# Probing RNA Structural Dynamics and Function by Fluorescence Resonance Energy Transfer (FRET)

RNA is a ubiquitous biopolymer with complex functions in the maintenance, transfer, processing, and regulation of genetic information. Its secondary structure provides a comparably stable scaffold onto which long-range tertiary interactions are built. RNA folding pathways describe the possible trajectories by which an RNA molecule may acquire a functional tertiary structure. Often, biological function of RNA is mediated by cyclic switching between two or more (meta-)stable arrangements of tertiary structure. Fluorophore labeling of RNA offers a unique view into these folding and conformational switching events, since a fluorescence signal is sensitive to its molecular environment and can be continuously monitored in real time to produce kinetic rate information. Many of these general aspects are discussed in *UNIT 11.8*. The current chapter complements *UNIT 11.8* by focusing on the practical implications of using fluorescence resonance energy transfer (FRET) to probe RNA structural dynamics and function. FRET is a particularly powerful fluorescence technique since, in addition to kinetic data, it provides insights into the structural basis of a conformational rearrangement.

The current unit provides protocols that describe how to postsynthetically label RNA for FRET (see Basic Protocol 1) and how to acquire and analyze FRET data (see Basic Protocol 2). Support Protocols describe methods for deprotecting synthetic RNA (see Support Protocols 1 and 2) and for purifying RNA by gel electrophoresis and HPLC (see Support Protocols 3 and 4, respectively). Considerations for selecting appropriate RNA, fluorophores, and labeling strategies are discussed in detail in the Commentary (see Critical Parameters).

*NOTE*: As for any experiments with RNA, care must be taken to avoid introducing ribonucleases (RNases) into the samples. Since most techniques described here involve commercially available synthetic RNA, the major source of RNases is postsynthetic contamination. This can be avoided by wearing gloves to avoid skin contact when handling samples and solutions; by using nuclease-free sterilized pipet tips, sample tubes, and other disposable plasticware (stored in clean, autoclaved, dust-free containers); by preparing all solutions from the highest purity (e.g., molecular-biology grade) components in doubly deionized water (18 MΩ conductivity); and by sterile filtering (0.22 μm) or autoclaving all solutions. When these suggestions are followed, inactivation of RNases using DEPC is not necessary and not particularly recommended because contaminating decay products may lead to fungal growth.

## POSTSYNTHETIC LABELING OF AMINO- OR THIOL-MODIFIED RNA

*BASIC PROTOCOL 1*

To perform FRET experiments, the RNA sample must be labeled with both a donor and an acceptor fluorophore. This can be accomplished by a variety of strategies that are discussed in detail in the Commentary (see Critical Parameters). In short, fluorophores can be added either during oligoribonucleotide synthesis or by postsynthetic conjugation to a modified oligoribonucleotide. This protocol describes a method for postsynthetic labeling of a modified RNA.

RNA modified with primary or secondary amino and thiol functionalities can be conveniently labeled under mild conditions with succinimidyl ester and maleimide derivatives of a fluorophore, respectively, available from companies such as Molecular Probes,

**Contributed by Nils G. Walter**
*Current Protocols in Nucleic Acid Chemistry* (2002) 11.10.1-11.10.23

Sigma-Aldrich, or Amersham Pharmacia Biotech. Since RNA purified by reversed-phase HPLC contains trace amounts of triethylamine that hydrolyze succinimidyl esters and, to a lesser extent, maleimides in a catalytic fashion, the labeling protocol consists of two steps: (1) chloroform extraction and ethanol precipitation to remove triethylamine and (2) coupling of the fluorophore derivative to the amino- or thiol-modified RNA. Excess fluorophore is then removed by C8-reversed phase HPLC purification (see Support Protocol 4).

This method can be used for single or double labeling of an RNA. Only one fluorophore need be added, for instance, if the other fluorophore was added during synthesis or if the donor and acceptor are conjugated to opposite strands of the RNA (see Critical Parameters). If the RNA already contains a fluorophore, it should be protected from light at all times to prevent photobleaching. If both donor and acceptor are to be added postsynthetically to the same strand, the RNA must contain both an amino and a thiol modification. In this case, the thiol functionality should be reacted first, since maleimides provide for higher selectivity than succinimidyl esters.

### *Materials*

Gel- and HPLC-purified RNA sample with amino or thiol functionality (see Support Protocol 4)
Chloroform, buffered (see recipe)
3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)
100% and 80% (v/v) ethanol
Succinimidyl ester (for amino-modified RNA) or maleimide derivative (for thiol-modified RNA) of fluorophore of choice (e.g., Molecular Probes, Sigma-Aldrich, Amersham Pharmacia Biotech; for selection of fluorophores, see Critical Parameters)
Dimethylsulfoxide (DMSO), anhydrous (e.g., Fisher)
100 mM sodium tetraborate, pH 8.5 (for amino-modified RNA, see recipe) or 100 mM HEPES-KOH, pH 7.0 (for thiol-modified RNA, see recipe)
100 mM ATP or GTP (*APPENDIX 2A*; recipe for dNTPs)

Speedvac evaporator (e.g., Savant)
Aluminum foil
Tube shaker (e.g., Fisher)

### *Chloroform extract and precipitate RNA*

1. Bring the RNA sample to be labeled up to 100 µL with water.

   *This volume is large enough to handle easily and small enough to ensure a good precipitation yield. Up to 100 µg RNA can be labeled in one reaction using this protocol. It is wise to keep 50% of the original material for a second labeling in case of unexpected loss.*

2. Extract RNA solution with 1 vol buffered chloroform, microcentrifuge briefly at maximum speed to separate phases, and transfer the top (aqueous) phase to a fresh tube.

3. Add 1/10 vol (10 µL) 3 M sodium acetate, pH 5.2, and 2.5 vol (250 µL) 100% ethanol, vortex, and precipitate at −70°C for 1 hr.

4. Microcentrifuge 30 min at maximum speed, 4°C, to collect RNA. Decant supernatant, wash with 80% ethanol, decant supernatant, and dry RNA in a Speedvac evaporator. Cover the evaporator with aluminum foil if the RNA already contains a fluorophore.

***Label RNA***

5. Dissolve 200 µg fluorophore succinimidyl ester or maleimide in 14 µL DMSO. If both an amino- and a thiol-modification will be used to attach two fluorophores, perform the reaction at the thiol functionality first.

   *In many cases it is easier to dissolve a whole vial of the fluorophore derivative at once than to weigh out a small amount of fluorophore. In this case, dispense fluorophore solution in 14-µL aliquots and store up to 6 months at –20°C wrapped in aluminum foil. For long-term storage, dissolve fluorophore derivative in methanol or acetonitrile, dispense in 200-µg aliquots per tube, dry in a Speedvac evaporator, and store up to 2 years at –20°C wrapped in aluminum foil.*

6a. *For amino-modified RNA:* Dissolve RNA pellet from step 4 in 11 µL water, add 75 µL of 100 mM sodium tetraborate, pH 8.5, and transfer to the tube containing the fluorophore succinimidyl ester stock solution from step 5.

6b. *For thiol-modified RNA:* Dissolve RNA pellet from step 4 in 11 µL water, add 75 µL of 100 mM HEPES-KOH, pH 7.0, and transfer to the tube containing the fluorophore maleimide stock solution from step 5.

7. Vortex tube, wrap in aluminum foil, and tumble on a tube shaker overnight (16 to 20 hr) at room temperature.

   *The exact incubation time and temperature may have to be optimized for a given reaction. For example, sterically hindered secondary amines (such as a 2′-amino modification on a ribose) tend to give higher labeling yields when incubated overnight at 4°C.*

8. Add 1/10 volume (10 µL) 3 M sodium acetate, pH 5.2, 1/40 volume (2.5 µL) 100 mM ATP or GTP as carrier, and 2.5 volumes (250 µL) ethanol, vortex, and precipitate at –70°C for 1 hr.

9. Microcentrifuge 30 min at maximum speed, 4°C, to collect RNA. Decant supernatant, wash twice with 80% ethanol, decant supernatant, and dry RNA in a Speedvac evaporator covered with aluminum foil.

10. Resuspend dried RNA in 90 µL water and store in the dark at –20°C (stable at least 2 years). Before use for FRET (see Basic Protocol 2), remove excess fluorophore by C8 reversed-phase HPLC (see Support Protocol 4).

11. *Optional*: For attachment of a second fluorophore at the amino functionality, repeat steps 5 to 10.

