignment with the genomes, the genome files in the format of '.genome' can be downloaded from the server or built by the users themselves. The anticipated results of `NAD.sort.bam` and `non.NAD.sort.bam` are shown in Fig. 5.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
2	The RNA band is not sharp	RNA is degraded during transcription, or the acrylamide/bis-acrylamide concentration of the gel is too low	Reduce the transcription time to 6 h to reduce RNA degradation and increase the acrylamide/bis-acrylamide concentration of the gel to 12% (wt/vol) or 15% (wt/vol)
3	Low yield of the modified product	4-Pentyn-1-ol is not dissolved, and NAD^+ is degraded to AMP	Completely mix the reaction tube and put on a shaker. Do not store NAD-RNA for too long
4	Low yield of RNA, lower than 10%	The model RNAs are lost using RNA Clean Kit 1 by adhering to the tube side wall instead of the	Turn down the centrifuge speed or replace the sample purification method with co-precipitation

Table continued

Table 1 (continued)

Step	Problem	Possible reason	Solution
		packing material due to a high centrifugation speed or because the model RNAs are short in length, which could be easily eluted from the packing materials	with linear acrylamide in 70% (vol/vol) EtOH, which is shown in Steps 8 and 9 in Box 1
9	No band for ligation product	ADPRC reactivity is low or 4-pentyn-1-ol is immiscible	Test ADPRC reactivity using NAD^+ standard and use new enzyme if it is inactive. Gently agitate the reaction occasionally
	The model NAD-RNA band is overlapped by dye	Xylene cyanol interferes with the RNA band	Prepare RNA dye without xylene cyanol
10	RNA bands are not sharp	RNA is degraded during sample storage	Re-prepare sample for RNA extraction and treat the pipettes and tips with RNase Away
38	Low number of reads is acquired	The flow cell is inappropriately stored or expires after over 3 months. The number of active nanopores on a flow cell decreases gradually during storage	Store flow cells at 4 °C and use them within 3 months. Purchase a new flow cell

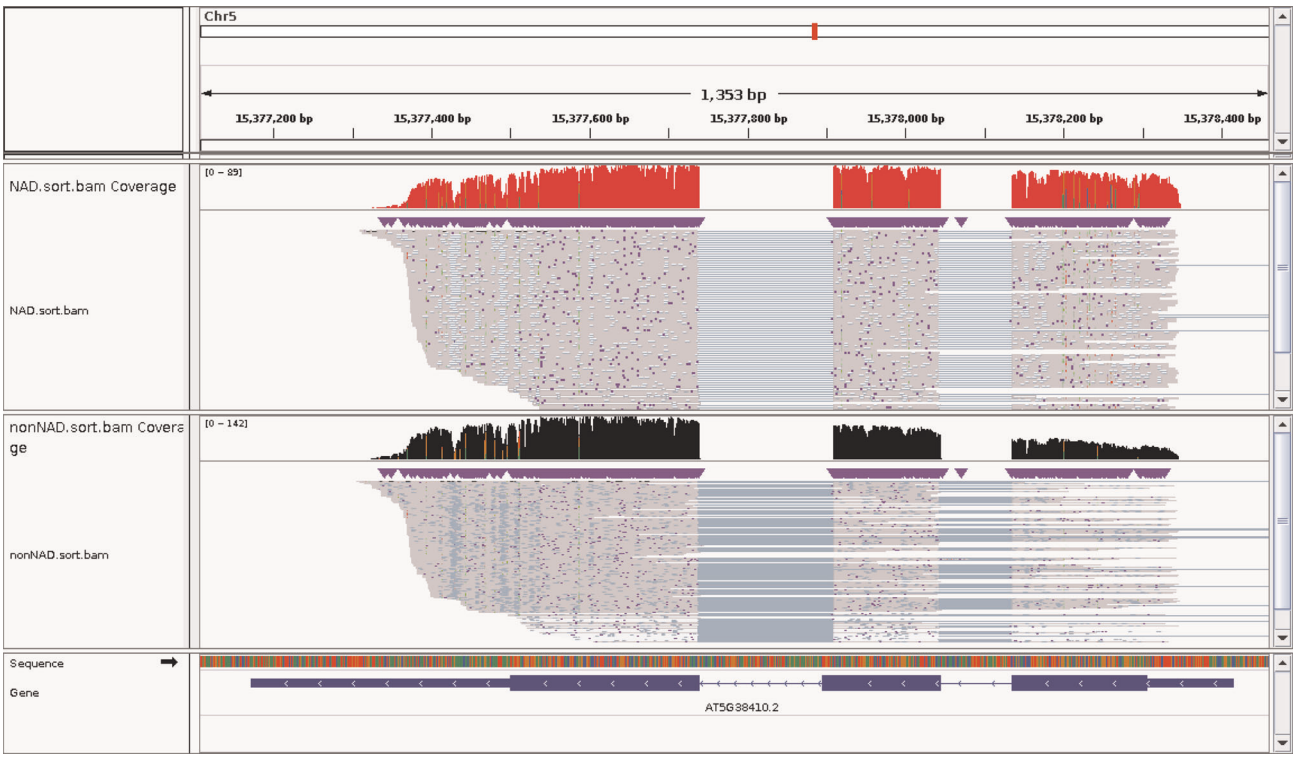

Fig. 5 | IGV screenshot showing a gene and its corresponding NAD-RNA and noncapped-RNA reads. The RNA reads are from the demo file. The gene encodes rubisco small chain, and it is aligned to the *A. thaliana* reference genome (AT5G38410). The NAD-RNA coverage track is marked red, and the non-NAD-RNA is black.

