Published in final edited form as:
Methods Mol Biol. 2012 ; 875: 271–295. doi:10.1007/978-1-61779-806-1_15.

Analysis of RNA Folding and Ribonucleoprotein Assembly by Single-Molecule Fluorescence Spectroscopy

Goran Pljevaljčić, **Rae Robertson-Anderson**, **Edwin van der Schans**, and **David Millar**
Department of Molecular Biology The Scripps Research Institute La Jolla, CA 92037 USA

Summary

To execute their diverse range of biological functions, RNA molecules must fold into specific tertiary structures and/or associate with one or more proteins to form ribonucleoprotein (RNP) complexes. Single-molecule fluorescence spectroscopy is a powerful tool for the study of RNA folding and RNP assembly processes, directly revealing different conformational subpopulations that are hidden in conventional ensemble measurements. Moreover, kinetic processes can be observed without the need to synchronize a population of molecules. In this chapter, we describe the fluorescence spectroscopic methods used for single-molecule measurements of freely diffusing or immobilized RNA molecules or RNA-protein complexes. We also provide practical protocols to prepare the fluorescently labeled RNA and protein molecules required for such studies. Finally, we provide two examples of how these various preparative and spectroscopic methods are employed in the study of RNA folding and RNP assembly processes.

Keywords

RNA folding; ribonucleoprotein assembly; Förster resonance energy transfer; single-molecule fluorescence spectroscopy; RNA labeling; RNA ligation; *in vitro* transcription of RNA; protein labeling

1. Introduction

Beyond their role as information carriers during gene expression, RNA molecules are involved in a broad range of other activities required for normal cellular function, including pre-mRNA splicing (1-3), translational regulation (4), metabolite sensing (5), protein synthesis (6) and protein targeting (7). RNA molecules also play a key role in viral infection processes (8-10). To execute their biological functions, RNA molecules must fold into specific tertiary structures and/or associate with one or more proteins to form ribonucleoprotein (RNP) complexes. There is often a close interplay between RNA folding and RNP assembly, since binding of one protein can induce an RNA folding transition that creates the binding site for a second protein. This coupling between protein binding and RNA folding events results in the hierachical assembly of large RNPs such as the ribosome, spliceosome and signal recognition particle (3, 6, 11). Once assembled, these large RNPs undergo a series of dynamic and reversible conformational changes during the course of their biological functions. Consequently, it is important to elucidate the mechanisms of RNA folding and RNP assembly, as well as the conformational dynamics of RNP complexes, in order to fully understand the diverse biological functions of RNA.

Corresponding author: David Millar, Department of Molecular Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037 USA. millar@scripps.edu..

Fluorescence spectroscopy offers a number of advantages as a method for the study of RNA folding and RNP assembly processes. Fluorescence measurements can be performed in solution under physiologically relevant conditions, without restrictions arising from the size of the molecules under study, and dynamic information is obtained over a wide range of time scales, from picoseconds to minutes. Various types of fluorescence parameters can be recorded, including emission intensity, polarization and lifetime, each of which reports different molecular properties. Owing to technical advances over the past decade, it is now possible to record fluorescence signals from individual biomolecules under physiologically relevant conditions (12-14). Single-molecule measurements are especially powerful, as they directly reveal different RNA conformational subpopulations that are hidden in conventional (ensemble-averaged) measurements. Moreover, kinetic processes can be observed without the need to synchronize a population of molecules. In principle, with these unique capabilities, it is possible to monitor the temporal order of protein-binding events during RNP assembly and the associated RNA conformational changes at each step. Despite this potential, very few such studies have been performed to date (15, 16). Single-molecule fluorescence methods can also be used to monitor autonomous RNA folding processes, such as occur during the catalytic cycle of various small ribozymes (17-19).

The purpose of this chapter is to describe some of the single-molecule fluorescence techniques that are useful in the study of RNA folding and RNP assembly processes. We also provide practical protocols to generate the necessary protein and RNA constructs. In addition, we present two examples from our own laboratory to illustrate how the methods are applied and the type of information that is forthcoming from single-molecule fluorescence studies of RNA folding and RNP assembly. It is hoped that this chapter will encourage the use of single-molecule fluorescence techniques in a broad range of RNA systems in the future.

1.1. RNA folding: Overview of methods

The spectroscopic phenomenon of Förster resonance energy transfer (FRET) has proven to be very informative as a tool to monitor the folding of individual RNA molecules (20-22). In the FRET process, the excitation energy of a donor fluorophore is transferred non-radiatively through space to a fluorescent acceptor, thereby reducing the fluorescence intensity and lifetime of the donor while giving rise to enhanced emission from the acceptor. The efficiency of FRET (E) is strongly dependent on the intervening donor-acceptor distance (R), as described by the Förster equation, $E=\left(1+\left(\frac{R}{R_0}\right)^6\right)^{-1}$, where R_0 is the Förster radius for the donor-acceptor pair. Hence, the FRET efficiency can be used to measure donor-acceptor distances (typically in the range from 30 to 70 Å) or to monitor changes in distance during macromolecular folding processes. To monitor the folding of RNA, the donor and acceptor dyes must be appropriately positioned to report on distance changes during the folding process. Suitable positions can often be deduced if the crystal structure of the folded RNA is known. Otherwise, the donor and acceptor can be incorporated in a variety of different positions and the resulting constructs tested under conditions in which the RNA is either completely folded or unfolded. Constructs that exhibit the largest differences in FRET efficiency between the folded and unfolded states are most suitable for detailed studies of the folding process. In addition, the donor and acceptor dye pair has to be selected so that its Förster radius is in the range of the anticipated distance change during the folding process.

A limiting factor for FRET experiments is the production of suitable doubly labeled RNA molecules. There are several different ways to obtain a RNA construct that contains donor and acceptor dyes at specific positions. In the case of relatively short RNA molecules

(typically less than 50 nt), both dyes can be directly incorporated at defined sites during solid phase oligonucleotide synthesis, either at strand termini or at internal positions. Alternatively, reactive amino or sulfhydryl groups can be incorporated during solid phase synthesis and subsequently modified with amine-reactive or sulfhydryl-reactive dye derivatives. In other cases, separate RNA oligonucleotides labeled with either donor or acceptor are annealed to obtain the desired doubly labeled construct for FRET measurements, as in the case of the hairpin ribozyme described later. However, these approaches are not feasible for labeling large RNA molecules that are beyond the practical size limit for solid phase synthesis (generally longer than 50 nt). In this case, dye-labeled DNA oligonucleotides can be annealed with the unlabeled RNA of interest (generated by *in vitro* transcription), usually within unstructured loop regions. Such an approach has been used to label ribosomal RNA for FRET studies (23).

In cases where an active, double-labeled RNA molecule cannot be constructed by simple annealing, short RNA oligonucleotides labeled with either donor or acceptor can be covalently joined to create longer molecules (24, 25). The donor and acceptor dyes are directly incorporated into each oligonucleotide during solid phase synthesis or by post-synthetic labeling of amino- or sulfhydryl-modified oligonucleotides. The two oligonucleotides are then annealed with a complementary DNA strand (splint), which positions the 5’ end of one oligonucleotide in close proximity to the 3’ end of the other. The two ends are then covalently joined in an enzymatic reaction catalyzed by RNA ligase 2. Since this reaction requires the presence of a 5’ phosphate group and a 3’ hydroxyl group, one of the oligonucleotides is first phosphorylated at the 5’ end using T4 polynucleotide kinase. A detailed protocol is presented in the next section.

Once suitable doubly labeled RNA constructs are in hand, FRET measurements can be performed on individual RNA molecules using either of two different detection formats. In one approach, individual RNA molecules are observed in a confocal microscope set up as they diffuse through a tightly focused laser beam (26). Single-molecule events are registered as discrete bursts of fluorescence emission. The number of donor and acceptor photons recorded during each burst is used to calculate the FRET efficiency. The results from many single-molecule events are compiled in the form of FRET efficiency histograms. These histograms can directly reveal different conformational subpopulations present during the RNA folding process, which appear as separate peaks in the histogram (19). Moreover, the shape of the histogram peaks can provide information on the rate of exchange between the different conformers. The advantage of this approach is that the RNA molecules are examined under natural solution conditions. However, the observation time is limited by the average period of time that the RNA molecule remains in the confocal volume, which is usually on the order of tens of milliseconds. Kinetic information on longer time scales is lost, although equilibrium populations can still be obtained. The necessary instrumentation, data acquisition and data analysis protocols are described in the next section.

