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Monitoring co-transcriptional folding of riboswitches through helicase unwinding

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Abstract

In the cell, RNAs fold and begin to function as they are being transcribed. In contrast, in the laboratory, RNAs are typically studied after transcription is completed. Co-transcriptional folding can regulate the function of riboswitches and ribozymes and dictate the order of ribonucleoprotein assembly. Methods to observe and investigate RNA folding and activity during transcription are therefore desirable, yet synchronizing RNA polymerases and incorporating labels at specific sites for biophysical studies can be challenging. A recent methodological advance has been to harness highly processive, engineered ‘super-helicases’ to unwind hybrid RNA-DNA duplexes, thereby releasing the RNA 5’ to 3’. When combined with single-molecule fluorescence detection, RNA folding and concomitant activity can be studied *in vitro* in a manner that mimics vectorial folding during transcription. Herein, we describe methods for designing and preparing fluorescently labeled RNA-DNA duplex substrates for sequential helicase-dependent RNA folding experiments.

Keywords

RNA structure; RNA folding; riboswitch; ribozyme; single molecule FRET; helicase; ligation; fluorescence

1. INTRODUCTION

The role of co-transcriptional folding in RNA function has been appreciated for four decades (Boyle, Robillard, & Kim, 1980; Nussinov & Tinoco, 1981; Pan & Sosnick, 2006). A small domain of RNA structure can typically fold in less time than is needed for synthesis of the next domain, given typical transcription speeds of 20 to 100 nt/s. As a result, the 5’-end of an RNA molecule begins to fold while the rest of the RNA is yet to be synthesized. The synthesis of the 5’ portion of a transcript prior to the complete synthesis of the entire transcript can temporally regulate the transcript’s folding and function. While co-transcriptional folding can regulate the activity of any RNA, since folding is important to the function of most transcripts, the best studied examples involve RNAs that regulate gene

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expression. For example, the misfolding or delayed folding of the 5' end of the transcript can decrease the activity of self-cleaving ribozymes, which in turn alters mRNA turnover or processing (Heilman-Miller & Woodson, 2003; Lazinski & Taylor, 1995; Pan, Artsimovitch, Fang, Landick, & Sosnick, 1999). Conversely, RNA folding during transcription is essential for riboswitches that regulate transcription termination and elongation in response to ligand binding (Jones & Ferré-D'Amaré, 2017), because the decision to stop or read through a terminator must be made before the polymerase passes by the terminator stem-loop. Elegant biophysical studies have shown that such riboswitches are "kinetically controlled" by the rates of strand synthesis, ligand binding, and RNA folding (Frieda & Block, 2012; Wickiser, Cheah, Breaker, & Crothers, 2005; Wickiser, Winkler, Breaker, & Crothers, 2005; Zhang, Lau, & Ferré-D'Amaré, 2010). Apart from better understanding how these RNA-based regulators function co-transcriptionally, investigating their mechanisms could provide insight into how to target them with inhibitors or engineer them for other purposes.

While measuring simultaneously the rates of RNA folding, ligand binding, and RNA synthesis for riboswitch or ribozyme function is technically challenging, individual rates have been determined using various techniques (Frieda & Block, 2012; Wickiser, Cheah, et al., 2005; Wickiser, Winkler, et al., 2005). Single-molecule Förster resonance energy transfer (FRET) can provide relative populations of individual states and transition rates between states under different experimental conditions, such as ion or ligand concentrations and temperature (Brenner, Scanlan, Nahas, Ha, & Silverman, 2010; Fiegand, Garst, Batey, & Nesbitt, 2012; Liberman et al., 2015; Wood, Ferré-D'Amaré, & Rueda, 2012). While the ideal experiment is to observe RNA molecules fold during synthesis by RNA polymerase at different polymerase rates, this is challenging due to the difficulty of introducing site-specific fluorescent labels during transcription. A common alternative is to study different lengths of transcripts representing different points of elongation to understand their activity at equilibrium. This approach is equivalent to measuring different time points of an elongating RNA at a very slow elongation rate (*i.e.*, assuming an equilibrium is reached). However, it may miss transient yet functionally important RNA structures.

