Incorporation of isotopic, fluorescent, and heavy-atom-modified nucleotides into RNAs by position-selective labeling of RNA

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Site-specific incorporation of labeled nucleotides is an extremely useful synthetic tool for many structural studies (e.g., NMR, electron paramagnetic resonance (EPR), fluorescence resonance energy transfer (FRET), and X-ray crystallography) of RNA. However, specific-position-labeled RNAs >60 nt are not commercially available on a milligram scale. Position-selective labeling of RNA (PLOR) has been applied to prepare large RNAs labeled at desired positions, and all the required reagents are commercially available. Here, we present a step-by-step protocol for the solid–liquid hybrid phase method PLOR to synthesize 71-nt RNA samples with three different modification applications, containing (i) a $^{13}C^{15}N$-labeled segment; (ii) discrete residues modified with Cy3, Cy5, or biotin; or (iii) two iodo-U residues. The flexible procedure enables a wide range of downstream biophysical analyses using precisely localized functionalized nucleotides. All three RNAs were obtained in <2 d, excluding time for preparing reagents and optimizing experimental conditions. With optimization, the protocol can be applied to other RNAs with various labeling schemes, such as ligation of segmentally labeled fragments.

INTRODUCTION

The overwhelming majority of RNA structures at atomic resolution in the Protein Data Bank were solved by NMR or X-ray crystallography. Both methods can be greatly facilitated by the availability of specifically labeled RNAs. For NMR, isotopic labeling at desired positions, especially for RNAs >50 nt, can simplify interpretation of otherwise very crowded spectra[1–4]. For X-ray crystallography, incorporation of heavy-atom-modified residues such as iodo-NTP and bromo-NTP at specific sites is a useful method to determine the phases of the diffraction data[5–7]. In addition, labeling RNAs with fluorophores at specific positions is a prerequisite for fluorescence-based techniques, as natural RNAs are nonfluorescent[8–10]. For example, single-molecule fluorescence resonance energy transfer (smFRET) can be used to observe the conformational behavior of fluorescently labeled RNAs, and provide dynamic and structural information at the single-molecule level. Finally, synthetic incorporation of modifications into RNAs can help illuminate the function of naturally occurring post-transcriptional modifications, and aid in engineering RNAs as diagnostics, sensors, and therapeutics[10–12].

The most popular method for position-specific labeling of RNA is chemical synthesis[13–15]. It has great capability and flexibility in generating chemically diverse RNAs but is limited in the size of the RNAs that can be practically made on a multimilligram scale. Generally speaking, RNAs >60 nt are not readily synthesized on a multimilligram scale by chemical synthesis because of low yields. However, combining chemical synthesis with enzymatic ligation, which allows larger RNAs to be assembled from smaller pieces, mitigates some of the size-limit issues[16,17]. Nevertheless, ligation yields are variable among RNAs and can be affected by many factors, such as ligation site, nucleotide type, and RNA sequence and structure[16,18]. Therefore, ligation is not a generally favored approach for assembling large RNAs with complex structures, and also cannot be practically used to achieve multiple discrete labeling using multiple ligation reactions.

In vitro transcription is the most frequently used method to prepare RNAs in large amounts, with no limits on RNA size because of the intrinsically high processivity of the polymerases. Unfortunately, in its conventional format, *in vitro* transcription cannot be used to incorporate probes at specific positions.

To address this limitation, we recently developed a hybrid solid–liquid phase transcription method, PLOR, with average initiation efficiency of ~40% and elongation efficiency of ~90%, for large-scale syntheses of a 71-nt RNA labeled at selected positions[19,20]. The capability of preparing position-specific labeled RNAs in multimilligram or even tens of milligram quantities is one unique advantage of PLOR. Such a capability has not been demonstrated and is perhaps not possible by other methods. PLOR combines the benefits of solid-phase chemical synthesis—incorporating labels at specific positions with great flexibility—with the known potential of highly processive RNA polymerases (RNAPs) to generate large quantities of specifically labeled RNAs of various sequences up to multiple kilobases in length.

Overview of the procedure

In PLOR, DNA templates are attached to beads to allow phase separation and reagent exchange; RNAP is used to transcribe the solid-phase attached DNA templates in a stepwise manner. A typical PLOR reaction is divided into three stages: initiation, elongation,

and termination (**Fig. 1**)[19]. In the initiation stage of PLOR, solid-phase DNA templates are mixed with RNAP, followed by initiation of transcription with the addition of three or fewer types of NTPs at 37 °C. Transcript extension pauses at the position where the missing NTP type is required. The residual NTPs from the previous reaction step are then removed by solid-phase extraction (SPE), in which the ternary complex immobilized on the beads is rinsed five times to avoid cross-contamination. SPE can be performed by filtering the reaction using either vacuum filtration or centrifugation. In subsequent elongation cycles, new NTP mixtures are added to resume transcription at 25 °C (or another empirically determined optimal temperature) and allow the transcript to be extended to the next point where the cognate NTP is absent, followed by three or more rinse cycles. Each pause/resumption step (also called an elongation cycle) is controlled by the composition of the added NTPs. Various NTP combinations in each cycle allow incorporation of nucleotides with desired probes into specific positions of the transcript. In principle, the reaction cycle can be repeated indefinitely, allowing modified or labeled NTPs to be introduced at multiple specific positions in nascent transcripts. Once the desired labeling is achieved, stoichiometric amounts of all four types of NTPs can be added in the termination stage to complete the transcription. The runoff transcript containing the desired labels is released from the template, collected, and kept at 4 °C before purification or a post-transcription sample treatment. The DNA-bearing beads can be recycled for future use after thorough rinsing using a reaction buffer to remove any residual reaction mixtures.

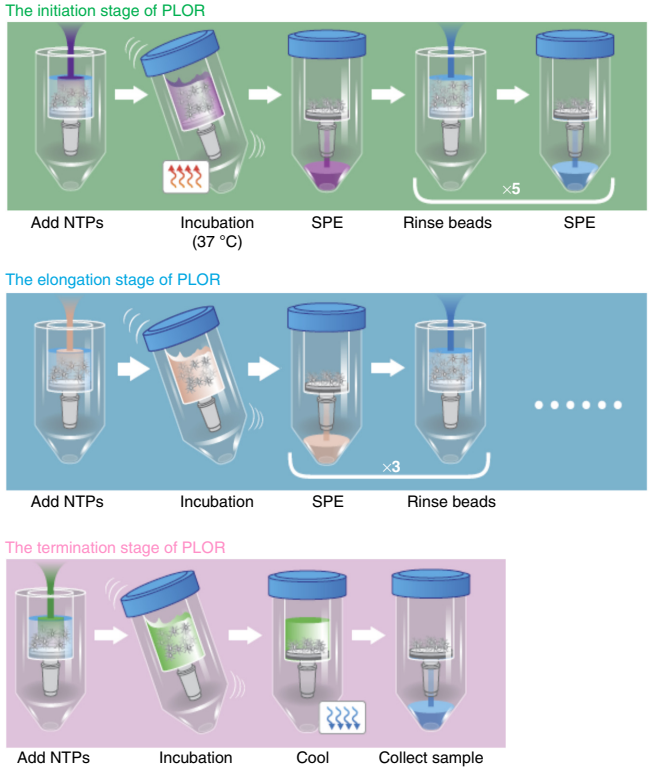

Figure 1 | Diagram of the PLOR synthesis. The solid-phase templates in PLOR are the biotin-tagged DNAs that are attached to neutravidin-coated agarose beads (gray dots). PLOR consists of three stages: initiation (green box), elongation (blue box), and termination (pink box). In initiation, an NTP mix (missing at least one NTP type), T7 RNAP, and DNA beads are added to the reactor and gently rotated for 15 min at 37 °C. T7 RNAP pauses at the position where the missing NTP is needed (see examples in **Figs. 3–5**). Solid-phase extraction (SPE) is performed to remove residual NTPs and abortive products, followed by multiple bead-rinsing cycles to remove the reaction mixture. In cycle 1 of elongation, transcription continues at ambient temperature for ~10 min with the addition of a new NTP mixture, followed by SPE and bead-rinsing cycles. The transcription pauses and resumes in elongation cycles 2 through *n* with various NTP combinations according to the desired labeling scheme. In the termination stage, all four types of needed NTPs are added to complete synthesis of the selectively labeled transcript. The amount of NTPs added is sufficient to complete transcription of the remaining sequence but not enough to start a new round of transcription.

Application of the method

We illustrate the PLOR protocol in detail using examples. They are the syntheses of TCV-RNA, a 104-nt RNA structural element located in the 3′ untranslated region of turnip crinkle virus[21–23], and riboA71, the 71-nt aptamer domain of the adenine riboswitch located in the 5′ untranslated region of its mRNA[24–26]. Although the steps, NTP compositions, and optimizations are specific to the cases demonstrated, they are readily generalizable to other sequences or labeling schemes.

Nucleotides with various modifications, including $^{2}H/^{13}C/^{15}N$ isotopes, fluorophores (Cy3, Cy5, Alexa Fluor 488, and Alexa Fluor 555), biotin, and other reactive functional groups have been introduced into riboA71 at specific positions by PLOR[19]. The labeled positions include a single region, multiple regions, a single position, and multiple discrete positions. Other probes, such as heavy atoms for crystallographic studies, spin labels (such as nitroxide) for EPR studies, photolabile groups, functional groups (e.g., azide, ethynyl, and thiol), or other modifications can, in principle, also be incorporated into RNAs by PLOR as long as they are well tolerated by the RNAP. In addition, in principle, there should be no size limits for PLOR synthesis, as the T7 RNAP used in PLOR has been routinely used to transcribe RNAs of different sizes, from a few tens of residues to many kilobases in length[1,27,28]. As it is currently implemented, PLOR is not without some limitations. The biggest limitation is its low efficiency in labeling RNAs with a large number of labeling steps. This is mainly due to inefficient washing and filtering processes. Such a limitation could be improved with new designs, such as column-based automation, that may allow liquid exchange more efciently, or a microfluidic-based approach that may improve washing and filtering efficiency. Another limitation is the intrinsic low initiation efficiency of T7 RNAP, ~40% (refs. 19,20). For this reason, introduction of any residues tagged with bulky functional groups during this stage is not advised.

Experimental design

In this section, we describe the general design of the solid-phase attached template, generalized optimization procedures, purification of transcripts, and three examples (labeling strategies for Lp2-CN-riboA71, U24Cy3-C55Cy5-B-riboA71, and U16-U19-I-riboA71).

General design of the solid-phase attached template. Biotinylated DNA templates are attached to neutravidin-coated agarose beads for PLOR synthesis. Covalent attachment can also be used but is not used in this protocol. Multisubunit RNAPs may have higher transcription fidelity than single-chain RNAPs, but the

Box 1 | Preparation of DNA templates for PLOR reactions ● TIMING ~2.5 h

PLOR is a single-round transcription method. As such, it requires more DNA templates than a conventional *in vitro* transcription method. DNA templates can be readily prepared by multiple rounds of PCR reactions. In addition, immobilized DNA templates in PLOR can be recycled and reused numerous times for subsequent transcription reactions.

We used the touchdown-PCR (TD-PCR) method[39] to amplify the DNA templates. In our experience, TD-PCR (described here) has better yields than regular PCR. The sequences for the primers and templates used for riboA71 are listed below.

Additional Materials

- Noncoding DNA template in PCR (Avetra Bioscience):

5′-biotin-*TCTGATTCAGCTAGTCCA***TAATACGACTCACTATA**GGGAAGATATAATCCT
AATGATATGGTTTGGGAGTTTCTACCAAGAGCCTTAAACTCTTGATTATCTTCCC-3′
(Linker and T7 promoter sequences are italic and bold, respectively.)

- Coding DNA template in PCR (Avetra Bioscience):

5′-mGmGGAAGATAATCAAGAGTTTAAGGCTCTTGGTAGAAACTCCCAAACCATATC
ATTAGGATTATATCTTCCC**TATAGTGAGTCGTATTA***TGGACTAGCTGAATCAGA*-3′
(Linker and T7 promoter sequences are italic, and bold, respectively.)