In the other approach, individual RNA molecules are tethered to a solid surface and visualized by means of total internal reflection fluorescence (TIRF) microscopy (27, 28). An evanescent field is created by total internal reflection of a laser beam at a quartz-water interface, using either an objective or prism-based optical system (28). Since the evanescent field penetrates only ~ 100 nm into the solution, only the fluorophores localized in the interfacial region are excited by the field and become capable of emitting fluorescence. The fluorescence from both donor and acceptor can be monitored for relatively long periods of time (tens of seconds), until one of the dyes is destroyed by photobleaching. Moreover, many individual RNA molecules can be monitored in parallel using a charge coupled device (CCD) camera. The FRET efficiency for each RNA molecule in the field of view can be tracked over time, directly revealing dynamic folding transitions on a molecule-by-molecule

basis. For this approach, biotinylated RNA molecules are immobilized by binding to a streptavidin-coated surface. Generally, the surface is also coated with polyethylene glycol (PEG) groups to suppress non-specific adsorption of RNA or protein molecules. The necessary protocols are provided in the next section. We also describe the instrumentation required for TIRF measurements, as well as the data acquisition and analysis protocols.

1.2. Ribonucleoprotein assembly: Overview of methods

During the process of RNP assembly, one or more proteins bind to a single RNA molecule, usually in a defined temporal order. During assembly, the conformation of the RNA molecule may remain fixed or else it may change in response to each protein-binding event. There are two types of information that can be obtained from single-molecule fluorescence studies of RNP assembly: (1) the temporal order of protein binding events, and (2) the nature of the associated RNA conformational changes (if any). To obtain temporal information, individual steps in the assembly pathway are directly visualized by TIRF microscopy as suitably labeled proteins bind one at a time to an immobilized RNA molecule. As there is no necessity to label the RNA in this approach, arbitrarily long RNA molecules can be employed, which are readily generated in an *in vitro* transcription reaction. The protocols used for protein labeling and *in vitro* transcription of RNA are described in the next section. Information on RNA conformational changes can be obtained by single-pair FRET measurements with donor-acceptor RNA, using either freely diffusing or immobilized RNA molecules. In this case, the proteins are unlabeled, although more sophisticated three-color FRET experiments can be devised in which both the RNA and proteins are labeled.

2. Methods

2.1 *In vitro* RNA transcription protocol

The following protocol is used to generate unlabeled RNA molecules for studies of RNP assembly.

Materials

1. 100 mg of plasmid DNA containing an insert coding for the desired RNA. To transcribe only the insert of interest, rather than the entire vector, the vector must be completely linearized with a suitable restriction endonuclease prior to the reaction. Following the restriction digestion, extract the linearized plasmid with phenol:chloroform:isoamyl alcohol, precipitate with ethanol, and resuspend in nanopure water that has been filtered through a 0.22 μm sterile filter.
2. Transcription Buffer (10x): 800 mM K-Hepes (pH 8.1), 10 mM spermidine, Triton X-100 (1% v/v).
3. FPLC Buffer A: 50 mM Tris-HCl (pH 7.8).
4. FPLC Buffer B: 50 mM Tris-HCl (pH 7.8), 2 M NaCl.
5. 100 mM ATP, divide into 120 mL aliquots and store at –20 °C.
6. 100 mM GTP, divide into 120 mL aliquots and store at –20 °C.
7. 100 mM CTP, divide into 120 mL aliquots and store at –20 °C.
8. 100 mM UTP, divide into 120 mL aliquots and store at –20 °C.
9. 1 M $MgCl_2$, store at room temperature.
10. 1 M DTT, dissolve in 0.01 M sodium acetate and store at –20 °C.

11. 0.5 M EDTA (pH 8.0), store at room temperature.
12. T7 RNA polymerase (50U/ml): divide into 40 mL aliquots and store at –20 °C.
13. 5 mL HiTrap Q High Performance (HP) column (Pharmacia/GE Healthcare).
14. 30,000 NMWL Centriprep Centrifugal Filter Unit (Millipore).
15. TE Buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

Methods

1. This protocol is for 2 × 1 mL transcription reactions. Each reaction requires 50 mg of linearized plasmid DNA (prepared as described in Materials).
2. Prepare the following reaction twice in two separate centrifuge tubes. All the RNA nucleotides should be thawed completely before adding to the reaction to ensure proper concentration. T7 polymerase should be kept on ice at all times and added to the reaction last.

100 μL	10x Transcription Buffer
60 μL	100 mM ATP
60 μL	100 mM GTP
60 μL	100 mM CTP
60 μL	100 mM UTP
25.3 μL	1 M $MgCl_2$
10 μL	1 M DTT
20 μL	T7 RNA polymerase
50 mg	linearized plasmid (volume depends on original concentration).
X μL	dH_20 (X is adjusted to bring the total reaction volume to 1 mL).

3. Incubate both reactions at 37 °C for 5 hours. After 5 hours the reactions should be cloudy, indicating that the reaction was successful.
4. Add 0.5 M EDTA to 50 mM (111 μL per reaction) to quench the reaction. At this point the reaction can be stored at −20 °C overnight, if necessary, prior to FPLC purification.
5. Purify the RNA from the plasmid using FPLC purification as follows. The FPLC column details are listed in Materials #13.
    a. Wash the column with nanopure water for 10 minutes.
    b. Equilibrate with FPLC buffer A for 10 minutes.
    c. Set the following program using a flow rate of 1 mL/min:

0 – 10 min	0% FPLC Buffer B
10 – 160 min	0 – 70% B
160 – 180 min	70 – 100% B
180 – 190 min	100% B
190 – 200 min	100 – 0% B

d. Combine both 1 mL reactions and load reactions onto column.

e. Run the program set in 5 c. Collect 3 mL fractions over the entire run. The RNA elutes before the plasmid.

f. Following the run, wash the column with 20% ethanol for 10 minutes. The column is stored in 20% ethanol at 4 °C.

g. Store all fractions at 4 °C before and during step 6.

6. Run an agarose gel (adjust percentage according to RNA length) on all of the fractions surrounding the RNA peak, with a relevant molecular weight marker, to determine which fractions contain pure RNA (no contaminating plasmid).

7. Combine all fractions containing pure RNA.

8. Concentrate and remove the salt from the RNA solution with a Centriprep Centrifugal Filter Unit (Material #14) as follows: Rinse the Centriprep with 15 mL of TE buffer. Load the RNA fractions into the Centriprep and concentrate to 1 mL by centrifugation. Add 15 mL of TE to the Centriprep and concentrate to 1 mL. Repeat twice. Ethanol precipitation can also be used in place of this step.

9. Aliquot the RNA into desired quantities.

10. Store in TE buffer at –20 °C, or dry the RNA using a speed vacuum concentrator and store at −20 °C.

2.2 RNA ligation protocol

This protocol is used to create large doubly labeled RNA molecules for FRET studies of RNA folding or RNP assembly. Since the enzymatic ligation reaction requires the presence of a 5’ phosphate group and a 3’ hydroxyl group, one of the oligonucleotides is first phosphorylated at the 5’ end using T4 polynucleotide kinase. One potential drawback of this approach is that the 5’-phophorylated RNA can potentially react with itself to form a cyclic ligation product. A simple way to prevent this undesirable side product is by using a dideoxy modification at the 3’-end.

Materials

1. Annealing Buffer: 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl. Filter through a 0.22 μm sterile filter. Store at room temperature.

2. T4 polynucleotide kinase buffer (1x): 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP and 5 mM dithiothreitol (DTT). Store at −20 °C.

3. T4 RNA ligase 2 buffer (1x): 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT and 400 μM ATP. Store at −20 °C.

4. DNase I buffer (1x): 10 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Store at −20 °C.

5. Soaking buffer: 50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 0.5 mM EDTA. Filter through a 0.22 μm sterile filter and store at room temperature.