An alternative approach to visualize the sequential folding of RNA in real time has recently been developed using engineered helicases (Hua, Panja, Wang, Woodson, & Ha, 2018). Crosslinked into their active unwinding conformation, these processive enzymes unwind thousands of base pairs of double-stranded DNA at ~140 nt/sec (Arslan, Khafizov, Thomas, Chemla, & Ha, 2015). The robust activity of these engineered helicases was exploited to observe the sequential folding of a ribozyme. In this assay, the helicase translocates 3' to 5' along the DNA strand of an RNA/DNA hybrid (Arslan et al., 2015; Hua et al., 2018). As the helicase unwinds the duplex, the RNA strand is released in the 5' to 3' direction, allowing for folding in a pseudo-co-transcriptional manner (Fig. 1A). This approach not only avoids the complex pausing behavior of cellular polymerases but also allows fluorescent labels to be attached to the RNA strand at any location in advance so that folding kinetics can be observed by single-molecule FRET. Alternative methods for incorporating a fluorophore involve starting transcription with an RNA fragment synthesized in advance or walking the polymerase along the template a few nucleotides at a time (Liu et al., 2015; Perez-Gonzalez, Lafontaine, & Penedo, 2016). In both cases the labeling sites are restricted to the 5' region of the RNA, which does not have an opportunity to fold during the transcription assay.

The helicase RNA folding assay is controlled by adding the reagents to the slide at specific times. The fluorescently labeled RNA-DNA duplex is hybridized with a biotinylated DNA oligomer and immobilized on slides doped with neutravidin (Fig. 1A). The helicase is pre-loaded onto a single-stranded 3' dT20 extension of the complementary DNA (T20-DNA). After unbound reagents are washed away, the unwinding reaction is started by adding ATP to the slide chamber. Other reagents, such as Mg^{2+} or riboswitch ligands, can be added to the slide at the same time. The folding kinetics of individual RNA molecules can be deduced from the changes in fluorescence intensity under a variety of conditions, including unwinding velocity. For example, the passing helicase enhances the fluorescence emission of Cy3, reporting the progress of unwinding. The folded state of the RNA can be inferred from the efficiency of FRET between a donor and acceptor pair of fluorophores attached to the RNA (Roy, Hohng, & Ha, 2008). Thus, ribozyme cleavage may be followed as the RNA folds and releases itself from the surface, and ribozyme or riboswitch folding may be observed as the RNA refolds in the presence of its ligand. In principle, this assay could be used to study any process for which directional RNA folding is necessary.

Herein we describe protocols for producing fluorescently labeled RNA-DNA hybrids for examining co-transcriptional folding or unwinding velocity using the helicase unwinding assay. We suggest methods for choosing and evaluating labeling sites, ligation and purification of fluorescently labeled oligonucleotides, and forming duplexes that serve as the substrates for unwinding experiments. A protocol for conducting helicase unwinding experiments is then described.

2. DESIGN STRATEGIES FOR LABELING SITES

The goal is to capture changes to RNA structure by observing correlated changes in judiciously placed fluorescence dyes that do not perturb the system. This can be achieved using dye pairs (*e.g.*, cyanine dyes Cy3 and Cy5) that undergo FRET (Roy et al., 2008) or by a single dye that fluctuates in intensity due to changes in its environment. In the latter case, interaction between the dye and helicase results in protein-induced fluorescence enhancement (PIFE), which is sensitive in the 0–4 nm distance range (Hwang & Myong, 2014). Since the helicase unwinds in a single direction in these experiments (Fig. 1 A), PIFE appears and disappears as the helicase passes by the Cy3 dye in the duplex.

2.1 Choice of Labeling Sites

As the RNA is extended in the hybrid duplex, placement of a Cy3 dye toward the 5' end of the RNA and the Cy5 dye at the 3' end will result in a low FRET efficiency for the initial duplex and increasing FRET efficiency as the RNA folds. Within the 5' half of the RNA, the ideal labeling spot is a bulge or loop residue that is flexible and not highly conserved. To reduce the costs of synthesizing a fluorescently labeled oligonucleotide, the labeling position may be near the 3' end of the RNA 5' fragment, such as the penultimate 3' nucleotide (*e.g.*, U17 of an 18-nt oligonucleotide).

Riboswitches are commonly studied using inline probing, a chemical probing method that uses the natural instability of RNA to determine which nucleotides are protected from ligand binding (Regulski & Breaker, 2008). Flexible nucleotides within loops and bulges tend to be

the most reactive, and paired or inflexible nucleotides tend to be less reactive. Inline probing is an alternative method to determine which nucleotides are ideal labeling sites. For riboswitches, this includes nucleotides that are reactive both in the presence and absence of ligand or across the range of conditions to be assayed. The choice of labeling site is greatly aided by a structural model of the RNA, to insure that the attached dye is unlikely to occlude a binding pocket or clash with nearby residues. The RNA's activity after fluorescent labeling should be evaluated by measuring the apparent dissociation constant of a ligand in single molecule experiments and comparing this value to a previously determined apparent dissociation constant for the unlabeled RNA. Alternatively, performing inline probing experiments with ^{32}P -labeled RNA with and without the fluorescent label can determine if the label perturbs binding and uses relatively little sample (Regulski & Breaker, 2008). If the RNA is a ribozyme, one should determine whether the attached fluorophores alter the ribozyme self-cleavage rate.