- Forward primer in PCR (Integrated DNA Technology):

5′-biotin-TCTGATTCAGCTAGTCCATAATACGACT

- Reverse primer in PCR (Integrated DNA Technology):

5′-mGmGGAAGATAATCAAGAGTTTAAGGCTCT (mG refers to 2′-*O*-methylguanosine)

A linker (italicized) was added upstream of the T7 promoter sequence (bold) to alleviate the potential steric hindrance between T7 RNAP and the bead surface. Two different linkers, an 18-nt linker (TCTGATTCAGCTAGTCCA) and a 23-nt linker (GCTATGACCATGATTAC-GAATTC), were tested in the PLOR system for TCV RNA, and gave indistinguishable yields. The shorter (18-nt) linker was chosen for the riboA71.

Procedure

1. Program the PCR machine. Each round of TD-PCR reaction contains two phases (see the table below): the touchdown phase and the PCR phase. We recommend adding the touchdown phase before the PCR phase, which may be helpful to prevent nonspecific binding between primers and templates. The touchdown phase has 20 cycles of melting at 95 °C for 30 s, with an extra 5 min in the first cycle, annealing at 75–45 °C for 45 s (the temperature should be reduced at 1.5 °C per cycle), and elongation at 72 °C for 1 min. The PCR phase has 25 cycles of melting at 95 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 1 min.

Phase	Cycle no.	Denature	Anneal	Extend	Final
TD	1	95 °C, 5 min			
	2–21	95 °C, 30 s	75 °C*, 45 s	72 °C, 1 min	
PCR	22–47	95 °C, 30 s	50 °C, 30 s	72 °C, 1 min	
	48				Hold, 4 °C

*Drop 1.5 °C per cycle.

2. Prepare the reaction mixture. The reagents used in a 10-ml TD-PCR reaction are listed in the following table:

Component	Volume	Final concentration
PCR buffer, 10×	1.0 ml	1×
DNA templates (10 μM)	50 μl	0.05 μM
Reverse primer (1 mM)	50 μl	5 μM
Forward primer (1 mM)	50 μl	5 μM
dNTP (2mM)	1.0 ml	0.2 mM
100× Taq polymerase (3.3g/l)	100 μl	1×
H_2O	7.75 ml	

3. Run the PCR reaction.

■ **PAUSE POINT** We recommend storing the PCR products at –20 °C before purification. We purified the PCR products by PAGE to remove the residual biotin-labeled primers. The purified products can be stored at –20 °C for years.

challenges of obtaining highly active multisubunit RNAPs and their relatively slow elongation rates keeps them from being used for this purpose. For the same reasons that bacteriophage T7 RNAP has been used for routine *in vitro* transcription—high activity, high specificity, and ease of preparation—we chose this single-subunit enzyme for PLOR. In principle, other similarly active single-subunit RNAPs (e.g., SP6 RNAP) can be used. Note that in the template DNA, the linker sequence (18 bp long and upstream of the T7 promoter) is included to prevent crowding near or at the bead surface and to alleviate potential steric hindrance between multiple transcribing T7 RNAPs. The biotinylated double-stranded DNAs were either obtained from commercial suppliers or prepared by PCR. One advantage of using PCR is that it can be used to produce longer DNAs in large quantity. The forward and reverse primers used in PCR contained biotin or 2′-*O*-methylguanosine (mG) at their 5′ ends, respectively. The

biotin group is used for the immobilization of DNAs on the beads[29], and the mG group is used to decrease nontemplated addition of nucleotides at the 3′ end of transcripts[30]. As an example, the PCR preparation of the DNA templates for riboA71 is detailed in **Box 1**.

Generalized optimization procedures. The experimental conditions, including buffers, reagent concentrations, temperature, and reaction time optimized for riboA71 are generally suitable for the synthesis of other types of RNA. Nevertheless, template sequences, template concentrations, usages of NTPs for the initiation and the elongation stages, and the T7 RNAP concentration at the initiation stage might need to be fine-tuned for each RNA to achieve optimal efficiency.

1. Optimization of template sequences. Aside from the well-known observation that a transcript should start with two or three consecutive guanosines at the 5′ end to increase transcription efficiency, a template should also be designed to minimize the number of pause sites necessary to achieve the desired labeling scheme while maintaining the integrity of the secondary and tertiary interactions. The number of elongation cycles depends on both sequences and type of labeling schemes. The number of cycles can be large if all four types of residues appear within a very short stretch of a sequence, even if the overall sequence is very short. On the other hand, the total number of cycles can be small if only two or three types of residues appear within a long stretch of nucleotides, even if the overall sequence is long. Appropriate mutation(s) that do not disrupt the overall secondary and tertiary structures may reduce the number of cycles substantially. Furthermore, the type of labeling scheme may also dictate the number of cycles. In general, labeling by a segment (such as the seven nucleotides continuous in sequence at Lp2, shown as a red arc in **Fig. 2a**) requires fewer cycles than discrete labeling at multiple isolated positions (such as the residues shown as blue dots in **Fig. 2a**); segmental labeling in the downstream regions requires more cycles than those upstream. Again, the proper mutations may reduce the number of cycles obviously. Such a template optimization is particularly feasible when the 3D structure of the RNA is known. For example, the sequence of riboA71 is that of the *add* A-riboswitch[25] with mutations at the termini to facilitate PLOR (**Fig. 2b**). In the case in which the 3D structure is unknown, multiple trials with various template sequences for transcription efficiency and structural integrity might be required. The transcription efficiency can be evaluated by gel electrophoresis and structural integrity can be evaluated by NMR spectroscopy.

Another requirement for a template is that the first ~13 residues, at the least, must be transcribed continuously to generate a stable ternary complex. This means that the first ~13 residues must consist of no more than three types of nucleotides. After the transcription of the first ~13 nt, T7 RNAP switches to processive transcription and forms a stable elongation complex with the DNA template and nascent RNA only[31,32]. The expected first pauses for the riboA71, TCV, and 47-nt RNAs were at +13, +17, and +9 nt (marked by red arrows in **Fig. 2**), upon addition of ATP/GTP/UTP, ATP/CTP/GTP, or ATP/CTP/GTP, respectively.

After completion of the synthesis, 0.5 µl of the final PLOR-generated products are separated on a 12% denaturing polyacrylamide gel (acrylamide concentrations expressed as percentages of wt/vol), and stained with SYBR Gold. Under the standard conditions,

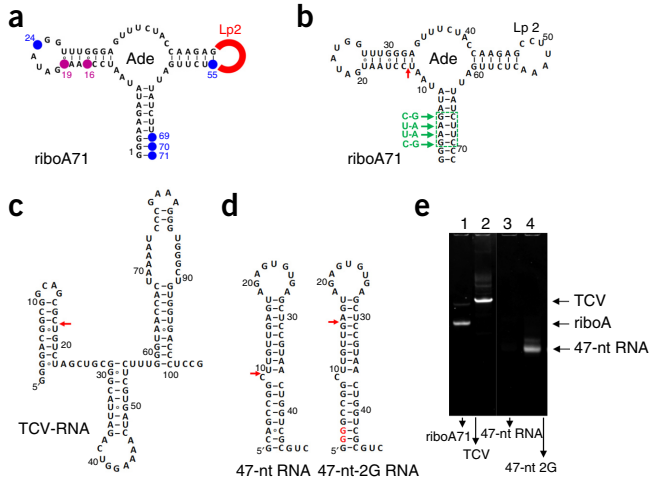

Figure 2 | PLOR for different RNAs. (**a**–**d**) Secondary structures of riboA71 (**a**; the segment to be labeled in sample Lp2-CN is shown as a red arc; the positions to be labeled in U16-U19-I, U24Cy3-U55Cy5-B are shown as magenta and blue dots, respectively), riboA71 (**b**; the nucleotides in the *add* A-riboswitch that differ from those of reference 25 are shown in green), TCV-RNA (**c**), and 47-nt RNA and its mutant, 47-nt-2G RNA (**d**; predicted by the mfold web server). The mutated residues are shown in red. The expected pause positions of RNAP in the initiation step for riboA71, TCV-RNA, and 47-nt RNA with the additions of ATP/GTP/UTP, ATP/CTP/GTP, and ATP/CTP/GTP, respectively, are marked by red arrows. (**e**) Gel image of the crude products of PLOR-generated riboA71 (Lane 1), TCV-RNA (Lane 2), 47-nt RNA (Lane 3), and 47nt-2G RNA (Lane 4). No products were detectable for 47-nt RNA (Lane 3), whose initial transcript was 9 nt in length. Introducing two mutations at the 5′ end (47-nt-2G RNA) extended the initial transcript to 15 nt, resulting in a significant increase in yield. For the PLOR reactions for all the RNAs, all four types of NTPs were added together to terminate the transcriptions after the initiation stage (elongation stages were skipped).

good transcript yields were observed in the riboA71 (Lane 1, **Fig. 2e**) and TCV (Lane 2, **Fig. 2e**) syntheses, but no 47-nt RNA was detected, even with sensitive staining (Lane 3, **Fig. 2e**). The yield of the 47-nt RNA was greatly improved (Lane 4, **Fig. 2e**) by mutating two residues at its 5′ end so as to shift the first pause position to +15 (47-nt-2G RNA) (**Fig. 2d**). This led us to conclude that an initiation transcript of at least 10 nt in length is a prerequisite for PLOR labeling schemes. Mutating the template is a straightforward and effective way to shift the initial pause position downstream so as to achieve the first pause after a sufficient number of nucleotides. A small-scale reaction (e.g., 25 µl in volume, 5 µM DNA template) of the simplest version of the PLOR (the PLOR without an elongation stage, as shown in **Box 2**) is recommended to test the feasibility of the PLOR initiation for a different RNA, which may take ~1 h.

2. Optimization of template concentrations. The concentrations of DNA templates should be optimized for different RNAs and different labeling schemes of the same sequence, because these DNA concentrations have a large effect on the PLOR yield. We suggest testing DNA concentrations between 0.1 and 40 µM to identify the optimum conditions. For riboA71, we found that the optimized concentrations of the DNA templates ranged from 5 to 40 µM, depending on the labeling scheme. The actual template concentration heavily depends on the efficiency of attachment through the biotin–neutravidin affinity chemistry and should be carefully measured (see Step 9 and **Box 3** for details). This

Box 2 | The PLOR procedure and reagent usages for testing initiation transcript length ● TIMING ~1 h

The PLOR procedure used for testing the initiation conditions for preparing RNAs contains only initiation and termination stages. The simplest versions of PLOR reactions, containing only initiation and termination stages, are straightforward to perform.
At initiation, mix T7 RNAP with the DNA beads at 37 °C, and incubate the three types of NTPs, along with the initiation buffer, for 15 min at 37 °C (the added NTPs vary with the initiation sequences of the RNAs), and then perform SPE five times to remove the excess NTPs.

In the termination step: incubate four types of NTPs with the beads for 12 min at 25 °C to complete the transcriptions. The amount of NTPs should be equal or less than the stoichiometrical amount of templates to avoid re-initiation of the reaction. Then, perform SPE and collect the eluate. The detailed reagent usages for individual RNAs are listed in the following table:

RNA	Reagent additions	
riboA71	Initiation:	5 μM T7 RNAP, 5 μM DNA beads for riboA71 480 μM ATP, 480 μM GTP, and 48 μM UTP
	Termination:	70 μM ATP, 65 μM CTP, 50 μM GTP, and 105 μM UTP
TCV-RNA	Initiation:	5 μM T7 RNAP, 5 μM DNA beads for TCV-RNA 160 μM ATP, 1.2 mM GTP, and 80 μM CTP
	Termination:	100 μM ATP, 100 μM CTP, 130 μM GTP, and 105 μM UTP
47-nt RNA	Initiation:	5 μM T7 RNAP, 5 μM DNA beads for 47-nt RNA 80 μM ATP, 320 μM GTP, and 64 μM CTP
	Termination:	30 μM ATP, 35 μM CTP, 65 μM GTP, and 60 μM UTP
47-nt-2G RNA	Initiation:	5 μM T7 RNAP, 5 μM DNA beads for 47-nt-2G RNA 240 μM CTP, 640 μM GTP, and 64 μM UTP
	Termination:	30 μM ATP, 35 μM CTP, 55 μM GTP, and 40 μM UTP

optimization process may take a few days for a labeling scheme with more than 15 elongation cycles.