6. Gel running buffer (5x): Tris-borate (54 g tris base and 27.5 g boric acid per 1 L) and 10 mM EDTA (pH 8.0).

7. PAGE loading buffer: 7.3 M Urea, 50% formamide and 9 mM EDTA (pH 8.0).

8. Nanopure water, filtered through a 0.22 μm sterile filter.

9. Quick Spin Columns (TE), (G-25 Sephadex Column, Roche Diagnostics). Store at + 2 to + 8 °C.
10. Illustra NAP-5 columns (Sephadex G25, GE Healthcare).
11. Polyacrylamide gel, SequaGel (National Diagnostics). Mini-gel system PROTEAN II (Biorad).
12. Two RNA oligonucleotides. One oligonucleotide is labeled with the FRET donor at a desired location, the other with the FRET acceptor. Synthetic RNA oligonucleotides labeled with fluorescent dyes can be obtained from commercial sources. Alternatively, synthetic oligonucleotides containing reactive amino or sulfhydryl groups can be labeled with succinimidyl ester or maleimide derivatives of the desired fluorescent dyes, respectively. Pure and homogeneous starting material should be used for the ligation. The RNA 5'-end at the ligation site has to be phosphorylated and the RNA 3'-end at the ligation site has to carry a hydroxyl group.
13. DNA oligonucleotide. The DNA splint is selected for efficient annealing with both RNA oligonucleotides. Longer splints might help prevent the formation of undesired RNA secondary/tertiary structures.

Methods

1. To phosphorylate the 5'-end of RNA, incubate RNA containing 5'-hydoxyl group (up to 300 pmol), T4 polynucleotide kinase buffer and T4 polynucleotide kinase (20U) in 50 μl for 1 h at 37 °C.
2. To denature T4 polynucleotide kinase, incubate the reaction mixture for 20 min at 65 °C.
3. To purify the RNA, use Quick Spin Columns (Roche). Homogenize the resin and let column run dry by gravity. Centrifuge the column for 2 min at 1100 × g. To equilibrate the column with water, add 200 ml nanopure water and centrifuge column for 2 min at 1100 × g. Load 50 ml (maximum) reaction mixture onto the column and centrifuge 4 min at 1100 × g. Collect the sample from flow through. The purified RNA is used in the following ligation reaction.
4. To anneal the two RNA's with the DNA splint, combine the two RNA strands (1 nmol each) and the DNA splint (1 nmol) in annealing buffer and heat the mixture for 3 minutes at 90 °C and let cool to room temperature.
5. To ligate the two RNA strands, add RNasin (40U/ml, 660 U/nmol RNA), T4 RNA ligase 2 buffer and T4 RNA ligase 2 (10 U/ml, 65 U/nmol RNA) to the solution and incubate for 20 hours at 37 °C.
6. To remove the DNA splint from the reaction mixture, add DNase I buffer and DNase I (10 U/ml, Roche, 660 U/nmol DNA) and incubate the reaction for 2 hours at 37 °C.
7. To digest the proteins remaining after the reaction, add Proteinase K (20 mg/ml, 1.3 mg per 1nmol RNA ligation reaction) and incubate 5 hours at room temperature.
8. To prepare the reaction mixture for purification, reduce the volume of the ligation mixture to 500 μl (if necessary) in a speed vacuum concentrator (Savant SC110).
9. To desalt the solution, use a NAP-5 (Sephadex G25, GE Healthcare) column, concentrate the sample with a speed vacuum concentrator and spin until sample is dry.

10. To purify the ligation product, dissolve the pellet in PAGE loading buffer and use a polyacrylamide gel (SequaGel, adjust polyacrylamide percentage for optimal separation) for separation of products. Pre-run the gel in TBE buffer (1x) for 30 min (300 V for mini-gel PROTEAN 2). Load the sample and bromophenol blue dye (to observe progress of gel run) onto the gel and run the gel in running buffer (at 300 V for Mini-gel system) until the dye runs out of the gel. Identify the ligation product either by UV shadowing or fluorescence and excise the corresponding RNA band. Crush and soak in soaking buffer at 4 °C overnight.
11. To desalt the ligated RNA, use a NAP-5 column.
12. To concentrate the sample, spin the sample in a speed vacuum concentrator until the sample is dry.
13. Record an UV absorption spectrum to determine the amount of the ligated RNA.

2.3 Biotinylation of RNA

The following protocol is used biotinylate RNA molecules (labeled or unlabeled) at the 3' end for subsequent attachment to a solid surface (required for TIRF measurements).

Materials

1. 1 nmol RNA.
2. 100 mM $NaIO_4$.
3. 3.3 M KCl.
4. 3 M NaOAc, pH 5.0.
5. 2.5 M NaOAc, pH 5.0.
6. EtOH stored at −20 °C.
7. 70% v/v EtOH in dH_20 stored at −20 °C.
8. 25 mg biotin hydrazide (Sigma B3770).
9. 1.53 mL DMSO.
10. Refrigerated centrifuge (4 °C) with rotor for 1.5 mL vials.

Methods

1. To oxidize the 3' cis diol into aldehydes, prepare the following reaction: 5 μM RNA, 100 mM NaOAc, pH 5.0, and 100 mM $NaIO_4$ in a volume of 200 μL. The $NaIO_4$ solution should be made freshly for each reaction.
2. Incubate this reaction for 1 hour on ice and protected from light.
3. Add 12.9 μL of 3.3 M KCl to precipitate IO_4^- ions as potassium periodate.
4. Incubate this reaction for 10 minutes on ice.
5. Spin this reaction in a centrifuge at 4 °C for 5 minutes at a speed of 16,000 ×g.
6. Following the spin, discard the resultant precipitate.
7. Precipitate the RNA in the supernatant with EtOH as follows:
    a. Add 20 μL of 3 M NaOAc and 500 μL EtOH to the supernatant.
    b. Incubate the reaction for 30 minutes at −20 °C.

c. Spin the reaction mixture at 4 °C for 30 minutes in a centrifuge at a speed of 16,000 ×g.

d. Discard the resultant supernatant and add 500 μL of cold 70% EtOH.

e. Spin the reaction mixture at 4 °C in a centrifuge for 5 minutes at a speed of 16,000 ×g.

f. Discard the resultant supernatant and dry the precipitated RNA. This can be done by incubating the open centrifuge tube at 37 °C for ~10 minutes.

8. Dissolve 25 mg biotin hydrazide in 1.53 mL DMSO and 170 mL dH_2O.

9. Add together 79.5 μL of dH_20 and 8 μL of 2.5 M NaOAc, pH 5. Keep this mixture on ice.

10. Keeping this reaction on ice, add 12.5 μL of the biotin hydrazide solution (step 8) and mix.

11. Add this reaction to the dried RNA and swirl to resuspend the RNA.

12. Incubate this reaction for 2 hours at room temperature.

13. Add 5 μL of the hydrazide solution and incubate at 4 °C overnight.

14. Precipitate the RNA by repeating step 7.

15. Store the dried RNA at −20 °C. Alternatively, the RNA can be resuspended in dH_20 or TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and stored at −20 °C.

2.4 Protein labeling protocol

The following protocol is used to prepare dye-labeled proteins for studies of RNP assembly. The protocol was developed for the HIV-1 Rev protein but should be applicable to any His-tagged protein containing a single cysteine residue. The HPLC purification and refolding steps are most suitable for relatively small proteins that can be reversibly unfolded and refolded.

Materials

1. Labeling Buffer: 50 mM Na_3PO_4 (pH 7.2) and 500 mM NaCl. Filter through a 0.22 μm sterile filter. Store at room temperature but keep on ice for the following protocol.

2. Elution Buffer: 50 mM Na_3PO_4 (pH 7.2), 500 mM NaCl and 250 mM imidazole. Filter through a 0.22 μm sterile filter. Store at room temperature but keep on ice for the following protocol.

3. Storage Buffer: 50 mM Tris-HCl (pH 7.2), 1.6 M NaCl and 1 mM EDTA. Filter through a 0.22 μm sterile filter. Store at 4 °C.

4. Resuspension Buffer: 50 mM Tris-HCl (pH 7.2), 1.6 M NaCl, 1 mM EDTA and 6 M guanidine HCl. Filter through a 0.22 μm sterile filter. Store at room temperature.

5. Nanopure water, filtered through a 0.22 μm sterile filter.

6. 1 M DTT solution in 0.01 M sodium acetate. Store at –20 °C.