2.2 Strategies for Obtaining RNAs for Ligation

The preparation of substrates for helicase RNA folding experiments may require the ligation of two or more oligonucleotides, at least one of which contains a fluorescent label, and annealing of the labeled RNA strand to a cDNA and a tether DNA to create a hybrid duplex (Fig. 1B). Fluorescent labels may be introduced during or after chemical synthesis, as described elsewhere (Hermanson; Walter & Burke, 2000). Using a DNA splint, as previously described (Moore & Query, 2000), the fluorescently labeled RNA 5' fragment is ligated to an *in vitro* transcribed RNA (the RNA 3' fragment) (Fig. 1B). As the 3' fragment must contain a 5'-monophosphate for the ligation to succeed, this RNA can be phosphorylated with PNK, phenol:chloroform extracted, and ethanol precipitated before the ligation (Fig. 1B). The DNA splint used to ligate the two RNAs can be the same DNA used later in annealing reactions (T20-DNA). The splint should anneal to as much of the RNA fragments as possible to maximize annealing while not being too close in size to the ligated RNA, which would result in band overlap during denaturing gel purification.

Unlabeled RNAs may be chemically synthesized or transcribed and purified following standard protocols (Ferré-D'Amaré & Doudna, 1996; Jones & Ferré-D'Amaré, 2014; Milligan & Uhlenbeck, 1989). An example template used for transcribing the ZTP riboswitch RNA 3' fragment contains the T7 promoter sequence, hammerhead ribozyme (italicized), and ZTP riboswitch sequence (only first 15 nt shown): 5'-TAA TAC GAC TCA CTA TA *ggg aga TAT AAC TGA TA ctg atg agt ccg tga gga cga aac ggt acc cgg tac cgt c* TATCAGTTATATGAC. The underlined 11-nt sequence is complementary to the first 11 nt of the ZTP riboswitch sequence and, upon base pairing, positions the ribozyme cleavage site. After transcription, the RNA product will contain the ZTP riboswitch sequence, a 5'-UU linker, and a 3' sequence (5'-CAU UAG CGG UAU UCC GGA AUU GCC GUA AUC GCG -3') for annealing to a biotinylated DNA tether (5'-Biotin/-CGC GAT TAC GGC AAT TCC GGA ATA CCG CTA ATG - 3') for attachment to the streptavidin slide surface. The template can be PCR-amplified with 2'-O-methyl modified primers as previously described (Kao, Zheng, & Rudisser, 1999), and the primer can be designed to create a template containing the 3'-biotin RNA sequence for attachment to the surface. For example, the reverse primer used for amplifying the ZTP riboswitch 3' fragment for ligation,

containing the 35-nt extension complementary to the DNA tether (underlined) and UU linker sequence (italicized), is 5'-mCm GCG ATT ACG GCA ATT CCG GAA TAC CGC TAA *TGA ACC GAC AAT CTA GGC TTG T* - 3', where the "m" indicates 2'-O-methylated residues. If the length of the RNA is similar in size to the 57-nt hammerhead RNA, the bands can overlap during separation *via* denaturing urea-PAGE, especially if the transcription yields are high. To improve separation, a stem-loop (*e.g.*, 5'-CAT GTG GAA ACA CAT GAA or 5'-CAT GTG TGT GTG GAA ACA CAC ACA CAT GAA - 3') may be inserted prior to the 11-nt complementary sequence in the hammerhead ribozyme to increase its length without disrupting cleavage. Note that if a hammerhead ribozyme is not used during transcription, the 5' triphosphorylated end must be dephosphorylated and re-phosphorylated (or converted into a monophosphate through the use of RNA 5'-pyrophosphohydrolase (Deana, Celesnik, & Belasco, 2008), available through NEB) prior to use and purified, and the ligation yields may be lower due to heterogeneity at the 5' end of the RNA.