3. Optimization of T7 RNAP concentrations. PLOR is different from conventional *in vitro* transcription, in which T7 RNAP transcribes a DNA molecule one time to generate one full-length RNA molecule. Therefore, in principle, the amount of active T7 RNAP should be equal to that of the available DNA template. We suggest optimizing the concentrations of T7 RNAP in the

Box 3 | Concentration measurements of the DNA on the solid-phase templates ● TIMING ~0.5–2 h

The DNA concentrations (C_{bound}) can be measured using option A or B, depending on whether the solid-phase templates are freshly prepared or repeatedly used. Option A is applied only for fresh DNA beads, and option B can be used for either fresh or used DNA beads. The former calculates the amount of the DNA attached to the beads on the basis of the concentrations of the total DNA used and the free DNA remaining unbound. The latter measures the amount of DNA directly by using a denaturing method to strip the DNA templates from the beads.

(A) Subtraction of the amount of the unbound DNA from the total DNA amount used to obtain the amount of the bound DNA

C_{bound} can be obtained by the formula: $C_{bound} = (M_{total}–M_{un\text{-}b})/V_{DNA–beads}$
where M_{total} is the total amount of DNAs incubated with the neutravidin beads and $M_{un\text{-}b}$ is the amount of the eluted DNAs collected. M_{total} and $M_{un\text{-}b}$ are calculated on the basis of the UV–visible absorbance at 260 nm. $V_{DNA–beads}$ is the volume of the DNA–bead solution.

(B) Dissociation of the DNA from the beads and measurement of concentration

1. Mix 1 μl of the DNA beads with 99 μl of formamide-EDTA buffer. Heat at 65 °C for 10 min.

▲ CRITICAL STEP Formamide-EDTA buffer can also be replaced by 10 M urea buffer. We recommend that the ratio of the volume of the DNA beads to the formamide-EDTA buffer (or 10 M urea buffer) be lower than 1:9 for efficient dissociation of DNA from the beads.

2. Filter. Collect the eluate.
3. Load ~1–2 μl of the eluate and standard DNA samples onto a 12% (wt/vol) PAGE analytical gel.

▲ CRITICAL STEP We advise using at least two standard DNA samples of different amounts for gel-band intensity calibration.

4. Run the gel using 1× TBE as running buffer at ~100 V for ~30 min to 1 h.
5. Stop running the gel. Stain the gel with Sybr Gold for ~10–30 s, following the manufacturer's protocol.
6. Automatically measure the concentrations of the bound DNA on the basis of the measured gel-band intensities in Image lab 4.1.

Options A and B gave very similar concentration measurements for the 106-nt DNA beads prepared in Step 9: ~85% of the added 106-nt DNAs were retained on the neutravidin beads.

Box 4 | Purification methods for PLOR-generated RNAs ● TIMING ~4 h–1 d

PAGE, phenol–chloroform extraction, or HPLC are used for RNA purification here. Phenol–chloroform extraction to remove the enzyme is simpler, quicker, and more efficient because the final products are generally pure and free of abortive short RNA fragments. However, PAGE is preferred for the PLOR reactions that contain detectable abortive RNAs at the termination stage. Reversed-phase HPLC is highly recommended to further purify the dye-labeled RNAs, as the presence of dyes does not introduce electrophoretic mobility shift on PAGE.

(A) Denaturing PAGE

1. Add 10% (wt/vol) APS and TEMED simultaneously to a 12% (wt/vol) PAGE solution, gently shake, pour the mixture into the gel plates, and let the gel polymerize overnight.

▲ **CRITICAL STEP** ~2 ml of 10% (wt/vol) APS, ~200 µl of TEMED, and 400 ml of PAGE solution are used for preparing the gel with a size of 3 mm × 32 cm × 40 cm. For higher recovery yields, gels with different sizes are suitable for different sample amounts. For riboA71, gels of the sizes 1 mm × 16 cm × 28 cm, 2 mm × 16 cm × 28 cm, 3 mm × 16 cm × 28 cm, and 3 mm × 32 cm × 40 cm are used to purify ~100, 200, 300, and 500 nmol of the RNA, respectively.

2. Place the gel onto the electrophoresis system, and prerun the gel using 1× TBE as running buffer at ~400 V for ~30 min.
3. Load the concentrated transcripts to the gel, and apply voltage until the RNA migrates 1/3 or further through the gel.
4. *Sample recovery*. Cut the darkest shadow band under UV light at 254-nm wavelength, and grind the band containing the RNA into fine pieces.
5. Shake the gel pieces in gel-soaking buffer overnight, filter, and collect the eluate. Concentrate the eluate and exchange the buffer for the desired buffer.

(B) Phenol–chloroform extraction

1. Add an equal volume of phenol–chloroform–isoamyl alcohol mixture to the RNA, vortex vigorously for ~1 min, centrifuge at 4 °C for ~15 min at 5,000*g*, and carefully transfer the upper aqueous phase to a clean tube.
2. *Ethanol precipitation*. Add a 1/10 volume of sodium acetate (final conc. is 0.3 M, pH 5.2), mix well, add 2.5 volumes of cold 100% ethanol, mix well, place the mixture at –80 °C for >30 min, centrifuge for 30 min at 4 °C at 15,000*g*, and remove the supernatant.
3. *Sample recovery*. Add ~2 ml of cold 70% ethanol to the pellet, gently wash the pellet, decant the supernatant, air-dry the pellet, and then resuspend the pellet in the desired buffer.

(C) HPLC elution

1. Run the sample on the following HPLC program: 2 min at 10% buffer B (90% buffer A) and then ramp from 10 to 50% buffer B over 30 min (flow rates are 0.35 ml/min).
2. *Sample recovery*. Collect and concentrate the eluate of the desired RNA, and exchange the buffer for the desired buffer.

range of 0.5*x*–2*x* (where *x* is the concentration of the DNA template). The specific activity of the T7 RNAP may vary from batch to batch, and it is strongly advised that it be tested using 25-µl reactions before any large-scale reactions. The optimization of the T7 RNAP concentration is straightforward but may take a few hours to complete.

4. Optimization of NTP concentrations in the initiation stage. T7 RNAP forms unstable initiation complexes with a high turnover rate, and the amount of NTPs added during initiation is critical to overall efficiency. High concentrations of NTPs in the initiation stage can drive T7 RNAP to form initiation complexes; however, too high an NTP concentration can favor misincorporation by T7 RNAP at the points where the RNAP is induced to pause by omitting an NTP type. In PLOR, NTP compositions and concentrations during initiation are dependent on the sequence of the residues to be transcribed. For example, if the missing residue type is a pyrimidine, the concentration of the other pyrimidine during initiation should be kept not much higher than that of the DNA template (because pyrimidine/pyrimidine or purine/purine misincorporation occurs more readily than purine/pyrimidine misincorporation[34]), ranging from 1*y* to 10*y*, and we recommend that the concentration of purines during initiation be optimized from 10*y* to 100*y* (where *y* is the concentration of the template multiplied by the number of occurrences of a particular NTP in the sequence to be transcribed). For example, in riboA71, the 13-nt initiation segment contains 6As, 4Gs, and 3Us; the corresponding *y* is 30 µM ATP/20 µM GTP/15 µM UTP for a 5 µM template, and we recommend optimizing the ATP and GTP starting in the ranges of 300–3,000 µM and 200–2,000 µM, respectively. For riboA71, the optimized concentrations for ATP and GTP are 16*y*, and 24*y*, respectively, in multiple labeling schemes[19]. Much lower concentrations of UTP are used to reduce misincorporation, and the recommended concentration range is 1*y*–10*y* (in riboA71, UTP concentrations were tested in the range of 15–150 µM with a 5 µM template, and the optimum concentration for UTP was 48 µM (3.2*y*), as shown in **Box 2**). For TCV-RNA, a 17-nt initiation segment containing 2As, 5Cs, and 10Gs, the optimum concentrations of ATP, GTP, and CTP are 16*y*, 24*y*, and 3.2*y*, respectively (shown in **Box 2**). For cases in which ATP is excluded from initiation, such as for the 47-nt-2G RNA, a different strategy is used to screen initiating NTP concentrations. As GTP is critical for initiation by T7 RNAP, too low a GTP concentration may result in inefficient transcription, so its addition is similar as in the group described earlier: GTP is screened in the range of 10*y*–100*y*, but either CTP or UTP is screened in the range of 1*y*–10*y* to reduce misincorporation. In 47-nt-2G RNA, the optimum concentration of both GTP and CTP is 16*y*, and the UTP concentration is reduced to 3.2*y*. Delicate NTP optimization in small-scale PLORs is strongly advised, and it may take from days to weeks to determine optimized NTP concentrations for initiation. However, once optimized, the same initiation conditions can be used with the same template sequence, even if different labeling schemes are used during elongation.

5. Optimization of NTP concentrations in the elongation/termination stage. NTP additions during individual elongation cycles vary with labeling schemes and the sequences to be transcribed. In general, NTP concentrations equimolar with their representation in the template segment being transcribed were optimal for maximizing yields. NTP concentrations that were too low led to reduced yields, as transcription was not able to proceed when NTPs were exhausted. However, no full-length products were detected if NTP concentrations were too high (≥tenfold in excess of their representation in the transcribed segment). This is probably because, with NTPs in large excess, misincorporation can occur at the pause step, resulting in abortive products containing misincorporated bases at the 3′ ends[34]. A stoichiometric NTP concentration in the segment being transcribed is optimal. For example, if a 5-nt segment containing 1 U, 2 Cs, and 2 As is being transcribed (with template concentration at 5 μM), then the corresponding NTP mix would be 5 μM UTP/10 μM CTP/10 μM ATP. Note that the NTP concentrations used in PLOR during elongation are much lower than in conventional transcription, in which NTPs are at millimolar concentrations. The concentrations of NTPs should be optimized in the range of 0.5*y*–2*y* (where *y* is the concentration of the template multiplied by the number of occurrences of a particular nucleotide in the sequence). NTP optimization at the elongation stage is relatively simpler than at the initiation stage, and may take as little as a few hours.

Purification of transcripts. The RNA products collected at the termination stage of PLOR contain almost no abortive products because abortive products from the initiation and the elongation stages are removed by SPE. PLOR-generated RNAs can be purified by either PAGE or phenol–chloroform extraction to remove T7 RNAP. Samples to be used for FRET, such as U24Cy3-C55Cy5-B-riboA71, should be further purified by reversed-phase HPLC. HPLC is more efficient in separating dye-labeled RNA from unlabeled RNA. The unlabeled RNA may be the result of misincorporation or insufficient washing before introducing the labeled residues. Purification details are given in **Box 4**.