Timing

Steps 1 and 2, verification of the production of model NAD-RNA: ~3 h
Steps 3–9, ligation of model NAD-RNA with RNA tag: ~4 h
Step 10, analysis of total RNA integrity on agarose gel: ~2 h
Steps 11–14, labeling of NAD-RNA with tagRNA-azide: ~2 h
Step 15, polyadenylation of RNA: ~3 h
Steps 16–18, purification using oligo $d(T)_{25}$ beads: ~1 h

Steps 19–20, preparation of DNA probe immobilized onto streptavidin resin beads: ~1 h
Steps 21–25, enrichment of tagged NAD-RNAs: ~2 h
Steps 26–38, library preparation and Nanopore sequencing: ~3 d
Steps 39–43, processing and analysis of sequencing reads: ~2 d
Box 1, in vitro transcription and purification of model NAD-RNA: ~2 d
Box 2, installing the dependent software and configuring system environments: ~3 h

Anticipated results

PAGE gel analysis of the synthesized model NAD-RNA

After purification from 10% (wt/vol) denaturing PAGE gel, the synthesized model NAD-RNA is aliquoted. A small aliquot of 50 ng is loaded to 10% (wt/vol) denaturing PAGE gel for verification of the products. As shown in Fig. 2, the model NAD-RNA appears as only one band, indicating that it is successfully synthesized and purified.

PAGE gel visualization of ligation products using model NAD-RNA

The ligation product of the model NAD-RNA with tagRNA-azide could be observed on a 10% (wt/vol) denaturing PAGE gel. The ADPRC^{-} reaction is used as a negative control. As shown in Fig. 3, the presence of a high-molecular-weight band (~63 nucleotides) only in the ADPRC^{+} lane demonstrates the successful labeling of the NAD-RNA with an RNA tag by the ADPRC-catalyzed reaction and click reaction.

Total RNA isolation

After RNA extraction and purification, the *A. thaliana* RNA integrity is verified using a 1% (wt/vol) agarose gel. As shown in Fig. 4, the RNA samples show five clear bands that correspond to 28S rRNA, 18S rRNA and 5S rRNA. The integrity of the RNA sample can be empirically assessed by observing the bands of 28S rRNA and 18S rRNA. Typically, a ratio of 1.5:1~2:1 could indicate that the RNA sample is intact.

Nanopore sequencing results visualized by IGV

The reads of the demo files are obtained from sequencing RNAs extracted from *A. thaliana* seedlings. The reads are processed with TagSeek to sort out the NAD-RNA and non-NAD-RNAs, which are then processed with TagSeqQuant module to align the reads to reference genome and reference transcriptome. The alignment results are then imported into IGV. As shown in Fig. 5, the structural differences between NAD-RNA and non-NAD-RNA gene coverage can be directly compared to show their respective alternative splicing patterns and 5′-untranslated regions. The NAD-RNA reads and non-NAD-RNA reads are aligned to AT5G38410.2 in *A. thaliana* transcriptome, and their count numbers are 89 and 142, respectively, which represents the relative ratio of NAD-RNA to non-NAD-RNA. Moreover, the quantification results of AT5G38410.2 can also be obtained using TagSeqQuant module in TagSeqTools.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data sets analyzed with the current protocol are available in the Gene Expression Omnibus repository under the accession number GSE127755. The web links for the raw data are https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127755, https://www.ebi.ac.uk/ena/data/view/PRJNA525282 and https://github.com/dorothyzh/TagSeqTools.

Code availability

All the software used in this protocol, including our Python script for sorting tagged and untagged RNA, are available in the 'TagSeqTools' repository under the Apache License v2.0 (https://github.com/dorothyzh/TagSeqTools) and as Supplementary Software.