2.3 Design of Complementary DNA and Tether DNA

The 3' end of the biotinylated DNA for tethering the complex to the slide may also be an ideal location for introducing a second dye (Cy5). This strategy (Fig. 1) is similar to placing a dye at the 3' of the RNA without having to perform an additional labeling reaction or ligation, and it also allows for the RNA sequence to be changed relatively easily while the biotinylated, Cy5-labeled DNA tether can be kept constant. For example, a series of experiments with a riboswitch would compare the RNA with and without the terminator hairpin, or with mutations to change the terminator stability or ligand binding site. Using the design above, only the transcribed RNA 3' fragment, reverse primer for amplifying the template to make that RNA, and unlabeled T20-DNA need to be synthesized to perform an additional series of experiments.

If folding brings the 5' and 3' ends of the RNA close together, a donor dye at the 5' end and an acceptor dye on the tether DNA will be brought in close proximity in the folded state. The resulting FRET efficiency for the folded state may approach 1 (ref. (Hua et al., 2018) and Hua, B., Jones, C.P., Ferré-D'Amaré, A.R., and Ha, T., unpublished observations). This could be a drawback if additional conformational changes occurring in the middle of the RNA are not observed because they do not disrupt pairing of the 5' and 3' ends.

For experiments exploiting multiple Cy3 PIFE peaks to measure helicase velocity, it is necessary to introduce more than one Cy3 dye into a single RNA prior to duplex annealing. This experiment takes advantage of the directional movement of the helicase across a Cy3 dye, which causes an increase in Cy3 fluorescence as the helicase approaches the dye during unwinding and a decrease in fluorescence as the helicase moves away (Hwang & Myong, 2014). The resulting peak can be used to indicate a location of the helicase on the template, and the observation of two peaks separated in time can be used to estimate the helicase unwinding rate under desired reaction conditions. Ligations involving more than two pieces of RNA can be performed simultaneously using the same protocols below although yields typically decrease as more fragments are introduced.

3. PREPARATION OF BIOTINYLATED, FLUORESCENTLY LABELED RNA/DNA HYBRID DUPLEXES

3.1 Equipment

Bio-Rad C1000 Thermal Cycler or equivalent device

Table-top centrifuges for microcentrifuge and PCR tubes

Typhoon Trio Phosphorimager (GE Healthcare) or equivalent fluorescent gel imaging system Slab gel electrophoresis apparatus, glass plates (16 cm wide, 25 cm long), 0.8 mm and 3 mm spacers, power supply

NanoDrop or equivalent UV-Vis spectrophotometer

Sterile razor blades

Whatman Elutrap electroelution system

Amicon Ultra centrifugal filters, regenerated cellulose (3,000 & 10,000 Da molecular weight cutoff), 0.5 mL (Millipore)

Amicon Ultrafree centrifugal filters, PVDF 0.22 μ m, 0.5 mL (Millipore)

Total internal reflection fluorescence microscope, equipped with a 532-nm laser and a 633-nm laser, as described in (Hua et al., 2014)

3.2 Reagents

T4 polynucleotide kinase (NEB), enzyme and buffers

T4 RNA Ligase 2 (NEB), enzyme and buffers

Hepes (Fisher), as a 0.5 M stock, adjusted to pH 7.4 with potassium hydroxide, sterile filtered Potassium chloride (Fisher), as a 1.5 M stock, sterile filtered

RepX helicase (e.g., 5 μ M stock), prepared as described previously (Arslan et al., 2015) Biotinylated bovine serum albumin (BSA, Sigma)

Tween-20 (Sigma)

Neutravidin (Fisher)

Trolox (Sigma)

Glucose (Sigma)

Glucose oxidase (Sigma)

Catalase (Sigma)

Quartz slides coated with dichlorodimethylsilane-Tween-20 (Hua et al., 2014) or polyethylene glycol-Tween-20 (Ha et al., 2002)

Custom fluorescently labeled and biotinylated oligonucleotides (e.g., Trilink)

Unlabeled RNA oligonucleotides for ligation (~2.5 nmol or ~0.1 mg, Integrated DNA Technologies)

Phenol, buffered to pH 8 (Sigma)

Chloroform (Fisher)

Sodium acetate (Fisher), as a 3 M stock, pH 5.1

100% ethanol, ice cold

70% v/v ethanol in RNase-free water, ice cold

Urea (Fisher)

40% 19:1 Acrylamide:bis-acrylamide (Fisher)

10X TBE buffer (0.9 M Tris base, 0.9 mM Boric acid, 20 mM ethylenediaminetetraacetic acid, pH 8.0)

30% w/v ammonium persulfate (APS) (Fisher)