7. Acetic acid.

8. Illustra NAP-10 column (GE Healthcare).

9. HPLC Buffer A: 0.1% TFA in dH_2O. Filter through a 0.22 μm sterile filter. Store at room temperature.
10. HPLC Buffer B: 0.1% TFA in acetonitrile. Filter through a 0.22 μm chemically resistant nylon filter. Store at room temperature.
11. 0.8 × 4 cm Poly-Prep chromatography column (BioRad).
12. Ni-NTA Superflow nickel-charged resin (Qiagen). The resin is stored in slurry of 50% resin and 50% storage buffer.
13. 250 × 4.6 mm HAISIL 300, C18 5 mm column (Higgins Analytical, Inc.) for HPLC purification.
14. Dialysis membranes: 3.5K molecular weight cut off (MWCO) Slide-A-Lyzer, 0.1 – 0.5 mL (Pierce Biotechnology), 3.5K MWCO Slide-A-Lyzer, 0.5 – 3 mL (Pierce Biotechnology), 3.5K MWCO Slide-A-Lyzer, 3 – 12 mL (Pierce Biotechnology).
15. Maleimide-based fluorophore such as Alexa Fluor® 555 C_2-maleimide (Invitrogen).

Methods

1. This protocol is for labeling 1 mL of His-tagged protein containing a single cysteine residue. The protein concentration should be > 75 mM.
2. Add 1 M DTT to protein to 5 mM (5 μL for 1 mL aliquot). Incubate overnight at 4 °C.
3. To remove DTT, pass protein through a NAP-10 column as follows: To equilibrate the column, add 15 mL (3 column volumes) of labeling buffer to the column 5 mL at a time. Once the labeling buffer has fully passed through column, add the 1 mL protein aliquot and allow to fully enter the gel bed. To elute the protein add 1.5 mL labeling buffer to the column. Collect elution product in a 1.5 mL centrifuge tube.
4. Dissolve 3x – 5x molar excess, compared to protein, of maleimide dye, such as Alexa Fluor® 555 C_2-maleimide (Invitrogen) in DMSO. Dye should be dissolved in DMSO to a final volume of less than 45 μL (DMSO content of the solution must be less than 3%). Add dye solution to protein (elution product from step 3). Wrap in foil and place on a rocker for ~ 3 hrs at 4 °C.
5. To quench the reaction, add 3 mL β-mercaptoethanol (2 mg/mL).
6. To remove free dye from the protein solution, run a 500 mL Ni:NTA column as follows:
    a. To prepare a 500 mL column, add 1 mL Ni:NTA resin slurry to column. Allow storage buffer to completely pass through column.
    b. Wrap the column in foil to protect the labeled protein from light.
    c. To wash the column, add 10 mL 0.2 μm sterile-filtered nanopure H_2O to the column. Allow water to fully pass through column.
    d. Add 4 mL labeling buffer to the column and allow to fully pass through the column.
    e. Add the labeled protein (1.5 mL) to the column and allow to fully enter the resin.

f. To remove free dye, add 5 mL labeling buffer. If the flow-through from the column is not clear (no visible fluorescence) after 5 mL, continue adding labeling buffer until flow-through is clear.

g. To elute the protein, add 1 mL elution buffer to the column and collect elution product in 1.5 mL centrifuge tube. Repeat until elution product and column resin is clear.

h. Combine the elution product from all the tubes with visible fluorescence (typically 2 – 5 mL).

7. If the labeling efficiency is sufficiently high (typically > 90%), the labeled protein can be used without further purification. In this case, dialyze the protein against storage buffer (step 14b) and concentrate using Centriprep Centrifugal Filter Unit. Small aliquots of protein are flash frozen in liquid nitrogen and stored at −80 °C.

8. If the labeling efficiency is low, it is necessary to separate the labeled and unlabeled fractions. In the following steps, the protein is denatured, purified by reverse phase HPLC and then refolded to the native state.

9. Dialyze labeled protein against 2 L of 2% acetic acid in nanopure H_2O. Pour solution into 2 L dialysis reservoir (Nalgene) with magnetic stir bar. Load labeled protein into a 3.5K MWCO Slide-A-Lyzer with the appropriate volume capacity, and place in the reservoir. Cover the reservoir in foil to protect the sample from light. Place reservoir on magnetic stirrer and dialyze at 4 °C for ~ 3 hrs. Switch to fresh dialysis buffer and continue dialysis overnight.

10. Remove protein from Slide-A-Lyzer.

11. Divide protein into 0.5 mL fractions in 1.5 mL centrifuge tubes. Place fractions in a speed vacuum concentrator (Savant SC110) and spin until protein fractions are dry.

12. Purify labeled protein from unlabeled protein using HPLC purification, as follows. The column details are listed in Materials #13.

a. Dissolve dry protein fractions in HPLC Buffer A to a total volume of 110 mL. Combine all fractions and mix. This volume is sufficient for a 10 mL analytical run, to check for elution time and to ensure everything is working correctly, and 2 × 50 mL preparative runs to purify the labeled protein.

b. Equilibrate at 5% HPLC Buffer B for 30 min.

c. Set the following program:

0 – 5 min	5% B
5 – 20 min	5 – 35% B
20 – 50 min	35 – 55% B
50 – 55 min	55 – 80% B
55 – 65 min	Wash 80% B
65 – 75 min	80 – 5% B
75 – 90 min	Equilibrate 5% B

d. Load 10 mL of protein solution onto the column and begin the program.

e. The labeled protein will elute after the unlabeled protein. Collect the elution product corresponding to the labeled protein. Fluorescence should be visible by eye during collection.

f. Repeat steps b – e with 50 mL of protein solution. Repeat with remaining protein.

g. During the final HPLC run stop the program when 80% B has been reached. The HPLC column should be stored in 80% B.

13. Combine collected labeled protein and dry using a speed vacuum concentrator, as described in Step 9.

14. Re-nature the protein as follows:

a. Dissolve the protein in chilled resuspension buffer. The concentration should be adjusted to prevent the protein from precipitating from solution when re-folding.

b. Prepare 2 L storage buffer for dialysis. Pour storage buffer into 2 L dialysis reservoir with magnetic stir bar. Store at 4 °C until buffer is chilled.

c. Load the protein into a 3.5K MWCO Slide-A-Lyzer of appropriate volume (typically 0.1 – 0.5 mL) and place in the dialysis reservoir.

d. Cover the reservoir in foil, place it on a magnetic stirrer, and dialyze at 4 °C for ~ 3 hrs. Switch to fresh pre-chilled storage buffer and continue dialysis overnight.

e. Remove protein from the Slide-A-Lyzer.

15. Divide protein into 20 mL aliquots and store at –80 °C.

2.5 Diffusion spFRET instrumentation

Diffusion single-pair FRET measurements are performed using an epi-illuminated confocal microscope setup (Fig. 1) (26). Lasers selected for excitation of donor and acceptor dyes are used as light sources. For alternating laser excitation (ALEX) (29), electro-optical modulators are used to rapidly switch between donor and acceptor excitation. The ALEX method automatically rejects RNA molecules labeled with only the FRET donor, which often arise from photobleaching of the acceptor. For ease of alignment the two laser beams are fed into a water immersion objective (60x, 1.2 NA) using an optical fiber. The emitted donor and acceptor fluorescence is collected through the same objective. A pinhole is used to select for the light coming from the confocal volume. A dichroic mirror and cutoff filters are used to spectrally separate the donor and acceptor emission, which are detected simultaneously on separate avalanche photo diodes (APD). The collected signals are acquired with a photon-counting card (National Instruments or Becker & Hickl) under the control of appropriate data acquisition software (e.g., Labview).

2.6 Diffusion spFRET data acquisition

Materials

1. Annealing buffer: as described in Section 2.2.

2. spFRET buffer: A buffer containing an oxygen scavenger should be used (Example: 50 mM TrisHCl (pH 7.5), 10 mM $MgCl_2$, 1 mM propyl gallate). Each stock solution should be filtered through a 0.22 μm sterile filter. The buffer solution should be prepared freshly before each measurement.

3. 8 chamber micro cuvettes (Nalgene).
4. 10 % Tween.