## MILD DEPROTECTION OF STANDARD RNA OLIGONUCLEOTIDES WITH $NH_4OH$/ETHANOL AND TRIETHYLAMINE TRIHYDROFLUORIDE

***SUPPORT PROTOCOL 1***

This protocol describes a mild deprotection scheme for RNA oligonucleotides synthesized using standard β-cyanoethyl phosphoramidites. Synthesis is as described in *UNIT 3.5* and *APPENDIX 3C*; phosphoramidites can be purchased from companies such as Glen Research, ChemGenes, Amersham Pharmacia Biotech, BD Biosciences (Clontech), CPG, Cruachem, Dalton Chemical Laboratories, or PE Applied Biosystems. This scheme is compatible with fluorophores (particularly fluorescein and the cyanine dyes) or linker modifications incorporated during synthesis. It uses a 3:1 mixture of concentrated ammonium hydroxide and ethanol to cleave the RNA from the solid support, perform a β-elimination of the cyanoethyl phosphodiester-protecting group, and remove the exocyclic *N*-acyl base-protecting groups. A second deprotection with triethylamine trihydrofluoride removes the *tert*-butyldimethylsilyl group on the 2′-hydroxyl functionality (also see *UNIT 3.6*). Many commercially available RNAs have already been cleaved from the support and deprotected at the base and phosphodiester moiety. If this is the case, the desilylation reaction is all that needs to be performed. If a fluorophore was already

attached during synthesis, the RNA should be kept protected from light as much as possible to avoid photobleaching.

***Materials***

RNA oligonucleotide attached to solid synthesis support (1-µmol scale), made using standard β-cyanoethyl phosphoramidites (see above for suggested suppliers and *UNIT 3.5* for synthesis protocols)
29% (v/v) ammonium hydroxide (e.g., Fisher)
100% and 80% (v/v) ethanol (e.g., Fisher)
Triethylamine trihydrofluoride (Aldrich or Acros)
*N,N*-Dimethylformamide (e.g., Fisher; optional)
1-Butanol (e.g., Fisher)

1.7-mL screw-top tube (e.g., Eppendorf Safe-Twist)
Parafilm (e.g., Fisher)
Aluminum foil
Heating block (e.g., Fisher)
14-mL Falcon centrifuge tube (e.g., Fisher)
Speedvac evaporator (e.g., Savant)
Tube shaker (e.g., Fisher)

***Cleave from support and remove cyanoethyl and N-acyl protecting groups***

1. Transfer the dried solid support beads with the attached RNA oligonucleotide from the synthesis cartridge to a 1.7-mL Safe-Twist screw-top tube.

   *Most commercial suppliers of RNA provide material that already has undergone steps 1 through 6. In this case, proceed directly to desilylation in step 7.*

2. Add 750 µL of 29% ammonium hydroxide and 250 µL 100% ethanol to the tube, screw the cap on tightly, wrap the top with Parafilm, and place in a heating block for 4 hr at 65°C. If a fluorophore is already attached to the RNA, cover with aluminum foil to protect from light. For RNA containing cyanine dyes, an even milder incubation (20 hr at 25°C) is preferable.

   *Due to its high vapor pressure, the concentrated ammonium hydroxide solution is easiest to pipet when stored in the freezer at –20°C until use. This precaution also minimizes a gradual decrease over time in ammonia concentration due to degassing.*

3. Remove the tube from the heating block, place it on ice, and wait 10 min for it to cool down.

   *This procedure avoids loss of contents when opening the tube.*

4. Microcentrifuge the tube 30 sec at maximum speed to compact the solid support beads, then pipet the supernatant into a 14-mL Falcon tube.

5. Add 1 mL water to the beads, vortex thoroughly, microcentrifuge for 30 sec, and add the supernatant to the Falcon tube. Repeat this wash a second time and again add the supernatant to the Falcon tube.

6. Evaporate the combined supernatants in a Speedvac evaporator. Cover the evaporator with aluminum foil if the RNA contains a fluorophore.

   *To avoid subsequent solubility problems, be careful not to overdry the RNA.*

***Remove tert-butyldimethylsilyl protecting group***

7. Add 800 µL triethylamine trihydrofluoride to the Falcon tube, wrap the top with Parafilm, and tumble on a tube shaker overnight (16 to 20 hr) at room temperature. Wrap in aluminum foil if the RNA contains a fluorophore.

   *To increase the solubility of long RNA (>30 nt), 25% dimethylformamide may be added to the reaction.*

8. Quench the desilylation reaction by adding 160 µL water to the Falcon tube.

9. Add 8 mL of 1-butanol and chill the solution at –20°C for 45 min.

10. Microcentrifuge 5 min at 3000 rpm, 4°C, and gently decant the 1-butanol from the precipitated RNA.

11. Wash RNA pellet with 80% ethanol, decant, and repeat wash. Evaporate remaining liquid from the Falcon tube in a Speedvac evaporator. Cover the evaporator with aluminum foil if the RNA contains a fluorophore.

    *Completely removing the 1-butanol by ethanol washes and evaporation is important to improve the separation during subsequent gel purification (see Support Protocol 3).*

    *If necessary, the dried RNA can be stored at –20°C until gel purification (stable at least 1 month). RNA recovery at this stage should be at least 50% of the total synthesis scale.*

## MILD DEPROTECTION OF 2′-ACE-PROTECTED RNA OLIGONUCLEOTIDES WITH ACETIC ACID

***SUPPORT PROTOCOL 2***

This protocol describes a specific deprotection scheme required for RNA oligonucleotides purchased from Dharmacon Research. Dharmacon uses a very different 5′-silyl-2′-orthoester protection chemistry, and typically supplies RNA after the support cleavage and base deprotection step. Mildly acidic conditions are then used to remove the 2′-orthoester protecting groups. Dharmacon provides a limited but increasing number of modifications (including 5′ fluorescein and cyanine dyes) that are all compatible with the deprotection protocol outlined below. Again, if a fluorophore was already attached during the synthesis, the RNA should be kept protected from light as much as possible to avoid photobleaching.

***Additional Materials*** *(see Support Protocol 1)*

RNA oligonucleotide made using 5′-silyl-2′-orthoester protection chemistry, already cleaved from solid support and base deprotected (Dharmacon Research)
100 mM acetic acid/TEMED, pH 3.8 (see recipe)

1. Briefly microcentrifuge the tube containing the RNA oligonucleotide to ensure that the RNA pellet is at the bottom.

2. Add 400 µL of 100 mM acetic acid/TEMED, pH 3.8, per 0.1 µmol synthesis material. Dissolve the pellet by pipetting up and down and vortexing. Centrifuge the solution to the bottom of the tube.

3. Wrap tube top with Parafilm and place the tube in a heating block for 30 min at 60°C. If a fluorophore is already attached to the RNA, cover tube with aluminum foil to protect from light.

4. Remove the tube from the heating block, place it on ice, and wait 10 min for it to cool down.

   *This procedure avoids loss of contents when opening the tube.*

5. Evaporate buffer in a Speedvac evaporator. Cover the evaporator with aluminum foil if the RNA contains a fluorophore.

*If necessary, the dried RNA can be stored at −20°C until gel purification (stable at least 1 month). RNA recovery at this stage should be at least 50% of the total synthesis scale.*

***SUPPORT PROTOCOL 3***

## GEL PURIFICATION OF RNA OLIGONUCLEOTIDES

Polyacrylamide gel electrophoresis (PAGE) is the most effective way of purifying a full-length synthetic RNA oligonucleotide from shorter synthesis byproducts. Since PAGE in general is described in *UNIT 10.4* and *APPENDIX 3B*, the following procedure focuses on a streamlined standard purification of a synthetic RNA oligonucleotide (up to 80 nt in length). The steps in preparative PAGE are: (1) preparation of the gel and gel apparatus, (2) electrophoretic separation, and (3) detection of the RNA by UV shadowing and elution from the gel.

*CAUTION*: Acrylamide is a known neurotoxin and should be handled with care. Wear safety goggles and gloves when handling solutions or a solidified gel. Work in a fume hood when weighing powdered acrylamide. To avoid the health risk from working with powdered acrylamide, it is advisable to purchase a premade acrylamide solution.

### *Materials*

Urea (e.g., Fisher)
38% (w/v) acrylamide/2% (w/v) bisacrylamide (e.g., Fisher; *UNIT 10.4*)
10× TBE electrophoresis buffer (*APPENDIX 2A*)
50% (w/v) APS (see recipe)
*N,N,N′,N′*-Tetramethylethylenediamine (TEMED; Fisher)
Deprotected RNA sample (see Support Protocol 1 or 2)
2× formamide loading buffer (see recipe)
Elution buffer (see recipe)
Chloroform, buffered (see recipe)
100 mM ATP or GTP (*APPENDIX 2A*; recipe for dNTPs)
100% and 80% (v/v) ethanol

Vertical slab gel electrophoresis apparatus (e.g., 20 × 16–cm system from CBS Scientific), including glass plates, 1-mm spacers, fitting seal, 1-mm one- or two-well comb, clamps, and aluminum plate
Power supply (e.g., Fisher)
60-mL syringe with bent 18-G needle (e.g., Fisher)
Heating compartment (e.g., Fisher) filled with copper shot (e.g., Fisher), *or* other temperature-controlled heating block, set at 95°C
Large-volume gel-loading pipet tips (e.g., Fisher)
Aluminum foil
Plastic wrap (e.g., Saran wrap)
20 × 20–cm TLC plate with fluorescent indicator (e.g., Fisher)
312- or 254-nm hand-held UV lamp (e.g., Fisher)
Empty Poly-Prep chromatography column (Bio-Rad)
Tube shaker (e.g., Fisher)
14-mL Falcon centrifuge tube (e.g., Fisher)
Speedvac evaporator (e.g., Savant)

***Prepare gel***

1. Assemble the gel plates, spacers, and seal following the manufacturer's instructions (see also *APPENDIX 3B*).
2. Combine in a beaker in the following order:

   24 g urea
   25 mL 38% acrylamide/2% bisacrylamide
   5 mL 10× TBE electrophoresis buffer
   5 mL water.