Tetramethylethylenediamine (TEMED) (Fisher)

SYBR Gold Nucleic Acid Gel Stain (Fisher), ethidium bromide, or equivalent nucleic acid stain

Ultrapure RNase-free water

4. PROTOCOLS

4.1 Ligation and Purification of Fluorescently Labeled RNA

1. All oligonucleotides should be resuspended in RNase-free water (e.g., at a concentration of 200 μ M and dilutions thereof) and checked for purity prior to ligation. For a two-piece ligation, this includes a RNA 5' fragment, a RNA 3' fragment, and a DNA splint complementary to both fragments (Fig. 1). As the ligated products will be separated from the starting materials and the DNA splint by denaturing PAGE, the splint should ideally be at least 10 nucleotides shorter or longer than the ligated RNA product. Purity at each step is extremely important, as undesired products can introduce heterogeneity later during annealing steps. Work should be performed in the dark when using fluorescently-labeled RNAs, which should be kept on ice unless otherwise noted.
2. Although RNA ligations often succeed at 1:1:1 mixtures of the three components, optimization is sometimes required. For pilot experiments to optimize annealing with the DNA splint and ligation of the two RNA fragments, set up reactions consisting of 1:2:1, 1:1:1, 1:1:2, and 2:1:1 ratios of RNA 5' fragment to RNA 3' fragment to DNA splint. In addition, set up control 1:1:1 reactions in which one or two of each of the three reagents are excluded. If the ligation is to be performed many times with different dye locations or mutated sequences, it may be beneficial to optimize the ligation first with unlabeled RNAs before proceeding with fluorescently labeled RNA. Omission of buffer and salt (50 mM Hepes, pH 7.4, and 150 mM KCl) diminished the efficiency of annealing (data not shown). While the ligation can also be performed with T4 DNA Ligase, inclusion of fluorescent labels near the ligation site greatly

decreases ligation yields using this enzyme in our hands. For ligations involving more than two fragments of RNA, the ratio of T20-DNA:RNA with all RNA pieces in an equimolar ratio can be optimized first. A sample analytical scale protocol for a 1:1:1 mixture is given in the next step.

3. Mix the following components by pipetting and centrifuge briefly.
 - 1 μ L of 10 μ M RNA 5' fragment (Cy3-labeled)
 - 1 μ L of 10 μ M RNA 3' fragment
 - 1 μ L of 10 μ M DNA splint
 - 2 μ L of 0.5 M Hepes-KOH, pH 7.4
 - 2 μ L of 1.5 M KCl
 - 13 μ L of RNase-free water
4. Anneal the mixture in a thermocycler by heating to 95 °C for 3 min and slowly cooling to 20 °C by decreasing the temperature 5 °C every 30 s. Save 2 μ L of the annealed mixture for non-denaturing PAGE analysis. Lower annealing temperatures may be used for ligating shorter RNAs.
5. Add 5 μ L of 10X T4 RNA Ligase 2 reaction buffer (containing ATP and MgCl₂) and bring the volume up to 50 μ L with RNase-free water. Add 1 μ L of T4 RNA Ligase enzyme, mix gently by pipetting, and centrifuge briefly. Incubate for 1 hr at 37 °C. Save an aliquot for denaturing PAGE analysis.
6. Check the success of the annealing step by running 2 μ L aliquots on an 8% polyacrylamide 1X TBE non-denaturing gel. Use a non-denaturing loading dye (25% glycerol and the refolding buffer with 0.001% w/v xylene cyanol, 0.001% w/v bromophenol blue). Image the gel with the fluorescence imager. The gel may also be stained with SYBR gold or ethidium bromide to identify unlabeled RNA fragments. If annealing is poor, the ligation is unlikely to succeed.
7. Check the success of the ligation reaction by running 2 μ L aliquots on an 8% polyacrylamide 8 M Urea 1X TBE denaturing gel. Use a denaturing loading dye (i.e., formamide with 0.001% w/v xylene cyanol, 0.001% w/v bromophenol blue) and heat samples at 95 °C for 5 min prior to loading. Image the gel with the fluorescence imager as before. Save successful ligations for purification of the ligated product.
8. Once an acceptable ligation yield has been achieved in the pilot reactions, the reactions may be scaled up 30 times and combined with successful small scale reactions for purification of the desired ligated product.
9. To purify the ligated RNA, prepare a preparative 8% polyacrylamide gel containing 8 M Urea and 1X TBE (3 mm spacers). Omit bromophenol blue or xylene cyanol from the loading mix if the dyes co-migrate with the RNA. Heat samples at 95 °C for 5 min prior to loading to completely denature the ligation complexes. Perform all steps in the dark to avoid photobleaching fluorophores.