Methods

1. To prepare the cuvettes for spFRET measurements, treat each sample chamber with Tween (10%) for 1 h at room temperature. Clean the sample chambers four times with distilled water and dry the chambers overnight.
2. To anneal the RNA, heat a stock solution of labeled RNA (1 mM) in annealing buffer to 90 °C for 3 minutes and let cool to room temperature.
3. For alignment and measurement, dilute the RNA solution twice in spFRET buffer to a final concentration of 1 nM (setup alignment) and 100 pM (measurement).
4. Load 200 μl of the 1 nM RNA solution into a sample chamber and place the cuvette onto the stage of the confocal microscope.
5. Focus the laser beam on the inner glass surface of the cuvette. Then move the objective so that the focus is located 35 μm into the solution.
6. Reduce the power of the excitation laser to avoid damage to the APD's. Choose a large pinhole (200 μm diameter) to align the APD's for maximum signal intensity. Reduce the pinhole size (75 μm diameter) and position the pinhole for maximum signal intensity.
7. To perform a measurement, load the 100 pM RNA sample and adjust the power of the excitation laser to approximately 200 μW (for Cy3/Cy5 dye pair).
8. To perform an ALEX measurement, the sample is excited with two excitation lasers that alternate rapidly (e.g. every 50 ms). The data acquisition is the same, regardless of the number of excitation lasers.
9. Count photons separately for donor and acceptor. For ALEX experiments an additional synchronization signal is recorded to be able to assign collected photons to the corresponding excitation laser during subsequent data analysis.

2.7 Diffusion spFRET data analysis

Isolated bursts of donor and acceptor fluorescence corresponding to single-molecule events are identified using a burst search algorithm (30). Only events that exceed a defined background threshold are used for further analysis. The FRET efficiency (E) for each single-molecule event is calculated using the equation $E = (1 + \gamma\ I_d/I_a)^{-1}$, where I_d and I_a represent the donor and acceptor intensities, respectively, and γ is a correction factor that accounts for differences in quantum yield and detection efficiency of the donor and acceptor (usually set to 1). A histogram containing the FRET efficiencies from many single-molecule observations (typically thousands of events are recorded during an acquisition time of a few minutes) is compiled. Conformational subpopulations can be directly identified as separate peaks in the FRET efficiency histogram (an example is shown in Fig. 2). Gaussian functions are used to fit these peaks and obtain the mean FRET efficiency, the width of the distribution and the area under each peak (using Origin or Kaleidagraph software). The mean FRET efficiencies are related to the average donor-acceptor distance (R) for each conformer, through the Förster equation, given above. The peak areas reflect the fractional populations of each conformer, and the width of the peak reflects conformational flexibility of a subpopulation if the theoretical shot noise width is exceeded. Hence, changes in the conformer populations and conformer properties can be followed in response to changes in

solution conditions, such as metal ion concentration (an example is shown in Fig. 2, bottom), ionic strength, temperature or the presence of specific RNA-binding proteins.

2.8 TIRF instrumentation

The prism-based setup used in our laboratory for TIRF measurements is illustrated in Fig. 3. The excitation laser is focused through the prism on a quartz slide. The incident angle is adjusted for total internal reflection at the quartz-water interface, yielding an evanescent field from the upper sample chamber surface on which the RNA molecules are immobilized. Fluorescence emitted by excited fluorophores is collected through a water immersion objective (63 ×, 1.2 NA) using dichroic and cut-off filters to select the fluorescence of the desired dye and reject scattered laser light. Fluorescence is detected with an intensified CCD camera (Andor model 897). For FRET experiments, the fluorescence from donor and acceptor dyes are spectrally separated and detected on different areas of the CCD camera using a Dual View optical system (Photometrics). Individual immobilized molecules appear as fluorescent spots whose intensity is recorded over time in form of a movie. The time resolution is determined by the frame rate of the CCD camera, which is adjustable from 5 msec to several hundred msec per frame.

2.9 RNA immobilization and surface treatment

The following protocol is used to immobilize biotinylated RNA molecules and to passivate the quartz surface with polyethylene glycol (PEG) groups.

Materials

1. 30 mm × 40 mm × 1 mm quartz microscope slides (TIRF Solutions), with holes drilled at each end to allow input and output of sample solutions.
2. 3 in × 1 in No. 1 glass microscope cover slips (Fisher Scientific).
3. Adhesive spacers (typically double-sided tape).
4. 50 mM Na_2CO_3 (pH 8.5). Bring the solution to pH 8.5 with acetic acid and filter through 0.22 μm sterile filter. This solution should be made no more than 1 week in advance due to an unstable pH. Store buffer at 4 °C.
5. mPEG-5000 (Laysan Bio, Inc.). Aliquot dry mPEG into ~100 mg quantities in centrifuge tubes. Seal tubes with parafilm and store in a dessicated jar at –20 °C.
6. Biotin-mPEG-5000 (Laysan Bio, Inc.). Aliquot into ~ 2 – 5 mg quantities in centrifuge tubes. Seal tubes with parafilm and store in a dessicated jar at –20 °C.
7. Dry acetone. To prepare, add molecular sieves to a 500 mL beaker. Add acetone to fill beaker. Prepare at least 1 day before use.
8. Acetone.
9. 3-Aminopropyltriethoxysilane (Pierce).
10. Immunopure streptavidin. Dissolve dry streptavidin stock in water to 1 mg/mL. Prepare 500 mL of 0.2 mg/ml streptavidin from stock prior to slide preparation.
11. 100 mM propyl gallate. Add propyl gallate to 100 mM in acetonitrile. Separate into 20 mL aliquots and store at –80 °C.

Methods

1. Place the desired number of quartz slides and glass cover slips in holders designed to separate all surfaces and keep all surfaces open to surrounding air or liquid.

2. Clean the slides and cover slips using a plasma cleaner as follows: Place the holders containing the slides and cover slips in the plasma cleaner (Harrick Scientific). Follow the manufacturer's directions to start cleaning process. Set the RF power to HIGH and run plasma cleaner for ~5 minutes.
3. Prepare a 2% solution of 3-aminopropyltriethoxysilane (Aminosilane) in dry acetone by adding 2 mL of Aminosilane to 98 mL of dry acetone in a 200 mL glass beaker. Following slide and cover slip cleaning, immerse each holder in the 2% Aminosilane solution for 60 seconds.
4. To rinse the slides and cover slips, immerse each holder in acetone for 60 seconds.
5. Dry all the slides and cover slips using a stream of N_2 gas.
6. Place the dry slides and cover slips into individual holders.
7. Dissolve an aliquot of mPEG-5000 (PEG) in 50 mM Na_2CO_3 to 150 mg/mL.
8. Dissolve an aliquot of Biotin-mPEG-5000 (Biotin-PEG) in 50 mM Na_2CO_3 to 10 mg/mL.
9. To prepare the quartz slide surface treatment, combine the Biotin-PEG solution and PEG solution to a ratio of 1:19 Biotin-PEG:PEG (e.g. 15 mL of Biotin-PEG and 285 mL of PEG).
10. Pipette 40 μL of the Biotin-PEG:PEG solution onto the center of each quartz slide, corresponding to the eventual location of the sample chambers.
11. Pipette 40 μL of the 150 mg/mL PEG solution onto the center of each glass cover slip.
12. Place the slides and cover slips in the dark and incubate at room temperature for 3 – 4 hours.
13. Rinse each slide and cover slip with nanopure water and dry using N_2 gas.
14. At this point the slides and cover slips can be stored for up to 3 days at –20 °C as follows: Seal each slide and cover slip holder with parafilm. Place sealed holders in a dessicated jar. Cover the jar in foil to protect it from light. Store at –20° C.
15. Pipette 40 μL of 0.2 mg/mL streptavidin on the center of each quartz slide. Incubate for 15 – 20 minutes at room temperature in the dark.
16. Rinse each slide and cover slip with nanopure water and dry using N_2 gas.
17. Assemble the sample chamber as follows: Attach an adhesive spacer to the treated side of each glass coverslip. Place the quartz slide on top of the cover slip such that the two treated surfaces are facing each other and the orientation of the slide is opposite that of the coverslip.