   Stir, heat in a microwave oven for 20 to 30 sec (*not longer*), and continue stirring until urea is dissolved.

   *This recipe produces a 20% acrylamide/8 M urea solution that should be prepared fresh for each gel. It can be scaled up for a larger gel or for several normal-sized gels. Degassing may be performed, if desired.*

   *If hexachlorofluorescein has been incorporated during RNA synthesis, urea should be left out of the gel mixture and 10 mL water added instead, as urea leads to loss of the chlorine substituents on the fluorophore.*

3. Make sure that acrylamide solution is stirring at room temperature. In quick succession add 35 µL of 50% APS and 35 µL TEMED to the solution and continue stirring for 10 sec.
4. Immediately pour gel solution between glass plates either from the beaker or using a syringe as described in *APPENDIX 3B*. With notched glass plate on top, hold plate sandwich at a 45° angle from the benchtop and slowly pour acrylamide solution between the plates down one side. Adjust angle of plates such that gel solution flows slowly and continuously without forming bubbles.
5. When solution reaches top of notched plate, lower gel sandwich to lie flat on an empty disposable pipet tip rack. Insert thin side of a 1-mm one- or two-well comb into the solution and slowly push in until it fits snugly with the notch of the upper glass plate. Be careful to avoid introducing bubbles. Let sit for 1 hr to polymerize.

   *For a typical 1-µmol standard RNA synthesis (see Support Protocol 1) or a 0.2-µmol synthesis from Dharmacon (see Support Protocol 2), one well of a two-well comb is sufficient for good separation. If more material is expected, use a one-well comb instead.*

   *Before continuing, make sure that the remaining acrylamide solution in the beaker, tilted for easier subsequent removal of polyacrylamide, has indeed polymerized.*

***Set up electrophoresis apparatus***

6. Once acrylamide is polymerized, remove seal or bottom spacer of gel sandwich. Thoroughly clean outside of glass plates under running tap water to remove any polyacrylamide and urea residue. Remove comb gently without tearing top of gel.
7. Place gel sandwich in electrophoresis apparatus and clamp plates to upper reservoir. Clamp aluminum plate for heat distribution on front plate.
8. Tilt gel apparatus and fill bottom reservoir with 1× TBE buffer. Adjust tilt of gel apparatus so that no buffer is spilled while air is displaced from bottom of gel. Fill lower reservoir so that gel plates are submerged 2 to 3 cm. Remove any air bubbles at bottom of gel by squirting buffer between plates from one side using a 60-mL syringe with a bent 18-G needle.
9. Fill upper reservoir of gel apparatus with 1× TBE buffer to ~1 cm from rim.
10. Prerun gel ~15 to 30 min at 25 W constant power.

    *In general, gels should be electrophoresed at ~40 V/cm.*

***Load and run gel***

11. Turn off power. Rinse well(s) with 1× TBE buffer just prior to loading gel to remove urea that has leached into the well. Use the same 60-mL syringe and bent 18-G needle used to remove bubbles from bottom of gel.

12. Dissolve the dried, deprotected RNA sample in 200 µL water and add an equal amount of 2× formamide loading buffer. Heat 1 min at 95°C, place in an ice water bath, and let chill.

13. Load whole sample in a single well using a gel-loading tip. Use a fresh tip for each sample to avoid cross-contamination.

    *If two samples are run on a two-well gel, mark them by placing labeled sticky tape on the outside glass plate. Fold an edge of the tape over so that it can be removed easily.*

14. Fill upper reservoir of gel apparatus with 1× TBE buffer to ~1 cm from rim. If the RNA contains a fluorophore, cover gel apparatus with aluminum foil to protect the fluorophore from light.

    CAUTION: *Obviously, the aluminum foil must not contact the electrophoresis buffer; modern buffer reservoirs have covers to prevent that.*

15. Run gel at 25 W constant power until bromphenol blue has reached an appropriate distance (typically ~1 to 3 cm) from the bottom of the gel (~90 to 120 min).

    *Bromphenol blue co-migrates with ~8-mer RNA.*

***Visualize RNA by UV shadowing***

16. Turn off power. Disassemble the gel apparatus and carefully open the gel sandwich from an unnotched corner using a spatula. Place gel on one end of a piece of plastic wrap and wrap the gel completely.

    *If labeled sticky tape is used to mark the sample(s), transfer it onto the plastic wrap.*

17. Wrap a 20 × 20–cm TLC plate with fluorescent indicator in plastic wrap. Place the wrapped gel on top of the TLC plate and visualize the RNA oligonucleotide under a 312-nm UV lamp held directly over the TLC plate.

    *The UV-absorbing RNA blocks excitation and thus fluorescence emission of the indicator and appears as a shadow.*

    *If sensitivity is not high enough, switch to a 254-nm UV lamp; minimize exposure to short-wavelength UV light to avoid photo-induced cross-linking. When RNA contains a fluorophore, its absorbance (color) and fluorescence emission will indicate the location of labeled RNA.*

18. Mark desired RNA band on the plastic wrap with a permanent marker. Remove from under the UV lamp.

    *Normally, the slowest migrating band will be the most prevalent main product. Sometimes, however, there are branched oligonucleotides present that migrate even more slowly. Depending on the coupling efficiency of the modifications introduced, there may be exceptionally strong bands that migrate faster than the main product.*

***Elute RNA from gel***

19. Slice the gel on the perimeter of the product band with a clean razor blade. Cut into ~2 × 2–mm pieces.
20. Transfer the excised gel fragments into an empty Poly-Prep chromatography column, add 4 mL elution buffer, and close tightly. If a fluorophore is already attached to the RNA, wrap column in aluminum foil.

    *If labeled sticky tape was used to mark the sample(s), transfer it onto the column.*

21. Tumble on a tube shaker overnight (16 to 20 hr) at 4°C.
22. Invert the Poly-Prep column, break off the bottom seal, uncap, and transfer the elution buffer into a 14-mL Falcon tube by gravity flow.

    *If yield is critical, a second gel elution typically yields an additional ~20% of the first elution.*

23. Extract SDS from the elution buffer by thoroughly vortexing with an equal volume of buffered chloroform and centrifuge 10 min at 13,000 × *g*, 4°C, to separate the phases. Transfer the top (aqueous) phase to a fresh Falcon tube.

    *If a pronounced interphase is carried over, a second chloroform extraction may be used to minimize it.*

24. Add 100 mM ATP or GTP to a final concentration of 1 mM, then add 2 to 2.5 vol of 100% ethanol, and precipitate RNA overnight at –20°C or 2 hr at −70°C.

    *Using ATP or GTP as a carrier increases the precipitation yield.*

25. Centrifuge 30 min at 13,000 × *g*, 4°C, to collect RNA precipitate. Decant supernatant, wash with 80% ethanol, decant supernatant, and dry RNA in a Speedvac evaporator covered with aluminum foil.

    *Dried RNA can be stored in a freezer at –20°C until HPLC purification (stable at least 1 month). Typical yields at this point are 20 to 100 nmol RNA from a 1-µmol scale synthesis.*

    *Save all supernatants until you know that RNA has been recovered.*

26. Resuspend dried RNA in 90 µL water for subsequent C8 reversed-phase HPLC (see Support Protocol 4).

## C8 REVERSED-PHASE PURIFICATION OF RNA OLIGONUCLEOTIDES

***SUPPORT PROTOCOL 4***

C8 reserved-phase HPLC is an efficient way to separate the desired RNA from not fully deprotected material and small organic molecule contaminants from previous steps. Reversed-phase HPLC of synthetic nucleic acids is described in general in *UNIT 10.5*. This protocol therefore focuses on the specific parameters optimized and streamlined for modified RNA.