At a 1.5 nmol ligation scale, the bands corresponding to unligated Cy3-labeled RNA and ligated Cy3-labeled RNA should be clearly visible by eye without UV shadowing (Fig. 2A). The gel should be run to maximize separation of the DNA splint and ligated fluorescently labeled RNA, allowing unreacted labeled RNA to run off the gel if necessary.

10. Cut out the ligated RNA product band (i.e., pink for Cy3) with a sterile razor blade. Elute the RNA from the gel slice overnight at 120 V in the dark in RNase-free 1X TBE buffer using an Elutrap. Note that contamination of the Elutrap with RNase is disastrous. Wash the Elutrap instruments thoroughly with RNase-free water before and after use, and store the Elutrap cells wrapped in Saran wrap when not in use, and use designated RNase-free tweezers for manipulating Elutrap cells and membranes. Never touch the inside surfaces of the Elutrap cells, breathe on the cells, or leave the cells in open air to collect dust for long. If contamination has occurred, extensive washing should be performed to remove RNase. The 1X TBE running buffer used for elution should be RNase-free inside the elution chamber. An intensely colored dot of fluorescently labeled RNA should appear in the elution chamber when transfer is complete (Fig. 2B).
11. Concentrate the labeled RNA in a centrifugal concentrator, wash several times with RNase-free water, filter with a 0.22 μm centrifugal filter, and measure the absorbance at both 260 nm and the dye absorption wavelength. Calculate the concentration of the labeled species using both the dye and RNA extinction coefficients. Concentrations can be calculated using the Beer-Lambert Law ($A = \epsilon cl$, where A is the absorbance, ϵ is the extinction coefficient at that wavelength, c is the concentration, and l is the cuvette path length). If the dye also absorbs at 260 nm, which is the case for Cy3, absorption of free dye can be measured to determine the correction value (b), which is the absorption at 260 nm (A_{260}) divided by the absorption at the fluorescence absorption wavelength (A_{fl}). The corrected RNA concentration at 260 nm can then be calculated by subtracting the contribution of the dye absorbance at 260 nm from the RNA absorbance at 260 nm ($A - bA_{fl} = \epsilon cl$). For longer RNAs, this correction may be small and within error of determining the RNA concentration. If the RNA concentration calculated from the absorbance at 260 nm is significantly higher than the concentration calculated from the absorbance of the dye, contaminating DNA is likely present, or the dye labeling efficiency is poor. For RNAs containing more than one dye, the dye concentration should reflect the number of dye molecules per strand.
12. Assess the purity of the fluorescently labeled RNA by analyzing a small aliquot by denaturing PAGE. Image the gel for fluorescence and again after staining with a sensitive nucleic acid stain (SYBR Gold) to visualize both fluorescently labeled and unlabeled species (Fig. 2C). The purity of the ligated RNA at this stage is extremely important for interpreting future pilot annealing reactions below. If more than one band is present, then the annealing mixture prepared below will be significantly more complicated.
13. Aliquot ligated RNA and store at $-20\text{ }^{\circ}\text{C}$ in the dark.