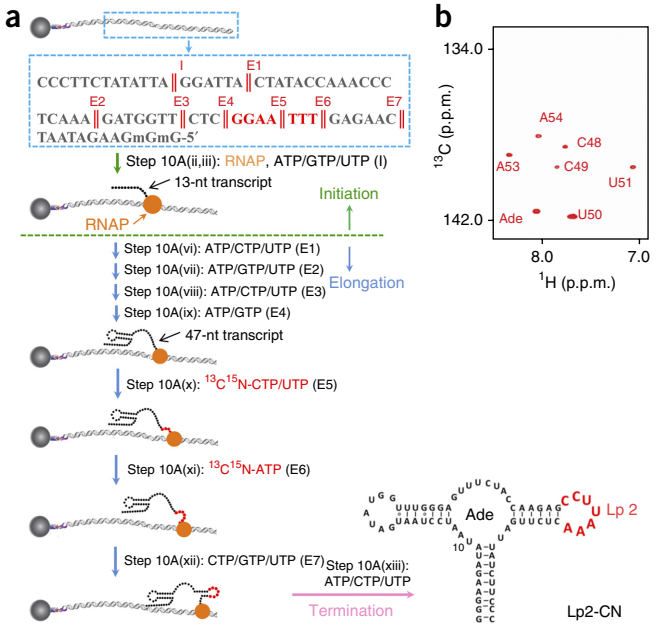

Figure 3 | Application of PLOR in generating an isotopic-labeled sample for NMR study. (**a**) Diagram of PLOR-generated Lp2-CN-riboA71 (Step 10A(iii–xiii)). The coding sequence of the template from 3′ to 5′ is shown in the blue box (the sequence to be transcribed with $^{13}C^{15}N$ labels is shown in red). In the initiation (I, Step 10A(ii,iii)), the DNA beads incubate with T7 RNAP, ATP, GTP, and UTP to generate the 13-nt transcript (black dots). T7 RNAP (orange circle) pauses at point I because of a lack of GTP. The expected paused positions of T7 RNAP at various steps are shown by red double lines in the template (the residues to be transcribed as Lp2 are in red). In the elongation, the addition of ATP/CTP/UTP at Step 10A(vi), ATP/GTP/UTP at Step 10A(vii), ATP/CTP/UTP at Step 10A(viii), and ATP/GTP at Step 10A(ix) generates a 47-nt transcript, paused at position E4. With the addition of $^{13}C^{15}N$-CTP/$^{13}C^{15}N$-UTP at Step 10A(x) and $^{13}C^{15}N$-ATP at Step 10A(xi), T7 RNAP continues to transcribe these $^{13}C^{15}N$-NTPs (red dots) into the Lp2 loop. CTP/GTP/UTP and ATP/CTP/UTP are added at Step 10A(xii) and Step 10A(xiii), respectively, to complete the transcription of full-length Lp2-CN-riboA71. (**b**) Aromatic regions of $^{1}H^{13}C$-transverse relaxation optimized spectroscopy (TROSY) spectrum of Lp2-CN-riboA71. **b** adapted from ref. 19, Springer Nature.

Examples of three applications

We provide details for three examples of the PLOR syntheses for three different applications. We use syntheses of riboA71 with various labeling schemes to illustrate the utility of PLOR. Specifically, the three examples illustrate the labeling strategies for the applications for NMR spectroscopy (Lp2-CN-riboA71), single-molecule FRET (U24Cy3-C55Cy5-B-riboA71), and crystallography phasing (U16-U19-I-riboA71).

Labeling strategy for Lp2-CN-riboA71. In the synthesis of Lp2-CN-riboA71, $^{13}C^{15}N$-labeled residues are incorporated into the Lp2 loop (**Fig. 3a** and **Table 1**). The whole procedure comprises initiation, seven elongation cycles, and termination. During initiation, a reactor containing T7 RNAP and DNA beads was gently rotated at 37 °C for ~10 min, enough time for binding of the RNAP to the promoter. ATP/GTP/UTP were then added to the reactor and incubated together with T7 RNAP and DNA beads at 37 °C for another 15 min. T7 RNAP paused at point I on the template (marked by red double line, **Fig. 3a**), where it needed CTP, resulting in the production of a 13-nt nascent transcript (shown as black dots, **Fig. 3a**). SPE and bead rinsing were then performed to remove residual NTPs from the reaction mixture. An elongation buffer containing ATP/CTP/UTP was then added to the reactor and incubated at 25 °C for 10 min in the first cycle of the elongation phase to allow transcription to continue until point E1, where GTP was required. Excess NTPs were removed and replaced with a new ATP/GTP/UTP set in cycle 2 of the elongation phase. After a 10-min reaction, the T7 RNAP paused at point E2. After SPE, ATP/CTP/UTP, and ATP/GTP were added in cycles 3 and 4, respectively, to extend transcription to point E4, next to the Lp2. In cycles 5 and 6, $^{13}C^{15}N$-CTP/$^{13}C^{15}N$-UTP and $^{13}C^{15}N$-ATP were added to allow the T7 RNAP to incorporate the $^{13}C^{15}N$-NTPs (red dots, **Fig. 3a**) into Lp2, and then transcription paused at point E6. CTP/GTP/UTP and ATP/CTP/UTP were added in cycle 7 of the elongation and termination phases, respectively, to complete transcription of the full-length RNA. The visible peaks at the aromatic region of the TROSY spectrum are from Lp2, indicating that the Lp2 loop is isotopic and labeled solely in Lp2-CN-riboA71 (**Fig. 3b**).

TABLE 1 | Reagent additions for 25-ml, 20 μM PLOR reaction to generate Lp2-CN-riboA71.

Component	Volume	Final concentration (μM)
Initiation stage (Step 10A(ii,iii))		
Initiation buffer, 5×	5 ml	1×
DNA beads (50 μM)	10 ml	20
T7 RNAP (400 μM)	1.25 ml	20
ATP and GTP (40 mM)	1.2 ml	1,920
UTP (4 mM)	1.2 ml	192
DEPC–H_2O	5.15 ml	
Elongation stage		
Cycle 1 (Step 10A(vi))		
Elongation buffer	19 ml[a]	1×
ATP, CTP, and UTP (40 mM)	25 μl	40
Cycle 2 (Step 10A(vii))		
Elongation buffer	19 ml	1×
ATP (40 mM)	37.5 μl	60
GTP (40 mM)	87.5 μl	140
UTP (40 mM)	100 μl	160
Cycle 3 (Step 10A(viii))		
Elongation buffer	19 ml	1×
ATP and CTP (40 mM)	37.5 μl	60
UTP (40 mM)	12.5 μl	20
Cycle 4 (Step 10A(ix))		
Elongation buffer	19 ml	1×
ATP (40 mM)	12.5 μl	20
GTP (40 mM)	25 μl	40
Cycle 5 (Step 10A(x))		
Elongation buffer	19 ml	1×
$^{13}C^{15}N$-CTP and $^{13}C^{15}N$-UTP (40mM)	25 μl	40
Cycle 6 (Step 10A(xi))		
Elongation buffer	19 ml	1×
$^{13}C^{15}N$-ATP (40 mM)	37.5 μl	60
Cycle 7 (Step 10A(xii))		
Elongation buffer	19 ml	1×
CTP (40 mM)	25 μl	40
GTP (40 mM)	12.5 μl	20
UTP (40 mM)	37.5 μl	60
Termination stage (Step 10A(xiii))		
Elongation buffer	19 ml	1×
ATP (40 mM)	25 μl	40
CTP (40 mM)	50 μl	80
UTP (40 mM)	62.5 μl	100

[a]The volume of elongation buffer = the total reaction volume (25 ml) – the static volume of DNA beads (6 ml).

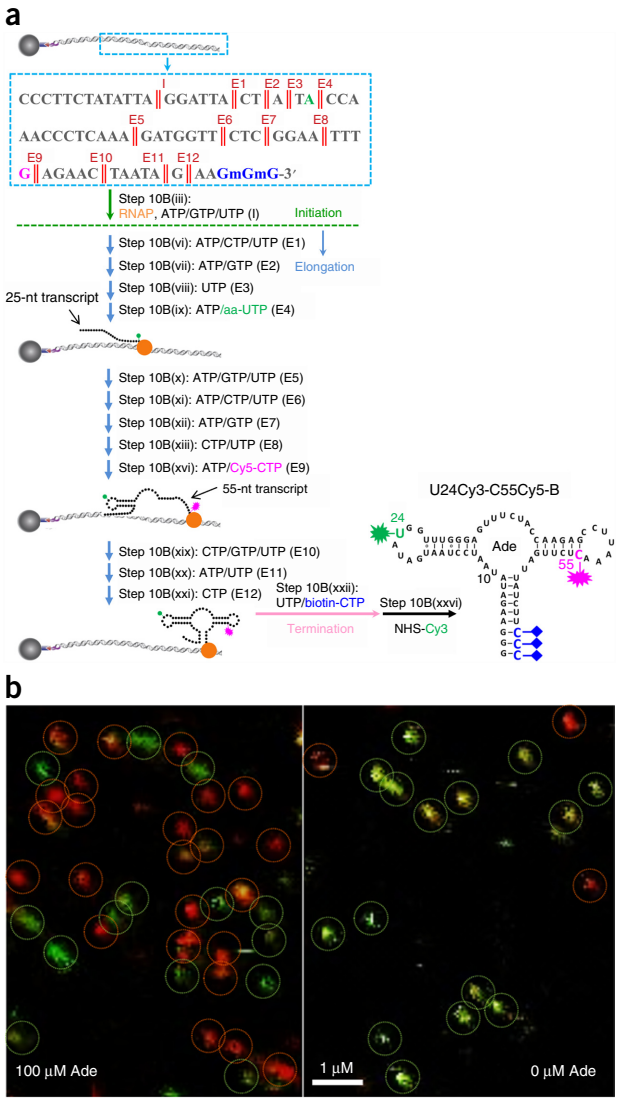

Figure 4 | Application of PLOR in generating a fluorescently labeled sample for FRET study. (**a**) Diagram of generation of U24Cy3-C55Cy5-B-riboA71 (Step 10B(ii–xxvi)). The coding sequence of the template from 3′ to 5′ is shown in the blue box (the positions to be transcribed or post-reacted with Cy3, Cy5, and biotin are shown in green, magenta, and blue, respectively). The pause points of T7 RNAP on the DNA template are marked by red double lines, and the sequence to be transcribed with probes is shown with non-black colors. With the addition of ATP/GTP/UTP at Step 10B(iii) (I, initiation), ATP/CTP/UTP at Step 10B(vi) (cycle 1, elongation), ATP/GTP at Step 10B(vii), and UTP at Step 10B(viii), T7 RNAP pauses at position E3. The addition of ATP/aminoallyl-UTP (aa-UTP, green dot) extends the nascent transcript to 25 nt, and introduces the aminoallyl group at position 24 (Step 10B(ix)). The addition of ATP/GTP/UTP at Step 10B(x), ATP/CTP/UTP at Step 10B(xi), ATP/GTP at Step 10B(xii), and CTP/UTP at Step 10B(xiii) makes the T7 RNAP pause at position E8. With the addition of ATP/Cy5-labeled CTP (magenta, Step 10B(xvi)), T7 RNAP resumes transcription and generates the 55-nt transcript, with Cy5 (magenta explosion) at position 55. The addition of CTP/GTP/UTP (Step 10B(xix)), ATP/UTP (Step 10B(xx)), CTP (Step 10B(xxi)), and UTP/biotin-CTP (Step 10B(xxii)) generates the full-length RNA with an aminoallyl group, Cy5, and biotins (blue diamonds). Post-transcription reaction between aminoallyl and NHS-Cy3 groups (Step 10B(xxvi)) is used to link Cy3 (green explosion) to position 24. (**b**) False-color image of surface-immobilized molecules reveals that RNA products contain all of the specified modifications and are able to form the high-FRET conformation in solution containing 1 mM Mg^{2+} and either 0 (right) or 100 (left) μM adenine (Ade). **b** adapted from ref. 19, Springer Nature.

TABLE 2 | Reagent additions for 3-ml, 5 μM PLOR reaction to generate U24aa-C55Cy5-B-riboA71.