***Materials***

Gel-purified RNA sample (see Support Protocol 3)
100 mM TEAA buffer, pH 7 (see recipe)
Acetonitrile (see recipe)

Centrifugal filtration unit (0.45-µm; Amicon)
HPLC system (*UNIT 10.5*) with 4.6 × 250–mm Microsorb 100 C8 analytical column (5-µm particle size; Varian) and optional guard column
Speedvac evaporator (e.g., Savant)
Aluminum foil
Spectrophotometer (220 to 800 nm)

Additional reagents and equipment for reversed-phase HPLC (*UNIT 10.5*)

**Table 11.10.1** Common Mobile Phase Gradients for Reversed-Phase HPLC Purification of RNA[a]

| Sample | Elapsed time (min) | % Mobile phase B at elapsed time |
|---|---|---|
| RNA without fluorophores | 0 | 0 |
| | 24 | 20 |
| | 34 | 40 |
| RNA with fluorophores | 0 | 0 |
| | 50 | 60 |
| RNA with Cy5 and Cy3 | 0 | 0 |
| | 20 | 20 |
| | 60 | 40 |
| | 70 | 60 |

[a]Gradient conditions are based on a flow rate of 1 mL/min using a 4.6 × 250–mm Microsorb 100 C8 column (5-µm particle size; Varian) at ambient temperature, and should be similar on other C8 reversed-phase columns.

1. For an analytical run, dilute 5 µL gel-purified RNA in 50 µL of 100 mM TEAA buffer, pH 7. For a preparative run, use 50% of the gel-purified RNA sample directly. Remove any particles with a centrifugal filtration unit.

   *It is always wise to first perform an analytical run to determine the elution volume of a newly synthesized RNA so that the fraction collector can be properly programmed for the preparative run. Keeping 50% of the RNA sample for a second preparative run is advised in case of unexpected loss.*

2. Start and equilibrate the HPLC system with 100% mobile phase A (100 mM TEAA buffer) according to manufacturer's instructions. Program the gradient system (see step 4) and fraction collector (see step 5).

   *A guard column may be used to prolong the life of the separation column.*

3. Inject the RNA sample, making sure that the sample loop volume is sufficiently large (typically 100 µL).

4. Increase the percentage of mobile phase B (acetonitrile) with time according to one of the gradients listed in Table 11.10.1, depending on the particular sample.

   *Eluted RNA is detected by UV absorbance at 254 nm. Using the gradients listed in Table 11.10.1 on a Microsorb C8 column leads to typical elution volumes between 12 and 18 min at a flow rate of 1 mL/min. If ATP or GTP was used as carrier for the ethanol precipitation, an additional peak is expected at ~10 min.*

   *An attached fluorophore considerably retards elution of an RNA (by several min) compared to the unlabeled control. If RNA is purified after a fluorophore labeling reaction, additional peaks at high elution times (typically >18 min) will originate from excess free fluorophore.*

5. Collect peak fractions and dry in a Speedvac evaporator. Cover the evaporator with aluminum foil if the RNA contains a fluorophore.

   *At a flow rate of 1 mL/min, three to four fractions should be collected per min.*

6. Dissolve each fraction in a suitable volume of water (20 to 200 µL) and combine all fractions that originate from the same HPLC elution peak.

7. Obtain a UV absorption spectrum of a 1:100 (v/v) diluted sample from 220 to 800 nm and calculate concentration from the peak absorption at 260 nm (1 A260 unit = 0.037 mg/mL RNA).

   *Typical yields are 10% to 50% of the starting material.*

   *Alternatively, the molar extinction coefficient at 260 nm ($\varepsilon_{260}$) of an RNA of given sequence can be estimated as the sum of extinction coefficients of the composing nucleotides, where $\varepsilon_{260}$ (U) = 9,900 L mol$^{-1}$ cm$^{-1}$, $\varepsilon_{260}$ (A) = 15,200 L mol$^{-1}$ cm$^{-1}$, $\varepsilon_{260}$ (C) = 7,050 L mol$^{-1}$ cm$^{-1}$, and $\varepsilon_{260}$ (G) = 12,010 L mol$^{-1}$ cm$^{-1}$.*

   *To calculate the concentration of a fluorophore-containing RNA, the additional absorbance of the fluorophore(s) at 260 nm should be subtracted. For fluorescein, $A_{260}/A_{492}$ = 0.3; for hexachlorofluorescein, $A_{260}/A_{535}$ = 0.3; for tetramethylrhodamine, $A_{260}/A_{554}$ = 0.49.*

8. Store purified RNA (stable at least 2 years) at –20°C. If a fluorophore is already attached to the RNA, wrap tube in aluminum foil to protect from light.

## DATA ACQUISITION AND ANALYSIS FOR STEADY-STATE FRET

***BASIC PROTOCOL 2***

Successful application of fluorescence methods in general hinges on paying close attention to experimental detail. There are numerous artifacts that can distort results and distract from obtaining meaningful results. Erroneous signals may arise from background fluorescence of solvents, stray light through the fluorometer, Raleigh and Raman scatter, higher-order light diffraction by the monochromator, and others. For more information on the origin of these artifacts, see Lakowicz (1999). In order to avoid these potential pitfalls, it is important to prepare samples and buffer solutions carefully, implement proper measurement and sample controls, and analyze data thoughtfully and on a case-by-case basis for each new RNA system. The following protocol describes generalized guidelines for: (1) preparation of samples and buffers, (2) getting started (the first steady-state fluorescence experiments), and (3) steady-state FRET data analysis.

To observe a conformational change by steady-state FRET, the system needs to be perturbed in a way that results in a distance change between the fluorophore pair. Steps 4 through 10 describe how this can be accomplished. Steady-state FRET refers to a continuous excitation of the donor as well as continuous recording of the donor and acceptor fluorescence signals. Such measurements can be done with widely available equipment to yield kinetic rate information, and should be the first experiments to be performed on a new RNA system. For analysis strategies in more sophisticated applications such as nanosecond time-resolved FRET for measurements of fluorophore distance distributions, please see Klostermeier and Millar (2001b).

### *Materials*

Fluorophore-labeled RNA sample of choice (see Basic Protocol 1)
Buffer of choice
Argon gas (optional)
Contrad 70 detergent (e.g., Fisher)

Water or oil pump connected to side-arm Erlenmeyer flask (optional)
Luer-tip syringe (e.g., Fisher)
Centrifugal filtration unit (0.45-µm; Amicon)
Quartz microcuvette (fill volume 120 to 150 µL; e.g., Starna)
Research-type spectrofluorometer (e.g., Thermo Spectronic AMINCO-Bowman Series 2 or equivalent instrument from Jobin Yvon, Hitachi, or others), ideally with temperature control and stopped-flow equipment for fast kinetics
Large-volume gel-loading tips (e.g., Fisher)

*NOTE*: If a circulating water bath is used with the spectrofluorometer, the temperature difference between the bath and the cuvette content should be calibrated.

***Prepare samples and buffers***

1. Use only highly purified RNA(s) (see Support Protocols 3 and 4) with fluorophores of choice attached (see Basic Protocol 1 and Commentary).
2. Choose the buffer of interest for the particular experiment. Prepare buffers using only high-quality nonfluorescent components.

   *Most buffer components will be compatible with fluorescence experiments. Exceptions include (1) high concentrations of transition metal ions, iodide, and some aromatic compounds that quench fluorophores; (2) components that themselves are fluorescent; or (3) components that make the solution very viscous or turbid. High viscosity complicates mixing and lowers fluorophore mobility, thus increasing fluorescence anisotropy. High turbidity leads to strong light scattering.*

3. Carefully remove oxygen from all buffer stock solutions used to make up the final sample by one or several of the following methods:

   a. Loosen container cap and heat solution in a microwave oven until close to boiling. Tighten cap, shake, loosen cap to release pressure, and repeat until no more bubbles emanate from the solution.

   b. Degas solution under partial vacuum by applying a water or oil pump to a side-arm Erlenmeyer flask that contains the solution in a bottle or tube with loose cap.

   c. For smaller volumes (e.g., up to 30 mL), load a luer-tip syringe with the solution, place Parafilm over the tip, and pull the plunger, allowing released air bubbles to move to the syringe tip. Remove the Parafilm and release all of the air. Repeat two to three times until no more air bubbles form.

   d. Bubble clean argon gas from a gas cylinder through the solution for 30 min.

***Get started: perform the first steady-state fluorescence experiments***

4. Anneal the fluorophore-labeled RNA in 145 µL buffer of choice by heating to 70°C for 2 min and cooling to room temperature over 5 min.

   *Fluorophore concentrations of 20 to 50 nM typically give a good signal-to-noise ratio. If separate RNA strands are annealed, unlabeled strands should be kept at an excess sufficient to saturate the labeled one. If donor and acceptor are coupled to two different segments of an RNA, the complex should be purified from any excess of the individual strands (see Commentary).*

   *Most standard fluorophores tolerate denaturation temperatures of 70°C. To anneal highly base-paired RNAs, higher temperatures may be necessary.*

5. Centrifuge filter to remove all particles that may scatter light.
6. Clean a quartz microcuvette with 5% (v/v) Contrad 70 or other detergent and rinse thoroughly with clean, autoclaved RNase-free water. Transfer RNA solution into the cuvette. Equilibrate at temperature of choice for 15 min.
7. Measure emission and excitation spectra of the sample. For the emission spectrum, excite the donor at its peak absorbance and scan emission from 10 to 150 or 200 nm above this wavelength. For the excitation spectrum, detect at the acceptor peak emission and scan the excitation from 150 or 200 to 10 nm below this wavelength. Make sure that emission and excitation peaks for the specific fluorophore pair appear at the expected wavelengths.