4.2 Duplex Annealing for Single Molecule FRET

1. Single-molecule FRET experiments are typically conducted at final concentrations of ~80 pM labeled duplex, so the reaction scales below will produce enough sample for many experiments. The annealing reaction for single molecule FRET involves three oligonucleotides—the labeled RNA purified above, a biotinylated DNA tether for attachment to the FRET surface, and a T20-DNA (Fig. 1A). The latter is complementary to the riboswitch RNA and has 20 T residues added to the 3' end for helicase binding prior to unwinding.
2. Anneal the complex by combining 1:0.9:10 ratio of RNA to biotinylated DNA to T20-DNA. Set up control reactions in which one or two reagents have been excluded, as well as controls containing only one reagent. To optimize annealing, it may necessary to titrate the ratio of T20-DNA. In the case of the ZTP riboswitch RNA, the primary source of heterogeneity is from the RNA, which dimerizes (Jones & Ferré-D'Amaré, 2015). Excess T20-DNA will be washed from the streptavidin-coated slide surface prior to initiating unwinding by adding ATP. An example recipe for annealing the complex is:
 - 1 µL of 2.5 µM ligated Cy3-labeled RNA
 - 0.9 µL of 2.5 µM Cy5-labeled biotinylated DNA
 - 1 µL of 25 µM T20-DNA
 - 2 µL of 0.5 M Hepes-KOH, pH 7.4
 - 2 µL of 1.5 M KCl
 - 13.1 µL of RNase-free water
3. Mix the reaction by pipetting and centrifuge briefly. In a thermocycler, heat the mixture at 95 °C for 3 min and slowly cool to 20 °C (i.e., decreasing temperature by 5 °C every 30 s).
4. Run an aliquot of the reaction and controls on an 8% polyacrylamide 1X TBE nondenaturing gel to check for annealing (Fig. 3). Fluorescence imaging should be performed with both dye channels to check if the Cy3 and Cy5 signals overlap as expected. Annealing with T20-DNA should shift the mobility of the RNA upward. An additional smaller upshift should be observed upon annealing with biotin-DNA. The sub-stoichiometric ratio of biotinylated DNA to RNA will result in two bands in the lane corresponding to the complexes with and without biotinylated DNA, one of which will not stick to the slide surface as it lacks a biotin. This band should be identified by its co-migration with the T20-DNA:RNA control annealing reaction lacking biotinylated DNA.
5. The control annealing mixture of RNA:biotin-DNA without T20-DNA should be saved for use as a control for FRET experiments described below as it represents the final equilibrium FRET state after unwinding (Fig. 1).
6. Store aliquots at -20 °C until further use.

4.3 Single Molecule Helicase Unwinding

1. From above, complexes now available for single molecule measurements include the T20-DNA:RNA-Cy3:biotin-DNA ternary complex and the RNA-Cy3:biotin-DNA control complex. Before conducting unwinding experiments, equilibrium measurements of the RNA-Cy3:biotin-DNA complex can be conducted to understand how the RNA folds in the absence of sequential folding.
2. A detailed protocol for the preparation of passivated quartz slides is available elsewhere (Hua et al., 2014). The slides used for these experiments have inlet and outlet holes at the ends, which can be attached to tubing and a syringe with epoxy (Roy et al., 2008) to allow for easy buffer washing and sample application. A single slide can contain multiple lanes made air-tight and separated from one another by double-sided tape. A ~1 cm piece of the wide end of a 200 μ L pipette tip (cut with a sterile razor blade) can be fixed to the slide with epoxy to create a small sample well of ~100 μ L around the slide inlet hole. Similarly, a ~1 cm piece of the small end of a 200 μ L pipette tip can be fixed to the outlet hole to create a port for syringe tubing. The buffer level in the sample well can be monitored as the buffer is drawn into the slide to avoid introducing air.
3. Wash the slide with 50 μ L of 0.2 g/L biotinylated BSA in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl (T50 buffer). Incubate for 5 min then wash out unbound BSA with T50 buffer.
4. Wash the slide with 0.25 g/L neutravidin in T50 buffer. Incubate for 5 min then wash out unbound neutravidin with T50 buffer.
5. Wash the slide with 60 pM fluorescently labeled complex freshly diluted in 50 mM HEPES-KOH, pH 7.4, 150 mM KCl, 10 mM MgCl₂ (RNA folding buffer), and incubate for 1 min. Wash out unbound nucleic acids with RNA folding buffer.
6. Check for the presence and density of the slide-bound complex by turning on lasers. Without imaging buffer, the dyes will photobleach rapidly. Turn off lasers, and move to a new region of the slide for subsequent data acquisition.
7. Wash the slide with 50 nM RepX in 10 mM Tris-HCl, pH 7.5, 0.3 M KCl, 10% v/v glycerol (RepX storage buffer). Incubate 1 min to allow RepX to bind. Wash out free RepX with RNA folding buffer.
8. Wash slide with RNA folding buffer containing 4 mM Trolox, 0.8% w/v glucose, 165 U/mL glucose oxidase, and 2170 U/mL catalase (imaging buffer). This buffer may be supplemented with suitable final reaction components (i.e., riboswitch ligand). Turn on lasers to check molecule density on the slide, laser brightness, and rate of dye photobleaching. Data may be collected at this point to observe the FRET values for intact complex molecules prior to unwinding. Based on the designs above (Fig. 1), the FRET efficiencies of the hybrid duplex should be ~0.