Component	Volume	Final concentration (μM)
Initiation stage (Step 10B(ii,iii))		
Initiation buffer, 5×	600 μl	1×
DNA beads (50 μM)	300 μl	5
T7 RNAP (400 μM)	37.5 μl	5
ATP and GTP (40 mM)	36 μl	480
UTP (10 mM)	14.4 μl	48
DEPC–H_2O	1.954 ml	
Elongation stage		
Cycle 1 (Step 10B(vi)), Elongation buffer	2.82 ml[a]	1×
ATP, CTP, and UTP (2 mM)	15 μl	10
Cycle 2 (Step 10B(vii)), Elongation buffer	2.82 ml	1×
ATP and GTP (2 mM)	7.5 μl	5
Cycle 3 (Step 10B(viii)), Elongation buffer	2.82 ml	1×
UTP (2 mM)	7.5 μl	5
Cycle 4 (Step 10B(ix)), Elongation buffer	2.82 ml	1×
ATP and 5-aminoallyl UTP (2 mM)	7.5 μl	5
Cycle 5 (Step 10B(x)), Elongation buffer	2.82 ml	1×
ATP (2 mM)	7.5 μl	5
GTP and UTP (2 mM)	45 μl	30
Cycle 6 (Step 10B(xi)), Elongation buffer	2.82 ml	1×
ATP and CTP (2 mM)	22.5 μl	15
UTP (2 mM)	7.5 μl	5
Cycle 7 (Step 10B(xii)), Elongation buffer	2.82 ml	1×
ATP (2 mM)	7.5 μl	5
GTP (2 mM)	15 μl	10
Cycle 8 (Step 10B(xiii)), Elongation buffer	2.82 ml	1×
CTP and UTP (2 mM)	15 μl	10
Cycle 9 (Step 10B(xvi)), Elongation buffer	2.82 ml	1×
ATP (2 mM)	22.5 μl	15
Cy5-CTP (1 mM)	15 μl	5
Cycle 10 (Step 10B(xix)), Elongation buffer	2.82 ml	1×
CTP and GTP (2 mM)	7.5 μl	5
UTP (2 mM)	22.5 μl	15
Cycle 11 (Step 10B(xx)), Elongation buffer	2.82 ml	1×
ATP (2 mM)	15 μl	10
UTP (2 mM)	22.5 μl	15
Cycle 12 (Step 10B(xxi)), Elongation buffer	2.82 ml	1×
CTP (2 mM)	7.5 μl	5
Termination stage (Step 10B(xxii))		
Elongation buffer	2.82 ml	1×
Biotin-11-CTP (1 mM)	45 μl	15
UTP (2 mM)	15 μl	10

[a]The volume of elongation buffer equals the total reaction volume (3 ml) – the static volume of solid-phase DNA beads (180 μl).

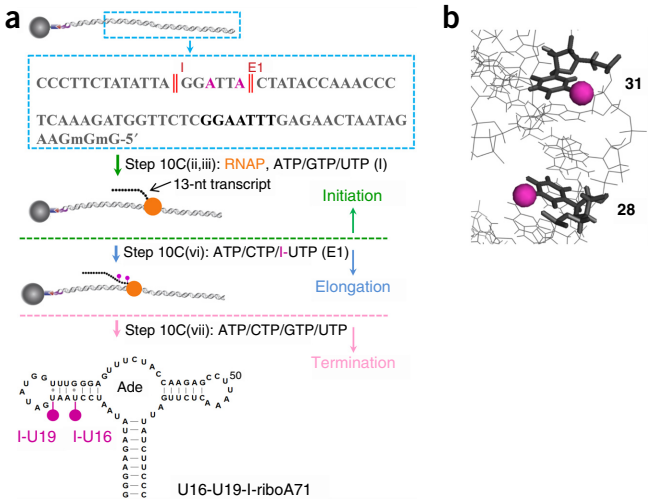

Figure 5 | Application of PLOR in generating a sample labeled with heavy atoms for crystallographic study. (**a**) Diagram of the generation of U16-U19-I-riboA71 (Step 10C(ii–vii)). The coding sequence of the template from 3′ to 5′ is shown in the blue box (the two positions to be transcribed with iodo-U residues are shown in purple). With the addition of ATP/GTP/UTP at Step 10C(iii) (I, initiation), and ATP/CTP/I-UTP at Step 10C(vi) (elongation), T7 RNAP pauses at position E1, and then introduces the iodo-uridines at positions 16 and 19 (purple dots). The addition of all four types of NTPs at Step 10C(vii) generates the full-length RNA of U16-U19-I-riboA71. (**b**) Close-up view of I-U16 and I-U19 as observed in the crystal structure of U16-U19-I-riboA71 (PDB ID: 5UZA), determined by anomalous phasing. The iodine atoms are shown as purple spheres.

TABLE 3 | Reagent additions for 5-ml, 10 μM PLOR reaction to generate U16–U19-I-riboA71.

Component	Volume	Final concentration (μM)
Initiation stage (Step 10C(ii,iii))		
Initiation buffer, 5×	1 ml	1×
DNA beads (50 μM)	1 ml	10
T7 RNAP (400 μM)	125 μl	10
ATP and GTP (40 mM)	120 μl	960
UTP (10 mM)	48 μl	96
DEPC–H_2O	2.0 ml	
Elongation stage		
Cycle 1 (Step 10C(vi)), Elongation buffer	4.4 ml[a]	1×
ATP, CTP, and I-UTP (2 mM)	50 μl	20
Termination stage (Step 10C(vii))		
Elongation buffer	4.4 ml	1×
ATP (40 mM)	15 μl	120
CTP (40 mM)	14 μl	110
GTP (40 mM)	12.5 μl	100
UTP (40 mM)	24 μl	190

[a]The volume of elongation buffer equals the total reaction volume (5 ml) – the static volume of solid-phase DNA beads (600 μl).

Labeling strategy for U24Cy3-C55Cy5-B-riboA71. The synthesis scheme for U24Cy3-C55Cy5-B (Cy3, Cy5, and biotins are labeled at positions 24, 55, and the 3′ terminus, respectively) is presented in **Figure 4a** and **Table 2**. It comprises an initiation step, 12 elongation cycles, and a termination step. The initiation step for preparing the sample was the same as described for Lp2-CN-riboA71. The procedure for individual elongation cycles was the same as described for Lp2-CN-riboA71, except for the NTP combinations added in individual cycles and the reaction temperature for introducing Cy5 into the RNA. The following NTP combinations were added to control the transcription pause/resumption in the elongation cycles: ATP/CTP/UTP (cycle 1); ATP/GTP (cycle 2); UTP (cycle 3); ATP/5-aminoallyl-UTP (aa-UTP) (cycle 4); ATP/GTP/UTP (cycle 5); ATP/CTP/UTP (cycle 6); ATP/GTP (cycle 7); CTP/UTP (cycle 8); ATP/Cy5-CTP (cycle 9); CTP/GTP/UTP (cycle 10); ATP/UTP (cycle 11); CTP (cycle 12); and biotin-CTP/UTP (termination). The expected points at which T7 RNAP pauses during elongation are marked by red double lines and labeled from E1 to E12 (**Fig. 4a**). Fluorescent groups are bulky, and incorporation of NTPs containing those groups by RNAP is generally less efficient. Therefore, we applied two strategies to improve efficiency. One is use of a higher reaction temperature for introducing Cy5-CTP into the RNA in cycle 9, and the other is to use a nucleotide labeled with a smaller reactive group that can first be specifically introduced into RNA (such as 5-aminoallyl-UTP, as used here in cycle 4) and then linked with a fluorescent group or functional group Cy3 via a post-transcription reaction. The smFRET image of the RNA indicates the incorporation of Cy3, Cy5 and biotin into U24Cy3-C55Cy5-B-riboA71 (**Fig. 4b**).

Labeling strategy for U16-U19-I-riboA71. The synthesis scheme for U16-U19-I-riboA71, in which iodo-uridines are incorporated at positions 16 and 19, is straightforward (**Fig. 5a** and **Table 3**). It comprises an initiation step, one elongation cycle, and a termination step. The initiation for this sample was the same as described for the other two samples. ATP/CTP/5-iodo-UTP (I-UTP) were used to elongate the transcripts in the second stage of PLOR, and all four types of NTPs were used to transcribe the remaining sequence in the termination stage.

MATERIALS

REAGENTS

- Neutravidin-coated agarose beads (Thermo Fisher Scientific, cat. no. 29204)
- Diethyl pyrocarbonate (DEPC; Sigma-Aldrich, cat. no. D5758) **! CAUTION** DEPC is highly toxic, and should be handled in a fume hood. DEPC can damage plastic, so a glass pipette should be used to transfer DEPC.
- Potassium chloride (Sigma-Aldrich, cat. no. P9333)
- Magnesium chloride (Sigma-Aldrich, cat. no. M8266)
- Tris-HCl buffer (KD Medical, cat. no. RGF-3360)
- 10× TBE (KD Medical, cat. no. RGF-3330)
- Sodium chloride (Sigma-Aldrich, cat. no. S7653)
- Potassium sulfate (EM Science, cat. no. PX1595-1)
- Magnesium sulfate (Sigma-Aldrich, cat. no. M7506)
- Potassium hydroxide (Sigma-Aldrich, cat. no. P5958)
- DL-dithiothreitol (DTT; Sigma-Aldrich, cat. no. D0632)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S6297)
- Sodium acetate (Sigma-Aldrich, cat. no. S2889)
- DMSO (Sigma-Aldrich, cat. no. D8418) **! CAUTION** DMSO can solubilize highly toxic compounds and help them traverse skin. It should be handled while wearing gloves.
- Phenol–chloroform–isoamyl alcohol mixture (Sigma-Aldrich, cat. no. 77618) **! CAUTION** The mixture is highly toxic and should be handled in a fume hood.
- SYBR Gold (Thermo Fisher Scientific, cat. no. S11494)
- Urea (Ambion, cat. no. AM9902)
- EDTA (KD Medical, cat. no. RGF-3130)
- Ammonium persulfate (APS; Bio-Rad, cat. no. 161-0700)
- TEMED (Bio-Rad, cat. no. 161-0801) **! CAUTION** This compound is toxic and should be handled in a fume hood.
- 30% Acrylamide/Bis solution (Bio-Rad, cat. no. 161-0156)
- Formamide (Thermo Fisher Scientific, cat. no. F-82) **! CAUTION** Formamide is highly toxic and irritating. It should be handled in a fume hood.
- Ethyl alcohol (Sigma-Aldrich, cat. no. 459844)
- Acetonitrile (Sigma-Aldrich, cat. no. 34998) **! CAUTION** This compound is toxic and should be handled in a be handled in a fume hood.
- Triethylamine (Sigma-Aldrich, cat. no. T-0886) **! CAUTION** This compound is toxic, and should be handled in a fume hood.
- Triethylammonium acetate (TEAA; Sigma-Aldrich, cat. no. 90358)
- HEPES (Sigma-Aldrich, cat. no. H4034)
- dNTPs (Invitrogen, cat. no. 10297-018)
- ATP (Sigma-Aldrich, cat. no. A7699)
- CTP (Sigma-Aldrich, cat. no. C1506)
- GTP (Sigma-Aldrich, cat. no. G8877)
- UTP (Sigma-Aldrich, cat. no. U6625)
- $^{13}C^{15}N$-ATP (Sigma-Aldrich, cat. no. 645702)
- $^{13}C^{15}N$-CTP (Sigma-Aldrich, cat. no. 645699)
- $^{13}C^{15}N$-UTP (Sigma-Aldrich, cat. no. 645672)
- 5-Aminoallyl uridine-5′-triphosphate (TriLink, cat. no. N1062)
- Cy3 mono NHS ester (GE Healthcare, cat. no. PA23001)
- Cy5-CTP (GE Healthcare, cat. no. 25801087)
- Biotin-11-CTP (Roche, cat. no. 04739205001)
- DNA templates and primers (**Box 1**)
- T7 RNA polymerase with His tag (prepared in-house[34])
- Taq DNA polymerase with His tag (prepared in-house: the expression procedure is as described by Pluthero[35] and the purification procedures are detailed in **Box 5**)
- Imidazole (Sigma-Aldrich, cat. no. 288-32-4)
- Lysozyme (Sigma-Aldrich, cat. no. 10837059001)
- Glycerol (Sigma-Aldrich, cat. no. 56-81-5)
- Triton X-100 (Sigma-Aldrich, cat. no. 9002-93-1)