   *If the signal is too weak, adjust excitation and emission slit widths and signal amplification; if this is insufficient, raise the RNA concentration.*

*If a strong signal is observed where it is not expected, make sure that it is not due to stray light (from light leaks in the fluorometer), Raleigh scattering (from a turbid solution), Raman scattering (will also be observed in a fluorophore-free control), or a second-order light diffraction in the monochromator (will have double the excitation wavelength). If a photomultiplier tube is used for detection, make sure it is not overloaded and damaged. If effects from the dependence of the monochromator transmission efficiency on sample polarization are to be avoided, e.g., for accurate donor-acceptor distance measurements, magic angle polarizer conditions need to be chosen at the expense of emission signal (Lakowicz, 1999).*

8. Excite the donor at its excitation peak wavelength, and record its fluorescence trace at the emission peak wavelength over a sufficient time course (initially this may be 10 to 30 min at 1 datum per second). Ideally, record the acceptor signal simultaneously. Make sure that both signals are stable over time.

   *If the signals are not stable, make sure that the solution is not visibly changing (i.e., forming a precipitate, evaporating), or ensure that a dust particle is not drifting through the light path. If one or both signals slowly decrease, fluorophore photobleaching may be the cause. This can be tested by decreasing the excitation slit width, i.e., lowering the excitation intensity. If the signal decrease becomes slower or less pronounced, photobleaching is likely the cause. In many cases photobleaching can be reduced by using a lower excitation intensity, by more completely removing oxygen (see above), or by adding a radical quencher and singlet oxygen scavenger such as 25 mM dithiothreitol.*

9. Start a new time course, wait for 100 sec to ensure that the fluorescence signal is indeed stable, then close the emission shutter and access the sample to add 5 µL of an additive that perturbs the RNA system. To achieve fast manual mixing, use a small-volume pipet with a sequencing gel-loading tip to add the additive to the bottom of the cuvette and quickly pipet up and down twice with a large-volume (200-µL) pipet with gel-loading tip. Be sure not to place air bubbles in the cuvette. Close the fluorometer and open the emission shutter to continue recording.

   *Possible additives include an additional RNA strand that binds to the fluorophore-labeled RNA (e.g., a substrate binding to a catalytic RNA), $Mg^{2+}$ to assist RNA folding, or EDTA to chelate $Mg^{2+}$ already present. The concentration of the additive should be in saturating excess so that simplified (i.e., pseudo-first order) reaction kinetics can be expected.*

   *If the FRET change is too fast for manual mixing, employ stopped-flow fluorescence equipment. This consumes considerably larger quantities of material, due to increased requirements both in concentration and volume.*

10. Record the complete time course for both donor and acceptor emission and repeat experiment to acquire multiple data sets for calculating a standard deviation.

    *Since all RNA molecules are synchronized with respect to the time of disturbance, any change in fluorophore distance and FRET in response to mixing will be observed as an ensemble-averaged relaxation to the new equilibrium.*

### *Analyze steady-state FRET data*

11. A meaningful steady-state FRET change is characterized by donor and acceptor signal changes in opposite directions. For a simple way to analyze these changes, calculate the ratio of acceptor to donor fluorescence over time as a relative measure for FRET efficiency. Discard any traces that contain artifacts or are particularly different from the majority. To extract rate constants, follow the procedure outlined in *UNIT 11.7* (protocol for determination of tertiary folding rates and activation parameters).

12. To understand the origin of the observed FRET change(s), design control experiments in which specific experimental parameters are systematically altered, such as concentration or nature of additive, temperature, sequence of the RNA, and so on. See Commentary for explicit examples that can be found in the literature.

## REAGENTS AND SOLUTIONS

*Use deionized, distilled, RNase-free water in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

***Acetic acid/TEMED, 100 mM, pH 3.8***

57.8 µL glacial acetic acid
5 mL water
Adjust to pH 3.8 with *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)
Bring to 10 mL with water
Store up to 1 year at –20°C

***Acetonitrile***

Sterile filter and degas HPLC-grade acetonitrile through a 0.2-µM, organic-solvent-resistant bottletop filter (e.g., Millipore Millicup HV). Store up to 1 year at room temperature.

***Ammonium persulfate (APS), 50% (w/v)***

5 g ammonium persulfate
Bring up to 10 mL with water
Store up to 1 year at –20°C

***Chloroform, buffered***

Mix 96 mL chloroform with 4 mL isoamyl alcohol. Overlay with ⅕ vol of 1× TE buffer (*APPENDIX 2A*) and mix again. Store up to 1 year at room temperature. For chloroform extraction, use organic (lower) phase.

***Elution buffer***

6.67 mL 7.5 M ammonium acetate (final 500 mM)
1 mL 10% (w/v) SDS (*APPENDIX 2A*; final 0.1%)
20 µL 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*; final 0.1 mM)
Bring to 100 mL with water
Sterilize by filtration
Store up to 6 months at room temperature

***Formamide loading buffer, 2×***

9 mL deionized formamide
1 mL 1× TBE electrophoresis buffer (*APPENDIX 2A*)
100 µL 2.5% (w/v) bromphenol blue
Store up to 1 year at –20°C

***HEPES-KOH, 100 mM, pH 7.0***

238.3 mg HEPES
8 mL water
Adjust to 7.0 pH with 1 M KOH
Bring to 10 mL with water
Store up to 1 year at –20°C

***Sodium tetraborate, 100 mM, pH 8.5***

381 mg sodium tetraborate decahydrate
8 mL water
Adjust to 8.5 pH with half-concentrated HCl
Bring to 10 mL with water
Store up to 1 year at –20°C

***Triethylammonium acetate (TEAA) buffer, 100 mM, pH 7***

1.8 L water
12.6 mL glacial acetic acid
28 mL triethylamine
Adjust to pH 7.0; when approaching pH 7.0, proceed slowly with addition of acetic acid or triethylamine so as not to overshoot
Bring to 2 L with water
Sterile filter and degas through a 0.22-μm bottletop filter
Store up to 1 year at room temperature

## COMMENTARY

### Background Information

The structure, dynamics, and function of several catalytic RNAs have been studied recently by FRET, including the hammerhead (Tuschl et al., 1994; Perkins et al., 1996; Bassi et al., 1997, 1999; Singh et al., 1999), hairpin (Murchie et al., 1998; Walter et al., 1998b, 1999, 2000, 2001; Pinard et al., 1999; Klostermeier and Millar, 2000, 2001a; Zhuang et al., 2002), hepatitis delta virus (Pereira et al., 2002), Varkud Satellite (Lafontaine et al., 2001a,b, 2002), and *Tetrahymena* ribozymes (Zhuang et al., 2000), as well as tRNA (Chan et al., 1999), a three-helix junction from *E. coli* 16S rRNA (Ha et al., 1999), and the RNA four-helix junction of U1 snRNA (Walter et al., 1998a). FRET techniques are applicable to any number of RNAs or RNA-protein complexes, and literature examples of their applications are increasing rapidly.

FRET is the nonradiative transfer through space of the excited-state energy of an excited donor fluorophore to an acceptor fluorophore. When the two fluorophores are covalently tethered to defined sites on a biopolymer or a macromolecular complex, FRET can be used as a molecular ruler to estimate the distance between them. The donor normally emits at a shorter wavelength (higher energy) than the acceptor, which makes it easy to optically distinguish and quantify their relative emissions. The energy transfer efficiency ($E_T$) strongly depends on the distance $R$ between the two interacting fluorophores:

$$E_T = \frac{R_0^6}{R^6 + R_0^6}$$

**Equation 11.10.1**

where $R_0$ is the Förster distance at which 50% of the donor energy is transferred. $R_0$ accounts for all factors besides distance that influence the rate of energy transfer, including the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the donor quantum yield, and the relative orientation of the donor and acceptor transition dipole moments:

$$R_0^6 = 8.79 \times 10^{-28} \times \Phi_D \times \kappa^2 \times n^{-4} \times J(\lambda)$$

$$(\text{in } \text{Å}^6)$$

**Equation 11.10.2**

where $\Phi_D$ is the donor fluorescence quantum yield in the absence of acceptor, $\kappa^2$ is the orientation factor of the interacting transition dipole moments, $n$ is the refractive index of the medium, and $J(\lambda)$ is the spectral overlap integral of donor emission and acceptor absorption.