2.10 TIRF data acquisition

Two different detection formats are typically used. For single-color measurements of protein binding during RNP assembly, unlabeled RNA is immobilized on the quartz surface and the labeled protein is present in solution. Binding or dissociation of the labeled protein to the immobilized RNA is registered as discrete jumps in fluorescence intensity from individual spots within the TIRF field of view (Fig. 4). For FRET measurements of RNA folding and/or RNP assembly, donor-acceptor labeled RNA molecules are immobilized on the quartz surface and the fluorescence signals from both donor and acceptor are monitored over time on separate segments of the CCD camera. Magnesium ions or unlabeled protein partners can also be added.

Materials

1. Recording buffer: Typically 50 mM Tris or HEPES (pH 7.2), supplemented with monovalent and divalent metal ions (concentrations depending on desired experimental conditions) and 1 mM propyl gallate or other oxygen scavenger. Filter through a 0.22 μm sterile filter. Store at 4 °C.
2. Sample of interest.

Methods

1. This protocol describes the general procedure to obtain TIRF movies using a prism based microscope setup.
2. Place the sample chamber onto the microscope stage, fill with buffer and align the excitation laser beam to obtain total internal reflection at the quartz/water interface. Focus the objective on the upper glass surface. Make sure to use glycerol at the contact site of the quartz slide and prism and water at the contact site between objective and cover slip.
3. Load biotinylated RNA molecules into the sample chamber. Generally, 1 mM propyl gallate or another oxygen scavenger is added along with the sample to hinder photobleaching.
4. Turn on the laser light source and adjust the focus to observe the immobilized fluorescent molecules. Adjust the excitation laser power to yield distinguishable fluorescent spots over background.
5. Record the intensity fluctuations over time as a movie file using the CCD camera software.
6. Introduce other sample components, such as monovalent or divalent ions or specific protein partners (unlabeled), as required, and continue data acquisition.

2.11 TIRF data analysis

Custom software is used to analyze the recorded TIRF movies. Individual fluorescent spots are first identified in the 2D images and their location is determined. In FRET experiments, individual spots in the donor channel are matched with corresponding spots in the acceptor channel. For each single spot, the time dependent intensity values are established after suitable background correction, and intensity trajectories (time traces) are constructed. These trajectories are analyzed using hidden Markov modeling to deduce the number of distinguishable states, the fluorescence intensity or FRET efficiency for each state and the transition rates connecting them (31).

3.1 Example: Folding of the hairpin ribozyme

Here we provide an example of an RNA folding study performed under equilibrium conditions using the diffusion spFRET method. The hairpin ribozyme is a small endonucleolytic RNA molecule responsible for reversible phosphodiester cleavage reactions (32). In order to attain catalytic activity, the ribozyme must fold into a compact structure that positions two internal loops (A and B) in close physical proximity. We monitored the folding of a hairpin ribozyme construct that contains donor and acceptor dyes on the two loop-bearing arms (Fig. 1) (19). The ribozyme was assembled from four synthetic RNA oligonucleotides, two of which were end-labeled with donor (Cy3) or acceptor (Cy5) dyes. Individual ribozyme molecules traversing a confocal volume give rise to isolated bursts of donor and acceptor fluorescence (Fig. 2, upper panel). A FRET efficiency histogram was compiled from many single-molecule events, directly revealing the presence of extended

and docked conformational subpopulations (Fig. 2, middle panel). The equilibrium distribution of extended and docked conformers can be readily determined as a function of Mgion concentration (or other solution conditions) from these spFRET measurements, revealing that the docked conformation is stabilized by one or more Mg-ions (Fig. 2, lower panel). Similar measurements performed with ribozymes containing different helical junction geometries (two-way or three-way junctions instead of the natural four-way junction) or specific mutations have identified the structural elements required for efficient docking (19). The same approach could be used to monitor folding of a doubly labeled RNA molecule in the presence of a cognate binding protein (unlabeled).

3.2 Example: Assembly of the HIV-1 Rev-RRE complex

Here we describe a TIRF-based study of RNP assembly, using the HIV-1 Rev-RRE system as an example. The Rev protein mediates export of unspliced and partially spliced viral mRNAs from the nucleus to the cytoplasm of an infected cell (8). To do so, Rev binds to a conserved region of the viral mRNA, known as the Rev response element (RRE), where it forms an oligomeric RNP consisting of multiple Rev proteins bound to a single RNA molecule. The detailed assembly mechanism of the Rev-RRE complex was investigated using the methods described in the preceding sections (16). A large fragment of the RRE generated by *in vitro* transcription was immobilized on a quartz slide. The Rev protein was mutated to change one of the two native cysteine residues to serine. The mutant protein was labeled at the remaining cysteine residue with Alexa Fluor555, purified by HPLC and refolded as described above. Single-color TIRF microscopy was used to monitor the binding of one or more labeled Rev proteins to the immobilized RRE molecules. A two-dimensional image of the surface-immobilized Rev-RRE complexes is shown in Fig. 4 (upper panel) and a typical fluorescence intensity trajectory for a single spot within this image is shown in the middle panel. Abrupt jumps between different fluorescence intensity levels are observed as individual Rev proteins bind to or dissociate from the immobilized RNA. Binding of up to four Rev monomers to a single truncated RRE molecule was observed. A jump size distribution compiled from thousands of such trajectories revealed that more than 90% of transitions correspond to binding or dissociation of single Rev monomers (16). Hence, the Rev-RRE complex assembles by a sequential monomer binding pathway, rather than by direct binding of performed Rev oligomers. The binding and dissociation rates for each Rev monomer were obtained from a statistical analysis of the dwell times in each intensity state. An example of a typical dwell time histogram is shown in Fig. 4 (bottom panel). While all the proteins are of the same type in this example, the experimental system could be readily extended to visualize the binding of different proteins, each labeled with a distinct dye, to a common RNA molecule. Multi-color TIRF detection could be used to monitor binding of the different proteins and to establish temporal correlations between individual binding events. In principle, this approach could be applied to a variety of RNPs, limited only by the ability to label the constituent proteins.

Acknowledgments

Work in the author's laboratory was supported by NIH grants RO1 GM058873, PO1 GM066669 and P50 GM082545.

References

1. Collins CA, Guthrie C. Nat. Struct. Biol. 2000; 7:850–4. [PubMed: 11017191]
2. Valadkhan S. Biol. Chem. 2007; 388:693–7. [PubMed: 17570821]
3. Wahl MC, Will CL, Lührmann R. Cell. 2009; 136:701–18. [PubMed: 19239890]
4. Winkler WC, Breaker RR. Annu. Rev. Microbiol. 2005; 59:487–517. [PubMed: 16153177]
5. Roth A, Breaker RR. Annu. Rev. Biochem. 2009; 78:305–334. [PubMed: 19298181]