EQUIPMENT

- Polypropylene conical tube (50 ml; Falcon, cat. no. 352098)
- Zeba spin desalting column (0.5 ml; Thermo Fisher Scientific, cat. no. 89882)
- Pierce centrifuge column (Pierce, 0.8 ml, cat. no. 89868; 2 ml, cat. no. 89896; 10 ml, cat. no. 89898)
- Corning vacuum filtration system (Corning, cat. no. 431096)
- C1000 Touch thermal cycler for PCR (Bio-Rad, cat. no. 184-0197)
- 96-well PCR plates (Fisherbrand, cat. no. 14230232)
- HB-500 Minidizer hybridization oven (UVP, part no. 95-0330-01)
- Paraflim (Parafilm, cat. no. PM996)
- Steriflip filter unit, 50 ml (Millipore, cat. no. SCNY00040)
- Steriflip vacuum filter unit (Fisher Scientific, cat. no. SCNY00040)
- Gel Doc EZ imager (Bio-Rad, cat. no. 170-8270)
- Electrophoresis equipment (CBS Scientific, cat. no. SG-400-33; Bio-Rad, cat. no. 1658003)
- Agilent Infinity series HPLC system (Agilent, model no. 1100) **▲ CRITICAL** The HPLC system should be equipped with a detector that can measure absorbance at 550 nm and 650 nm, for detecting Cy3 and Cy5, respectively.
- HPLC column (In our laboratory, an Agilent PLRP-S column (Agilent, cat. no. PL1912-5501) with 5-µm particle size and 30-nm pore size was used. The inner diameter of the column is 2.1 mm, and it has a length of 250 mm).
- Freeze dryer (Labconco, model no. FreeZone 4.5)
- Amicon Ultra-15C centrifugal filter units (Millipore, cat. no. UFC900324)
- Spectrometer (Beckman, model no. DU-60)
- Homogenizer (VWR Scientific, model no. Sonifier 250)
- Image lab software (cat. no. 1709690)

REAGENT SETUP

DEPC–H_2O Add 4 ml of DEPC to 4 liters of ddH_2O, stir overnight, and autoclave or heat at 120 °C for 40 min twice to remove residual DEPC. The solution is ready for use after cooling to room temperature (~25 °C). **▲ CRITICAL** Autoclaving destroys DEPC, leaving CO_2 and ethanol. DEPC-treated H_2O is RNase-free, and is highly recommended to replace regular ddH_2O for preparation of the buffers in this protocol.

PCR buffer, 10× For 100 ml of buffer, mix 3.73 g of potassium chloride, 0.24 g of magnesium chloride, and 10 ml of Tris-HCl buffer (1 M, pH 8.0) in DEPC–H_2O. Adjust the pH to 8.4 with 2 M potassium hydroxide. Filter the buffer before use. This buffer can be stored at room temperature for months.

Incubation buffer For 500 ml of the buffer, mix 20 ml of Tris-HCl buffer (1 M, pH 8.0), 8.7 g of potassium sulfate, and 0.36 g of magnesium sulfate in DEPC–H_2O. Adjust the pH to 8.0 with 2 M potassium hydroxide. The buffer can be stored at room temperature for months. **▲ CRITICAL** DEPC–H_2O is acidic; pH adjustment is required.

Initiation buffer, 5× For 100 ml of the buffer, mix 20 ml of Tris-HCl buffer (1 M, pH 8.0), 8.7 g of potassium sulfate, 0.36 g of magnesium sulfate, and 0.77 g of DTT in DEPC–H_2O. Shake the buffer to make sure that the DTT is dissolved and adjust the pH to 8.0 with 2 M potassium hydroxide. The buffer can be stored at −20 °C for several weeks.

Elongation buffer For 500 ml of the buffer, mix 20 ml of Tris-HCl buffer (1 M, pH 8.0), 0.36 g of magnesium sulfate, and 0.77 g of DTT in DEPC–H_2O. Adjust the pH to 8.0 with 2 M potassium hydroxide. The buffer can be stored at −20 °C for weeks.

Washing buffer For 500 ml of the buffer, mix 20 ml of Tris-HCl buffer (1 M, pH 8.0) and 0.36 g of magnesium sulfate in DEPC–H_2O. Adjust the pH to 8.0 with 2 M potassium hydroxide. The buffer can be stored at room temperature for months.

ATP (40 mM) For 10 ml of the reagent, mix 0.22 g of ATP and 0.1 ml of Tris-HCl buffer (1 M, pH 8.0) in DEPC–H_2O, dissolve using 1 M HCl, and adjust the pH to 7.5 using 2 M potassium hydroxide. The reagent can be stored at −20 °C for months.

CTP (40 mM) For 10 ml of the reagent, mix 0.21 g of CTP and 0.1 ml of Tris-HCl buffer (1 M, pH 8.0) in DEPC–H_2O, dissolve using 1 M HCl, and adjust the pH to 7.5 using 2 M potassium hydroxide. The reagent can be stored at −20 °C for months.

GTP (40 mM) For 10 ml of the reagent, mix 0.21 g of GTP and 0.1 ml of Tris-HCl buffer (1 M, pH 8.0) in DEPC–H_2O, dissolve using 1 M HCl, and adjust the pH to 7.5 using 2 M potassium hydroxide. The reagent can be stored at −20 °C for months.

UTP (40 mM) For 10 ml of the reagent, mix 0.22 g of UTP and 0.1 ml of Tris-HCl buffer (1 M, pH 8.0) in DEPC–H_2O, dissolve using 1 M HCl,

Box 5 | Purification procedures for His-tagged Taq DNA polymerase

● TIMING ~1 d

This protocol was adapted from the original protocol by Frey and Görlich (http://wwwuser.gwdg.de/~jgrossh/protocols/recombinant-proteins/Taq_polymerase.html) from a list of protocols (http://wwwuser.gwdg.de/~jgrossh/protocols/) assembled by Jörg Großhans (http://wwwuser.gwdg.de/~jgrossh/) with minor modifications. The purification procedures contain high-temperature incubation and Ni^{2+}-resin purification. The reagents used here are quantified for the cell pellet of Taq DNA polymerase extracted from a1-liter cell culture.

High-temperature incubation purification of His-tagged Taq DNA polymerase.

1. Thaw the frozen cell pellet of Taq DNA polymerase in warm water.
2. Resuspend the cell pellet in 10-ml of suspension buffer with 50 mg of lysozyme (final concentration of lysozyme is 5 mg/ml).
3. Incubate for 15 min at room temperature.
4. Break the cells by sonication three times using a Sonifier 250 sonicator for 2 min each time.

▲ CRITICAL STEP Put the cell container into ice for 5 min during sonication intervals to avoid overheating of the cells.

5. Put the cell solution into a water bath at 80 °C for 15 min.

▲ CRITICAL STEP The high temperature used here is to denature the proteins except for the Taq DNA polymerase.

6. Take the cell solution from the water bath and put into ice for ~1 h.
7. Centrifuge the cell solution at 20,000 r.p.m. for 1 h.

! CAUTION Set the centrifugation temperature at 4 °C to avoid overheating of the cell solution.

8. Pour the supernatant into a clean container.

■ PAUSE POINT The supernatant can be stored at 4 °C for a short time or at –80 °C for years.

Ni^{2+}-resin purification of His-tagged Taq DNA polymerase.

9. Mix the supernatant with the pre-equilibrated Ni^{2+} resin (5-ml bed volume), and incubate at 4 °C for 1 h.

▲ CRITICAL STEP Incubation for 1h is done to bind the His-tagged DNA Taq polymerase in the supernatant to the Ni^{2+} resin.

10. Transfer the resin mixture (the mixture of the Ni^{2+} resin and the supernatant) to a Steriflip vacuum filter unit, and evacuate the unbound mixture from the resin.
11. Wash the resin with 30 ml of suspension buffer, and evacuate the buffer from the resin.
12. Wash the resin with 30 ml of buffer C, and evacuate the buffer from the resin.
13. Wash the resin with 30 ml of buffer D, and evacuate the buffer from the resin.
14. Add 4 ml of elution buffer to the resin, and evacuate the buffer from the resin.
15. Collect the elution, and mix the elution with an equal volume of buffer E to prepare 100× Taq polymerase.
16. Store 100× Taq polymerase at –80 °C for up to a few months.

and adjust the pH to 7.5 using 2 M potassium hydroxide. The reagent can be stored at −20 °C for months.

Gel-soaking buffer For 100 ml of buffer, add 0.41 g of sodium acetate and 0.4 ml of EDTA (0.5 M, pH 8.0) to DEPC–H_2O. This buffer can be stored at 4 °C for months.

12% (wt/vol) PAGE solution For 400 ml of solution, mix 40 ml of 10× TBE, 160 ml of 30% acrylamide/Bis solution, and 100 ml of water. Dissolve 192 g of urea in the mixture, and heat at 50 °C to dissolve the urea completely. Add ddH_2O until the total volume is 400 ml. Filter the solution before use. This solution can be covered with aluminum foil and stored at room temperature for weeks.

10% APS (wt/vol) For 2 ml of buffer, add 0.2 g of APS to 2 ml of ddH_2O, and vortex ~10 s to dissolve. The buffer can be stored at 4 °C for 1 week.

Buffer A For 250 ml of buffer, mix 25 ml of TEAA with 225 ml of DEPC–H_2O. Adjust the pH to 7.0 with triethylamine. Filter using a Corning vacuum filter and vacuum-degas the buffer before use. The buffer can be stored at room temperature for months but must be degassed before use.

Buffer B For 200 ml of buffer, mix 20 ml of TEAA and 150 ml of acetonitrile with 30 ml of DEPC–H_2O. Degas the buffer before use. The buffer can be stored at room temperature for months.

Formamide–EDTA buffer Add 0.2 ml of EDTA (0.5 M, pH 8.0) to 9.8 ml of formamide. Adjust the pH to 8.0 with 2 M potassium hydroxide. The buffer can be stored at room temperature for months.

10 M urea buffer Add 6.0 g of urea to 10 ml of DEPC–H_2O and heat at 50 °C to dissolve the urea completely. The buffer can be stored at room temperature for weeks. This buffer can be heated before use if crystallization occurs.

$NaHCO_3$ buffer Dissolve 0.25 g of sodium bicarbonate in 10 ml of DEPC–H_2O. Prepare freshly before use.

Suspension buffer For 100 ml of the buffer, mix 5 ml of Tris-HCl buffer (1 M, pH 8.0), 1.76 g of sodium chloride, 20 µl of EDTA (0.5 M, pH 8.0), 0.068 g of imidazole, and 0.154 g of DTT. Prepare freshly before use.

Buffer C For 100 ml of the buffer, mix 5 ml of Tris-HCl buffer (1 M, pH 8.0), 1.17 g of sodium chloride, 20 µl of EDTA (0.5 M, pH 8.0), 0.0068 g of imidazole, 0.077 g of DTT, and 5 ml of glycerol. Prepare freshly before use.

Buffer D For 100 ml of the buffer, mix 5 ml of Tris-HCl buffer (1 M, pH 8.0), 0.585 g of sodium chloride, 20 µl of EDTA (0.5 M, pH 8.0), 0.0068 g of imidazole, 0.077 g of DTT, and 25 ml of glycerol. Prepare freshly before use.

Elution buffer For 100 ml of the buffer, mix 5 ml of Tris-HCl buffer (1 M, pH 8.0), 0.585 g of sodium chloride, 20 µl of EDTA (0.5 M, pH 8.0), 2.04 g of imidazole, 0.154 g of DTT, and 25 ml of glycerol. Prepare freshly before use.

Buffer E For 100 ml of the buffer, mix 5 ml of Tris-HCl buffer (1 M, pH 8.0), 0.585 g of sodium chloride, 20 µl of EDTA (0.5 M, pH 8.0), 0.031 g of DTT, 75 ml of glycerol, and 2 ml of Triton X-100. Prepare freshly before use.

PROCEDURE

Generation and purification of DNA templates ● TIMING ~8 d

1| Prepare the 106-nt DNA template for the riboA71 synthesis by PCR.