For most donor-acceptor pairs $R_0$ has a value of 15 to 90 Å. FRET can be used to estimate the distance between the two fluorophores when that distance is in the range of ~0.5 to 2 times $R_0$. This feature makes FRET an ideal tool to complement other techniques that are applied to biological macromolecules to measure global architectures and their changes, such as NMR spectroscopy and electron microscopy, which are most sensitive at smaller and larger distances, respectively.

The accessible range of rate constants that can be measured by FRET is limited only by the available fluorometer equipment and its dead time. Manual mixing has a typical dead time of 5 sec, limiting the measurable rate constants to less than ~5 $min^{-1}$. Rapid-mixing (stopped-flow) techniques reach dead times of 2 msec and below. Continuous-flow mixing techniques reach microsecond dead times. Relaxation techniques such as laser-induced temperature jumps can reach nanosecond dead times. All of these techniques can be coupled with FRET detection of structural changes.

## Critical Parameters

Strategic planning is critical for a successful application of FRET to RNA, since each system is quite idiosynchratic, making generalizations difficult. The effort invested in planning is often more than compensated by the unique insights gained from a successful experiment. Figure 11.10.1 summarizes a practical strategy to adapt a new RNA system to the use of FRET techniques.

### *Selection of RNA sequence*

First, one has to decide on the RNA sequence to study. Often, it might be useful to start with a wild-type or previously characterized sequence, but it is advisable to computationally check whether this sequence is predicted to fold homogeneously, e.g., by using Michael Zuker's online RNA folding software mfold version 3.1 (*http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi*) or Douglas Turner's PC version RNAstructure (*http://rna.chem.rochester.edu*; see *UNIT 11.2*; Mathews et al.,

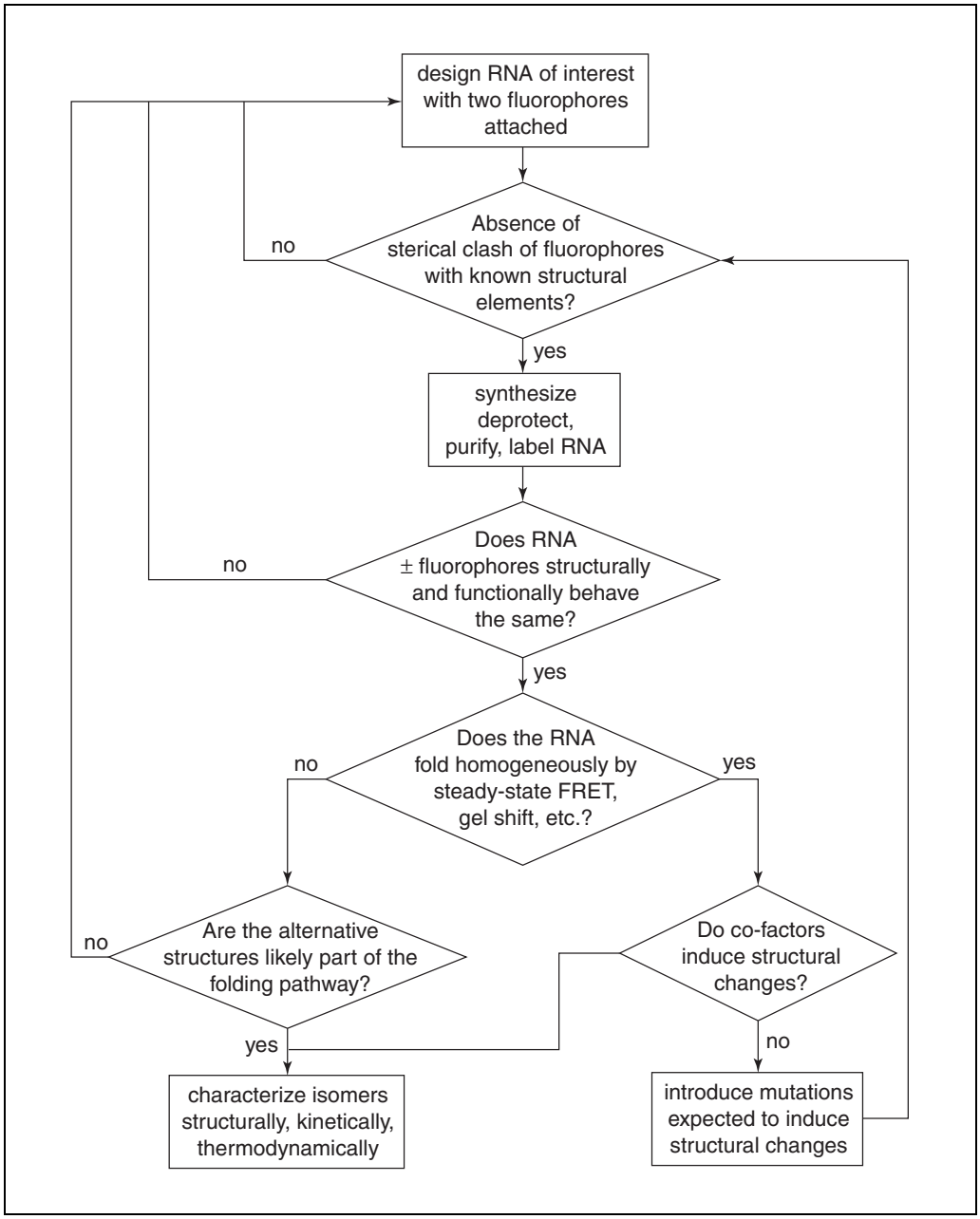


**Figure 11.10.1** Iterative procedure to adapt a new RNA system to the use of FRET for studying structural dynamics. Adapted from Walter (2001) with permission from Academic Press.

1999). In case of the hairpin ribozyme, for example, self-complementarity of the wild-type substrate was discovered and could be eliminated by careful redesign of the substrate and substrate-binding strands, under retention of all conserved base positions (Butcher et al., 1995; Walter and Burke, 1997). This modification led to improved catalytic (Esteban et al., 1997) and structural behavior (Hampel et al., 1998), which, in turn, allowed for a detailed characterization by FRET (Walter et al., 1998b, 1999; Zhuang et al., 2002). A simple computational check of all RNA strands for undesired secondary structures becomes particularly important when dividing a large RNA into several smaller strands that are synthesized to site-specifically introduce fluorophores.

### *Fluorescent labeling strategies: Labeling during or after synthesis*

Unlike proteins, most RNAs do not contain intrinsic fluorophores (Walter and Burke, 2000). The most efficient way to site-specifically introduce the two fluorophores necessary for FRET is by synthesizing the fluorophore-containing RNA strand(s). The fluorophore can be added during or after synthesis; in the latter case, a functional group is introduced during synthesis that allows for subsequent coupling to a reactive fluorophore derivative. Detailed descriptions of the many possible synthetic strategies are given elsewhere (Qin and Pyle, 1999; Walter and Burke, 2000). In general, solid-phase RNA synthesis in the 3′→5′ direction based on phosphoramidite chemistry (*UNIT 3.5* and *APPENDIX 3B*) can be modified to introduce:

1. On the 5′ end: Fluorophore derivatives that are resistant to RNA deprotection chemistry (e.g., fluorescein or cyanine phosphoramidites).
2. On the 3′ end or internally: Fluorophores that are resistant to both the coupling and deprotection chemistries (e.g., fluorescein), using column supports and nucleotide phosphoramidites, respectively, modified with linkers carrying the fluorophore.
3. On the 5′ or 3′ ends or internally: Aliphatic amino or thiol linkers that can be postsynthetically coupled under mild conditions with derivatives (e.g., amino-reactive succinimidyl esters or thiol-reactive maleimides) of chemically sensitive fluorophores (e.g., rhodamines).

New variations of these themes are continuously being developed, for example, the use of a site-specific phosphorothioate modification for internal labeling (Konarska, 1999). Assembling the RNA on an automated DNA/RNA synthesizer (e.g., PE Applied Biosystems; *http://www.appliedbiosystems.com*) is quite straightforward using β-cyanoethyl phosphoramidites supplied by companies such as Glen Research (*http://www.glenres.com*), ChemGenes (*http://www.chemgenes.com*), Amersham Pharmacia Biotech (*http://www.apbiotech.com*), BD Biosciences (*http://www.clontech.com*), CPG (*http://www.cpg-biotech.com*), Cruachem (*http://www.cruachem.com*), Dalton Chemical Laboratories (*http://www.dalton.com*), or PE Applied Biosystems. More expensive, yet very convenient, is the option to buy from commercial suppliers of synthetic RNA such as Dharmacon Research (*http://www.dharmacon.com*), Xeragon (*http://www.xeragon.com*), Midland Certified Reagent Co. (*http://www.mcrc.com*), IBA GmbH (*http://www.iba-go.com*), the Keck Foundation's Biotechnology Resource Laboratory at the Yale University School of Medicine (*http://www.info.med.yale.edu/wmkeck/oligos*), Oligos Etc. (*http:// www.oligosetc.com*), CPG, or Cruachem. Some of these suppliers, such as Dharmacon and Xeragon, use alternative 2′-OH protection chemistries to increase synthesis yield and maximum length of the synthesized RNA. Many reactive fluorophore derivatives for postsynthetic labeling of RNA are available from Molecular Probes (*http://www.probes.com*) or Sigma-Aldrich (*http://www.sigma-aldrich.com*). Succinimidyl esters of the cyanine dyes may be currently obtained only from Amersham Pharmacia Biotech.