6. Staley JP, Woolford JL. Curr. Op. Cell Biol. 2009; 21:109–18. [PubMed: 19167202]
7. Keenan RJ, Freymann DM, Stroud RM, Walter P. Annu. Rev. Biochem. 2001; 70:755–775. [PubMed: 11395422]
8. Pollard VW, Malim MH. Annu. Rev. Microbiol. 1998; 52:491–532. [PubMed: 9891806]
9. Iglesias N, Stutz F. FEBS Lett. 2008; 582:1987–96. [PubMed: 18394429]
10. Boulo S, Akarsu H, Ruigrok RWH, Baudin F. Virus Res. 2007; 124:12–21. [PubMed: 17081640]
11. Sykes MT, Williamson JR. Annu. Rev. Biophys. 2009; 38:197–215. [PubMed: 19416066]
12. Nie S, Zare RN. Annu. Rev. Biophys. Biomol. Struct. 1997; 26:567–96. [PubMed: 9241430]
13. Moerner WE, Orrit M. Science. 1999; 283:1670–6. [PubMed: 10073924]
14. Joo C, Balci H, Ishitsuka Y, Buranachai, Ha T. Annu. Rev. Biochem. 2008; 77:51–76. [PubMed: 18412538]
15. Stone MD, Mihalusova M, O'Connor CM, Prathapam R, Collins K, Zhuang X. Nature. 2007; 446:458–61. [PubMed: 17322903]
16. Pond SJ, Ridgeway WK, Robertson R, Wang J, Millar DP. Proc. Natl. Acad. Sci. USA. 2009; 106:1404–8. [PubMed: 19164515]
17. Tan E, Wilson TJ, Nahas MK, Clegg RM, Lilley DM, Ha T. Proc. Natl. Acad. Sci. USA. 2003; 100:9308–13. [PubMed: 12883002]
18. Zhuang X, Kim H, Pereira MJ, Babcock HP, Walter NG, Chu S. Science. 2002; 296:1473–6. [PubMed: 12029135]
19. Pljevaljcić G, Millar DP, Deniz AA. Biophys. J. 2004; 87:457–67. [PubMed: 15240479]
20. Bokinsky G, Zhuang X. Acc. Chem. Res. 2005; 38:566–73. [PubMed: 16028891]
21. Pljevaljčić G, Millar DP. Meth. Enzymol. 2008; 450:233–52. [PubMed: 19152863]
22. Alemán EA, Lamichhane R, Rueda D. Curr. Op. Chem. Biol. 2008; 12:647–54.
23. Dorywalska M, Blanchard SC, Gonzalez RL, Kim HD, Chu S, Puglisi JD. Nucleic Acids Res. 2005; 33:182–9. [PubMed: 15647501]
24. Moore MJ, Query CC. Meth. Enzymol. 2000; 317:109–23. [PubMed: 10829275]
25. Bullard DR, Bowater RP. Biochem. J. 2006; 398:135–44. [PubMed: 16671895]
26. Mukhopadhyay S, Deniz AA. J. Fluoresc. 2007; 17:775–83. [PubMed: 17641956]
27. Roy R, Hohng S, Ha T. Nat. Meth. 2008; 5:507–16.
28. Axelrod D. Meth. Cell Biol. 1989; 30:245–70.
29. Kapanidis A, Lee NK, Laurence TA, Doose S, Margeat E, Weiss S. Proc. Natl. Acad. Sci. USA. 2004; 101:8936–41. [PubMed: 15175430]
30. Eggeling C, Berger S, Brand L, Fries JR, Schaffer J, Volkmer A, Seidel CA. J. Biotechnol. 2001; 86:163–80. [PubMed: 11257530]
31. McKinney S, Joo C, Ha T. Biophys. J. 2006; 91:1941–51. [PubMed: 16766620]
32. Walter NG, Burke JM. Curr. Op. Chem. Biol. 1998; 2:24–30.

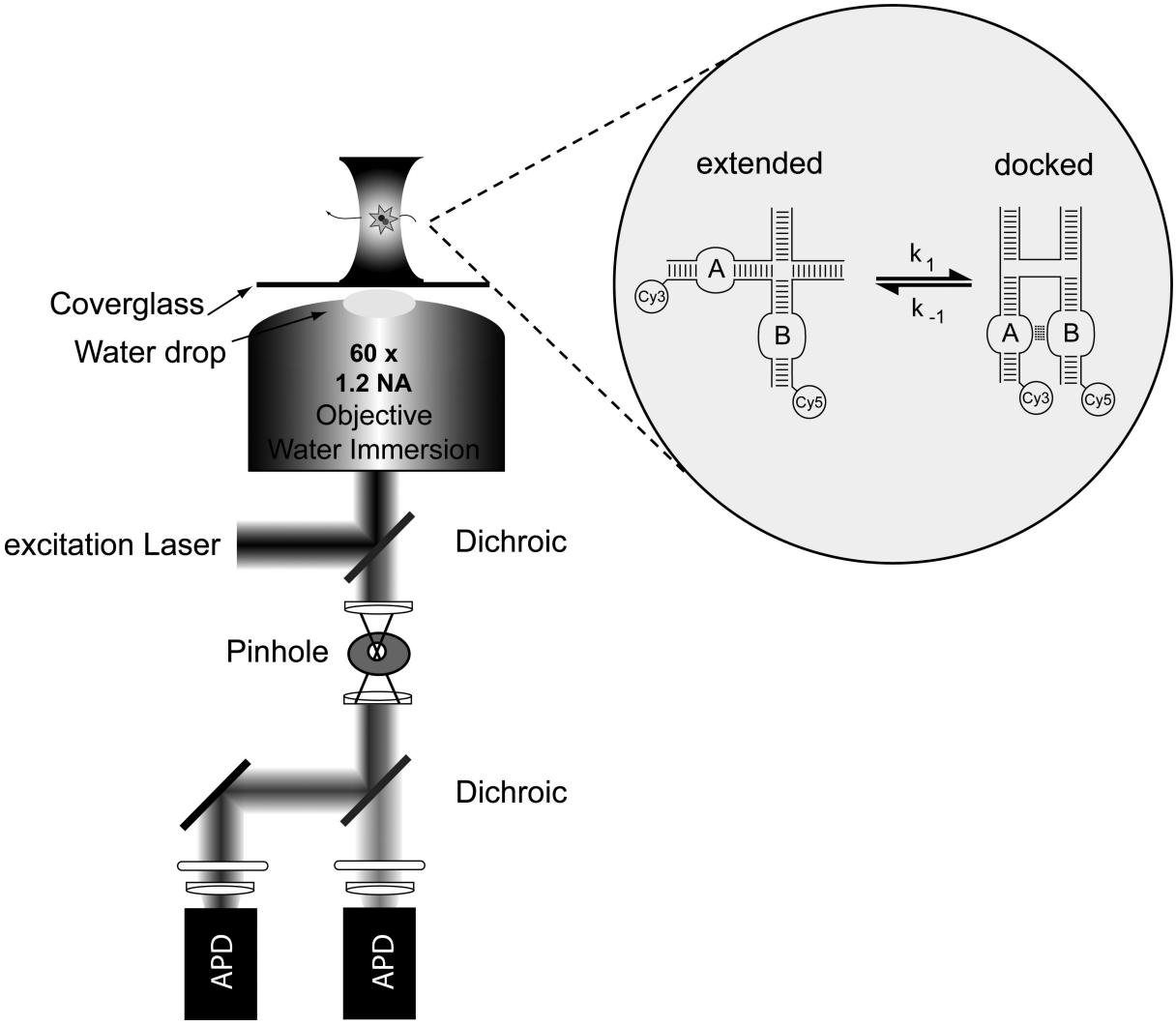

Figure 1.

Experimental set up for diffusion spFRET measurements. A single hairpin ribozyme molecule labeled with donor (Cy3) and acceptor (Cy5) dyes is shown traversing the excitation volume of a tightly focused laser beam. The ribozyme can exist in two conformations (extended or docked), which can be distinguished by their different FRET efficiencies (see Fig. 2). The fluorescence emitted by both donor and acceptor is collected by the same objective used for laser excitation, transmitted through a small pinhole to reject emission of molecules outside the confocal volume, spectrally separated with dichroic mirrors and bandpass filters, and finally detected on separate avalanche photodiode (APD) detectors. For ALEX measurements, two different lasers are used to alternately excite the donor and acceptor.