▲ CRITICAL STEP The sequences of the DNA templates and primers used for PCR reactions are listed in **Box 1**. The biotin group on the forward primer is for DNA immobilization on the neutravidin-coated beads, and the mG groups on the reverse primer are for minimizing nontemplated nucleotide addition in transcription. The PCR protocol is listed in **Box 1**. For template preparation, one can choose to prepare a relatively small amount of template by PCR, and repeatedly use the same solid-phase immobilized DNA template to perform PLOR multiple times to obtain a desired amount of RNA, or, alternatively, prepare a relatively large amount of template by PCR to generate a large amount of RNA in fewer runs. Here, we preferred to use a large quantity of template by PCR. A 10-ml PCR reaction in 96-well PCR plates was repeated ~40 times to obtain ~0.5–1.0 μmol of double-stranded 106-nt DNA after PAGE purification. We finished the PCRs in ~6 d by using two PCR machines for 8–10 h per day.

2| Purify the PCR products by 12% (wt/vol) PAGE.

▲ CRITICAL STEP This step is important to remove biotinylated primers that are not used up in PCR. The residual biotinylated primers may compete with biotinylated templates for binding to neutravidin sites on the beads, resulting in lower density of the templates on the beads. Refer to **Box 4** for more advice regarding purification.

3| Immerse the crushed gel band in gel-soaking buffer, and mix overnight at 4 °C with rotation. Filter to remove the crushed gel using a Corning vacuum filter.

4| Recover the 106-nt DNAs from the soaking buffer and obtain ~0.6 μmol of 106-nt DNAs. Replace the buffer of the DNAs with incubation buffer using Amicon Ultra-15C centrifugal filter units.

■ PAUSE POINT The DNA can be stored at 4 °C for months or at –20 °C for years.

Equilibration of neutravidin beads ● TIMING ~1.5 h

5| Add 12 ml of neutravidin bead slurry (50% (vol/vol); the static volume of the neutravidin beads is ~6 ml) to a 50-ml polypropylene conical tube; centrifuge for ~2–5 min; and pour off the supernatant.

▲ CRITICAL STEP Throughout the PROCEDURE, the centrifugation temperature should be 4 °C, speed should be 200*g*, and time should be ~2–5 min, except where it is noted otherwise. A higher centrifuge speed may damage the beads.

6| Rinse the beads with 5 ml of DEPC–H_2O and then three times with 5 ml of incubation buffer. To do this, add buffer/water to the beads, and then gently shake to resuspend the beads in buffer/water. Remove the buffer/water using either Steriflip filter units (for beads in polypropylene conical tubes) or a centrifuge (for beads in Pierce centrifuge columns).

▲ CRITICAL STEP Bead rinsing is necessary to remove contamination and/or exchange the buffer for the beads. As the agarose beads are porous and may retain unwanted contaminants inside the beads, make sure to allow sufficient time and cycles for rinsing and buffer exchange.

■ PAUSE POINT The beads can be stored at 4 °C for months.

Immobilization of DNA on neutravidin beads ● TIMING ~2 d

7| Add 0.6 μmol of 106-nt DNA dropwise to the rinsed neutravidin beads (the static volume of the beads is ~6 ml). Gently rotate at 4 °C for 2 d.

▲ CRITICAL STEP Rotation is needed to improve the efficiency of biotinylated template binding to neutravidin beads and for even distribution of DNA on the bead surface.

8| Filter the DNA beads, followed by rinsing with incubation buffer (5 ml, as described in Step 6). Repeat for a total of five filtration and rinse cycles.

▲ CRITICAL STEP The separation of the DNA beads from the incubation buffer can be done quickly by using a Steriflip unit with vacuum assistance, following the manufacturer's protocol. Thorough rinsing is necessary to remove any unbound DNA templates. Usually, five cycles of rinse and filtration are sufficient to remove >99% of unbound DNAs. Failure to remove unbound DNA templates may result in overestimating the template DNA concentration. The presence of the free template leads to nonproductive ternary complexes and reduced yields.

9| Add ~4 ml of incubation buffer to the DNA beads and calculate the DNA concentration using the methods described in **Box 3**.

▲ CRITICAL STEP Accurate measurement of the DNA concentration is critical to PLOR yields. We obtained 50 μM DNA for DNA beads in 10.2 ml using both methods listed in **Box 3** for concentration measurement.

? TROUBLESHOOTING

■ **PAUSE POINT** The DNA beads can be stored at 4 °C for >1 year. After storage, however, Steps 8 and 9 should be repeated before use to remove any templates that may have dissociated from the beads.

Preparation of the RNAs

10| The DNA beads are now ready for use. Refer to options A, B, and C for the procedures to prepare Lp2-riboA71, U24Cy3-C55Cy5-B-riboA71, and U16-U19-I-riboA71, respectively.

(A) Preparation of PLOR-generated Lp2-riboA71 ● TIMING **~5–6 h**

(i) The following procedure is for a 25-ml, 20 μM (500 nmol) synthesis scale (synthesis scale refers to the DNA template in PLOR). Refer to the reagents and volumes listed in **Table 1**. Optimize the concentrations for T7 RNAP and NTPs, referring to the advice in the Experimental design section.
▲ CRITICAL STEP The concentrations of the template, T7 RNAP, and NTPs should be optimized in small-scale trials before large-scale synthesis.

(ii) Add DNA beads and T7 RNAP to a 50-ml polypropylene conical tube (also referred to as 'the reactor' in the following steps (Step 10A(iii and xiv)), then gently mix the DNA beads and T7 RNAP in a hybridization oven at 37 °C for 10 min.
▲ CRITICAL STEP To improve the contact between the solid-phase DNAs and the liquid-phase reagents, gentle rotation of the tube is needed. 50-ml polypropylene conical tubes can be used as reactors for ~5–50 ml of synthesis. T7 RNAP may become denatured and appear as a sticky white foam if rotation is too intense.

(iii) Add ATP, GTP and UTP, 5× initiation buffer, and DEPC–H_2O to the reactor. Gently rotate at 37 °C for 15 min.
▲ CRITICAL STEP To avoid a sudden temperature shift when adding reagents, all solutions used in the initiation stage should be preheated to 37 °C.

(iv) Take the reactor out of the hybridization oven. Adjust the temperature of the hybridization oven to 25 °C.
■ **PAUSE POINT** Our hybridization oven, with its temperature set at 25 °C, was located in a refrigerator or a cold room. The reaction mixture can be left overnight at 4 °C, if needed.

(v) Filter the reaction mixture using a 50-ml Steriflip filter unit, following the manufacturer's protocol to remove the liquid phase from the reactor. Rinse the solid-phase bead complexes five times with 10 ml of washing buffer.
▲ CRITICAL STEP Rinsing the beads thoroughly is necessary to remove residual NTPs, unbound T7 RNAP, and abortive transcripts from the reaction system, and to avoid cross-contamination. We recommend using 50-ml Steriflip filter units to achieve SPE in cases in which 50-ml polypropylene conical tubes are used as the reactors. We recommend recovering the T7 RNAP from the elution of PLOR reactions using nickel (or cobalt) affinity chromatography.

(vi) Add ATP, CTP, and UTP with the elongation buffer to the reactor. Gently rotate in the hybridization oven at 25 °C for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.
▲ CRITICAL STEP Except where noted, all reagents used in the elongation stage should be pre-incubated at 25 °C before adding to the reactor.

(vii) Add ATP, GTP, and UTP with the elongation buffer to the reactor. Gently rotate in the hybridization oven at 25 °C for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.

(viii) Add ATP, CTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.

(ix) Add ATP and GTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.

(x) Add $^{13}C^{15}N$-CTP and $^{13}C^{15}N$-UTP with the elongation buffer to the reactor. Gently rotate for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.
▲ CRITICAL STEP $^{13}C^{15}N$-NTPs are used to introduce isotopic labels into the Lp2 loop.

(xi) Add $^{13}C^{15}N$-ATP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.

(xii) Add CTP, GTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the bead complexes three times with 10 ml of washing buffer.

(xiii) Add ATP, CTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 12 min.

(xiv) Filter and collect the eluate. Rinse the DNA beads approximately six to eight times with 10 ml of incubation buffer to remove all the reagents and products from the reaction for future use, and then store the beads at 4 °C for months.

(xv) The eluate contains the final RNA product and T7 RNAP. Recover T7 RNAP from the eluate by nickel (or cobalt) affinity chromatography.

▲ CRITICAL STEP In cases in which the RNA product is sensitive to heavy metal ions such as Ni^{2+} or Co^{2+}, we recommend skipping the T7 RNAP recovery from the final elution.

(xvi) Purify the RNA by 12% denaturing-PAGE or directly by phenol–chloroform extraction/ethanol precipitation (**Box 4**).

(xvii) Assess the purity of the RNA by denaturing PAGE gel or mass spectroscopy: one sharp band on the gel or a correct molecular mass by mass spectroscopy represents a pure sample.

(xviii) Calculate the RNA concentration from the UV-absorbance measurement at ~260 nm using a Beckman DU-60 spectrometer.

? TROUBLESHOOTING

(B) Synthesis of U24Cy3-C55Cy5-B-riboA71 ● TIMING ~2 d

(i) The following procedure is for PLOR-generated U24aa-C55Cy5-B-riboA71 (labeled with an aminoallyl group, Cy5, and biotin at positions 24, 55, and 3′ terminus, respectively). The following steps (Step 10B(ii–xxiii)) take ~8 h and the procedure is for 3 ml of 5 μM U24aa-C55Cy5-B-riboA71 (15-nmol synthesis scale); refer to **Table 2** for details. First, optimize the experimental conditions.

▲ CRITICAL STEP The optimization of experimental conditions should be performed in small-scale reactions before the 3-ml synthesis.

(ii) Add neutravidin bead–DNAs and T7 RNAP to a 10-ml Pierce centrifuge column (also referred to as 'the reactor' in the following steps (Step 10B(iii–xxii)), and gently mix the DNA beads and T7 RNAP in the hybridization oven at 37 °C for 10 min.

▲ CRITICAL STEP Pierce centrifuge columns of different sizes are suitable reactors for ~0.1- to 10-ml synthesis. A 0.8-ml column (Pierce, cat. no. 89868), a 2-ml column (Pierce, cat. no. 89896), and a 10-ml column (Pierce, cat. no. 89898) are suggested for synthesis scales of ~0.1–0.5 ml, 0.5–2 ml, and 2–10 ml, respectively.

(iii) Add ATP, GTP and UTP, 5× initiation buffer, and DEPC–H_2O to the reactor. Gently rotate at 37 °C for 15 min in the hybridization oven.

▲ CRITICAL STEP All solutions should be preheated to 37 °C.

(iv) Take the reactor out of the hybridization oven. Adjust the temperature of the hybridization oven to 25 °C.

(v) Remove the twist-off bottom from the reactor; separate the liquid phase from the beads by centrifugation. Rinse the DNA–bead complexes five times with ~2–3 ml of washing buffer. Remove the liquid phase from the reactor by centrifuging as described in Step 5.

▲ CRITICAL STEP Centrifugation is used to remove the liquid phase from the reactor, and ~2–3 ml of washing buffer is used to rinse the beads in the following steps (Step 10B(vi–xxi)), except where noted otherwise.

(vi) Cover the bottom of the reactor with the cap provided by the vendor, and also seal the bottom using Parafilm to avoid potential leakage. Add ATP, CTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times as described in Step 6.

▲ CRITICAL STEP Except where noted, the bottom of the reactor should always be sealed before NTP additions.

(vii) Add ATP and GTP with elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(viii) Add UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(ix) Add ATP and 5-aminoallyl-UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(x) Add ATP, GTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(xi) Add ATP, CTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(xii) Add ATP and GTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(xiii) Add CTP and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min.

(xiv) Take the reactor out of the hybridization oven. Adjust the temperature of the hybridization oven to 37 °C.

▲ CRITICAL STEP 37 °C is recommended for introducing nucleotides with bulky fluorescent groups into RNA.

(xv) Filter the DNA–bead complexes. Rinse the beads three times.

(xvi) Add ATP and Cy5-CTP with the elongation buffer to the reactor. Wrap the reactor in aluminum foil. Gently rotate at 37 °C for 10 min.