### *Fluorophore labeling strategies: Coupling sites and fluorophores*

At present, the length of a synthetic RNA is restricted to ~50 to 80 nt due to limited coupling efficiency per nucleotide cycle. One way to overcome this limitation is to change the RNA strand connectivity; in the case of the hairpin ribozyme, this led to the use of a two-partite ribozyme with fluorophores on the 5′ and 3′ ends of the 32-nt 5′ segment. The ribozyme assembles by hybridization with a 21-nt 3′ segment and a 14-nt substrate (Walter et al., 1998b). Other approaches have used donor and acceptor on two different hybridized segments of an RNA; in this case, either the complex must be purified from any excess of individual strands (Bassi et al., 1997; Murchie et al., 1998; Lafontaine et al., 2001a) or the analysis must discriminate against an excess of (typically) the acceptor-labeled strand (Klostermeier and Mil-

lar, 2000, 2001a). In an elegant single-molecule FRET study, the donor fluorophore was coupled to an external substrate, while the acceptor was introduced through a DNA oligonucleotide complementary to a 3′ extension of the *Tetrahymena* ribozyme (Zhuang et al., 2000). The DNA oligonucleotide was also surface bound, which allowed any excess donor-labeled substrate to be washed away. Alternatively, a long contiguous RNA strand bearing an internal FRET fluorophore pair can be constructed by ligating, e.g., a synthetic fluorophore-labeled strand with one or two RNAs derived from natural sources or by in vitro transcription using T4 DNA ligase and a DNA splint (for reviews see Moore, 1999; Qin and Pyle, 1999). Additional enzymatic and chemical ligation methods are currently being developed.

The choice of the two labeling sites, fluorophores, tether lengths, and adjacent RNA sequences depends on the unique features of the system to be studied. In general, the following considerations should be taken into account:

1. Available information on folding pathways and three-dimensional structures should be used to choose labeling sites expected to minimally interfere with biological function.

2. If structural transitions are to be observed, the attachment sites of the two fluorophores should be chosen to maximize the expected distance changes.

3. Each donor-acceptor pair is characterized by a specific Förster distance, $R_0$ (Wu and Brand, 1994; Lakowicz, 1999). According to Equation 11.10.1, changes in FRET efficiency are at a maximum for distance changes that approximate $R_0$. Hence, choosing a fluorophore pair whose $R_0$ is close to the measured distance (e.g., within approximately two fold) will increase the sensitivity toward distance changes.

4. For certain requirements, specific donor-acceptor pairs may be better suited than others. The following criteria need to be weighed against each other to choose fluorophores for a given problem. If quantity is an issue, high labeling yields can be obtained with fluorophores incorporated during synthesis. A large separation of the donor and acceptor emission peaks simplifies their optical distinction, if required by the available detection filters. High absorption, fluorescence quantum yield, and photostability lead to enhanced sensitivity, as required, e.g., for single-molecule FRET applications. Some fluorophore pairs that have been successfully used are listed below (see discussion of fluorophore pairs for FRET).

5. The labeling chemistry has to allow for site-specific incorporation of the two fluorophores. There are four options to accomplish such specificity. (1) Both fluorophores can be incorporated during the synthesis of a single RNA strand. (2) One fluorophore can be incorporated during and one after synthesis of a single RNA strand. (3) Both fluorophores can be incorporated after synthesis of a single RNA strand that carries two modifications allowing for distinct and specific (orthogonal) labeling chemistries, such as one thiol and one amino functionality. (4) The fluorophores can be incorporated into separate RNA strands that are assembled by hybridization or ligation. The choice of approach depends in part on the fluorophores used (see discussion of fluorophore pairs for FRET below).

6. A sufficient length of the fluorophore tether is necessary to assure high conformational dynamics. Such fast isotropic motion of the fluorophores is important for absolute distance measurements between donor and acceptor from Equation 11.10.1, since the Förster distance, $R_0$, depends on the orientation factor $\kappa^2$ of the interacting transition dipole moments (see Equation 11.10.2).

The following values are normally well defined for a given system: $\Phi_D$, the donor fluorescence quantum yield in the absence of acceptor; $n$, the refractive index of the medium; and $J(\lambda)$, the spectral overlap integral of donor emission and acceptor absorption. In principle, $\kappa^2$ can range from 0 to 4 and is defined for a fixed relative fluorophore orientation; however, this is difficult to achieve in a dynamic solution-based system. The only other case in which $\kappa^2$ adopts a well-defined value (of $^2/_3$) is if the transition dipole moments of the two interacting fluorophores have an isotropic (random) relative orientation, i.e., show low fluorescence anisotropy after excitation with polarized light due to high conformational dynamics (Lakowicz, 1999; Klostermeier and Millar, 2001b; Parkhurst et al., 2001). In this case, $R_0$ for a given FRET pair can be determined from Equation 11.10.2 so that Equation 11.10.1 yields the fluorophore distance.

7. Often, the emission of an excited fluorophore is quenched by a proximal nucleobase through an outer-sphere electron transfer between the two species. According to Marcus theory (Marcus, 1964), the rate of quenching is governed by the frequency of diffusional encounters of the excited fluorophore with the quenching base, as well as the activation barrier of their redox chemistry (Seidel et al., 1996).

Therefore, the RNA sequence immediately adjacent to the fluorophore (and, to some extent, the fluorophore tether length; Dapprich et al., 1997) has an influence on the extent of quenching. For example, both fluorescein (Walter and Burke, 1997) and tetramethylrhodamine (Widengren et al., 1997), which constitute a popular FRET pair, are quenched by guanine. Such nucleobase-specific quenching has been utilized to observe RNA secondary structure formation (Walter and Burke, 1997; Walter et al., 1998b), yet it is best avoided to allow for absolute distance measurements by time-resolved FRET (Walter et al., 1999; Walter and Burke, 2000).

***Fluorophore pairs for FRET***

Typical examples for fluorophore pairs used on RNA are:

1. Fluorescein ($ex_{max}$ = 490 nm; $em_{max}$ = 520 nm; quantum yield, qy = 71%) and hexachlorofluorescein ($ex_{max}$ = 538 nm; $em_{max}$ = 551 nm). Both can be incorporated during or after synthesis. Pinard et al. (1999); Walter et al. (1998b, 2000, 2001).

2. Fluorescein and tetramethylrhodamine ($ex_{max}$ = 554 nm; $em_{max}$ = 573 nm; qy = 28%). Any of a number of other rhodamines can also be used, but this is probably the most widely used and photophysically best understood FRET pair. Tetramethylrhodamine should be incorporated after synthesis. Clegg (1992); Tuschl et al. (1994); Perkins et al. (1996); Chan et al. (1999); Singh et al. (1999); Walter et al. (1999); Klostermeier and Millar (2000).

3. Fluorescein and cyanine 3 (Cy3; $ex_{max}$ = 554 nm; $em_{max}$ = 573 nm; qy = 14%). Cy3 can be incorporated on the 5′ end during synthesis or anywhere after synthesis. Bassi et al. (1997, 1999); Murchie et al. (1998); Ha et al. (1999); Lafontaine et al. (2001a,b, 2002).

4. Tetramethylrhodamine and Cy5 ($ex_{max}$ = 652 nm; $em_{max}$ = 672 nm; qy = 18%). Cy5 can be incorporated on the 5′ end during synthesis or anywhere after synthesis. The photophysics of Cy5 seem to involve fast photobleaching and dark states that are not yet well understood. Deniz et al. (1999).

5. Cy3 and Cy5 (Klostermeier and Millar, 2001a). This FRET pair is particularly useful for single-molecule experiments (Zhuang et al., 2000, 2002).