9. To initiate unwinding, draw imaging buffer containing 1 mM ATP into the slide, and wait 1 min for unwinding to complete. Turn on lasers and collect data at various regions of the slide until a satisfactory number of molecules have been imaged.
10. Alternatively, for real-time imaging of duplex unwinding, begin recording movies a few seconds before drawing ATP into the slide. As only a single region of the slide will be available for data collection, it is essential to find an optimally dense region of the slide to maximize the number of molecules observed. Unwinding will proceed rapidly after ATP addition, and traces should complete within ~10–20 seconds for the ~100-bp RNA/DNA hybrid duplexes described herein. Data collection should continue until the majority of molecules have photobleached to discriminate single molecules from multiple overlapping molecules.
11. Traces are recorded and analyzed using custom code as previously described (Hua et al., 2018), with intensity corrections performed by plotting uncorrected FRET efficiency against uncorrected stoichiometry (Hellenkamp et al., 2018). A fraction of molecules may remain in a no-FRET state throughout the measurement due to either the absence of RepX on the duplex or the reannealing of T20-DNA immediately after unwinding.

5. SUMMARY

These protocols describe how to produce fluorescently labeled substrates for performing helicase unwinding experiments, an orthogonal approach to probing sequential RNA folding as it might occur during transcription. In this experiment, synthesis by RNA polymerase has been replaced with helicase-facilitated vectorial liberation from a hybrid duplex consisting of a fluorescently labeled RNA and unlabeled T20-DNA. An advantage of this approach is the ease with which substrates can be prepared, the ability to study ligands that might otherwise modulate RNA polymerase activity, and the recreation of transcription-like dynamics in a systematic manner. The DNA strand can be easily changed to incorporate base modifications that stabilize or destabilize the duplex, creating a potential avenue for future exploration.

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Fig. 1.

Work-flow of duplex preparation. (A) An example helicase unwinding event displaces the T20-DNA strand (black) to allow for the RNA strand (gray and colored) to sequentially fold in a 5' to 3' direction. After complete unwinding, only the Cy3-labeled RNA strand and Cy5-labeled biotinylated DNA strand remain. RNA folding should be observed as a decrease in the distance between the Cy3 and Cy5 dyes, increasing FRET efficiency. For riboswitches, folded states may contain mutually exclusive paired regions (pink and green) observed by changes in FRET. (B) The RNA 3' fragment containing a 3' region complementary to the biotinylated DNA is *in vitro* transcribed and purified. After phosphorylation at the 5'-end and purification, the 3' fragment is ligated with T4 RNA Ligase 2 to the chemically synthesized Cy3-labeled 5' fragment via annealing to a DNA splint (black). After purification to remove the splint and unligated fragments, the ligated RNA is annealed to a T20-DNA (black) containing the dT20 helicase binding site at the 3'

end and a biotinylated, Cy5-labeled DNA tether for attachment to the streptavidin-coated slide surface.

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Fig. 2.

Preparation of ligated Cy3-labeled RNA. (A) Example 1.5 nmol scale ligation purification denaturing PAGE gel viewed by eye (left) and by UV shadowing (right). Note the pink band for Cy3-labeled RNA (arrowed) running above the DNA splint. Lanes 1 and 3 contain a 128-nt marker, and lanes 2 and 4 contain the ligation mixture. (B) An Elutrap cell collection chamber contains labeled RNA (arrowed) after elution. (C) Post-ligation 8% denaturing PAGE imaged for fluorescence prior to staining (lanes 1–3) and after staining with ethidium bromide (lanes 4–6). As lanes 1 and 2 contain no fluorescent marker, the two gel images were aligned by eye. Lanes 1 and 4 contain a 150-nt marker, lanes 2 and 5 contain a 76-nt marker, and lanes 3 and 6 contain the 129-nt ligated Cy3-labeled RNA.

Fig. 3.

Example duplex annealing gel. The same gel was imaged twice to detect Cy3 (lanes 1–6) and Cy5 (lanes 7–12). In this experiment, the RNA contains a Cy3 label, the biotinylated DNA contains a Cy5 label, and the T20-DNA also contains a Cy3 label, and the three were mixed in a 1:0.9:10 ratio. Lanes 1 and 7 contain all three oligonucleotides, lanes 2 and 8 contain RNA and biotinylated DNA, lanes 3 and 9 contain RNA and T20-DNA, lanes 4 and 10 contain biotinylated DNA and T20-DNA, lanes 5 and 11 contain RNA only, and lanes 6 and 12 contain T20-DNA only. Note that in this annealing reaction, a higher migrating species is present in the absence of biotinylated DNA, likely due to a greater than 1:1 interaction between the RNA and T20-DNA. Xylene cyanol dye in the nondenaturing gel loading buffer is also visible in lanes 7–12.