▲ CRITICAL STEP Cy3 and Cy5 groups are light sensitive. Covering the reactor with aluminum foil and running the reaction in a dark room are suggested to avoid light exposure. Preheat ATP and Cy5-CTP at 37 °C before adding to the reactor.

(xvii) Take the reactor out of the hybridization oven. Adjust the temperature of the hybridization oven back to 25 °C.

(xviii) Filter the DNA–bead complexes by centrifugation as described in Step 5. Rinse the beads three times.

(xix) Add CTP, GTP, and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(xx) Add ATP and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(xxi) Add CTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min. Filter and rinse the beads three times.

(xxii) Add biotin-11-CTP and UTP with the elongation buffer to the reactor. Gently rotate at 25 °C for 10 min.

(xxiii) Separate the liquid and solid phases by centrifugation (200g, 4 °C, ~2–5 min). Purify the liquid phase by 12% denaturing-PAGE or phenol–chloroform extraction (refer to **Box 4**) to obtain the RNA product, U24aa-C55Cy5-B-riboA71. Recover the RNA and exchange the buffer for DEPC–H_2O.

? TROUBLESHOOTING

■ **PAUSE POINT** The RNA product can be stored at –20 °C for months.

(xxiv) *Post-transcription reaction to introduce Cy3 into the RNA.* (Step 10B(xxiv–xxvi) takes ~1 d). Lyophilize the U24aa-C55Cy5-B-riboA71 sample using a Labconco FreeZone 4.5 freeze dryer.

(xxv) Dissolve the lyophilized RNA in 10 µl of DEPC–H_2O. Add 5 µl of $NaHCO_3$ buffer.

(xxvi) Add 0.2 mg of Cy3 mono NHS (dissolved in 2 µl of DMSO). Vortex at 37 °C for 1 h, mix at 25 °C overnight, and then mix at 37 °C for 1 h in a hybridization oven. Exchange the buffer for the desired buffer by filtering with Amicon Ultra-15C centrifugal filter units (following the manufacturer's protocol).

▲ **CRITICAL STEP** One vial of Cy3 mono NHS is 1 mg; we dissolved the whole vial in 10 µl of DMSO, and transferred 2 µl of dissolved dye (containing 0.2 mg of Cy3 mono NHS) to perform the reaction. 0.2 mg of Cy3 mono NHS is excessive, and the unreacted Cy3 mono NHS reagent can be removed using Zeba spin desalting columns. The column usage follows the manufacturer's protocol. Usually, three to five columns are needed to completely remove free dye (no red color is observed in the elution).

? TROUBLESHOOTING

■ **PAUSE POINT** The RNA product can be stored at –20 °C for months.

(xxvii) Purify the RNA product by analytical-scale HPLC. Collect the eluate containing U24Cy3-C55Cy5-B-riboA71.

▲ **CRITICAL STEP** Reversed-phase HPLC is used to remove the unreacted U24aa-C55Cy5-B-riboA71 from the U24Cy3-C55Cy5-B-riboA71 (refer to **Box 4**). Only U24Cy3-C55Cy5-B-riboA71 has absorbance at both 550 nm and 650 nm, and hence we advise collecting the eluate with an absorbance reading >0.1 at both wavelengths. We recommend keeping the collection volume small enough (for example, ~0.2 ml) to obtain concentrated fraction(s) for future use.

? TROUBLESHOOTING

(xxviii) Lyophilize the eluate using a Labconco FreeZone 4.5 freeze dryer and exchange the buffer for the desired buffer by using Amicon Ultra-15C centrifugal filter units.

■ **PAUSE POINT** The purified RNA, U24Cy3-C55Cy5-B, can be stored at –20 °C for months.

(C) Preparation of PLOR-generated U16-U19-I-riboA71 ● **TIMING ~1–2 h**

(i) The following procedure is for a 5-ml, 10 µM (50 nmol) synthesis scale; refer to details listed in **Table 3**. First, optimize the experimental conditions.

(ii) Add DNA beads and T7 RNAP to a 50-ml polypropylene conical tube (also referred to as 'the reactor' in the following steps (Step 10C(ii–vi)), and gently mix the DNA beads and T7 RNAP in a hybridization oven at 37 °C for 10 min.

▲ **CRITICAL STEP** 50-ml polypropylene conical tubes are suitable reactors for ~5- to 50-ml synthesis.

(iii) Add ATP, GTP and UTP, 5× initiation buffer, and DEPC–H_2O to the reactor. Gently rotate the reaction tube at 37 °C for 15 min.

(iv) Take the reactor out of the hybridization oven. Adjust the temperature of the hybridization oven to 25 °C.

■ **PAUSE POINT** The reaction mixture can be left overnight at 4 °C, if needed.

(v) Filter the reaction mixture using a 50-ml Steriflip filter unit to remove the liquid phase from the reactor. Rinse the solid phase five times with 5 ml of washing buffer.

(vi) Add the elongation buffer and ATP, CTP, and I-UTP to the reactor. Gently rotate in the hybridization oven at 25 °C for 10 min. Filter and rinse the bead complexes three times with 5 ml of washing buffer.

(vii) Add the elongation buffer and ATP, CTP, GTP, and UTP. Gently rotate in the hybridization oven at 25 °C for 12 min.

(viii) Filter and collect the elution. Rinse the DNA beads approximately six to eight times with 5 ml of incubation buffer and store the beads at 4 °C for up to several months.

▲ **CRITICAL STEP** Purify the RNA product by phenol–chloroform extraction/ethanol precipitation (**Box 4**).

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
9	Failure of DNA bead generation	The biotin group may not be attached to the PCR primers	Check the molecular mass of the biotinylated primers by mass spectrum
10A(xviii), 10B(xxiii), and 10C(viii)	No final product detectable	T7 RNAP is de-activated	Check the activity of T7 RNAP by using conventional *in vitro* transcription
		Initiation failed	Load the elution product of SPE from the initiation and elongation stages to a small gel for analysis. Initiation may fail if abortive products are observed at the initiation stage only, instead of at both stages. Mutate the DNA sequence to extend the initiation length
		DNA concentrations may not be correct	The concentrations of DNA in PLOR are much higher than in conventional *in vitro* transcription in order to obtain a large amount of products, because PLOR is a single-round transcription. For the riboA71 system, we observed that high concentrations (~5–40 μM) are optimal for detection by PAGE using Sybr Gold
10A(xviii), 10B(xxiii), and 10C(viii)	Low yield of final product	DTT may be oxidized	Prepare the buffer with fresh DTT
		T7 RNAP has low activity because of long or inappropriate storage	Use freshly prepared T7 RNAP
		DNA dissociates from repeatedly used beads and its concentration is lower than expected	Re-measure the DNA concentrations (refer to **Box 3**)
10B(xxvi)	Low efficiency of Cy3 labeling	Reagents may have been stored for too long	Prepare fresh $NaHCO_3$ and Cy3-NHS
10B(xxvii)	Low efficiency of Cy5 labeling	Concentration of Cy5-UTP may be too low	Repeat synthesis with a higher amount of Cy5-UTP, for example, 1.5×, 2×, or 3×

● TIMING

Steps 1–4, generation and purification of DNA templates: ~8 d
Steps 5 and 6, equilibration of neutravidin beads: ~1.5 h
Steps 7–9, immobilization of DNA on neutravidin beads: ~2 d
Step 10A, preparation of PLOR-generated Lp2-riboA71: ~5–6 h
Step 10B, synthesis of U24Cy3-C55Cy5-B-riboA71: ~2 d
Step 10C, preparation of PLOR-generated U16-U19-I-riboA71: ~1–2 h
Box 1, preparation of DNA templates for PLOR reactions: ~2.5 h
Box 2, the PLOR procedure and reagent usages for testing initiation transcript length: ~1 h
Box 3, concentration measurements of the DNA on the solid-phase templates: ~0.5–2 h
Box 4, purification methods for PLOR-generated RNAs: ~4 h–1 d
Box 5, purification procedures for His-tagged Taq DNA polymerase: ~1 d

ANTICIPATED RESULTS

Typical yields

The scales of the three PLOR reactions described in the PROCEDURE were 15, 50, and 500 nmol to obtain enough sample for performing FRET, X-ray crystallographic, and NMR experiments, respectively. The examples also show that the PLOR reactions can be performed at a wide range of scales. The transcription yield of PLOR for Lp2-CN-riboA71 is calculated

by the equation: Yield $= I \times E^n$ where I and E are the efficiencies of initiation and elongation (or termination), respectively, and n is the number of cycles used in elongation and termination[19]. The average I and E values for the riboA71 system are 46.0% and 87.8%, respectively (please refer to ref. 19 for detailed determination of yields). The estimated yields for Lp2-CN-riboA71, U24Cy3-C55Cy5-B, and U16-U19-I-riboA71 are 16.2%, 8.5%, and 35.5%, respectively. A total of two rounds of the same PLOR reactions were performed to get an NMR sample of adequate concentration. One round of the PLOR reaction yielded 1.2 mg of Lp2-CN-riboA71, with an average yield of one round of synthesis, 10.2% ([product]/[template]). A second round of PLOR was performed using the same DNA beads used in the first-round reaction to obtain an NMR sample (1.7 mg after purification).

~0.5 nmol of U24aa-C55Cy5-B was obtained from one round of PLOR, ~40% of the calculated yield, possibly due to the relatively poor tolerance of T7 RNAP for the bulky Cy5 fluorophore.

~0.43 mg of U16-U19-I-riboA71, with an actual yield of ~37.5%, was generated by PLOR, which was then purified by phenol–chloroform extraction/ethanol precipitation and used for crystallographic studies.

NMR and FRET studies

NMR spectra have been used to identify the $^{13}C^{15}N$-labeled positions in Lp2-CN-riboA71. The H6/H8–C6/C8 region of the TROSY spectrum collected using Lp2-CN-riboA71 is shown in **Figure 3b**[19]. The visible peaks in the spectrum match the residues in the Lp2 loop, which suggests that the labeled residues are located only in the Lp2 loop. smFRET is ideal for the visualization of single molecules and, in this case, to track the structural dynamics of the two positions labeled with dyes upon binding of the adenine ligand. **Figure 4b** is a false-color image of surface-immobilized molecules, revealing that all of the specified modifications are successfully incorporated into the U24Cy3-C55Cy5-B-riboA71 sample. It also indicates that the kissing loop interaction between Lp1 and Lp2 is present with or without the ligand. But a greater population of the high-FRET conformation is observed in the presence of adenine[19,32,34].

X-ray crystallography studies

The selective incorporation of iodo-uridine into the adenine riboswitch aptamer by PLOR represents a general strategy for heavy-atom derivatization of RNA for the purpose of anomalous phasing of crystallographic data. The results demonstrate that incorporation of a small number of halogen atoms (in this case, two sites in a 71-nt RNA) generates enough anomalous phasing power for *de novo* structure determination, while minimizing RNA structural perturbations and negative effects on crystal quality. Diffraction data for U16-U19-I-riboA71 crystals were collected at the 19BM beam line, Advanced Photon Source, Argonne National Lab, at a photon energy of 8 keV. The data were processed to 2.2 Å using HKL2000 (ref. 36) in the orthorhombic space group $P2_12_12$, with unit-cell dimensions very similar to those of the nonlabeled riboA71 crystals[37]. The crystal structure clearly shows that the iodo-uridines are located at the expected 16 and 19 positions (**Fig. 5b**), with peak heights of 24 and 19 sigma for I-U16 and I-U19, respectively (ANODE)[38].

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AUTHOR CONTRIBUTIONS Y.L. performed RNA synthesis, NMR experiments, and X-ray crystallography experiments, and wrote the manuscript; E.H. and D.N. designed and performed smFRET experiments; P.Y. performed enzyme preparation; R.S. provided critical advice on PLOR; J.R.S., K.T. and X.Z. performed X-ray crystallography experiments and data analysis; and Y.-X.W. designed PLOR. All authors revised the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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