***Preparation of RNA and initial experiments***

Once the RNA sequence, strand connectivity, and modifications are chosen, the RNA is synthesized, deprotected, and purified. Post-synthetically attached fluorophores are coupled at this stage. After a final HPLC purification step, the fluorophore-labeled RNA should be compared to unlabeled RNA to ensure that the fluorophore modification does not interfere with biological function (Fig. 11.10.1). If there are specific activity assays available, such as those for catalytic RNAs, they should be performed and compared in the presence and absence of the fluorophores. In principle, any biophysical or biochemical technique that generates a signature of proper folding and function may be used. As a broadly applicable method, unimpaired native folding may be tested on a nondenaturing polyacrylamide gel as described in *UNIT 11.4*, and may be detected either by autoradiography of radiolabeled RNA and/or by gel-based FRET analysis of fluorophore-labeled RNA (Ramirez-Carrozzi and Kerppola, 2001; Pereira et al., 2002). Such initial experiments may well yield the first evidence for RNA structural changes. For example, native gels may reveal alternate tertiary structures as part of an RNA folding pathway, if their interconversion rate is slow (Emerick and Woodson, 1994; Juneau and Cech, 1999; Pan et al., 2000; Pinard et al., 2001). Such structures may then be further characterized by FRET. Alternatively, RNA structural changes may be induced by the addition of co-factors (Emerick and Woodson, 1994) or substrates (Walter et al., 1998b), or by the introduction of mutations known to interfere with activity (Bassi et al., 1996; Pinard et al., 2001). By following the strategic plan in Figure 11.10.1 and combining complementary biochemical and biophysical techniques with FRET, one is likely to capture the most relevant and functionally important RNA structural changes.

## Anticipated Results

A typical result for a steady-state FRET assay is exemplified in Figure 11.10.2 for the hairpin ribozyme-substrate complex. A minimal reaction pathway of this ribozyme that cleaves an external substrate is composed of three reversible steps: substrate binding, cleavage, and product dissociation (Hegg and Fedor, 1995; Esteban et al., 1997; Walter et al., 1997). Previous linker insertion studies of the junction between the two independently folding domains A and B of the hairpin ribozyme-substrate complex (Feldstein and Bruening, 1993; Komatsu et al., 1994) suggested that a conformational change, docking of the two domains, occurred after binding and before the chemical step of the reaction. To observe this structural

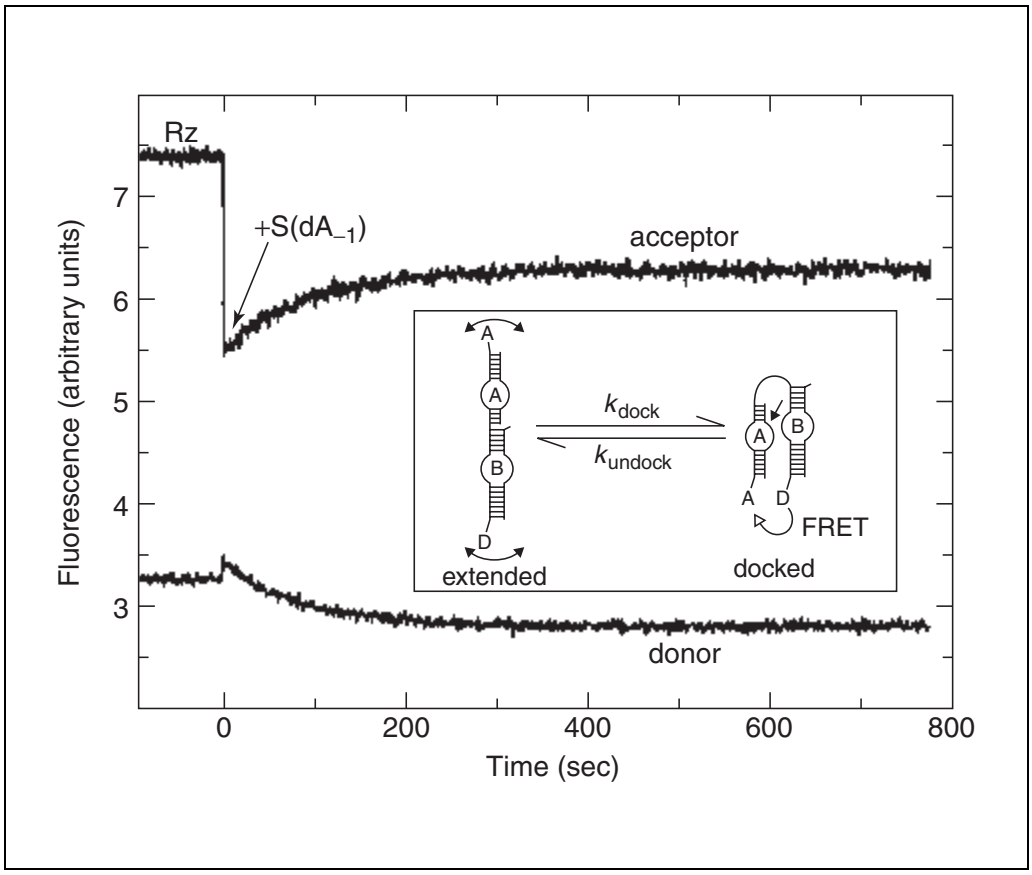


**Figure 11.10.2** Fluorescence signals over time as a result of tertiary structure folding of the hairpin ribozyme-substrate complex. The doubly labeled ribozyme displays a strong signal for the acceptor (A) and a weaker one for the donor (D) fluorophore. Upon manual addition of a ten-fold excess of noncleavable substrate analog S(dA$_{-1}$), significant quenching of the acceptor fluorescence is observed due to rapid ribozyme-substrate complex formation. Subsequently, the acceptor signal increases, while the donor signal decreases at the same rate, reflecting the reversible transition from a flexible extended (low-FRET) to a docked (high-FRET) and catalytically active conformation (inset; short arrow, catalytic site). Adapted from Walter (2001) with permission from Academic Press.

transition by FRET, a two-strand version of the hairpin ribozyme was utilized and labeled with a 5′-hexachlorofluorescein acceptor and a 3′-fluorescein donor. A ten-fold molar excess of the 3′ ribozyme segment was annealed with the 5′ segment by heating to 70°C for 2 min, followed by cooling to room temperature over 5 min, to saturate the fluorescently labeled strand.

Steady-state fluorescence spectra and intensities were recorded as described in Basic Protocol 2 on an AMINCO-Bowman Series 2 spectrofluorometer from Thermo Spectronic in a cuvette with 3-mm excitation and emission path lengths (120-µl minimal fill volume). This instrument allows for the parallel detection of both fluorophores during a time course by continually shifting the emission monochromator back and forth. A noncleavable substrate analog with a deoxy modification at the cleavage site, S(dA$_{-1}$), and the assembled ribozyme were preincubated separately for 15 min in standard buffer (50 mM Tris·Cl, pH 7.5, 12 mM $MgCl_2$, 25 mM DTT), at 25°C. Fluorescence data acquisition was started, and hairpin ribozyme-substrate complex was formed by manually mixing 145 µl ribozyme solution in the fluorometer cuvette with 5 µl substrate stock solution (supplying a saturating ten-fold substrate excess). Fluorescence emission values (one datum per second) for both donor ($F_{515}$ nm) and acceptor ($F_{560}$ nm) were recorded using the fluorometer software package. Upon substrate addition, a strong acceptor quench and slight donor dequench were observed (Fig. 11.10.2). At the same time, an increasing fluorescence anisotropy of the acceptor revealed decreasing fluorophore mobility upon substrate binding (Walter et al., 1998b). Under the conditions used, substrate binding was fast and

occurred within the manual mixing time (Walter and Burke, 1997). Because the fluorescence decrease was observed only with cognate substrate, the authors concluded that the rapid acceptor quench is mostly due to quenching of hexachlorofluorescein in the ribozyme-substrate complex, presumably by a base-specific electron transfer mechanism involving the 3′-terminal uracils of the substrate (Walter and Burke, 1997).

Subsequently, the donor fluorescence decreased over several minutes, while the acceptor fluorescence increased at the same rate. This observation strongly suggested that the underlying molecular process involves increasing FRET between the two fluorophores, as expected for their approach upon domain docking in the ribozyme-substrate complex (inset of Fig. 11.10.2). From the temporal change of the ratio $Q = F_{560}/F_{515}$, the rate constant of the transition between the extended and docked conformations was extracted and its dependence on RNA and buffer modifications was studied (Walter et al., 1998b). It should be noted that for a reversible docking step, the observed docking rate constant $k_{dock,obs}$ of $0.64 \pm 0.04$ $min^{-1}$ under standard conditions is a linear combination of the elementary docking and undocking rate constants: $k_{dock,obs} = k_{dock} + k_{undock} \times k_{dock,obs}$ can only be dissected further by an independent measurement of either the docking equilibrium constant (possible by time-resolved FRET; Klostermeier and Millar, 2001b) or the undocking rate constant (possible in single-molecule experiments; Zhuang et al., 2002).

These studies on the hairpin ribozyme illustrate some of the principles that can be utilized to probe RNA structural dynamics and function by FRET. Each unique RNA system will require specific modification of these procedures to provide novel and often unanticipated results.

## Time Considerations

Typically, 2 to 3 weeks are needed from the design of an RNA construct to beginning the first steady-state FRET experiments with the deprotected, purified, and fully labeled material. Most of this initial work will require only a few hours of effort each day. Steady-state FRET experiments, once they are set up, will be more time consuming and will be best accomplished in 4- to 6-hr time blocks.

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