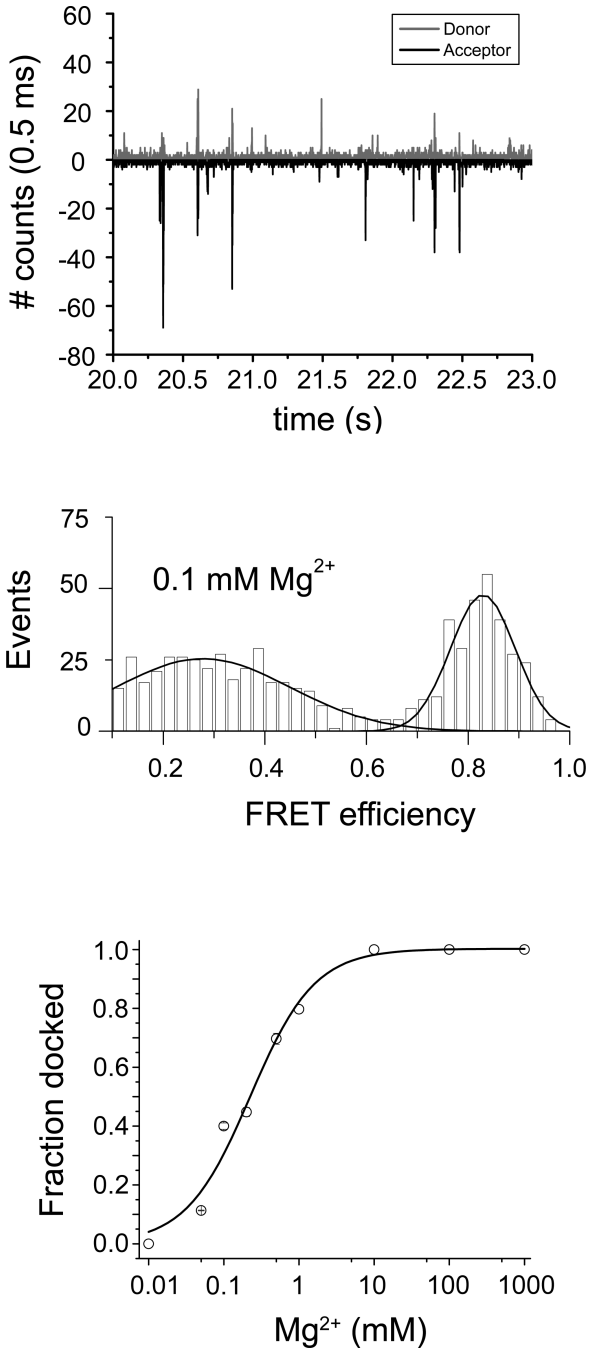

Figure 2.

Example of diffusion spFRET data for a doubly labeled hairpin ribozyme molecule. Upper panel: Typical bursts of fluorescence emission from donor and acceptor observed as single ribozyme molecules diffuse through the confocal volume. Middle panel: FRET efficiency histogram compiled from thousands of single-molecule events. The two well resolved peaks correspond to extended ($E \sim 0.3$) and docked ($E \sim 0.85$) ribozyme conformations. The smooth lines are fits to Gaussian functions. Lower panel: Fraction of docked ribozymes as a function of magnesium-ion concentration. Increasing Mg-ion concentrations favor formation of the docked conformation. The solid line is a fit to the Hill equation.

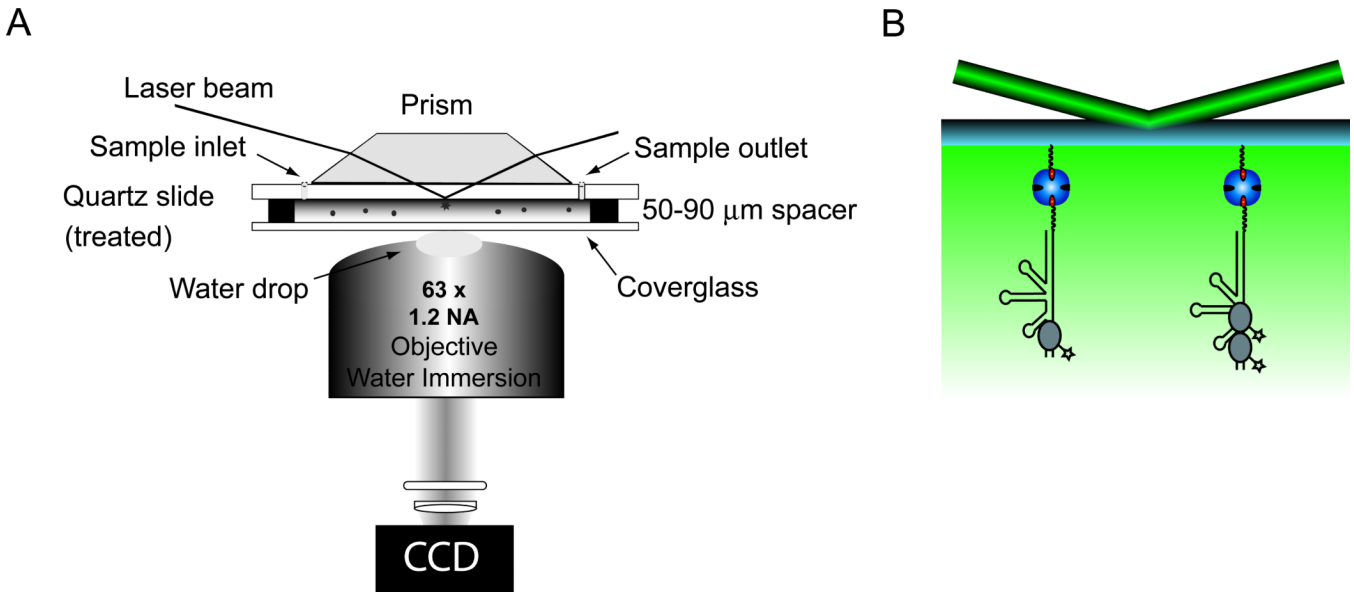

Figure 3.

Experimental set up used for single-molecule TIRF measurements. Left panel: A laser beam enters a prism at an incident angle above the critical angle for total internal reflection. The resulting evanescent field transmitted into the aqueous medium excites fluorescently-labeled molecules immobilized on the adjacent quartz surface. The resulting fluorescence emission is collected though a water immersion objective and imaged on a CCD camera. Right panel: Close up view of individual RRE molecules immobilized on a quartz slide by means of biotin-streptavidin bonds. One or two fluorescently labeled Rev molecules are shown bound to the immobilized RRE molecules.

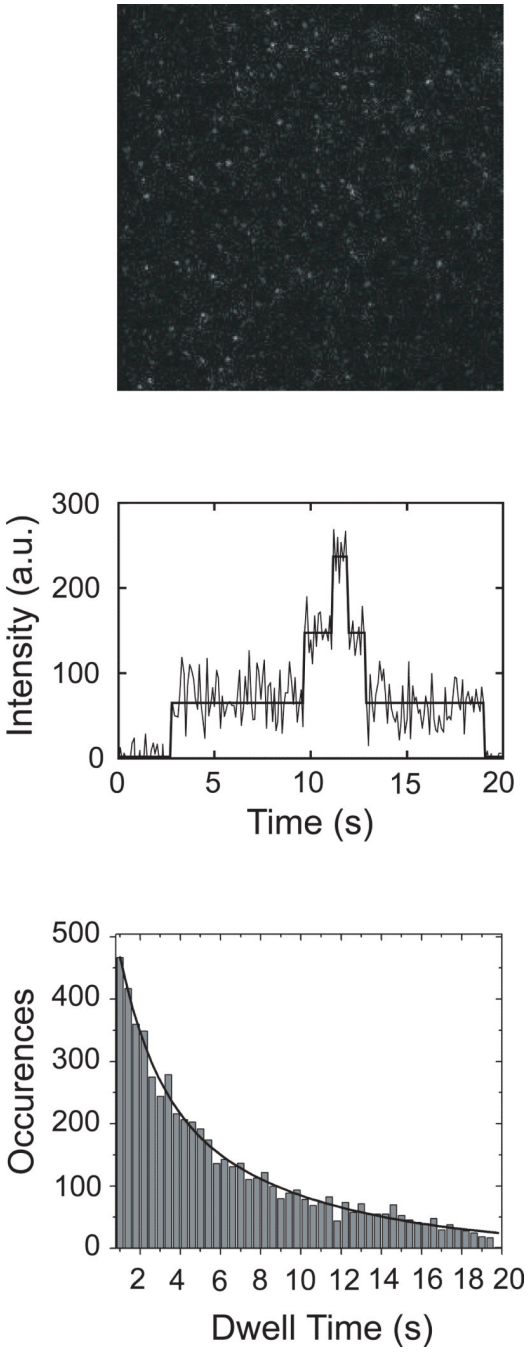

Figure 4.

Visualizing Rev-RRE complex assembly by single-molecule TIRF microscopy. Upper panel: Typical image of surface-immobilized Rev-RRE complexes. The discrete spots correspond to individual Rev-RRE complexes containing various numbers of bound Rev monomers. Middle panel: Intensity trajectory for a single fluorescent spot, revealing discrete Rev binding and dissociation events, manifested as abrupt jumps in the fluorescence intensity. Lower panel: Histogram of dwell times spent in the intensity state corresponding to zero Rev monomers before transition to the intensity state corresponding to a single Rev monomer. The histogram is fitted to an exponential function to obtain the association rate

for the first Rev monomer. A similar analysis was performed for each Rev binding and dissociation event (16).