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Author contributions

All authors contributed to the design of the experiments; H. Zhang and X.S. performed experiments; H. Zhong and Z.Y. performed data analysis; all authors analyzed experimental results and contributed to the writing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Key reference using this protocol
Zhang, H. et al. *Proc. Natl Acad. Sci. USA* **116**, 12072–12077 (2019): https://doi.org/10.1073/pnas.1903683116

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Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Image Lab Software 6.0.1 for acquisition of gel image when using ChemiDoc XRS+; MinKNOW 19.6.8, with base-caller of Guppy embedded, from Oxford Nanopore Technology
Data analysis	Image J 1.50i for image adjustment; Ubuntu 18.04.3 LTS, Linux-based operating system (https://ubuntu.com/download); Python 2.7 (http://www.python.org/download/); R 3.2.1 (https://cran.r-project.org/); Biopython 1.16.6 (https://biopython.org/wiki/Download); Regex 2020.2.20 (https://pypi.org/project/regex/); FastQC 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/); Minimap2 (v2.12) (https://github.com/lh3/minimap2); Samtools 1.7 (http://samtools.sourceforge.net/) to translate the sam file to bam file and obtain its bam.bai file; Two homemade python scripts called TagSeek.py and TagSeqQuant.py, that is included in TagSeqTools repository; Integrative Genomics Viewer 2.7.2 (IGV; https://software.broadinstitute.org/software/igv/); Minimap2 to align the sequenced RNA to genome or transcriptome databases for interpretation of the RNA identities;

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The datasets analyzed with the current protocol are available in the GEO Omnibus repository with the accession number of GSE127755.
The web links for the raw data are https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127755, https://www.ebi.ac.uk/ena/data/view/PRJNA525282, and https://github.com/dorothyzh/TagSeqTools.

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Sample size	Not applicable.
Data exclusions	Not applicable.
Replication	Not applicable.
Randomization	Not applicable.
Blinding	Not applicable.

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All studies must disclose on these points even when the disclosure is negative.

Study description	*Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, hierarchical), nature and number of experimental units and replicates.*
Research sample	*Describe the research sample (e.g. a group of tagged Passer domesticus, all Stenocereus thurberi within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.*
Sampling strategy	*Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.*
Data collection	*Describe the data collection procedure, including who recorded the data and how.*
Timing and spatial scale	*Indicate the start and stop dates of data collection, noting the frequency and periodicity of sampling and providing a rationale for these choices. If there is a gap between collection periods, state the dates for each sample cohort. Specify the spatial scale from which the data are taken*
Data exclusions	*If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.*
Reproducibility	*Describe the measures taken to verify the reproducibility of experimental findings. For each experiment, note whether any attempts to repeat the experiment failed OR state that all attempts to repeat the experiment were successful.*
Randomization	*Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.*
Blinding	*Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.*

Did the study involve field work? ☐ Yes ☐ No

Field work, collection and transport

Field conditions	*Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).*
Location	*State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).*
Access and import/export	*Describe the efforts you have made to access habitats and to collect and import/export your samples in a responsible manner and in compliance with local, national and international laws, noting any permits that were obtained (give the name of the issuing authority, the date of issue, and any identifying information).*
Disturbance	*Describe any disturbance caused by the study and how it was minimized.*

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used — *Describe all antibodies used in the study; as applicable, provide supplier name, catalog number, clone name, and lot number.*

Validation — *Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer's website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.*

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) — *State the source of each cell line used.*

Authentication — *Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.*

Mycoplasma contamination — *Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.*

Commonly misidentified lines (See ICLAC register) — *Name any commonly misidentified cell lines used in the study and provide a rationale for their use.*

Palaeontology

Specimen provenance — *Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).*

Specimen deposition — *Indicate where the specimens have been deposited to permit free access by other researchers.*

Dating methods — *If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.*

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals — *For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals.*

Wild animals — *Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.*

Field-collected samples — *For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.*

Ethics oversight — *Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics — *Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*

Recruitment — *Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*

Ethics oversight — *Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	*Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.*
Study protocol	*Note where the full trial protocol can be accessed OR if not available, explain why.*
Data collection	*Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.*
Outcomes	*Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.*

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links *May remain private before publication.*	*For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.*
Files in database submission	*Provide a list of all files available in the database submission.*
Genome browser session (e.g. UCSC)	*Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.*

Methodology

Replicates	*Describe the experimental replicates, specifying number, type and replicate agreement.*
Sequencing depth	*Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.*
Antibodies	*Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.*
Peak calling parameters	*Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.*
Data quality	*Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.*
Software	*Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.*

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	*Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.*
Instrument	*Identify the instrument used for data collection, specifying make and model number.*
Software	*Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.*

Cell population abundance	*Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.*
Gating strategy	*Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.*

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type	*Indicate task or resting state; event-related or block design.*
Design specifications	*Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.*
Behavioral performance measures	*State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).*

Acquisition

Imaging type(s)	*Specify: functional, structural, diffusion, perfusion.*
Field strength	*Specify in Tesla*
Sequence & imaging parameters	*Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.*
Area of acquisition	*State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.*

Diffusion MRI ☐ Used ☐ Not used

Preprocessing

Preprocessing software	*Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).*
Normalization	*If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.*
Normalization template	*Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.*
Noise and artifact removal	*Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).*
Volume censoring	*Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.*

Statistical modeling & inference

Model type and settings	*Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).*
Effect(s) tested	*Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.*

Specify type of analysis: ☐ Whole brain ☐ ROI-based ☐ Both

Statistic type for inference (See Eklund et al. 2016)	*Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.*
Correction	*Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).*

Models & analysis

n/a	Involved in the study
☐	☐ Functional and/or effective connectivity
☐	☐ Graph analysis
☐	☐ Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.